Original Article

Assay Method Development And Validation Of Drug In Its Formulation By Hplc

Heera Karemore[1]* ,Hemlata More[1], Shubhangi Choudhry[1], Shubham Shende[1], Deepak Karemore[2]

[1*]School of Pharmacy,G H Raisoni University, Saikheda, MP
[2]Kamla Nehru College of Pharmacy, Butebori, MH

*Corresponding Author: Heera Karemore

*School of Pharmacy,G H Raisoni University Saikheda MP, Email Id: heerakaremore1997@gmail.com
DOI: 10.47750/pnr.2022.13.S10.560

Abstract

Lamivudine is a nucleoside reverse transcriptase inhibitor that is widely used for the treatment of HIV-1 infection in combination with other antiretroviral. It is a highly effective agent that can be dosed once or twice daily due to its long intracellular half-life. High performance liquid chromatographic (HPLC) method for the assay of 100-mg Lamivudine tablets. The chromatographic conditions of the method employ a Phenomenex C-08-04 (5µm),150x4.60mm column, isocratic elution with (pH 3.0): ACN: phosphate buffer (65:35 % v/v) as the mobile phase at a flow rate of 1.5 ml/min, a 20 µl injection volume, and Detection Wavelength is 274nm. The active was analysed at ambient column temperature, using peak area responses.

Keywords: Lamivudine, Liquid chromatography, Assay Development, Validation, Potency.

INTRODUCTION

Chromatography is probably the most powerful and versatile analytical technique available to the modern chemist, its power arises from its capacity to determine quantitatively many individual components present in mixture in one, single analytical procedure. Its versatility comes from its capacity to handle a very wide variety of samples that may be gaseous, liquid or solid in nature. The sample can range in complexity from a single substance to multicomponent mixture containing widely differing chemical species. The beginning of chromatography started with the work of botanist Michael Tswett in the year 1896. Tswett defined chromatography as the method in which the components of a mixture are separated on an adsorbent column is a flowing system. Recently, the IUPAC has defined chromatography as: "A method used primarily for the separation of the components of a sample, in which the components are distributed stationary phase may be a solid or a solid or a liquid supported on a solid or a gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid".

a) Adsorption chromatography: The stationary phase is a solid; the mobile phase containing the dissolved solutes passes over the surface of the stationary phase.

b) Partition chromatography: Results from a Thermodynamic distribution between two liquid (or liquid like) phases taking into consideration the relative polarities of the stationary and mobile phases, partition chromatography can be divided into normal phase and reverse and revers phase chromatography.

c) Ion exchange chromatography: Involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charges are attracted.

d) Size-exclusion chromatography (Gel chromatography): Involves a solid stationary phase with controlled pore size. Solutes are separated according to molecular, size, with the large molecules unable to enter the pores elute first.

High Performance Liquid Chromatography (HPLC)

HPLC is a popular method of analysis because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. Modern HPLC has many applications including separation, identification, purification, and quantification of various compounds. Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical and biochemical research as well as for the pharmaceutical industry, these fields currently comprise only about 50% of HPLC users. Currently HPLC is used by a variety of fields including cosmetics, energy, food, and environmental industries.

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open column chromatography, paper chromatography, and thin layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography.
High pressure liquid chromatography was developed in the mid-1970's and has quickly improved with the development of column packing materials and the additional convenience of on-line detectors. New methods including reverse phase liquid chromatography allowed for improved separation, identification, purification and quantification for above the previous techniques. Computers and automation added to the convenience of HPLC. Improvement in type of columns and thus reproducibility were made as terms such as micro-column, affinity columns and fast HPLC began to emerge.

Advantages of HPLC

The major advantages of HPLC include:

1. High resolving power.
2. Speed of separation.
3. Continuous monitoring of column effluent.
4. Accurate quantitative measurement.
5. Repetitive and reproducible results can be obtained.
6. Automation of analytical procedure and data handling.
7. Amenable to diverse sample.
8. It offers advantages over gas chromatography in analysis of many polar, ionic substances, high molecular weight substances, metabolic products and thermo labile as well as non-volatile substances.

Most Commonly Used Method in HPLC

Normal phase chromatography:

Normal phase HPLC was the first kind of HPLC chemistry used and separates analysts based on polarity. This method uses a polar stationary phase and a nonpolar mobile phase and is used when the analyst of interest is fairly polar in nature. The polar analyst associates with and is retained by the polar stationary phase. Absorption strengths increase with increase in analysts' polarity, and the interaction between the polar analysts and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyst molecules, but also on steric factors and structural isomers is often resolved from one another. Use of more polar solvents in the mobile phase will decrease the retention time of the analysts while more hydrophobic solvents tend to increase retention times. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is somewhat particular to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).
Mechanism: Retention by interaction of the stationary phase polar surface with polar parts of the sample molecules.

1. **Stationary phase:** It is a bonded siloxane with polar functional group like SiO2, Al_2O_3, $-NH_2$, -CN, $-NO_2$, -Diol.
2. **Mobile phase:** Nonpolar solvents like heptane, hexane, cyclohexane, chloroform, ethyl ether, dioxane.
3. **Application:** Separation of non-ionic, nonpolar to medium polar substances.
4. **Sample elution order:** Least polar components are eluted first.

