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Optimizing Antibiotic Analysis: A Comprehensive Guide to HPLC Process of Assessment and Quantification

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Abstract

The history and uses of chromatography are thoroughly covered in this work, with an emphasis on the use of High-Pressure Liquid Chromatography (HPLC) in pharmaceutical analysis. Since its discovery by Russian botanist Mikhail Tswett in the 19th century, chromatography has undergone tremendous developments that have given rise to a variety of chromatography types and applications. The story follows the evolution historically, emphasizing Tswett's groundbreaking breakthrough in plant color classification and the birth of real chromatography. Chromatography types began to evolve in the 1940s, but Csaba Harwath's invention of the first liquid chromatographic apparatus in the 1960s was a turning point. The introduction of a flow cell for a UV detector by Kirkland in the late 1960s was a pivotal factor in the development of High-Performance Liquid Chromatography (HPLC). The paper covers the fundamentals of chromatography, the benefits of high-performance liquid chromatography (HPLC), and the different kinds of separation it provides, such as reverse and normal phase chromatography. The importance of buffers in HPLC separations is underlined, and the apparatus of HPLC is examined. The significance of solubility, absorbance, and water in mobile phases is addressed in detail, along with the selection of solvents, columns, and mobile phases in HPLC. In addition, the research explores degassing methods and emphasizes the need of system appropriateness criteria for method validation. The final section of the paper delves deeply into the creation of HPLC analytical methods, addressing important factors such mobile phase optimization, detector selection, and chromatography mode.

Keywords: Chromatography, High-Pressure Liquid Chromatography (HPLC), Pharmaceutical Analysis, Analytical Method Development, Gas Chromatography (GC)

INTRODUCTION

Chromatography, known as "color writing," was initially discovered by Russian botanist Mikhail Tswett in the 19th century, who termed it paper chromatography. Its primary application at that time was in categorizing plant colors, particularly chlorophyll. While various chromatography-like techniques existed in the 19th century, true chromatography, as we know it today, emerged with Tswett's work. In the 1940s, new chromatography types evolved, broadening its applicability for different partition processes.

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In the late 1950s, S. Moore and W.S. Stein explored the multidimensional aspect of liquid chromatography for amino acid analysis. By the late 1960s, the fundamental principles of chromatography had been extensively studied. Continuous advancements and diverse perspectives fueled improvements in liquid chromatography instrumentation. The first liquid chromatographic equipment was created by Csaba Harwath in 1964 during his time at Yale University. In 1968, Kirkland's incorporation of a flow cell for a UV

detector played a crucial role in advancing High-Performance Liquid Chromatography (HPLC) for various applications.

In the pharmaceutical industry, gas chromatography (GC) gained prominence in the mid-1950s. Enhanced detectors, such as the flame ionization detector evolving into the nitrogen-phosphorus identifier, significantly improved sensitivity and selectivity. The electron capture detector further boosted sensitivity for electro-active compounds. Transitioning from packed columns to capillary columns required additional advancements for effective separations. These developments have shaped chromatography into a versatile and powerful analytical technique used widely today.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

Analytical chemistry encompasses the scientific and artistic pursuit of devising sensitive, reliable, and accurate methodologies for discerning the elements or compounds present in a substance. In the realm of pharmaceutical analysis, the focus is on determining the identity, strength, quality, and purity of drug substances, as well as associated chemicals. This field is crucial for bio-pharmaceutical and pharmacokinetic investigations, addressing bulk compounds, dosage forms, and, more recently, biological samples [1].

Pharmaceutical analysts play a pivotal role in the development of new drugs, ensuring that rigorous procedures are in place to maintain the quality and stability of a novel drug product throughout its shelf life.

High-Pressure Liquid Chromatography (HPLC), alternatively referred to as High-Performance Liquid Chromatography or High-Cost Liquid Chromatography, serves as a separation method utilized to analyze diverse chemical substances and ions. HPLC relies on methods such as adsorption, partition, ion exchange, or occasionally size exclusion, contingent upon the stationary phase utilized. In the HPLC setup, a stainless-steel column is typically filled with a mobile liquid phase and a solid stationary phase. The components of a solution separate due to variations in the relative distribution ratios of solutes between these two phases [2].

To separate, identify, and measure analyte molecules, high performance liquid chromatography (HPLC), especially in the form of column chromatography, is frequently used in disciplines like analytical chemistry and biochemistry. A solid stationary phase is poured into a column, and the mobile phase is pushed through it alone or in combinations. The retention durations of the molecules are then indicated by a detector; these times are affected by interactions between the stationary phase, the molecules being studied, and the solvents employed [3].

DEFINITION OF CHROMATOGRAPHY

Chromatography achieves the separation of components within a mixture by establishing an equilibrium distribution between two phases. This specific chromatographic method relies on variations in the rates at which components of a mixture traverse a porous medium termed the stationary phase, influenced by a solvent or gas, known as the mobile phase [4].

In the chromatographic process, a sample or its extract is dissolved in a mobile phase, which can be a gas, liquid, or occasionally a supercritical fluid. The solid stationary phase, immobile and immiscible, is then compelled through the mobile phase under pressure. The choice of phases is based on their distinct solubilities for the sample components at each stage. A component significantly soluble in the stationary phase will traverse it more slowly than a component highly soluble in the liquid mobile phase but not at all in the stationary phase. As the sample components progress through the stationary phase, their separation occurs due to these differences in mobility [5].

