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Review Article

A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION

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High-Performance Liquid Chromatography (HPLC) is an advanced analytical technique used for separating, identifying, and quantifying components in complex mixtures. It operates based on the principle of differential interaction of analytes with a stationary phase and a liquid mobile phase under high pressure. These interactions enable precise separation of compounds based on their chemical or physical properties such as polarity, molecular size, or charge. The HPLC method can be used to analyze the majority of drugs in multicomponent dosage forms. HPLC method development and validation are critical in new drug discovery, development, and manufacturing, as well as a variety of other human and animal studies. Validation of analytical methods is required during drug development and manufacturing to ensure that these analytical methods are fit for their intended purpose. To meet GMP requirements, pharmaceutical industries should have an overall validation policy that details how validation will be carried out. This article is primarily concerned with the optimization of HPLC conditions.

Keywords: High-Pressure Liquid Chromatography (HPLC), Method validation, Method development

INTRODUCTION

HPLC is renowned for its versatility and efficiency, making it an essential tool in various fields, including pharmaceuticals, biotechnology, environmental science, and food analysis. It is widely employed in drug formulation, quality control, biomolecular analysis, and detecting contaminants in environmental samples. High-performance liquid chromatography (HPLC) stands as a powerful analytical tool in modern chemistry. It excels at identifying, measuring, and separating components within liquid-dissolved samples. Widely employed in pharmacological product analysis, HPLC is prized for its precision in both quantitative and qualitative assessments, contributing significantly to advancements in analytical chemistry.[1] In high-performance liquid chromatography (HPLC), a sample solution (stationary phase) is injected into a porous column. A liquid (mobile phase) is then pumped through the column at high pressure. Components in the sample exhibit different migration rates through the column due to partitioning between stationary and mobile phases. This leads to elution at distinct times, allowing separation. HPLC's precision arises from nuanced component behaviors during partitioning, offering a robust method for

analyzing diverse samples in fields like pharmaceuticals and analytical chemistry.[2]

In high-performance liquid chromatography, a compound with lower affinity for the stationary phase travels faster and covers a longer distance, while a compound with higher affinity moves slower and covers a shorter distance. This differential migration facilitates effective separation and analysis of sample components. High-performance liquid chromatography (HPLC) proves invaluable in pharmaceutical analysis, efficiently isolating and quantifying major medications, reaction impurities, synthesis intermediates, and degradants. As a preeminent analytical tool, HPLC excels in identifying, measuring, and separating diverse sample components soluble in liquid. Its precision is paramount for both quantitative and qualitative drug product analysis, playing a pivotal role in determining drug product stability. By offering a meticulous approach to characterizing pharmaceutical samples, HPLC stands as an indispensable technique in ensuring the quality and safety of medicinal formulations in the field of analytical chemistry.[3]

Principle of HPLC

The principle of High-Performance Liquid Chromatography (HPLC) is based on the differential partitioning of analytes between a stationary phase and a mobile phase under high pressure. When a mixture of compounds is introduced into the HPLC system, it is transported by the liquid mobile phase through a column containing the stationary phase.[4]

Types of High-Performance Liquid Chromatography (HPLC)[5-6]

HPLC can be classified based on the nature of the stationary phase, the separation mechanism, or the type of mobile phase used. Below are the main types of HPLC:

❖ **Normal-Phase HPLC (NP-HPLC)**

Principle: Separation is based on polarity.

Stationary Phase: Polar (e.g., silica).

Mobile Phase: Non-polar solvents (e.g., hexane, chloroform).

Applications: Used for separating polar compounds, such as vitamins, amino acids, and pharmaceuticals.

❖ **Reverse-Phase HPLC (RP-HPLC)**

Principle: Separation is based on hydrophobic (non-polar) interactions.

Stationary Phase: Non-polar (e.g., C18, C8, or phenyl groups bonded to silica).

Mobile Phase: Polar solvents (e.g., water with acetonitrile or methanol).

Applications: Widely used for separating organic compounds, peptides, and proteins.

❖ **Ion-Exchange HPLC (IE-HPLC)**

Principle: Separation is based on the ionic interactions between charged analytes and the oppositely charged stationary phase.

Stationary Phase: Ionic resins (cation or anion exchangers).

Mobile Phase: Aqueous buffers with varying pH or ionic strength.

Applications: Used for separating charged species like amino acids, proteins, and nucleotides.

❖ **Size-Exclusion HPLC (SEC-HPLC)**

Principle: Separation is based on molecular size, with larger molecules eluting first.

Stationary Phase: Porous beads (e.g., cross-linked polymers or silica).