Reverse phase chromatography:

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. Reversed phase chromatography is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification.
RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyses, and the non-polar stationary phase. The driving force in the binding of the analyst to the stationary phase is the decrease in the area of the non-polar segment of the analyst molecule exposed to the solvent.
Mechanism: Retention by interaction of the stationary phase non-polar hydrocarbon chain with nonpolar parts of sample molecules.

1. **Stationary phase:** It is bonded siloxane with nonpolar functional group like n-octadecyl (C-18) or n-octyl (C-8), ethyl, phenyl, $-(CH_2)n$-diol, -CN.
2. **Mobile phase:** polar solvents like methanol, acetonitrile, water or buffer (Sometimes with additives of THF or dioxane).
3. **Applications:** Separation of non-ionic and ion forming nonpolar to medium polar substances (carboxylic – acids hydrocarbons).
4. **Sample elution order:** Most polar components are eluted first.

COMPONENTS OF THE HPLC SYSTEM

HPLC Gradient Mixers
HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low-pressure systems this requires great precision in the operation of the miniature mixing valves used and low dispersion flows throughout the mixer. For multi-pump high-pressure systems it requires a very precise control of the flow rate which making very small changes of the flow rate.

HPLC Pumps
Because of the small particles used in modern HPLC column packing, modern LC pump need to operate reliably and precisely at pressures of 10,000 p. s. i. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pump usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical proposes, HPLC pumps should have flow rates that ranges from 0 to 10 ml/min, but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rate.

Pump pressure
Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 6000 $1bf/in^2$ (~40 MPa, or about 400 atmospheres). Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2 micrometers). These "Ultra High Performance Liquid Chromatography" systems or UHPLCs can work at up to 15,000 $1bf/in^2$ (~100 MPa or about 1000 atmospheres). Note that the term "UPLC", sometimes found instead is a trademark of Waters Corporation and not the name for the technique in general.

HPLC Columns
Column is often referred to as the heart of the HPLC separation process. HPLC columns are packed with very fine particles (Usually a few microns in diameter) to attain the low dispersion that give the high plate counts expected of modern HPLC. LC column, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase.

C_{18} and C_8 Columns
1. Classic reversed-phases for all general – purpose applications.
2. Excellent peak shape and efficiency compared to competitive columns.
3. Classic reversed-phase retention and selectivity.
4. C_{18} generally more retentive than the C_8.

HPLC Detectors
The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. HPLC detectors use the same detection principles with extra care being given to the same solute elution volumes that result from the combination of high column efficiencies with small volumes. In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then peaks that appeared are with flat tops as the whole be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell must also decrease. This is of course at odds for the requirement of detector to maintain high sensitivity, as this is usually dependent on having a larger cell volume. Again, this requires the very careful design of modern detectors,

Types of Detectors:
Liquid chromatographic are of two basic types. Bulk property detectors respond to a mobile phase bulk property such as density, refractive index, di-electric constant that is modulated by presence of solutes. In contrast, solute property detectors respond to some property of solute such as UV absorbance, fluorescence, diffusion current, that is not possessed by mobile phase.
The list of most common detectors for HPLC and some of their most important properties following table:

Table no.1: List of detector and their important properties

No.	Detector	Analysts	Comments
1.	UV-Visible	Any compound with chromosphere	It has a degree of selectivity and is useful for many HPLC applications

2	Fluorescence	Fluorescent compounds	Highly selective and sensitive. Often used to analyze derivatives compounds
3.	Refractive Index (RI)	Compounds with different RI of the mobile phase	Virtually universal detector but has limited sensitivity
4.	Conductivity	Charged or polar compounds	Excellent for Ion exchange methods
5.	Electrochemical	Readily oxidized or reduced compounds	Very sensitive and sensitive

Method Development in HPLC

Methods for analyzing drugs by HPLC can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character, pKa values and the solubility parameter. An exact recipe for HPLC method development cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.

The water soluble active pharmaceutical ingredients is further differentiated as ionic or nonionic which can be separated by reverse-phase. Similarly, the organic soluble API can be classed as polar and non-polar and equally separated by reverse phase. In some cases the non-polar API may have to be separated using adsorption or normal phase HPLC, in which mobile phase would be non-polar organic solvent.

Role of different parameters for selection of mobile phase

1. pH

pH is a critical factor which affects the selectivity of the separation of the separation process in reversed-phase HPLC. It is necessary to select the proper buffer pH to reproducibly separate ionisable compounds by reversed-phase HPLC. Selecting an improper pH for ionisable analyse often leads to asymmetric peaks that are board with tailing, spitting or shouldering of peak. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviation (RSD) between injections, and reproducible retention times. Sample retention increases when the analyst is more hydrophobic.

1. Buffer

In reversed-phase liquid chromatography, the mobile phase pH values are usually between 2.0 and 7.5. Buffers are needed when an analyst is ionisable under reversed-phase conditions or the sample solution is outside this pH range. Analysts ionisable under Reversed-Phase conditions often have amine or acid functional groups with pKa between 1.0 and 11.0.

