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THE COMPREHENSIVE STUDY: AN OVERVIEW FOR UNDERSTANDING THE CONCEPT OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

HPLC is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries.[1, 2] It is Today 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.[3, 4] 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. The reversed-phase means the mobile phase is relatively polar, and the stationary phase is relatively non-polar. HPLC instrumentation includes a Solvent reservoir, pump, injector, column, detector, and integrator or acquisition and display system. The heart of the system is the column where separation occurs. The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound. The major applications are in the area of Pharmaceuticals, food, research, manufacturing, forensics, and bio-monitoring of pollutants.

KEYWORDS : *Column; Mobile phase; Separation; Stationary phase*

➢ INTRODUCTION TO HPLC

High-performance liquid chromatography (HPLC), formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify specific components in mixtures. The mixtures can originate from food, chemicals, pharmaceuticals, biological, environmental and agriculture, etc., which have been dissolved into liquid solutions.High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) a specific form of column chromatography generally used in bio-chemistry and analysis to separate, identify and quantify the active compounds.It relies on high pressure pumps, which deliver mixtures of various solvents, called the mobile phase, which flows through the system, collecting the sample mixture High-performance liquid chromatography on the way, delivering it into a cylinder, called the column, filled with solid particles, made of adsorbent material, called the stationary phase.

Each component in the sample interacts differently with the adsorbent material, causing different migration rates for each component. These different rates lead to separation as the species flow out of the column into a specific detector such as UV detectors. The output of the detector is a graph, called a chromatogram.

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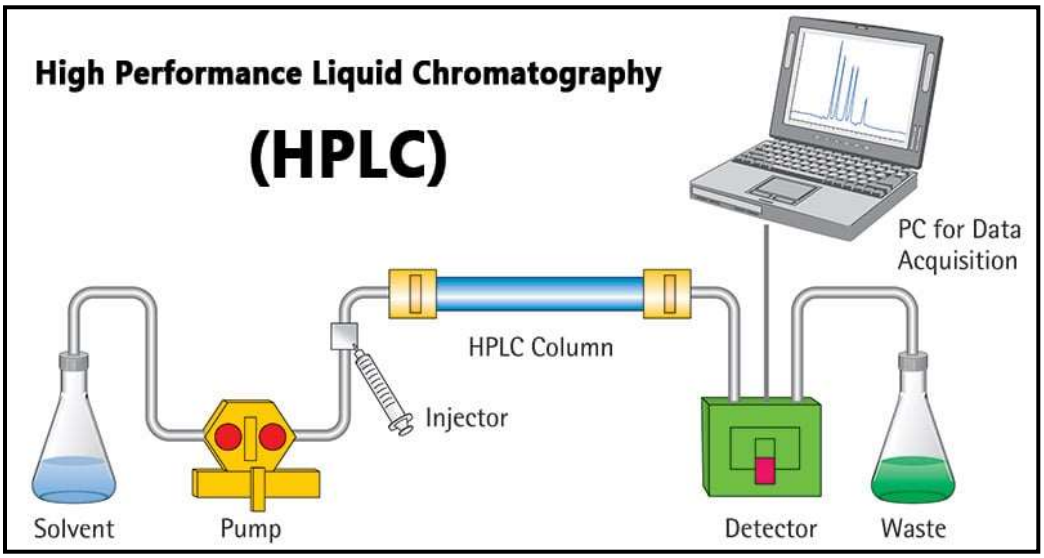

Fig:- 1. HPLC

Fig:- 2. A modern self-contained HPLC.

- **Principle/ Operation**

HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50–~1400 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the packed column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter, and 30–250 mm length.[5] Also, HPLC columns are made with smaller adsorbent particles (1.5 -- 50 µm in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique

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The principle involved in HPLC can be either adsorption or partition.[5] Chromatographic separation involved in HPLC is the result of interaction of the sample with both stationary phase and mobile phase. The analytes are injected into the flow of the mobile phase, just in front of the separation column. The outlet of the column is connected to a detector where the eluted substances are detected.[5] The separation is achieved in the column packed with stationary phase material of low particle size and the liquid mobile phase is pumped through the column. The reliable flow rate of the mobile phase with appropriate pressure is applied. The sample mixture is interacted between the stationary phase and mobile phase.

- **Instrumentation**
- Degassing
- Pumps
- Precolumn
- Sample injector
- Column
- Column packing material
- Detectors

A line diagram of the HPLC unit is shown in Fig. below. To attain reasonably high flow rates and yet keep particle size of packing very low (3-10 um), pumping pressures of several hundred atmospheres (2000-8000 psi) are required. Thus, the equipment for HPLC is quite elaborate though simple.[6]

