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A REVIEW ON: RP-HPLC

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ABSTRACTS

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 micrometre-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.

KEYWORDS: HPLC, RP-HPLC, Analytical methods, Chromatographic parameters.

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] 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.[4]

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.[5 6,7]

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.[8]

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 hydrophobic binding interaction itself is a matter of heated debate.[9] but the conventional wisdom assumes the binding interaction to be the result of a favourable entropy effect. The initial mobile phase binding conditions used in 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, the degree 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.[10] **(Figure 1).**

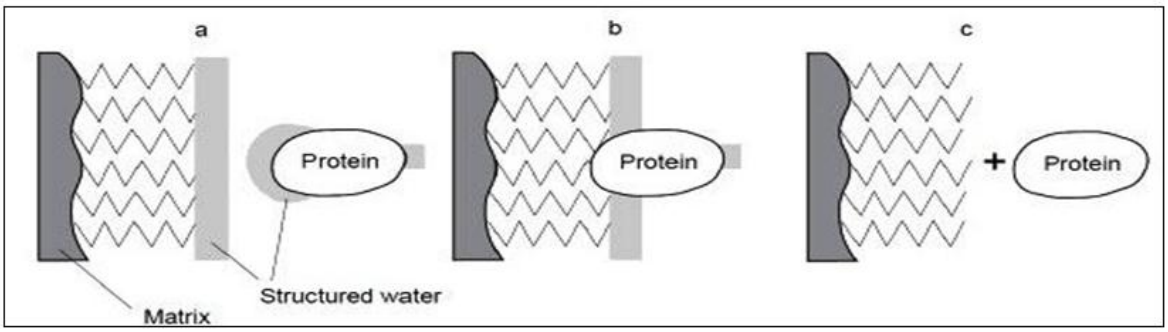

Figure 1: Interaction of a Solute with a Typical Reversed Phase Medium.

Water adjacent to hydrophobic regions is postulated to be 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 depend on the reversible adsorption/desorption of solute molecules 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**.

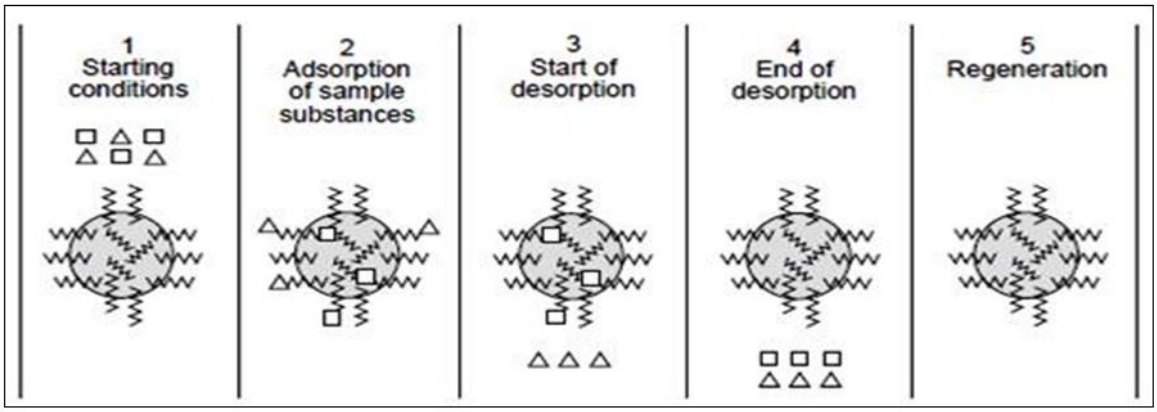

Figure 2: Principle of Reversed Phase Chromatography with Gradient Elution.

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:

1) The unique requirements of the application, including scale and mobile phase conditions
2) The molecular weight, or size of the sample components.
3) The hydrophilicities of the sample components.
4) 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. 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.

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.

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.[11,12] The analytical strategy for HPLC method development contains a number of steps,[13] as shown in **figure 3**.

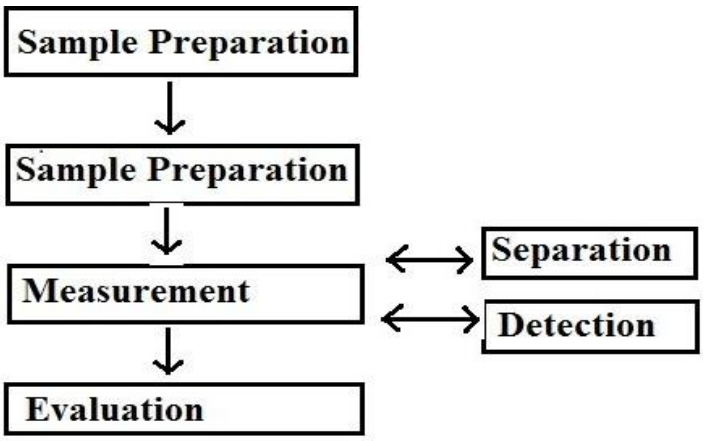

Figure 3: A Typical Strategy for HPLC Method Development.

