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CHROMATOGRAPHIC MASTERY: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TECHNIQUE

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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 column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. 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 used. 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 (come 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). Separation has been done to vary the mobile phase composition during the analysis; this is known as 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.

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 column chromatography are better resolution of the promised substances, faster separation times and the increased delicacy, perfection and perceptivity with which the promised substances may be quantified.

HISTORY AND DEVELOPMENT

Prior to HPLC, scientists used benchtop column liquid chromatography techniques. Liquid chromatographic systems were largely inefficient due to the flow rate of solvents being dependent on gravity.

Taking after on the original work of Martin and Synge in 1941, it was anticipated by Cal Giddings, Josef Huber, and other 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 150µm 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, 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 quantitation.

By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification, and quantification far above those obtained using previous techniques. Computers and automation added to the convenience of HPLC. Additional column types giving better reproducibility were introduced and such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry, in actual facts these fields currently comprise only about 50% of HPLC users. Currently HPLC is used in a variety of fields and industries including the cosmetics, energy, food, and environmental industries.

Chromatography: It is a technique for separating mixtures into their components in order to analyze, identify, purify, and quantify the mixture or components.

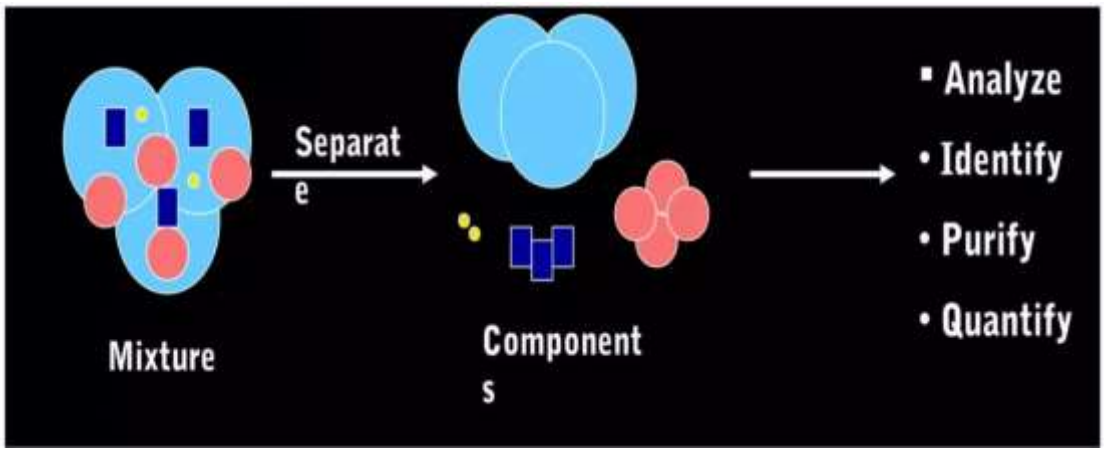

Fig. 1: Chromatography.

Invention of Chromatography

Micheal Tswett invented the chromatography in 1901 during his research on plant pigments.

He used the technique to separate various plant pigments such as chlorophyl, xanthophylls, carotenoids.

Components of Chromatography

Mobile Phase: gas or liquid that carries the mixture of components through the stationary phase.

Stationary Phase: the part of the apparatus that holds the components as they move through it, separating them.

Uses for Chromatography

Chromatography is used by scientists to

Analyze – examine a mixture, its components, and their relations to one another.

Identify: determine the identity of a mixture or components based on known components.

Purify: separate components in order to isolate one of interest for further study.

Quantify: determine the amount of the mixture and the components present in the sample.

Real life examples of uses for chromatography

1. Pharmaceutical Company
2. Hospital
3. Law Enforcement
4. Environmental Agency
5. Manufacturing Plant

Types of Chromatography

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

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 height of 50 cm to 1 mwith a tap at the bottom.