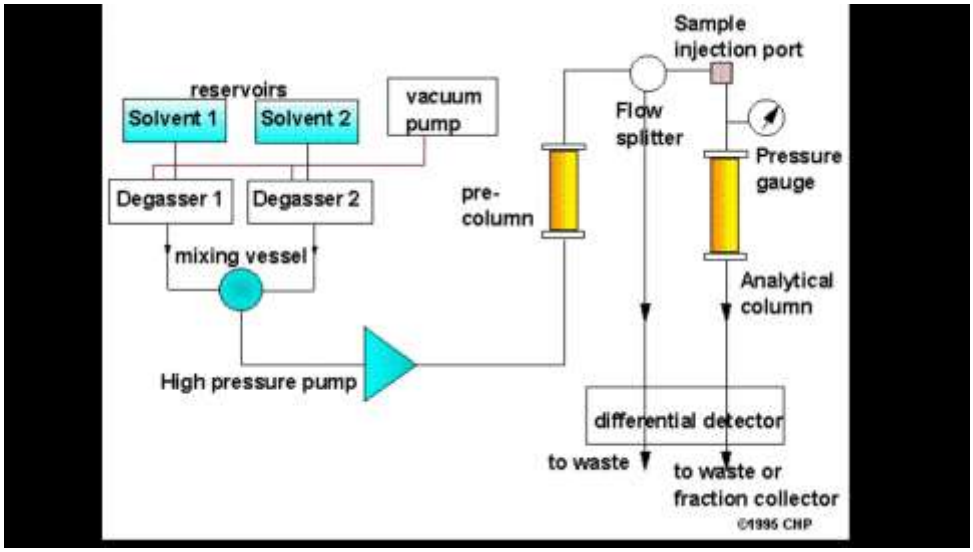

Fig:- 3. Line diagram of HPLC

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, containing 500 ml or more of solvent.[6] The reservoirs are often equipped by means of removing dissolved gases, usually 02 and N2, that interfere by forming bubbles in the columns and detector systems. These bubbles cause band spreading; in addition, they interfere with the performance of the detector.[6]

- **Degassing**

It is necessary to remove the dissolved gases present in the mobile phase solvent.
Different techniques are followed for degassing which are discussed below.[7,8]

(a) External Vacuum Degassing:

In this method, the solvent in a container is kept in an ultrasonic bath. Ultrasonication is done under vacuum using a vacuum pump.[7] This process will remove the dissolved gases from the solvents and then can be used for HPLC. This technique is more useful for solvents, which can absorb gases like carbon dioxide and also useful for eluent blanketed with an inert gas like helium.

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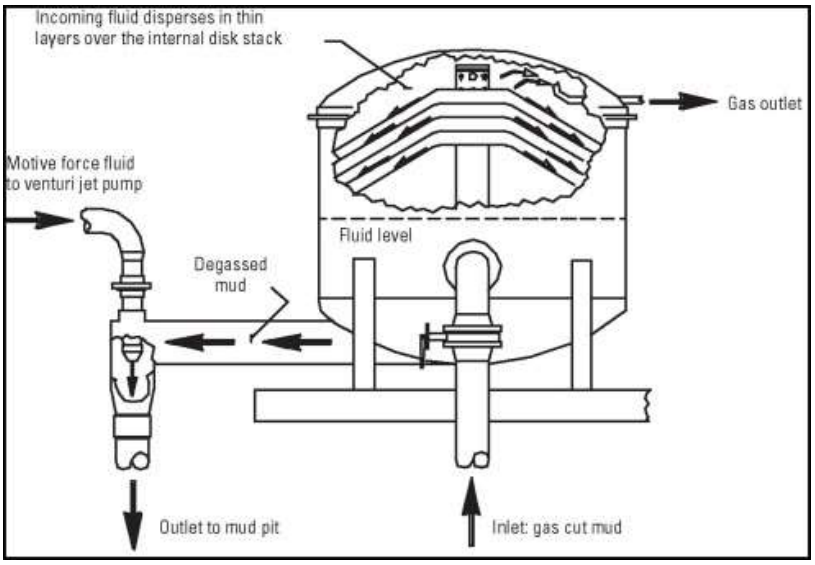

Fig:- 4. External vacuum degassing.

(b) Helium Sparging

In this technique, helium is bubbled into the solvent which will remove the other dissolved gases[8]. The volume and time of application of helium should be decided. Helium is insoluble in the mobile phase so it escapes out without interfering with the chromatographic process. This process is called helium sparging. This can be done online if the helium tank is equipped with the HPLC unit or the process can be done offline. However, the limitations are that Helium may selectively volatilize the more volatile solvent, thus changing the composition of the premixed solvents, more expensive and required in large quantities.

