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HPLC METHOD DEVELOPMENT AND VALIDATION: A REVIEW

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ABSTRACT

HPLC is the most commonly used separation technique for detecting, separating, and quantifying drugs. To optimize the method, several chromatographic parameters were investigated, including sample pretreatment, mobile phase selection, column selection, and detector selection. The purpose of this article is to go over the method development, optimization, and validation processes. Because of its advantages such as rapidity, specificity, accuracy, precision, and ease of automation, 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.

1. INTRODUCTION

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.[3] 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 productanalysis, playing a pivotal role in determining drug product stability. By offering a meticulous approach to characterizing pharmaceutical samples, HPLC stands as an indispensable techniquein ensuring the quality and safety of medicinal formulations in the field of analytical chemistry.[4]

HPLC principle

High-performance liquid chromatography (HPLC) relies on the distribution of the analyte between a stationary phase and a mobile phase (eluent), typically within the column's packing material. The chemical structure of the analyte dictates its movement rate through the stationary phase, forming the basis for separation. This principle enables precise separation and analysis of diverse compounds, making HPLC a fundamental technique in analytical chemistry, particularly in pharmaceutical and chemical industries.

Types of HPLC

The choice of High-Performance Liquid

Chromatography (HPLC) method for analysis is dictated by the employed phase system. Normal Phase HPLC, or normal phase chromatography (NP-HPLC), classifies analytes based on polarity. In NP- HPLC, a non-polar mobile phase and a polar stationary phase interact with polar analytes, retaining them. Elution time increases with rising analyte polarity due to this interaction. NP-HPLC provides effective separation, revealing insights into sample composition based on polarity, making it a valuable analytical tool, particularly in characterizing compounds with different polarities in diverse fields such as chemistry, pharmaceuticals, and environmental analysis.[5,6]

Classification of HPLC can be done as

1. HPLC is classified into analytical and preparatory categories based on the scale of operation.
2. Various chromatographic techniques include size exclusion, affinity, and adsorption chromatography.
3. Chiral phase and ion exchange chromatography are categorized based on the principle of separation.
4. Isocratic and gradient separation methods distinguish chromatography based on elution technique.
5. Chromatography operates in normal and reverse phases, determined by modes of operation.[7,8]

1. Size exclusion chromatography

Size Exclusion Chromatography (SEC), also known as gel permeation or gel filtration chromatography, separates particles based on size. It is utilized to determine the quaternary and tertiary structures of amino acids and proteins. This technique is commonly employed for assessing the molecular weight of polysaccharides, providing valuable insights into their structural characteristics in various scientific and analytical applications.

2. Ion exchange chromatography

Ion-exchange chromatography relies on the retention of solute ions attracted to charged sites on the stationary phase. Ions with similar charges are repelled. This method finds application in water purification, protein ion-exchange chromatography, ligand-exchange chromatography, and high-pH anion-exchange chromatography of carbohydrates and oligosaccharides. Its versatility makes it a crucial technique in various fields, allowing selective separation and analysis based on the charged characteristics of different substances.[9,10]

3. Bio-affinity chromatography

Affinity chromatography relies on reversible ligand-protein interactions for separation. Proteins interacting with the column-bound ligands are immobilized by covalently attaching the ligands to a solid support on a bio-affinity matrix. This selective binding allows for precise separation and purification of target proteins based on specific interactions, making affinity chromatography a valuable technique in biochemistry and protein purification processes.[11]

4. Normal phase chromatography

In normal phase chromatography, the stationary phase is polar, and the mobile phase is non-polar. This causes the polar analyte to be retained by the stationary phase. Higher polarity in solute molecules leads to longer elution times and enhanced adsorption capacity. Chemically modified silica, such as cyanopropyl, aminopropyl, and diol, serves as the stationary phase. Typical columns measure 150-250 mm in length and have an internal diameter of about 4.6 mm. As the mixture traverses the column, polar compounds adhere longer to the polar silica, causing non-polar compounds to pass through more rapidly.[12]

5. RP-HPLC (Reversed-Phase HPLC)

In Reverse Phase High-Performance Liquid Chromatography (RP-HPLC), the mobile phase is polar or somewhat polar, while the stationary phase is non-polar.

