

# LECTURE # 2

## Introduction to HPLC

### **HPLC-**

- Originally referred to

#### **High pressure liquid chromatography**

- High pressure to be able to use small particle size to allow proper separation at reasonable flow rates.

- Laterally referred to

#### **High performance liquid chromatography**

- High performance due to its reproducibility.

- Currently refers to

#### **High precision chromatography**

- High precision due to its precise results.

It was developed from classical column liquid chromatography that pumps a sample mixture or analyte in a solvent (known as mobile phase) at a high pressure through a column with chromatographic packing material. HPLC mainly utilizes a column that holds packing

material (stationary phase), a pump that moves the mobile phase through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecule being analyzed, and the solvent used. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is related by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. Common solvents used include any miscible combination of water or organic liquids (the most common are methanol and acetonitrile). Separation has been done to vary the mobile phase composition during the analysis, this is known as gradient elution. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.

## **1. Definition**

It is a chromatographic technique used to separate components of mixture for the purpose to identify, quantify or purify the individual components of the mixture. This is widely used in field of biochemistry and analytical chemistry.

## 2. Principle of HPLC

Principle of HPLC is based on Van Deemter equation which relates the efficiency of the chromatographic column to the particle size of the column, molecular diffusion and thickness of stationary phase.

The Van Deemter's equation is given as:

$$H \text{ or HETP} = A + B/\mu + C\mu$$

**A** = eddy diffusion

**B** = molecular diffusion

**C** = rate of mass transfer

$\mu$  = flow rate

## 3. Types of HPLC techniques

- Based on mode of chromatography
- Based on principle of separation
- Based on elution technique
- Based on scale of operation
- Based on type of analysis

## ➤ **Based on modes of chromatography**

### **Normal phase chromatography:**

Also known as normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacts with and is retained by the polar stationary phase. Adsorption strength increases with increased analyte polarity, and the interaction between the polar analyte and polar stationary phase increases the elution time.

### **Reverse phase chromatography:**

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

## ➤ **Based on principle of separation:**

### **Adsorption chromatography:**

In this separation of components take place due to the differences in affinity of compounds toward stationary phase.

### **Ion-exchange chromatography:**

In ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to stationary phase. Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc.

### **Size exclusion or gel permeation chromatography:**

Size exclusion chromatography (SEC), also called gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides.

## ➤ **Based on elution technique:**

### **Isocratic separation:**

In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

### **Gradient 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.

## ➤ **Based on the scale of operation:**

### **Analytical HPLC:**

In which only analysis of the samples are done. Recovery of samples is not done.

### **Preparative HPLC:**

In which the individual fractions of pure compound can be collected using fraction collector. The collector samples are reused.

## ➤ **Based on the type of analysis:**

### **Qualitative analysis:**

It is used to identify the compound, detect the impurities, to find the number of components, etc.

### **Quantitative analysis:**

It is used to determine the quantity of the individual or several components in a mixture. This can be done by comparing peak area of the standard and sample.

## **Advantages of HPLC**

- High resolution and speed of analysis.
- HPLC columns can be reused without replacing or regeneration.
- Greater reproducibility due to close control of the parameters affecting the efficiency of separation.
- Easy automation of instrument operation and data analyses.
- Adaptability to large scale, preparative procedure.
- Two major advances, stationary supports with very small particle sizes and large surface area.
- Appliance of high pressure to solvent flow.

- Rapid and precise quantitative analysis.
- High sensitivity detection.

## **Advantages of partition HPLC over other techniques**

- The partition chromatography provides large resolving power than the other techniques.
- The high pressure techniques are generally suitable for low concentration of mixture.
- The partition chromatography basically depend on solubility of two liquid, mole difference in molecular weight will influence partition, that is why partition is prefer for homologes series of compound.
- The partition co-efficient in such techniques is independent of concentration over great range of concentration.
- The partition /high pressure technique develop relationship between structure and constituent and their effects.
- Faster and efficient separation of mixtures (high resolution power).
- Suitable for analyzing very complex mixtures.



- Accurate quantitative measurements (qualitative analysis is also possible).
- Repetitive and reproducible analysis using the same column.
- It is possible to analyze multiple components of a mixture in a single analysis.

## **Advantages of HPLC over Gas chromatography**

High performance liquid chromatography and gas chromatography both are the separation techniques for the components in analytical chemistry and they are well established from overtime. Each technique has many advantages and some disadvantages over each other.

### **➤ Mobile Carrier Phase:**

GC evaporates the sample and it is taken with the system by an inert gas the nitrogen and helium. The use of hydrogen gas gives improved efficiency and separation. But due to the flammable nature of gases, many labs prevent the use of these gases. While, using liquid chromatography, samples are solid or liquid forms and travel through high pressure through solvents or mobile phases such as methanol, acetonitrile and water, etc through the column. The solvent composition can be modified as per the requirement of samples /

complex mixture of components gives better separation, but in GC a gas passes through the column at constant proportion.

### ➤ **Column Types:**

The GC column has an extremely small internal diameter and 10 to 45 meters in length. These are silica-based columns and it can be heated up to 150 °C. Columns of HPLC are also made up of silica-based columns covered with stainless steel outer surface and are made up of 50 to 250 cm in length. These columns are used under ambient temperature on high pressure.

### ➤ **Compound Stability:**

In GC, the sample is introduced into the injector and vaporizes it at a specific temperature and travels through the column. Thus the compound should be capable of withstanding the heat at high temperatures without degradation. On the contrary, in the high-performance liquid chromatography system you allow the analysis of stable or less stable compounds because the heating to sample is not necessary.

➤ **Sample Requirement:**

Sample by GC required volatile compounds but in HPLC both volatile and non-volatile samples are analyzed.