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Instrumental Handling and Applications of 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 movable phases. The difference in the partition coefficients of the two phases serves as the basis for the separation of constituents. The word "chromatography" comes from the Greek words "chroma" (colour) and "graphein" (to write). For measuring pharmaceutical and environmental sample quality and quantity, high performance liquid chromatography (HPLC) is a crucial technique. It is the most adaptable, secure, dependable, and quick chromatographic approach for determining the quality of medicinal ingredients. The analytical chemistry method of high-performance liquid chromatography (HPLC; formerly known as high-pressure liquid chromatography) is used to separate, recognise, and quantify each component in a mixture. One type of liquid chromatography, HPLC, uses a liquid as the mobile phase. The most used kind of HPLC is reversed-phase HPLC. In a reversed-phase system, the stationary phase is relatively non-polar while the mobile phase is relatively polar. A solvent reservoir, pump, injector, column, detector, and integrator or acquisition and display system are components of HPLC instrumentation. The column where separation takes place is the brain of the system. HPLC can be used to identify, quantify, and resolve a compound, among other pieces of information.

Keywords: Chromatography, Pressure Liquid Chromatography, Types, Instrumentation, Application, Advantages & Disadvantages of HPLC

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INTRODUCTION

Chromatography refers to the methods used to separate, recognize, and quantify the chemical components present in complicated mixtures.

Similar to spectroscopy, this method is widely used and extremely effective for both analytical and preparative methods. By using this technique, high-grade pure compounds can be produced. Chromatography's definition is as follows: “It is the technique in which the components of a mixture are separated based upon the rates at which they are carried or moved through a stationary phase (column) by a gaseous or liquid mobile phase.[1]

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries. HPLC has over the past decade become the method of choice for the analysis of a wide variety of compounds. Its main advantage over GC is that the analytes do not have to be volatile, so macromolecules are suitable for HPLC analysis.[2]

HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of the stationary phase.

The separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase. Column length No. of theoretical plates per unit area Resolving power Column length Particle size Surface area [3]

In HPLC this partitioning is affected by the relative solute/stationary phase and solute/mobile phase interactions. Thus, unlike GC, changes in mobile phase composition can have an enormous impact on your separation. Since the compounds have different mobilities, they exit the column at different times; i.e., they have different retention times. [4]

The retention time is the time between injection and detection. Thus, HPLC is most often used when one is performing a target compound analysis, where one has a good idea of the compounds present in a mixture so reference standards can be used for determining retention times.

HPLC has gained its quality primarily because of its reliability (use of pressure-driven liquid support) and suppleness (possibility of adjusting the composition of every mobile and stationary phase). The activity mode or separation mechanism depends on the final

interactive relationships between the stationary half, the mobile half, and additionally the analyte.

Particle-packed columns with either entirely porous- or the newly developed core-shell particles and monolithic columns are utilized in normal or miniaturized HPLC. Quantitative analysis is often accomplished with HPLC. An automatic injector providing reproducible injection volumes is extremely beneficial and is standard on modern commercial systems. HPLCs are rather simple. Good separation of a given pair of compounds by HPLC depends on the choice of column and efficiency of the overall system.[5]

The relative position of the various components in the sample on the chromatogram is affected by a solute-solvent type of interaction with the column substrate competing with a solute-solvent interaction with the mobile phase. Column efficiency is concerned with the broadening of an initially compact band of solutes as it passes through the column.

The broadening is a result of column design and column operating conditions. For samples with a broad range of retention times, it is often desirable to employ solvent programming, whereby the mobile phase composition is varied continuously or in steps as the separation proceeds. The analysis of mixtures of widely varying compositions frequently leads to a very widespread retention time.[6]

HPLC is just one type of liquid chromatography, meaning the mobile phase is a liquid. Reversed-phase HPLC is the most common type of HPLC. What reversed-phase means is that the mobile phase is relatively polar, and the stationary phase is relatively non-polar.