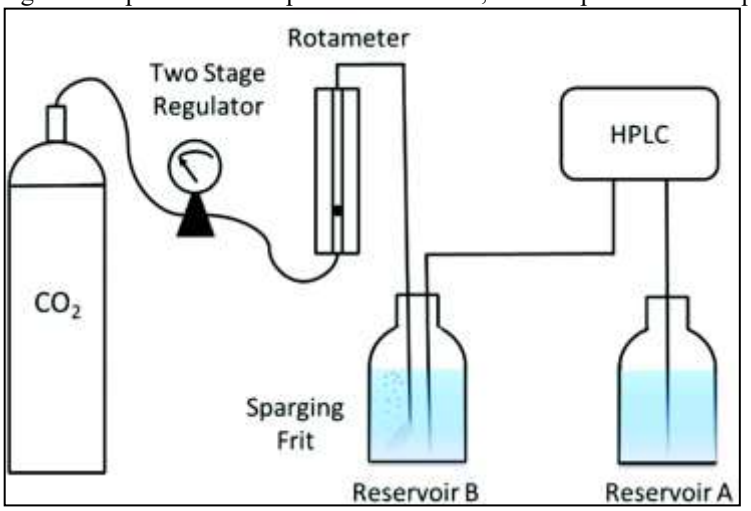

Fig:- 5. Helium sparging degassing

(c) Online Degassing

In this method, a vacuum pump is equipped with the HPLC and vacuum is applied on the semi-permeable tubes in which the solvents run. The air from the solvents is removed and goes to the waste collecting container.[9] This approach is shown in Fig. below:

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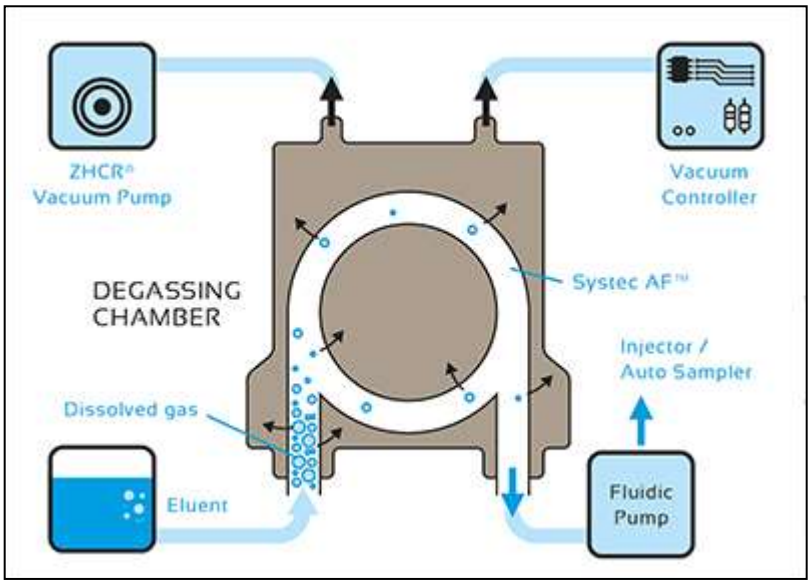

Fig:- 6. Online degassing

d) Filters:

Other than the above methods filters are also used to remove the dust and other matters from the solvents.[10] Membrane filters of 0.45 are usually used. The mobile phase filtered through these filters using Buchner funnel under vacuum followed by ultrasonication.

(a) a vacuum pumping system.

(b) a distillation system.

(c) devices for heating and stirring the solvents.

(d) device for sparing in which the dissolved gases are swept out of solution by fine bubbles of an inert gas of low solubility.

- **Pumps**

The pumps are used to pass mobile phase through the column at high pressure and controlled flow rate.

Based on the mechanism of working the pumps can be classified into**:**

1. Syringe Pump/Displacement pumps.
2. Reciprocating piston pumps.
3. Constant pressure pumps.

1. Syringe Pumps:

A syringe pump consists of a large barrel syringe with a plunger connected to a digital stepping motor or precision-screw drive. As the plunger moves forward, it drives the fixed volume of solvent through the chromatograph with a pulseless flow. These pumps are known for their pulseless flow of solvents. The flow rates are less than 100 l/min.

Flow is independent of viscosity and column back pressure. However, the run-time is limited by the volume of the syringe and no flow occurs during the refill step. It has limitations such as low solvent capacity (200-500 ml) and it is not easy to change solvent during gradient elution.

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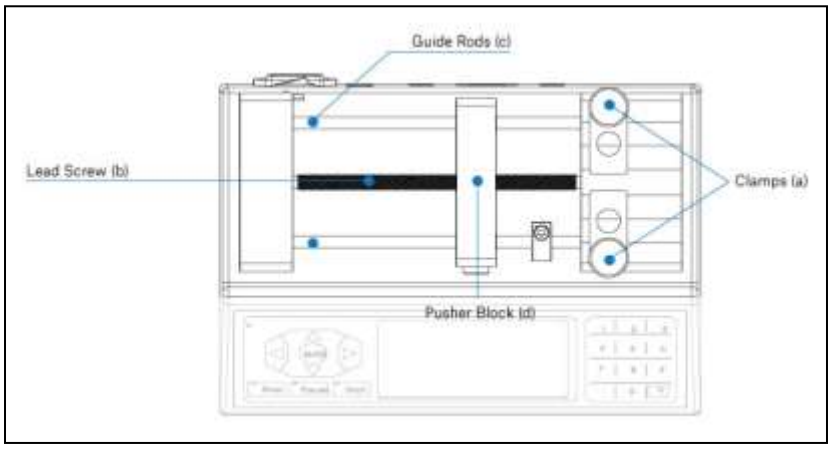

Fig:- 7. Syringe pump.

