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CHROMATOGRAPHIC MASTERY: HPLC TECHNIQUES AND INNOVATIONS IN PHARMACEUTICAL ANALYSIS

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ABSTRACT

High performance liquid chromatography (HPLC) is a type of column chromatography which is commonly used in biochemistry and analysis to separate or identify & quantify the active chemicals. HPLC is the most used separation technology for detecting, separating & quantifying the drugs. The accuracy, specificity, linearity, range, limit of detection, the limit of quantification, robustness, and system suitability testing are all included in the validation of an HPLC techniques according to ICH guidelines. Chromatography is used in all research laboratories and pharmaceuticals industries universally. The chromatography Is very popular techniques and it is mostly used analytically. There are different types of chromatography techniques namely paper chromatography, gas chromatography, liquid chromatography, thin layer chromatography (TLC), ion exchange chromatography And lastly high performance liquid chromatography (HPLC). This review article mainly focuses on the chromatography types and HPLC, parameters of HPLC, instrumentation of HPLC, validation method, pump, detectors and types of detectors , application of HPLC .

KEYWORDS: Chromatography, mobile phase, stationary phase, analyte, HPLC, validation, pump, detectors.

INTRODUCTION

High performance liquid chromatography (HPLC) is a specific form of a column chromatography. It's generally used in the biochemistry and analysis to separation, identification and quantify the active emulsion.

Separation has been done to vary the mobile phase composition during the analysis; these is known as grade elution.[2,3] HPLC substantially utilizes a column that holds quilting material, a pump that moves the mobile phases throughout the column and a sensor that shows the retention times of the motes.[1]

The liquid phase Is pumped at a constant rate to the column packed with the stationary phase. Before entering the column the analysis sample is fitted into the carrier sluice on reaching the column the sample factors are widely retained on the base of physicochemical commerce between the analyte patch and the stationary phase. The mobile phase moving at a steady rate elutes the factors grounded on the operating condition discovery ways are employed for discovery and quantification of the eluted factors.[4]

The fashion Is grounded on the same modes of separation as classical column chromatography, i.e. adsorption, partition(including reversed- phase partition), ion exchange and gel saturation, but it differs from column chromatography in that the mobile phase is pumped through the packed column under high pressure. The top advantages of HPLC compared to classical (graveness- feed) column chromatography are bettered resolution of the promised substances, faster separation times and the increased delicacy, perfection and perceptivity with which the promised substances may be quantified.[7]

History

Prior to HPLC scientist used standard liquid chromatographic ways. Liquid chromatographic system were largely hamstrung due to the inflow rate of detergent being dependent on graveness. Separation took numerous hours, and occasionally days to complete. Gas chromatography at the time was more important than liquid chromatography, still it was believed that gas phase separation and analysis of veritably polar high motes weight biopolymer was insolvable.[5]

Taking after on the original work of Martin and Synge in 1941, it was anticipated by Cal Giddings, Josef Huber, and others in the 1960s that LC could be worked in the high-

proficiency mode by dwindling the pressing patch dimension freehandedly beneath the run of the shop LC (and GC) position of 150 μm and exercising pressure to expand the protean stage haste. These prospects endured broad trial and refinement all through the 60s into the 70s. Early experimental disquisition started to enhance LC patches, and the invention of Zipax, an externally passable patch, was promising for HPLC technology. The 1970s achieved multitudinous advancements in outfit and instrumentation. Specialists started exercising pumps and injectors to make a simple configuration of a HPLC system. Gas amplifier pumps were perfect since they worked at harmonious pressure and didn't bear release free seals or check faucets for steady inflow and great quantitation.[5]

While instrumentational advancements were important, the literal background of HPLC is basically about The history and development of patch technology. After the donation of passable sub caste patches, there has Been a steady pattern to reduced patch size to enhance effectiveness. Still, by dwindling patch size new Issues arrived. The disadvantage from the gratuitous pressure drop is anticipated to drive protean liquid through The member and the trouble of setting up a livery pressing of to a great degree fine accoutrements. Every time patch size is lowered altogether, another round of instrument advancement typically should do to handle the pressure.

Chromatography

Chromatography is an analytical method in which compounds are physically separated prior to measurement. The main purpose of chromatography is to separate and quantify the target sample in the matrix. The first developer of chromatography was M. Tswett.

