



A Review on High Performance liquid Chromatography

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ABSTRACT:

Chromatography is described as a group of methods used to separate components in a mixture. There are two phases to this technique: stationary and mobile phases. The difference in the partition coefficients of the two phases serves as the basis for the separation of constituents. The words chroma (color) and graphene are the Greek roots of the word "chromatography" (to write). Chromatography is a relatively common technology that is mostly utilized for analytical purposes. Paper chromatography, Gas chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography, and High-Performance Liquid Chromatography are some of the numerous types of chromatographic procedures (HPLC). The HPLC technology, including its theory, varieties, apparatus, and applications, is the major subject of this paper. High-pressure liquid chromatography, sometimes known as HPLC, is a type of liquid chromatography. The preferred approach for testing the peak purity of new chemical entities, keeping track of reaction changes during scale-up or synthesis processes, assessing new formulations, and performing quality control and assurance on finished pharmaceutical products is HPLC. In essence, HPLC is a significantly improved version of column chromatography. Solvent is externally driven through a column at high pressures of up to 400 atm rather than dripping through it while only being affected by gravity. This greatly speeds up the chromatographic procedure. Additionally, it permits the use of column packing material with very fine particle sizes, providing a lot more surface area for interactions between the stationary phase and the molecules passing through it. As a result, it makes it possible to separate the mixture's components better. In high-performance liquid chromatography (HPLC), the mobile phase is pushed quickly across the column. Because much smaller adsorbent or support particles may now be used, the analysis time is lowered by 1-2 orders of magnitude compared to traditional column chromatography, significantly enhancing column efficiency.

Key Words: Chromatography, HPLC, Resolution, Column Efficiency.

INTRODUCTION TO CHROMATOGRAPHY:

1)Introduction of chromatography^[1,4]

At the start of the 20th century, Russian-Italian botanist Mikhail Semyonovich Tswett devised chromatography, a physicochemical method for dividing composite mixtures. Chromatography is a non-destructive method for separating a multi-component mixture into its component parts, whether they are trace, minor, or substantial. Solids, liquids, and gases can all be modified in various ways. Chromatography is primarily a separating tool, though it can also be used quantitatively.

Thin layer chromatography and ion exchange chromatography were two types of chromatography that were established as separation methods in the 1930s. Partition and paper chromatography were first proposed by Martin and Synge in 1941. In 1952, gas chromatography was first used. The routine use of chromatography as a separation technique spread to many fields of study throughout the following ten years, including chemistry, biology, and medicine. It is becoming a possible technology for the preparation of extremely pure compounds, such as in the pharmaceutical business or the production of pure chemicals, in addition to its usage in analysis. The chromatographic methods of biomolecule separation are totally responsible for the recent amazing advancements in the biosciences.

Definition of Chromatography^[1]

Chromatography is a process that divides a mixture of components into their separate components by the distribution of equilibrium between two phases.

Various types of chromatographic techniques

Table 1 : types of chromatographic technique

<u>Sr.no</u>	<u>Technique</u>	<u>Stationary phase</u>	<u>Mobile phase</u>
1	Column chromatography or adsorption chromatography	Solid	Liquid
2	partition chromatography	Liquid	Liquid
3	paper chromatography	Liquid	Liquid
4	Thin layer chromatography	Liquid or solid	Liquid
5	Gas liquid chromatography	Liquid	Gas
6	Gas solid chromatography	Solid	Gas
7	Ion exchange chromatography	solid	liquid

Classification of chromatographic methods ^[2]

The chromatographic method methodology can be divided and summed up in three different ways as follows: 1) Rely on the stationary phase's form. such as column and planar chromatography. 2) Rely on both the fixed and mobile phase's physical conditions. such as liquid and gas chromatography. 3) Rely on how the stationary and mobile phases interact for instance, size exclusion chromatography, ion exchange, partition, and affinity.