2. Temperature

Although the effect of temperature is less significant in liquid chromatography, it may play a significant role in the optimization for difficult separations. Temperature variations over the course of a day have quite significant effect on HPLC separations. This can even occur in air conditioned rooms. While temperature is a variable that can effect the selectivity, its effect is relatively small. Also retention time generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analysts. Snyder reported that an increase of 1^0 C will decrease time by 1 to 2%.

3. Flow rate

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased, to increase the resolution slightly, there is a corresponding increase in the run time.

4. Selection of detector

Detector is the eye of the HPLC system and measures the compounds after their separation on the column. There are basically two types of detectors the bulk property detector and solute property detectors. Detectors, in order of their popularity are UV, fluorescent, conductivity and refractive index detectors. UV detectors is the first choice because of its convenience and applicability in case of most of the samples.

5. Sample preparation

The sample prepared should be homogenous. It should be completely should in the selected solvents, usually the solvent used to dissolve the sample should be the mobile phase itself or any solvent miscible with the mobile phase.

6. Chromatographic separation

After achieving a resolution with a pre-optimized solvent system, to obtain reproducible results following criteria must be satisfied.

1. Monitoring flow rate.
2. Keeping the solvent composition intact.

3. Solvent system must be covered before storage.
4. Monitoring column temperature.

SYSTEM SUITABILITY PARAMETERS

1. Theoretical Plate (N)

It is also called as column efficiency. A column can be considered as being made of large number of theoretical plates where distribution of sample between liquid-liquid or solid-solid phase occurs. The number of theoretical plates in given by the following relationship

$$N = 16\ (t / w)^2$$

Where, is the retention time and w is the width at the base of the peak.

$$HETP = L/N$$

Where, L is the length of column

Theoretical plates should be more than 2000.

2. Height equivalent to theoretical plate (h)

The efficiency of the column can be expressed as the height equivalent of theoretical of theoretical plates (HETP). Lower the HETP, higher is the efficiency of the column i.e. higher the theoretical plate, more efficient the column.

$$h = L / n$$

Where,

h = height equivalent to theoretical plate (HETP)
L = Length of the column,
n = Number of theoretical plates

3. Retention time (Rt)

t is the time in between which the sample is injected and the chromatographic peak is recorded.

4. Resolution (Rs)

It is a measure of quality of separation of adjacent bands in a chromatogram; obviously overlapping bands have small Rs values. It is calculated from the width and retention time of two adjacent peaks.

$$Rs = \frac{(t_2 - t_1)}{W_1 + W_2}$$

Where,

t_1 and t_2 are the retention time of first and second adjacent bands, whereas, W_1 and W_2 are their baseline bandwidths. Reliability of calculation is poor if Rs is < 1.0.

5. Capacity factor (K')

It is the measure of the position of a sample peak in the chromatogram, being specific for a given compound. K' depend on the stationary phase, mobile phase, temperature, and quality of column packing.

For good chromatographic performance with isocratic separation, K' value should be in the range of 1 – 10.

$$K' = \frac{R_1 - {}_0}{t_0}$$

$$K' = \frac{R_2 - {}_0}{t_0}$$

Where,

t_{R1} and t_{R2} total retention time of components 1 and 2 respectively.
t_0 is non-adsorbent time.

6. Separation factor (Selectivity) (a)

It is a measure of peak spacing and is expressed as:

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

7. Tailing factor (T)

It is the measure of peak symmetry & is unity for perfectly symmetrical peaks and its value increases as tailing become more pronounced.

$$T = \frac{W_{0.05}}{2F}$$

Where,
$W_{0.05}$ is the width of peak at 5% height and
F is the distance from the peak maximum to the leading edge of the peak height from the baseline.

1.2. VALIDATION OF ANALYTICAL TECHNIQUES

Introduction to validation

Validation is a concept that has been evolving continuously since its first formal appearance in United Status in 1978. The concept of validation has expanded through the years to encompass a wide range of activities from analytical methods used for the quality control of drug substances and products to computerized system for clinical trial, labelling or process control. Validation is the overall expression for a sequence of activities in order to demonstrate and document that a specific product can be reliably manufactured by the designed processes, usually, depending on the complexity of today pharmaceutical products, the manufacturer must ensure; "that products will be consistently of a quality appropriate to their intended use".

ICH guideline **Q2** (**R1**) Method Validation Parameters are as follows:-

1. Accuracy
2. Precision
3. Specificity
4. Linearity
5. System suitability
6. Ruggedness
7. Robustness

1. Accuracy

It is the measure of how close the experimental value is to the true value. It is measured as the % of analyst recovered by assay or by spiking samples in a bling study. Accuracy should be established across the specified range (that is, line of working range) of the analytical procedure. For the assay of the drug substance, accuracy measurements are made by comparison of the results with the analysis of a standard reference material or to compare the results obtained from a second well-characterized independent procedure, the accuracy of which is stated and / or defined.

The % recovery was then calculated by using formula.

$$\% \text{ Recovery} = \frac{A+B}{C \times 1OO}$$

Where,
A : % Total amount of drug estimated
B :% Amount of drug found on reanalysed basis.
C : % Amount of pure drug added

2. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous samples. It is expressed as standard deviation or coefficient of variation.

Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision.