2. Reciprocating-Piston Pump:

A reciprocating pump is the most common design used in modern HPLC. The mechanism is similar to the constant displacement pump. The pump head consists of check valves and seal-piston assembly. The check valves regulate the flow of solvents from the reservoir to the pump chamber and further to the column. Two strokes namely fill stroke and delivery stroke are involved in the functioning. During fill stroke, the solvent is able to enter the liquid chamber from the solvent reservoir only. During the delivery stroke, the piston moves into the liquid chamber and pressurizes the liquid and the inlet check valve is forced to close. When the pressure inside the pump head exceeds the pressure on the column side of the pump, the outlet check valve opens and the mobile phase flows towards the column.

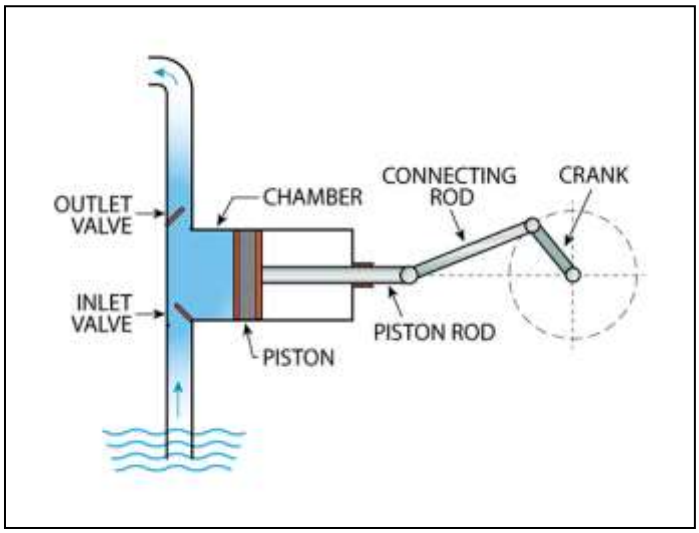

Fig:- 8. Reciprocating-Piston Pump

3.Constant Pressure Pumps:

In these pumps, high pressure from gas is introduced through a large piston which drives the solvent from the pump chamber to the column. The solvent chamber volume is around 70 ml (Fig. 15.9). Since the pressure on the solvent is proportional to the ratio of the area of the two pistons, usually between 30:1 and 50:1, a low-pressure gas source of 1 atm can be used to generate high liquid pressures (1-400 atm). An intermediate solvent can be used to reduce the interference of dissolved gas by entering to solvent. The rapid refill of the solvent chamber is facilitated by a valve. This system provides a continuous and pulseless pumping, and high flow rates for preparative applications. This type is used for packing columns; however it is inconvenient for gradient elution.

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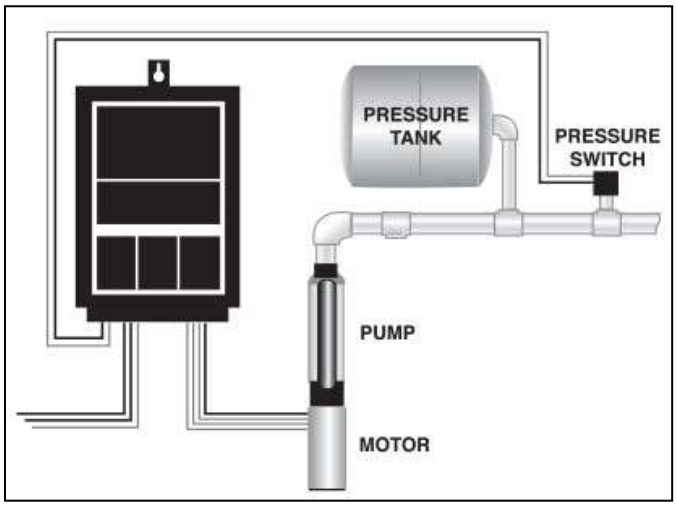

Fig:- 9. Constant pressure pump

Pump Pulsations:

Pump pulsations may be a problem during the trace analysis of analytes due to the baseline noise. It can be minimized by proper selection of pumps or by the use of cam design or pulse dampeners. The flow delivered by a single-piston pump is relatively pulsating, so single piston-pumps are rarely used to deliver eluent.[11]

Cam Design:

This set up consists of a two pump head design and a non-circular cam. The non-circular cam rotates and drives the piston so that when one liquid chamber is emptying, the other is refilling, thus, the two processes occur simultaneously. This produces a pulseless flow. The pistons are 180° out of phase.[12]