Thus non-polar compounds will be more retained (i.e. have longer retention times) than a polar compounds. In normal phase HPLC, the mobile phase is relatively non-polar and the stationary phase is relatively polar. Other more general types of HPLC include partition, adsorption, ion-exchange, size-exclusion, and thin-layer chromatography.[7]

Principle

In a separation column between a stationary and a mobile phase, the purification happens. A separation column contains a granular substance with incredibly small porous particles as the stationary phase. On the other hand, the mobile phase is a solvent or solvent mixture that is pushed through the separation column under high pressure. The sample is injected into the mobile phase flow from the pump to the separation column via a valve with a connected sample loop, which is a tiny tube or a stainless-steel capillary. [8]

As a result of interactions with the stationary phase, the various components of the sample are retained to variable degrees, which cause them to migrate across the column at various rates. After leaving the column the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer. At the end of this operation a

chromatogram in the HPLC software on the computer is obtained, which allows the identification and quantification of the different substances**.** Though HPLC retains major of the credits for the analytical side, the earlier one of simple Liquid Chromatography still find application for the preparative purposes figure.

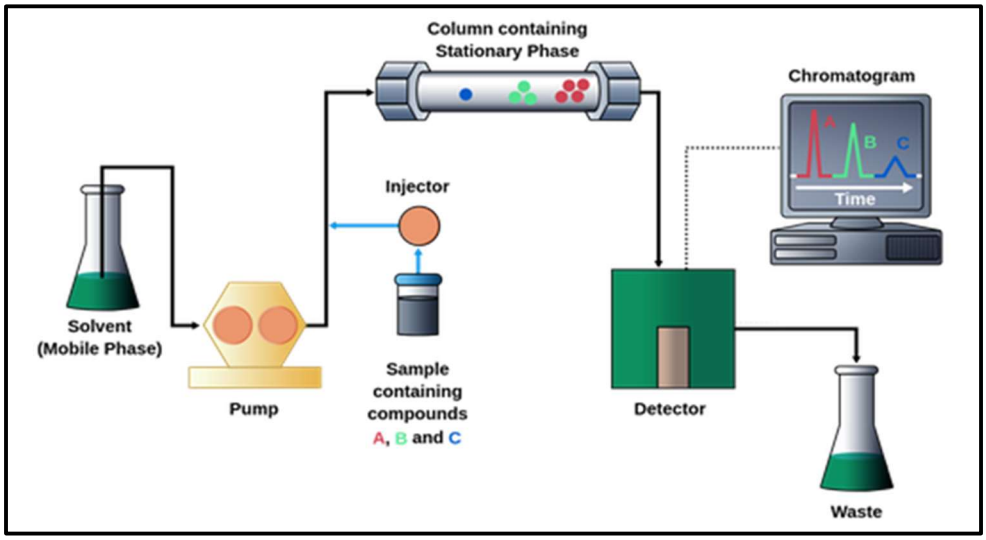


Figure 1: Component of HPLC

The table shows relation between various parameters of HPLC.

• Trendline

Column length ↑	No. of theoretical plates per unit area ↑
Resolving power ↑	Column length ↑
Particle size ↓	Surface area ↑

- Stationary phase have small particulate size and high surface areas.
- Columns: 20 cm or less
- Mobile phase pumped at high pressures of 400Bar, 6000 psi.
- Flow rates: 1-3 cm3 per min [9]

INSTRUMENTATION

Components of HPLC:

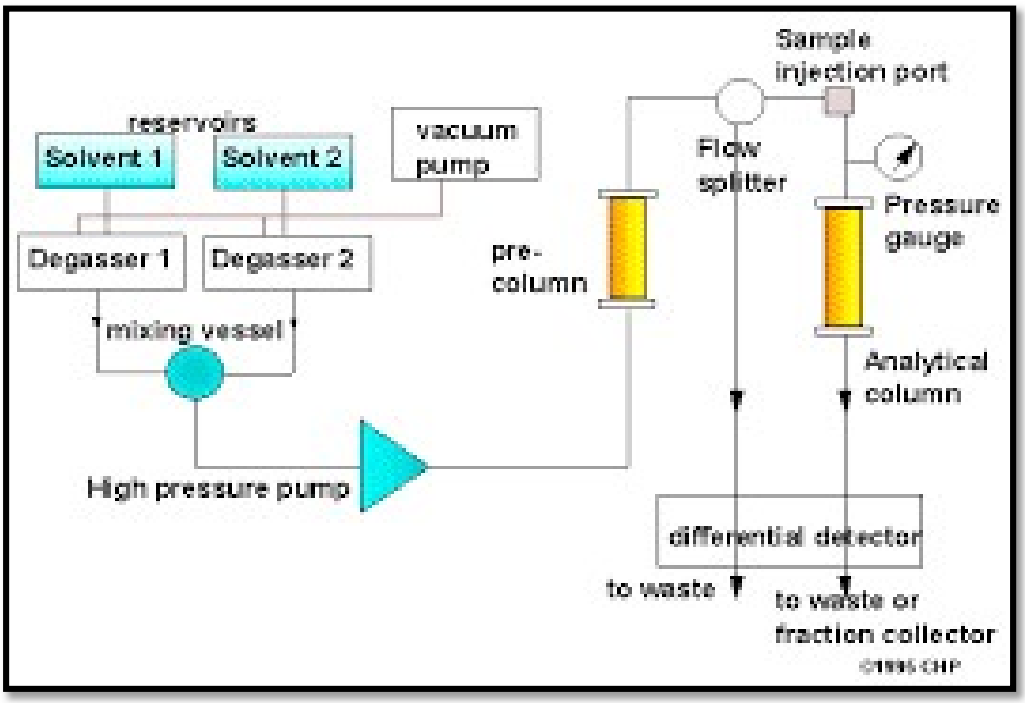
1. Solvent Reservoir
2. Pump
3. Sample Injection System
4. Columns
5. Detectors
6. Data Collection Devices
7. Degasser