The separation principle relies on hydrophobic interactions. Less polar analytes in a mixture are retained longer by the non- polar stationary phase, causing the most polar component to elute first. RP-HPLC is widely used for its ability to separate compounds based on hydrophobicity, providing effective analysis in various fields, including pharmaceuticals, biochemistry, and environmental science.[13]

Instrumentation of High-Performance Liquid Chromatography (HPLC)

Analytical separation in High-Performance Liquid Chromatography (HPLC) involves the high-pressure flow of liquid through a column containing a stationary phase, which can be either liquid (L) or solid (LSC/LLC). Injected compounds segregate as they traverse the column, and electronic detection occurs as they elute at the column's end. While less flexible than Gas Chromatography, HPLC offers an extensive selection of mobile and stationary phases, enhancing its versatility for precise compound analysis in various applications, such as pharmaceuticals, environmental analysis, and biochemistry.[14]

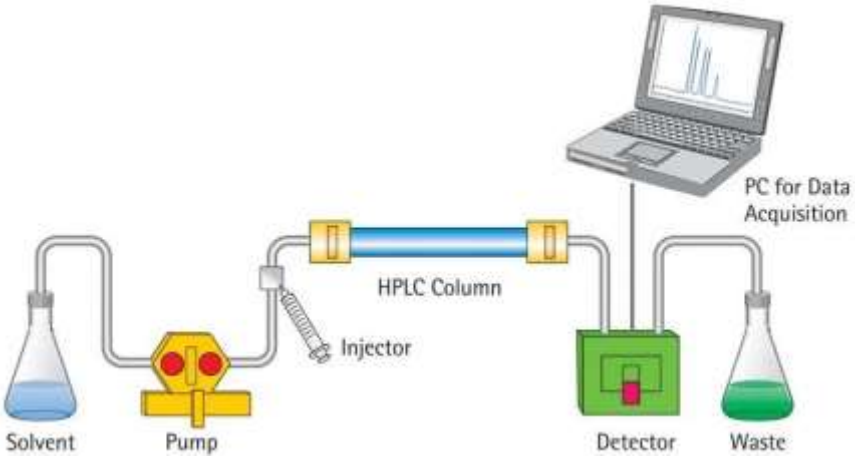

Figure 1: HPLC.

The HPLC technique has the characteristics listed below

High-Pressure Liquid Chromatography (HPLC) employs significantly greater mobile phase pressure, small-diameter stainless steel or glass columns, facilitating fast analysis and precise control over mobile phase flow rates. This configuration enhances efficiency and resolution, making HPLC a powerful analytical tool for rapid and controlled separation of compounds in diverse applications.[15]

HPLC has many advantages, including: High-resolution, simultaneous analysis, extreme sensitivity, excellent repeatability, limited sample size, mild analysis conditions, and ease of fractionation and purification make High-Performance Liquid Chromatography (HPLC) a versatile and advantageous technique in various analytical applications.[16]

HPLC Method Development: Developing and validating analytical methods is crucial in pharmaceutical research, development, and production. These methods ensure the identification, purity, potency, and effectiveness of pharmaceutical products. Method development involves considering factors like physicochemical characteristics (pKa, log P, solubility) for selecting the appropriate detection mode, especially in UV detection. The validation of an HPLC method for stability indication is a significant aspect of analytical development. It focuses on separating and quantifying the primary active ingredient, reaction impurities, synthetic intermediates, and degradants, ensuring the reliability and accuracy of the analytical process in pharmaceutical quality control.[17]

HPLC method development

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.[18]