ADVANTAGES OF HPLC

- *Efficiency:* Many analyses can be completed within 20 minutes or even less in some cases.
- *Sensitivity:* A wide range of detectors can be employed, contributing to high perceptiveness.

- *Enhanced Resolution:* Various stationary phases can be utilized, leading to improved resolution.
- *Reusable Analytical Columns:* Although columns may be expensive, they can be employed for a wide range of analytical procedures.
- Suitability for Compounds with Minimal Volatility.
- Clear and Concise Sample Recovery, Handling, and Maintenance Procedures.
- *Time and Labor Efficiency:* The instrumentation allows for automation and quantitation, reducing time and labor.
- *Extremely Efficient and Reproducible:* Ensures consistent and reliable results.
- *Calculations Performed by Instrument Software:* The instrument software streamlines the calculation process.
- *Suitable for Preparative Liquid Chromatography:* Applicable for larger-scale separations in preparative chromatography.

TYPES OF SEPARATION IN HPLC

In total, there are four categories of HPLC chromatography, distinguished by various factors [6]:

1. According to Modes of Separation:
   - Normal Phase Chromatography (N/P)
   - Reverse Phase Chromatography (R/P)
2. Based on the Type of Elution Process:
   - Isocratic Elution Technique
   - Gradient Elution Technique
3. Depending on the Scale of Operation:
   - Analytical HPLC
   - Preparative HPLC

INSTRUMENTATION OF HPLC

The main components of HPLC instrument are follows-as shown in the Figure 1.

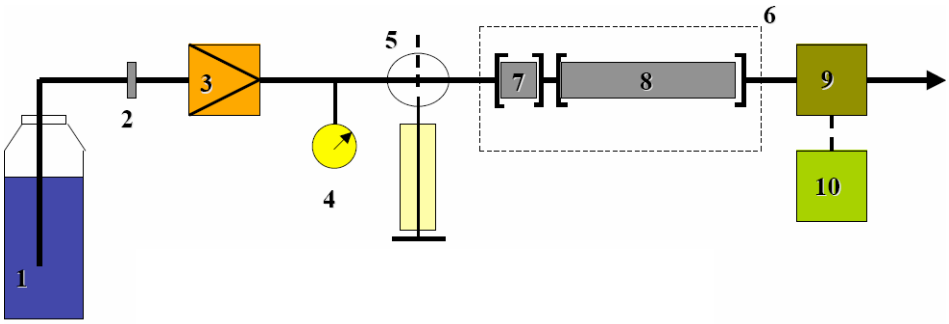

Figure 1. Schematic representation of HPLC instrument.

1	*Eluent reservoir*	***6***	*Column oven*
2	*Filter*	***7***	*Guard column*
3	*High pressure pump with dampener*	***8***	*Column*
4	*Pressure gauge*	***9***	*Detector*
5	*Sample injection valve with syringe*	***10***	*Recorder*

WORKING OF HPLC

High-performance liquid chromatography (HPLC) is an analytical technique that is widely used for the separation, identification, and quantification of components in a mixture. Here is a quick rundown of how HPLC operates:

1. Sample Injection: The HPLC system is injected with a tiny volume of the sample for analysis.
2. Mobile Phase: A liquid mobile phase—typically a solvent or a combination of solvents—transports the sample through the apparatus.

3. Column: The stationary phase, which is usually a chromatographic substance (like silica or polymer) packed within a stainless steel or glass column, is passed through by the mobile phase.
4. Separation: Depending on their chemical makeup, the sample's constituents interact with the stationary phase in different ways.
5. Detector: Separated components go via a detector as they leave the column. The presence of these elements is tracked by the detector using variables like absorbance and fluorescence.
6. Data Analysis: After analyzing the detector's data, a chromatogram is produced. Different components in the sample are represented by distinct peaks in the chromatogram.
7. Quantification: Quantitative analysis is possible because the area under each peak in the chromatogram corresponds to the quantity of the relevant component in the sample.

Because of its great adaptability, HPLC is useful in many different fields, such as medicines, food and beverage analysis, environmental monitoring, and more. The method is prized for its sensitivity, accuracy, and capacity to extract different substances from mixtures.

HPLC SEPARATION MODES

In the context of High-Performance Liquid Chromatography (HPLC), "separation modes" refer to different methods or techniques employed to separate components within a mixture as they pass through the chromatographic column. These modes are not part of the instrumentation; instead, they represent the principles or strategies applied to achieve separation based on specific properties of the analytes.

Generally, three fundamental characteristics of chemical compounds can be employed to establish HPLC separations:

1. Polarity
2. Electrical Charge
3. Molecular Size

To begin, let's examine polarity and delve into the two main separation modes that capitalize on this characteristic - normal phase (NP) and reversed phase (RP) chromatography [6].

Separations Based on Polarity

Organic molecules are classified into distinct groups based on the primary functional group(s) present in their structure. The type and positioning of these functional groups play a crucial role in determining the chromatographic retention of different molecules when utilizing a polarity-based separation mode. An analogy often used is the immiscibility of oil and water, representing non-polar and polar substances, respectively. In contrast to magnetism, where opposite poles exhibit attraction, polarity-based chromatography operates on the principle of "Similar Entities Attract."

