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Analytical Methods Development and Validation of HPLC & RP-HPLC

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Abstract

Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases stationary and mobile phases. The separation of constituents is based on the difference between partition coefficients of the two phases. The chromatography term is derived from the Greek words, namely chroma (colour) and graphein (to write). Chromatography is a very popular technique and it is mostly used analytically. There are different types of chromatographic techniques, namely Paper Chromatography, Gas Chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography and lastly, High-Performance Liquid Chromatography (HPLC). This review mainly focuses on the HPLC technique, its principle, types, instrumentation and applications. High-performance liquid chromatography is the most accurate method extensively used for the qualitative and quantitative analysis of drug products. Analytical method development and validation play vital role in the drug discovery, Drug development and manufacture of pharmaceuticals. A simple, precise, accurate, specific and RP-HPLC method was developed for the determination of drugs in pharmaceutical formulation.

Keywords*: - HPLC, RP-HPLC, Chromatography, Chromatographic Conditions, Abacavir (ABAC), Lamivudine (LAMI).*

INTRODUCTION

High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High-performance liquid chromatography (HPLC) is the most accurate analytical method widely used for the quantitative as well as qualitative analysis of drug products. [1]

The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from the different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. [2]

The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity, which travel faster and for a longer distance. [3]

The High Performance Liquid Chromatography is more versatile than gas chromatography since

a) It is not limited to volatile and thermally stable samples, and
b) The choice of mobile and stationary phases is wider. [4]

Reversed-phase chromatography (RP-HPLC) separates molecules on the basis of differences in their hydrophobicity. The components of the analytic mixture pass over stationary-phase particles bearing pores large enough for them to enter, where interactions with the hydrophobic surface remove them from the flowing mobile-phase stream.

The strength and nature of the interaction between the sample particles and the stationary phase depend on both hydrophobic interactions and polar interactions. As the concentration of organic solvent in the eluant increases, it reaches a critical value for each analyte, which desorbs it from the hydrophobic stationary-phase surface and allows it to elute from the column in the flowing mobile phase. [5]

AIM & OBJECTIVES

The sample mixture to be separated and analyzed is introduced in a small discrete volume (typically microliters) into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase).

The velocity of each component depends on its chemical nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analyte.

Objectives

A simple, rapid, accurate, precise, and reproducible validated reverse-phase high-performance liquid chromatography (HPLC) method was developed for the determination of Abacavir (ABAC) and Lamivudine (LAMI) in bulk and tablet dosage forms.

The objective of this article is to review the method development, optimization and validation. HPLC method development depends on the chemical structure of the molecules, synthetic route, solubility, polarity, pH and pKa values, and functional group activity etc.

NEED FOR ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

- Existing methods may be too much error, contamination prone or they may be unreliable.

- There may be a need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

- There may not be a suitable method for a particular analyte in the specific sample matrix.

- Existing methods may not provide adequate sensitivity.

- For regulatory requirements, it is required.

- Available method may be too expensive, time-consuming or energy-intensive, or that may not be easily automated.[7]

