
athshala पाठशाला

MHRD Govt. of India

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| Description of Module | |
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| **Subject Name** | Biochemistry |
| **Paper Name** | 12 Biochemical Techniques |
| **Module Name/Title** | 13 HPLC |

## 1. Objectives

**1.1. To understand the basic concept and principle of HPLC**

**1.2. To understand the components and techniques of HPLC**

**1.3. To know alternatives to HPLC**

**2. Basic Concept & Principle of HPLC**

A good chromatography needs to meet certain performance parameters, like high resolution between two peaks in a chromatogram, a large number of theoretical plates in a column, a low theoretical plate height and high values for the separation factor between two analytes. One possibility for improving the performance of a chromatography is decreasing the particle size of stationary phase, which increases the surface area, thereby increase in the number of theoretical plates or equilibrations among analyte, stationary and mobile phases, resulting into high performance. However, decreasing the particle size of stationary phase increases the resistance to mobile phase, backpressure in the column, which leads to a damage to the stationary phase and finally decreasing the eluent flow and resolution. The main solution to such problems due to small particle size is to select or prepare small particles (5-10μm) that can withstand the pressures of 40MPa, and the requirement of high pressure to increase the flow rate. Such chromatography with small particle size of stationary phase and the use of high pressure is called high performance or high-pressure liquid chromatography (or) HPLC is simply a separation of biomolecules with high performance parameters under high pressure by using appropriate instrumentation.

3. **HPLC instrumentation or components**

Different parts of HPLC are mobile phase reservoirs, stainless steel tubing, pumps, gradient mixer, injector, guard column, main column, detectors and fraction collector. There components illustrated in the figure 1.