When constructing a chromatographic separation system, selecting a mobile phase and a stationary phase with different polarities stimulates competition for various molecules in the sample. Sample compounds that are polar in relation to the stationary phase attract the particles more strongly, resulting in a slower or lag in motion. Conversely, compounds that are similarly polar to the mobile phase are preferentially attracted and move through the column faster. As mobile phase molecules vie for the coveted stationary phase sites, analytes are displaced, accelerating the analytes' flow through the column and decreasing retention [7, 8].

The surface of silica, constituting the stationary phase, features acidic silanols, akin to alcohol, creating an active and hydrophilic (water-attracting) structure. By chemically bonding less polar functional groups to the silica surface, known as bonded phase, the polarity or activity of the surface can be selectively altered. When selecting a mobile phase for a specific stationary phase, analysts must consider retaining the target analytes without overly strong retention that could hinder elution. While

"Like Attracts Like," the process of separating substances based on polarity requires a deep understanding of the sample, experience with diverse analytes, and knowledge of retention times [9].

Normal Phase HPLC

The renowned scientist Tswett successfully separated plant extracts using a polar stationary phase (chalk in a glass column) and a mobile phase of significantly lower polarity. This traditional chromatographic technique is commonly known as normal phase chromatography.

Reverse-Phase (RP-HPLC)

Reverse phase chromatography, a chromatography mode, operates in stark contrast to normal phase. In this configuration, a hydrophilic mobile phase (polar) is combined with a hydrophobic stationary phase that is non-polar. Unlike typical phase chromatography, the blue dye, being more non-polar, now exhibits the highest retention due to its increased affinity for the non-polar stationary phase.

In this scenario, the polar, aqueous mobile phase, adhering to the principle of like attracts like, moves swiftly across the bed and elutes first. This competitive mechanism leads to the limited retention of the polar yellow dye. Table 1 provides a condensed summary of the phase characteristics for the two primary HPLC separation modes based on polarity. Table 2 provides the chromatography parameters. It's crucial to bear in mind that the principle of like attracts like is fundamental to these polarity-based modes.

Table 1. Modes of separations.

Separation mode	Stationary Phase(particle)	Mobile phase Solvent
Normal phase	POLAR	NON-POLAR
Reversed phase	NON-POLAR	POLAR

Table 2. Showing Chromatographic conditions as per USP-NF.

Name of the parameter	Limits as per USP
Mobile phase	Selected M/P for the method development & validation. Minor components- ±30%, if NMT ±10% absolute
pH	± 0.2 units
Buffer conentration	± 10% as far as the allowed change in the pH value
Column	Type of Column selected based on nature of separation
Flow rate	± 50% or more sometimes, provided linear flow velocity which remains same
Injection volume	The injection volume can be adjusted as far as, it is consistent with the allowed precision, linearity, and detection ranges.
Column temperature	This can be adjusted by to a great extent as ±10°.
Wavelength	0, There is no allowance for deviations from the specified wavelengths in the procedure. The detector manufacturer's prescribed procedure or another validated method used to confirm the detector wavelength error must not exceed ±3 nm.

USE OF BUFFERS IN HPLC SEPARATIONS

When dealing with samples containing ionizable components, the mobile phase becomes crucial. One of the pivotal factors influencing retention in reverse phase HPLC (RP-HPLC) separations is the pH. However, uncontrolled pH can pose significant challenges throughout the analysis. Given that many

pharmaceutical compounds examined via RP-HPLC contain acidic or basic functional groups, maintaining pH becomes essential. Buffers are frequently employed in HPLC analysis for this specific purpose [13].

Buffers are essential in reverse-phase HPLC separations (RP-HPLC) since they influence the ionization level of sample molecules as pH changes. While this difference is minor for non-polar molecules, it becomes noteworthy for polar molecules featuring acidic or basic groups in their chemical composition. Adjusting the pH, particularly in cases of closely spaced eluting peaks, proves to be the most effective method for peak separation. Therefore, employing buffers to control pH enhances the separation of peaks that are closely spaced or overlapping [14].

The use of buffers for pH adjustment relies on several critical parameters, which include:

Selection of Buffer

- Buffer pH
- Buffer Concentration
- Buffer solubility

SELECTION OF SOLVENT IN HPLC

The choice of solvents for the mobile phase in HPLC analysis is a crucial stage in the development of a technology. There is no one solvent that works for all situations; rather, a variety of solvents are selected in accordance with the particular needs of the analysis. The stationary phase of the column and the physical characteristics of the sample have an impact on the solvent selection. The appropriateness of solvents for use as the mobile phase is determined by a number of important criteria [15].

Solubility

Complete solubility of the sample in mobile phase systems is imperative. The presence of even a slight degree of insolubility can lead to phase separations or suspensions, introducing additional operational challenges for the instrument.

Absorbance

Detectors employed in HPLC commonly rely on the capacity of sample components to absorb light. It is crucial that the intrinsic absorbance of mobile phase components within the specified wavelength range does not interfere with the sample's relative absorbance to its constituents. The ideal solvent for the mobile phase should exhibit no absorbance at the critical wavelength [15].

