SENNOS

Journal of Drug Delivery and Biotherapeutics

Journal homepage: https://sennosbiotech.com/JDDB/1

Review Article

Advances in High-Performance Liquid Chromatography (HPLC) Method Development and Validation: A Comprehensive Review

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ARTICLEINFO

ABSTRACT

High-Performance Liquid Chromatography (HPLC) stands as the cornerstone technique for the detection, separation, and quantification of drugs in various matrices. Method optimization necessitates a thorough investigation of multiple chromatographic parameters, encompassing sample pretreatment, mobile phase composition, column selection, and detector configuration. This article aims to comprehensively address the processes of method development, optimization, and validation within the realm of HPLC. The inherent advantages of HPLC, including its rapidity, specificity, accuracy, precision, and automation feasibility, render it an indispensable tool for analyzing a wide spectrum of drugs in complex multi-component dosage forms.

Furthermore, HPLC method development and validation hold paramount importance across the spectrum of drug discovery, development, and manufacturing, as well as in diverse human and animal studies. The validation of analytical methods during drug development and manufacturing is imperative to ensure their fitness for the intended purpose. Adhering to Good Manufacturing Practice (GMP) requirements, pharmaceutical industries must establish comprehensive validation policies outlining the validation procedures. This article primarily delves into the optimization of HPLC conditions, emphasizing the critical role of method refinement in ensuring robust and reliable analytical methods.

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

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Received date: 15-May-2024 Revised date: 10-Jun-2024, Accepted date: 23-Jun-2024

Crossref DOI: https://doi.org/10.61920/jddb.v1i02.138

1.Introduction

High-Performance Liquid Chromatography (HPLC) stands as the cornerstone technique in pharmaceutical analysis, offering unparalleled capabilities in the detection, separation, and quantification of drugs across a diverse range of matrices. Its versatility and precision make it indispensable in both research and industrial settings, where accurate and reliable analytical methods are essential for drug development, formulation, and quality control [1].

The optimization of HPLC methods is a multifaceted process that involves meticulous scrutiny of various chromatographic parameters. These parameters, including sample pretreatment, mobile phase composition, column selection, and detector configuration, play pivotal roles in determining the efficacy and reliability of the analytical method. By systematically investigating and fine-tuning these parameters, analysts can enhance the sensitivity, selectivity, and efficiency of HPLC assays, thereby facilitating the accurate quantification of drug compounds in complex matrices.

In addition to method optimization, the validation of analytical methods is a critical aspect of pharmaceutical analysis, particularly in the context of drug development and manufacturing. Regulatory agencies such as the FDA and EMA mandate the validation of analytical methods to ensure their suitability for intended use and to uphold the quality and safety standards of pharmaceutical products. Validation encompasses a comprehensive assessment of method performance characteristics, including specificity, accuracy, precision, linearity, and robustness, among others.

Furthermore, compliance with Good Manufacturing Practice (GMP) guidelines necessitates the establishment of robust validation policies by pharmaceutical industries. These policies outline the procedures and criteria for method validation, ensuring consistency, reproducibility, and regulatory compliance throughout the drug development and manufacturing process [2].

Considering the critical importance of HPLC method development and validation in pharmaceutical analysis, this review aims to provide a comprehensive overview of the methodologies, strategies, and best practices involved in optimizing and validating HPLC methods. Through a thorough examination of the latest advancements and industry standards, this review seeks to elucidate the key considerations and challenges encountered in the development, optimization, and validation of HPLC methods, thereby offering valuable insights for researchers, analysts, and industry professionals engaged in pharmaceutical analysis [3].

2. HPLC principle

The principle of High-Performance Liquid Chromatography (HPLC) is founded on the equilibrium distribution of analytes between two phases: a stationary phase and a mobile phase, commonly housed within a column packed with stationary phase material. As analytes traverse through the column, they interact with the stationary phase based on their chemical properties, leading to differential retention and ultimately separation.

The movement of analytes through the stationary phase is governed by factors such as polarity, size, and affinity for the stationary phase. Analytes with stronger interactions with the stationary phase will exhibit slower movement, while those with weaker interactions will elute more rapidly. This differential retention results in the separation of analytes according to their chemical characteristics, facilitating precise quantification and identification [4].

HPLC's versatility stems from its ability to accommodate a wide range of stationary phases and mobile phase compositions, allowing for tailored separations to suit specific analytical requirements. By optimizing parameters such as column type, mobile phase composition, and flow rate, analysts can achieve optimal separation efficiency and resolution.