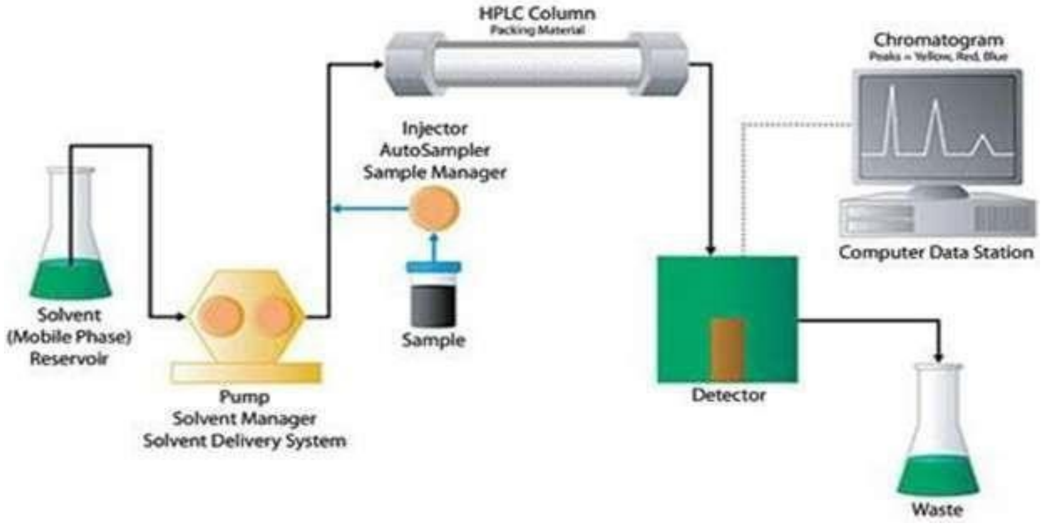

Figure 1: Instrumentation of HPLC

MATERIAL & METHOD

Analytical method development finally results in official test methods. Consequently, quality control laboratories used these methods to check the efficacy, identity, purity, safety as well as the performance of products of the drug. Regulatory authorities give utmost importance to analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods.

Modern pharmaceutical analysis needs the following requirements.

1) The analysis should take minimal time and should be economical
2) The accuracy of the analysis must accept the guidelines of Pharmacopoeia.
3) The chosen method should be precise and selective. [12]

Steps in Method Development [13]

- Physicochemical Properties of Drug Molecule
- Selection of Chromatographic Conditions
- Developing the approach of analysis
- Preparation of Sample
- Method Optimization
- Method Validation

A. *Physicochemical Properties of Drug Molecule:*

a) Chemical Properties

Chemical structure of the known and expected product chemical structures are good inputs for initiating the method development and it will give a scientific approach for the method development. Draw the comparative difference between impurities, starting materials, by-products intermediate and degradation products with final products. Solubility study at different pH values information for all targeted molecules is the best inputs for selecting the common diluent for all molecules. Selecting the polar/non-polar HPLC column the information of polarity of molecules is important.

b) pH and pKa Value of Compound

The nature and polarity of the compound are assumed based on pH and pKa values. The compound is half ionized when pH is equivalent to pKa. Almost all the pH related change occurs within the ± 1.5 units of the pKa values. Outsides the range, the compound is either ionized or non-ionized, and its retention does not change much with pH. [14]

B. Selection of Chromatographic Conditions

During initial method development, a set of initial conditions (column, mobile phase and detectors) is selected. In most cases, these are based on reversed-phase separations on a C18 column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point.

a) Selection of Column

The principle part of an HPLC system is the column. Changing a column will have a great effect on the resolution of analytes during method development. An appropriately selected column can produce a good chromatographic separation and it provides accurate and reliable analysis.[15]

Reversed-phase separation employs a polar eluent and a non-polar (hydrophobic) stationary phase. The hydrophobic layer (or phase) is bonded or coated onto rigid support that can endure the high pressure commonly used in HPLC. Until recently, about 80% of all HPLC methods specified silica-based stationary phases.

- C18 and C8 bonded phases are the best for initial rapid method development with typical sample types
- Choosing the most sample appropriate bonded phase and using special, targeted bonded phases, such as SB-Aq for polar, difficult to retain compounds can decrease method development time.
- Rapid resolution columns are needed to reduce the method of development time.
- Rapid resolution columns reduce both gradient and isocratic analysis time and permit high throughput rapid analysis [16].

b) Chromatographic Conditions

The selection of appropriate chromatographic conditions is the next step in the method development. This includes a selection of temperature, selection of the composition of the mobile phase, pH of mobile phase and flow rate etc. A decision of developing either an isocratic or a gradient method should be made at this point. In most cases, these are based on reversed-phase separations on a C18 column with UV detection [17].

c) Optimization of Mobile Phase

When samples contain ionizable compounds, the mobile phase pH can be one of the most important variables in the controls in the control of retention in a reversed-phase HPLC (RP- HPLC) separation. However, if it is not controlled properly, pH can be a source of many problems. Since most compounds analyzed by RP-HPLC contain one or more acidic or basic functional groups, most mobile phases require pH control. For this reason, buffers are widely used [18].

d) Column Temperature

The use of temperature in HPLC method development presents a challenge because it can have unpredictable effects on selectivity.