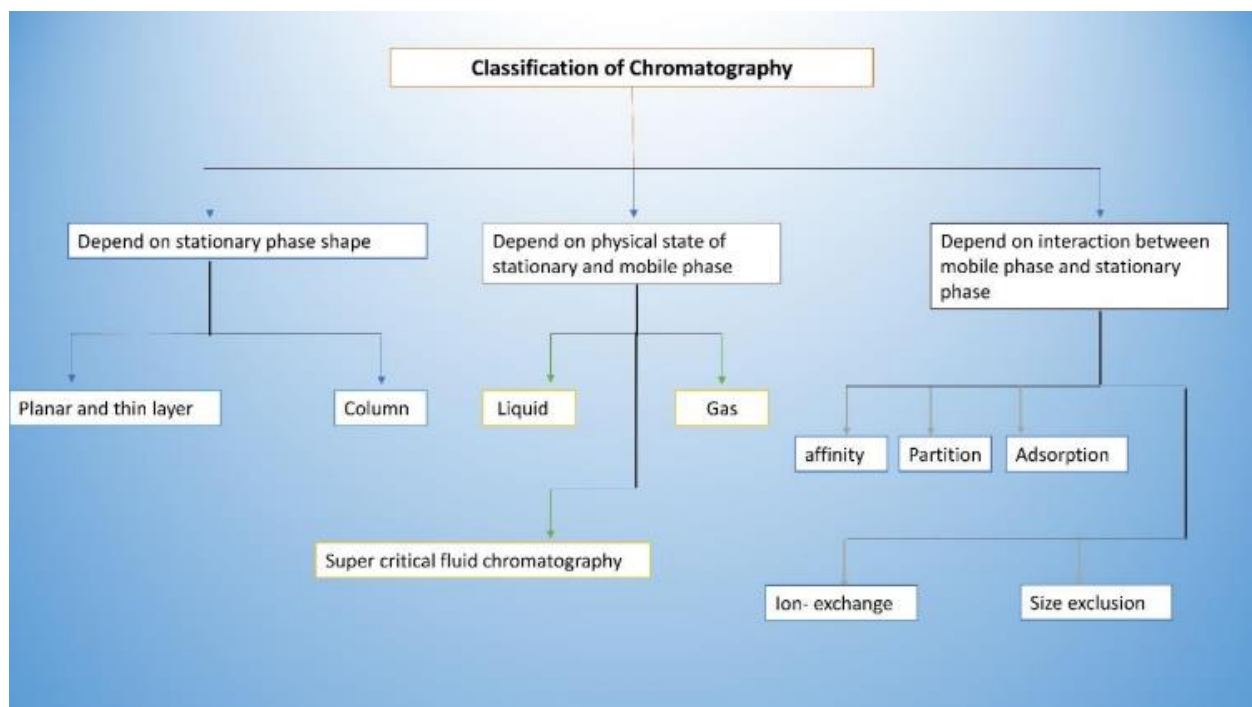


Figure 1: classification of chromatography

Planar chromatography

Here, the stationary phase is a solid that contains silica gel or alumina (thin layer chromatography), while the mobile phase is a liquid solution that flows through it via capillary action or gravity.

Paper chromatography (PC)

In partition chromatography, the polar adsorbed water in the paper serves as the stationary phase in a 2D plate. Both the stationary phase and the mobile phase are liquids. The dissolving sample is placed in a tiny location on the filter paper, half an inch from the edge, and allowed to dry. The solvent contacts the dry patch closest to the sample and travels up or down by capillary action. The solvent is stored at the front end in a closed chamber saturated with atmosphere. We eliminated the uncolored patches that had separated in the paper when the mobile phase mixture reached the end of the paper and used a method called retention factor or rate flow to measure each

$$\text{Retention factor} = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

zone that had separated (Rf).

Thin-layer chromatography (TLC)

Solid-liquid adsorption occurs when the stationary phase interacts with the liquid mobile phase through a high surface area interaction. The stationary phase is moved higher by capillary action as the mobile phase. The polarity of the material, solid phase, and solvent all have an impact on this upward motion rate.

Macromolecules such amino acids, active substances, and preservatives in medications and pharmacological preparations can be purified using thin-layer chromatography.

