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A Comprehensive Review on Analytical Method Development and Validation Using High Performance Liquid Chromatography (HPLC)

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Abstract: *Chromatography, while fundamentally a separation technique, is extensively utilized in chemical analysis, with High-Performance Liquid Chromatography (HPLC) being one of the most versatile methods available. In HPLC, analytes are separated by passing them through a column packed with micrometer-sized particles. Among the various HPLC techniques, reversed-phase chromatography (RP-HPLC) has become the most commonly used. This popularity stems from its simplicity, versatility, and ability to handle compounds of diverse polarity and molecular mass.*

Reversed-phase chromatography is particularly valued in both analytical and preparative applications within biochemical separation and purification. It is especially effective for molecules with hydrophobic characteristics, such as proteins, peptides, and nucleic acids, offering excellent recovery and resolution.This review highlights the critical role of RP-HPLC in analytical method development, exploring strategies for optimizing chromatographic parameters to achieve efficient method development. Key parameters, such as mobile phase composition, column selection, flow rate, temperature, and detection wavelength, are discussed to provide a comprehensive understanding of the process. The review aims to elucidate the significance of RP-HPLC in developing robust and reliable analytical methods, ensuring accurate and precise chemical analysis.

Keywords: HPLC, RP-HPLC, Analytical methods, Chromatographic parameter

I. INTRODUCTION

Modern analytical chemistry, playing a terminus role related to chemical innovation, began in the 18th century, especially in many aspects of chemistry such as chemical synthesis, qualitative and quantitative analysis [1]. Nowadays, analytical chemists are working on different instruments such as mass 4211spectroscopy (MS) Nuclear Magnetic Resonance (NMR) inductively coupled plasma, gas chromatography, HPLC and more recently UPLC. These analytical methods are not only useful for chemistry laboratories but also helpful for environmental and biological laboratories and have gained excellent benefits [2] . Amongst all the above analytical methods, HPLC has become most widely used analytical tools. In 1970s, there were various advancements in equipment and instrumentation. HPLC has started a revolution in biological, pharmaceutical chemistry and other fields of science [3]. The first commercially available UPLC system was demonstrated in 2004 [4]. Today ultra-performance liquid chromatography has overtaken HPLC as the standard platform [5].

High performance liquid chromatography (HPLC) has proven to one of the most and predominant technology used in analytical laboratories for the analysis of drugs worldwide during the past 30- plus years [6, 7].

One of the basic concerns for the growth of this technique is the packing material which effects the separations. In this separation mechanism the principal apply is Van Demeter equation, with which any student of chromatography is intimately familiar.

Smaller plate height value corresponds to greater peak efficiency, as more plates can occur over a fixed length of column [8,9]

Shorter diffusion path length of smaller particles allows a faster movement of the solute in and out of the particles. Because of this the solute/ analyte spends less time inside the particle where the peak diffusion occurs [10-11] (Figure 2)

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It has been noticed that using a shorter column length allows much higher sample throughput without losing chromatographic quality of the analytical method [12].

$$H=A+B/v+Cv \quad [13]$$

The above equation is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP, or column efficiency). And, since particle size is one of the variables, a Van Deemter curve can be used to investigate chromatographic performance. Where A, B and C are constants and v is the linear velocity, the carrier gas flow rate.

A= Eddy mixing
B =Axial diffusion
C=Solute's mass transfer

The A term is independent of velocity and represents "eddy" mixing. It is smallest when the packed column particles are small and uniform. The B term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by v. The C term is due to kinetic resistance to equilibrium in the separation process. The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus this term is proportional to v [14-16].

TYPES OF HPLC

Types of HPLC generally depend on phase system used in the process. Following types of HPLC generally used in analysis

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. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time. [17]

Reversed phase chromatography:

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.[18]

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. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides.[19]

Ion exchange chromatography: In Ionexchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, Ligandexchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc. [20]

Bio-affinity chromatography: Separation based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio-affinity matrix, retains proteins with interaction to the column-bound ligands Proteins bound to a bioaffinity column can be eluted in two ways:

- **Biospecific elution:** inclusion of free ligand in elution buffer which competes with column bound ligand.