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.[12]

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 final method.[12]

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 behavior between mobile liquid phase and stationary phase.[13,14]

Table 1: Sample Pretreatment options.

S.no.	Option	Comment
1.	Sample collection	Obtain representative sample using statistically valid processes
2.	Sample storage and preservation	Use appropriate inert, tightly sealed containers; be especially careful with volatile, unstable, or reactive materials; biological samples may require Freezing.
3.	Preeliminary 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
4.	Weighing or volumetric dilution	Take necessary precautions for reactive, unstable, or biological materials; for dilution, use calibrated volumetric glasswares.
5.	Alternative sample processing methods	Solvent replacement, desalting, evaporation, freeze drying, etc.
6.	Removal of particulates	Filtration, solid-phase extraction, centrifugation.
7.	Sample extraction	Different methods used for liquid samples and solid samples
8.	Derivatization	Used mainly to enhance analyte detection; sometimes used to improve Separation.

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.[15] 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.[16]

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 three fold, 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.[11,12]

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.[5,12,13,17,18] Some important factors need to be considered while selecting column in RP- HPLC are summarized in **table 2**.

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.

Table 2: factors affecting column efficience.

Factor(s)	Effect on column efficiency
Column length	*Choose longer columns for enhanced resolution *Choose shorter column for shorter analysis time, lower back pressure and fast equilibration and less solvent consumption
Column internal diameter	*Choose wider diameter column for greater sample loading *Choose narrow column for more sensitive and reduced mobile phase consumption
Particle shape	*Choose spherical particles for lower back pressure, column stability and greater stability *Choose irregular particles when high surface area and high capacity is required
Particle size	*Choose smaller particle (3-4 µm) for complex mixture with similar components *Choose larger particle (5-10 µm) for sample with structurally different compounds *Choose very large particle (15-20 µm) for preparative separation
Pore size	*Choose a pore size of 150?or less for sample with molecular weight less than 2000 *Choose a pore size of 300?or less for sample with molecular weight greater than 2000
Surface area	*Choose end capped packing to eliminate unpredictable secondary interaction with the base materials *Choose non-end capped phase for selectivity differences for polar compounds by controlling secondary interaction
Carbon load	*Choose high carbon loads for greater column capacities and resolution *Choose low carbon loads for fast analysis

The column should provide,

- Reasonable resolution in initial experiments,
- Short run time,
- An acceptable pressure drop for different mobile phases.[12]

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.

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.

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 ortho- phosphoric acid. These acids maintain a low Ph environment and suppress the ionisation of the acidic groups in the solute molecules. Varying the concentration of strong acid components in the mobile phase can change the ionisation of the solutes and, therefore, their retention behavior.

The major benefit of ion suppression in reversed phase chromatography is the elimination of mixed mode retention effects due to ionisable silanol groups remaining on the silica gel surface. The effect of mixed mode retention is increased retention times with significant peak

broadening (**Figure 4**).

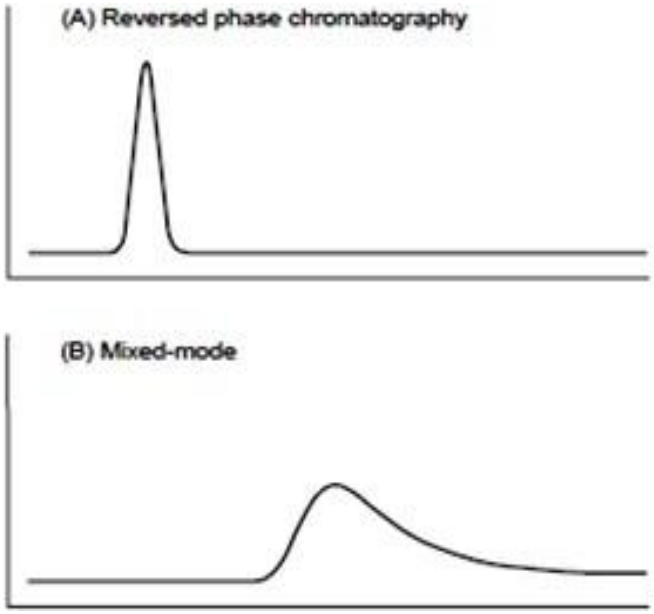

Figure 4: Typical effects of mixed-mode retention.