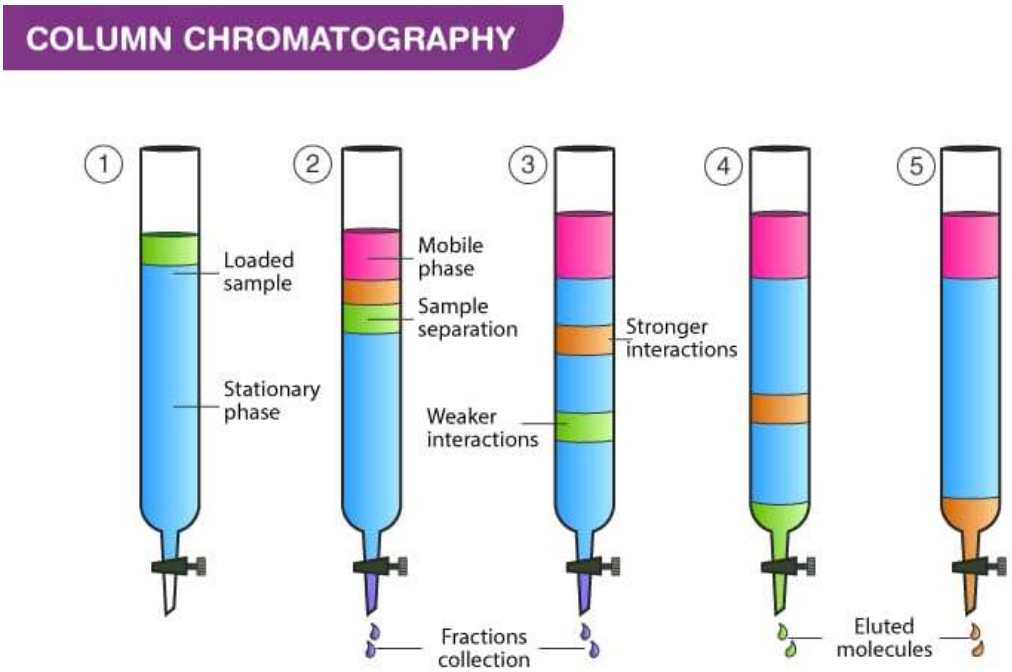

Fig.2: Column chromatography.

2) Paper Chromatography

It's a logical fashion for separating and relating fusion 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; it's an important tutoring tool. Two-way paper chromatography, also called dimensional chromatography, involves using two detergents and rotating the paper 90° in between. This is useful for separating complex fusion of analogous composites, for illustration, amino acids.

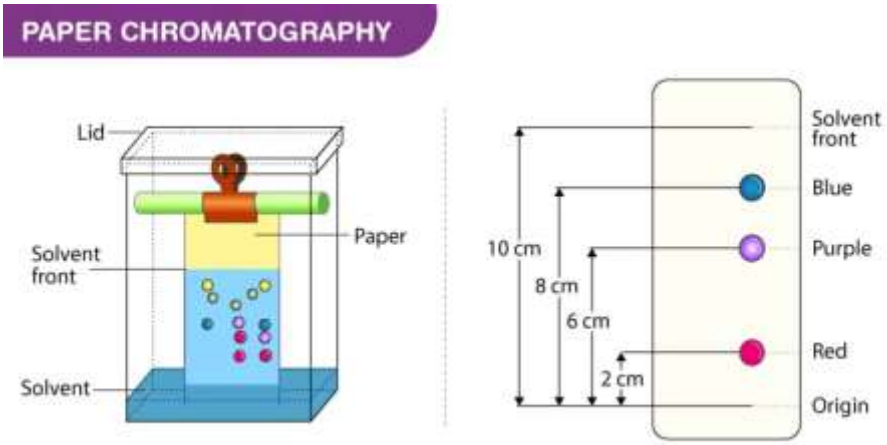

Fig. 3: Paper chromatography.

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 gets between the mobile liquid phase and the stationary phase.