Types of chromatography

There are twelve types of chromatography technique.[16]

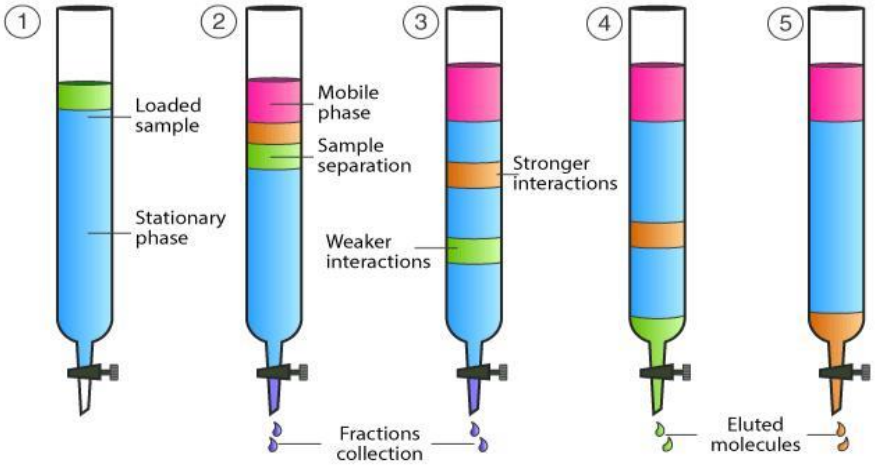
The twelve types are

1. Column Chromatography
2. Paper Chromatography
3. Thin Layer Chromatography
4. Gas Chromatography
5. High Performance Liquid Chromatography
6. Fast Protein Liquid Chromatography
7. Supercritical Fluid Chromatography

8. Affinity Chromatography
9. Reversed Phase Chromatography
10. Two Dimensional Chromatography
11. Pyrolysis Gas Chromatography and
12. Counter Current Chromatography.

1) Column chromatography

It is a preparative application of the chromatography. It is used to obtain the pure chemical compounds from a mixture of compounds on a scale from micrograms up to kilograms using large industrial columns. The classical preparative chromatography column is a glass tube with a diameter from 5 to 50 mm and a height of 50 cm to 1 m with a tap at the bottom.



2) Paper chromatography

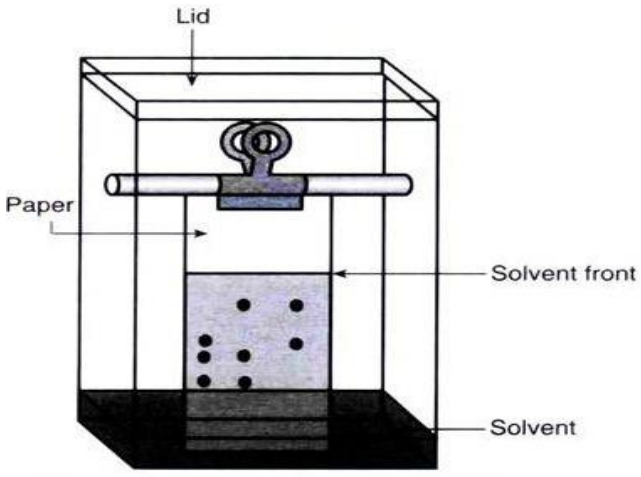


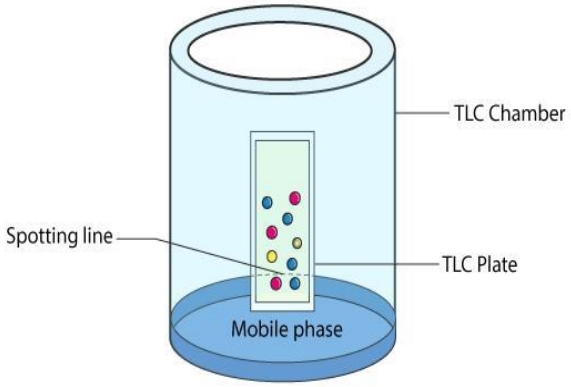
Fig. Schematic diagram of paper chromatography

It's an logical fashion for separating and relating fusions that are or can be colored, especially colors. This can also be used in secondary or primary seminaries in essay trials. This system has been largely replaced by thin layer chromatography; still it's still a important tutoring tool. Two- way paper chromatography, also called two- dimensional chromatography, involves using two detergents and rotating the paper 90 ° in between. This is useful for separating complex fusions of analogous composites, for illustration, amino acids.