Column chromatography

The column is a three-dimensional shape model with a geometrical structure that can be packed or open tubular. When a room is packed, the stationary phase is particularly full and takes up the entire column's wall space. However, the stationary phase is with the column sites in the open tubular.

Liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography, is based primarily on the employment of a packing material-filled column. (Stationary phase), a pump to move the mobile phase(s) across the column, and a detector to show the retention times of the molecules. The sample is often injected to the mobile phase stream, where it interacts chemically or physically with the stationary phase to slow it down. Based on the analyte's affinity for the mobile phase, the gradient divides analyte mixtures. The selection of the mobile phase, additives, and gradient are influenced by the characteristics of the stationary phase and the sample.

Separation techniques^[3]

The goal of all chromatographic techniques is to separate two or more components from a mixture. These techniques are carried out by dividing the components between the stationary phase and the mobile phase. The following methods can be used for chromatographic separation:

1. Elution Analysis: -

It is a technique that is frequently used in column chromatography. In this procedure, the mobile phase is allowed to flow through the column while a tiny amount of the mixture to be separated is introduced to the top of the column. The mixture delivered to the column separates into zones as the mobile phase descends the column because the combination's components have been adsorbed to the column material to varying degrees. Each component of the mixture is eluted out as a distinct component upon further passage of the mobile phase.

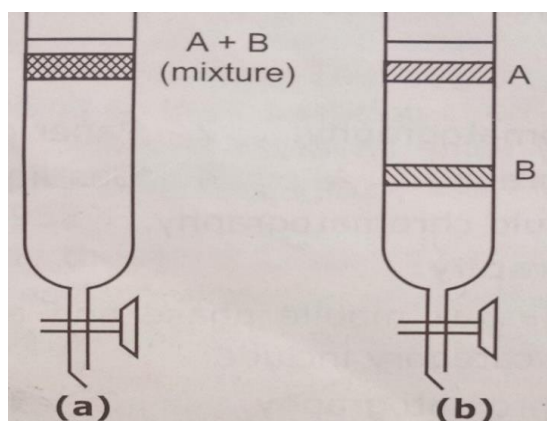


figure 2: elution analysis

The graph shows separated out amount against volume of elute fractions, this is elution analysis.

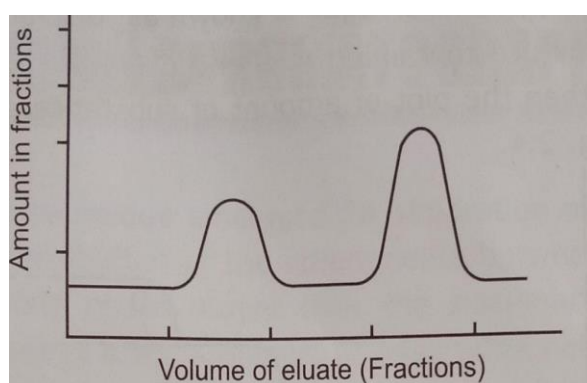


Figure 3: graph showing separated amount against volume of elute fractions

2. Frontal Analysis: This method constantly adds sample mixture solution to the column. The formation of the column does not employ a mobile phase (solvent).

On the column, a combination made up of A, B, and C is added. If component A is least adsorbed, component B is intermediately adsorbed, and component C is most strongly adsorbed to the column material, the least adsorbed component A will flow quickly down the column, component B will reach an intermediate stage, and component C will stay at the top of the column as the mixture flows through it. As more sample mixture is sent through the column, the first few fractions of the eluate will include A, followed by fractions containing A+B, and finally, fractions containing A+B+C. A is only partially separated from B and C.