The use of elevated temperatures will: Reduce mobile phase viscosity and back-pressure. This can allow you to operate at higher flow rates or to use longer columns with smaller particle sizes. Reduce elution time. Improve method reproducibility (as opposed to operating at room temperature).

e) Selection of wavelength and detector

All listed molecules UV/Visible and FT-1R spectrums are required to select the UV detector nm for all molecules. FTIR spectral data is the main source for understanding the functional group activity.

C. Sample Preparation

The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually affect the separation so long as the volume of the sample loaded is small compared to the column volume. When large sample volumes are applied, the only effect may be an extra peak or two eluting in the void volume after sample injection. Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column. The aim of sample preparation is a sample aliquot that is relatively free of interferences, Will not damage the column, and Is compatible with the intended HPLC method; that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution.

D. Method Optimization

The experimental conditions should be optimized to get the desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

E. Method Validation

Definition: Analytical method validation is "A Documented evidence, which provides a high degree of assurance that a specific process will consistently produce, a product meeting its pre-determined specifications and quality attributes."

Figure 2: HPLC Equipment

VALIDATION METHOD AND CHARACTERISTICS

Parameters of analytical method validation:

1) Accuracy
2) Precision
    a) Repeatability
    b) Intermediate Precision
    c) Reproducibility
3) Specificity
4) Detection Limit
5) Quantitation limit
6) Linearity
7) Range
8) Stability
9) Robustness
10) Ruggedness
11) System Suitability

1) Accuracy

Accuracy of an analytical method may be defined as "The closeness of test results obtained by that method to the true value. This accuracy should be established across its range.

2) Precision

Definition: It expresses the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

3) Specificity

Definition: Specificity is the ability to assess the analyte unequivocally in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

ICH divides the term specificity into two separate categories:

Identification: to ensure the identity of an analyte.

Impurity Tests: to ensure that all the analytical procedures performed to allow an accurate statement of the content of impurities of an analyte, i.e., related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): to provide an exact result allows an accurate statement on the content or potency of the analyte in a sample. Analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation. It is not always possible to demonstrate that a single analytical procedure is specific for a particular analyte. Specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimized for the best selectivity.

4) Detection Limit (LOD) and quantitation limit (LOQ)

LOD of an analytical procedure is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated as an exact value, whereas LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. ICH guidelines describe three methods for determining LOD and LOQ that include:

Visual evaluation

It may be used for both non-instrumental and instrumental methods. The LOD and LOQ are determined by analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected or quantified with acceptable accuracy and precision, respectively.

Signal to noise ratio approach

This method can only be applied to analytical procedures that exhibit baseline noise. It is determined by comparing measured signals from samples of known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. An S/N ratio of 3:1 is considered acceptable for estimating LOD (with Relative Standard Deviation (RSD) $\leq$ 10%) LOQ, an S/N ratio of 10:1 is considered appropriate (with Relative Standard Deviation (RSD) $\leq$ 3%).

The LOD and LOQ may be expressed as:

LOD = 3.3 x σ/S

LOQ = 10 x σ/S

Where σ = the standard deviation of the response

S = the slope of the calibration curve of the analyte

5) Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. [31] Linearity is determined by a series of five to six injections of five or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

6) Range

The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy and linearity. The range is normally expressed in the same units as the test results (for example, percentage, parts per million) obtained by the analytical method.

