

# High Performance Liquid Chromatography

There are four type of chromatographic methods which uses liquid as mobile phase. These are :

- (i) Partition Chromatography.
- (ii) Adsorption Chromatography.
- (iii) Ion exchange Chromatography.
- (iv) Size exclusion Chromatography.

In most of the liquid chromatographic methods, including the original method followed by Tswell, was carried out in glass column with diameter of 1 to 5 cm. and length of 50 to 500 cm. To assure reasonable flow rates, the diameter of particles of solid stationary phase was usually in 150 to 200  $\mu\text{m}$  ranges. Even then, flow rates were low, amounting to flow length of milliliter per minute. Thus separation times were long often several hours in some cases..

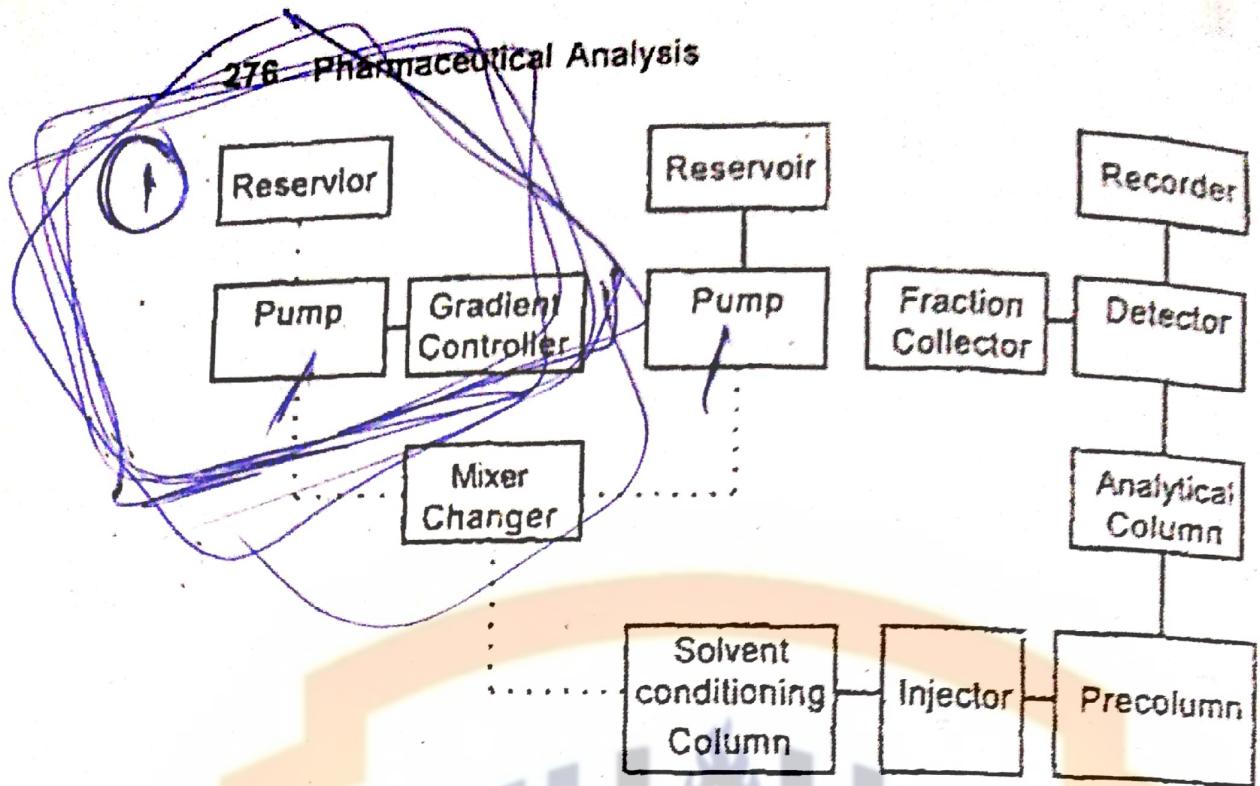
So, there was a need of important in speeding up this process without affecting efficiency. This is done either by :

- (i) Passing the mobile with pressure through column.

or

- (ii) Decreasing the particle size (as small as 3 to 10  $\mu\text{m}$ ).