3) Thin layer chromatography

Thin- layer chromatography (TLC) is a chromatographic fashion that's useful for separating organic composites. It involves a stationary phase conforming of a thin subcaste of adsorbent material, generally silica gel, aluminium oxide, or cellulose paralyzed onto a flat, inert carrier distance. A liquid phase conforming of the result to be separated dissolved in an applicable detergent is drawn through the plate via capillary action, separating the experimental result.

When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent force. The promised spots are imaged with ultraviolet light or by placing the plate in iodine vapour. The different factors in the admixture move up the plate at different rates due to differences in their portioning geste between the mobile liquid phase and the stationary phase.



4) Gas chromatography

It's a type of chromatography In which the mobile phase is a carrier gas, generally an inert gas similar as helium or an unreactive gas similar as nitrogen, and the stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside glass or metal tubing, called a column. The instrument used to perform gas chromato-graphic separations is called a gas chromatograph (also aerograph, gas division).

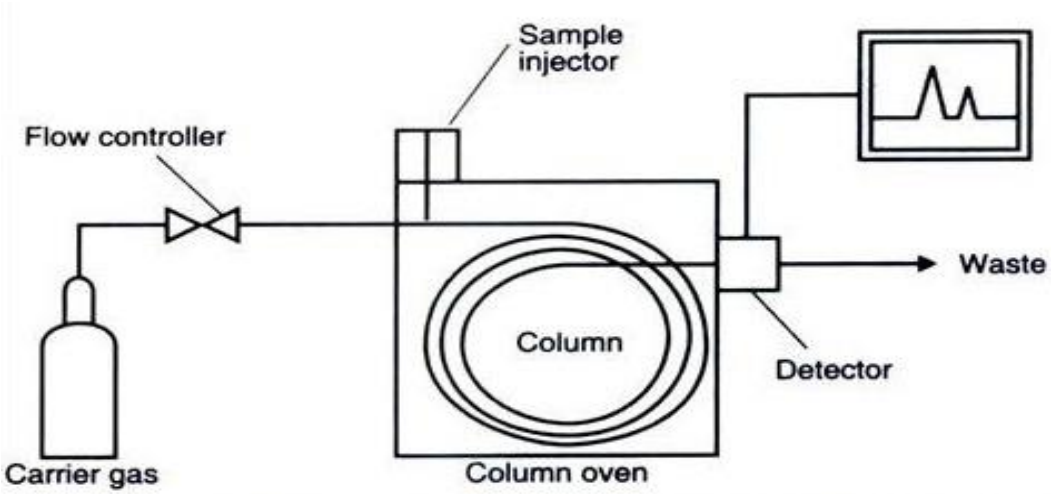


Fig Diagram of a gas chromatograph

5) High performance liquid chromatography

High- performance liquid chromatography(HPLC) is a form of column chromatography used constantly in biochemistry and analytical chemistry. It's also occasionally referred to as high-pressure liquid chromatography. HPLC is used to separate factors of a admixture by using a variety of chemical relations between the substance being anatomized(analyte) and the chromatography column.

6) Fast protein liquid chromatography

Fast Protein Liquid Chromatography, generally appertained to as FPLC, is a form of column chroma-tography used to separate or purify proteins from complex mixtures. It's generally used in biochemistry and enzymology. Columns used with an FPLC can separate macromolecules grounded on size, charge distribution, hydrophobicity, or bio-recognition(as with affinity chromatography).

7) Supercritical fluid chromatography

It's a robust and easy- to- use form of normal phase chromatography immaculately suited to the analysis and sanctification of low to moderate molecular weight, thermally labile molecules. It's especially suited to the separation of chiral composites. Analogous to high performance liquid chromatography(HPLC), SFC generally utilizes carbon dioxide as the mobile phase; thus, the entire chromatographic inflow path must be pressurized.

In addition, SFC metering pumps bear that the pump head be kept cold in order to maintain the carbon dioxide in a supercritical state, where it can be effectively metered at some

specified inflow rate. The druggist sets mobile phase inflow rate, composition, and column temperature. In addition, SFC provides an fresh control parameter, pressure, which the druggist also sets through the keyboard. From an functional viewpoint, SFC is as simple and robust as HPLC.

