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Recent Trends In Hplc Method Development: A Review

Authors: - Nandini P. Khirade*, Madhuri Theng

Gawande College of Pharmacy, Lavala Road, Sakharkherda

Tq: Sindlhedraja , Dist : Buldhana (MS) India

Author For Correspondence: -

Miss. Nandini P. Khirade

ABSTRACTS: -

Chromatography is the backbone of separation science and is being used in all research laboratories and pharmaceutical industries universally. High Performance Liquid Chromatography (HPLC) is the dominant separation technique to detect, separate and quantify the drug. A number of chromatographic parameters were analyzed to optimize the method like sample pretreatment, choosing mobile phase, column, detector selection. 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. The creation of an HPLC technique is influenced by the chemical structure of the molecules, the synthetic pathway, solubility, polarity, pH and pKa values, and the activity of functional groups, among other factors. Accuracy, accuracy, specificity, linearity, range, limit of detection, the limit of quantification, robustness, and system suitability testing are all included in the validation of an HPLC technique according to ICH Guidelines.

Keywords: - Chromatography, HPLC, Method validation, Method development.

INTRODUCTION: -

High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High performance liquid chromatography (HPLC) is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product. [1] The principle is that a solution of the sample is injected into a column of a porous material

(stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. [2] The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance. [3] The High Performance Liquid Chromatography is more versatile than gas chromatography since

(a) it is not limited to volatile and thermally stable samples, and

(b) the choice of mobile and stationary phases is wider. [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. 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. NPHPLC 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]

HPLC has numerous advantages like

- ✓ Simultaneous Analysis
- ✓ High Resolution
- ✓ High Sensitivity
- ✓ Good repeatability
- ✓ Small sample size
- ✓ Moderate analysis condition.
- ✓ Easy to fractionate the sample and purify.