Mobile Phase: Typically aqueous or organic, depending on the analyte.

Applications: Used for molecular weight determination and separation of biomolecules like proteins, polymers, and oligosaccharides.

❖ **Affinity HPLC (A-HPLC)**

Principle: Separation is based on specific interactions between the analyte and a ligand attached to the stationary phase.

Stationary Phase: Ligand-immobilized resin or matrix.

Mobile Phase: Buffer systems tailored to enhance or disrupt binding.

Applications: Used for purifying biomolecules like enzymes, antibodies, and nucleotides.

❖ **Chiral HPLC**

Principle: Separation is based on stereochemical differences (chirality) of enantiomers.

Stationary Phase: Chiral selectors, such as modified polysaccharides or proteins.

Mobile Phase: Varies depending on the analyte (polar or non-polar solvents).

Applications: Used for separating enantiomers in pharmaceuticals and natural products.

HPLC Method Development:

Analytical method development and validation are critical steps in the discovery, development, and manufacturing of pharmaceuticals. These techniques are used to ensure the identity, purity, potency, and performance of pharmaceutical products. When developing methods, there are numerous factors to consider. In the case of UV detection, they first gather information about the analyte's physiochemical properties (pKa, log P, solubility) and determine which mode of detection would be suitable for analysis. The majority of the analytical development effort is spent validating an HPLC–method for indicating stability. The purpose of the HPLC method is to separate and quantify the main active drug, any reaction impurities, all available synthetic intermediates, and any degradants.[7-9]

The following is a step in HPLC method development

Establishing an analytical strategy involves selecting chromatographic settings and understanding the physicochemical properties of drug molecules. The subsequent steps include sample preparation, refining the method, and validating it to ensure accuracy and reliability in pharmaceutical analysis.[10-12]

Recognizing the Physicochemical Properties of Drug Molecules

When developing an analytical method for a medicinal molecule, understanding its physicochemical characteristics is essential. Initial considerations include the drug molecule's pH, polarity, solubility, and pKa. Polarity, a key physical characteristic, guides the choice of solvent and mobile phase composition. Molecular solubility, linked to polarity, adheres to the principle "like dissolves like." Selection of mobile phase or diluents is influenced by analyte solubility, ensuring compatibility. Analytes must not react with components and be soluble. Parameters like pH and pKa are critical in High-Performance Liquid Chromatography (HPLC) method development, influencing solvent selection and overall method success. pH equals $-\log_{10}[H_3O^+]$

Choosing Chromatographic Conditions

During the initial development of a method, a set of conditions, including the detector, column, and mobile phase, is chosen to generate the sample's initial "scouting" chromatograms. Commonly, reversed- phase separations using a C18 column with UV detection are employed. At this stage, the decision arises on whether to develop a gradient method or opt for an isocratic approach, each offering distinct advantages depending on the specific separation requirements and characteristics of the analytes in the sample.[13]

Selection of Column

The column is the cornerstone of a chromatograph, playing a pivotal role in achieving reliable and accurate analyses. A well-chosen column ensures good chromatographic separation, contributing to trustworthy results. Conversely, improper column selection can lead to inadequate and confusing separations, rendering results invalid or challenging to interpret. In High-Performance Liquid Chromatography (HPLC) systems, the column is central, and altering it significantly influences analyte resolution during method development. Considerations like particle size, retention capacity, stationary phase chemistry, and column dimensions are crucial for selecting the ideal column tailored to a specific analytical application.

Selection of Chromatographic mode

Chromatographic modes are dictated by the analyte's polarity and molecular weight. Reversed-phase chromatography (RPC) takes precedence in case studies, especially for small organic compounds. RPC is extensively utilized for separating ionizable substances, such as acids and bases, employing ion-pairing reagents or buffered mobile phases to prevent analyte ionization.[14]

Optimization of Mobile phase[15-16]

- **Buffer Selection**

Various buffers, including acetate, sodium phosphate, and potassium phosphate, were

evaluated based on overall chromatographic performance and system suitability criteria. Through a series of experiments, potassium dihydrogen phosphate emerged as the most suitable buffer for successful separation of all peaks. Test concentrations of 0.02 M, 0.05 M, and 0.1 M were examined. Interestingly, altering the buffer concentration did not significantly impact the elution pattern and resolution, although the 0.05 M concentration enhanced the sensitivity of the technique without substantial changes in the separation characteristics.