The applications of HPLC are extensive, spanning various industries including pharmaceuticals, chemicals, food and beverages, environmental analysis, and more. Its capability to separate and quantify diverse compounds with high sensitivity and accuracy has made it an indispensable tool in analytical chemistry [5].

In pharmaceutical and chemical industries, HPLC plays a crucial role in quality control, drug development, and regulatory compliance. It enables the precise quantification of active pharmaceutical ingredients (APIs), impurities, and degradation products in pharmaceutical formulations, ensuring product safety, efficacy, and consistency.

Overall, the principle of HPLC, based on the equilibrium distribution of analytes between stationary and mobile phases, underpins its efficacy as a versatile and indispensable technique in analytical chemistry, with widespread applications across diverse industries [6].

3. Types of HPLC

High-Performance Liquid Chromatography (HPLC) encompasses several types, each tailored to specific analytical needs and applications. One such type is Normal Phase Chromatography (NPC), where the stationary phase is polar, typically composed of materials like silica, while the mobile phase is non-polar, often comprising organic solvents. NPC separates analytes based on polarity differences, with polar compounds interacting more strongly with the stationary phase and eluting later. In contrast, Reverse Phase Chromatography (RPC) employs a non-polar stationary phase, such as C18 or C8, and a polar mobile phase, like water or an aqueous buffer. Analytes are separated based on hydrophobic interactions with the stationary phase, with more hydrophobic compounds eluting later. RPC finds extensive use in separating polar and moderately polar compounds, including pharmaceuticals, peptides, and proteins. Another important type is Ion-Exchange Chromatography (IEC), which separates analytes according to their charge differences. In IEC, the stationary phase contains charged functional groups, such as ion-exchange resins, while the mobile phase consists of an electrolyte solution. Analytes with opposite charges interact with the stationary phase, leading to their separation based on charge characteristics [7].

3. HPLC classification

HPLC classification encompasses several dimensions, each delineating different aspects of the technique's operation and application:

Scale of Operation: HPLC can be categorized into analytical and preparatory scales based on the quantity of analyte processed. Analytical HPLC is employed for routine analysis and quantification of samples, typically at lower volumes, while preparatory HPLC handles larger sample quantities for purification or isolation purposes [8].

Chromatographic Techniques: HPLC encompasses various chromatographic techniques, including size exclusion, affinity, and adsorption chromatography, each tailored to different analyte properties and separation objectives. Size exclusion chromatography separates analytes based on size, while affinity chromatography relies on specific interactions between analytes and ligands immobilized on the stationary phase. Adsorption chromatography separates analytes based on their affinity for the stationary phase surface [9].

Principle of Separation: HPLC techniques such as chiral phase and ion exchange chromatography are categorized based on the underlying principle of separation. Chiral phase chromatography resolves enantiomers by utilizing chiral stationary phases, while ion exchange chromatography separates analytes based on their ionic interactions with the stationary phase [10].

Elution Technique: Isocratic and gradient separation methods distinguish chromatography based on the elution technique employed. Isocratic chromatography maintains a constant mobile phase composition throughout the analysis, while gradient chromatography utilizes varying mobile phase compositions to elute analytes based on their differing interactions with the stationary phase [11].

Modes of Operation: HPLC operates in normal and reverse phases, determined by the polarity of the stationary phase relative to the mobile phase. In normal phase chromatography, the stationary phase is polar, while the mobile phase is non-polar, whereas in reverse phase chromatography, the stationary phase is non-polar, and the mobile phase is polar [12].

3.1 Size Exclusion Chromatography (SEC)

Size Exclusion Chromatography (SEC), also referred to as gel permeation or gel filtration chromatography, operates on the principle of separating particles according to their size. Unlike other chromatographic techniques that rely on interactions between analytes and the stationary phase, SEC separates molecules solely based on their size. Larger molecules elute earlier, while smaller ones are retained longer within the stationary phase matrix. SEC finds extensive utility in elucidating the quaternary and tertiary structures of amino acids and proteins, crucial for understanding their functional properties and biological activities. Moreover, SEC is widely employed for determining the molecular weight of polysaccharides, offering valuable insights into their structural characteristics and polymerization states in diverse scientific and analytical contexts. Its versatility and accuracy make SEC an indispensable tool in various fields, including biochemistry, biotechnology, pharmaceuticals, and polymer science, where precise

characterization of macromolecules is paramount [13].