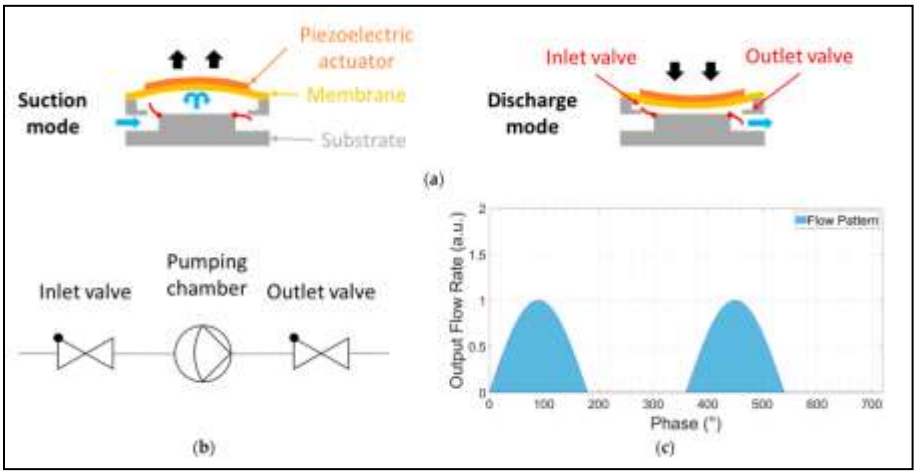

Fig:- 10. Cam driven, dual head reciprocating pump capable of delivering constant flow with relatively low pulsation. Flow rate is controlled by cam rotation frequency.

Pulse Dampers:

These are the useful set up in addition to cam designers. Most reciprocating pumps incorporate pulse dampers. Pulse dampers consist of long and narrow tubings folded back on them many times and placed between the pump and the injector. They receive the solvent during the delivery stroke and discharge during the refilling stroke through a restrictor. This setup minimizes the fluctuations and results in pulseless flow. The limitation of setting up of pulse dampers is the increase in system volume which makes the solvent change over particularly in the gradient elution, making it inconvenient.

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- **Precolumn**

Some HPLC instruments are equipped with a precolumn, which contains a packing chemically identical to that in the analytical column. Particle size is large; hence the pressure drop across the precolumn is negligible with respect to the analytical column. The precolumn is mainly used to remove the impurities from the solvent and thus prevent contamination of the analytical column

- **Sample Injectors**

Often the limiting factor in the precision of liquid chromatographic measurements lies in the reproducibility wherewith samples can be introduced into the column packing. It must be noted that overloading of the sample causes band broadening. Therefore, a minimum amount of samples must be introduced. It is convenient to introduce the sample without depressurizing the system. The sample is usually injected at the head of the column with minimum disturbance of the column material.[13]
There are different types of sample injection systems in practice.

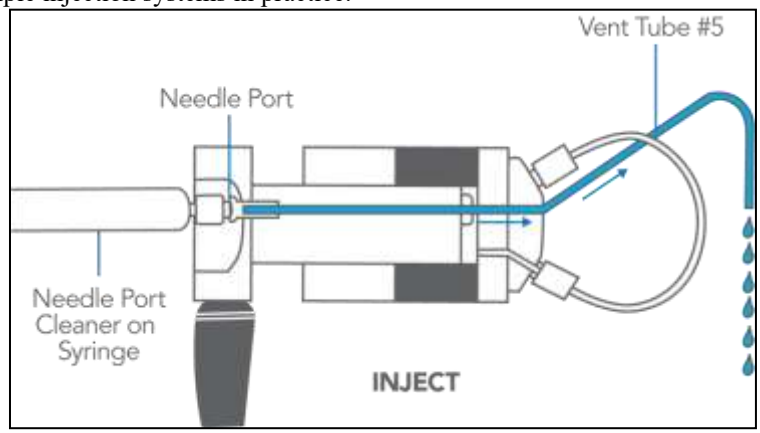

Fig:- 11. Sample injectors

Nowadays the above methods are replaced by the incorporation of valves for injection.
There are two types of valves:
1. External loop valve injector
2. Internal loop valve injector
1. External Loop:
It has 6 ports, out of which 2 ports connected with a fixed volume sample-loop and other 4 ports are used to carry the mobile phase and sample in and out of the valve. The valve consists of two positions namely 'load' and 'inject'. At load position, sample is loaded into the loop using a microlitre syringe and the position is rotated to inject position to inject the fixed volume of sample contained in the loop into the column along with mobile phase. The minimum volume of this loop starts from 10 ul. These are expensive but give precise results.

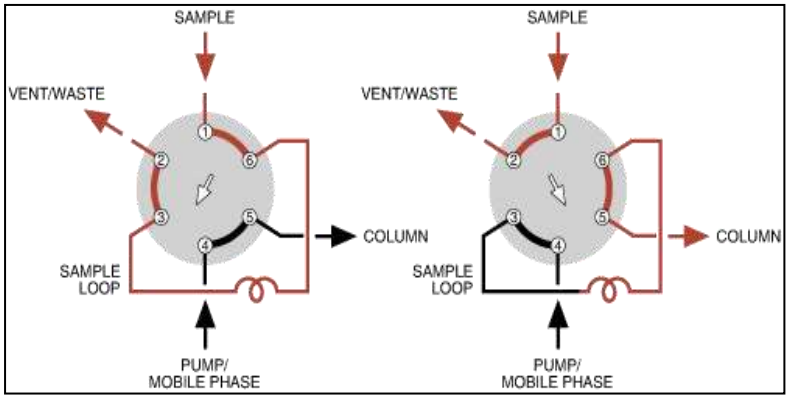

Fig:- 12. External loop

2. Internal Loop
This type of valve consists of 4 ports with an internal loop. In this, the sample loop is an engraved slot in the body of the valve. These types are used for small volume injection. This also has the same functioning as an external loop valve.

