High Performance Liquid Chromatography (HPLC)

HPLC can be discussed by following headings.

⇒ Introduction
⇒ Basic principle
⇒ Instrumentation
- The solvent reservoir
- The pumping system
- The injector
- The column
- The detector
- The data system

⇒ Separation modes of HPLC / Types of HPLC
⇒ Advantages of HPLC
⇒ Applications of HPLC

⇒ Introduction to HPLC

HPLC is an abbreviation for High Performance Liquid Chromatography. It is also called as Ultra performance liquid chromatography. HPLC was first time described by Kirkland and Huber b/w 1967 & 1969. HPLC has made significant contribution to pharmaceutical, clinical, biochemical and environmental samples where analysis takes place b/w 5–30 minutes because of application of high pressure of 1000–6000 psi. HPLC can readily be used for fractionation of
- Thermally unstable compounds
- Pesticides
- Polymers
- pharmaceutical drugs
- Highly polar substances including ions.
- Heavy hydrocarbons
- Enzymes etc.

Date: / /

⇒ Basic Principle :-

HPLC is a separation technique that involves the injectio of a small volume of liquid sample into a tube (the column) packed with tiny particles (3-5 um in diameter called stationary phase where individual components of the sample are moved down the column with a liquid mobile phase forced through column by high pressure delivered by a pump. The components in the sample get separated from one another due t their different physical & chemical interactions b/w the stationary & mobile phase. These separated components are detected at the exit of the colum by a detector that can also measure their amount. An output from this detector is calle a liquid chromatogram.

⇒ Instrumentation :-

i. The Solvent Reservoir.

The choice of mobile phase is very important in HPLC a the eluting power of mobile phase is determined by u overall polarity, the polarity of stationary phase and the nature of sample components. For normal phase HPLC, non-polar solvents are used with polar stat phase but reverse is used for reverse phase HPL It is very important to use

- de-aerated mobile phase to avoid pressure fluctuati
- HPLC grade solvents in order to remove UV-absorbi impurities.

The most widely used non-polar solvents include hexane, chloroform, toluene and the polar solvents

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include water + miscible organic solvent e.g methanol, ethanol, acetonitrile, THF etc.

ii. The Pump

The role of the pump is to force the liquid mobile phase through the column at a specific flow rate, expressed in milliliters per minute (ml/min). Normal flow rates in HPLC are in the 1-2 ml/minute range. The pumping system can generate a pressure of about 6000 psi depending upon column dimensions, particle size of stationary phase, flow rates & the composition of mobile phase. There are four important types of pumps which have been used in HPLC to propel the liquid mobile phase through the system. These are

a) Pneumatic pumps
b) Syringe type pumps
c) Reciprocating pumps
d) Hydraulic amplifier pump.

During the chromatographic operation, a pump can deliver the mobile phase to the column by either of the two ways.

i. Isocratic elution
ii. Gradient elution.

In isocratic elution the composition of mobile phase does not change but during gradient elution the composition of mobile phase changes with the passage of time. Most modern HPLC chromatograph are equipped with a computer or a microprocessor that runs the system by either isocratic elution or by gradient elution according to defined programme.

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iii. The Injector :-

The sample solution is usually introduced into the flowing mobile phase at or near the head of column using an injector based on injection valve design which can operate at high pressure. The typical injector used are syringe injector that can serve Sometimes manual injector and autoinjector.

iv- The Column :-

HPLC columns are usually constructed from smooth bore polished stainless steel tubing. HPLC columns are Sometimes made from heavy walled glass tubing and polymer tubing tubing such as polyetheretherketone (PEEK). In addition stainless steel columns lined with glass or PEEK are also available.

most HPLC columns range from 5-25cm long: Straight Columns are invariably used. Sometimes length is added by coupling two or more columns. The inside diameter of analytical column is often 3-5 mm, with most common particle size of packings is 3 or 5 μm. The most common columns are 10 or 15 cm long, 4.6 mm internal diameter and packed with 5 μm ~~diamete~~ particles.

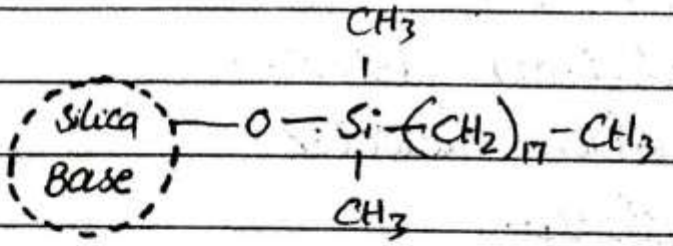
Usually a short Guard column is introduced before the analytical column for removing not only particulate matter and contaminants from solvents but also sample components that bind irreversibly to the stationary phase. The composition of the guard column is similar to that of analytical column.

Two basic types of packed particles have been used in HPLC columns i.e pellicular & porous.

