HPLC METHOD DEVELOPMENT AND VALIDATION: A REVIEW

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ABSTRACT

High-Performance Liquid Chromatography (HPLC) is a type of column chromatography commonly used in biochemistry and analytical chemistry to separate, identify, and quantify active compounds. HPLC is the most frequently employed separation technology for detecting, separating, and quantifying drugs. Method development and validation of HPLC are essential in new drug discovery, development, and manufacturing, as well as various other human and animal studies. The purpose of this article is to review the method development, optimization, and validation processes of HPLC. To optimize the method, several chromatographic parameters were investigated, including sample pretreatment, mobile phase selection, column selection, and detector selection. The HPLC method can be used to analyze the majority of drugs in multicomponent dosage forms due to its advantages such as rapidity, specificity, accuracy, precision, and ease of automation.

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

1. INTRODUCTION

High-Performance Liquid Chromatography, also known as High-Pressure Liquid Chromatography, is a type of column chromatography commonly used in biochemistry and analytical chemistry to separate, identify, and quantify active chemicals. It is a popular analytical technique for separating, identifying, and quantifying each element of a mixture. HPLC is a sophisticated column liquid chromatography technology.

In HPLC, the solvent normally flows through the column due to gravity, but in this process, the solvent is pushed under high pressures of up to 400 atmospheres. This allows the sample to be separated into different constituents based on differences in relative affinities. HPLC generally comprises a column containing packing material (stationary phase), a pump that drives the mobile phase(s) through the column, and a detector that detects molecule retention times. The retention time is affected by the interactions between the stationary phase, the molecules being analyzed , and the solvent(s) utilized. The samples to be analyzed are added in small quantities to the mobile phase stream and are slowed by specific chemical or physical interactions with the stationary phase. The amount of retardation is determined by the nature of the analyte as well as the composition of both the stationary and mobile phases. The retention time is the time it takes for a certain analyte to elute.

Any miscible combination of water or organic liquids is a common solvent. Gradient elution has been used to change the mobile phase composition during the analysis. The gradient separates analyte mixtures based on the analyte's affinity for the current mobile phase. The nature of the stationary phase and the analyte influence the choice of solvents, additives, and gradients .

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.

In 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.

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.

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.

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 theanalyte 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.

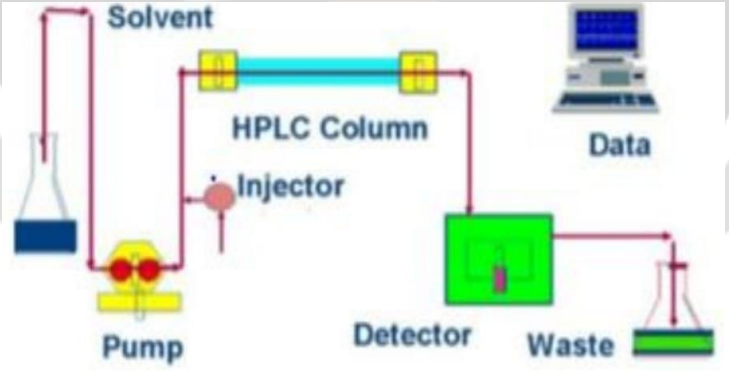

Figure 1: Flow Diagram of HPLC

TYPES OF HPLC

HPLC can be classified as follows: Based on a scale of operation Preparative HPLC and analytical HPLC 7 Based on the principle of separation Affinity chromatography, adsorption chromatography, size exclusion chromatography, ion-exchange chromatography, chiral phase chromatography.8 International Journal of Pharmaceutical Sciences Review and Research Available online at www.globalresearchonline.net 23 ©Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or

in part is strictly prohibited. Int. J. Pharm. Sci. Rev. Res., 74(2), May - June 2022; Article No. 03, Pages: 23-29 ISSN 0976 – 044X Based on the elution technique Gradient separation and isocratic separation Based on modes of operation Normal phase chromatography and reverse-phase chromatography.