Water

Whether employed independently or in combination with other solvents, water holds a significant role as one of the mobile phases in reverse phase chromatography. Commercial water purifying systems are available in the market, and it is advisable to utilize water generated by these systems to prevent any degradation in quality during storage. Due to the high sensitivity of the HPLC detector to variations in the mobile phase's composition, it is necessary to filter solvents through a 0.45-m filter to eliminate any solid suspensions [16].

Additionally, degassing is a crucial step, as even a small amount of dissolved air can lead to flow restrictions or the generation of inaccurate peaks.

COLUMNS

An essential part of an HPLC device is a column. It will produce highly reliable results in routine analysis if it is properly handled and used.

Adjusting operating flow rates and pressures gradually will prevent any disruption of the stationary phase packing. Use of a column oven is strongly advised in order to maintain a steady column temperature for consistency in flow rate and column performance because the flow of mobile phase is

based on the temperature parameter [17]. The column length and diameter can vary widely, as shown in Figure 1, depending on the chromatographic separation and kind of separations.

TYPES OF COLUMNS

The prevalent HPLC/UPLC packing columns commonly incorporate long-chain alkyls like C18 or C8. While both materials can be utilized for most separations, they exhibit slight variations in overall retention, with shorter-chain alkyl packings being less stable and less retentive [18].

Among the HPLC/UPLC columns, C18 or C8 bonded phase silica columns stand out as the most frequently employed (Figure 2) (Table 3). These columns deliver promising results in terms of retention, selectivity, durability, and operating pressure. In most cases, HPLC analysis using either C8 or C18 proves equally effective. Notably, when comparing "same" columns from different manufacturers, variations in selectivity are often more significant than when comparing "different" columns from the same source [19].

Silica remains the most popular column material due to its superior effectiveness and repeatability compared to polymeric packing columns. Silica-based columns are particularly advantageous when a wide pH range is necessary or when interactions with silanol cause excessive tailing. The study of sample factors influencing the properties of the bound phase in the column is known as column chemistry.

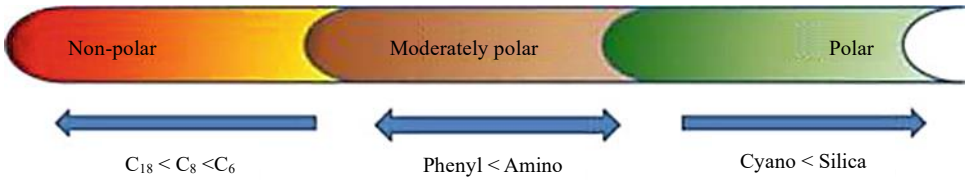

Figure 2. Shows different column packings.

Table 3. Shows different column packing's.

Type of the column	Comments
C 18 (OCTADECYL, ODS)	Rugged, highly retentive & most widely used
C 8 (OCTYL)	Same as C18, but slightly less retentive when compared t C18
C3 & C4	Less retentive & least stable; mainly used for peptides & protein analysis
C 1 (TRIMETHYLSILYL)	Less retentive & least stable
PHENYL & PHENETHYL	Intermediate retentive, selectively change
CN (CYANO)	Intermediate retentive, also normal phase
NH2 (AMINO)	Weakly retentive; most often used for Normal phase & less stable
POLYSTYRENE	stable for 1<pH<13, good peak shapes & lifetime; selectively change & often less efficient

HPLC COLUMNS

- To optimize column performance and lifespan, especially with UHPLC/HPLC columns, consider the following recommendations [20]:
- Ensure the use of freshly prepared aqueous mobile phases to prevent bacterial growth.
- Filter all samples, standards, and mobile phases consistently, employing a 0.2-m filter, for instance.
- Incorporate an in-line filter system into the setup at all times.
- Cleanse contaminated samples as required to eliminate impurities.

HPLC MOBILE PHASES

The primary purpose of the mobile phase is to transport the injected sample to the column and subsequently, after component separation in the column, convey the components individually to the

detector for identification and quantification. It is advisable to begin the analysis with a freshly prepared mobile phase and HPLC-grade solvents, ensuring the solvents are free from suspended particles or contaminants. Initial filtration of the solvents can be performed using 0.45 Whatman filter paper under vacuum [21].

To eliminate any remaining suspended particles or contaminants, additional use of online filters is recommended. Both the sample injected into the mobile phase and the solvents used in the mobile phase should be completely miscible. It is crucial to prevent buffers from drying up inside the system when utilizing them as mobile phase components. This is because the accumulation of salts can obstruct the free flow of the mobile phase and damage the pump's components [22].

TYPES OF M/P

Reverse phase (RP-HPLC) mobile phases commonly consist of an organic modifier and water or an aqueous solution, often with the addition of an aqueous buffer. For the analysis of ionizable substances, buffers and other additives may be introduced into the aqueous phase to regulate retention and peak shapes.

Typically, a single organic modifier, known for its lower polarity, is incorporated. As a result, the hydrophobic analyte is less strongly attracted to the adsorbent, leading to a shorter retention period in the stationary phase and, consequently, an earlier elution. This enhances the chromatographic "strength" of the modifier, positively influencing retention or elution [23]. With an increasing amount of organic modifier in the mobile phase, the analyte's retention time decreases. Figure 1 provides a more detailed description of typical organic modifiers, including their Snyder polarity index values that measure solvent polarity. Additionally, μ° values are presented, serving as an indicator of the relative elution intensity (the values provided are for elution on C18).