3. Specificity

It is the ability to the analyst in the presence of components, which may be expected to be present in the sample under consideration. This might include degrades, impurities, matrices, excipients etc.

4. Linearity

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyse in the sample within a given range. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and / or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyse concentration or content. If there is a linear relationship, test result should be evaluated by appropriate statistical methods.

5. System suitability

System suitability is a Pharmacopeia requirements and is used to verify, whether the resolution and reproducibility of the chromatographic system is adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard solutions.

6. Ruggedness

Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories and different days, etc.

7. Robustness

The robustness of analytical methods is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.
Perform experiments by changing conditions such as temperature (±5^{0}C), change in wavelength (±2), and ionic strength of buffers, level of activities to mobile phase.

Table No.2: Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy/Trueness	Recovery98-102%(individual)with 80,100,120% spiked
Precision	RSD <2%
Repeatability	RSD <2%
Intermediate precision	RSD <2%
Specificity/Selectivity	No interference
Detection limit	S/N >2 OR 3
Quantitative limit	S/N > 10
Linearity	Correlation coefficient r^2>0.999
Range	80-120%
Sample solution stability	>24 h or >12 h

Advantages of analytical method validation

- ✓ The biggest advantages of method validation are that it builds a degree of confidence, not only for the developer but also to the user.
- ✓ Although the validation exercise may appear costly and the consuming, it result inexpensive, eliminated frustrating repetitions and leads to better time eliminate in the end.
- ✓ Minor condition absorbs the shock of such conditions and pays for more than invested on the process.

LAMIVUDINE

Lamivudine, commonly called 3TC, is an antiretroviral medication used to prevent and treat HIV/AIDS. It is also used to treat chronic hepatitis B when other options are not possible. It is effective against both HIV-1 and HIV-2. It is typically used in combination with other antiretroviral such as zidovudine and abacavir. Lamivudine may be included as part of post-exposure prevention in those who have been potentially exposed to HIV. Lamivudine is taken by mouth as a liquid or tablet.

Common side effects include nausea, diarrhea, headaches, feeling tired, and cough. Serious side effects include liver disease, lactic acidosis, and worsening hepatitis B among those already infected. It is safe for people over three months of age and can be used during pregnancy. The medication can be taken with or without food. Lamivudine is a nucleoside reverse transcriptase inhibitor and works by blocking the HIV reverse transcriptase and hepatitis B virus polymerase.

EXPERIMENTAL AND RESULT

PREPARATION OF STANDERD AND BUFFER SOLUTIONS

➢ Standard stock solution (A1)

An accurately weighed about 10.0mg LAMIVUDINE was transferred in a 10.0ml volumetric flask, dissolved in sufficient quantity and volume was made up to the mark with mobile phase. (Conc.1000µg/ml)

➢ Working stock solution (A2)

A 1.0ml of stock solution (A1) was transferred in 10.0ml volumetric flask and volume was made up the mark with mobile phase. (Conc. 100µg/ml)

➢ Working standard solution (A3)

The working stock solution (A2) was appropriate diluted with mobile phase to get the final concentration of 20µg/ml.

➢ Phosphate buffer (pH-3):

Dissolved 1.36g of potassium dihydrogen orthophosphoric acid and 2ml of triethylamine in 800 ml of water, adjust the pH to 3.0 with orthophosphoric acid and add sufficient water to produce 1000 ml.

Preliminary optimization of mobile phase and other chromatographic conditions

In order to achieve the optimized chromatographic condition, one or two parameters were modified at each trial and chromatograms were recorded with all specified chromatographic conditions. Various mobile phases were tried by permutation and combination and also by change in flow rate, buffer and its pH.

The standard solution of drug was prepared in different mobile phases and various trials were taken out. The respective chromatograms and observation of last two trials are summarised in Fig. No.1 and 2.