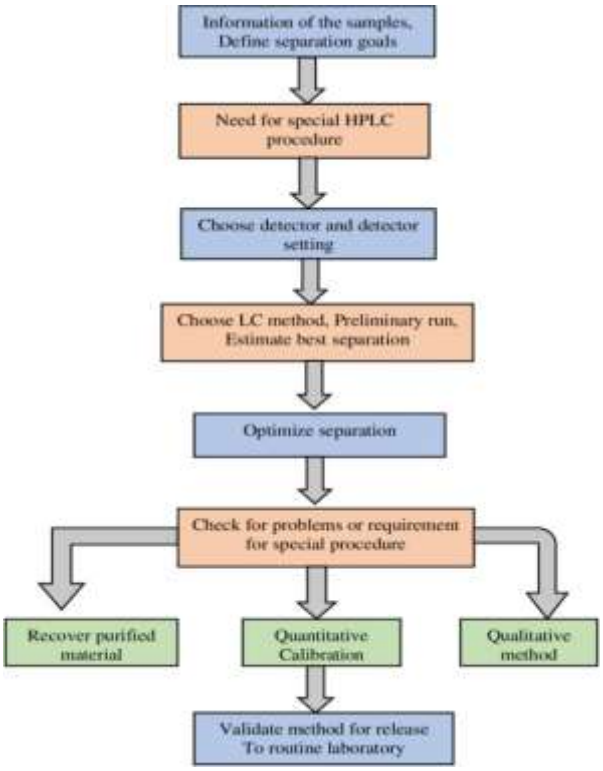

Figure 2: Steps involved in HPLC Method development.

1. 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 besoluble. Parameters like pH and pKa are critical in High-Performance Liquid Chromatography (HPLC) method development, influencing solvent selection and

overall method success. pH equals -log10[H3O+]

In High-Performance Liquid Chromatography (HPLC), achieving sharp and symmetrical peaks is often a result of optimizing the pH for ionizable analytes. Sharp, symmetrical peaks are crucial for obtaining low detection limits, low relative standard deviations between injections, and repeatable retention durations in quantitative analysis, ensuring the precisionand sensitivity required for accurate measurements and reliable results.[19]

2. Choosing ChromatographicConditions

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 advantagesdepending on the specific separation requirements and characteristics of the analytes in the sample.

2.2.1 Selection of Column

The column is the cornerstone of a chromatograph, playing a pivotal role inachieving 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. In an HPLC column,the three essential components are the hardware, matrix, and stationary phase. Matrices, such as alumina, zirconium,polymers, and most commonly silica, support the stationary phase. Silica matrices are favored for their strength, consistent spherical size, ease of derivatization, and resistance to compression under pressure. When selecting the ideal column, considerations encompass particle size, retention capacity, stationary phase chemistry, and column dimensions. These factors collectively influence the efficiency and effectiveness of the column in achieving accurate and reliable separations for specific analytical applications.[20,21]

In an HPLC column, the key components include hardware, matrix, and stationaryphase. Various matrices, including alumina, zirconium, polymers, and most commonly silica, support the stationary phase. Silica matrices, widely used, offer strength, consistent spherical size, ease of derivatization, and resistance tocompression under pressure. These characteristics contribute to the efficiency and reliability of HPLC columns in achieving precise separations for analyticalapplications.

Silica is chemically stable in the majority of organic solvents and low pH environments.However, a drawback is that traditional silica-based solid supports disintegrate above pH 7. Recently developed silica- supported columns allow operation at higher pH levels. The composition, shape, and particle size of silica contribute to effective separation, with larger particle sizes increasing the number of theoretical plates. The suitability of a column for normal-phase or reverse-phase chromatography is determined by the type of stationary phase employed, highlighting the versatility of silica-based columns in various chromatographic applications.[22,23]

Normal phase chromatography involves using a polar stationary phase and a non- polar mobile phase, where polar moleculestypically elute later than non-polar ones. Inreverse phase chromatography, the choice of columns is crucial. For instance, propyl (C3), butyl (C4), and pentyl (C5) phases arebeneficial for large molecules and peptides with hydrophobic residues, particularly in ion-pairing chromatography (C4). However, C3–C5 columns generally retain non-polar solutes less effectively compared to C8 or C18 phases. These variations in column chemistry enable tailored separations for diverse compounds in chromatographic analyses.[24,25]

Examples like YMC-Pack C4, Luna C5,and Zorbax SB-C3 represent reverse-phase columns, each with specific characteristics. However, columns with shorter alkyl chains, like C4 and C5, may have lower resistance to hydrolysis compared to those with longer chains. Octyl (C8) phases find utility in various applications, offering advantages for compounds like steroids, nucleosides, and medications, although they are less retentive than C18 phases. Theselection of the stationary phase or columnis pivotal in method development, aswithout a reliable and high-performing column, it's challenging to establish a repeatable procedure. Stability and reproducibility are critical to avoid issues insample retention during method development.[26,27]

The separation selectivity in HPLC can vary between columns from different manufacturers and even within batches from the same manufacturer. Key factors influencing this variability include parameters of the bonded stationary phase, properties of the silica substrate, and column diameters. Silica-based packing is commonly preferred in modern HPLCcolumns due to its favourable physical features, offering versatility and efficiency in achieving diverse and precise separationsfor analytical applications.[28]

2.2.2 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 bufferedmobile phases to prevent analyteionization.[29]

2.2.3 Optimization of Mobile phase

❖ **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.[30]

❖ **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 pHof 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.[31]

❖ **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.[32]

2.2.4 Selection of detector andwavelength

After chromatographic separation, the target analyte is identified using appropriate detectors. Common detectors in liquidchromatography (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 reliabilityof the analysis.[33,34]

3. 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 resolutiongreater 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.