8. Column Heater

SOLVENT RESERVOIR (MOBILE PHASE) :

Mobile stage substances are contained in a glass reservoir. The versatile stage, or dissolvable, in HPLC is typically a blend of polar and non-polar liquid segments whose particular fixations are changed relying upon the arrangement of the specimen.[10]

- Measures: microfilter, degasser
- Degassing:
- -Vacuum filtration, Ultrasonication
    - Warming
    - Stirring vigorously with magnetic stirrer
    - Sparge with inert gas (N2 or He)



HPLC PUMP

A pump suctions the versatile stage from the dissolvable reservoir and drives it through the framework's column and detector. Contingent upon various components including column measurements, molecule size of the stationary stage, the stream rate and synthesis of the versatile stage, working weights of up to 42000 kPa (around 6000 psi) can be created.

Ideal pump

- Deliver high pressure (upto 50MPa)
- Deliver pulse free flow
- Constant volume delivery
- Deliver high volumes (flow rates) of solvent (to 10 mL/min)
- Solvent replacement is easy

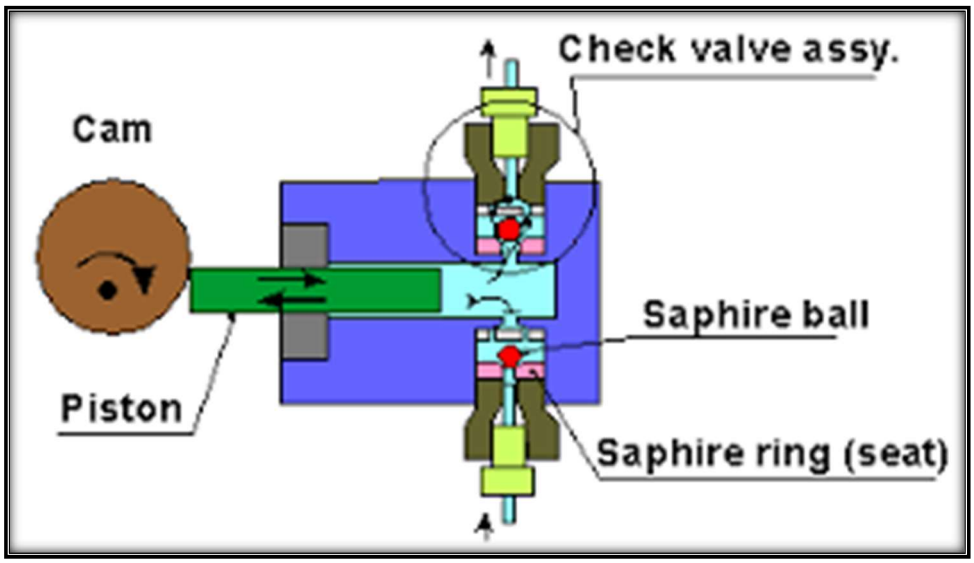
TYPES OF HPLC PUMP

Constant Pressure

- A steady pump pressure
- (usually about 1000–2000 psi) is needed to ensure reproducibility & accuracy.