This process required sophisticated instrument, the block diagram of which is given in Fig 20.1. The name high performance



**Fig. 20.1. Block diagram of HPLC : The items connected by dashed lines are necessary only for gradient elution.**

liquid chromatography (HPLC) is employed to distinguish newer procedures from basic methods.

### 1. HPLC System

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

Typical HPLC system consists of following main components:

The solvent reservoirs (glass or stainless steel containers) are capable of holding up 1 L of mobile phase which may consist of pure organic solvents or aqueous solutions of salts or buffers.

The solvents used to prepare the mobile phase should be the highest quality since contaminant eventually deposited on the column and ~~disturb~~ the resolution in chromatography. The mobile phase is filtered to remove particulate matter which may clog the system and they also are degassed using vacuum, sonification or sparging with helium to eliminate outgassing in the pump or detector.

Since the particles (stationary phase) which are used to pack HPLC columns, are small enough ( $250 \mu\text{m}$ ) to prevent solvent flow by gravity, pumps which develop pressure up to 5000 psi are essential to force the mobile phase through the column. Two types are available:

(a) Mechanical type, which deliver mobile phase at constant flow rate.

(b) Pneumatic type, which produce constant pressure.

Reciprocating pumps are mostly used mechanical type pump. It consists of small chamber in which the solvent is pumped by the back and forth motion of a motor driven piston (Fig. 20.2)

Two ball check valves, which open and close alternately, control the flow of solvent. The solvent is in direct contact into piston. Reciprocating pump has disadvantage of producing a pulsed flow, hence it must be damped by providing pulse damping device because its presence is manifested as base line noise in the chromatogram. The advantage of reciprocating pumps include their small internal

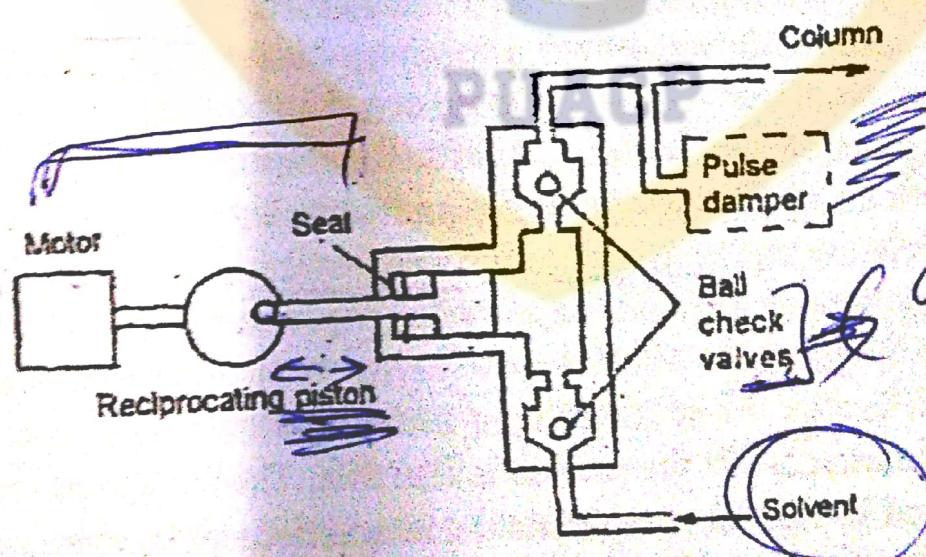


Fig. 20.2. Diagrammatic representation of reciprocating pump.

volume (35 to 400 ml), their high output pressure (up to 10000 psi), their ready adaptability to gradient elution and constant flow rate.

**Pneumatic Pump** : In the simple pneumatic pump; the mobile phase is contained in collapsible container housed in a vessel that can be pressurized by a compressed gas. Pumps of this type are in expensive and pulse free; they suffer from limited capacity and pressure output as well as a dependence of flow rate on solvent viscosity and column back pressure. In addition, they are not amenable to gradient elution and limited to pressure less than about 2000 psi.

**Gradient Analyser (Optional)**: If gradient analysis is necessary to achieve a particular separation, the most common way of forming the gradient is to include a second reservoir and pump and a gradient controller. This is electronic device, which synchronizes the operation of two pump to provide a mobile phase mixing of desired concentration. For examples if a 50-50 mixtures of the solvent in the two reservoirs is desired at an overall flow rate of 1.0 ml/min, the controller will adjust the rate of delivery of each pump to 0.5 ml/min. the individual solvents then are combined in the mixing chamber and delivered to the chromatograph.

