# 18. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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## 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.

**COMPARISON OF CLASSICAL COLUMN CHROMATOGRAPHY WITH HPLC**

| Parameter | Classical Column Chromatography | HPLC |
|---|---|---|
| Stationary Phase - Particle size | Large 60-200μ | Small 3-20μ |
| Column size length x int.diameter | Large 0.5-5m x 0.5-5cm i.d. | Small 5-50cm x 1-10mm i.d. |
| Column material | Glass | Mostly metal |
| Column packing pressure | Slurry packed at Low pressure - Often gravity | Slurry packed at High pressure > 5000 psi |
| Operating pressure | Low (< 20 psi) | High (500-3000 psi) |
| Flow rates | Low to very low | Medium - High (often > 3ml $min^{-1}$) |



| Parameter | Classical Column Chromatography | HPLC |
|---|---|---|
| Sample load | Low to Medium (g/mg) | Low to very low (μg) |
| Column efficiency i.e. Resolving power | (Low) < 500 theoretical plates per meter | (High) often > 1,00,000 plates per meter |
| Cost | Low - Few Hundreds | High - Few Lakhs |
| Detector flow cell volume | Large - 300 to 1000μl | Low 2 to 10μl |
| Types of Stationary phases available | Limited Range | Wide range |
| Scale of operation | Preparative Scale | Analytical and Preparative scale |

### 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.



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**2. Reverse phase mode:** In reverse phase technique, a ***non-polar stationary phase*** is used. The ***mobile phase*** is ***polar*** in nature. Hence 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 $C_{18}$, $C_8$, $C_4$, 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 resin is used. The principle of ion exchange separation, techniques and factors affecting ion exchange separations as discussed in the chapter on Ion exchange chromatography is applicable to ion exchange chromatography by HPLC.



#### 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 Pentane or Hexane or Heptane or Octane sulphonic acid sodium salt, Tetramethyl 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.



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**D. *Based on the scale of operation***

**Analytical HPLC:** where only analysis of the samples are done. 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**

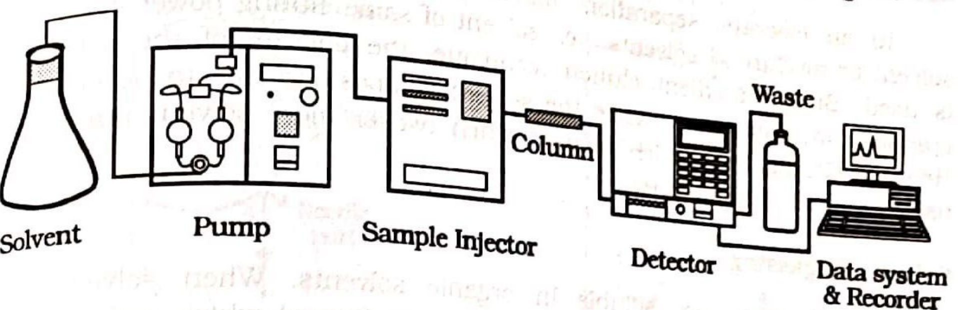
The principle of separation in ***normal phase mode*** and ***reverse phase mode*** is **adsorption**. When a mixture of components are introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent, 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



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 uses 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 unit, 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.



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## Gradient controller

In an isocratic separation, mobile phase is prepared by using pure solvent or mixture of solvents. i.e., solvent of same eluting power or polarity is used. But in gradient elution technique, the polarity of the solvent is 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 gases 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 causes 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

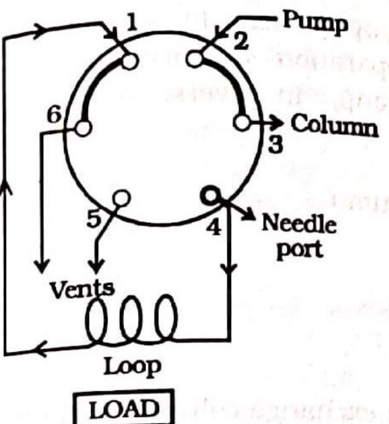
a. **Septum injectors** - for injecting the sample through a rubber septum. This is not common, since the septum has to withstand high pressure.

b. **Stop flow (on line)** - 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 or 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 of the HPLC technique which decides the efficiency of separation. There are several stationary phases available depending upon the technique or mode of separation used.

**Column material**: The columns are made up of either stainless steel, glass, polyethylene and PEEK (Poly ether 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.



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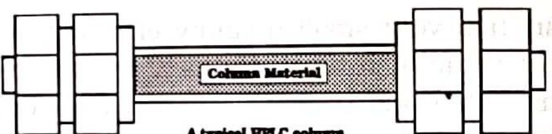
**Surface area:** 1 gram of stationary phase provides surface area ranging from 100 - 860 sq.m with an average of 400sq.m.

**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 modes of chromatography, ion exchange columns, gel columns, chiral columns, affinity chromatographic columns, etc are available. A model of column is given below.