Constant displacement Pump

- Reciprocating pump: constant flow rate through the column
- Slight cyclical variation in pressure pulse dampeners.



HPLC PUMP OPERATING MODE

Isocratic elution:

A separation that employs a single solvent or solvent mixture of constant composition with single pump throughout the run. Uses: Simple separation & compound with similar structures & retention time.[11]

Gradient elution:

Here two or more solvent systems that differ significantly in polarity with separate pumps.

- the ratio of the solvents is varied in a programmed way,
- sometimes continuously and
- sometimes in a series of steps.
- Separation efficiency is greatly enhanced by gradient elution.

Uses: complex separations

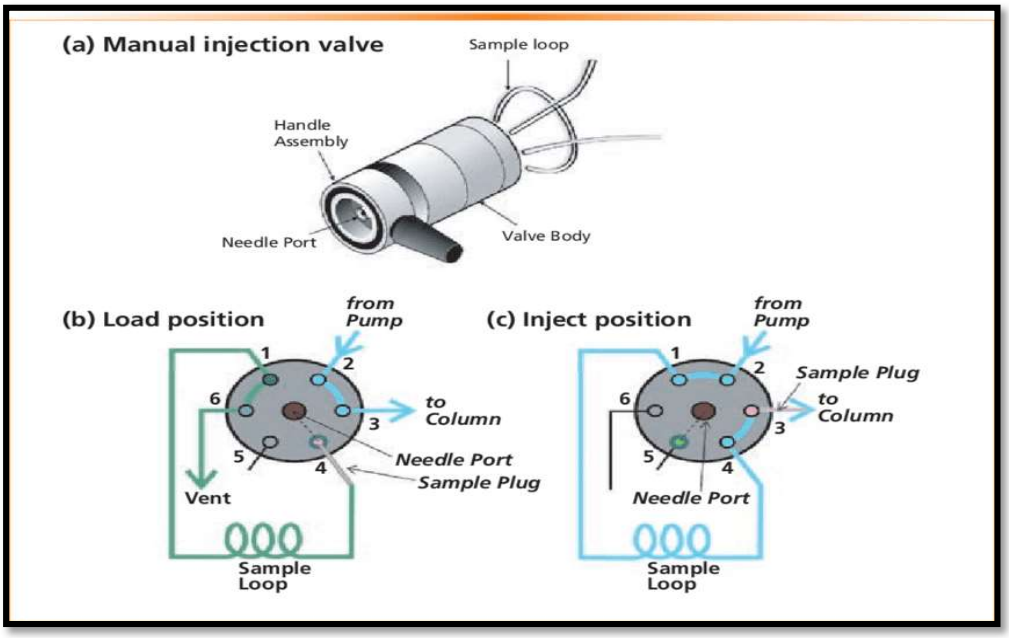
SAMPLE INJECTION SYSTEM

The injector can be a solitary infusion or a mechanized infusion framework. An injector for a HPLC framework ought to give infusion of the liquid specimen inside the scope of 0.1–100 mL of volume with high reproducibility and under high weight (up to 4000 psi).

Manual Injector:

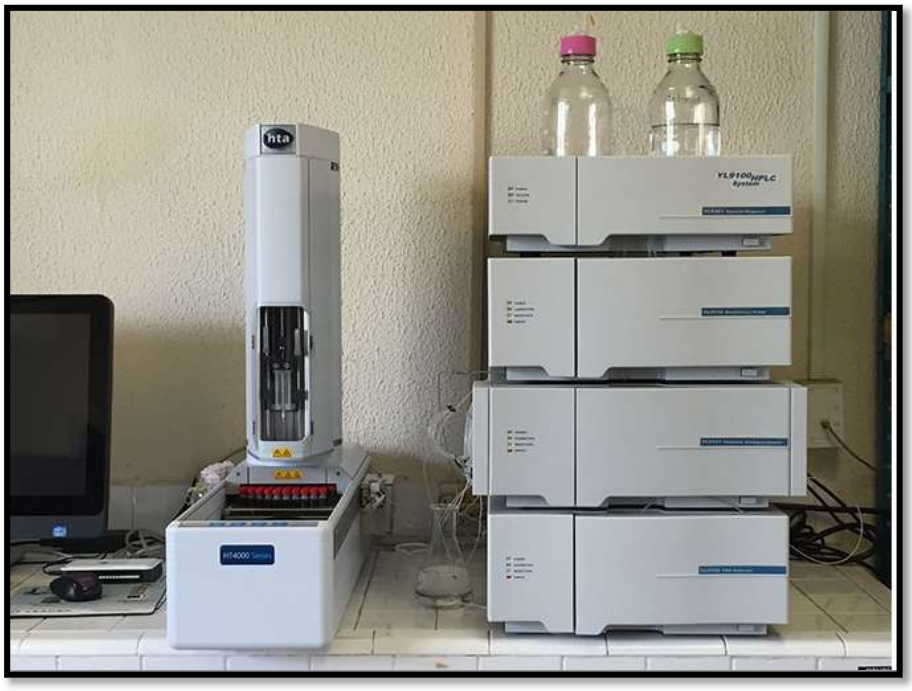
- Manually load sample into the injector using a syringe.