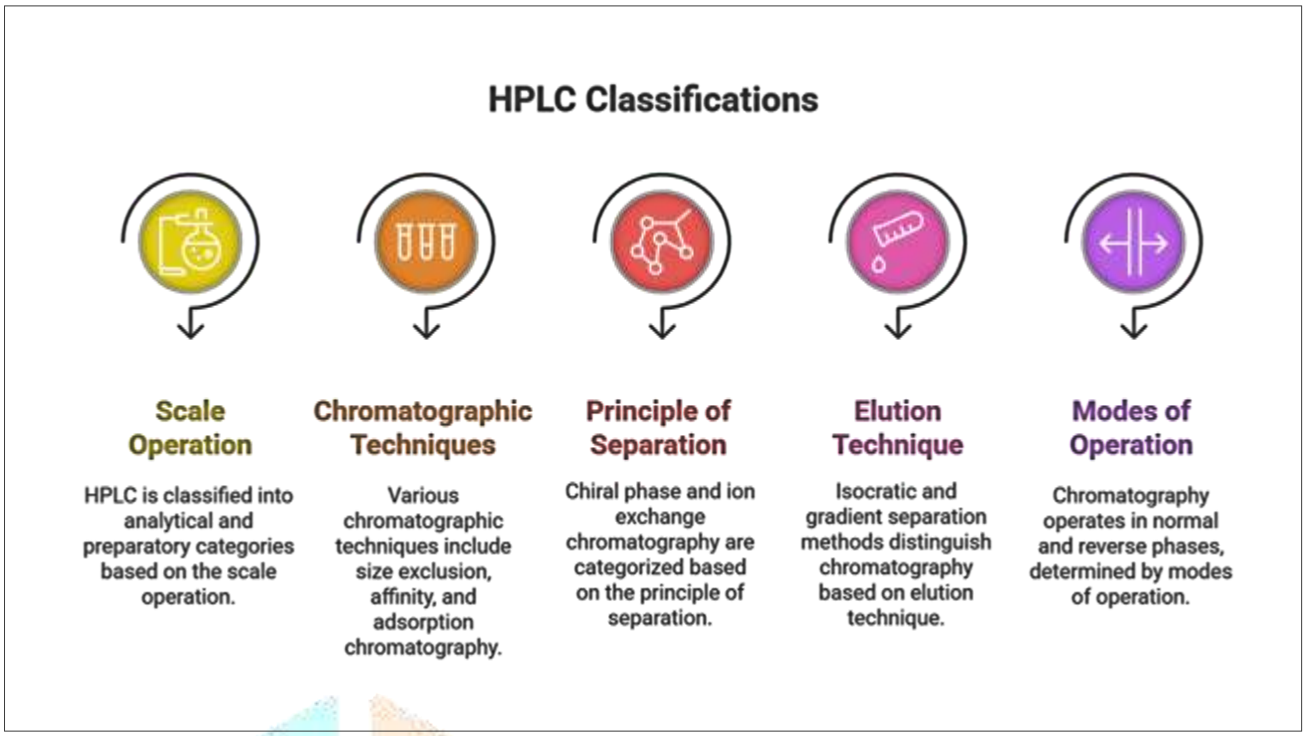

Figure 01: HPLC Classification

In high-performance liquid chromatography, a compound with lower affinity for the stationary phase travels faster and covers a longer distance, while a compound with higher affinity moves slower and covers a shorter distance. This differential migration facilitates effective separation and analysis of sample components. High performance liquid chromatography (HPLC) proves invaluable in pharmaceutical analysis, efficiently isolating and quantifying major medications, reaction impurities, synthesis intermediates, and degradants. As a preeminent analytical tool, HPLC excels in identifying, measuring, and separating diverse sample components soluble in liquid. Its precision is paramount for both quantitative and qualitative drug product analysis, playing a pivotal role in determining drug product stability. By offering a meticulous approach to characterizing pharmaceutical samples, HPLC stands as an indispensable technique in ensuring the quality and safety of medicinal formulations in the field of analytical chemistry. [7]

Liquid Chromatographic Separation Modes [8,9,10]

- **Normal phase chromatography**

Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. In normal phase chromatography, mobile phase is non-polar and stationary phase is polar. Hence, the station phase retains the polar analyte. An increase in polarity of solute molecules increases the adsorption capacity leading to an increased elution time. Chemically modified silica (cyanopropyl, aminopropyl and diol) is used as a stationary phase in this chromatography. For example, A typical column has an internal diameter of around 4.6 mm, and a length in the range of 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick longer to the polar silica than the non-polar compounds. Therefore, the non-polar ones will pass more quickly through the column.

- **Reversed phase chromatography**

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase In a mixture of components those analytes which are relatively less polar will be retained by the non-polar stationary phase longer than those which are relatively more polar. Therefore, the most polar component will elute first.

Based on principle of separation

- **Adsorption chromatography**

The principle of separation is adsorption. Separation of components takes place due to difference in the affinity between nonpolar stationary phase and polar mobile phase. This principle is seen in both normal phase and reverse phase mode, where adsorption takes place.

- **Ion exchange chromatography**

The principle of separation is ion exchange, which is reversible exchange of functional groups. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions. The retention is based on the attraction between solute ions and charged ions bound to the stationary phase. Cationic and anionic exchange resins are used where similar ions are excluded and opposite charge ions are retained. Thus, this is used for separation of charged molecule only. pH and ionic strength are used to control elution time.

- **Ion pair chromatography**

This technique is also called as RP Ion Pair Chromatography or Soap Chromatography. A reverse phase column is converted temporarily into a ion exchange column by using ion pairing agents like pentane or hexane or heptanes or octane sulphonic acid sodium salt, tetra methyl or tetraethyl ammonium hydroxide, etc. Strong acidic and basic compounds can be separated by this method by forming ion pairs with suitable counter ions.

- **Size exclusion chromatography**

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size using gels. The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. Soft gels like dextrose, agarose or polyacrylamide are used. Semi rigid gels like polystyrene, alkyl dextran in aqueous medium are also used. The mechanism of separation is by stearic and diffusion effects. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids.

- **Affinity chromatography**

Affinity chromatography uses the affinity of sample with specific stationary phases involving highly specific biochemical interaction for separation. This technique is mostly used in the field of Biotechnology, microbiology, Biochemistry, etc. It can be used to isolate proteins, enzymes, even antibodies from complex mixtures. Bio-affinity chromatography: This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The separation is based on specific reversible interaction of proteins with ligands, which are covalently attached to solid support on a bio-affinity matrix. It retains proteins by the interaction to the column-bound ligands. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipoledipole interaction, hydrophobic interaction, and the hydrogen bond. Proteins bound to a bioaffinity column can be eluted in two ways:

- Biospecific elution: it includes inclusion of free ligand in elution buffer which competes with column bound ligand.
- Aspecific elution: the change in pH, salt, etc. which weakens interaction of protein with column-bound substrate.

- **Chiral phase chromatography**

It involves separation of enantiomers, mainly optical isomers using chiral HPLC column that is packed with a chiral stationary phase. Different principles operate for different types of stationary phases and different samples. Enantiomers are separated based on the number and type of each interaction that occurs during their exposure to the chiral stationary phase.

Based on elution technique

Isocratic elution

A separation in which the mobile phase composition remains constant throughout the process is termed isocratic (meaning constant composition). In isocratic elution, peak width increases with retention time, linearly so peaks get very flat and broad. The same polarity or elution strength is used throughout the process.

Gradient elution

A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. In this technique a mobile phase combination of lower polarity or elution strength are used followed by gradually increasing the polarity or elution strength.

Based on scale of operation

Analytical HPLC

Where only the analysis of the samples are done. Recovery of the samples is not done because only very low quantity samples used

Preparative HPLC

Where the individual fractions of pure compound can be collected using fraction collector. The collected samples are reused.