8) Affinity chromatography

It is a chromatographic method of separating biochemical mixtures, based on a highly specific biologic interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography with the ability to design a stationary phase that reversibly binds to a known subset of molecules.

9) Reversed phase chromatography

Reverse-phase chromatography (RPC) includes any chromatographic method that uses a non-polar stationary phase. All of the mathematical and experimental considerations used in other chromatographic methods apply (i.e., separation resolution proportional to the column length and inversely proportional to the column width).Reversed-phase column chromatography is widely used in the pharmaceutical, chemical, and biochemical industry for separating molecules of small molecular weight. In more recent years RPC has been used to separate larger molecules.

10) Two dimensional chromatography

By using an additional physicochemical (Chemical classification) criterion for separation of the mixture of analytes (sample), the resolution and quality of chromatographic separation can be increased. As a result, higher specificity concerning the separational capability of the chromato-graphic technique is obtained, allowing separation and preparation or analysis of compounds indis-tinguishable by one-dimensional chromatography. In Gas-Phase Chromatography, two-dimensional separation is achieved by coupling a second, short column to the first long column. Coupling is achieved by different techniques, for example, shock-freezing the elutes in order of elution from the first column at fixed time-intervals, and then reheating them in order of elution, releasing them into the second column. The time of traversal through the second column needs to be shorter than the time remaining until the next sample is reheated to prevent compound build-up and to fully exploit the separational capability.

11) Pyrolysis gas chromatography

Pyrolysis-chromatography is a potent analytical tool able to thermally crack (fragment) essentially non-volatile molecules into fragments suitable for chromatographic analysis. The technique enables a reproducible and characteristic "fingerprint" to be generated of a non-volatile sample. The tech-nique can be applied to such varied tasks as bacterial strain differentiation and forensic characterization of paints, polymers and fiber cross-matching.

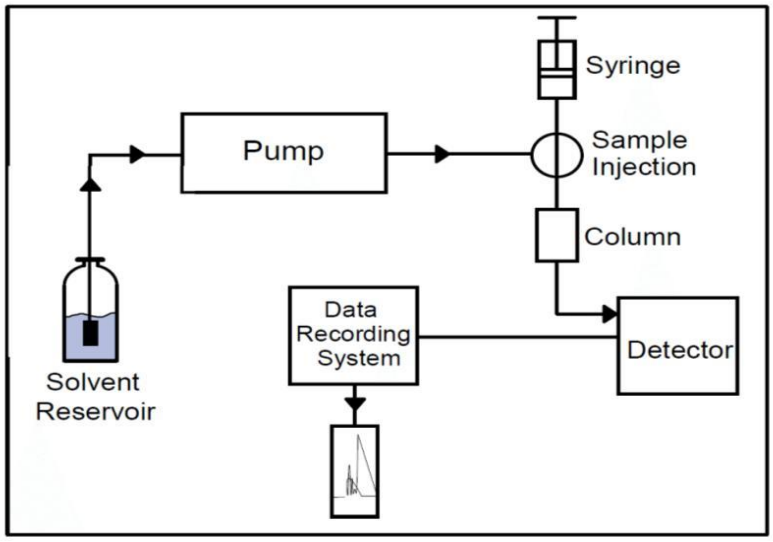
12 Counter current chromatography

CCC or Partition Chromatography is a category of liquid-liquid chromatography techniques. Chro-matography in general is used to separate components of a mixture based on their differing affini-ties for mobile and stationary phases of a column. The components can then be analyzed separately by various sorts of detectors which may or may not be integrated into an apparatus. In liquid-liquid chromatography, both the mobile and stationary phases are liquid. CCC can be thought of as occurring in three stages: mixing, settling, and separation (although they often occur continuously). Mixing of the phases is necessary so that the interface between them has a large area, and the analyte can move between the phases according to its partition coefficient.

Type of Chromatography	Applications in the Real World	Why and What is it
Liquid Chromatography	testing water samples to look for pollution	Used to analyze metal ions and organic compounds in solutions. It uses liquids which may incorporate hydrophilic, insoluble molecules.
Gas Chromatography	detecting bombs in airports, identifying and quantifying such drugs as alcohol, being used in forensics to compare fibres found on a victim	Used to analyze volatile gases. Helium is used to move the gaseous mixture through a column of absorbent material.
Thin-Layer Chromatography	detecting pesticide or insecticide residues in food, also used in forensics to analyze the dye composition of fibres	Uses an absorbent material on flat glass plates. This is a simple and rapid method to check the purity of the organic compound.
Paper Chromatography	separating amino acids and anions, RNA fingerprinting, separating and testing histamines, antibiotics	The most common type of chromatography. The paper is the stationary phase. This uses capillary action to pull the solutes up through the paper and separate the solutes.