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- **Aspecific elution:** change in pH, salt, etc. which weakens interaction protein with column-bound substrate. Because of specificity of the interaction, bio affinity chromatography can result in very high purification in a single step (10 - 1000-fold).[21]

II. PARAMETERS

For the accurate analysis of a compound, there are some parameters which are used as a standard for a particular compound. If there is a change occurs in the parameters the result may be affected greatly. The most commonly used parameters are internal diameter, particle size, pore size, pump pressure. For different compounds the parameters can be changed according to their nature and chemical properties.

Internal diameter:

The internal diameter (ID) of an HPLC column is a critical aspect that determines quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity. [22]

Particle size:

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.[23]

Pore size:

Many stationary phases are porous to provide greater surface area.Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface. [24]

Pump pressure:

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2 micrometres).[25]

III. APPLICATION

The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound. Preparative HPLC refers to the process of isolation and purification of compounds. This differs from analytical HPLC, where the focus is to obtain information about the sample compound.

Chemical Separations

It is based on the fact that certain compounds have different migration rates given a particular column and mobile phase, the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.[26]

Purification

Purification is defined as the process of separating or extracting the target compound from a mixture of compounds or contaminants. Each compound showed a characteristic peak under certain chromatographic conditions. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound. [27]

Identification

Generally assay of compounds are carried using HPLC. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed.[28]

Instrumentation of HPLC [29-50]

HPLC is a fast growing analytical technique for the analysis of drugs. Its simplicity, specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids. The rate of

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distribution of drugs between stationary and mobile phase is controlled by diffusion process. If diffusion is minimized, a faster and effective separation can be achieved. The technique of high performance liquid chromatography is so called because of its improved performance when compared to conventional column chromatography. Advances in column technology, high pressure pumping system and sensitive detectors have transformed liquid column chromatography into high efficient, accurate and highly resolved method of separation.

Different part of HPLC instrument are described here with as follows:

- Solvent delivery system
- Pumps
- Sample injection system
- Column
- Detectors
- Data system

Solvent Delivery System: The mobile phase is pumped under presser from or severs reservoir and flow through the column at constant rate. It is available to use directed mobile phase solvent mixture using a vacuum pump or other suitable means of direction that has no effect on composition of mixture. The choice of mobile phase is important in HPLC and the eluting power of mobile phase is determined by its overall polarity of stationary phase and nature of a sample components. For normal phase separations eluting power decrease with increase solvent polarity, optimum separation condition can often be achieved by making use of mixture of two solvents and gradients elution is frequently use where sample components vary widely in polarity.

Pump: Another most important component in HPLC is the pump, because it performance directly affect the retention time, Reproducibility and Detector sensitivity, Most of work in analytical HPLC is done using the presser between about 4000-1500 psi, but for analytical application in which the column 25-50cm in length (4.0-10mm id) and packed with particles of 5-10um. There are four important type of pump which have been used in HPLC to propel the liquid mobile phase through the system, these are

1) Pneumatic pump
2) Reciprocating pump
3) Displacement pump

Sample Injected System: The amount of the sample to be injected into column, after the final preparation step, can be determined by the fact that the component of interest have to be detected with adequate accuracy alter separation on the column the amount depends two main factors. The sensitivity of detector for these components .The extent of dilution undergone in the column.

Columns: Typical LC columns are 10, 15 and 25 cm in length and are filled with extremely small diameter (3,5or 10mm) particles. The internal diameter of the columns is usually or 0.4 mm; this is considered the best compromise among sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected, larger diameter column may be needed. For many applications, precise control of column temperature is not necessary, and HPLC separations are performed under ambient conditions. However, temperature control can enhance chromatographic reproducibility and afford opportunities to improve separation efficiency, and UHPLC techniques typically require column heating. Modern instruments can be equipped with column heaters/ovens that control column temperature to a few tenths of a degree from near ambient to 150°C. Occasionally, column chillers are also employed, with some column compartments spanning controlled temperature ranges of 4–100°C

Detector (S): By passing the column effluent through the detector, some chemical or physical property of the analyse transduces to an electrical signal, and the solutes are monitored as they are eluted from the column. The electrical signal, which can be amplified and manipulated by suitable electronics, is proportional to the level of some property of the mobile phase or solutes HPLC detectors are classified as either bulk property detectors, which respond to a bulk property of the eluent (e.g., refractive index (RI) or conductivity), or solute property detectors, which respond to some property of the analytic (e.g., UV absorbance). In either case, the response of the detector is modulated by the presence and amount of the analytic. Solute property detectors tend to be more sensitive than bulk property detectors, on the

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order of 1000 times or more. Ideal characteristics of an HPLC detector are high sensitivity, good stability, linearity, short response time, reliability, non-destructiveness, ease of use, and low dead volume. It should be noted that HPLC detectors may be used in-line with each other, allowing for the use of multiple detection techniques in a single analysis. Many types of analytical techniques have been applied to HPLC with varying degrees of success.