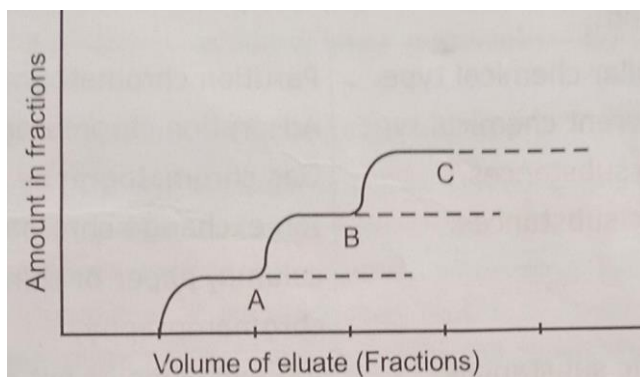


Figure 4: frontal analysis

2. Displacement Analysis: -

In a displacement analysis, a tiny amount of mixture is poured into the column, and a solvent containing a solute with a high affinity for the column's substance is used to elute the mixture. The solute from the mobile phase displaces the mixture's adsorbed components. Every solute in the mixture pushes aside a less strongly adsorbed solute. The component that is least adsorbed is pushed out of the column. The method is characterized as "displacement analysis" because the material employed in the mobile phase is referred to as a "displacer."

In separation of mixture containing A, B and C (with $A < B < C$ adsorption), if D is used as displacer, then the plot of amount of substance against volume of eluate will be as shown in fig

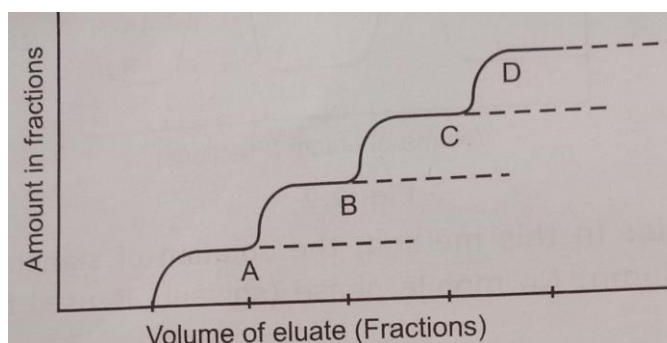


Figure 5: displacement analysis

This technique is mainly used in preparative work; hence, not suitable for analysis since there are chances of overlapping forasmuch as zones are not distinctly separated.

APPLICATIONS OF CHROMATOGRAPHY ^[5]

1) **PHARMACEUTICAL AND CLINICAL TESTING:** -

Chromatography is crucial to the security of medications. Chromatography is used by pharmaceutical businesses to quantify and examine substances for impurities. For instance, chiral substances have two distinct forms as a

result of the tiny spatial variation of their atoms. There is evidence that some chiral chemicals can be harmful. The safe version of the chiral chemical can be distinguished from the harmful form using chromatography.

2) **FOOD AND BEVERAGE: -**

Chromatography can be used to implement quality control in the food and beverage sector. Chromatography is a technique used in the food business to separate and evaluate ingredients, vitamins, proteins, amino acids, and other nutritional components in food products. Chromatography can also be used to identify the quantity of organic acids present and to identify any potentially dangerous poisons that may have been added to the food item in order to determine expiration dates. In the food business, the 2013 horsemeat crisis is a well-known illustration of chromatography in action.

3) **ENVIRONMENTAL AND CHEMICAL INDUSTRY: -**

The chemical sector is required to follow several environmental safety measures. PFAs, also known as perfluoroalkyl compounds, have developed into a constant hazard to both the environment and human health. PFAs can be found in a variety of products, including electronics, firefighting foams, and protective coatings on clothing and shoes. Although the exceptional durability of these materials makes them useful for products, their continued accumulation poses a threat to the environment.

4) **DRUG TESTING: -**

Chromatography can be particularly helpful in clinical toxicology reports and drug testing. Urine sample components can be separated and analyzed using chromatography. Chromatography analyses a urine sample to identify the compounds that have been consumed, determining whether any dangerous or illegal drugs have been used, whether conducting a clinical toxicology report, drug testing a new hire, or testing a professional athlete for performance enhancing drugs.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

❖ **INTRODUCTION:^[6]**

- High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography.
- It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography.
- The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures upto 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities.
- In HPLC, pumps will be utilized to move the pressured liquid solvent and sample combination into a column made of solid adsorbent material. Each sample component will interact differently, which results in different flow rates for each component and, in the end, leads to the separation of column components. Adsorption is a component of the mass exchange process that makes up chromatography. Pumps are used in

HPLC to pressurize a fluid and a sample blend through an adsorbent-filled section, causing the specimen segments to separate.

