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A REVIEW ON : HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD DEVELOPMENT AND VALIDATION.

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ABSTRACT::

HPLC is the primary technique employed for the detection, separation, and quantification of pharmaceuticals. The development and validation of HPLC methodologies are essential components in the realms of novel drug discovery, development, and manufacturing, as well as in various studies involving both human and animal subjects. This review examines the numerous processes associated with the development and validation of HPLC techniques. The development of HPLC methods is influenced by factors such as the chemical structure of the compounds, the synthetic pathway, solubility, polarity, pH and pKa values, and the activity of functional groups. The HPLC method is applicable for analyzing a wide range of drugs within multicomponent dosage forms. To comply with Good Manufacturing Practice (GMP) standards, pharmaceutical companies should establish a comprehensive validation policy that outlines the procedures for validation. This article focuses primarily on the optimization of HPLC conditions.

Keywords: Pressure Liquid Chromatography, Chromatography, Method validation, Method devlopment.

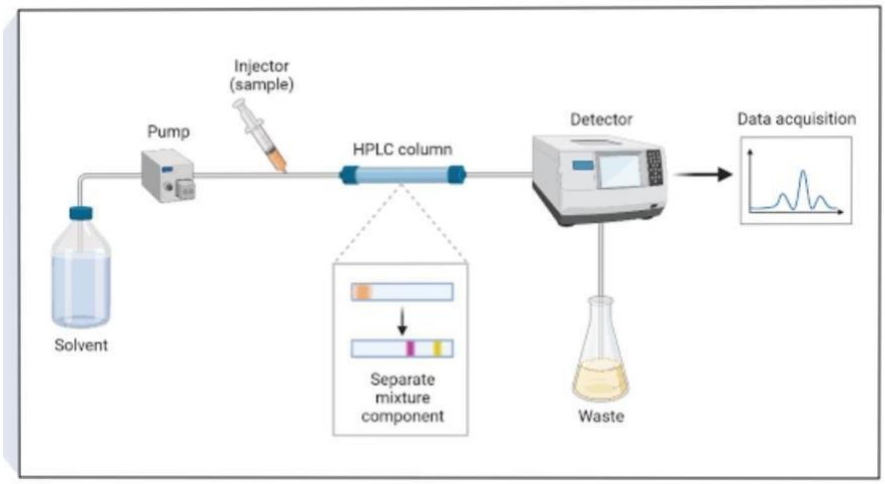

Figure 1: Diagram of HPLC.

INTRODUCTION:

High-performance liquid chromatography (HPLC) stands as one of the most precise analytical techniques available for the quantitative and qualitative analysis of drug products. This method typically involves a column filled with packing material (the stationary phase), a pump that propels the mobile phase(s) through the column, and a detector that measures the retention times of the molecules. A sample compound exhibiting a greater affinity for the stationary phase will move more slowly and cover a shorter distance compared to a compound with a lesser affinity, which will travel more quickly and over a longer distance. PLC has numerous advantages like

- Simultaneous Analysis
- High Resolution
- High Sensitivity
- Good repeatability

- Small sample size
- Moderate analysis condition

CLASSIFICATION/TYPES OF HPLC :

Liquid chromatography encompasses several techniques, categorized as follows:
I. Preparative HPLC and analytical HPLC, differentiated by the scale of operation.
II. Affinity chromatography, adsorption chromatography, and size exclusion chromatography, classified according to the separation principle.
III. Ion exchange chromatography and chiral phase chromatography, based on the underlying separation mechanisms.
IV. Gradient separation and isocratic separation, distinguished by the elution technique employed.
V. Normal phase chromatography and reverse-phase chromatography, categorized by their operational modes.[9]

1. **Size exclusion chromatography:**

SEC, also known as gel permeation or gel filtration chromatography, is a technique for separating particles based on their size. It is also capable of determining the tertiary and quaternary structures of proteins and amino acids. This method is commonly used to calculate polysaccharide molecular weight.

2. **Ion exchange chromatography:**

Retention in ion-exchange chromatography is based on the attraction of solute ions to charged sites. Bound to the stationary phase. Lons with the same charge are not allowed. This chromatography technique is widely used in water purification, ligand-exchange chromatography, protein ion- exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and other applications.[5.6]

3. **Bio-affinity chromatography:**

Separation based on reversible protein-ligand interactions. Ligands are an abio-affinity matrix, which keeps proteins that interact with the column-bound ligands in place. [5]

4. **Normal Phase Chromatography:**

In normal phase chromatography, the mobile phase is characterized as non-polar, whereas the stationary phase is polar. Consequently, polar analytes are retained by the stationary phase. The heightened polarity of the solute molecules enhances the adsorption capacity, leading to an extended elution time. This technique typically employs a stationary phase composed of chemically modified silica, such as cyanopropyl, aminopropyl, and diol. A standard column generally features an internal diameter of approximately 4.6 mm and a length that varies between 150 and 250 mm. When a mixture containing polar compounds is introduced into the column, these compounds adhere to the polar silica for a longer duration compared to their non-polar counterparts, resulting in the latter moving through the column at a faster rate.