In simultaneous estimation of two components, the detection wavelength is typically chosen at an isobestic point. Following this, the linearity of the drug is assessed to determine the concentration range exhibiting a linear pattern. The established approach for simultaneous estimation is further validated by analyzing a laboratory combination. Subsequently, the commercial product is diluted to match the linearity concentration range for analysis. This systematic process ensures the practicality, accuracy, and reliability of the method for simultaneous estimation in liquid chromatography.[35,36]

4. 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 preparationis 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.[37,38]

5. 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.

6. 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).[39]

Method Validation

Validation is the process of laboratory testing to demonstrate that the performance characteristics of an analytical method meet the requirements of the intended analytical application. Whether used by multiple operators with the same equipment in the same or different laboratories, any new or updated method must be validated to ensure it consistently produces repeatable and reliable results. The specific method and its intended uses determine the type of validation program required, ensuring that the analytical process is robust and fit for its purpose in various settings.

Method validation results are a crucial aspect of any robust analytical procedure, providing an evaluation of the quality, consistency, and reliability of analytical results. Essential to the validation process is the use of equipment that meets specifications, is correctly calibrated, and is operating and functional. The validation process ensures that analytical methods are thoroughly assessed and either validated for use or invalidated if they do not meet the required criteria. This ensures the accuracy and dependability of analytical results in various applications.[40,41]

The following are typical parameters recommended by the FDA, USP, and ICH.

1. Specificity
2. Linearity & Range
3. Precision

I. Method precision (Repeatability)
II. Intermediate precision (Reproducibility)

4. Accuracy (Recovery)
5. Solution stability
6. Limit of Detection (LOD)
7. Limit of Quantification (LOQ)
8. Robustness
9. Range
10. System suitability

1. Specificity

Selectivity and specificity are often used interchangeably in the context of method validation. Specificity refers to the ability to unequivocally assess the analyte in the presence of other components that may be present. This is the capacity to distinguish the analyte with absolute certainty in the presence of potentially interfering substances.

To determine specificity, a comparison is made between test results from an analysis of samples containing contaminants, degradation products, or placebo ingredients and those from an analysis of samples without such elements. This comparison controls and evaluates the method's ability to selectively identify and quantify the analyte of interest amidst potential interferences, ensuring the reliability and accuracy of the analytical method.[42,43]

2. Linearity and range

Linearity in an analytical process refers to its ability to produce test results that are directly proportional to the concentration of the analyte in the sample, within a specified range. It is crucial to evaluate this linear relationship across the spectrum of the analytical technique. The suggested approach involves diluting a normal stock solution containing the constituent parts of the medicinal product to directly demonstrate linearity on the drug substance.

In establishing linearity, the confidence interval around the slope of the regression line is commonly employed. According to ICH recommendations, a minimum of five concentrations is proposed for establishing linearity. The range of an analytical method is defined as the interval between the higher and lower values that have been demonstrated to be determined with precision, accuracy, and linearity using the method. This comprehensive assessment ensures the reliability and validity of the analytical method over a defined concentration range.[44,45]

3. Precision

Precision in the context of analytical methods represents the degree of agreement or scattering between a series of measurements made under specific conditions from several samplings of the same homogeneous material. Precision is a critical parameter for assessing the entire analytical process's reproducibility.

Precision consists of two components: repeatability and intermediate precision. Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variance introduced by the sample preparation procedure and that caused by the instrument or system. During validation, numerous replicates of an assay composite sample are analyzed using the analytical procedure to determine repeatability, and a recovery value is calculated.