DEGASSING OF M/P

In the laboratory, solvents and surrounding gaseous components are brought into balance. Henry's law says that "the quantity of a gas dissolved in a solution is directly proportional to the gas's partial pressure above the solution." This applies to a liquid's gas solubility. When solvents are well combined, air becomes less soluble than it would be in pure solvents by the same percentage, which increases the risk of excessive air departing the solution and bubbling. Most people are aware that the term "outgassing" or "degassing" refers to the process of dissolved gases rising out of a solution.

Furthermore, the presence of air bubbles can impede the flow of solvents in the mobile phase through the column by generating dead volumes. To mitigate the issues arising from bubble formation in HPLC analysis, it is highly advisable to degas the mobile phase system [24]. The problems associated with bubble formation during solvent mixing can be effectively prevented through the degassing of the mobile phase.

Degassing Techniques

Commonly used degassing practices for HPLC mobile phase are as follows:

- Helium purging
- Vacuum degassing
- Ultrasonication

While boiling is the most effective method for completely eliminating dissolved air, it is not advisable due to the potential loss of volatile components and gases, as well as the extended time required to bring the mobile phase to the necessary ambient temperature conditions.

Analytical method development encompasses showcasing the appropriateness of a formulated chromatographic method for the manufacturing of a pharmaceutical drug substance and a pharmaceutical drug product. The subsequent enumeration outlines the essential separation techniques and principles utilized in the development of analytical methods employing HPLC and UPLC:

- Selection of chromatography mode
- Selection of detector
- Selection of column (stationary phase)
- Selection and optimization of mobile phase
  - Buffer and its strength
  - pH of buffer
  - Mobile-phase composition
- Selection of organic modifiers
- Selection of ion-pair reagents
- Selection of flow rate
- Selection of solvent delivery system (elution mode)
- Selection of diluent
- Methods of extraction
- Samples to be used
- Experimentation to finalize the method
- Selection of test concentration and injection volume
- Forced degradation studies (stress resting)
- Evaluation of stress testing
- Mass balance study
- Finalization of wavelengths
- Stability of solution
- System suitability
- Robustness of the method
- Relative response factor
- Quantification methods

Validation Parameters

Establishing proof that the method accurately, consistently, and reliably carries out its specified functions is the main objective of method validation [25]. The following is a description of the validation parameters in compliance with ICH guidelines:

SYSTEM SUITABILITY PARAMETERS

System Suitability

The system suitability test holds significant importance in the methodology, ensuring the appropriate functioning of the chosen chromatographic system

Efficiency / Column efficiency / Plate count (N):

The effectiveness of a chromatographic peak can be defined as an assessment of the spreading of the analyte band as it moves through the HPLC system and enters the column. Ideally, chromatographic peaks should resemble fine pencil lines, but the impacts of analyte molecules on the column result in their well-known "Gaussian" form. The peak dispersion of the HPLC column, indicative of column performance, is quantified by the plate number, denoted as "N. The distance between each plate, representing one equilibrium between the stationary and mobile phases of the column for each analyte component, is the plate. Consequently, the quality of separation improves with the number of theoretical plates present within a column [27].

Figure 3 explains how to calculate the number of theoretical plates (N), which is a measure of a chromatography column's efficacy.

$$N = 16\left(\frac{t_R}{w_b}\right)^2 = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

Where,

N = The no. of theoretical plates per meter i.e., Plate number or plate count

t_R = retention time, or the baseline's separation from the greatest of the peak of interest between the injection site and the perpendicular.

w1/2 = the peak of interest's width, measured in the same units as tR and determined at half peak height.

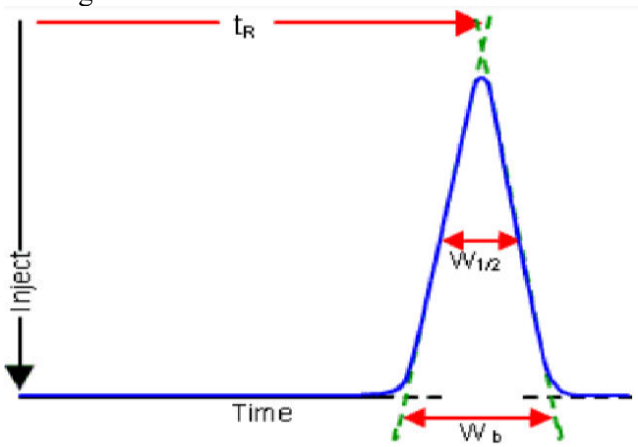

Figure 3. Sample peak showing Column efficiency.

The column plate number increases with the following factors:

- Well-packed columns ("quality" of the column)
- Lengthier columns
- Low flow rates (but not too low)
- Small column-packing particles
- Reduce mobile-phase viscosity and higher temperature
- Small sample molecules.

HETP (High Equivalent Theoretical Plates)

Similar to this idea, a fractionating tower of a certain length (L) will arrange its plates closer together as the number of plates increases. When high-efficiency separations are needed, this leads to a low plate height (H) and a high plate number (N), which indicate the distance between each plate.