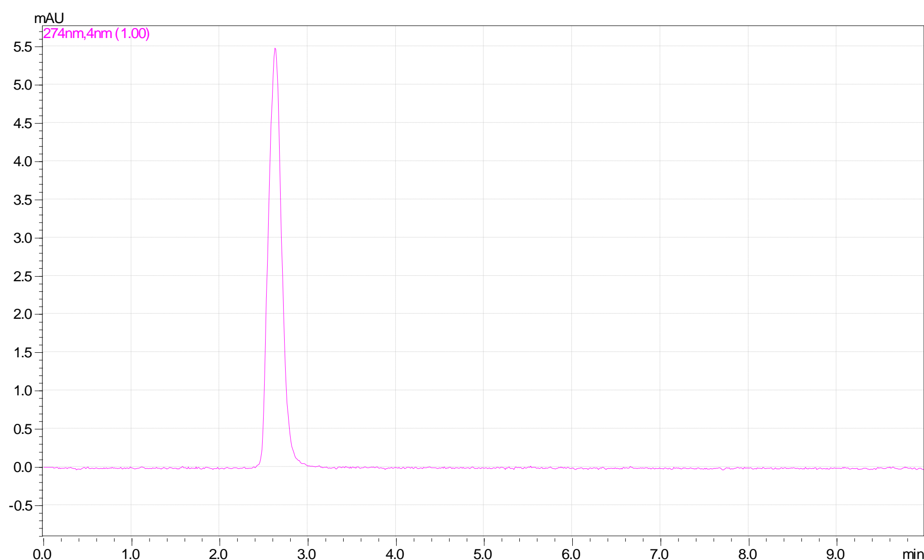

Fig.No.1: A chromatogram of standard during trial 1

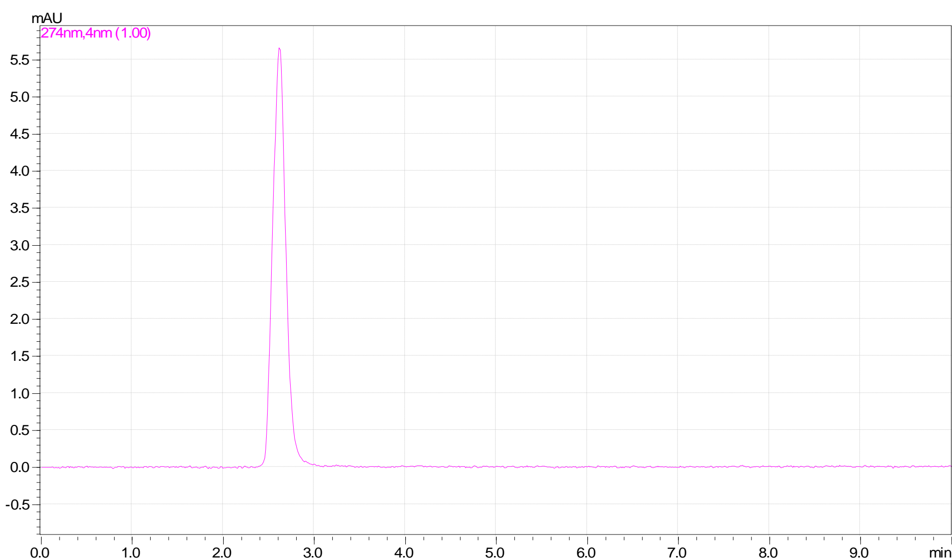

Fig.No.2: A chromatogram of standard during trial 2

The observations for last two trial are summarized in Table No. 3.

Table No.3: Selection of Mobile Phase

Trial	Mobile phase (%V/V)	Tailing Factor	Flow rate (mL/Min)	Retention time (Min)	Remark
I.	ACN:Phosphate buffer pH-3 (65:35v/v)	1.452	1	2.643	Sharp Peak
II.	ACN:Phosphate buffer pH-3 (70:30v/v)	1.81	1	2.428	PeakSlightly Broad

Finally, selected mobile phase and other chromatographic parameters for Lamivudine were found suitable for resolution of intact drug, free from interference of excipients.

Table No.4: Optimized chromatographic parameters

System	Shimadzu HPLC series 1100
Stationary phase	Phenomenex C-08-04 (5µm),150x4.60mm
Mobile phase	ACN: phosphate buffer (65:35 % v/v)

Detection wavelength	274
Flow rate	1.5ml/min
Ph	3
Temperature	Ambient
Injection volume	20µL
Diluent	Mobile phase

Procedure:

The chromatographic condition were set as per the optimized parameters mobile phase was allowed to equilibrate with stationary phase as indicate by steady baseline. A 20µL of solution was injected through manual injector and chromatogram was recorded. A mobile phase containing ACN: phosphate buffer, pH 3 (65:35 % v/v) gave well-resolved peak and reasonable retention time as shown in Fig. No. 3.

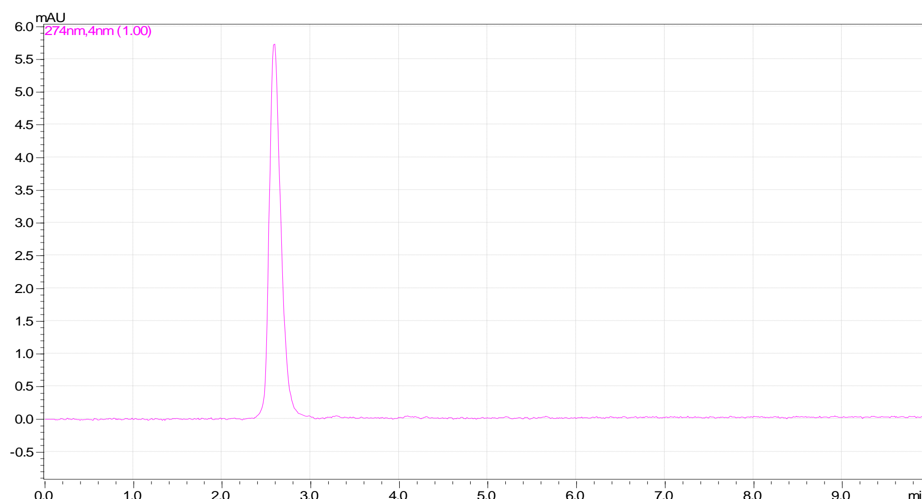

Fig. No.3: A chromatogram for standard Lamivudine Study of system suitability parameters

After equilibration of column with mobile phase, working standard solution (A3) 2.0 ml portion was further diluted to 10ml to get the concentration about 20µg/ml, was injected through the manual injector five times and the chromatograms were recorded and the peak area was measured. The recorded results of system suitability parameters are shown in Table No.5.