Intermediate precision refers to the fluctuation that occurs within a laboratory on different days, with different instruments, and involving different analysts. These components of precision assessment ensure a comprehensive understanding of the reliability and reproducibility of the analytical method under varying

conditions and across different operators.[46,47]

4. Accuracy

Accuracy is the extent to which a measured value aligns with the true or accepted value. In practice, accuracy refers to the discrepancy between the true value and the mean value obtained. To calculate accuracy, the method is applied to samples with known analyte concentrations, which are then compared to blank and standard solutions to ensure there is no interference. Accuracy is computed as a percentage of the analyte recovered by the assay based on the test results. It is commonly expressed as the assay-based recovery of known, additional analyte levels, providing a measure of how well the analytical method reflects the true values.[48,49]

5. Solution stability

During validation, the stability of standards and samples is assessed under various conditions, including normal settings, standard storage conditions, and sometimes within the instrument. This evaluation helps determine whether specific storage conditions, such as refrigeration or protection from light, are necessary to maintain the stability of standards and samples. Understanding the impact of storage conditions is crucial for ensuring the reliability and integrity of analytical results over time, and it informs the appropriate handling and storage practices for the substances involved in the analysis.[50,51]

6. Limit of Detection (LOD)

The detection limit of a single analytical method is the most basic measure of an analyte in a sample that can be identified but not accurately quantified. This limit represents the lowest concentration at which the presence of the analyte can be reliably detected, providing a fundamental indicator of the method's sensitivity.[52]

7. Limit of Quantification (LOQ)

The quantitation limit of a specific analytical system is the lowest quantity of analyte in a sample that can be precisely and accurately measured quantitatively. This limit serves as a quantitative test parameter for assessing low levels of analytes in test matrices. The quantitation limit is crucial in identifying impurities and/or contaminants in samples and provides a threshold for reliable quantitative measurements in analytical methods.[53]

8. Robustness

The robustness of an analytical procedure is a measure of its reliability under typical conditions and its ability to withstand small but intentional alterations in method parameters. This quality assessment ensures that the analytical method remains dependable and produces consistent results even when subjected to minor variations or deliberate adjustments in its parameters. Robust methods are less sensitive to changes and variations, contributing to the method's reliability in real-world analytical applications.[54,55]

9. Range

The range of an analytical method refers to the interval between the higher and lower values of an analyte that have been demonstrated with sufficient linearity, precision, and accuracy. This range is typically determined based on a linear or nonlinear response curve and is expressed in the same units as the test findings. Establishing a defined range is essential for accurately assessing and reporting results within the method's validated and reliable concentration limits.[56,57]

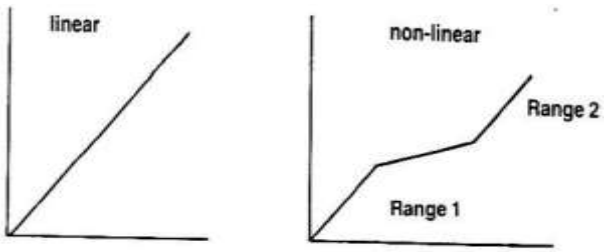

Figure 3: Range determination.

10. System Suitability

System suitability tests are a standard practice in liquid chromatographic procedures. They serve as a guarantee that the chromatographic system's repeatability, resolution, and detection sensitivity are sufficient for the intended analysis. These tests are based on the concept that the tools, electronics, processes involved in the analysis, and the samples to be examined are all components of a larger system that can be assessed as a whole.

Key parameters such as peak resolution, the number of theoretical plates, peak tailing, and capacity are examined during system suitability tests to assess the adequacy and performance of the employed analytical method. This comprehensive evaluation ensures the reliability and fitness of the chromatographic system for accurate and reproducible analyses.[58,59]

CONCLUSION

In the field of pharmaceutical analysis, there has been significant interest in the recent development of analytical methods for drug identification, purity assessment, and quantification. This paper provides an overview of the development and validation of High-Performance Liquid Chromatography (HPLC) methods. It addresses the construction of HPLC methods for compound separation in a generic and fundamental manner. Understanding the physicochemical characteristics of the primary compound is essential before creating an HPLC procedure.

The selectivity for separation is greatly influenced by the organic and pH makeup of the buffer and mobile phase. Finally, optimization can be achieved for the gradient slope, temperature, flow velocity, and the type and concentration of mobile phase modifiers. The optimized method is then validated using various characteristics, such as specificity, precision, accuracy, detection limit, linearity, among others, in accordance with International

Council for Harmonisation (ICH) criteria. This thorough process ensures the reliability and robustness of the developed HPLC methodfor pharmaceutical analysis.

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