HPLC - High-pressure liquid-solid chromatography (HPLC) is rapidly becoming the method of choice for separations and analysis in many fields. Almost anything that can be dissolved can be separated on some type of HPLC column. However, with this versatility comes the necessity to think about the separation desired and the best way to achieve it. HPLC is not now and probably never will be a turn-key, push-button type of operation. Many dedicated

system-in-a-box packages are sold for specific separations, but all of these still offer wide possibilities for separation. Changing the column and the flow rate lets you change the separation and the amount of sample you can inject. This is not the worst thing in the world, for it does create great opportunity for the chromatographer and a great deal of job security for the instrument operator.[9]



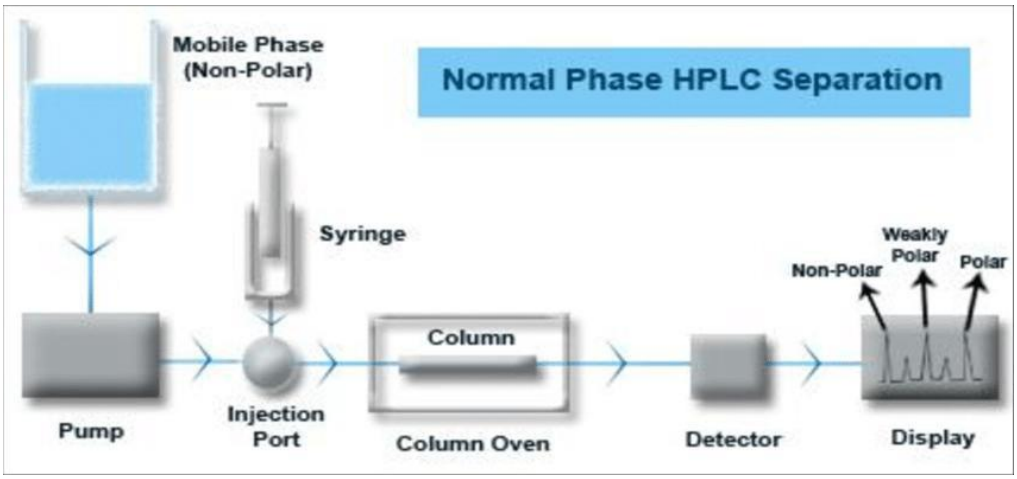
Types of HPLC

Types of HPLC generally depend on phase system used in the process. Following types of HPLC generally used in analysis[10,11,14,15]

1) Normal phase chromatography
2) Reversed phase chromatography
3) Size exclusion chromatography
4) Ion exchange chromatography
5) Bio affinity chromatography.