- The adsorbent, which makes up the section's dynamic segment, is often a granular substance comprised of solid particles that range in size from 2 μ m to 50 μ m, such as silica or polymers. The components of the sample blend or mixture



Figure 6 :- HPLC instrument

Its structure and temperature have a significant impact on the connections that develop between the sample segments and the adsorbent, which is how the partition process works.

Since HPLC operates at considerably higher pressures (50 bar to 350 bar), it can be distinguished from conventional liquid chromatography, which frequently relies on gravity to move the portable stage through the segment. Scientific HPLC isolates very small amounts of sample, hence column section measurements range from 2.1 mm to 4.6 mm in width and 30 mm to 250 mm in length. Additionally, smaller sorbent particles are used to create HPLC segments. This makes HPLC a popular chromatographic technique by giving it great determining or resolving power (the ability to detect components when isolating mixtures).

❖ PRINCIPLE:^[7]

The distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase is the foundation of the HPLC separation principle (packing material of the column). The molecules travel through the stationary phase more slowly depending on the chemical makeup of the analyte. The duration of a sample's "on-column" time is determined by the specific intermolecular interactions between the sample's molecules and the packing material. As a result, different components of a sample elute at various periods. Thus, the sample ingredients are successfully separated. After leaving the column, the analytes are recognized by a detecting equipment (such a UV detector). A data management system (computer software) converts and records the signals, which are subsequently shown in a chromatogram. The mobile phase may then be subjected to further detector units, a fraction collecting unit, or the waste after passing the detection unit. A solvent reservoir, a pump, an injection valve, a column, a detector unit, and a data processing unit are the typical components of an HPLC system. The pump circulates the solvent (eluent) throughout the system at a high pressure and steady speed. As little drift and

noise as possible are maintained in the detector signal by a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve.

❖ TYPES OF HPLC:^[8]

• Normal Phase HPLC

- This technique uses polarity to separate materials. Hexane, chloroform, and diethyl ether are employed as the non-polar stationary phase while silica serves as the primary polar stationary phase. On a column, the polar samples are kept.

• Reverse Phase HPLC

HPLC is used in reverse to normal phase. The stationary phase is hydrophobic or non-polar while the mobile phase is polar. The non-polar character will be kept more the more of it there is.

• Size-exclusion HPLC

The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.

• Ion-exchange HPLC

The stationary phase has a surface that is electrically charged in the opposite direction of the sample charge. Aqueous buffer is utilized as the mobile phase and will regulate the pH and ionic strength.

❖ INSTRUMENTATION:

The various components that are present in HPLC equipment are:^[4]

