INTRODUCTION

The technique of high performance liquid chromatography is so called because of its **improved performance** when compared to classical column chromatography. It is also called as **high pressure** liquid chromatography since **high pressure** is used when compared to classical column chromatography.

The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional larger particle sizes.

1960's - 40 to 60μ

1970's - 10 to 20µ

1980's - 5 to 10µ

1990's - 1 to 3µ

A porous particle of 5μ offers a surface area of 100-860 sq.metres/gram with an average of $400m^2/g$. These offer very high plate counts upto 1,00,000/metre.

TYPES OF HPLC TECHNIQUES A Based on Modes of Chromatography

There are two modes viz - Normal phase mode and Reverse phase mode. These modes are based on the polarity of stationary and mobile phase. Before explaining the modes, it is important to know the interactions which occur between solute, stationary and mobile phase.

Polar-Polar - interaction or affinity is more

Nonpolar-Nonpolar - interaction or affinity is more

Polar-Nonpolar - interaction or affinity is less

These hold good, whether the interaction is between solute and stationary phase or solute and mobile phase.

1. Normal phase mode: In normal phase mode, the stationary phase (eg. Silica gel) is **polar** in nature and the **mobile phase** is **non-polar**. In this technique, non-polar compounds travel faster and are eluted first. This is because of less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because of more affinity towards stationary phase and takes more time to be eluted from the column. This is not advantageous in pharmaceutical applications since most of the drug molecules are polar in nature and takes longer time to be eluted and detected. Hence this technique is not widely used in pharmacy. 2. Reverse phase mode: In reverse phase technique, a non-polor stationary phase is used. The mobile phase is polar in nature. Here polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, which is advantageous. Different columns used are ODS (Octadecyl silane) or C_{la} Cs. C4, etc.

B. Based on principle of separation

- 1. Adsorption chromatography
- 2. Ion exchange chromatography
- 3. Ion pair chromatography
- 4. Size exclusion or Gel permeation chromatography
- 5. Affinity chromatography
- 6. Chiral phase chromatography

Each of the above technique is described in brief as follows:

1. Adsorption chromatography

The principle of separation is **adsorption**. Separation of components takes place because of the difference in affinity of compounds towards stationary phase. This principle is seen in Normal phase as well as Reverse phase mode, where adsorption takes place.

2. Ion exchange chromatography

The principle of separation is ion exchange, which is *reversible exchange of functional groups*. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions. For cations, a cation exchange resin is used. For anions, an anion exchange factors affecting ion exchange separations as discussed in the chapter on lon exchange chromatography is applicable to ion exchange chromatography

3. Ion pair chromatography

In ion pair chromatography, a reverse phase column is converted temporarily into ion exchange column by using ion pairing agents like temporation Hexane or Heptane or Octane sulphonic acid sodium salt, retramethyl or Tetraethyl ammonium hydroxide, etc.

4. Size exclusion or gel permeation chromatography

In this type of chromatography, a mixture of components with different molecular sizes are separated by using gels. The gel used acts as molecular sieve and hence a mixture of substances with different molecular sizes are separated. Soft gels like dextran, agarose 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 mostly 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, 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.

D. Based on the scale of operation

Analytical HPLC: where only analysis of the samples are $d_{One.}$ Recovery of the samples for reusing 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: which 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** is **adsorption**. When 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.

INSTRUMENTAL 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 columns
- 6. Detectors
- 7. Recorders and integrators

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



- 1. **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.
- 2. **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.
- 3. **Presence of impurities:** This can be seen by the presence of additional peaks when compared with a Reference standard or reference material. The percentage of impurities may also be calculated from peak areas.
- 4. **Quantitative analysis:** The quantity of a component can be determined

b. Calibration curve method

In calibration curve method, series of standards are used to determine their peak areas. A calibration curve of peak area Vs concentration of the drug is plotted. From the peak area of the unknown sample, by intrapolation, the concentration of the sample can be determined. This method has the advantage that errors, if any, are minimised.

c. Internal standard method

In this method, a compound with similar retention characteristics is used. A known concentration of the internal standard is added to the sample solution whose concentration is not known. The chromatogram is recorded and their peak areas are determined. By using formula, the concentration of the unknown solution is determined.

- 5. **Multicomponent analysis or Determination of mixture of drugs:** Similar to the quantification of a single drug, multicomponent analysis can also be done easily. The quantity of each component is determined by using any one of the above methods. Marketed formulations which contain several drugs, can be determined quantitatively for each component.
- 6. **Isolation and identification of drugs** or metabolites in urine, plasma, serum etc can be carried out.
- 7. **Isolation and identification of mixture** of components of natural or synthetic origin.
- 8. Biopharmaceutical and Pharmacokinetic studies.
- 9. Stability studies.
- 10. **Purification** of some compounds of natural or synthetic origin on preparative scale.