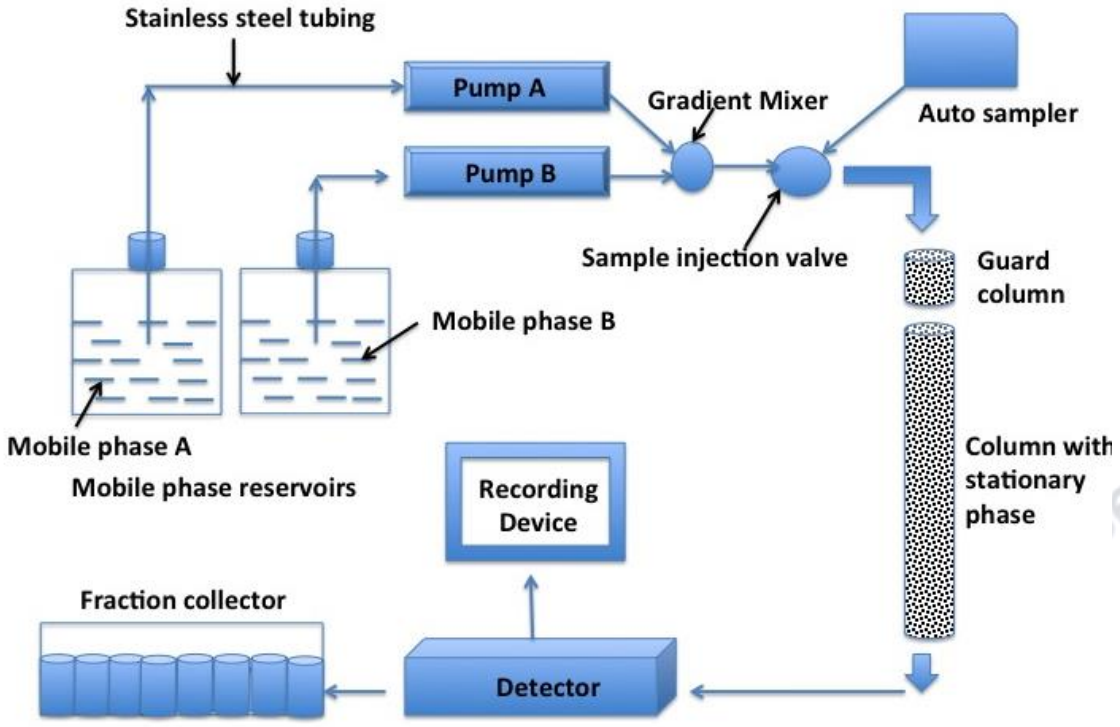



**Figure 1. Components of HPLC**

### 3.1. HPLC mobile phase

The HPLC mobile phase usually comprises of two or more solvents (e.g., water and acetonitrile) for the separation of biomolecules. The solvents should be highly purified, called HPLC grade, and they should be chemically unreactive. If these solvents are not pure, the impurities may interfere in detection system, especially at a wavelength of less than 200 nm. In addition, the solvents should be degassed before using them in HPLC. It is imperative, specifically, for those eluents with aqueous ethanol and methanol, because high pressure results in bubbling of gas or air present in solvents and those damage stationary phase. Several degassing methods, like warming, vigorous stirring with magnetic stirrer, vacuuming, ultrasonification and bubbling helium gas through eluent reservoir, can be used. However, the most common one is vacuuming.

Selection of HPLC mobile phase depends on the type of separation and the components in the sample. Usually, polar and non-polar solvents are used in certain ratio and they should not interfere in the detection mechanism. Based on the composition of mobile phase throughout HPLC experiment, HPLC elution can be classified as isocratic or gradient elution. In isocratic elution, the mobile phase composition is constant throughout the HPLC separation. The advantages of isocratic

elution are, it can be used for simple mixtures, and the system and column are equilibrated all the time and does not suffer from fast chemical changes. In gradient elution, mobile phase composition is gradually changing in the ratio of polar to non-polar compounds during the sample run. Gradient elution is useful for the separation of complex mixtures.

### 3.2. HPLC Stationary phase

Based on the interactions among sample analytes, mobile and stationary phases, the HPLC technique can be classified as normal phase HPLC and reverse phase HPLC. In normal phase HPLC, the stationary phase is more polar (e.g., Alumina or silica) than mobile phase. Hence, the first compound to be eluted is less polar. In reverse phase HPLC, the stationary phase is less polar (e.g., Alkyl, aliphatic or phenyl bonded phase) than mobile phase. The first compound to be eluted is more polar in nature and the retention time of a compound increases with decreasing polarity of the particular analyte.

The stationary phase particles in HPLC are rigid rather than soft gel as in open column chromatography. The particles are spherical and in uniform size to reduce space for diffusion. Mainly, there are three types of particles; micro porous particles, pellicular particles and bonded phase particles.

#### 3.2.1. Micro porous particles

These are of 5-10µm in diameter composed of silica or aluminum and contain microscopic pores running through the particle (Figure 2A) to increase the surface area for more the interaction with analytes and mobile phase.

e.g., Porous poly vinyl chloride (Fractogel TSK-HW-55) gel filtration of proteins.

#### 3.2.2. Pellicular particles

These are also called as superficially porous particles. They are of 40 µm in diameter. In has an inert core, on which the pores are coated (Figure 2B). Inert core is made up of glass or some other hard material. The porous material could be made up of polymethacrylates or poly(styrene-divinylbenzene). These particles display excellent pH stability (pH 2-12) with high mechanical stability.

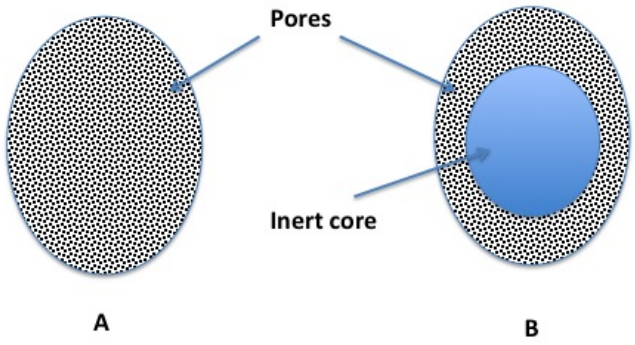



**Figure 2. Microporous (A) and pellicular (B) stationary phase particles**

### 3.2.3. Bonded phase particles

These particles also contain an inert core made up of silica. The stationary phase is chemically bonded on that inert core by different linkages (Figure 4).