A. Normal phase chromatography: The mobile phase in normal phase chromatography is non polar, whereas the stationary phase is polar. As an outcome, the polar analyte is retained by the station phase.10 The increased polarity of solute molecules improves adsorption capacity, resulting in a longer elution time. In this chromatography, a

stationary phase of chemically modified silica (cyanopropyl, aminopropyl, and diol) is used 11. As an example, A typical column has an interior diameter of around 4.6 mm and a length ranging from 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick to the polar silica for a longer period than non-polar compounds. As a result, the non-polar ones will go quickly through column 12 B. RP-HPLC (Reversed-phase HPLC): The stationary phase of RP-HPLC is non-polar, and the mobile phase is polar or moderately polar. The notion of hydrophobic interaction underpins RP-HPLC 13. The non polar stationary phase will hold analytes that are comparatively less polar in a combination of components for a longer period than those that are substantially more polar. As a result, the most polar component elutes the first.

Classification of HPLC can be done as

1. HPLC is classified into analytical and preparatory categories based on the scale ofoperation.
2. Various chromatographic techniques include size exclusion, affinity, andadsorption 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 ofoperation.[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. Thistechnique is commonly employedfor assessing the molecular weight of polysaccharides, providing valuable insights into their structural characteristics in various scientific and analytical

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 variousfields, allowing selective separation and analysis based on the charged characteristics of different substances.

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 preciseseparation and purification of targetproteins basedon specific interactions, making affinity chromatography a valuable technique in biochemistry and protein purification processes.

METHOD DEVELOPMENT ON HPLC

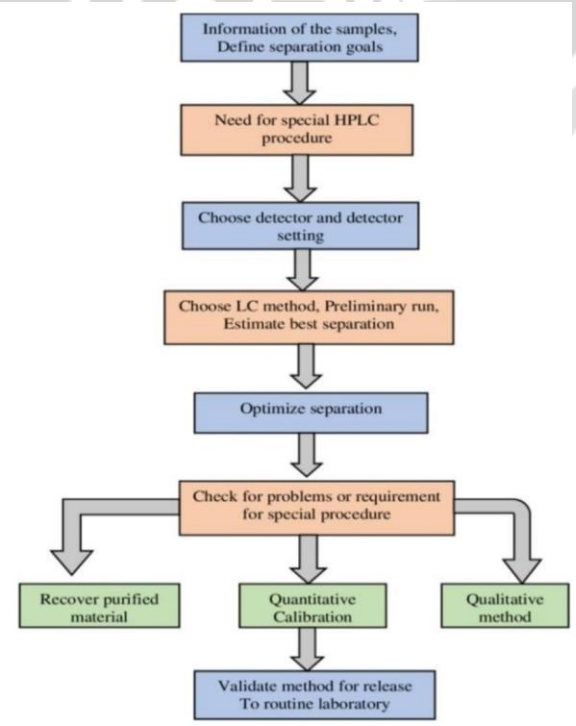

Figure 1: Steps involved in HPLC Method development

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 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 -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 precision and sensitivity required for accurate measurements and reliable results.

1. 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 todevelop a gradient method or opt for an isocratic approach, each offering distinct advantages depending on the specific separation requirements and characteristicsof the analytes in the sample.

2.2.1 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, renderingresults 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, supportthe stationary phase. Silica matricesare 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 phasechemistry, and column dimensions. These factors collectively influence the efficiency and effectiveness of the column in achieving accurate and reliable separationsfor specific analytical applications.[20,21]

In an HPLC column, the key components include hardware, matrix, and stationary phase. 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 to compression under pressure. These characteristics contribute to theefficiency and reliability of HPLC columns in achieving precise separations for analytical applications.

Silica is chemically stable in the majority of organic solvents and low pH environments. However, a drawbackis that traditional silica-based solid supports disintegrate above pH 7. Recently developed silica- supportedcolumns 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-phasechromatography is determined by the type of stationary phase employed, highlighting the versatility of silica- based columns in various chromatographic applications.

Normal phase chromatography involves using a polarstationary phase and a non- polar mobile phase, where polar molecules typically 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 are beneficial 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, andmedications, although they are less retentive than C18 phases. The selection of the stationary phase or column is pivotal in method development, as without a reliable and high-performing column, it's challenging to establish a repeatable procedure. Stability and reproducibility are critical to avoid issues in sample retention

during method development.

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 HPLC columns due to its favourable physical features, offering versatility and efficiency in achieving diverse and precise separations foranalytical applications.[28]

2.2.2 Selection of Chromatographic mode Chromatographic modes are dictated by the analyte'spolarity and molecular weight. Reversed-phasechromatography (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.