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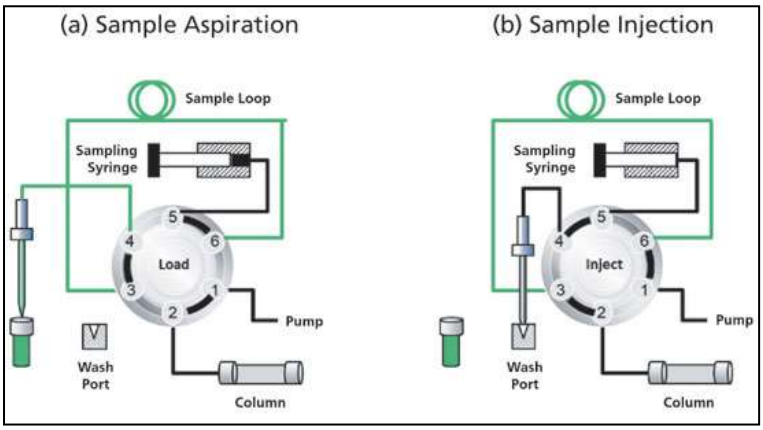

Fig:- 13. Internal loop

- **Liquid Chromatographic Columns**

They are usually constructed from smooth bore stainless steel tubing or heavy-walled glass tubing. If prepared from heavy walled glass tubing, then pressure is restricted to lower than 600 psi. Occasionally, you may come across coiled columns, but their use is very limited.
The columns are of two types:
(a) Analytical
(b) Preparative.
For analytical columns:
Size: Length: 25-100 cm with internal diameter of 2-6 mm.
For preparative columns:
Size: Length: 25-100 cm and internal diameter of 06 mm or more.
The common particle size is 5-10 um; recently manufacturers have been producing high speed, high performance micro-columns which have smaller dimensions.
Length: 3-7.5 cm and internal diameter of 1-4.6 mm; particle size: 3 or 5 um.
The main advantage of these columns is speed and minimum solvent consumption.
These columns should be provided with a system for temperature control to withstand high pressure.

- **Requirements for an Ideal HPLC Column**

An ideal HPLC column should fulfill the following conditions.
1. It should have uniform column packing.
2. It should have spherical particles.
3. The particle diameters should be ranging from 3 to 10 um.
4.The porosity of the particles should be in the range 50-70%, extending to 80% for the size-exclusion chromatography.
5. The column should withstand the pressure during operation.[14]
6. It should give reproducible results.
7. It should be easy to handle.
8. The particles should not shrink or swell with the nature of the eluent.[14]
9. It should have a uniform particle pore size distribution.
10. Particles should be available with a range of mean pore diameters of 60-100 A°.
11. Column packing should be chemically inert.
12. It should provide reproducible results.
13. It should be easily available and cost effective.

- **Column Packing Materials**

Construction Materials of Column:
Most columns are constructed from 316 stainless steel, Glass, Teflon and PEEK. Columns are also available for use with more aggressive mobile phases like HCl or solutes like proteins that may adsorb to the stainless steel. Polymeric columns are more common for ion-exchange packings while glass is commonly used for protein separations.
Types of Columns:

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(i) Guard Column: Guard column is the column placed before the analytical column. It contains a packing chemically identical to that in an analytical column with large particle size. The pressure drop across the precolumn is negligible as compared to that in the analytical column. These columns are otherwise called pre columns.

They are used to protect the analytical column from the impurities and other contaminants from solvent. It also removes the components that bind irreversibly to the stationary phase. The guard columns are compulsorily used during the bioanalytical studies to protect the analytical column from biological matrix.[15]

(i) Analytical Column: Analytical column is considered as the heart of an HPLC system.
The reason is that it is the part where the separation of the mixture takes place. The efficiency of the separation purely depends on the column.

Types of Analytical Columns:
(a) Small-bore Columns: Small-bore or microbore is the term used for HPLC columns having a diameter less than about 2 mm. They are also known as 'microcolumns'.
(b) 3 x 3 Columns: Short (3.3 x 4.6 mm) columns packed with 3 um bonded silica stationary phases have sufficient efficiency for many separations. They are commonly called 3 x 3 columns and compared to the conventional columns, they offer the following advantages.
(c) Monolithic Columns: It is a type of column used in HPLC that has porous channels rather than being packed with beads. They have a structure different from that of the traditional columns. Their construction is more similar to a rod with random channeling and outcroppings.