- For Assay - 80 to 120% of test concentration
- Content uniformity - 70 to 130% of test concentration
- Dissolution - Q- 20% to 120%
- Impurities - reporting level- 120% of impurity specifications limit
- Assay & Impurities: Reporting level to 120% of assay-specific. Pm

7) Stability

Solution stability is the stability of standard and extracted sample solution (ready to inject) from the sample or matrix and analyzed as per specified method, and it should be stored properly in room temperature and refrigerated condition depending upon the stability of the sample and standard solution. The stability of standard and sample solution should be established at room temperature and refrigerated; if refrigerated before analyzing, it should be thawing to room temperature. A minimum of two preparation of standard and sample solution should be prepared and analyzed as per the specified method. The analyzed solutions are stored in necessary condition and the stability can be established for two days or solution stability can be established on an hourly basis depending upon the nature of the product.

8) Robustness

The robustness of an analytical procedure 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.

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers
- Temperature
- Flow rate

The factors chosen for all the drugs under investigation were the flow rate, mobile phase composition, pH of a mobile phase and using a different lot of LC column. The observation shall be summarized and critical parameters shall be listed out in the validation report. System suitability parameter must be within the limit of acceptance criteria as mentioned in the method.

9) Ruggedness

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 normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of influence on the test results of operational and environmental variables of the analytical method. For the determination of ruggedness, the degree of reproducibility of the test result is determined as a function of the assay variable. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness analytical method.

10) System Suitability

According to the USP, system suitability tests are an integral part of chromatographic methods. 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 is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (%RSD retention time and area of repetitive injection) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability "sample" that is a mixture of main components and expected by-products. See below **figure 3**.

ADVANTAGES

- Simultaneously Analysis
- High Resolution
- High Sensitivity
- Good repeatability
- Small sample size
- Moderate analysis condition
- Easy to fractionate the sample and purify
- The biggest advantage of method validation is that it builds a degree of confidence not only for the developer but also for the users.
- Although the validation exercise may appear costly and time-consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end. [6]