Method development involves the following steps:

- ✓ Physicochemical Properties of the drug molecule.
- ✓ Selection of chromatographic conditions.
- ✓ Developing the approach of analysis.
- ✓ Sample preparations
- ✓ Method optimization
- ✓ Method validation

The physicochemical qualities of a therapeutic molecule are critical in method development. To develop a method, one must first evaluate the physical properties of the drug molecule, such as solubility, polarity, pKa, and pH. A compound's polarity is a physical property. It supports an analyst in evaluating the solvent and mobile phase composition. The polarity of molecules can be used to explain molecular solubility. Polar solvents, such as water, and nonpolar solvents, such as benzene, do not combine. In general, like dissolves like, which means that elements with comparable polarities dissolve in one other. The analyte's solubility is used to select diluents. The pH value is commonly used to define a substance's acidity or basicity. Choosing the correct pH for ionizable analytes frequently results in symmetrical and crisp peaks in HPLC. The pH value is defined as the negative of the logarithm to base 10 of the hydrogen ion concentration

$$pH = - \log10[H3O+].$$

Selecting an appropriate pH for ionizable analytes frequently results in symmetrical and sharp peaks in HPLC. In quantitative analysis, sharp, symmetrical peaks are required to achieve low detection limits, low relative standard deviations between injections, and predictable retention durations

Selection of chromatographic conditions

During the early stages of method development, a set of beginning conditions (detector, column, and mobile phase) is chosen to generate the sample's first "scouting" chromatograms. These are typically based on reversed phase separations on a C18 column with UV detection. At this point, a choice should be taken between establishing an isocratic or a gradient method.

Selection of Column

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

Buffer Selection

Different buffers, such as potassium phosphate, sodium phosphate, and acetate, were tested for system compatibility factors and overall chromatographic performance.

Selection of Mobile Phase

The mobile phase influences resolution, selectivity, and efficiency. The composition of the mobile phase (or the strength of the solvent) is critical in RP-HPLC separation. Acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) are regularly used solvents in RP-HPLC, with UV cut-offs of 190, 205, and 212nm, respectively. These solvents are miscible with water. During technique development, an acetonitrile-water mixture is the ideal initial choice for the mobile phase

Selection of Chromatographic mode

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

Developing the approach of analysis

The initial stage in developing an analytical method for RPHPLC is to select various chromatographic parameters such as mobile phase, column, mobile phase flow rate, and mobile phase pH. All of these characteristics are chosen based on trials, and they are then compared to the system suitability parameters. Typical system suitability parameters include, for example, a retention time of more than 5 minutes, a theoretical plate count of more than 2000, a tailing factor of less than 2, a resolution of more

than 5, and a percent R.S.D. of the area of analyte peaks in standard chromatograms of no more than 2.0 %. In the case of simultaneous estimation of two components, the detection wavelength is usually an isosbestic point. The laboratory combination is also analyzed to determine the practicability of the suggested method for simultaneous estimation. Following that, the marketed formulation is analyzed by diluting it up to the concentration range of linearity[11,12]

Method optimization

Identify the method's "weaknesses" and optimize the method using experimental design. Understand how the approach performs under varied settings, with different instrument setups, and with different samples. The majority of HPLC technique development optimization has been focused on the optimization of HPLC conditions. The compositions of the mobile phase and stationary phase must be considered. Optimization of mobile phase parameters is always prioritized above optimization of stationary phase parameters since it is considerably easier and more comfortable. Only the parameters that are likely to have a substantial effect on selectivity in the optimization must be studied to reduce the number of trial chromatograms involved. The different components of the mobile phase defining acidity, solvent, gradient, flow rate, temperature, sample quantities, injection volume, and diluents solvent type are primary control variables in the optimization of liquid chromatography (LC) procedures[13]

Method Validation

Validation is the evaluation and provision of objective evidence that the specified requirements for a given intended application are met. A way of assessing method performance and demonstrating that it fits a specific condition. In other words, it understands what your method is capable of producing, especially at low concentrations. Analytical methods need to be validated or revalidated. Before their introduction into routine use; Whenever the conditions change for which, the method has been validated

Components of Method Validation [14-17]

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 discovered. 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 value. 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. 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. The standard deviation or relative standard deviation of a sequence of data is commonly used to express the precision of an analytical technique. 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 laboratory.

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. 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. 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. 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.

$$LOD = 3.3 \times S / SD$$

and

$$LOQ = 10 \times S / SD$$

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

CONCLUSION

This article gives an idea that how to develop a method, what is validation, importance of validation, types of validation, how to perform validation process and its parameters to prove that the method is suitable for its intended use. Chromatography is a separation technique used to separate the individual compound from a mixture using a stationary and mobile phase. The discovery of chromatography is a millstone event in biomedical research. Chromatographic separation is based on the principles of adsorption, partition, ion exchange, molecular exclusion, affinity and Chirality. HPLC is a highly assertive analytical technique which uses sophisticated technologies that have been extensively practiced from decades. Modernizations such as ultrahigh-pressure liquid chromatography, nano liquid chromatography, liquid chromatography-mass spectrometry, chiral phase separations, core-shell columns, and novel stationary phases have helped HPLC to acquire higher performance levels; in diverse factors, yielding faster speed, higher resolution, greater sensitivity, and increased precision. The practice of HPLC is restricted to analyzers, but is now widely performed by students, chemists, biologists, production workers, and other novices in academia, research, and quality control laboratories.

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