Fig:- 14. Types of column

Types of Packing used in HPLC Columns:
Different types of packing used in HPLC are as follows:
(a) Porous
(b) Pellicular
(c) Bonded phases.
(a) Porous Packings: Porous microparticles are the most commonly used stationary phase particles in modern HPLC. They consist of fully porous particles that can be either irregular or spherical in shape. The diameters are ranging from 3 to 10 um. The pores provide the surface with which the sample interacts.[16] Particles with smaller pore size exhibit a larger surface area and therefore have greater retention. Large particles like proteins require a large pore size.[17] The particles are composed of silica, alumina, the synthetic resin polystyrene-divinylbenzene, or an ion-exchange resin. Silica is by far the most common packing used in LC.
(b) Pellicular Packings: Pellicular materials consist of a solid spherical bead of relatively large inner diameter with a thin outer layer of stationary phase.[17] The original pellicular particles were spherical, non-porous, glass or polymer beads with typical diameters of 30 to

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40 um. A thin, porous layer of silica, alumina, a polystyrene-divinylbenzene synthetic resin, or an ion-exchange resin is deposited on the surface of these beads.

- **Detectors**

A detector is required to sense the presence, and the amount of sample component in the column effluent. A detector that measures property possessed by both mobile phase and solute is called bulk property detector, e.g. Refractive Index detector.
If the solute possesses the property e.g. absorption of UV/visible light of electrochemical property, the detectors are called a solute property detector.
A good detector should have the following features:
(a) It should respond to all components of the mixture in a wide range of mobile phases.
(b) It should not respond to the mobile phase.
(c) It should be unaffected by changes in temperature and flow rate.
(d) It should have high sensitivity, i.e. larger detector signal for smaller amount of solute.
(e) Low noise and a wide linear response to solutes present.
(f) It should not constitute zone spreading.
(g) Non-destructive, inexpensive, reliable and easy to use.
Generally two types of detectors are used:

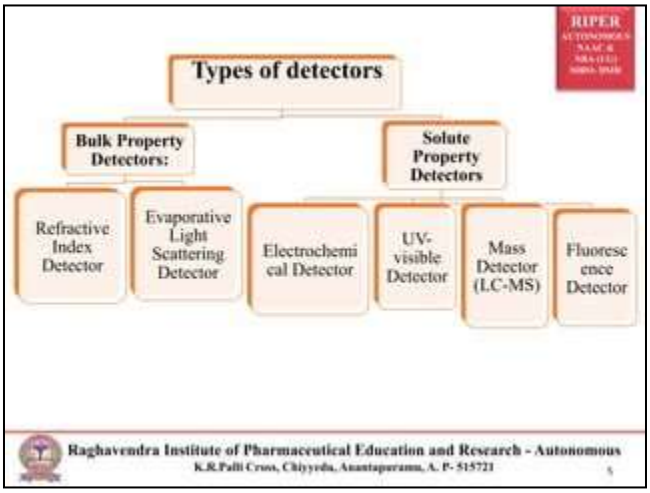

Fig:- 15. Types of detectors

- Bulk property detectors and Solute property detectors.

1. Refractive index monitors detectors:

Since every compound has its own refractive index, this property becomes a universal indicator. A differential refractometer continuously monitors the difference in Ri between the pure mobile phase (reference stream) and the column effluent. The advantages of these detectors are: (a) They respond to nearly all solutes. (b) They are reliable and unaffected by flow rate. (c) They do not require any double bond or aromaticity to be. present in the structure for elucidating a response while disadvantage is that there must be a difference between the refractive index of the solutes and of the mobile phase. Besides, this is not a sensitive detector.
Construction of Refractive Index monitor detector:
1. Several different designs of a refractive index detector have been used in HPLC, one of which is the deflection refractometer.

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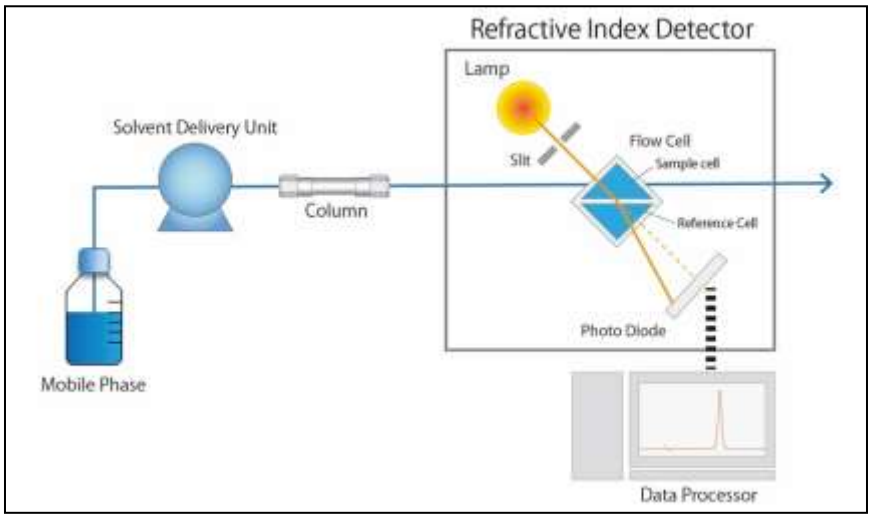