Data System: Since the detector signal is electronic, use of modern data acquisition techniques can aid in the signal analysis. In addition, some system can store data in a retrievable from for highly sophisticated computer analysis at a later time. The main goal in using electronic data system is to increase analysis accuracy and precision, while reading operation attention [29-50]

Other applications of HPLC:[50-65]

Pharmaceutical applications

- Tablet dissolution study of pharmaceutical dosages form.
- Shelf-life determinations of pharmaceutical products
- Identification of active ingredients of dosage forms
- Pharmaceutical quality control

Environmental applications

- Detection of phenolic compounds in Drinking Water
- Identification of diphenhydramine in sedimented samples
- Bio-monitoring of pollutant

Forensics

- Quantification of the drug in biological samples.
- Identification of anabolic steroids in serum, urine, sweat, and hair
- Forensic analysis of textile dyes.
- Determination of cocaine and metabolites in blood

Clinical

- Quantification of ions in human urine Analysis of antibiotics in blood plasma.
- Estimation of bilirubin and bilivirdin in blood plasma in case of hepatic disorders.
- Detection of endogenous neuropeptides in extracellular fluids of brain

Food and Flavor

- Ensuring the quality of soft drink and drinking water.
- Analysis of beer.
- Sugar analysis in fruit juices.
- Analysis of polycyclic compounds in vegetables.
- Trace analysis of military high explosives in agricultural crops.

Analytical method development using RP-HPLC:

Methods of analysis are routinely developed, improved, validated, collaboratively studied and applied. Compilations of these developed methods then appear in large compendia such as USP, BP and IP, etc. In most cases as desired separation can be achieved easily with only a few experiments. In other cases a considerable amount of experimentation may be needed. However, a good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result(s). The development of a method of analysis is usually based on prior art or existing literature using almost the same or similar experimentation. The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final need or requirement of the method. [66-70]

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Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why. In the HPLC method development stage, decisions regarding choice of column, mobile phase, detectors, and method quantitation must be considered. So development involves a consideration of all the parameters pertaining to any method. [71]

Therefore, development of a new HPLC method involves selection of best mobile phase, best detector, best column, column length, stationary phase and best internal diameter for the column.[72] The analytical strategy for HPLC method development contains a number of steps . [73-75]

Sample collection and preparation:

The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually effect the separation so long as the volume of the sample loaded is small compared to the column volume. The only effect when large sample volumes are applied may be an extra peak or two eluting in the void volume after sample injection.

Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column. The aim of sample preparation is a sample aliquot that,

- Is relatively free of interferences,
- Will not damage the column, and
- Is compatible with the intended HPLC method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution.[76]

Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column and encompasses the various operations. All of these operations form an important part of sample preparation and have a critical effect on the accuracy, precision, and convenience of the final method.

Measurement: The measurement of a given analyte can often be divided into a separation step and a detection step.

Separation: Analytes in a mixture should preferably be separated prior to detection. Simple LC consists of a column with a fritted bottom containing the stationary phase in equilibrium with a solvent. The mixture to be separated is loaded on to the top of the column followed by more solvent. The different components in the column pass at different rates due to difference in their partitioning behaviour between mobile liquid phase and stationary phase.[77-85]

Detection: It is essential to use reagents and solvents of high purity to ensure minimum detection limits for optimum sensitivity. All organic solvents and many additives, such as ion pairing agents, absorb in the UV range and the detection limit is related to the wavelength. A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles for detecting the analytes after the chromatographic separations. However, only about twelve of them can be used effectively for LC analysis and, of those twelve, only four are in common use. The four dominant detectors used in LC analysis are the UV detector (fixed and variable wavelength), the electrical conductivity detector, the fluorescence detector and the refractive index detector. These detectors are employed in over 95% of all LC analytical applications. The choice of detector depends on the sample and the purpose of the analysis.[85-90]

Critical Parameters in Reversed Phase Chromatography

Classifying the sample:

The first step in method development is to characterize the sample as regular or spherical. Regular samples are a mixture of small molecules that can be separated using more or less standardized starting conditions. Separations in regular samples respond in predictable fashion to change in solvent strength (%B) and type (Acetonitrile, methanol) or temperature. A 10% decrease in %B increases retention by about threefold, and selectivity usually changes as either %B or solvent type is varied.