Table No.5: Observations of system suitability parameters

Sr. No.	Wt. of std. drug taken in (mg)	Peak Area (µV)
1	~10.0	1501396
2		1511800
3		1514049
4		1523686
5		1529592
6		1532481
	Mean	**1518834**
	±SD	**11848.83**
	%RSD	**0.78**
	Retention time (Mean)	**2.645**
	Tailing Factor	**1.546**
	HETP	**44.46**

Study of linearity

Aliquots of working standard solution (A3) were diluted in range of 1-5 mL in 10.0mL Volumetric flask with mobile phase and volume was made up to mark mobile phase to obtain concentration ranging from 1-5µg/mL of Lamivudine.

Procedure:

The mobile phase was allowed to equilibrate with stationary phase till steady baseline was obtained. Each of the final solution was injected separately and recorded the chromatogram. The observations of area under curve are shown in Table No.6.

Table No.6 : Observational of linearity study

Sr. No.	Conc. (µg/mL)	AUC (mV)	Retention Time
1	5	4937516	2.760
2	10	9594179	2.779
3	15	14912392	2.840
4	20	19980891	2.848
5.	25	25208853	2.775

A graph plotted between concentrations Vs. area under curve is shown in Fig.No.4 the correlation coefficient was found to be 0.9992.

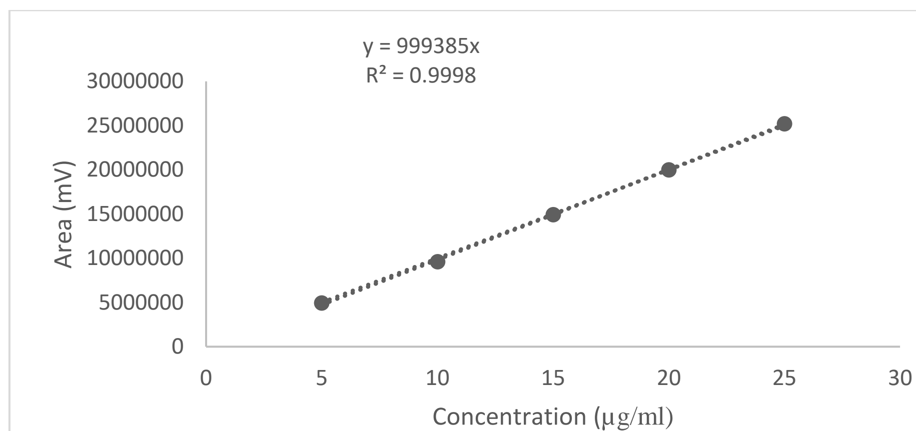

Fig. No.4: Calibration Curve of Lamivudine

APPLICATION OF PROPOSED METHOD FOR ASSAY OF MARKETED FORMULATION

Preparation of sample solution

Twenty tablets were weighed and average weight was calculated. The tablets were triturated thoroughly and mixed. An accurately weighed quantity of tablet powder equivalent to 20mg of Lamivudine was transferred to 25 mL volumetric flask and volume was made up to the mark with mobile phase. The content was sonicated for 15 min and filtered through whatmann filter paper. A 1.0 mL portion of filtrate was diluted to 10.0 mL with mobile phase. A 2.0mL portion of filtrate was further diluted to 10.0mL with mobile phase. Five such samples were prepared. After equilibrations of column with mobile phase 20µL volume of the final diluted solutions were injected in the system, the representative chromatograms were recorded is shown in Fig.5.

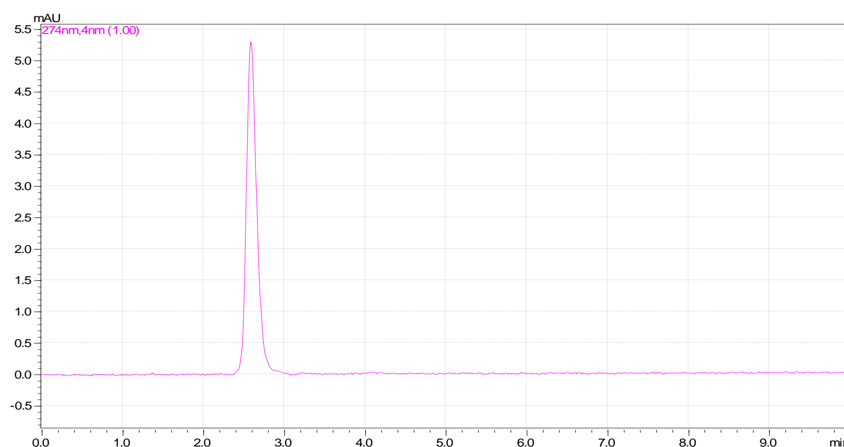

Fig. No.5: A chromatogram of Laboratory mixture of Lamivudine

The content of Lamivudine in each sample was calculated by comparing the peak area of sample with that of standard using formulae,

$$\text{Amt of drug estimation} = \frac{\text{Au x Cs x Dilution factor x vol of stock}}{\text{As}}$$

$$\%\text{Label claim} = \frac{\text{Amt.of drug estimation x Avg.Wt}}{\text{Wt.to be taken x Label claim}}$$

Where,

Au = peak area of sample
As = peak area of standards
Avg. wt. = Average weight of lab prepared mixture (mg)
L.C = Label claim of drug (mg)

The obtained results and statistical data are shown in Table No.7.

