4. Size exclusion or gel permeation chromatography

In this type of chromatography, a mixture of components with different molecular sizes is separated by using gels. The gel used acts as molecular sieve and hence a mixture of substances with different molecular sizes is separated. Soft gels like dextran, agrarose or polyacrylamide are used. Semi rigid gels like polystyrene, alkyl dextran in non aqueous medium are also used. The mechanism of separation is by steric and diffusion effects.

5. Affinity chromatography

Affinity chromatography uses the affinity of the sample with specific stationary phases. This technique is used in the field of Biotechnology, Microbiology, Biochemistry, etc.

6. Chiral phase chromatography

Separation of optical isomers can be done by using chiral stationary phases. Different principles operate for different types of stationary phases and for different samples. The stationary phases used for this type of chromatography are mostly chemically bonded silica gel.

C. Based on elution technique

Isocratic separation: In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

Gradient separation: In this technique, a mobile phase combination of lower polarity or elution is used followed by gradually increasing the polarity or elution strength.

D. Based on the scale of operation

Analytical HPLC: Where only analysis of the samples are done. Recovery of the samples for resulting is normally not done, since the sample used is very low. eg. µg quantities.

Preparative HPLC: Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. eg. Separation of few grams of mixtures by HPLC.

E. Based on the type of analysis

Qualitative analysis: Which is used to identify the compound, detect the presence of impurities, to find out the number of components, etc. This is done by using retention time values.

Quantitative analysis: This is done to determine the quantity of the individual or several components in a mixture. This is done by comparing the peak area of the standard and sample.

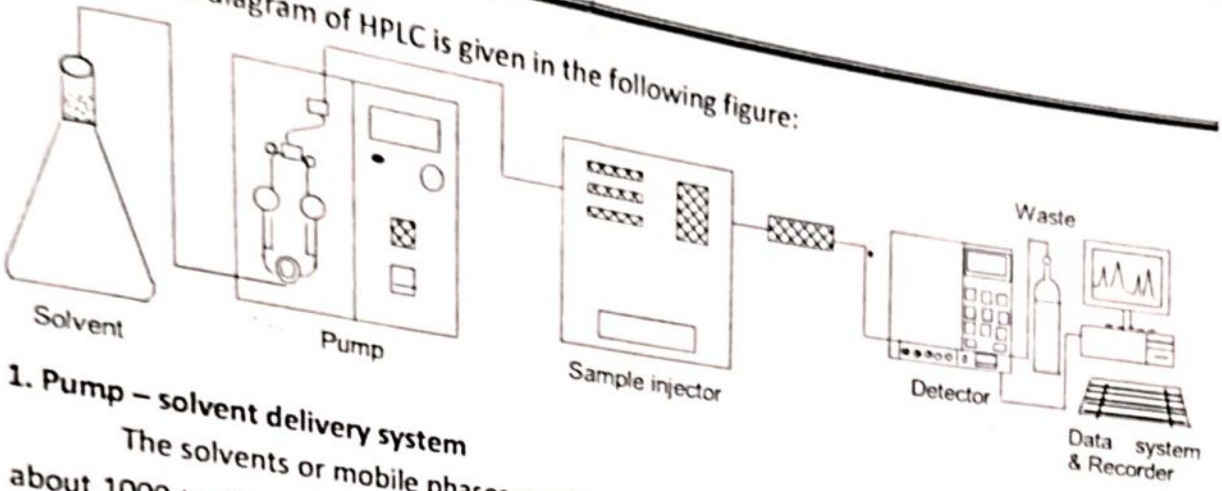
Principle of separation in HPLC:-

The principle of separation in normal phase mode and reverse phase mode in adsorption. Where a mixture of components are introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent, travels slower. The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.

INSTRUMENTATIONAL REQUIREMENTS

1. Pumps-solvent delivery system
2. Mixing unit, gradient controller and solvent degassing
3. Injector-Manual or auto injectors
4. Guard column
5. Analytical column
6. Detectors
7. Recorders and integrators.

The schematic diagram of HPLC is given in the following figure:



1. Pump – solvent delivery system

The solvents or mobile phases used must be passed through the column at high pressure at about 1000 to 3000psi. This is because as the particle size of stationary phase is few μ (5-10μ), the resistance to the flow of solvent is high. Hence such high pressure is recommended. There are different types of pumps available. They are mechanical pumps and pneumatic pumps. Mechanical pumps operate with constant flow rate and use a sapphire piston. This type of pump is used in analytical scale. Pneumatic pumps operate with constant pressure and use highly compressed gas. The solvents used must be of high purity, preferably HPLC grade and filtered through 0.45μ filter.