3.2 Ion exchange chromatography

Ion exchange chromatography exploits the interactions between solute ions and charged sites on the stationary phase, selectively retaining or eluting ions based on their electrostatic interactions. This technique is pivotal in various applications, including water purification, protein ion-exchange chromatography, ligand-exchange chromatography, and high-pH anion-exchange chromatography of carbohydrates and oligosaccharides. By leveraging the charged characteristics of different substances, ion exchange chromatography enables precise separation, purification, and analysis across diverse industries and disciplines, ranging from biochemistry and biotechnology to environmental science and pharmaceuticals, making it an indispensable tool for research, development, and quality control purposes [14].

3.3 Bio-affinity chromatography

Bio-affinity chromatography, a subset of affinity chromatography, operates on the principle of reversible ligand-protein interactions to achieve separation. In this method, proteins within a sample selectively bind to column-bound ligands, which are immobilized onto a solid support within a bio-affinity matrix. Through covalent attachment, the ligands establish specific interactions with target proteins, facilitating their immobilization while non-target components pass through the column. This selective binding mechanism enables the precise separation and purification of target proteins based on their specific affinity for the immobilized ligands. Bio-affinity chromatography finds widespread utility in biochemistry and protein purification processes, offering a valuable tool for isolating and characterizing biomolecules with high specificity and efficiency [15].

3.4 Normal phase chromatography

In normal phase chromatography, the stationary phase exhibits polarity, contrasting with the non-polar mobile phase. This configuration results in the retention of polar analytes by the stationary phase, wherein higher polarity in solute molecules correlates with longer elution times and increased adsorption capacity. Chemically modified silica, such as cyanopropyl, aminopropyl, and diol, commonly serves as the stationary phase in normal phase chromatography. Typical columns utilized measure between 150-250 mm in length with an internal diameter of approximately 4.6 mm. As the sample mixture traverses the column, polar compounds adhere more strongly to the polar silica, leading to slower elution, while non-polar compounds pass through more rapidly. This differential retention facilitates the separation of analytes based on their polarity characteristics, making normal phase chromatography a valuable technique in analytical chemistry and chromatographic applications [16].

3.5 RP-HPLC (Reversed-Phase HPLC)

In Reverse Phase High-Performance Liquid Chromatography (RP-HPLC), the mobile phase exhibits polarity, while the stationary phase is non-polar, constituting a reversal of the typical chromatographic configuration. This technique capitalizes on hydrophobic interactions as its separation principle, whereby less polar analytes within a mixture adhere more strongly to the non-

polar stationary phase, resulting in longer retention times. Conversely, the most polar component elutes first due to weaker interactions with the stationary phase. RP-HPLC is renowned for its capability to effectively separate compounds based on their hydrophobicity, rendering it indispensable in various fields such as pharmaceuticals, biochemistry, and environmental science. Its versatility and efficiency make RP-HPLC a cornerstone technique for analytical and preparative chromatography applications, facilitating precise and reliable analysis of diverse analytes across a broad spectrum of samples [17].

4. Instrumentation of High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) relies on sophisticated instrumentation to achieve precise analytical separations. The process involves the high-pressure flow of liquid through a column packed with a stationary phase, which can be either liquid (L) or solid (LSC/LLC). As compounds are injected into the system, they segregate and interact differently with the stationary phase, leading to separation based on their chemical properties. Detection of eluted compounds occurs electronically as they exit the column. Although HPLC is generally considered less flexible than Gas Chromatography (GC), it compensates with a diverse array of mobile and stationary phases, enhancing its versatility for analyzing compounds in various fields such as pharmaceuticals, environmental analysis, and biochemistry. The precise control over parameters and the ability to tailor conditions to specific analytes make HPLC a cornerstone technique in analytical chemistry, offering reliable and accurate results crucial for research, quality control, and regulatory compliance in numerous industries [18].