Table No.7: Observation and Results of estimation of Lamivudine

Sr.No.	Wt. taken (mg)	AUC of standard (µV)	AUC of sample (µV)	% Labelled Claim
1	63.2	1998089	3276652	102.18
2	63.4		3222501	100.32
3	63.3		3176652	99.37
4	63.4		3286721	102.32
5	63.1		3184941	99.47
			Mean	**100.732**
			±SD	**1.4349**
			%RSD	**1.42**

Recovery study

It was carried out by standard addition method (SAM)

Preparation of sample solution

An accurately weighed quantities of laboratory powder excluding drug was transferred in a series of 10.0mL of different volumetric flask and add to it known quantities of standard drug was added at three different level (weights are recorded in Table No. 8. To each flask mobile phase was added, -the content were shaken and sonicated for 15 min and filtered through o.45µm nylon filter and volume was made up to the mark with mobile phase. A 1.0mL of above filtrate was diluted to 25.0mL with mobile phase. A 20µL volume of each final dilution solution was injected separately and the chromatogram was recorded.

Amount contributed by marketed preparation and % recoveries were calculated using the following formula

$$\text{Amt. contributed by} = \frac{\text{Wt. Taken}}{\text{Avg.Wt.}} \times \text{Amt. of drug present in Avg}$$

Marketed preparation Wt. as per mean % estimated

$$\%\text{Recovery} = \frac{\text{Total Amt of drug estimation} - \text{Amt. contributed}}{\text{Amt. of pure drug added}} \times 100$$

The observation and result are shown in Table No.8.

Table No.8: Result of estimation under recovery study

Sr. No.	Wt. of lab prepd. powder (mg)	Amt. of pure drug added (mg)	Standard Area (µV)	Sample Area (µV)	Amount estimated	% Recovery
1	63.1	10.2	1998089	4854901	30.372	101.68
2	62.3	20.3		6473556	40.498	100.93
3	63.4	30.2		8041836	50.310	100.36
					Mean	**100.99**
					±SD	**0.6620**
					%RSD	**0.66**

Validation of method

Validation of the proposed method was carried out as per ICH guidelines.

Accuracy

Accuracy of the proposed method was ascertained on the basis of recovery studies performed by standard addition method. Result are 1.31%RDS.

Precision

Precision of any analytical method was expressed as SD and %RSD of series of measurements. Precision of estimation of Lamivudine by proposed method was ascertained by replicate analysis of homogeneous sample. Results are shown in Table.9.

Table N0.9: Observations and results for precision

Sr. No.	Wt. of powder taken (mg)	AUC of Standard (µV)	AUC of Sample (µV)	% Labelled claim

1	61.9	1998089	3105656	99.03
2			3116443	99.37
3			3161443	100.81
4			3144327	100.26
5			3134379	99.95
			Mean	**99.88**
			±SD	**0.7065**
			%RSD	**0.71**

Linearity and Range

An accurately weighed Tablet powder equivalent to about 80-150% of label claim was taken and dilutions were made as described under the laboratory mixture. Then each solution was injected and chromatograms were recorded. The correlation coefficient was found to be and results are recorded in Table No.10.

Table No.10: Observation for Linearity and range study

Sr. No.	Wt. of tablet powder (mg)	Wt. of tablet powder taken equivalent to % label claim	Area (mV)
1	**10mg**	80	914168
2		90	1006652
3		100	1126678
4		110	1235421
5		120	1330033
		Coefficient of correlation	0.9983

A graph of % of label claim Vs. Area was plotted as shown in Fig. No.6.

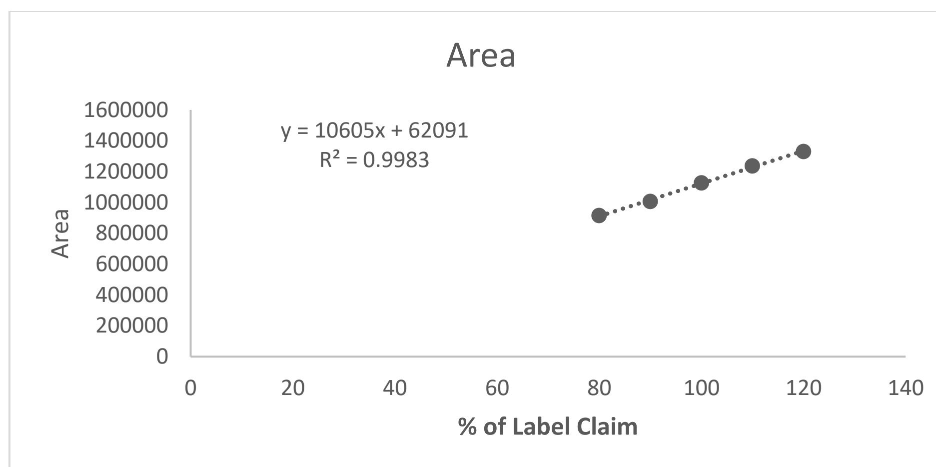

Fig. No.6: Linearity curve of Lamivudine Ruggedness

The ruggedness study was carried out by using different analyst and instruments.