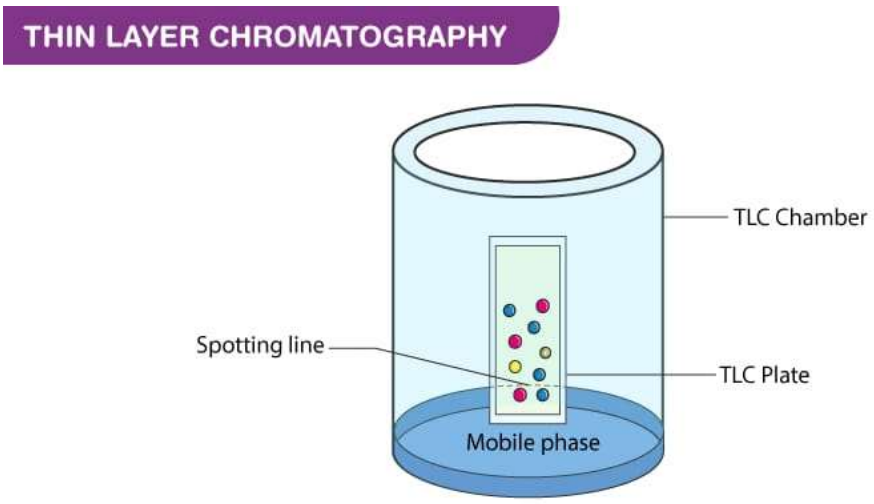

Fig.4: Thin layer chromatography.

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.

Principle of Gas Chromatography

The sample solution injected into the instrument enters a gas stream which transports the sample into a separation tube known as the "column". (helium or nitrogen is used as the so-called carrier gas). The various components are separated inside the column.

The **Flame Ionization Detectors (FID)** is the most common detector used in gas chromatography.

Solvents i.e. Ethyl acetate, hexane, methanol, dichloromethane, and acetone are used.

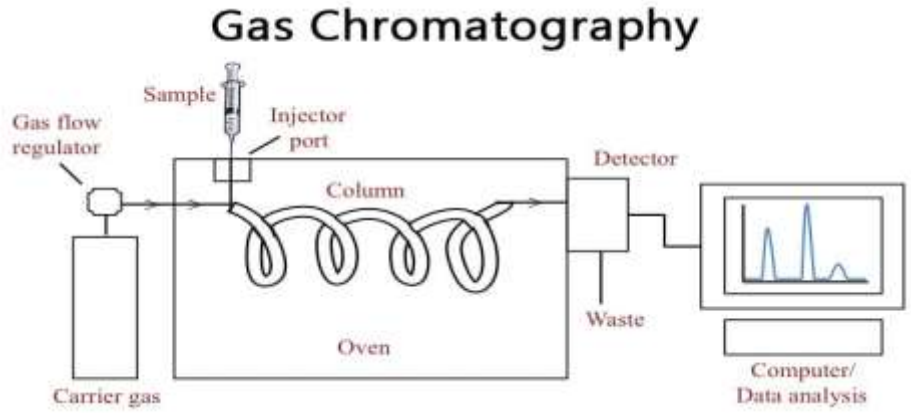

Fig. 5: Gas chromatography.

5) High Performance Liquid Chromatography (HPLC): 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 an admixture by using a variety of chemical relation between the substance being anatomized and the chromatography column.

Principle of HPLC

A separation column separates the stationary and mobile phase during purification.

In a separation column, the stationary phase is a granular substance with very small porous particles.

The mobile phase is a solvent or solvent combination that is pushed through the separation column under high pressure.

The sample is loaded into the mobile flow regime from the pump to the separation column using a syringe through a valve with a linked sample loop, i.e. a tiny tube or capillary made of stainless steel.