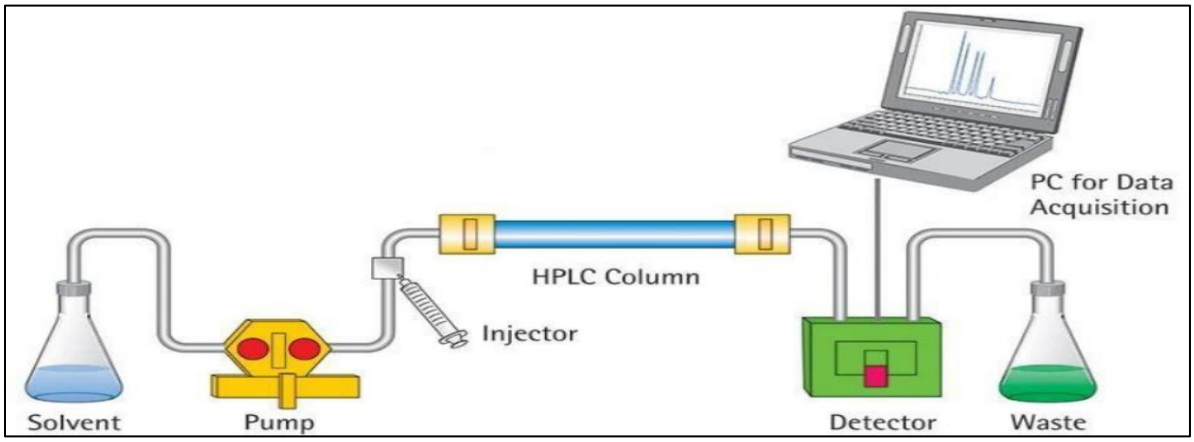

Fig. 1: Instrumentation of High-Performance Liquid Chromatography (HPLC)

6. HPLC method development

It plays a pivotal role in pharmaceutical research, development, and production by ensuring the identification, purity, potency, and effectiveness of pharmaceutical products. This process involves meticulous consideration of various factors, including physicochemical characteristics such as pKa, log P, and solubility, which inform the selection of the appropriate detection mode, particularly in UV detection. Validation of an HPLC method for stability indication constitutes a significant aspect of analytical development, focusing on the separation and quantification of the primary active ingredient, reaction impurities, synthetic intermediates, and degradants. This validation process ensures the reliability and accuracy of the analytical process in pharmaceutical quality control, thereby safeguarding the safety and efficacy of pharmaceutical products throughout their lifecycle [18].

6.1 Recognizing the Physicochemical Properties of Drug Molecules

When embarking on the development of an analytical method for a medicinal molecule, a thorough understanding of its physicochemical properties is imperative. Fundamental considerations encompass aspects such as the molecule's pH, polarity, solubility, and pKa. Polarity, a fundamental characteristic, dictates the selection of solvent and mobile phase composition. Molecular solubility, closely linked to polarity, adheres to the principle of "like dissolves like." Consequently, the choice of mobile phase or diluents is guided by analyte solubility to ensure compatibility. Furthermore, analytes must exhibit solubility and refrain from reacting with components. Parameters such as pH and pKa hold paramount importance in High-Performance Liquid Chromatography (HPLC) method development, significantly influencing solvent selection and overall method success. For instance, achieving sharp and symmetrical peaks in HPLC often entails optimizing the pH for ionizable analytes. Such peaks are vital for attaining low detection limits, minimal relative standard deviations between injections, and consistent retention times in quantitative analysis. These attributes are indispensable for ensuring the precision and sensitivity requisite for accurate measurements and reliable analyses [19].

6.2 Choosing Chromatographic Conditions

In the initial stages of method development, selecting appropriate chromatographic conditions is paramount. This includes determining the detector, column, and mobile phase to generate initial "scouting" chromatograms of the sample. Typically, reversed-phase separations utilizing a C18 column coupled with UV detection are employed as a starting point. At this juncture, a crucial decision arises regarding the development of a gradient method or the adoption of an isocratic approach. Each option presents distinct advantages based on the specific separation requirements and characteristics of the analytes present in the sample. Gradient methods offer enhanced resolution and flexibility by adjusting the composition of the mobile phase over time, while isocratic methods provide simplicity and reproducibility by maintaining a constant mobile phase composition throughout the analysis. The selection between these approaches hinges on factors such as analyte complexity, elution profile, and desired chromatographic performance, with careful

consideration needed to optimize separation efficiency and peak resolution for accurate and reliable analyses [20].

6.3 Optimization of Mobile phase

Optimizing the mobile phase is a fundamental aspect of method development in High-Performance Liquid Chromatography (HPLC), crucial for achieving efficient and reliable separation of analytes. Several parameters must be carefully considered to fine-tune the mobile phase composition effectively. Firstly, the selection of solvents depends on the polarity of the analytes and the stationary phase, often involving a combination of polar and non-polar solvents to achieve the desired elution profile. Buffers may also be added to control pH and improve peak shape, particularly for ionizable compounds. Adjusting the pH of the mobile phase is essential for optimizing peak shape and resolution, especially in reversed-phase chromatography. The addition of salts to the mobile phase can influence the retention and selectivity of analytes, requiring careful adjustment of ionic strength to improve peak symmetry and resolution, especially for charged compounds. Moreover, gradient elution programs, which involve changing the composition of the mobile phase over time, provide precise control over elution profiles and separation conditions. Flow rate optimization is crucial for achieving the desired chromatographic performance while maintaining system compatibility and efficiency. Additionally, temperature control can impact the viscosity and stability of the mobile phase, necessitating optimization to enhance separation and peak resolution, especially for thermally labile compounds. Through systematic adjustment and evaluation of these parameters, analysts can optimize the mobile phase to achieve the desired separation outcomes, ensuring reproducibility, accuracy, and reliability in HPLC analyses [21].