Check valves: these are present to control the flow rate of solvent and back pressure.

Pulse dampners: these are used to dampen the pulses observed from the wavy baseline caused by the pumps.

2. Mixing uint, gradient controller and solvent degassing

Mixing unit is used to mix solvents in different proportions and pass through the column. There are two types of mixing units. They are low pressure mixing chamber which uses helium for degassing solvents. High pressure mixing chamber does not require helium for degassing solvents. Mixing of solvents is done either with a static mixer which is packed with beads or a dynamic mixer which uses magnetic stirrer and operates under high pressure.

Gradient controller

In an isocratic separation, mobile phase is prepared by using pure solvent or mixture of solvent of same eluting power or polarity is used. But in gradient elution technique, the polarity of the solvent in gradually increased and hence the solvent composition has to be changed. Hence a Gradient controller is used when two or more solvent pumps are used for such separations.

Solvent degassing:

Several gasses are soluble in organic solvents. When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process, steady baseline and the shape of the peak. Hence degassing of solvent is important. This can be done by using any one of the following technique.

a. Vacuum filtration- Which can remove the air bubbles. But it is not always reliable and complete.

b. Helium purging- i.e. by passing helium through the solvent. This is very effective but Helium is expensive.

C. Ultrasonication- by using ultrasonicator, which converts ultra high frequency to mechanical vibrations. This cases the removal of air bubbles.

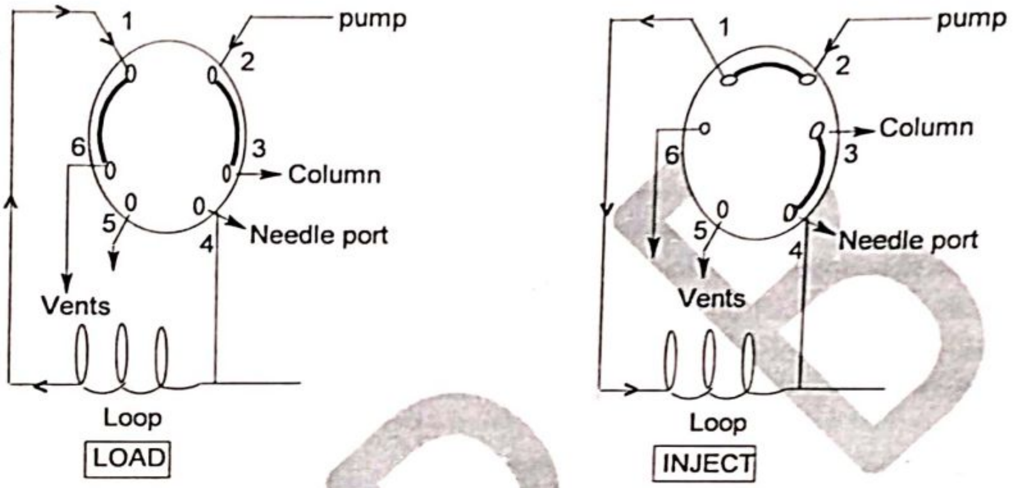
3. Injector-Manual or auto injectors

Several devices are available either for manual or auto injection of the sample. Different devices are

a. Septum injectors- for injecting the sample through a rubber septum. This is not common, since the septum has to withstand high pressure.

b. Stop flow (online)- in which the flow of mobile phase is stopped for a while and the sample is injected through a valve device.

c. Rheodyne injector (Loop valve type)- It is the most popular injector. This has a fixed volume loop like 20µl or 50µl more. Injector has two modes, i.e., **load position** when the sample is loaded in the loop and **inject mode,** when the sample is injected. The diagram of the Rheodyne injector is shown below.



Rheodyne injector

4. Guard column:

Guard column has very small quantity of adsorbent and improves the life of the analytical column. It also acts as a prefilter to remove particulate matter, if any, and other material. Guard column has the same material as that of analytical column. Guard column does not contribute to any separation.

5. Analytical columns:

Analytical column is the most important part to the HPLC technique which decides the efficiency of separation. There are several stationary phases available depending upon the technique or made of separation used.