➤ HPLC Solvent

➤ Pump

➤ Injector

➤ Column

➤ Detector

➤ Data handling device and microprocessor control

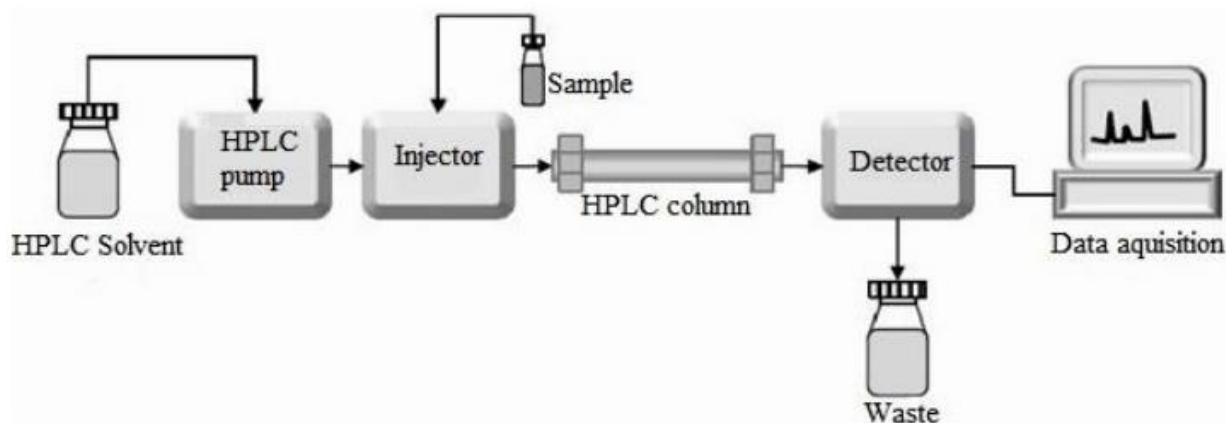


Figure 7:-schematic instrumentation of HPLC

HPLC Solvent^[9]

The solvent reservoir is another name for this component. Here, we keep mobile phase. We use water with a resistivity of 18.2 M cm at 25°C and highly purified solvents such as HPLC grade solvents to prepare the mobile phase.

Pump^[4]

1. A mobile phase flow rate, commonly measured in milliliters per minute, is generated and metered by a high-pressure pump (solvent delivery system or solvent manager). The mobile phase is drawn from the solvent reservoir by the pump, forced into the column, and then passed on to the detector. The column's dimensions, particle size, flow rate, and mobile phase composition all affect the operating pressure. In HPLC, flow rates typically vary from 1 to 2 ml/min. Normal pumps have a pressure range of 6000 to 9000 psi (400-to 600- bar).

1. Displacement pump: It produces a flow that tends to independent of viscosity and back pressure and also output is pulse free. But it possesses limited capacity (250 ml).

2. Reciprocating pump: Its internal capacity is tiny (35 to 400 l), its output pressure is high (up to 10,000 psi), and its flow rates are constant. However, it results in a pulsed flow.

3. The capacity of pneumatic or constant pressure pumps is constrained, and their flow rate is dependent on the viscosity of the solvent and the column back pressure. They can only be used at pressures under 2000 psi.

Injector

There are septum injectors available, which are used to inject the sample solution. The ability to introduce the sample into the continuously circulating mobile phase stream that transports the sample onto the HPLC column is provided by an injector (sample manager or autosampler). The combination of a loop injector and a new, sophisticated rotary valve can result in repeatable outcomes. Sample quantities typically range from 5 to 20 microliters (μ l).

There are three important ways of introducing the sample into injection port.

1. Loop injection: Using a fixed volume loop injector, a fixed volume of volume is introduced.

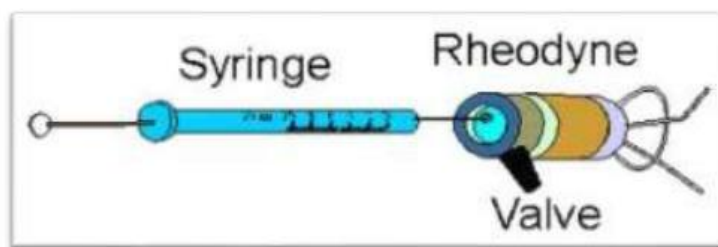


Figure 8:- diagram of loop injection

2. Valve injection: A variable volume is introduced by using an injection valve in this technique.
3. On column injection: A syringe is used to inject a changeable volume via a septum.

Columns^[9]

It is the location of the real separation. The chromatographic packing material required for the separation is present in the column. Due to the fact that the column hardware keeps this packing material in place, it is known as the stationary phase. It is a stainless steel tube with an internal diameter of 2.46 cm and a length of 5 to 25 cm. The packing material is either completely porous or only slightly porous.

Modern HPLC uses packing made of tiny, stiff particles with a limited particle size distribution. In HPLC, there are three different forms of column packing. Porous, polymeric beds.

1. Porous layer beds
2. Totally porous silica particle

Detector^[9]

The detector is capable of identifying the specific molecules that elute from the column. In order for the chemist to quantitatively examine the sample components, a detector measures the quantity of those molecules. A recorder or computer receives an output from the detector, which produces the liquid chromatogram (i.e., the graph of the detector response). When a substance has travelled through the column, it can be determined in a number of different ways. UV spectroscopy is typically used to detect the particular chemicals. Numerous organic substances are UV-absorbing at various wavelengths. The amount of a specific substance that is travelling through the beam at any one time will determine how much light is absorbed.

