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Importance of RP-HPLC in Analytical Method Development: A Review

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ABSTRACT: *Chromatography, although primarily a separation technique, is mostly employed in chemical analysis in which High-performance liquid chromatography (HPLC) is an extremely versatile technique where analytes are separated by passage through a column packed with micrometer-sized particles. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass. Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography with excellent recovery and resolution. This review covers the importance of RP- HPLC in analytical method development and their strategies along with brief knowledge of critical chromatographic parameters need to be optimized for an efficient method development.*

I. INTRODUCTION

Chromatography is probably the most powerful analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in mixture by single analytical procedure [1, 2]. High-performance liquid chromatography (HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture [3]. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. [4] The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile). [5, 6] Separation has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. [6]

II. TYPES OF HPLC

Types of HPLC generally depend on phase system used in the process. [6, 7] Following types of HPLC generally used in analysis.

1. **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.

2. **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-

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polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

3. **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. Ion exchange chromatography: In Ion exchange 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, Ligand exchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc. [6, 7]
4. **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.
   - Aspecific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.

Because of specificity of the interaction, bioaffinity chromatography can result in very high purification in a single step (10 - 1000-fold).

III. RP HPLC

Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography with excellent recovery and resolution [9]. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed-phase mode. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass [9, 10, 11].

1. **Theory of Reversed Phase Chromatography:**
   Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character can be separated by reversed phase chromatography with excellent recovery and resolution.[12]
   The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e., the stationary phase. The actual nature of the hydrophobicbinding interaction itself is a matter of heated debate [13] but the conventional wisdom assumes the binding interaction to be the result of a favourable entropyeffect. The initial mobile phase binding conditions usedin reversed phase chromatography are primarily aqueous which indicates a high degree of organised water structure surrounding both the solute molecule and the immobilised ligand.
   As solute binds to the immobilised hydrophobic ligand, the hydrophobic area exposed to the solvent is minimised. Therefore, thedegree of organised water structure is diminished with a corresponding favourable increase in system entropy.In this way, it is advantageous from an energy point of view for the hydrophobic moieties, i.e., solute and ligand, to associate.[14]

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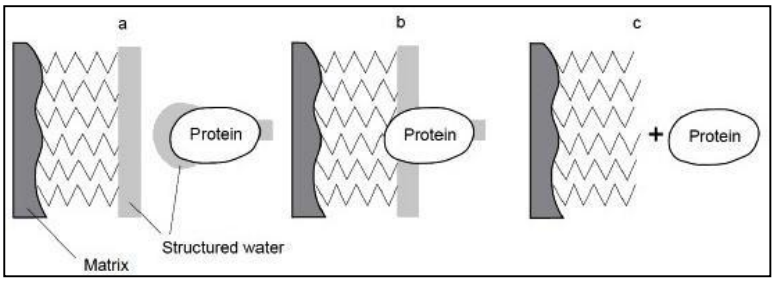

FIGURE 1: INTERACTION OF A SOLUTE WITH A TYPICAL REVERSED PHASE MEDIUM

Water adjacent to hydrophobic regions is postulated tobe more highly ordered than the bulk water. Part of this 'structured' water is displaced when the hydrophobic regions interact leading to an increase in the overall entropy of the system. Separations in reversed phase chromatography dependon the reversible adsorption/desorption of solutemolecules with varying degrees of hydrophobicity to a hydrophobic stationary phase. The majority of reversed phase separation experiments are performed in several fundamental steps as illustrated in Figure 2.

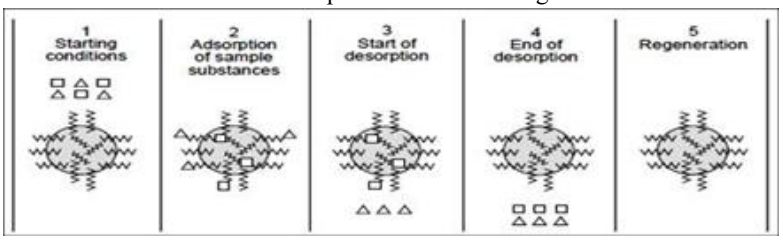

FIGURE 2: PRINCIPLE OF REVERSED PHASE CHROMATOGRAPHY WITH GRADIENT ELUTION

Choice of Separation Medium: The proper choice of reversed phase medium is critical for the success of a particular application. This choice should be based on the following criteria:

- The unique requirements of the application,including scale and mobile phase conditions.
- The molecular weight, or size of the samplecomponents.
- The hydrophobicity of the samplecomponents.
- The class of sample components.

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. Thedevelopment 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.

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

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. [15,16] The analytical strategy for HPLC method development contains a number of steps [17], as shown in figure 3.