Fig:- 16. Construction of Refractive Index monitor detector

2. UV-visible absorption detector

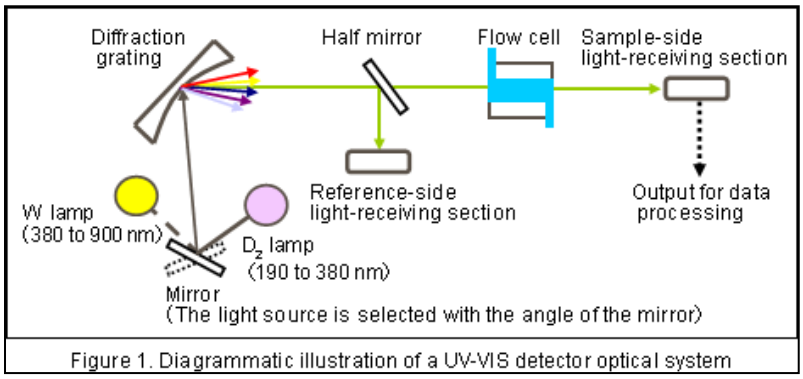

Fig:- 17. Flow cell for UV/visible absorbance detector

UV Absorption Detectors with Filters:

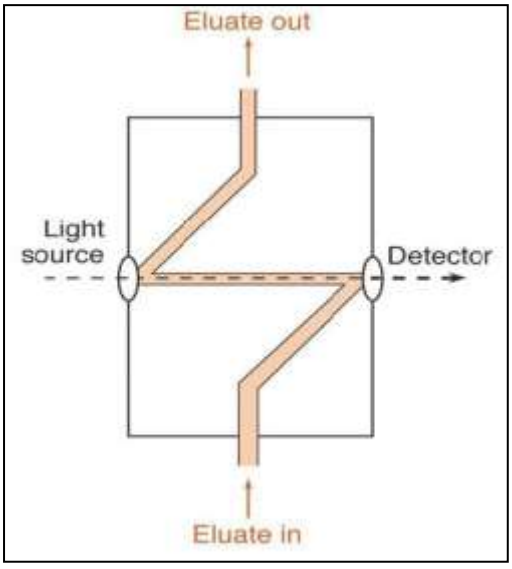

Fig:- 18. A typical Z-shaped UV flow-through cell

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Absorption Detectors with Scanning Capabilities:
These detectors are provided with scanning spectrophotometer with grating optics. This provides various benefits such as single wavelength analysis and multi-wavelength analysis.
Best wavelength for each eluent can be selected in a mixture and analysed.

3. Photodiode Array (PDA):
These are the most powerful UV-visible detectors. The detection is carried out in the UV-visible region. A DAD has multiple photodiode arrays. Using this detector a wide range of wavelengths can be employed at a time. At an interval of 1 second or less the spectra of analytes are recorded. In addition to retention time the comparison of spectra will give the proper identification of the analyte. The light from the lamps directly approach the flow cell, dispersed by the diffraction grating, and the amount of the dispersed light is estimated for each wavelength in a photodiode array.

4. Fluorescence Detectors:
Fluorimetric detectors used In HPLC are similar in design to the fluorometers and spectrofluorometers used for fluorometry. In most of these, fluorescence is observed by a photoelectric transducer located at 90° to the excitation beam. Mercury excitation source or xenon source and monochromator to isolate the fluorescence radiation are used. The advantage of the fluorimetric detectors is their higher sensitivity.

5. Amperometric Detectors:
These detectors are based on measurement of electrochemical detection. The detector has three types of electrodes which include, reference electrode, working electrode and auxiliary electrode. The potential of the working electrode is set relative to the reference electrode. When the analyte, which is an electroactive substance, reaches the detector, it is subjected to a reaction of either oxidation or reduction in the electrodes. This reaction creates some current flow in the electrode due to the transfer of electrons. The electrodes are connected to an electronic circuitry and the electric current signal is amplified and measured as a signal.
Other detectors:
(1) Infrared Absorption Detectors
IR spectrophotometer and FTIR spectrophotometer have been used for HPLC.[18] IR detector cells are similar in construction to those used in the UV instruments, except that the IR cuvettes are made up of Sodium chloride or Calcium fluoride. Cell path lengths range from0.2 to 1.0 mm and volumes from 1.5 to 10 uL. A major limitation in the use of the IR detectors is the low transparency of many useful solvents.[19] Also, the use of aqueous mobile phases is restricted.

(2) Evaporative Light Scattering Detector (ELSD):
In this, the column effluent is converted into a fine mist by a flow of nitrogen or air using a nebulizer. The fine-droplets are passed through a heated tube where the mobile phase evaporates,leaving fine particles of the analyte. This is then passed through a laser beam.

(3) Mass Spectrometric Detectors
Nowadays the highly sophisticated mass spectrometric detectors are widely used due to their sensitivity and reliability. When a mass spectrometer is used as a detector for LC (LC-MS), it can greatly aid in identifying species as they elute from a chromatographic column. Various interfaces are used to couple LC with MS.[20]

- **Recorder**

There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.[21]

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