International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 4(4): July-August 2014

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Pharmaceutical Sciences **Review Article……!!!**

Received: 20-06-2014; Revised; Accepted: 16-07-2014

A REVIEW ON HPLC AND RP-HPLC ANALYSIS METHOD

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Keywords:

HPLC, Elution, Detector, Reverse phase, Column

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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. High performance liquid chromatography (HPLC) is an important qualitative and quantitative technique, generally used for the estimation of pharmaceutical and biological samples. It is the most versatile, safest, dependable and fastest chromatographic technique for the quality control of drug components. 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 article was prepared with an aim to review different aspects of HPLC, such as principle, types, instrumentation and application.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.

INTRODUCTION

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. [1] 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. [2] 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). [2,3] Sepration has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. [3] The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.

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 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[4-6].

TYPES OF HPLC

Types of HPLC generally depend on phase system used in the process. [3, 7] 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.

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.

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. [3, 7]

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).

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.

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.

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.

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). [3, 7]

INSTRUMENTATION

Injection of the sample: Septum injectors are available; using which sample solution is injected. Sample can be injected when the mobile phase is flowing or it is stopped. A new advanced rotary valve and loop injector can be used to produce reproducible results.

The detector: There are several ways of detecting when a substance has passed through the column. Generally UV spectroscopy is attached, which detect specific compounds.

Many organic compounds absorb UV light of various wavelengths. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.

Interpreting the output from the detector: The output is recorded as a series of peaks, each one representing a compound in the mixture passing through the detector and absorbing UV light. The area under the peak is proportional to the amount of substance, which is passed through detector, and this area can becalculated automatically by the computer linked to the display. [3, 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]. 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.

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 application, including scale and mobile phase conditions.

2) The molecular weight, or size of the sample components.

3) The hydrophobicities 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].

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 ffecting sample retention or resolution[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].

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 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 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 [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]

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 dimethylsilane (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 theoreticalplate 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.

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 ionization 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 pHenvironment 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 behaviour. 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 .

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[12,17].

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 [18].

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.

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.

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 separated from extraneous peaks at detection levels which assay will be performed.

Other applications of HPLC: Other applications of HPLC includes

Pharmaceutical applications [19-21]

• Tablet dissolution study of armaceutical dosages form.

• Shelf-life determinations of parmaceutical products

• Identification of active ingredients of dosage forms

• Pharmaceutical quality control

Environmental applications [22-25]

• 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.

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 [26-31]

• 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.

CONCLUSION

It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules.

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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