- and then injected sample → into the flowing mobile phase →which transports the sample into the beginning (head) of the column, which is at high pressure.



Autosampler injector:

- User loads vials filled with sample solution into the autosampler tray (100 samples)
- Autosampler automatically
- Takes appropriate sample volume,
- injects the sample,
- then flushes the injector to be ready for the next sample, until all sample vials are processed.[12]



Columns :

Columns are generally made of cleaned stainless steel, are in the vicinity of 50 and 300 mm long and have an inside distance across of in the vicinity of 2 and 5 mm. They are normally loaded with a stationary stage with a molecule size of 3–10 µm. Columns with interior distances across of under 2 mm are regularly alluded to as microbore HPLC columns. In a perfect world the temperature of the portable stage and the column ought to be kept steady amid an examination.

- Control of column temperature as constant temp is required.
- Achieved by: – Column chambers
  - Water jackets
  - Temperature controlled blankets
  - Heating/cooling blooks
- Stable column temperature is required to generate reproducible retention time.

It is a vital component and should be maintanied properly as per supplier instructions for getting reproducibility sepration efficiency run after run. The type of columns are:

Guard columns:

A guard column is introduced before the analytical column to increase the life of the analytical column by removing not only particulate matter and contaminants from the solvents but also sample components that bind irreversibly to the stationary phase. The guard column serves to saturate the mobile phase with the stationary phase so that losses of this solvent from the analytical column are minimized. The composition of the guard-column packing is similar to that of the analytical column; the particle size is usually larger. When the guard column has become contaminated ,it is repacked or discarded and replaced with a new one.

Analytical columns:

It is the heart of High-performance liquid chromatography. Liquid-chromatographic columns range in length from 10 to 30 cm. normally, the columns are straight, with added length, where needed, being gained by coupling two or more columns together. The inside diameter of liquid columns is often 4 to 10 mm; the most common particle size of packing is 5 or 10 m. The most common column currently in use is one that is 25 cm in length, 4.6 mm inside diameter, and packed with 5 mm particles. Columns of this type contain 40,000 to 60,000 plates/meter Figure .

HPLC columns are mostly made up of smooth bore stainless steel. HPLC columns are sometimes made from heavywalled glass tubing and polymer tubing, suc

HPLC columns are mostly made up of smooth bore stainless steel. HPLC columns are sometimes made from heavywalled glass tubing and polymer tubing, such as polyether either ketone.[14]