Column material: The columns are made up of stainless steel, glass, polyethylene and PEEK (Poly ethyl ether ketone.) Most widely used are stainless steel which can withstand high pressure. Latest ones are PEEK columns.

Column length: Varies from 5cm to 30cm

Column diameter: Ranges from 2mm to 50mm

particle size: From 1µ to 20µ

Particle nature: Spherical, uniform sized, porous materials are used.

Surface area: 1gm of stationary phase provides surface area ranging from 100 – 860 sq.m.

Functional group: The functional group present in stationary phase depends on the type of chromatographic separation. In normal phase mode it contains the silanol groups (hydroxy group). In reverse phase mode it contains the following groups:

C_{18} - Octa Decyl silane (ODS) column

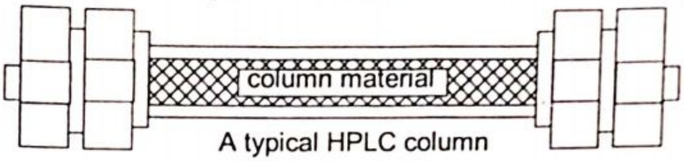
C_8 - Octyl column

C_4 - Butyl column

CN- Nitrile column

NH_2 - Amino column

For other mode of chromatography, ion exchange columns, gel columns, chiral columns, affinity chromatography columns, etc are available. A model of column is given below.



A typical HPLC column

Detectors:

Detectors used depend upon the property of the compounds to be separated. Different detectors available are

a. UV. Detector: This detector is based upon the light absorption characteristics of the sample. Two types of this detector are available. One is the fixed wavelength detector which operates at 254nm where most drug compounds absorb. The other is the variable wavelength detector which can be operated from 190nm to 90nm.

b. Refractive index detector: This is a non specific or universal detector. This is not used for analytical applications because of low sensitivity and specificity.

c. Flour metric detector: This detector is based on the fluorescent radiation emitted by some class of compounds. The excitation wavelength and emission wavelength can be selected for each compound. This detector has more specificity and sensitivity. The disadvantage is that some compounds are not fluorescent.

d. Conductivity detector: Based upon electrical conductivity, the response is recorded. This detector is used when the sample has conducting ion like anions and cations.

e. Amperometric detector: This detector is based on the reduction or oxidation of the compounds when a potential is applied. The diffusion current recorded is proportional to the concentration of the compound eluted. This is applicable when compounds have functional groups which can be either oxidised or reduced. This is a highly sensitive detector.

f. Photodiode array detector (PDA detector): This is a recent one which is similar to UV which operates from 190-600nm. Radiations of all wavelengths fall on the detector simultaneously. The resulting spectra are a 3-D or three dimensional plot of Response Vs Time Vs Wavelength. The advantage is that the wavelength need not be selected, but the detector detects the response of all the compounds.

Recorders and integrators: Recorders are used to record the response obtained from detectors after amplification, if necessary. They record the baseline and all the peaks obtained, with respect to time. Retention time for all the peaks can be found out from such recordings, but the area of individual peaks cannot be known.

Integrators: Integrators are improved version of recorders with some data processing capabilities. They can record the individual peaks with retention time, height and width of peaks, peak area, percentage of area, etc. Integrators provide more information on peaks than recorders. Now a days computers and printers are used for recording and processing the obtained data and for controlling several operations.

Applications of HPLC:

a. Qualitative analysis: It is nothing but identification of a compound. This is done by comparing the retention time of the sample as well as the standard. Under identical conditions, the retention time of the standard and the sample are same. If there is a deviation, then they are not the same compound.

b. Checking the purity of a compound: By comparing the chromatogram of the standard and that of the sample, the purity of the compound can be inferred. If additional peaks are obtained, impurities are present and hence the compound is not pure. From the percentage area of the peaks obtained, the percentage purity can also be known.

c. Presence of impurities: This can be seen by the presence of additional peaks when compared with a reference standard or reference material. The percentage impurities may also be calculated from peak areas.

d. Quantitative analysis: The quantity of a component by several methods can be determined like Direct comparison method, calibration curve method internal standard method.

e. Isolation and identification of drugs or metabolites in urine, plasma, serum etc can be carried out.

f. Isolation and identification of mixture of components of natural or synthetic origin.

g. Biopharmaceutical and pharmacokinetic studies.

h. Stability studies.

I. Purification of some compounds of natural or synthetic origin on preparative scale.