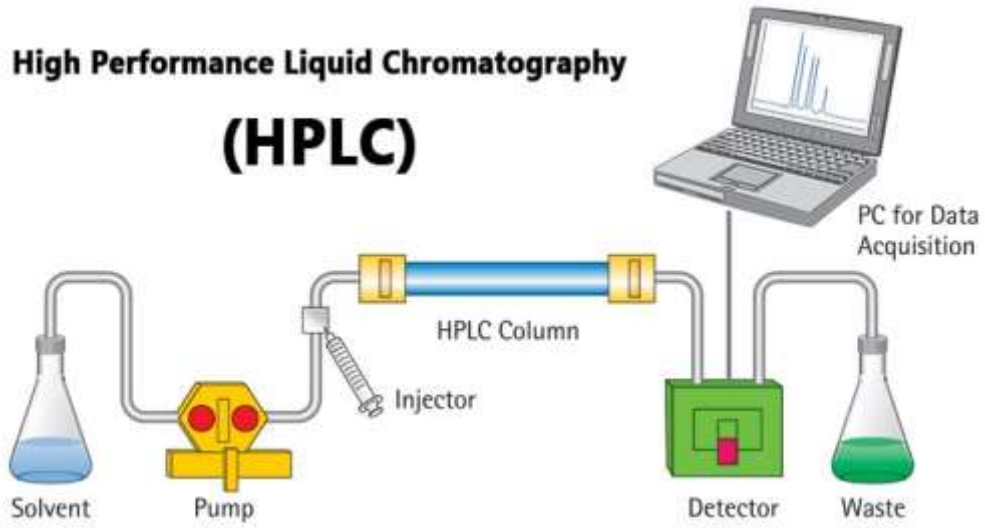

Fig.6: High performance liquid chromatography.

6) Fast Protein Liquid Chromatography

Fast Protein Liquid Chromatography, generally appertained to as FPLC, is a form of column chromatography 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.

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, 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 head 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 fresh control parameter, pressure, which the druggist also sets through the keyboard. From 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 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 includes any chromatographic method that uses a non-polar stationary phase. All of the mathematical and experimental considerations used in other chromatographic method 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 additional physicochemical 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 chromate-graphic technique is obtained, allowing separation and preparation or analysis of compounds indistinguishable 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 technique 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. Chromatography in general is used to separate components of a mixture based on their differing affinities for mobile and stationary phase 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 phase are liquid. CCC can be thought of as occurring in three stages: mixing, settling, and separation. 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 coefficient.

HPLC: High pressure liquid – solid chromatography (HPLC) is rapidly becoming the method of choice for separation and analysis in many fields. Almost that can be dissolved can be separated on some types 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.

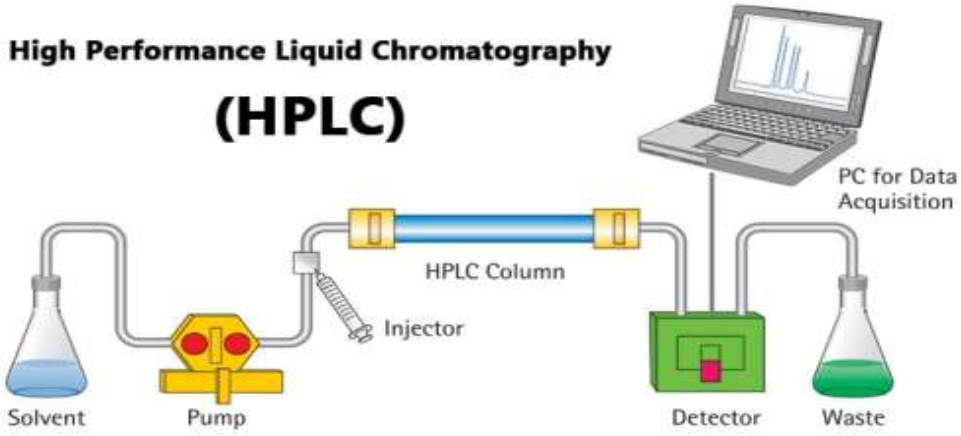

Fig.7: High performance liquid chromatography.

Types of HPLC

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

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.