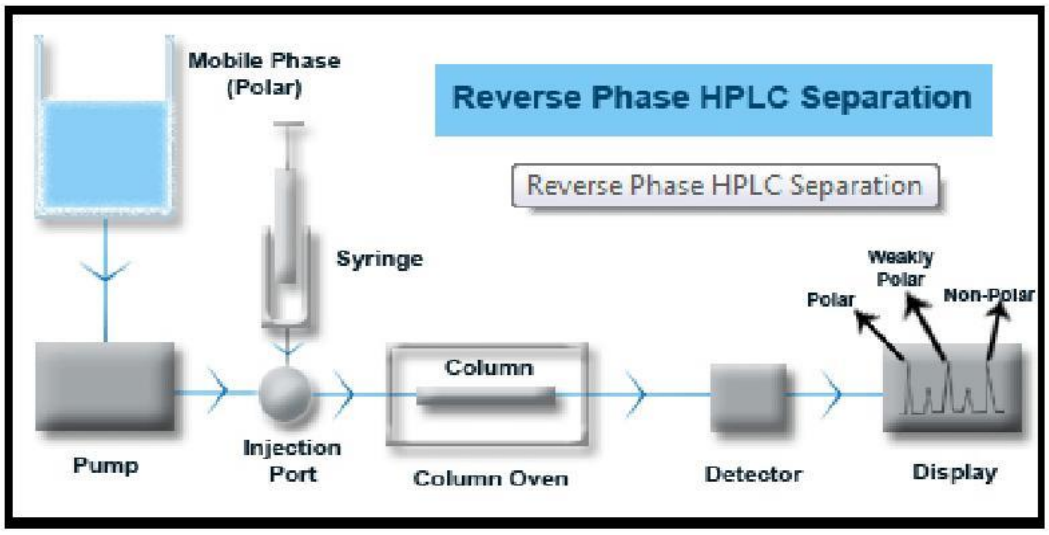
1) Normal phase chromatography

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 stationary phase is polar, mostly Silica is used and the non-polar phase used is hexane, chloroform and diethyl ether. The polar samples are Retained on column.[12]



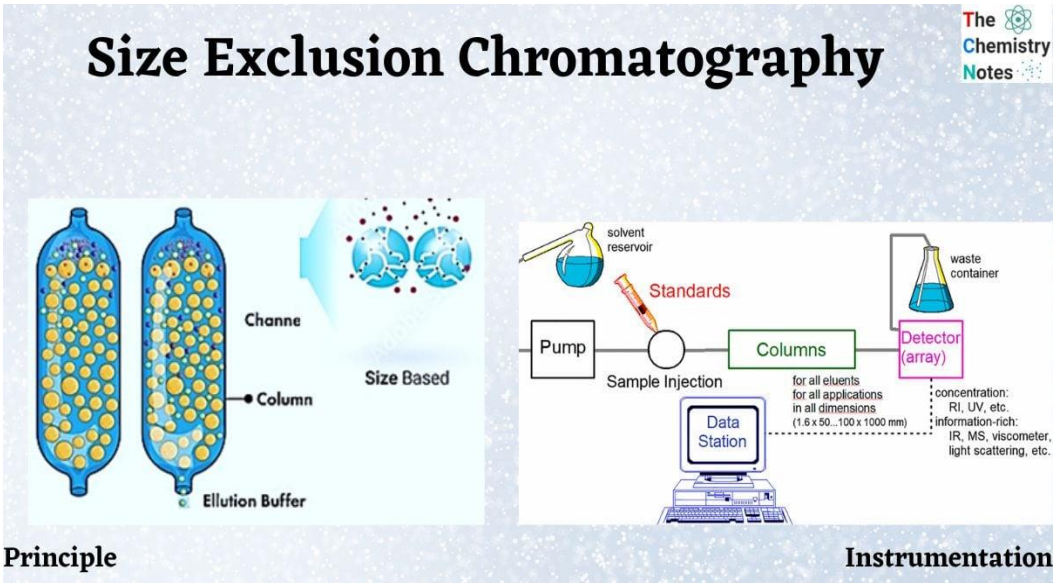
2) Reversed phase chromatography

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. The mobile phase is polar and the stationary phase is non polar or hydrophobic. The more is the non-polar nature the more it will be retained.



3) Sized exclusion chromatography

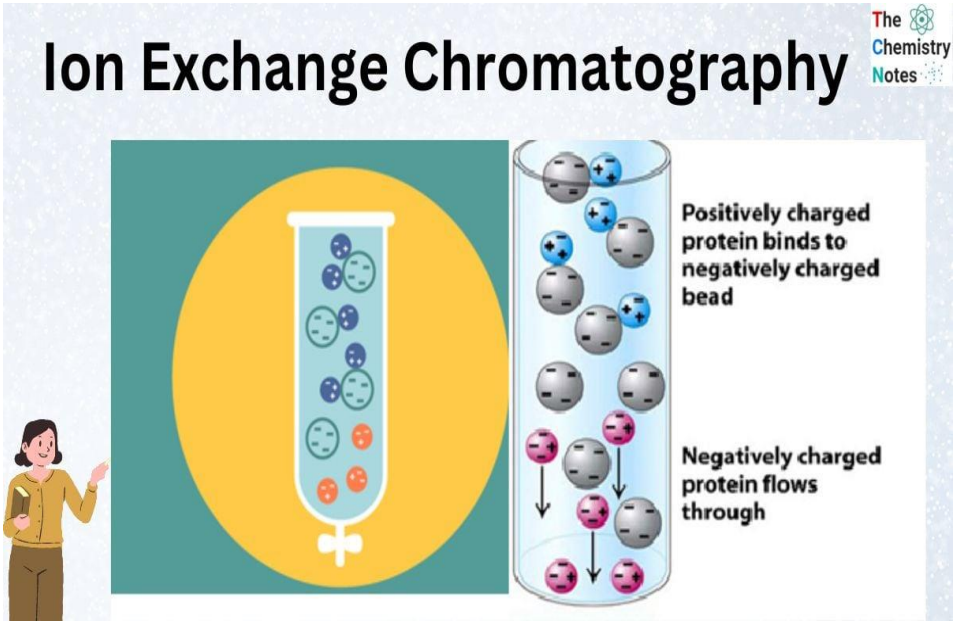
Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. Based on the difference in molecular sizes the separation of constituents will occur.