7. Method optimization

Method optimization in High-Performance Liquid Chromatography (HPLC) is a meticulous process crucial for enhancing separation efficiency, resolution, and sensitivity while minimizing analysis time and solvent consumption. It involves careful adjustment of various parameters to achieve optimal chromatographic performance. Firstly, selecting the appropriate column based on factors such as stationary phase chemistry, particle size, and dimensions is essential for achieving the desired separation. Mobile phase composition plays a critical role, and optimization involves balancing solvent type, buffer concentration, pH, and ionic strength to achieve optimal analyte retention and peak shape. Gradient elution programs may be utilized to further enhance separation efficiency. Additionally, adjusting the flow rate of the mobile phase influences retention time, peak width, and resolution, ensuring optimal chromatographic performance. Temperature control is also important, especially for thermally labile compounds, as it can impact analyte retention and peak shape. Finally, optimization of injection volume ensures adequate sample introduction without overloading the system, balancing sensitivity with peak broadening and system suitability. Through systematic adjustment of these parameters, analysts can optimize HPLC methods to achieve accurate, reliable, and efficient chromatographic separations for a wide range of analytes and applications [22].

8. Method Validation

Method validation is a critical process in analytical chemistry, ensuring that an analytical method is suitable for its intended purpose and delivers reliable and accurate results. Several parameters are typically evaluated during method validation to assess the performance and robustness of the method. These parameters include:

Specificity: Specificity evaluates the ability of the method to differentiate the analyte of interest from other components in the sample matrix. It ensures that the method accurately measures only the target analyte without interference from impurities, degradation products, or matrix components [23].

Accuracy: Accuracy measures the closeness of the test results to the true or accepted reference value. It is typically assessed by analyzing reference standards or spiked samples at known concentrations and comparing the measured values to the true values [24].

Precision: Precision evaluates the repeatability and reproducibility of the method. It includes assessing both within-day (repeatability) and between-day (reproducibility) variations in measurements. Precision is often expressed as the relative standard deviation (RSD) of replicate measurements [25].

Linearity: Linearity assesses the ability of the method to produce results that are directly proportional to the analyte concentration within a specified range. It is typically evaluated by analyzing standard solutions at different concentrations and plotting a calibration curve.

Range: The range of the method defines the concentration range over which the method is validated to provide accurate and precise results. It encompasses the lowest and highest concentrations that can be reliably measured with acceptable accuracy and precision.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ represent the lowest concentration of the analyte that can be reliably detected and quantified, respectively. They are determined based on the signal-to-noise ratio and are indicative of the method's sensitivity [26].

Robustness: Robustness evaluates the method's ability to remain unaffected by small variations in experimental conditions such as pH, temperature, and flow rate. It demonstrates the method's reliability under slightly altered conditions [27].

System Suitability: System suitability tests assess the performance of the chromatographic system, including column efficiency, resolution, and peak symmetry. These tests ensure that the chromatographic system is functioning properly and is suitable for the intended analysis [28].

9. Conclusion

In conclusion, the development and validation of High-Performance Liquid Chromatography (HPLC) methods play a vital role in pharmaceutical analysis. This paper has provided an insightful overview of the fundamental aspects involved in constructing and validating HPLC methods for compound separation. An essential initial step emphasized is the thorough understanding of the physicochemical characteristics of the primary compound, setting the foundation for method development. The selectivity for separation, a critical factor, is heavily influenced by the organic and pH composition of the buffer and

mobile phase. Furthermore, optimization efforts targeting gradient slope, temperature, flow velocity, and mobile phase modifiers are essential for fine-tuning the method's performance. Subsequent rigorous validation, encompassing parameters such as specificity, precision, accuracy, detection limit, and linearity, among others, in adherence to International Council for Harmonisation (ICH) criteria, ensures the reliability and robustness of the developed HPLC method for pharmaceutical analysis. This thorough validation process underscores the integrity and accuracy of analytical results, thereby instilling confidence in the pharmaceutical industry regarding the quality and safety of drug products.

Conflict of Interest

The authors declare no conflicts of interest associated with the completion and publication of this work. There are no financial or personal relationships with other people or organizations that could potentially bias our research, influence the interpretation of the results, or create undue influence on the decision to publish. This work has been conducted with transparency and integrity, and any potential conflicts of interest have been appropriately addressed. We are committed to maintaining the highest ethical standards in research and publication.

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