e.g., Silica C-18 for reverse phase chromatography of peptides/proteins

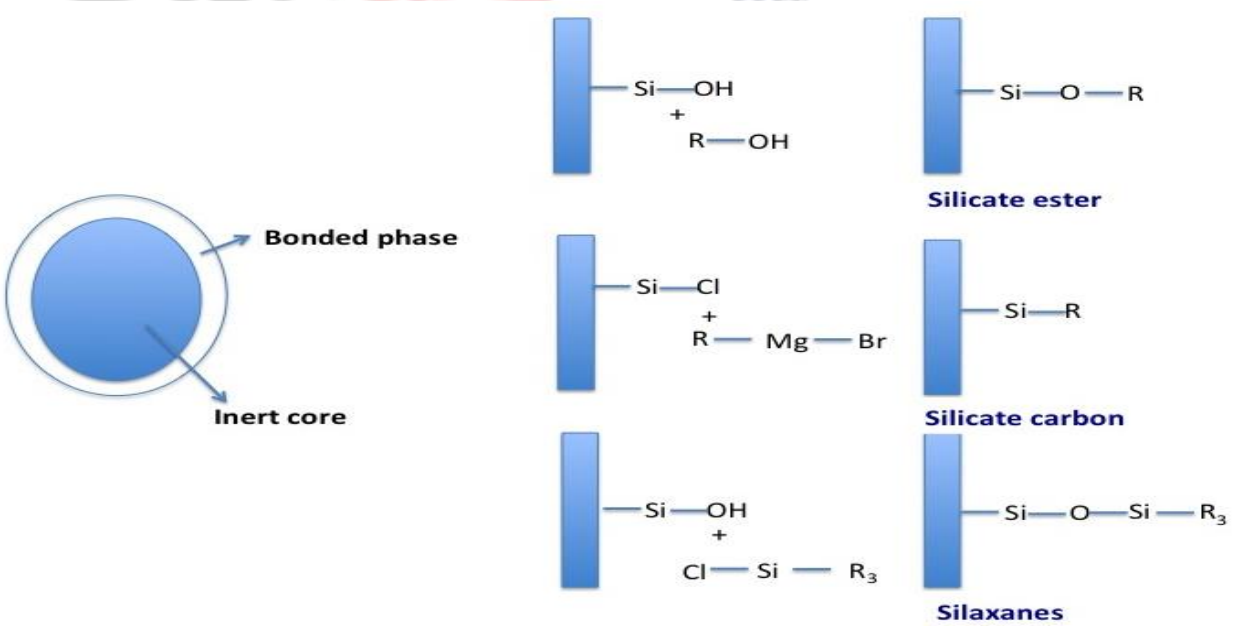



**Figure 3. Bonded phase particles and their chemical linkages.**

### 3.2.4. HPLC columns to hold stationary phase

#### 3.2.4.1. Conventional HPLC columns

These are made of stainless steel to withstand 50MPa pressure. They are generally 3-25 cm in length and 2-5mm in diameter. The flow rate of mobile phase in this column is 1-10ml/min.

#### 3.2.4.2. Capillary columns

The inner diameter of these columns is 100µm-1mm and the flow rate is 0.4-200µl/min.

#### 3.2.4.3. Nanobore columns

The inner diameter of these columns is 25-100µm and the flow rate is 25-4000nl/min. These columns are helpful to use at the interface with mass spectrometry. These columns are important in situations to analyze minute quantities of proteins –proteomics.

### 3.2.5. Stationary phase packing

The stationary phase is packed into a HPLC column by high pressure slurring procedure. In this procedure, the stationary phase and solvent for slurry need to be in equal density and the slurry can be pumped into a column by high pressure. By this method, a dense, continuous bed of stationary phase without any cracks and other imperfections will be formed.

## 3.3. HPLC pumps

An important requirement in HPLC is the flow of mobile phase should be very stable (i.e., pulse free). The following two types of pumps can reasonably attain this.

### 3.3.1. Constant pressure pumps

These pumps maintain constant pressure irrespective of stationary phase resistance to mobile phase flow. If the resistance increases in stationary phase, the flow rate decreases automatically to maintain constant pressure.

### 3.3.2. Constant volume pumps

These pumps maintain constant flow rate through stationary phase. The pumps increase the pressure if there is an increase in stationary phase resistance. However, increasing pressure is within the pre-set limits of the pump. If the pressure is required more than the preset limits, the pumps are inactivated automatically by safety-cut off mechanism.

These pumps are more popular in HPLC. An example for common constant volume pump is reciprocating pump, which uses a piston to deliver a fixed volume of solvent onto the column in repeated cycles of filling and emptying (pulses). In order to avoid pulses while the flow of mobile phase pulse dampners are built into the pumps.