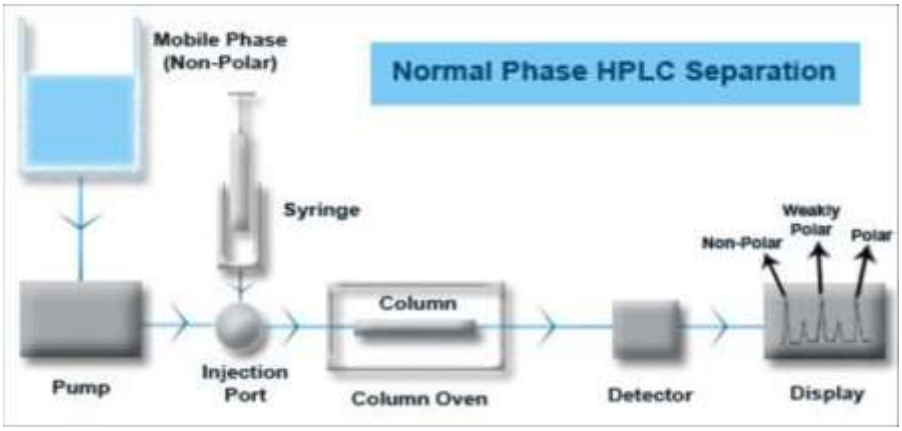

Fig.8: Normal phase HPLC separation.

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.

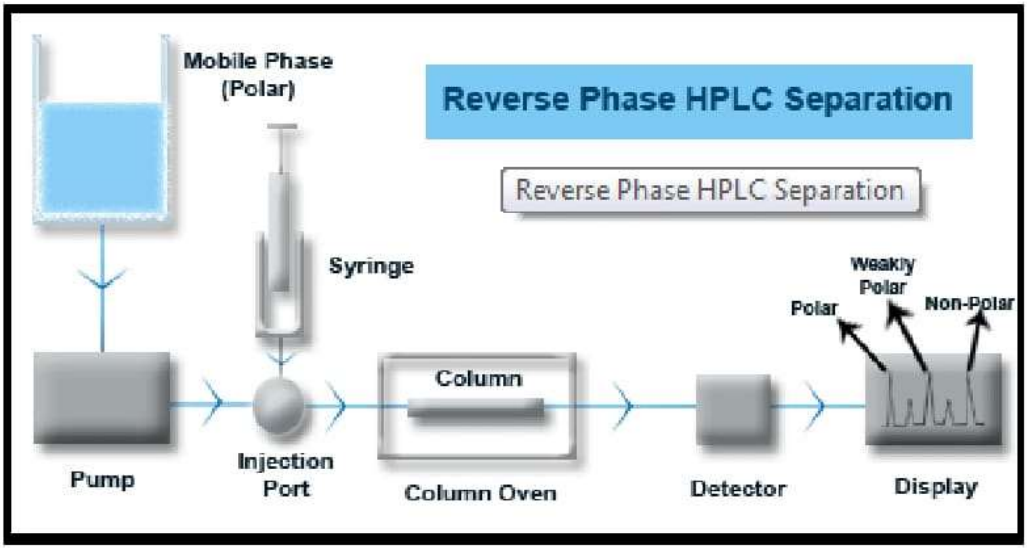

Fig. 9: Reverse phase HPLC separation.

3) Sized exclusion chromatography

Sized exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basic 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.

Sized exclusion chromatography has been the main chromatographic method used to determine the size of proteins, and in particular the distribution of aggregates in final DP.

Buffers with pH between 6.0 and 8.0 are normally used in SEC applications since many proteins are stable in that pH range.