In general, "plate height" is abbreviated as "HETP," or "Height Equivalent to a Theoretical Plate."

Generally speaking, the number of theoretical plates a column has is commonly used to determine its efficiency for a given approach. In the event that the plate number drops below a predetermined threshold, the chromatographer has the option to declare the specific process operational.

Retention Time (R_t)

The duration between the injection point and the elution point from a column is termed the retention time during detection. It signifies the time elapsed from injection until detection. Depending on the chemical composition, each compound within a sample comprising multiple compounds will exhibit a unique retention time in the column. Retention times are commonly quantified in seconds or minutes.

Retention Volume (V_r)

Multiplying the retention period by the flow rate yields the retention volume, which is the mobile phase volume required to elute 50% of the component from the column.

Retention volume (V_r) = Retention time X Flow rate

Relative Retention Time (RRT):

RRT, or relative retention time, serves as an indication of how long a sample has been retained in relation to the retention time of the standard. It is determined by comparing the retention times (RTs) of different compounds. The calculation for relative retention (RRT) is expressed in the following formula.

RRT = Standard RT / Sample RT

Retention / Capacity factor (k) / Mass distribution ratio (D_m):

The retention duration of the analyte sample molecule in relation to the column dead volume is measured by the capacity factor (k2) (Figure 4).

By dividing the retention time of an analyte on the column by the retention time of a non-retained molecule, one can find the retention factor, also known as the capacity factor.

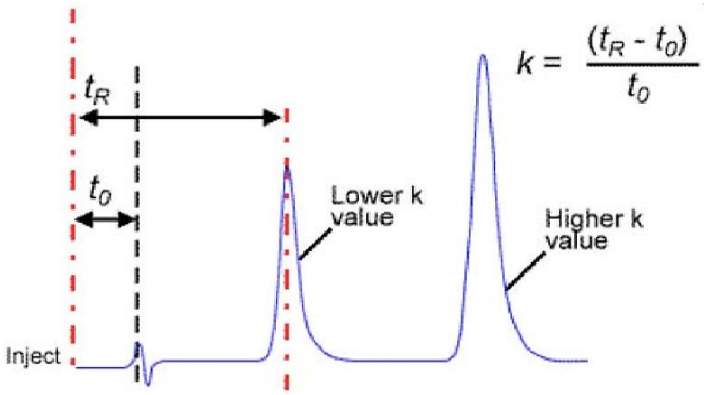

Figure 4. Determination of retention factor.

In Fig.4 ,

k= Retention factor or Capacity factor

t_R = retention time of the solute molecule

t_0 = retention time of an unretained component

Selectivity may be harmed if the peak elutes near the solvent front, as indicated by a low k value. It is advised to have a minimum k value of 1 for the peak of interest.

By altering the relative amount or composition of solvents in the mobile phase, the retention duration of the test material can be adjusted.

Shorter retention times on normal phase columns (NP-HPLC) and longer retention times on reverse phase columns (RP-HPLC) are often the results of increasing the amount of a more polar solvent.

Resolution Factor (R_s)

It quantifies the degree of separation between two compounds and the achievement of baseline separation. In HPLC analysis, the primary objective is to achieve optimal resolution in the shortest possible time.

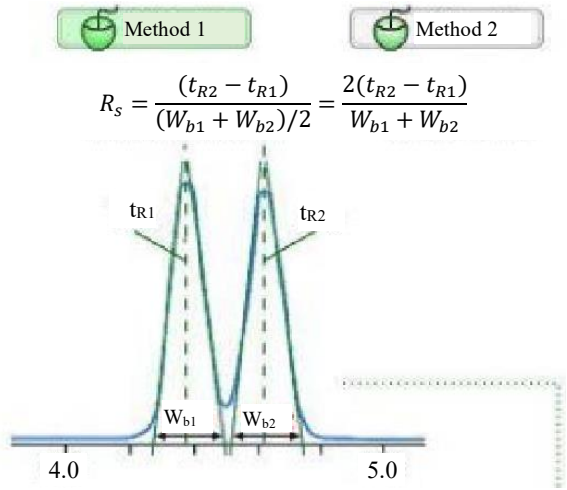

Figure 5. Calculation of chromatographic resolution (R_s).

The formula above can be used to determine the resolution between two peaks in a chromatogram that are similar in height (Figure 5).

Where,

***t*R1** = retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of first peak

***t*R2** = retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the second peak

***W*b1 and *W*b2** = the respective peak widths determined at half peak height and also measured in the same units as t_{R1} and t_{R2}.

For a baseline separation between peaks of comparable height, Rs must have a value of at least 2. (explained in **Figure 6).**

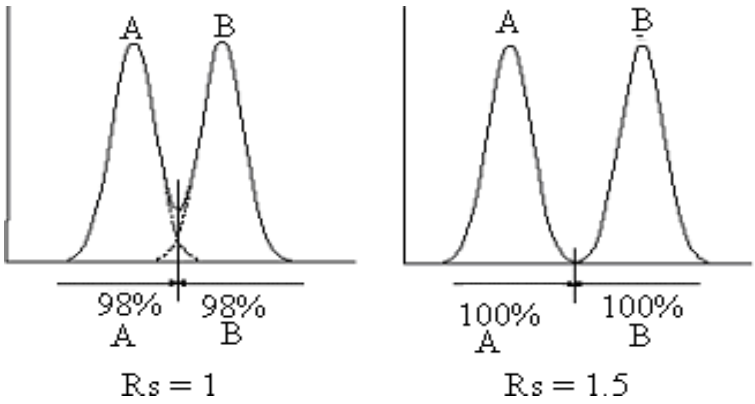

Figure 6. Extent of resolution peak asymmetry factor (A_s).