4) 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.

The stationary phase is having ionically charged surface opposite to the sample charge. the mobile phase used is aqueous buffer which will control pH and ionic strength.[13]



5) 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.

Instrumentation of HPLC

The HPLC instrumentation involves pump, injector, column, detector, and integrator and display system**.**

1) Pump

The pump suctions the mobile phase from the solvent reservoir and forces it to the column and then passes to the detector. 42000 KPa is the operating pressure of the pump. This operating Pressure depends on column dimensions, particle Size, flow rate and composition of mobile phase.[17]

Dual-piston reciprocating pumps produce an almost pulse-free flow because the two pistons are carefully phased so that as one is filling the other is pumping. These pumps are more expensive than single- piston pumps but are of benefit when using a flow-sensitive detector such as an ultraviolet or refractive index detector.[7]

2) Injector

An injector for an HPLC framework should give an infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

Modern injectors are based on injection valves which allow the sample at atmospheric pressure to be transferred to the high-pressure mobile phase immediately before the column inlet. The design of different valves varies widely but a typical arrangement.[7]

3) Column

Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm.[18]

4) Detector

The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, mass spectrometric and electrochemical identifiers.[19]

It should also have a low dead volume to reduce further band-broadening of the components in the detector and good stability to prevent fluctuations of the response. The most commonly used detectors in the HPLC analysis of pharmaceutical substances are described below.[7]

5) Integrator

Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic Information.[20]

Sample preparation for HPLC

Samples in liquid form can be analyzed directly, after a suitable clean-up to remove any particulate materials or after a suitable extraction to remove matrix interferents. In determining polyaromatic hydrocarbons (PAH) in wastewater, For example, an initial extraction with CH2Cl2 serves the dual purpose of concentrating the analytes and isolating them from matrix interferents. Solid samples must first be dissolved in a suitable solvent, or the analytes of interest must be brought into solution by extraction. For example, an HPLC analysis for the active ingredients and degradation products in a pharmaceutical tablet often begins by extracting the powdered tablet with a portion of the mobile phase.

Organic isocyanates in industrial atmospheres can be determined in this manner by bubbling the air through a solution of 1-(2-methoxyphenyl) piperazine in toluene. Reacting the isocyanates with 1-(2-methoxyphenyl) piperazine serves the dual purpose of stabilizing them against degradation before the HPLC analysis while also forming a derivative that can be monitored by UV absorption.[29]

Advantages of HPLC

- Separations are fast and efficient (high-resolution power).
- Continuous monitoring of the column effluence
- It can be applied to the separation and analysis of very complex mixtures
- Accurate quantitative measurements.

- Repetitive and reproducible analysis using the same column.
- Adsorption, partition, ion exchange, and exclusion column separations are excellently made.
- HPLC is more versatile than GLC in some respects because it has the advantage of not being restricted to volatile and thermally stable solute and the choice of mobile and stationary phases is much wider in HPLC.
- Aqueous and non-aqueous samples can be analyzed with little or no sample pre-treatment.
- A variety of solvents and column packing are available, providing a high degree of selectivity for specific analyses.
- It provides a means for the determination of multiple components in a single analysis and etc.[30]

Disadvantages of HPLC

*Column performance is very sensitive, which depends on the method of Packing.

- Further, no universal and sensitive detection system is available.
- Very costive, have low sensitivity for certain compounds, and some cannot be detected as they are irreversibly adsorbed.[30]

Validation method

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics Of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the Planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are Intended to be used for analyzing any clinical samples will need to be validated.[21]

The main typical analytical performance characteristics which may be tested during methods validation are System Suitability, Accuracy, Precision, Repeatability, Intermediate precision, Linearity, Detection limit, Quantification limit, Specificity, Range, Robustness, System Suitability determination, Forced degradation studies and Solution stability studies.

