High Performance Liquid Chromatography (HPLC)

HPLC is a type of liquid chromatography, in which components of mixture partitioned between the stationary phase by liquid mobile phase moving with high pressure.

Gas chromatography is not useful for the separation of compounds which do not vaporize or unstable at high temperature. For analysis of such compounds HPLC is very good technique.

Principle

Compounds are separated by injecting a sample mixture onto the column. Components of sample solution get distributed between stationary and mobile phase while traveling through the column. Depending on the affinity of components for stationary and mobile phase, they travel with different rate causing their separation.

Types of HPLC

HPLC with solid stationary phase

Solid particles having 5-50 micrometer size are used as stationary phase. Due to small particle size pressure is required to push the mobile phase through the column. Mobile phase is liquid flowing with high pressure (30- 200 atm). This technique is based on adsorption principle. (Different components adsorbed differently on solid stationary phase.)

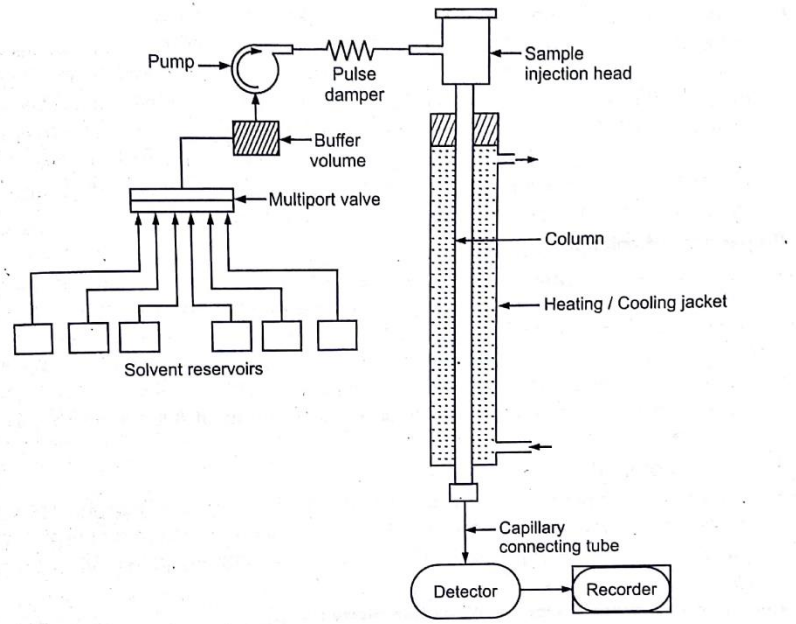
It is generally used for non-polar compounds having high molecular weight.

HPLC with liquid stationary phase

Liquid is coated on solid support act as stationary phase and another liquid act as mobile phase. Separation is based on partition principle.

It is generally used for polar compounds having low molecular weight.

Instrumentation



HPLC consist of following parts

1) **Mobile phase / Solvent reservoir**
2) **Injection valve and sample loop**
3) **Column**
4) **Detector**
5) **Recorder**

Mobile phase / Solvent reservoir

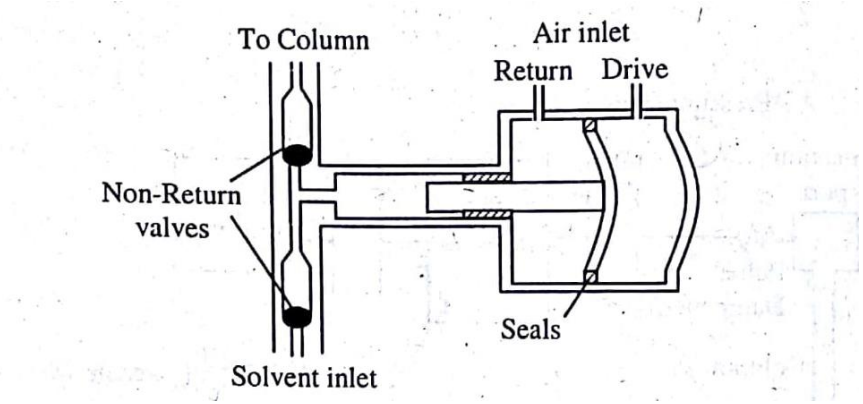
Solvent may be single or mixture of many solvents having different polarity and pH. Depending on the component to be separated either Isocratic or Gradient elution system is used

Isocratic system: same mobile phase is used throughout the separation

Gradient separation: stepwise or continuous change in mobile phase composition.

For HPLC highly pure solvents are required to avoid damage of detector.

High pressure pump



Pump provide uniform stream of solvent from reservoir to detector through the column. Most of the HPLC operate with 400-1500 psi pressure.

Types of pumps:

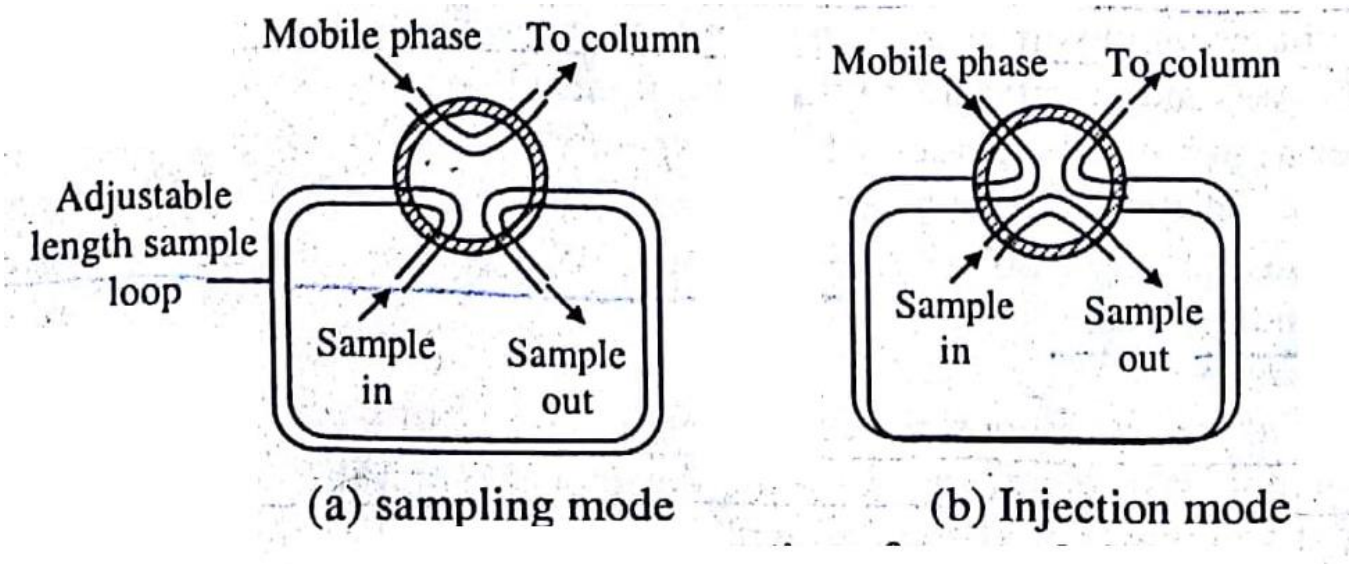
1) Gas displacement pump

It works similar to syringe and drive the mobile phase with high pressure.

2) Reciprocating pump

3) Pneumatic pump

Sample injection valve



It consists of different ports, one of which goes to column. Two ports are connected to external sample loop while remaining three open segments are connected to external port.

Column

Column is made up of stainless steel or heavy wall glass in order to withstand high pressure. Generally 10-50 cm long column with internal diameter 4-10 mm. is used. It is packed with fine particles having 5-25 micrometer diameter. Al_2O_3, Silica gel, Ion exchange resin are some important stationary phases used for packing of column. For some column temperature control is necessary, hence cooling jacket is placed at the end of the column.

Detectors

Detector used in HPLC has very high sensitivity and can detect the component in ppm and ppb level. Detector monitors the mobile phase when it comes out of the column.

Commonly used detectors in HPLC:-

- U.V.-Visible absorption detector
- Refractive index detector
- Diode array detector
- Fluorescence detector
- Amperometric detector

U.V. Visible absorption detector

It is highly sensitive detector having sensitivity about 10^{-8} g/ml (0.01ppm). It work on the principle of Lambert Beer law. It operate at fixed wavelength of 254 nm.

$$A = € .b. C$$

Where, A= Absorbance, b= path length,

C= concentration, € = Molar extinction coefficient

When solute that absorb u.v. radiation come with mobile phase, detector show absorbance which is directly proportional to the concentration of solute. Deuterium or tungsten lamp is used as a source of light.

Limitations:-

1.It can not be used when mobile phase have absorbance in u.v. region.

2.It can not be used when solute does not show absorbance in u.v. region.

Refractive index detector

It is called as universal detector. This measure the refractive index of eluent that coming out from column. (Mixture of solvent and solute component) Refractive index values of mobile phase and mixture of solvent and solute component are different, from which solute component can be identified by using standard values.

Limitation

1) It can detect only concentration up to 10^{-5} to 10^{-6} (1 to 10 ppm)
2) Sensitivity of detector is less

Detectors

iii) Florescence detector

This is useful for detecting naturally fluorescent compounds and their derivatives. Sensitivity better than U.V. detector.

iv) Amperometric detector

This detector is useful for electroactive substances. It consists of three electrodes i.e. working, auxiliary and reference. It used in various biological estimation processes like separation and detection of catechol amines from Bain.

v) Diode Array detector

This detector is useful for analysis of mixtures which absorb in the same wavelength region .It is capable of resolving the overlapping spectra.

Recorder and integrators

Recorder records the responses obtain from the detector. It record the base line and all the peaks obtained with respect to time

Stationary phase used in HPLC

It can be solid, liquid or bonded phase. In bonded phase stationary phase is chemically bound to solid support.

Normal phase chromatography

Combination of highly polar stationary phase (Ethylene glycol, water etc.) and non-polar mobile phase (n-hexane) is called normal phase chromatography. In this type of column less polar component of sample mixture elute first.

Reverse phase chromatography

Combination of non-polar stationary phase (Hydrocarbon.) and polar mobile phase (water, methanol etc.) is called reverse phase chromatography. In this type of column more polar component of sample mixture elute first.

Working of HPLC:

The mobile phase is taken up by pump through the filter. Pressure of solvent is indicated by the pressure guage which is about 2000-10000 psi. Sample in suitable solvent is injected through the rotary valve and slowly loaded on the column through the injection loop. The detector give signal to the recorder and a chromatogram is obtained similar to that in gas chromatography.

Applications of HPLC

HPLC can be used for separation of number of compounds by using different Column, mobile phase and detectors.

1) Pesticide analysis: Analysis of various pesticide resides can be done by using HPLC.

2) Determination of poly nuclear aromatic hydrocarbon. (PAH) Poly nuclear aromatic hydrocarbons are those which contain two or more benzene rings fused together. Poly nuclear aromatic hydrocarbon. (PAH) can also determined by GC or GC-MS but HPLC superior is methods.