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster, and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. There are two types of mobile phases: Isocratic and gradient.

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In isocratic elution compounds are eluted using constant mobile phase composition. All compounds begin migration through the column at onset. However, each migrates at a different rate, resulting in faster or slower elution rate. This type of elution is both simple and inexpensive, but resolution of some compounds is questionable and elution may not be obtained in a reasonable amount of time.

In gradient elution different compounds are eluted by increasing the strength(polarity) of the organic solvent. The sample is injected while a weaker mobile phase is being applied to the system. The strength of the mobile phase is later increased in increments by raising the organic solvent fraction, which subsequently results in elution of retained components. This is usually done in a stepwise or linear fashion. Compared with isocratic elution, resolution and separation are improved, and bandwidths are nearly equal.

Guard pump (optional): The next component, a solvent conditioning/guard column is used only under special circumstances. Most of the HPLC column packing materials are prepared from silica gel which will dissolve slowly in solvents whose pH values are below 2 or above 7.

This results in shrinkage of packing material giving rise to void space in which separated solutes remix or are diluted, thereby leading to loss of resolution. Therefore to minimize this occurrence and to protect the expensive silica based packing material, a small column (5 to 10 cm) packed with similar HPLC grade silica gel as in analytical column is inserted into the liquid stream after pump. The material in this column is dissolved preferentially, saturating the mobile phase and preserving the analytical column. Although there is some slight dissolution even in the pH range of 2 to 7, conditioning column need not be used always and may be disadvantage if fractions are collected with the object of recovering the solute since dissolved silica is different to remove from the solute.

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Sample injector: The solute mixture is introduced into the chromatograph by means of a suitable injection device. Septum

~~injections are available in which sample solution is injected through a self-sealing rubber or Teflon disk using a micro liter syringe. This may be done while mobile phase is flowing or while it has been stopped temporarily.~~ Although these devices are inexpensive and easy to use, it is difficult to achieve reproducible injections and automate their operation. Therefore sample introduction is done mainly by using rotatory valve and loop injector. This consist of stainless steel and Teflon block which has been drilled to provide two alternate paths for solvent flow each selectable by rotating valve. When the valve is in fill position, the solvent flows through one path directly into column. In other path, there is fixed volume (20 to 1000  $\mu\text{L}$ ) loop of narrow bore stainless steel tubing, which is filled with the sample solution using a syringe or suction. When the valve is moved to the "inject" position, the mobile phase path is diverted through the loop and washes its content into the column. The results are reproducible and the injector can be automated.

**Precolumn (optional):** It may be used for either of two reasons. When stationary phase consist of a thin layer of a liquid coated on a solid support, the liquid slowly dissolves in the mobile phase causing a degradation of resolution. In this case, precolumn will contain solid support coated with higher percentage of liquid phase than the analytical column in order to saturate the mobile phase and retard dissolution. Since most stationary phases used currently in HPLC are bonded permanently and not subject to dissolution, the precolumn is used mainly to protect the main column by trapping particulate matter and retaining substances which would be irreversibly absorbed on the analytical column. In this case, it is usually called guard column. The guard column is packed with a stationary phase identical to that in the main analytical column except its particle size may be larger so that it will not restrict the flow. The larger material is relatively inexpensive and easy to pack. Because of its short length (2 to 10 cm), it does not effect the separation.

**Analytical column:** It is the column in which the actual separation take place, is a stainless steel tube usually 25 cm. in length with an internal diameter of 2 to 4.6 mm. It is packed with the stationary phase in one of two ways.

For materials of larger particle ( $< 30 \text{ mm}$ ), the dry packing is introduced in small amounts through a funnel while the outside of column is vibrated or tapped to ensure setting of materials into a firm bed. For the more commonly used stationary phases with particle size less than  $10 \text{ mm}$ , the packing is slurred in a solvent or solvent mixture and then forced into column under pressure of up to  $6000 \text{ psi}$ . This method is superior to dry packing since it gives a tighter, more uniform bed.