Every chromatographic peak should ideally be symmetrical, or Gaussian. The formula to calculate Peak Asymmetry is shown in the **Figure 7**.

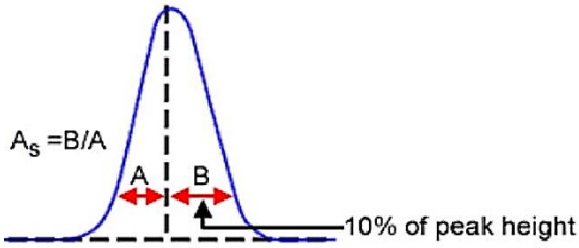

Figure 7. Determination of Peak asymmetry.

Reasons for Asymmetrical peaks:

- Effects of instrument dead-volume
- Adsorptive effects of the stationary phase
- Quality of the column packing
- Peaks may often show a tailing behaviour.

A peak is said to be tailing if its tail part (shown as distance 'B' in the image below) is wider than its front portion (shown as distance 'A' in the diagram below). Furthermore, in cases when the column is damaged, the sample concentration is excessive, or the sample contains "channels," a fronting peak form may appear (as explained in **Figure 7).**

Tailing Factor (T)

Peak symmetry is indicated by the Tailing Factor (T), where a perfectly symmetrical peak has a T value of unity. The T value rises with increasing tailing, and values smaller than 1 may be seen in certain cases. Integration precision may become less dependable as peak asymmetry increases (Figure 8).

$$T = \frac{W}{2 \times F}$$

Where,

T= Tailing factor
W = width of peak at 5% peak height
F = Time from width start point at 5% of peak height to the Rt
Rt = Retention time
Limit: d 2

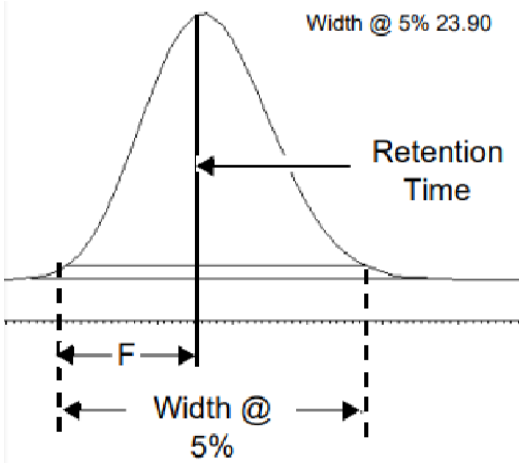

Figure 8. Calculation of tailing factor.

DEFINITIONS OF VALIDATION PARAMETERS

CALIBRATION CURVE or STANDARD CURVE using Calibration standards The calibration curve represents the correlation between the instrumental response and the known concentrations of the analyte. Also known as a standard curve, it should ideally exhibit a simple monotonic pattern, either strictly increasing or decreasing, to ensure reliable measurements and high accuracy. To construct a

calibration curve, it is recommended to use the same biological matrix as the samples in the expected study. This involves spiking the matrix with known concentrations of the analyte, referred to as standards.

QC Samples

Quality control (QC) samples are typically created in pairs at three distinct concentration levels, including one near 3 × LLOQ (low QC), one at the mid-range (middle QC), and one in proximity to the high end (high QC). These QC samples must be included in each assay run. The outcomes of the QC samples serve as the basis for approving or disapproving the run.

SPECIFICITY

The bioanalytical method's capacity to measure, estimate, and distinguish analytes in the presence of various interfering components, including metabolites, contaminants, degradants, and matrix constituents, defines its specificity.

SENSITIVITY

Sensitivity is characterized as the minimum analyte concentration measurable with acceptable accuracy and precision, commonly known as the Limit of Quantitation (LLOQ).

LINEARITY VS RANGE

Linearity

The lowest analyte concentration that can be measured with a reasonable degree of accuracy and precision—also known as the Limit of Quantitation (LLOQ)—determines sensitivity. The ability of the analytical method to yield test findings that are exactly proportionate to the concentration or amount of the analyte in the sample is what defines linearity, according to the ICH definition. A method's linearity is evaluated by looking at how closely the response vs concentration calibration plot resembles a straight line with respect to the standard curve..

Range

The range of the analytical techniques shows that the expected levels of precision, accuracy, and linearity are displayed. It represents the range between the higher and lower concentrations of the analyte in a sample.

ACCURACY

Measurement accuracy is defined by the extent to which the measured value mirrors the true value. In a high-accuracy study design, a sample with a known "real value" is assessed, and the measured value closely aligns with the true value.

PRECISION

The proximity of agreement among a series of measurements derived from multiple samplings of an identical homogeneous sample under comparable analytical conditions indicates the precision of an analytical method, which can be categorized into three groups [29].