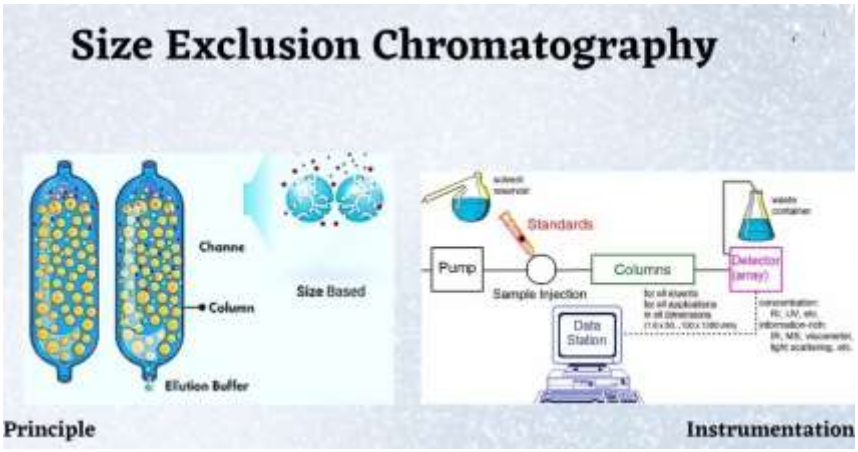

Fig. 10: Size exclusion chromatography.

4) Ion exchange chromatography

Ion exchange chromatography is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.

The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on-ion exchangers.

In this process, two types of exchangers i.e.,

1) **Cationic exchangers:** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.
2) **Anionic exchangers:** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials.

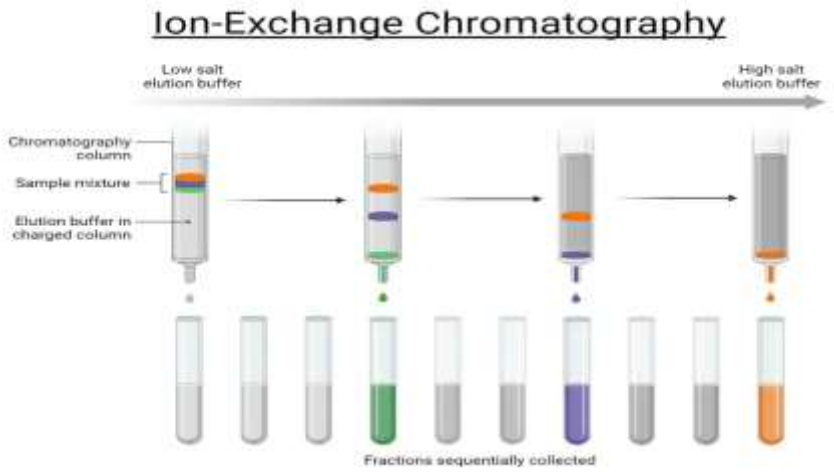

Fig.11: Ion exchange chromatography

5) Bio affinity chromatography

Bio affinity chromatography is adsorption chromatography, based on the exceptional ability of biologically active substances to bind specifically and reversibly complementary substances.

Instrumentation of HPLC

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

The Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses".

Injector

- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.
- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless-steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate.
- The eluent used for LC varies from acidic to basic solvents.

- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes.

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline.
- Degasser uses special polymer membrane tubing to remove gases.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions.

- For some analysis such as sugar and organic acid better resolutions can be obtained at elevated temperatures (50 to 80°C).
- The columns are generally kept inside the column oven (column heater).

Sample Preparation for HPLC

High-performance liquid chromatography (HPLC) is a analytical chemistry technique used to separate, quantify and identify each component in a mixture. Components are mixed in a pressurized liquid solvent which pass the sample through a HPLC column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column. The sample is mixed with solvents in varying dilutions and can be pre-filtered before the sample is put into the column to ensure purity of the results, where a sample has to pass through the column at a regular or fast speed a HPLC pump is used.