There are basically two types of detectors

1. Bulk property detectors:- It contrasts the overall alterations in a mobile phase physical characteristic with and without an eluting solute. such as density, dielectric constant, or refractive index..

2. Solute property detectors: It reacts to a solute physical characteristic that the pure mobile phase does not display. for instance, diffusion current, fluorescence, or UV absorption.

Data handling device and microprocessor control^[9]

Each peak in the output is a different compound in the mixture that has passed through the detector and absorbed UV light. The area beneath the peak, which is proportionate to the quantity of drug detected, can be determined automatically by the computer connected to the display.

❖ ADVANTAGES:^[10]

- HPLC provides a quick, automated, and incredibly accurate way to identify specific chemical components in a sample.
- Using high-performance liquid chromatography, a quantitative analysis is quick and accurate.
- A gradient solvent system may be used in specific procedures.
- HPLC can be upgraded to mass spectroscopy (MS).
- The HPLC is very rapid, efficient, and delivers high resolution as compared to other chromatographic techniques, such as TLC, column chromatography, and paper chromatography.
- Manages all areas of analysis to increase productivity

❖ DISADVANTAGES:^[10]

- HPLC can be an expensive method, it required a large number of expensive organics, needs a power supply, and regular maintenance is required.
- It can be complicated to troubleshoot problems or develop new methods.
- The lack of a universal detector for HPLC, however, the UV-Vis detector only detects chromophoric compounds.
- The separation in High-performance liquid chromatography has less efficiency than GC.
- It is more difficult for the beginner.
- HPLC pump process reliability relies on of cleanliness of the sample, mobile phase, and proper operation of the system.

APPLICATION:^[11]

Pharmaceutical applications

There is a wide variety of applications throughout the process of creating a new drug from drug discovery to the manufacture of formulated products that will be administered to patients.

This Process to create a new drug can be divided into 3 main stages

1)Drug discovery 2)Drug development 3)Drug manufacturing.

LC-MS is the best tool for compound identification and characterization. It may be used as a measurement tool during high throughput screening. Preparative HPLC is also used to isolate and purify hits and lead compounds as required. E.g.: a combinatorial synthesis.

1. Tablet dissolution study of the pharmaceutical dosage form.
2. To control drug stability, Shelf-life determination.
3. Identification of active ingredients.
4. Pharmaceutical quality control.
5. Tablet dissolution of pharmaceutical dosage forms.

Food and Flavor analysis

1. Rapid screening and analysis of components in nonalcoholic drinks.
2. Measurement of quality of soft drugs and water.
3. Sugar analysis in fruit juices.
4. Analysis of polycyclic compounds in vegetables.
5. preservative analysis.
6. Multiresidue analysis of lots of pesticides in food samples by LC triple quadrupole MS.

Environmental applications

1. Detection of phenol compounds in drinking water.
2. Identification of diphenhydramine in sedimented samples.
3. Bio-monitoring of pollutant.
4. Rapid separation and identification of carbonyl compounds by HPLC.
5. LC/MS/MS solution for pharmaceuticals and personal care products in water, sediment, soil and biosolids by HPLC/MS/MS.
6. Determination of 3-mercaptopropionic acid by HPLC

Forensics applications

1. Quantification of the drug biological samples.
2. Identification of anabolic steroids in serum, urine, sweat & hair.
3. Forensic analysis of textile dyes.

4. Determination of cocaine and other drugs of abuse in blood, urine, etc.

5. Determination of benzodiazepines in oral fluid using LC/MS/MS.

Clinical applications

1. Catecholamines such as epinephrine and dopamine are highly important for many biological functions. Analyzing their precursors and metabolites can provide diagnosis of diseases such as Parkinson's disease, heart disease, and muscular dystrophy.