❖ Effect of pH

For ionizable analytes, determining the appropriate mobile-phase pH is crucial, guided by the analyte's pKa. This ensures that the target analyte is either in a neutral or ionized form. The ability to adjust the pH of the mobile phase is a powerful tool in the chromatographer's toolkit. This capability allows simultaneous modifications to retention and selectivity, providing a strategic means to optimize separation conditions, particularly for critical pairs of components in the sample. pH adjustment plays a vital role in tailoring chromatographic conditions to achieve desired separation outcomes.

❖ Effect of organic modifier

Selecting the organic modifier for reverse- phase HPLC is typically straightforward, with acetonitrile and methanol being the most popular choices (occasionally THF). Achieving optimal elution for every component in complex multicomponent samples under isocratic conditions, where the solvent strength remains constant, can be challenging. Hence, gradient elution is often employed, allowing for varying solvent compositions between k (retention factor) 1 and 10. This dynamic approach enhances separation efficiency and is particularly useful in handling intricate mixtures in high-performance liquid chromatography.

Selection of detector and wavelength

After chromatographic separation, the target analyte is identified using appropriate detectors. Common detectors in liquid chromatography (LC) include UV, fluorescence, electrochemical, refractive index (RI), and mass spectrometry (MS). The choice of detector is influenced by the nature of the sample and the analytical objectives. For example, in multicomponent analysis, the absorption spectra may shift to longer or shorter wavelengths than those of the parent chemical, influencing the choice of a suitable detector for accurate and selective identification. Detector selection plays a crucial role in achieving the desired sensitivity and specificity in LC analysis. In UV detection, the spectra of the target analyte and contaminants must be acquired at various levels, superimposed, and then normalized. Selecting a wavelength is crucial to ensure a sufficient

response for the majority of analytes, allowing for accurate and reliable detection in liquid chromatography. The careful consideration of UV spectra at different levels ensures that the analytical method is sensitive to all relevant components in the sample, contributing to the precision and reliability of the analysis.[17]

Creating an analytic approach

The initial stage in developing an analytical method for Reverse Phase High- Performance Liquid Chromatography (RP- HPLC) involves selecting various chromatographic parameters such as the mobile phase, column, mobile phase flow rate, and mobile phase pH. Through trials, each characteristic is optimized and then compared against system suitability parameters. Typical parameters include a retention time of more than five minutes, a theoretical plate count exceeding 2000, a tailing factor less than two, a resolution greater than five, and a percent Relative Standard Deviation (R.S.D.) of the area of analyte peaks in standard chromatograms not exceeding two percent. These parameters ensure the reliability and precision of the RP-HPLC method.[18]

Sample preparation

Sample preparation is a crucial step in High-Performance Liquid Chromatography (HPLC) analysis, ensuring a homogeneous and repeatable solution for injection onto the column. The goal of sample preparation is to create an interference-free aliquot that is column-compatible and compatible with the desired HPLC method. This involves selecting a sample solvent that dissolves in the mobile phase without compromising retention or resolution. The initial steps in sample preparation involve sample collection and injection into the HPLC column, laying the foundation for accurate and reliable chromatographic analysis.[18]

Method optimization

Identify the weaknesses in the approach and employ experimental design to enhance it. Assess the impact of the approach on various samples, equipment configurations, and environmental factors. This iterative process helps refine the methodology, ensuring robustness, reliability, and applicability across diverse conditions in High-Performance Liquid Chromatography (HPLC) analysis.

Validation

Validation is the systematic process of assessing and providing objective evidence that specific requirements for a particular intended use are met. It involves evaluating a method's performance and demonstrating its capability to meet specific criteria. Essentially, validation provides a thorough understanding of what your technique can reliably produce, particularly when dealing with low doses or challenging conditions in analytical methods like High-Performance Liquid Chromatography (HPLC).[19-20]

The following are typical parameters recommended by the FDA, USP, and ICH.[21-22]

- Specificity
- Linearity & Range
- Precision
  - Method precision (Repeatability)
  - Intermediate precision (Reproducibility)
- Accuracy (Recovery)
- Solution stability
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)
- Robustness
- Range
- System suitability

CONCLUSION:

The development of analytical methods for drug identification, purity evaluation, and quantification has received a lot of attention in the field of pharmaceutical analysis in recent years. This review provides a general overview of HPLC method development and validation. A general and very simple approach to developing HPLC methods for compound separation was discussed. Before developing an HPLC method, it is critical to understand the physicochemical properties of the primary compound. The composition of the buffer and mobile phase (organic and pH) has a significant impact on separation selectivity. Finally, the gradient slope, temperature, and flow rate, as well as the type and concentration of mobile-phase modifiers, can be optimized. The optimized method is validated using various parameters (e.g., specificity, precision, accuracy, detection limit, linearity, and so on) following ICH guidelines.

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