### 3. 4. Sample application

Most common sample injector in HPLC is loop injector (Figure 4), which is operated manually. Some advanced systems have automatic sample injectors.

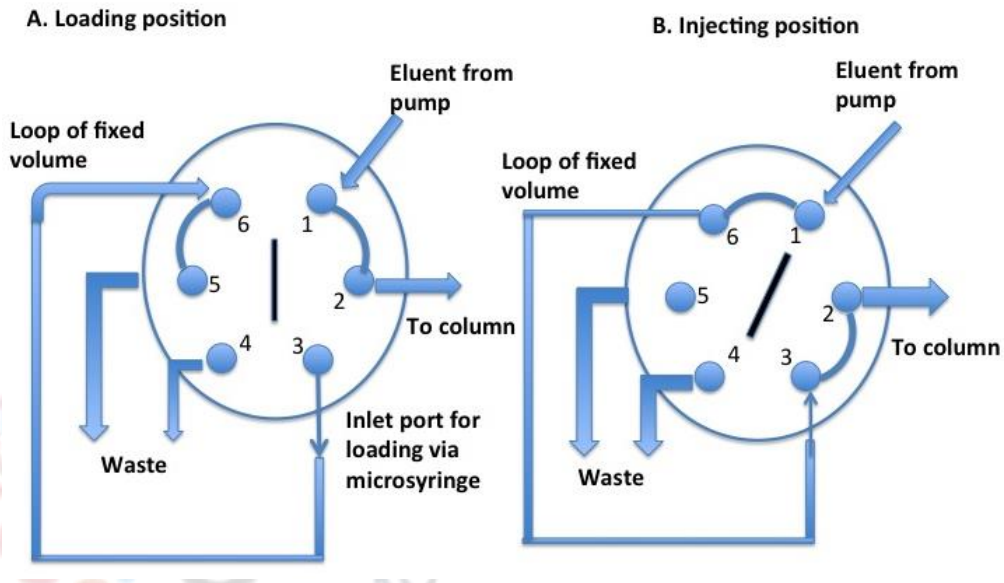


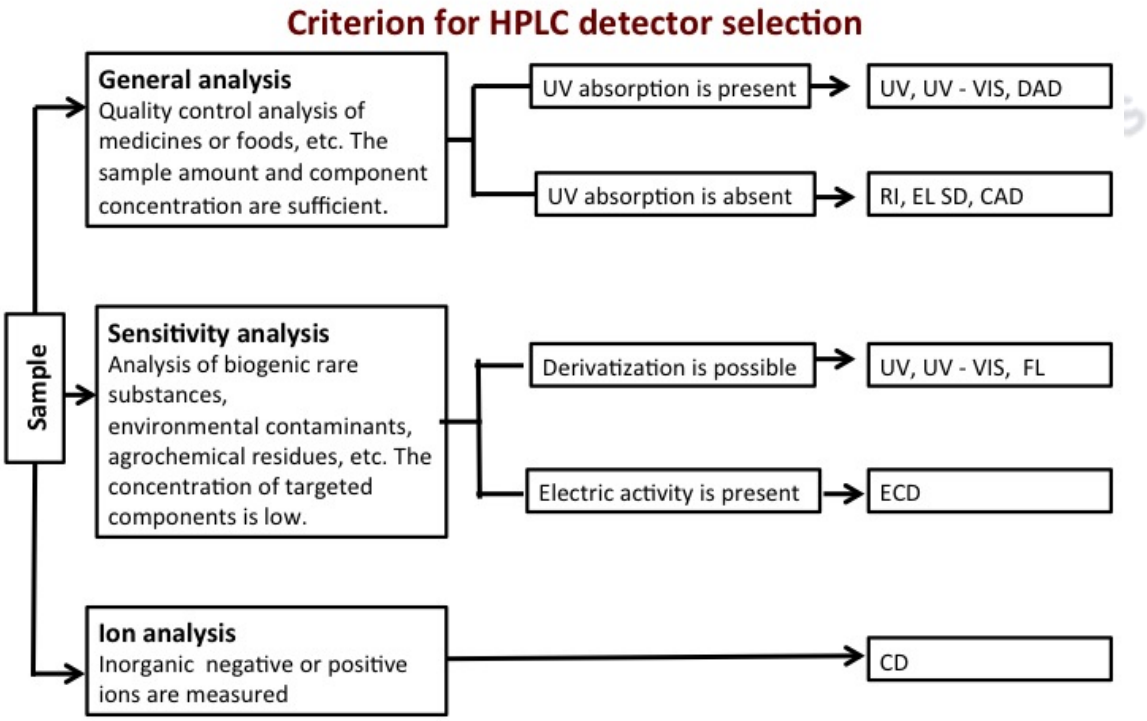

**Figure 4. HPLC loop injector.** In the loading position of injector (A), the sample can be loaded into the loop of fixed volume through port 3, and the excess sample is lost though port 5. At this position, the eluent is directly passed to the column through ports 1 and 2. In the injecting position (B), a connection will be established between ports 1 and 6, then the eluent passes through loop, ports 3 and 2 to the column.

### 3.5. Guard column

It is a small column between injector and main HPLC column. This column is mainly used to protect the main column from impurities.

### 3.6. HPLC detectors

Two major detection mechanisms, such as optical and electrical, are broadly involved in HPLC detectors. Optical detection based detectors are variable wavelength detectors (UV-Vis), scanning wavelength detectors (DAD), fluorescence detectors (FL), refractive index detectors (RI) and evaporative light-scattering detectors (EL). Electrical based detectors are Electrochemical detectors (ECD), conductivity detector (CD). Additionally, mass spectrometer detectors and NMR spectrophotometer detectors can be integrated with HPLC. A criterion for selection of a specific detector for specific analysis is illustrated is the following figure 5.




### 4.0. HPLC limitations

1. High cost of generation of high pressure
2. High pressure imposes some constraints on instrumentation
3. It has relatively low flow rates. Hence, the experiment is time taking.

Probable solutions for above limitations

1. Decreasing the pressure and increasing the flow rate for comparable resolution of analytes by HPLC. It is possible with perfusion chromatography.

2. Increasing the instrumentation that can withstand high pressure by considering cost economics for better resolution than HPLC. It is possible with UPLC.

4. HPLC is especially useful in the separation and analysis of low molecular weight biomolecules. But it does not usually useful for the purification of complex biopolymers (e.g., proteins) in active form.