Different analyst

The sample solution was prepared as per the procedure described under marketed formulation. Then sample solutions were analysed using proposed method by different analysts. Results are shown in Table.11

Table No.11: Observations and results of Analyst-to- analyst Variation

Analyst	Wt. of tablet powder taken (mg)	AUC of Std. (µV)	AUC of Sample (µV)	%Labelled Claim
Analyst 1	58.6	1998089	2961152	99.74
Analyst 2	58.8		2967108	99.60
Analyst 3	58.7		2961443	99.54
			Mean	**99.626**
			±SD	**0.1026**
			%RSD	**0.1**

Intra-day variation

The sample was prepared by using earlier mentioned procedure. After equilibrium of stationary phase, sample solutions were injected separately at 1, 2, and 3 Hr. and the chromatograms were recorded. The chromatograms so recorded and result were calculated. The content of Lamivudine were calculated by comparing the peak area of sample with that of standard using formula given under laboratory mixture formulation. The observation and result are recorded in Table No.12 for intra-day study respectively.

Table No.12: Observation and results for intraday study

Time (hr.)	Wt. of tablet powder taken (mg)	Standard Area (µV)	Sample Area (µV)	% Labelled claim
1 hr	59.9	1998089	3030635	99.86
2 hr			3076652	101.38
3 hr			3042550	100.26
			Mean	**100.5**
			±SD	**0.7879**
			%RSD	**0.78**

Robustness

The robustness of the method was evaluated by injection 100.81 the sample at deliberately varying the chromatographic condition viz. composition of organic phase in mobile phase by 10%, pH of buffer by +0.2unit, varying flow rate and change in wavelength by +5nm. The system suitability parameters were evaluated at each varied condition and the observations are tabulated in Table No.13.

Table No.13: Observation for robustness study

Parameters	Wt. of powder taken (mg)	Retention time (min)	Theoretical plate	Tailing factor
Standard condition	63.104	2.663	44.39	1.583
Wavelength at 269nm		2.633	44.94	1.588
Wavelength at 279nm		2.659	44.30	1.540
Phosphate buffer:ACN (65:35)		2.643	44.31	1.575
Phosphate buffer:ACN (55:45)		2.645	45.49	1.529
Flow rate (1.00mL/min)		2.630	45.16	1.544
Flow rate (1.2mL/min)		2.135	45.20	1.546
		Mean	**44.605**	**1.5578**
		%RSD	**0.84**	**1.51**
		Mean RSD	**1.175**	

Limit of Detection (LOD) and Limit of Quantification (LOQ):

There are several terms that have been used to define LOD and LOQ. In general, the LOD is taken as the lowest concentration of an analyst in a sample that can be detected, but not necessarily quantified, under the state condition of the test. The LOQ is the lowest concentration of an analysts in a sample that can be determined with acceptable precision and accuracy under the stated condition of test.

Table No. 14: Result of LOD and LOQ

PARAMETERS	LAMIVUDINE
LOD	0.266
LOQ	0.808

SUMMARY AND CONCLUSION

Summary

The objective of present work is to develop Assay method for the determination lamivudine in the tablet dosage form that would provide helpful information to the manufacturers.

In the present research work attempts were made to develop simple, accuracy, precise, and rapid analytical methods for the estimation of Lamivudine from its tablet are as follows.

Estimation of LAMIVUDINE by HPLC Methods

Shimadzu HPLC series 1100 was used for quantitative estimation of Lamivudine from its Tablet Formulation.

The analysis was performed using Phenomenex C-08-04 (5µm), 150x4.60mm, mobile phase used was Acetonitrile: Phosphate buffer (65:35 % v/v) and selected wavelength was 274 nm at which drug showed sharp peak. The recovery studies were performed by standard addition method and the results of estimation of Lamivudine in laboratory mixture and recovery studies are summarized in Table No.15

Table No.15: Summary of the results of estimation and recovery by HPLC

Laboratory Mixture	%Recovery (274nm)
Mean	102.41
±SD	1.3466
%RSD	1.31

Validation by proposed methods

Validation was performed to assure the reliability of the proposed method and was carried out as per ICH guidelines for the following parameters.

I. Accuracy
II. Precision
III. Linearity and range
IV. Ruggedness
V. Robustness

The results and statistical data of validation parameters for Lamivudine by HPLC methods are summarized in Table No.16. Respectively.

Table No.16: Summary of validation parameters using HPLC

Parameters	Results
Accuracy (% RSD)	1.31
Precision (% RSD)	0.71
Linearity and range (% RSD)	0.9983
Analyst (%RSD)	0.1
Intraday (%RSD)	0.78
Robustness (%RSD)	1.175
LOD	0.266
LOQ	0.808

CONCLUSION

The results obtained by RP-HPLC methods for determination of Lamivudine are reliable, accurate and precise. The method does not have any interference of excipients while determining Lamivudine its laboratory prepared mixture. The developed HPLC method was found to be superior with respect to resolution of drug from its prepared mixture can be employed for routine quality control analysis of Lamivudine in tablet dosage form.

CONFLICT OF INTEREST – None

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