5. **Reversed-Phase HPLC (RP-HPLC):**

In RP-HPLC, the stationary phase is non-polar, while the mobile phase is either polar or moderately polar. The principle of hydrophobic interaction is fundamental to this method. The non-polar stationary phase retains analytes that are relatively less polar within a mixture for a longer duration than those that exhibit significantly higher polarity. Consequently, the most polar component is eluted first.

HPLC method of development:

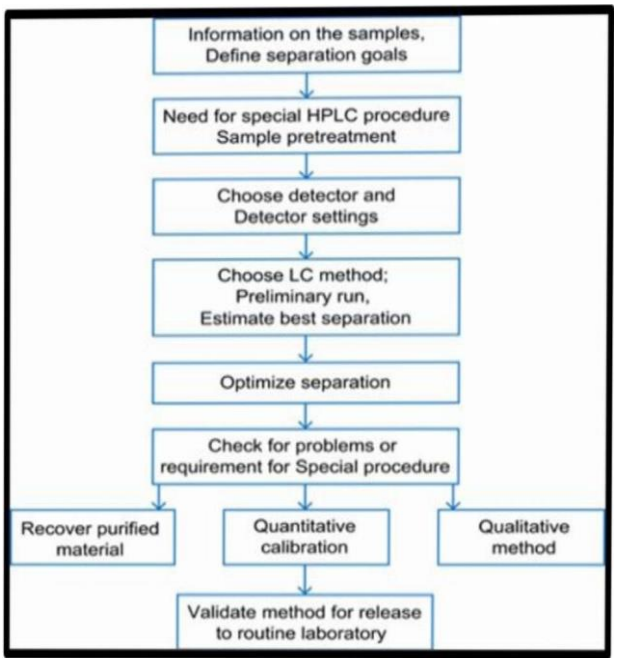

Figure 2: steps involved in method development of HPLC

Steps involve in method development are:

1. Understand the physicochemical properties of drug molecule.
2. Selection of Chromatographic conditions.
3. Preparation of sample solution for method development.
4. Method optimization.
5. Validation of method

Understanding the Physicochemical Properties of the drug molecule:

1. The comprehension of the physicochemical characteristics of a pharmaceutical compound is essential for method development. Initially, it is necessary to assess the physical attributes of the drug molecule, including solubility, polarity, pKa, and pH. Polarity is classified as a physical property, characterized by a polar covalent bond where one atom exhibits a stronger attraction for electrons than its counterpart.

The solubility of molecules can be understood through their polarity; for instance, polar solvents like water do not mix with nonpolar solvents such as benzene. Generally, substances with similar polarities tend to dissolve in one another, leading to the principle that "like dissolves like." The choice of diluents is determined by the solubility of the analyte. The pH value serves as a standard measure of a substance's acidity or basicity. Selecting the appropriate pH for ionizable analytes often results in well-defined and symmetrical peaks in high-performance liquid chromatography (HPLC). The pH is mathematically defined as the negative logarithm (base 10) of the hydrogen ion concentration.

pH = -log10[H3O+].

The acidity or basicity of a substance is most commonly characterized by its pH value. The appropriate selection of pH for ionizable analytes is crucial, as it often results in the formation of symmetrical and sharp peaks in High-Performance Liquid Chromatography (HPLC). In quantitative analysis, the presence of sharp and symmetrical peaks is essential for achieving low detection limits, minimizing relative standard deviations between injections, and ensuring predictable retention times.

2. Selection of chromatographic conditions:

In the initial phases of method development, a preliminary set of conditions, including the detector, column, and mobile phase, is established to produce the first "scouting" chromatograms of the sample. It is vital to consider the influence of pH on analyte retention, the type and concentration of buffer to be utilized, as well as the solubility of the organic modifier and its impact on detection during the development of reversed-phase chromatography (RPC) methods for ionic analytes.

3. Buffer selection:

The selection of a buffer is primarily determined by the target pH. For reversed-phase chromatography utilizing silica-based packing, the typical pH range is between 2 and 8. It is crucial that the buffer possesses a pKa that is close to the desired pH, as buffers are most effective at maintaining pH levels near their pKa. A general guideline is to select a buffer with a pKa value that is less than 2 units from the intended pH of the mobile phase.