– Reason 1: Some HPLC formats like reverse phase chromatography needs organic solvents which denatures proteins

– Reason 2: Although large capacity HPLC columns are available, they are expensive. Small capacity columns are quite common.

**Probable solution:** Development of another format for biopolymers- FPLC

**5.0. HPLC supplements**

**5.1. Perfusion chromatography**

With small particle size, high resolutions can be possible in short times with high flow rates in perfusion chromatography. In this chromatography, the stationary phase particles contain not only longitudinal pores through out the particle, but also diffusive pores connecting the longitudinal pores (e.g., POROS particles). According to VanDeemter's curve, the height of theoretical plate inversely proportional to the diffusion coefficient of mobile phase. At high values of flow rate, the connective term is much important than diffusion term of mobile phase between the particles. Hence, theoretical plate height becomes largely independent of flow rate, but dependent on stationary particle diameter when the stationary phase particles have both longitudinal and diffusive pores. Therefore, inter-particle diffusion becomes unimportant relative to intra-particle convection in perfusion chromatography. These kinds of stationary phase particles have large surface area for interacting with mobile phase and analytes.

**5.2. Ultra performance liquid chromatography (UPLC)**

Decreasing the particle size and increasing the capacity of instruments by Waters Corporation developed this kind of chromatography. It has stationary phase particle size of 1.7 µm diameter. The stationary phase particles are made up of Bridged Ethylsilixane Silica Hybrid (BEH). This chromatography is 10 times faster than conventional HPLC.

### 5.3. Fast protein liquid chromatography (FPLC)

It is useful for biopolymer (e.g., proteins, DNA) separation. Major difference between HPLC and FPLC is low pressure in FPLC (approx. 1-2 MPa). Reciprocating pumps with two pistons are used in FPLC to avoid pulses. The columns in FPLC are made up of glass or plastic because of low pressure. The stationary phase in FPLC is usually compatible with aqueous buffers and salts, and hence it is useful for biopolymer separations.

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