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 systemsuitability criteria. Through a series of experiments, potassium dihydrogen phosphate emerged as the mostsuitable buffer for successful separation of all peaks. Testconcentrations of 0.02 M, 0.05 M, and 0.1 M were examined. Interestingly, altering the buffer concentrationdid 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 appropriatemobile-phase pH is crucial, guided by the analyte's pKa. This ensures that the target analyte is either in a neutralor ionized form. The ability to adjust the pHof the mobilephase 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 criticalpairs of components in the sample. pH adjustment playsa 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 incomplex multicomponent samples under isocraticconditions, 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 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 superimposed, and then normalized. Selecting awavelength 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.[33,34]

2. 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.

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.

3. 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.

4. 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.

5. 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 itspurpose in various settings.

Method validation results are a crucial aspect of anyrobust 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 theydo not meet the required criteria. This ensures the accuracy and dependability of analytical results in variousapplications.

COMPONENTS OF METHOD VALIDATION

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 discovered40. 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 value41. It is calculated by applying the procedure to samples containing known levels of analyte. To confirm that there is no interference, these should be compared to standard and blank solutions. The accuracy is then computed as a percentage of the analyte recovered by the assay based on the test findings. It is frequently expressed as the recovery of known, added amounts of analyte by test. The accuracy is then computed as a percentage of the analyte recovered by the assay based on the test findings. It is frequently expressed as the recovery of known, added amounts of analyte by test.

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.43 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.44 The standard deviation or relative standard deviation of a sequence of data is commonly used to express the precision of an analytical technique.45 Precision can refer to the reproducibility or repeatability of an analytical method under normal conditions. Intermediate precision (also known as ruggedness) expresses variability within laboratories, for as on different days or with different analysts or equipment within the same laboratory46. 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.

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.48 Linearity is typically stated as the confidence limit around the regression line's slope. A linear relationship should be investigated across the analytical procedure's range. The proposed approach is used to show it directly on the drug substance by dilution of a standard stock solution of the drug product components. Linearity is typically stated as the confidence limit around the regression line's slope. 16 18 The ICH recommendation recommends a minimum of five concentrations for the establishment of linearity.

Limits of detection and quantitation

An individual procedure's limit of detection (LOD) is the smallest amount of analyte in a sample that can be detected but not necessarily quantitated as an accurate number. In analytical techniques with baseline noise, the LOD might be based on a signal-to-noise (S/N) ratio (3:1),
which is commonly stated as the analyte concentration in the sample. The limit of quantitation (LOQ) is defined as the lowest analyte concentration in a sample that can be measured with acceptable precision and accuracy under the method's stated operational circumstances. ICH recommends a signal-to-noise ratio of 10:1 for LOQ 50,51. LOD and LOQ can alternatively be computed using the standard deviation of the response (SD) and the calibration curve(s) slope at values close to the LOD using the formulae listed below.

LOD = 3.3 × S /SD and LOQ = 10 × S /SD

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. The following are the ramifications of this definition: Identification: the process of ascertaining the identity of an analyte. Purity tests are used to guarantee that all analytical processes are performed to allow for accurate characterization of an analyte's impurity concentration.

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

Range

The method's range is the interval between an analyte’s upper and lower levels obtained with appropriate precision, accuracy, and linearity

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 interchangeablyin 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.

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.

3. Precision

Precision in the context of analyticalmethods 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.

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.

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 theimpact 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.

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 presenceof the analyte can be reliably detected, providing a fundamental indicator of the method's sensitivity.

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 analyticalmethods.

8. Robustness

The robustness of an analytical procedure isa 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.

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

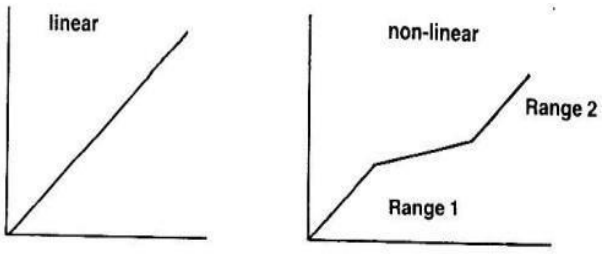

Figure 3: Range determination.

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.

CONCLUSION

In the field of pharmaceutical analysis, there has been significant interest in the recent development of analyticalmethods 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 compoundis 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 gradientslope, 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 method for pharmaceutical analysis.

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