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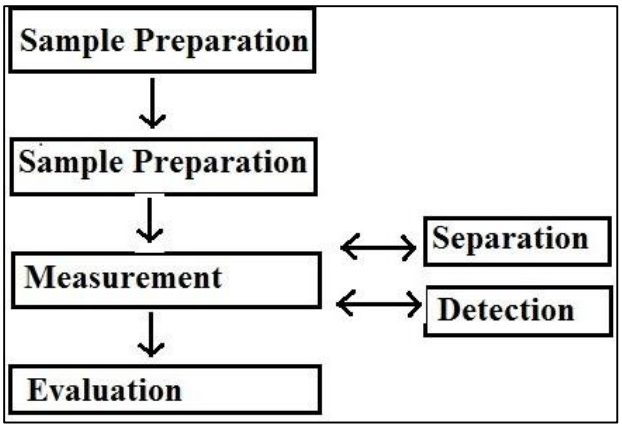

FIGURE 3: A TYPICAL STRATEGY FOR HPLC METHOD DEVELOPMENT

Sample Collection and Preparation:

The sample shouldideally 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 affect 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 HPLCanalysis, intended to provide a reproducible andhomogenous 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 thatis, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution.

Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column and encompasses the various operations summarized in **table 1**. All of these operations form an important part of sample preparation and have a critical effect on the accuracy, precision, and convenience of the finalmethod.

Measurement: The measurement of a given analytecan 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 inthe column pass at different rates due to difference in their partitioning behavior between mobile liquid phase and stationary phase. [17,18]

IV. SAMPLE PRETREATMENT OPTIONS

- **Sample Collection:** Obtain representative sample using statistically valid processes.
- **Sample Storage and Preservation:** Use appropriate inert, tightly sealed containers; be especially careful with volatile, unstable, or reactive materials; biological samples may require freezing.
- **Preliminary Sample Processing:** Sample must be in a form for more efficient sample pretreatment (e.g., drying, sieving, grinding, etc.); finer dispersed samples are easier to dissolve or extract.
- **Weighing or Volumetric Dilution:** Take necessary precautions for reactive, unstable, or biological materials; for dilution, use calibrated volumetric glassware.
- **Alternative Sample Processing Methods:** Solvent replacement, desalting, evaporation, freeze drying, etc.
- **Removal of Particulates:** Filtration, solid-phase extraction, centrifugation.

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- **Sample Extraction:** Different methods used for liquid samples and solid samples.
- **Derivatization:** Used mainly to enhance analyte detection; sometimes used to improveseparation.

Detection: It is essential to use reagents and solventsof 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.[19] 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. [20]

V. 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 (<2000 Daltons) 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.

The Column/Stationary Phase: Selection of thestationary 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 [21, 22].

VI. FACTORS AFFECTING COLUMN EFFICIENCY

- **Column Length:** Choose longer columns for enhanced resolution and choose shorter column for shorter analysis time, lower back pressure and fast equilibration.
- **Column Internal Diameter:** Choose wider diameter column for greater sample loading.
- **Particle Shape:** Choose spherical particles for lower back pressure, column stability and greater stability.
- **Particle Size:** Choose larger particle (5-10 µm) for sample with structurally different compounds.
- **Pore Size:** Choose a pore size of 150? or less for sample with molecular weight less than 2000.
- **Surface Area:** Choose end capped packing to eliminate unpredictable secondary interaction with the base materials.
- **Carbon Load:** Choose high carbon loads for greater column capacities and resolution.

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. It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. Thisis due to the fact that the siloxane linkage area cleaved below pH 2.0; while at pH valued above 8.0 silica may dissolve. [23]

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Absorbance: An UV-visible detector is based on theprinciple of absorption of UV visible light from the effluent emerging out of the column and passed through a photocell placed in the radiation beam. UV detector is generally suitable for gradient elution work.Most compounds adsorb UV light in the range of 200- 350 A°. The mobile phase used should not interfere in the peak pattern of the desired compound hence it should not absorb at the detection wavelength employed. [24]

Selectivity: Selectivity (α) is equivalent to the relative retention of the solute peaks and, unlike efficiency,depends strongly on the chemical properties of thechromatography medium.

Temperature: Temperature can have a profound effecton reversed phase chromatography, especially for low molecular weight solutes such as short peptides and oligonucleotides. The viscosity of the mobile phase used in reversed phase chromatography decreases with increasing column temperature. Since mass transport of solute between the mobile phase and the stationary phase is a diffusion-controlled process,decreasing solvent viscosity generally leads to moreefficient mass transfer and, therefore, higher resolution. Increasing the temperature of a reversed phase column is particularly effective for low molecularweight solutes since they are suitably stable at the elevated temperatures [8].

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 detector, mass. Wide linear dynamic range (this simplifiesquantitation).

- Low dead volume (minimal peak broadening).
- Cell design that eliminates remixing of theseparated bands.
- Insensitivity to changes in type of solvent, flowrate, and temperature.
- Operational simplicity and reliability.
- Tunability, so that detection can be optimizedfor different compounds.
- Large linear dynamic range.
- Nondestructive to sample.

VII. APPLICATIONS

- Designing a biochemical purification
- Purification of platelet-derived growth factor(PDGF)
- Purification of cholecystokinin-58 (CCK-58) frompig intestine
- Purification of recombinant human epidermalgrowth factor
- Process purification of inclusion bodies.

VIII. CONCLUSION

Analytical methods development playsimportant roles in the discovery, development and manufacture of pharmaceuticals. RP-HPLC is probably the most universal, most sensitive analytical procedureand is unique in that it easily copes with multi- component mixtures. While developing the analytical methods for pharmaceuticals by RP-HPLC, 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 theeffort should be spent in method development andoptimization as this will improve the final method performance.

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