Sample preparation is vital to high quality reproducible and specific results, often overlooked methodical sample preparation can improve efficiency in the laboratory. Sometimes seen as a laborious task sample preparation isn't always viewed as the most efficient use of time in the laboratory. HPLC and increasingly UHPLC is one of the most commonly used high precision analytical methods in today's fast paced modern laboratory. A sample needs to be prepared so that it can be directly injected into a HPLC column. First the sample needs to be dissolved in an appropriate solvent; methanol and acetonitrile are favourite choices, for a variety of reasons; there are many guides available as to the proportion on solvent in relation to your sample; some methods are tried and tested some are just a matter of trial and error. Chromatography forums provide a wealth of experience that is freely shared via the Internet. Depending on the sample to be analysed, whether it be for animal biological fluids, drinks, food, human biological fluids, petrochemical residues, pharmaceutical analysis, toxicological residues, waste waters, the sample will need to be filtered prior to injection. Solid Phase Extraction (SPE) tubes provide an excellent clean up method or a syringe filter with a suitable membrane to ensure that your sample is free of particles that may cause interference during detection and will prevent blockages to your column. Syringe filters are the most efficient method of pre-filtering the sample for sample with complex properties or for small volumes, they come in a variety of membranes and differing parasites. It is important that the

sample is a clean as possible before it is introduced to the column, taking time at this stage to ensure that the sample is a clean as it can be is worth the extra time.

Problems with peaks, particularly Ghost Peaks can be detected either with the first injection or problems may present themselves during the course of your analysis leading to a gradual deterioration in your results. A poorly prepared sample may lead to you having to repeat the whole process, it may even involve you having to clean the column if the sample is too contaminated. Taking extra time to clean the sample will save valuable laboratory time in the long run.

A clean sample will help to ensure.

- High sensitivity of the HPLC Column
- Accurate, reproducible results
- Fewer false-positive peaks
- Low background noise
- No blockages in the column

Using ultra-pure water can also have its advantage particularly for pharmaceutical analysis where the need for the purest water is paramount. Scientific research has proved that more than 80% of problems experienced during HPLC analysis are directly attributable to water purity. Bio-solve Ultra-Pure water is supplied by Greyhound Chromatography in the UK and to laboratories world-wide. With less than 0.0002%w/w residues after evaporation, the HPLC grade water from Bio solve is filtered through 0.2µm and bottled under inert gas.

Some scientists prefer to use an automated sample preparation methods to eliminate human error, samples can become unreliable where dilution has been carried out manually use hand to eye determination of sample size and not a regulated reproduction of a sample using an electronic pipette, for example. Automated sample preparation can aid productivity by speeding up the analysis process, this is particularly useful for UHPLC where speed of analysis under high pressure is essential. Triple quad systems provide increased sensitivity at lower analyte concentrations, allowing high throughput of samples. The laboratory environment is increasingly complex, particularly in the field of biological research and analysis. The need for research and drug development in the area of Cancer research alone is driving new developments every day at the edge of treatment development.

Advantages of HPLC

1. HPLC has high resolution and speed of analysis.
2. High surface area.
3. It has high pressure gradient.
4. It has wide range of stationary phases.
5. Precise flow rate control.
6. Sensitive detection methods.
7. Low sample method requirement.
8. Accurate peak identification using HPLC.

Disadvantages of HPLC

1. HPLC has high cost.
2. High quality components are needed.
3. The solvents and columns used in HPLC are expensive.
4. Regular maintenance and calibration are needed which add extra cost.
5. Sophisticated software is required for data analysis.
6. Research and development cost.

CONCLUSION

The primary medication and any reactive contaminants must be separated and quantified using the HPLC technique. The mobile phase in HPLC is a liquid. The most used kind of HPLC is reversed-phase HPLC. Reversed-phase refers to a situation in which the stationary phase is relatively non-polar and the movable phase is substantially polar. Consequently, compared to polar chemicals, non-polar compounds will be kept more and have longer retention durations. The stationary phase is relatively polar in normal phase HPLC, while the mobile phase is generally non-polar. Column packing, which involves various chemical and/or physical interactions between the components' molecules and the packing particles, keeps these parts apart from one another. At the departure of a column, a low-through device (detector) that measures their quantity detects these separated components. Principle-wise, LC and HPLC operate in a similar manner, but HPLC has far better speed, efficiency, sensitivity, and ease of use. The output from this detector is known as a "HPLC." Also, it is the most reliable analytical technique frequently used to assess the stability of drug goods and conduct quantitative and qualitative analyses of drug products.

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