1.General Considerations for Buffer Selection

Phosphate exhibits greater solubility in methanol/water compared to acetonitrile/water or THF/water mixtures. Certain salt buffers possess hygroscopic properties, which may lead to alterations in chromatographic performance. Trifluoroacetic acid is subject to degradation over time and is a volatile compound that absorbs at low UV wavelengths. Additionally, microbial growth can rapidly occur in buffered mobile phases. When the pH exceeds 7, phosphate buffers can enhance the dissolution of silica, thereby significantly shortening the lifespan of silica-based HPLC columns. It is advisable to utilize organic buffers at pH levels above 7 whenever feasible. Ammonium bicarbonate buffers are generally susceptible to pH fluctuations and maintain stability for only 24 to 48 hours. Due to the release of carbon dioxide, the pH of this mobile phase tends to shift towards a more basic level. Following the preparation of buffers, it is essential to filter them using a 0.2-micron filter. Degassing of mobile phases is also necessary.

2.Selection of Detectors

The detector serves as a vital element in HPLC systems. The choice of detector is influenced by the chemical composition of the samples under investigation, potential interferences, required detection limits, availability of detectors, and associated costs. The selected detector must provide the high sensitivity necessary for routine UV-based applications, including the identification of low-level impurities and quantitative analyses. The Photodiode Array (PDA) Detector offers sophisticated optical detection capabilities for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optical innovations ensure high chromatographic and spectral sensitivity.

3.Selection of Mobile Phase

The mobile phase influences resolution, selectivity, and efficiency. The composition of the mobile phase (or the strength of the solvent) is critical in RP-HPLC separation. Acetonitrile (CAN), methanol (Me OH), and
tetrahydrofuran (THF) are regularly used solvents in RP HPLC, with UV cut-offs of 190, 205, and 212nm, respectively.

Mobile Phase Reservoirs

Inert container with inert lines leading to the pump are required. Reservoir filters (2-10 mm) at reservoir end of solvent delivery lines

- Degassed solvent
- Vacuum filtration

- Spurge with inert gas (N_2 or He)
- Ultrasonic under vacuum.

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography. The mobile phase consists of an aqueous buffer and a non-UV active water miscible organic Solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed Will affect the analysis of the drug molecule.

METHOD OPTIMISATION :

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. After adequate selectivity has been attained, this is utilized to discover the optimal balance between resolution and analysis time. Column dimensions, column-packing particle size, and flow rate among the parameters at play. These parameters are
changeable without impacting the capacity factor or selectivity.

METHOD OF VALIDATION :

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use
Analytical methods need to be validated or revalidated. Before their introduction into routine use;
Whenever the conditions change for which, the method has been validated Whenever the method is changed.

1. Accuracy

The accuracy of an analytical procedure expresses the degree of agreement between the value acknowledged as a conventional true value or an approved reference value and the value discovered. The closeness of a measured value
to the true or accepted value is defined as accuracy. In practice, accuracy denotes the difference between the mean value discovered and the genuine value.

2. Precision

An analytical procedure's precision expresses the degree. Of agreement (degree of scattering) between a series of Measurements acquired from multiple samplings of the same homogenous sample under the required conditions. An analytical procedure's precision expresses the degree of
agreement (degree of scattering) between a series of measurements acquired from multiple samplings of the same homogenous sample under the required conditions. Precision is classified into three categories: repeatability,
intermediate precision, and reproducibility. An analytical procedure's precision is determined by assaying a sufficient number of aliquots of a
homogeneous. Sample to derive statistically accurate estimates of standard deviation or relative standard deviation".

3. Linearity

The capacity of an analytical process to produce test results that are directly proportional to the concentration. Of analyte in the sample (within a certain range) is referred to as linearity. If the method is linear, the test findings are proportional to the concentration of analyte in samples. Within a given range, either directly or through a well-defined mathematical transformation.

4. Specificity

Specificity is the ability to assess the analyte unequivocally in the presence of components that are expected to be present. Impurities, degradants, matrices, and so on are examples of these. An individual analytical method's lack of specificity may be compensated for by another supporting analytical procedure."

5. Robustness

The capacity of an analytical method to remain unaffected by minor but deliberate adjustments in method parameters (e.g., pH, mobile phase
composition, temperature, and instrumental settings) is characterized as robustness, and it indicates its reliability during typical operation.

6. Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

7. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

CONCLUSION :

This article provides an overview of the RP-HPLC Technique method development and validation. Method development and validation are continuous and interconnected activities that measure a parameter as
intended and determine the measurement's performance limits. The makeup of the buffer and mobile phase (organic and pH) has a significant impact on separation selectivity.
The definitions of method validation parameters are well explained. Validation as per ICH guidelines. The knowledge of the pKa, pH and solubility of the primary compound is of utmost importance prior to the HPLC method development. Final optimization can be performed by changing the
temperature, gradient slope, and flow rate as well as the type and concentration of mobile-phase modifiers.

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