Repeatability

This denotes precision under identical operating conditions and by the same analyst within a brief timeframe. Repeatability reflects the analytical consistency under consistent operating conditions during a short period (within assay, intra-assay) (Figure 9).

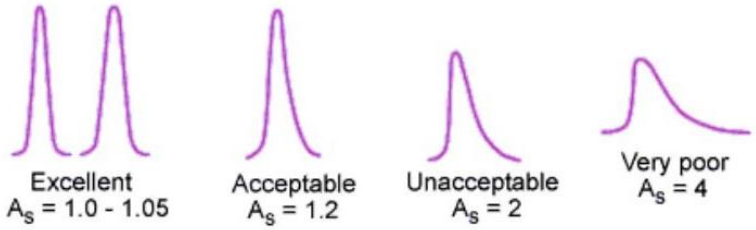

Figure 9. Different types of peaks - good & poor peak shapes.

Reproducibility

This refers to inter-laboratory studies, where reproducibility represents the precision observed between two or more laboratories, often termed collaborative or interlaboratory studies. A widely used statistical measure for precision is the sample standard deviation (S), as illustrated in the equation below:

$$\sqrt{\frac{(x-\mu)^{22}}{N}}$$

Where,

x = dataset
μ = Mean
N = no. of determination
• = Sum of the variables

The square of standard deviation is called as Variance (S2).

It is typically measured as coefficient of variation (%CV) or as relative standard deviation (RSD) of the replicate measurement.

%CV= Standard deviation/Mean X 100

RECOVERY

Recovery is characterized as the extraction efficiency within an analytical process, quantified based on the quantity or amount of the analyte transported through the sample extraction and processing phases [30].

LIMIT OF DETECTION (LOD)

Recovery is defined as the efficiency of the extraction process in an analytical procedure, indicating how much of the analyte successfully passes through the sample extraction and processing stages.

The following is the formula for calculating LOD:

LOD = 3.3 ′ /S

Where,

′ = Standard deviation of intercepts of calibration plots.
S = Slope of linearity plot.

LIMIT OF QUANTITATION (LOQ)

The lowest observed drug concentration (LOQ) in a sample is determined with the necessary accuracy and precision under predetermined experimental conditions. Similar to LOD, ICH also suggests the next four techniques for estimating LOQ.
The following is the formula for calculating LOD:

LOQ = 10 ′ /S (8)

Where,
′ = standard deviation of response
S = Slope of calibration plot

The lowest amount of analyte in a sample that permits a dependable and repeatable quantitative evaluation with the necessary precision and accuracy is known as the Lower Limit of Quantification, or LLOQ.

Quantification's lower bound (LLOQ) It is only appropriate to use the signal-to-noise ratio (S/N) to determine the lower limit of quantification (LLOQ) in chromatographic procedures when baseline noise is present. Generally speaking, a signal-to-noise ratio of 10:1 is seen to be enough for separating the analyte from the background noise.

Maximum quantification threshold (ULOQ) The highest analyte concentration in a sample that can be determined with reasonable accuracy and precision is known as the upper limit of quantification, or ULOQ.

Quantification Range

The concentration range encompasses samples at both the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), allowing for a dependable and consistently reproducible estimation with appropriate accuracy and precision utilizing a concentration-response relationship, typically represented by a calibration curve.

ROBUSTNESS

It is assessed by evaluating the ability of an analytical method to withstand minor, intentional adjustments in the parameters employed for method development, particularly in chromatographic conditions. In HPLC analysis, these procedural variables encompass factors such as flow rate, column temperature, sample temperature, pH, and the composition of the mobile phase, among other elements.

RUGUDNESS

The term robustness in an analytical method refers to the extent of uniformity in test results when thoroughly analyzing identical samples across diverse standard test conditions. An investigation into the robustness of the method involves introducing variations in experimental conditions. This includes scenarios such as transitioning from one column to another of a similar type and conducting diverse operations within the same laboratory while employing different equipment, reagent sources, chemicals, solvents, and involving various laboratories and analysts.

CONCLUSION

In conclusion, there has been a notable evolution in the field of chromatography from its beginnings with Mikhail Tswett to the complex uses of High-Pressure Liquid Chromatography (HPLC) in contemporary pharmaceutical analysis. Tswett's groundbreaking research established a method that is now a vital resource across numerous scientific fields. Chromatography's versatility and efficiency have increased due to ongoing developments, particularly in HPLC instrumentation and methodology.

With its several separation modes, including Reverse Phase Chromatography and Normal Phase Chromatography, HPLC is a very potent analytical tool. The precision with which solvents, columns, and mobile phases are chosen, along with the method's development and validation, highlights the accuracy and dependability of HPLC in pharmaceutical applications.

As this paper explains, chromatography adoption in pharmaceutical analysis is essential to guaranteeing the quality, potency, and purity of medicinal ingredients. This document provides a thorough guide for researchers, pharmaceutical analysts, and scientists involved in the creation of analytical methods. It covers everything from the investigation of separation principles to the in-depth analysis of system suitability criteria and validation parameters.

All things considered, the discipline of chromatography—and especially HPLC in particular—remains vibrant, influencing analytical chemistry and making a substantial contribution to advances in pharmaceutical sciences and other related fields.

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