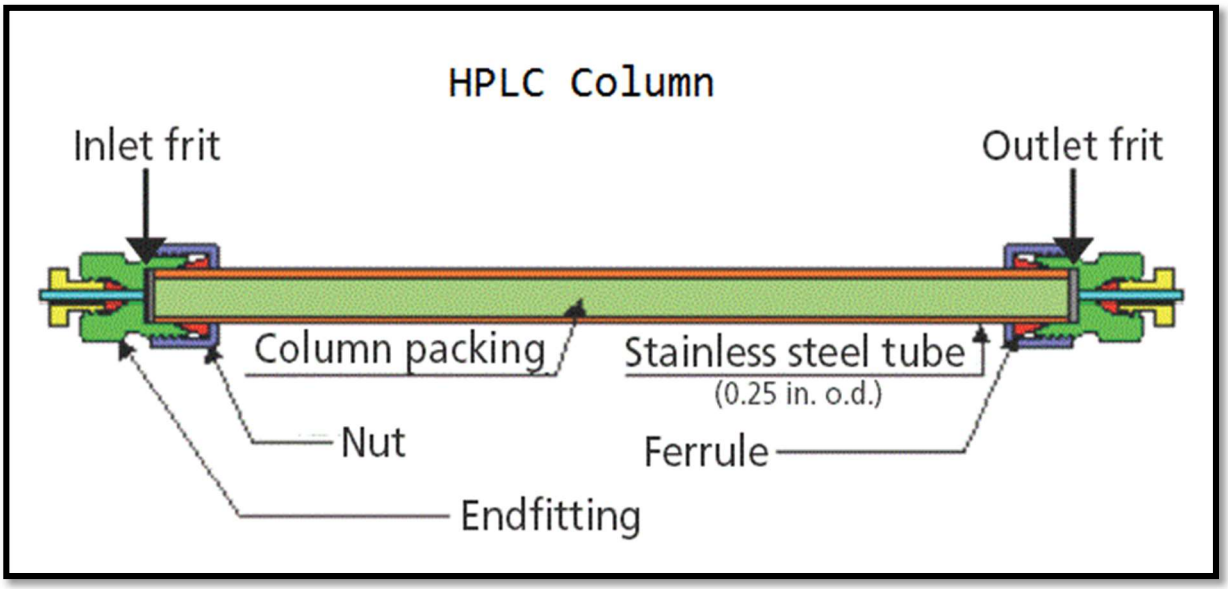


Figure : Typical HPLC column.

Column temperature control:

For some applications, close control of column temperature is not necessary and columns are operated at room temperature. Often, however, better, more reproducible chromatograms are obtained by maintaning constant column temperature .Three way the temperature of a column could be controlled are oven heater block and water bath but now a day modern instruments may need a higher temperature with limitations.[15]

Detector:

The HPLC indicator, situated toward the finish of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized finders are UV spectroscopy, fluorescence, mass-spectrometric and electrochemical indicators.

The detector can see (detect) the individual molecules that come out (elute) from the column.

- A detector serves to measure the amount of thosemolecules so that the chemist can quantitatively analyze the sample components.
- The detector provides an output to a recorder or computer that results in the liquid chromatogram(i.e., the graph of the detector response).[16]

THE IDEAL CHARACTERISTICS

- ✓ Adequate sensitivity for the particular task.
- ✓ Good stability and reproducibility.
- ✓ The wide linear dynamic range of response.

- ✓ Short response time that is independent of flow rate.
- ✓ Insensitive to changes in a solvent, flow rate, and temperature.
- ✓ Cell design that eliminates remixing of the separated bands.
- ✓ High reliability and ease of use.
- ✓ Non-destructive for the sample.

Type of detector

- Mass Spectrometers
- Refractive Index
- Fluorescence
- UV Detectors
- Electrochemical
- IR Detectors

Mass spectrometer :

- Universal detector
- An MS detector senses a compound eluting from the HPLC column First by ionizing it then by measuring its mass and/or fragmenting the molecule into smaller pieces that are unique to the compound.
- The MS detector can sometimes identify the compound directly since its mass spectrum is like a Fingerprint and is unique to that com.[17]

Refractive Index:

- The refractive index (RI) detector uses a monochromator and is one of the least sensitive LC detectors.
- This detector is extremely useful for detecting those compounds that are non-ionic, do not absorb ultraviolet light and do not fluoresce.[18]
- e.g. sugar, alcohol, fatty acid and polymers.

Fluorescence :

- Fluorescence rays emitted by the sample after absorbing incident light are measured.
- A xenon arc lamp is used to produce light for excitation, High precision and sensitivity.
- Only suitable for compounds that can produce fluorescence.
- Some compounds are not stable under fluorescent stimulation.[19]

UV Detectors:

- Based on electronic transitions within molecules.
- A most common type of detector for LC

- The fixed wavelength, Hg lamp 254 nm (π = > π*) • The tunable wavelength is selectable for specific wavelengths, monochromators,or filters.
- Still limited to single wavelengths.
- Solvent limitations with UV-vis abs. Detectors
- Z-shape, flow-through cell (V, 1 ~ 10 µL and b, 2 ~ 10 mm)
- Spectrophotometer: more versatile.[20]

Electrochemical Detectors:

- Based on the amperometric response of analyte to electrode usually held at a constant potential.
- If the analyte is electro-active, can be highly sensitive since the response is based on a surface phenomenon rather than a solution bulk property (e.g. UV-vis absorbance) .
- simplicity, convenience, and wide-spreading application
- The thin-layer flow cell of Teflon: 50µm thick, 1 ~ 5 µL volume.
- Indictor E: Pt, Au, C
- Multi-electrode: simultaneous detection or sample purity indication.[21]

IR Detectors:

- Filter instrument or FTIR
- Similar cell (V, 1.5 ~ 10 µL and b, 0.2 ~ 1.0 mm)
- Limit: no suitable solvent, special optics
- FT-IR allows for spectrum records of flowing systems analogous to the diode array system.
- Water/alcohols can be major interferences to solute detection
- LOD 100 ng (limit of detection)[22]

Data Collection Devices:

Electronic signals generated by detectors are recorded inthe form of chromatograghic peak at varied function of time. Peak Area, height, retention time, base width of chromatograghic peak is measured to compute analyte concentration of each peak.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are nonvisible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. 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. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). [23]

Column Heater

The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep the consistent temperature conditions. Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50 to 80°C). It is also important to keep stable temperature to obtain repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. Thus, columns are generally kept inside the column oven (column heater).[24]

Advantage Of HPLC

- Separations are fast and efficient (high-resolution power).
- Continuous monitoring of the column effluent
- 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 etc.[25]

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.
- Need a skill to run the instuments.
- Solvent consuming.[26]

APPLICATIONS

The identity, quantification, and resolution of a compound are all pieces of information that can be discovered via HPLC. The isolation and purification of chemicals is known as preparative HPLC. This contrasts with analytical HPLC, where learning more about the sample substance is the primary goal.

The following are the main applications:

Pharmaceuticals :

In order to determine API and related compounds in a single run, highperformance liquid chromatography delivers dependable quantitative precision and accuracy in addition to a high linear dynamic range. The dispersion of samples in water or aqueous solutions that have been modified with acetonitrile or methanol is a practical method for solid dosage form manufacturing. There are numerous ways to separate chiral compounds into their individual enantiomers using HPLC. Precolumn derivatization to create diastereomers is one of them. You might also employ custom columns made with cyclodextrins or unique chiral moieties as stationary phases. To put it briefly, reverse phase HPLC the pharmaceutical industry's most widely used option for quantitative analysis is HPLC. The following are typical application areas for pharmaceutical analysis:[27]

Analytical Method Validation :

Analytical method validation is a documented process that proves an analytical method is suitable for its intended purpose by ensuring the reliability and consistency of its results. It involves assessing key parameters like accuracy, precision, specificity, linearity, and robustness to confirm the method’s quality, safety, and efficacy. This process is critical in regulated industries such as pharmaceuticals to meet requirements from agencies like the FDA.

To control drug stability :

To control drug stability, pharmaceutical manufacturers and distributors must control factors such as temperature, humidity, light, and oxygen, as well as use proper formulation, packaging, and storage practices. Stability testing during drug development is also essential to ensure a product remains safe and effective over its shelf life.

Tablet dissolution study of the pharmaceutical dosage form.

- **Compound Identification :** Compound identification in the pharmaceutical industry involves a combination of spectroscopic, chromatographic, and biological techniques to confirm the identity, purity, and structure of compounds, including active pharmaceutical ingredients (APIs) and impurities. Techniques like Mass

Spectrometry (MS), Nuclear Magnetic Resonance (NMR), Infrared (IR) spectroscopy, and High-Performance Liquid Chromatography (HPLC) are crucial for this process.