1) System suitability

System suitability testing originally believed by the industry of pharmaceuticals to decide whether a chromatographic system is being utilized day today in a routine manner in

pharmaceutical laboratories where quality of results is most important which is suitable for a definite analysis. The parameters used in the system suitability tests (SST) report are: Number of theoretical plates or Efficiency (N), Capacity factor (K), Separation or Relative retention (α), Resolution (Rs), Tailing factor (T), Relative Standard Deviation (RSD).[22-25]

1) Number of theoretical plates-: In a specified column, efficiency is defined as the measurement of the degree of peak dispersion and it should have the column characteristics. The efficiency is conveyed in terms of number of theoretical plates. The formula of calculation of N is illustrated bellow in the following.

N = Number of theoretical plates.

tR = Retention time or retention distance.

H = Peak height.

Wb = width of the peak at the base line.

$$N = 16\left(\frac{t_R}{W_b}\right)^2 = 5.54\left(\frac{t_R}{W_{\frac{1}{2}}}\right)^2$$

The plate number depends on column length. Theoretical plate number is the measure of column efficiency. As stated by plate theory, the Analyte will be in instant equilibrium with stationary phase and column has to be divided into number of hypothetical plates and each Plate consists of a fixed height and analyte spends finite time in the plate. Height equivalent to theoretical plate (HETP) is given by following formula:

$$HETP = L/N,$$

Where, L = length of column. N = plate number

2) Capacity ratio or capacity factor (k): The capacity factor (k') is a means of measuring the retention of an analyte on the chromatographic column.

$$K' = \frac{t_R - t_M}{t_M}$$

Generally the value of k' is > 2.

3) Relative retention or separation factor: The selectivity (or separation) factor (α) is the ability of the chromatographic system to 'chemically' distinguish between sample components.

$$\alpha = \frac{(t_{R2} - t_M)}{(t_{R1} - t_M)}$$

T1= the retention time from the point of injection of reference peak defined.

T2= Retention time calculated from point of injection.

TM= Unretained peak time (Retention time (tR) of an inert component not retained by the column).

4) Resolution (Rs)-: Resolution is the capability of the column to separate 2 components in 2 individual peaks or chromatographic zones and it is improved by enhancing column length, reduction of particle size and rising temperature, altering the eluent or stationary phase. It can be told in terms of ratio of separation of the apex of two peaks by the tangential width average of the peaks. by using the following formula resolution is calculated.

$$Rs = \frac{(t_{R2} - t_{R1})}{(0.5(t_{wb1} - t_{wb2}))}$$

5) Resolution factor (R)-: Resolution is a function of capacity factor, function of selectivity and a function of efficiency (or) number of theoretical plates (N). In Order to separate any two peaks you must have right capacity factor ideally between 2 and 10, but appropriate selectivity is required i.e., Ideally 1.2 and enough efficiency i.e., number of theoretical plates (more than 2000 theoretical plates).

6) Tailing factor or asymmetry factor: Chromatographic peak assumed to have a Gaussian shape under ideal conditions. However in practical conditions, there is always a deviation from normal distribution which indicates non- uniform migration and non-uniform distribution process. The asymmetry factor and tailing factor are roughly same and rarely accurate and equal in most cases. Values should normally between 1.0-1.5 and values greater than 2 are unacceptable.[26]

2) Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

3) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

4) Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three Levels: repeatability, intermediate precision and reproducibility.

5) Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intraassay precision.

6) Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

7) Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

8) Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value.

9) Quantification limit

The quantification limit is a parameter of quantitative assays for low levels of com-Pounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

10) Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample.

11) Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

12) Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

13) Force degradation studies

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products.[27]

14) Solution stability studies

During validation the stability of standards and samples is established under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions are necessary, for instance, refrigeration or protection from light.[28]

CONCLUSION

It Can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic techniques. The advantage of HPLC technology were it's great selectivity, sensitivity, low detection limit and expensive cost, it give and accurate and pricesses results. HPLC techniques used for the evaluation of pharmaceutical and biological sample. It can be used both laboratory and clinical sciences. The only disadvantages of HPLC is high cost and complex methodology. It includes all fundamental aspects of chromatographic techniques.

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