2. Quantification of ions in human urine analysis of antibiotics in blood plasma.

3. Estimation of bilirubin & biliverdin in blood plasma in case of hepatic disorders.

4. Detection of endogenous neuropeptides in extracellular fluids of the brain.

CONCLUSION:

One of the most popular analytical techniques is HPLC. It has several benefits over traditional chromatographic methods. HPLC makes precise and quick identification and determination of a variety of natural and synthetic substances possible because of its ease of use and effectiveness. In terms of quantitative and qualitative estimation, it has several applications in a variety of sectors, including pharmaceutical, environmental, forensic, food and flavor, clinical, and many others. It can be used in both laboratory and clinical science. Essentially, it is a greatly enhanced kind of column chromatography. Solvent is externally driven through a column at high pressures of up to 400 atm rather than dripping through it while only being affected by gravity. This greatly speeds up the chromatographic procedure. Additionally, it permits the use of column packing material with very fine particle size, providing a lot more surface area for interactions between the stationary phase and the molecules passing through it. As a result, it makes it possible to separate the mixture's components better. The cost of HPLC is its lone drawback.

REFERENCES:

1. Gurdeep R. Chatwal, Anand Sharma Instrumental method of Analysis 5th edition, Himalaya publishing house PVT.LTD, 2002 pp 2.566-2.587.
2. Mohamed Ahmed Sayed, "A review of chromatography: principles, classification and application", *Researchgate*, **2021**, 1-15.
3. Dr. A. V. Kasture, et al. pharmaceutical analysis Instrumental Methods, Nirali prakashan, 21st edition, 2013, pp 2.4-2.
4. SS Dubey, C Sudhakar, "Application of HPLC and UPLC Techniques- A Short Review", *Inter. Advanced Research J. in Sci, Engi. and Tech*, **2020**, 7(5), 10-20.
5. MR Bansal, et al. "HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A SHORT REVIEW", *J of Global Pharma Tech.*, **2010**, 2(5),22-26.

6. Ramole Rina, et al. "A Review: Analytical Method Development and Validation", *Syst. review Pharmacy*, **2021**,12(8): 450-454.
7. Kate Monks, Juliane Bottcher, et al. "HPLC Basic principles and parameters", <https://www.knauer.net/en/Systems-Solutions/Analytical-HPLC-UHPLC/HPLC-Basics---principles-and-parameters#:~:text=The%20separation%20principle%20of%20HPLC,while%20passing%20the%20stationary%20phase>
8. Mukthi Thammana, "A Review on High Performance Liquid Chromatography", *Research & Reviews: J. of Pharm. Anal.*, **2016**, 5(2), 22-28.
9. Gita Chawla, K K Chaudhary, "A review of HPLC technique covering its pharmaceutical, environmental, forensic, clinical and other applications", *Inter. J. of Pharmaceutical Chem and Analysis*, **2019**, 6(2),27-39.
10. M. Patil, "Advantages and disadvantages of HPLC", march 2019, <https://chrominfo.blogspot.com/2019/03/advantages-and-disadvantages-of-hplc.html?m=1>
11. P. R. Shankar, Sai Snehalata, et al. "Applications of HPLC in Pharmaceutical Analysis", *International J. of pharm. Sci. Reviews and Research*, **2019**, 59(1), 117-124.
12. PV Sujani, et al. "Validated RP-HPLC Method for the estimation of Simvastatin and Sitagliptin", *Scholars Academic journal of Pharmacy*, **2014**,3(3), 265-270.
13. P.B. Miniyar, et al. "RP-HPLC Method Development and Validation for estimation of Stavudine by DoE Approach", *Pharmaceutical Resonance*,**2018**,1(1), 1-7.
14. M W Ibrahim, et al. "Application of HPLC Method in Determination of Miconazole nitrate in Environmental Sample from el-gharbia governorate in Egypt", *J. of Anal. and Pharm. Research*, **2019**,8(4), 160-164.
15. C Garcia, et al. "Validation of an Analytical method by HPLC applicable to the Cuban mangiferin", *Ars Pharmaceutica*, **2018**,59(4),227-233.