It is possible to separate many regular samples just by varying solvent strength and type. Therefore, RPC method development for all regular samples (both neutral and ionic) can be carried out initially in the same way

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The column/Stationary phase: Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended.[90-95]

Some important factors need to be considered while selecting column in RP-HPLC.

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C2), butylsilane (C4), octylsilane (C8), octadecylslane (C18), base deactivated silane (C18) BDS phenyl, cyanopropyl (CN), nitro, amino, etc. Generally longer columns provide better separation due to higher theoretical plate numbers. As the particle size decreases the surface area available for coating increases. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability.

- Reasonable resolution in initial experiments,
- Short run time,
- An acceptable pressure drop for different mobile phases

Mobile phase: In many cases, the colloquial term used for the mobile phases in reversed phase chromatography is "buffer". However, there is little buffering capacity in the mobile phase solutions since they usually contain strong acids at low pH with large concentrations of organic solvents. Adequate buffering capacity should be maintained when working closer to physiological conditions. [96]

Organic solvent: The organic solvent (modifier) is added to lower the polarity of the aqueous mobile phase. The lower the polarity of the mobile phase, the greater its eluting strength in reversed phase chromatography. Although a large variety of organic solvents can be used in reversed phase chromatography, in practice only a few are routinely employed. The two most widely used organic modifiers are acetonitrile and methanol, although acetonitrile is the more popular choice. Isopropanol (2-propanol) can be employed because of its strong eluting properties, but is limited by its high viscosity which results in lower column efficiencies and higher backpressures. Both acetonitrile and methanol are less viscous than isopropanol. All three solvents are essentially UV transparent. This is a crucial property for reversed phase chromatography since column elution is typically monitored using UV detectors. Acetonitrile is used almost exclusively when separating peptides. Most peptides only absorb at low wavelengths in the ultra-violet spectrum (typically less than 225 nm) and acetonitrile provides much lower background absorbance than other common solvents at low wavelengths. [97]

Ion suppression: The retention of peptides and proteins in reversed phase chromatography can be modified by mobile phase pH since these particular solutes contain ionisable groups. The degree of ionisation will depend on the pH of the mobile phase. The stability of silica-based reversed phase media dictates that the operating pH of the mobile phase should be below pH 7.5. The amino groups contained in peptides and proteins are charged below pH 7.5. The carboxylic acid groups, however, are neutralised as the pH is decreased. The mobile phase used in reversed phase chromatography is generally prepared with strong acids such as trifluoroacetic acid (TFA) or orthophosphoric acid. These acids maintain a low pH environment and suppress the ionisation of the acidic groups in the solute molecules.[98]

pH: pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Reversed phase separations are most often performed at low pH values, generally between pH 2-4. The low pH results in good solubility of the sample components and ion suppression, not only of acidic groups on the sample molecules, but also of residual silanol groups on the silica matrix. Acids such as trifluoroacetic acid, heptafluorobutyric acid and ortho-phosphoric acid in the concentration range of 0.05 - 0.1% or 50 - 100 mM are commonly used. Mobile phases containing ammonium acetate or phosphate salts are suitable for use at pH's closer to neutrality. Note that phosphate buffers are not volatile.[99]

Detectors: A large numbers of detectors are used for RP-HPLC analysis. However, among these the five dominant detectors used in LC analysis are the electrical conductivity detector, the fluorescence detector, the refractive index

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detector, mass spectrometry detector and the UV detector (fixed and variable wavelength). These detectors are employed in over 95% of all LC analytical applications.[100]

IV. CONCLUSION

Analytical methods development plays important roles in the discovery, development and manufacture of pharmaceuticals. RP-HPLC is probably the most universal, most sensitive analytical procedure and is unique in that it easily copes with multi-component mixtures. While developing the analytical methods for pharmaceuticals by RPHPLC, must have good practical understanding of chromatographic separation to know how it varies with the sample and with varying experimental conditions in order to achieve optimum separation. To develop a HPLC method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance.

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