- **Pharmaceutical quality control :** Pharmaceutical quality control is the process of ensuring that drug products are safe, effective, and meet all required quality standards through systematic testing of raw materials, intermediates, and finished products. It involves analytical testing, documentation, and ensuring compliance with regulations to guarantee identity, purity, and potency. This is a product-focused activity that acts as a final checkpoint to identify defects and implement corrective measures.
- **Stability Studies :** Stability studies evaluate how a product's quality, safety, and efficacy change over time under various environmental conditions like temperature, humidity, and light. These studies are crucial in industries like pharmaceuticals to determine a product's shelf life, establish appropriate storage conditions, and ensure it meets regulatory requirements for its entire lifespan**.**
- **Assay :** An assay is an analytical procedure to determine the presence, amount, and/or functional activity of a specific substance in a drug product. It's a critical quality control test to ensure a drug is safe, effective, and meets label claims for its active pharmaceutical ingredient (API).
- **Related Substances :** Related substances in pharma are structurally similar impurities that can be found in a drug substance or product, arising from manufacturing processes, starting materials, or degradation. They are crucial to monitor because they can affect a drug's safety and efficacy, and their presence must be controlled within strict limits defined by regulatory bodies like the FDA and ICH guidelines. Analytical methods like high-performance liquid chromatography (HPLC) are used to identify and quantify these substances to ensure the quality and consistency of the final product.
- **Working Standards :** A pharmaceutical working standard is a calibrated reference material of high purity used for routine quality control and analytical testing in a lab. It is prepared by comparing it against a more expensive, primary reference standard to ensure consistency and accuracy in day-to-day operations, making it a cost-effective alternative for frequent use. Working standards require regular re-calibration to confirm their accuracy over time.[28]

Food:

In the area of food analysis, high-performance liquid chromatography has brought about a number of desired benefits. Food matrices are typically complex, making analyte extraction a difficult operation. The fact that both desired and unwanted components are frequently

present in trace quantities further complicates issues, and traditional extraction and analysis do not offer the necessary levels of accuracy and precision. Due to the numerous stationary phase and mobile phase possibilities, HPLC provides workable solutions.[29]

Common applications in foods are:

- Fat-soluble vitamins (A, D, E, and K)
- Water-soluble vitamins (B-complex vitamins such as B1 , B2 , B3 , B6 , Folic acid, Pantothenic acid, B12, Vitamin C)
- Residual pesticides such as 2, 4-D, and Monochrotophos.
- Antioxidants such as TBHQ, BHA, and BHT.
- Sugars: Glucose, Fructose, Maltose, and other saccharides
- Cholesterol and sterols
- Mycotoxins such as Aflatoxins B1 , B2 , G1 , G2 , M1 , M2 , and ochratoxin
- Amino acids
- Residual antibiotics
- Steroids and flavanoids
- Aspartame and other artificial sweeteners.

Manufacturing:

Both in the laboratory and in the field of clinical science, HPLC has several uses. Since it is a reliable method for obtaining and ensuring product purity, it is a common approach employed in pharmaceutical development. Despite the fact that HPLC can create products of incredibly high quality (purity), it isn't always the main technique employed in the manufacturing of bulk medicinal ingredients. Only 15.5% of syntheses, according to the European Pharmacopeia, use HPLC. In contrast, it's involved in 44% of the syntheses in the US Pharmacopeia. Given that HPLC can be an expensive procedure when used on a big scale, this might be the result of different time and financial restrictions. Unfortunately, an increase in cost is correlated with an increase in HPLC's specificity, precision, and accuracy.

Research:

Similar assays can be performed for research purposes, detecting concentrations of potential clinical candidates like anti-fungal and asthma drugs. This technique is obviously useful in observing multiple species in collected samples, as well, but requires the use of standard solutions when information about species identity is sought out. It is used as a method to confirm the results of synthesis reactions, as purity is essential in this type of research.

Medical:

HPLC can be used for medication analysis in medicine, however nutritional analysis is more closely associated with this use. Blood serum is the sample used for the majority of medical

HPLC tests, even though urine is the most used medium for assessing drug concentrations. HPLC has been compared against other approaches, especially immunoassays, for the detection of compounds useful for clinical studies. In one instance, the sensitivity of HPLC and competitive protein binding assays (CPBA) for the detection of vitamin D was evaluated. It was discovered that while this CPBA was helpful for identifying vitamin D deficiency in children, its sensitivity and specificity only reached 40% and 60%, respectively, of HPLC's capacity. HPLC is a costly tool, yet it has almost unmatched accuracy**.**

Other application of HPLC includes

Environmental Applications

1. Detection of phenolic compounds in drinking water.
2. Bio-monitoring of pollutants.

Applications in Forensics

1. Quantification of drugs in biological samples.
2. Identification of steroids in blood, urine, etc.
3. Forensic analysis of textile dyes.
4. Determination of cocaine and other drugs of abuse in blood, urine etc.

Applications in Clinical Tests

1 Urine analysis, antibiotics analysis in blood.

2 Analysis of bilirubin, biliverdin in hepatic disorders.

3 Detection of endogenous neuropeptides in the extracellular fluid of the brain, etc.

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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