A typical HPLC column

## 6. Detectors

Detectors used depends 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 600nm.

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

c. **Flourimetric detector:** This detector is based on the flourescent radiation emitted by some class of compounds. The exitation 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 flourescent.



d. **Conductivity detector:** Based upon electrical conductivity, the response is recorded. This detector is used when the sample has conducting ions 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 detector which operates from 190-600nm. Radiations of all wavelength fall on the detector simultaneously. The resulting spectra is 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 responses of all the compounds.

## 7. Recorders and integrators

***Recorders*** are used to record the responses 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.

## 8. Parameters used in HPLC

Refer all the parameters as given in Chapter on Gas liquid chromatography.

## APPLICATIONS OF HPLC

HPLC is being more widely used in several fields. Apart from its use in Pharmaceutical field, it is used in Chemical and Petrochemical industry,



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Environmental applications, Forensic applications, Biochemical separations, Biotechnology, Food analysis, etc. In fact there is no field where HPLC is not being used. It is a versatile and sensitive technique which can be used in several ways. Some of them are listed below:

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 by several methods like

**a. Direct comparison method**

By injecting a sample and standard separately and comparing their peak areas, the quantity of the sample can be determined.

Area of the peak = peak height × width of peak at the half height

$$\frac{A_1}{A_2} = \alpha \frac{W_1}{W_2}$$

where $A_1$ and $A_2$ are peak area of sample and standard

$W_1$ and $W_2$ are weight or concentration of sample and standard

$\alpha$ is the response factor



**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.



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Although an exhaustive list with hundreds or thousands of compounds which are analysed by HPLC can be prepared, only few of the pharmaceutical applications are described below:

**A. Identification of drugs**

| Drug | Column | Mobile phase | Detection |
|---|---|---|---|
| Betamethasone valarate ointment | RP-18 | Water : Methanol (53:47) | UV 238nm |
| Cefatoxime sodium injection | RP-18 | Phosphate buffer : Methanol : Water | UV 254nm |
| Mephalan injection | RP-18 | Ammonium carbonate : Methanol : Glacial acetic acid | UV 254nm |
| Levodopa and Carbidopa tablets | RP-8 | Sodium dihydrogen phosphate : Sodium decane sulphonate : Phosphoric acid : Water | UV 280nm |

**B. Identification of related substances**

| Drug | Column | Mobile phase | Detection |
|---|---|---|---|
| Atenolol | Normal phase | Hexane : Ammonia : Ethanol | UV 226nm |
| Ceftazidime | RP-18 | Phosphate buffer : pH 7 | UV 235nm |
| Diltiazem HCl | RP-18 | d-10-Camphor sulphonic acid : Sodium acetate : Acetonitrile : Methanol | UV 240nm |
| Cyanocobalamin | RP-8 | Disodium hydrogen phosphate : Methanol : Phosphoric acid | UV 361nm |

**C. Identification of specific impurities**

| Drug | Column | Mobile phase | Detection |
|---|---|---|---|
| Adrenaline bitartarate injection | RP-18 | Tetramethyl hydrogen sulphate : Sodium heptane sulphonate:MeOH | UV 205nm |
| Aspartame | RP-18 | Citric acid : NaOH : Methanol | UV 254nm |
| Carbidopa | RP-8 | Potassium dihydrogen phosphate : Methanol | UV 282nm |



**d. Uniformity of content**

| Drug | Column | Mobile phase | Detection |
|---|---|---|---|
| Alprazolam tablets | Normal phase | Acetonitrile : $CHCl_3$ : 1-Butanol : Water : Glacial acetic acid | UV 254nm |
| Enalapril maleate tablets | RP-8 | Phosphate buffer : Water : Acetonitrile | UV 215nm |
| Folic acid tablets | RP-18 | Phosphate buffer : Acetonitrile | UV 283nm |
| Haloperidol tablets | RP-18 | Ammonium acetate : Acetonitrile | UV 247nm |

**e. Assay of drugs**

| Drug | Column | Mobile phase | Detection |
|---|---|---|---|
| Alprazolam | Normal phase | Acetonitrile : $CHCl_3$ : 1-Butanol : Water : Glacial acetic acid | UV 254nm |
| Benzhexol | RP-18 | Acetonitrile : Water : Triethylamine : phosphoric acid | UV 210nm |
| Cefadroxil | RP-18 | Phosphate buffer : Acetonitrile | UV 230nm |
| Methotrexate | RP-18 | Acetonitrile : Phosphate buffer | UV 230nm |
| Norfloxacin | RP-18 | Acetonitrile : Phosphoric acid | UV 275nm |
| Omeprazole | RP-18 | Phosphate buffer | UV 302nm |
| Paracetamol | RP-18 | Sodium butane sulphonate in water : Methanol : Formic acid | UV 243nm |

**RP-18 - ODS or Octa Decyl Silane or $C_{18}$ column (5-10μ)**

**RP-8 - Octyl or $C_8$ column (5-10μ)**

**Normal Phase - Porous silica column (5-10μ)**



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