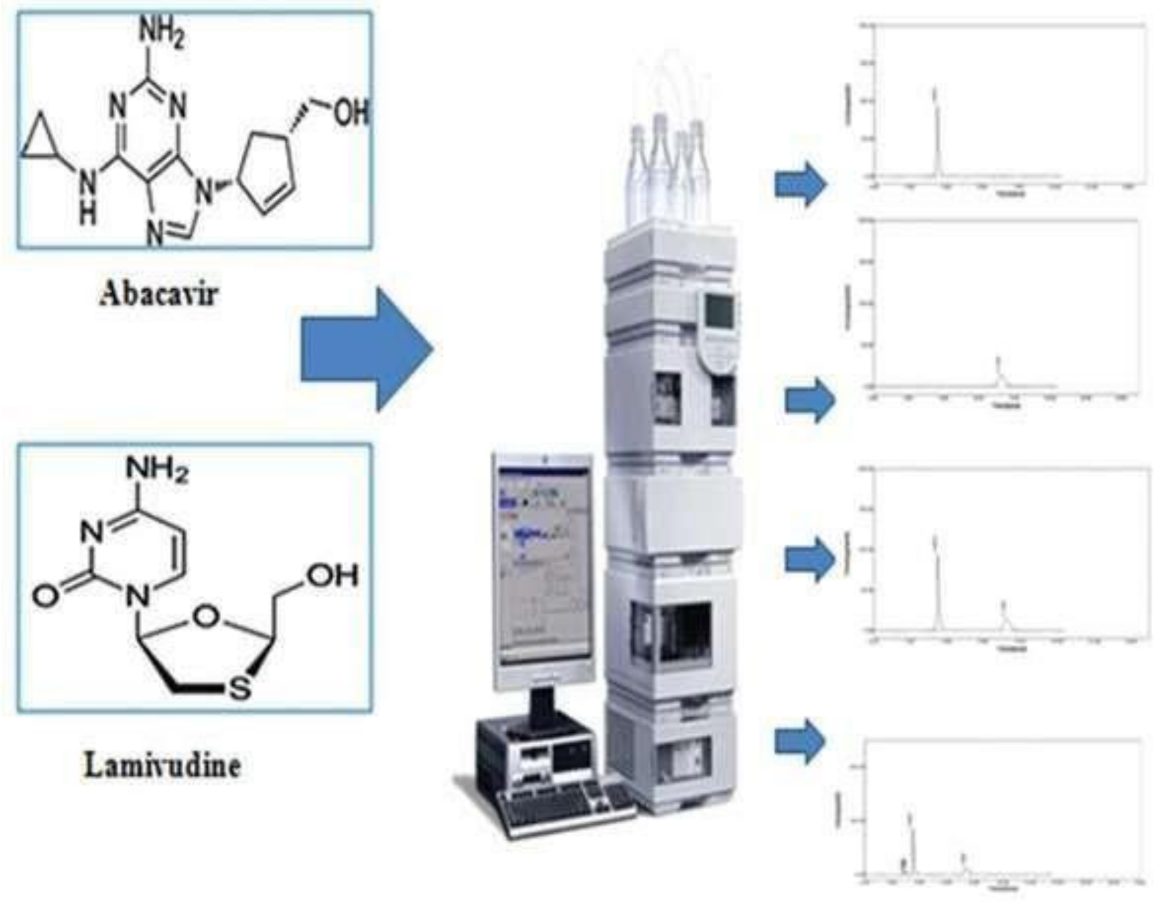

Figure 3: Development and validation of HPLC

APPLICATIONS

The HPLC has several applications in the fields of pharmacy, forensic, environment and clinical. It also helps in the separation and purification of the compound. [9-11]

- Pharmaceutical Applications: The pharmaceutical applications include controlling drug stability, dissolution studies and quality control.
- Environmental Applications: Monitoring of pollutants and detecting components of drinking water.
- Forensic Applications: Analysis of textile dyes, quantification of drugs and steroids in biological samples.
- Food and Flavour Applications: Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives.
- Clinical Applications: Detecting endogenous neuropeptides, analysis of biological samples like blood and urine.
- Qualitative analysis
- Checking the purity of a compound
- Presence of impurities
- Determination of the mixture of drugs

SUMMARY

Some drug can be determined by this, both HPLC & RP-HPLC Analytical method below:

Table 1: Analytical method through determine the drug

Sr. No.	Drug	Method
1.	MIRABEGRON	HPLC, RP-HPLC
2.	POTASSIUM GUAIACOL SULFONATE	HPLC
3.	CLOZAPINE	HPLC
4.	CRIZOTINIB	HPLC
5.	DICLOFENAC POTASSIUM	RP-HPLC
6.	ROSUVASTATIN	HPLC, RP-HPLC
7.	PALIPERIDONE	HPLC, RP-HPLC
8.	ABACAVIR	RP-HPLC
9.	LAMIVUDINE	RP-HPLC
10.	MICONAZOLE	HPLC
11.	EPTIFIBATIDE ACETATE	HPLC, RP-HPLC,
12.	ITRACONAZOL	RP-HPLC, HPLC
13.	SITAGLIPTIN PHOSPHATE	RP-HPLC
14.	ARTESUNATE	HPLC
15.	DIMETHOATE	

CONCLUSION

This review describes about RP-HPLC Technique. The method development and validation are continuous and interrelated processes that measure a parameter as intended and establish the performance limits of the measurement. The selection of Column, buffer, detector and wavelength and other conditions composition (organic and pH) plays a dramatic role in the separation selectivity. The advantages of the HPLC technique were high selectivity, sensitivity, economical, less time consuming and low limit of detection. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. The optimized method is validated with various parameters (e.g., specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

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ABBREVIATIONS

HPLC	: High Performance Liquid Chromatography
RP-HPLC	: Reverse Phase High Performance Liquid Chromatography
TLC	: Thin Layer Chromatography
M.P.	: Mobile Phase
S.P.	: Stationary Phase
V/V%	: Percentage Volume By Volume
C18	: Carbon 18
ABAC	: Abacavir
LAMI	: Lamivudine
LOD	: Detection Limit
LOQ	: Quantitation Limit
NM	: Nanometer