The original pellicular particles were spherical, non-porous glass or polymer beads upon which a thin layer of silica, alumina, ~~Polyvinyl~~ polystyrene-divinylbenzene synthetic resin or ion-exchange resin is deposited. The porous particles

There are many types of stationary phases used in HPLC that include

- Unmodified Silica, alumina or porous graphite which constitute normal phase HRLC - where separation is based on differences in adsorption and desorption.
- a variety of chemically modified supports ~~prepared~~ deposited on ~~from~~ polymers, Silica, alumina or porous graphite. This constitute reverse phase HPLC where separation is based on partition of molecules b/w mobile phase & Stationary phase. The most widely used Supports include
  - octyl $Si-(CH_2)_7-CH_3$ C_8
  - octadecyl $Si-(CH_2)_{17}-CH_3$ C_{18}
  - Cyanopropyl $Si-(CH_2)_3-CN$
  - aminepropyl
  - diol $Si-(CH_2)_3-CH(OH)-CH_2-OH$



- Resins or polymers with acid or basic groups that constitute ion-exchange HPLC
- Natural or synthetic gels for Size exclusion HPLC.

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v. The detector :-

The function of the detector is to monitor the liquid mobile phase for presence of sample molecules as they emerge from the column. The detectors for HPLC are of two types

⇒ Bulk property detectors which compare an overall change in a physical property of the mobile phase with and without an eluting solute e.g

- Refractive index detector
- Conductivity detector

⇒ Solute property detector which responds to a physical property of the solute which is not exhibited by pure mobile phase. Examples include

- Photodiode assay detector (PAD)
- UV-Visible detector
- IR detector
- Evaporative light scattering detector.
- multi-angle light scattering detector
- mass spectrometric detector
- Florescence detector
- chemiluminescence detector
- Optical Rotation detector
- photo-ionization detector

vi- The data system..

A computer called the data system, not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (Retention time)

of the Sample components (qualitative analyses) and the amount of sample components (quantitative analysis)

⇒ Separation Modes in HPLC.

There are four major separation modes that are used to separate most compounds

- Normal phase HPLC
- Reverse phase HPLC
- Ion-EXchange HPLC
- Size EXClusion HPLC.

Let us briefly look at each mode.

★ **Normal phase HPLC**

In this mode, the column packing is polar e.g Silica Gel, ~~Cyanopropyl bonded~~ alumina and mobile phase is non-polar (hexane, iso-octane, DCM). The technique is useful for water sensitive compounds, chiral compounds, Geometric isomers.

★ **Reverse phase HPLC**

In this mode, the column packing is non-polar e.g C_8, C_{18}, phenyl and mobile phase is water + miscible solvent e.g methanol, acetonitrile. It is the most popular mode and over 90% of Chromatographers use this mode.

★ **Ion-EXchange HPLC**

In IE HPLC, the column packing contains ionic groups such as Sulfonic, (SO_3H), tetra-alkylammonium (R_4N^+) on polymers formed by copolymerization of styrene and divinylbenzene and the mobile phase is an aqueous buffer (Phosphate, formate). This mode is well suited for

- separation of inorganic & organic anions & cations
- ionic dyes, amino acids, proteins (RNA polymerase, chymotrypsinogen, Lysozyme).

★ Size EXclusion HPLC

In Size exclusion HPLC, the column packing comprises of some natural or synthetic gel having pores in it and mobile phase may be aquous or non-aquous. The molecules in the sample are separated on the basis of their sizes and shapes. Molecules larger than the pore opening do not diffuse and elute first while the smaller molecules penetrate in pore openings of gel and elute later. This mode of HPLC is mainly used for polymer characterization and for proteins.

⇒ **Advantages of HPLC:-**

- Separation is fast and efficient.
- Column effluents are continuously monitored.
- applicable for analysis of very complex mixture.
- accurate quantitative measurements are possible.
- analytical procedure can be easily automated.
- Adsorption, partition, ion-exchange and size exclusion phenomena are excellently made.
- very little sample pre-treatment are required.
- HPLC is method of choice for thermally labile and high molecular weight compounds.
- versatile technique due to availability of variety of solvents and column packings.

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⇒ Disadvantages of HPLC :-

- Need a skill to run the instrument
- High volumes of solvents are used which are costly.

⇒ Applications of HPLC :-

1. Pharmaceutical industry

- To control drug stability
- To quantify antibiotics, sedatives, steroids, analgesics etc in a pharmaceutical dosage form.
- To determine the quantity of a drug in biological fluids.

2. Environmental Sciences.

To determine the presence of pesticides, phenols, PCBs, condensed aromatics, surfactants, propellants and other pollutants in samples like water, soil, tissues, biological fluids etc.

3. Forensic Science.

- To determine the concentration of steroids, poisons, alcohol, narcotics etc in blood, urine, sweat etc
- To detect the psychotropic drugs in plasma.

4. Clinical Science

- To monitor the presence of bile acids, drug metabolites, estrogens in biological samples for diagnosis of diseases.

5. Food Industry :-

- To determine the presence of artificial sweeteners, anti-oxidants, afflatoxins, preservatives and other additives in food items such as colas, fruit juices, Jams, Jellies, fruits, etc.

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