(Peaks are broader and skewed, and retention time increases)

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. This is due to the fact that the siloxane linkage area cleaved below pH 2.0; while at pH valued above 8.0 silica may dissolve.[12,19]

Absorbance

An UV-visible detector is based on the principle 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.[20]

Selectivity

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

The selectivity, α, for two peaks is given by; $\alpha = k2'/k1' = V2 - V0/V1 - V0 = V2/V1$

Where V1 and V2 are the retention volumes, and k2′

/k1′ are the capacity factors, for peaks 1 and 2 respectively, and V0 is the void volume of the column. Selectivity is affected by the surface chemistry of the reversed phase medium, the nature and composition of the mobile phase, and the gradient shape (**Figure 5**).

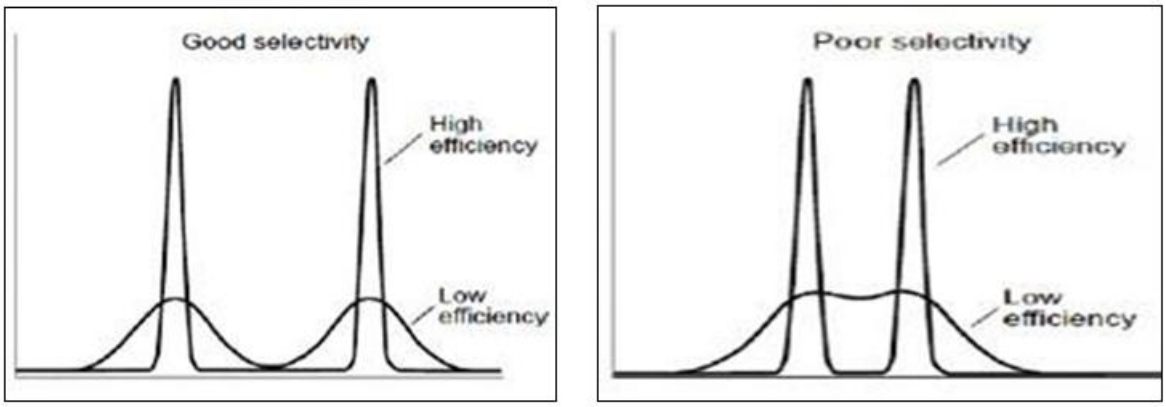

Figure 5: The effect of selectivity and efficiency on resolution.

Both high column efficiency and good selectivity are important to overall resolution. However, changing the selectivity in a chromatographic experiment is easier than changing the efficiency. Selectivity can be changed by changing easily modified conditions like mobile phase composition or gradient shape.

Viscosity

Solvent of lowest possible viscosity should be used to minimize separation time. An added advantage of low viscosity is that high efficiency theoretical plate (HETP) values are usually lower than with solvents of higher viscosity, because mass transfer is faster. Viscosity should be less than 0.5 centipoise, otherwise high pump pressures are required and mass transfer between solvent and stationary phase will be reduced.

Temperature

Temperature can have a profound effect on 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 more efficient mass transfer and, therefore, higher resolution. Increasing the temperature of a reversed phase column is particularly effective for low molecular weight 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 spectrometry detector and the UV detector (fixed and variable wavelength). These detectors are employed in over 95% of all LC analytical applications.[21-22]

The detector selected should be chosen depending upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction, etc. Characteristics that are to be fulfilled by a detector to be used in HPLC determination are:

- High sensitivity, facilitating trace analysis.
- Negligible baseline noise to facilitate lower detection.
- Low drift and noise level.
- Wide linear dynamic range (this simplifies quantitation).
- Low dead volume (minimal peak broadening).
- Cell design that eliminates remixing of the separated bands.
- Insensitivity to changes in type of solvent, flow rate, and temperature.
- Operational simplicity and reliability.
- Tunability, so that detection can be optimized for different compounds.
- Large linear dynamic range.
- Non destructive to sample.

Applications

- Designing a biochemical purification.

- Purification of platelet-derived growth factor (PDGF).
- Purification of cholecystokinin-58 (CCK-58) from pig intestine.
- Purification of recombinant human epidermal growth factor.
- Process purification of inclusion bodies.

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 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 the effort should be spent in method development and optimization as this will improve the final method performance.

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