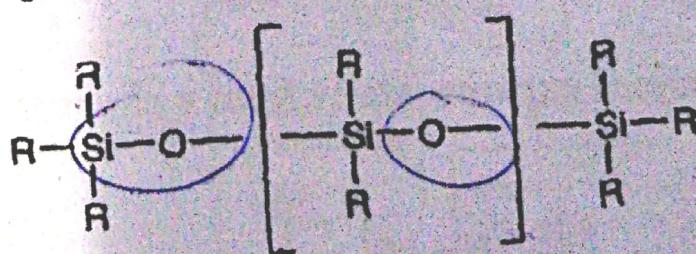
The material used to pack the column are of two types: superficially porous or (pellicular) and totally porous.

The pellicular substance consists of a layer of porous stationary phase coated on a solid core, usually a glass bead. Solute molecule can penetrate the surface layer but not the solid support. Because of size of core, the particles are relatively large ( $37$  to  $50 \mu\text{m}$ ) and they are less efficient than the smaller particles which are totally porous. Pellicular packing materials are used mainly as the support in conditioning pre and guard column.

The typical porous packing for the liquid chromatography consist of porous micro particle having diameter ranging from  $3$  to  $10 \mu\text{m}$ . The particles are composed of silica, alumina or an ion exchange resin with silica being by far more common. Silica particles are synthesized by agglomerating sub-micron-size silica particles having highly uniform diameters. The resulting particles are often coated with thin organic films, which are chemically or physically bonded to the surface.

Table 21.1 enlists the most widely used stationary phases for packed column.

Five of liquids listed in Table 21.1 are polydimethyl silioxanes that have the general structure.



In the first polydimethyl siloxane, the  $-\text{R}$  groups are all  $-\text{CH}_3$ , giving a liquid that is relatively non polar. In other polysiloxanes

Table 21.1. Stationary Phase

Substances	Common applications
Polydimethyl silioxane	Hydrocarbons, polynuclear aromatics
Poly (phenylmethyl dimethyl) silioxane	Fatty acid, alkaloids.
Poly (phenylmethyl) silioxane	Drug, Steroids, pesticides
Poly (trifluoropropyl dimethyl) silioxane	Chlorinated aromatics.
Polyethylene glycol	True acids, alcohols, ethers, essential oils.
Poly (dicyaroallyl dimethyl) silioxane	Polyunsaturated fatty acids.

shown in Table 21.1, a fraction of methyl group is replaced by function group such as phenyl  $-C_6H_5$ , cyanopropyl  $(-C_3H_6CN)$  and trifluoropropyl  $(-C_3H_6CF_3)$ . These substitution increase the polarity of liquids to various degrees. The fifth entry in Table 21.1 is polyethylene glycol having the structure  $HO - CH_2 - (O - CH_2 - CH_2)_n - OH$ . It is used for separating polar species.

Bonding involves attaching a monomolecular layer of Stationary phase to the silica surface of the column by a chemical reaction.

**Column efficiency:** A chromatographic column is divided into  $N$  theoretical plates. A thermodynamic equilibrium of analytes between the mobile and stationary phase occurs with each plate. The efficiency of the column is thus expressed as the number of theoretical plates:

$$N = \frac{L}{H}$$

L: length of column packing (cm)

H: Plate height

N is determined experimentally from a chromatogram using the equation:

$$N = 16 \left[ \frac{t}{w} \right]^2$$

t = retention time

w = peak width along the baseline of peak

**Detectors:** The ideal detector should possess all the properties as in gas chromatography with the exception that the liquid chromatography need not be responsive over as great temperature range. In addition, an HPLC detector should have minimal internal volume in order to reduce zone broadening.

**Data acquisition and control system:** It is a computer based system that controls all parameters of HPLC instrument and acquires data from the detector and monitor system.

## 2. Detectors

HPLC uses two types of detectors:

- (a) **Bulk property detector** responds to a mobile phase bulk property such as refractive index, dielectric constant, or density that is modulated by the presence of solutes.
- (b) **Solute property detectors** responds to some property of solutes such as uv absorbance, fluorescence or diffusion current that is not possessed by the mobile phase.

~~There is no one highly sensitive, universal detector used for HPLC. The selection of detector is based on the need, such as detection limit, expense etc. A summary of detection methods, which are used with HPLC separation, can be seen in Fig. 21.3. and some methods are discussed here.~~

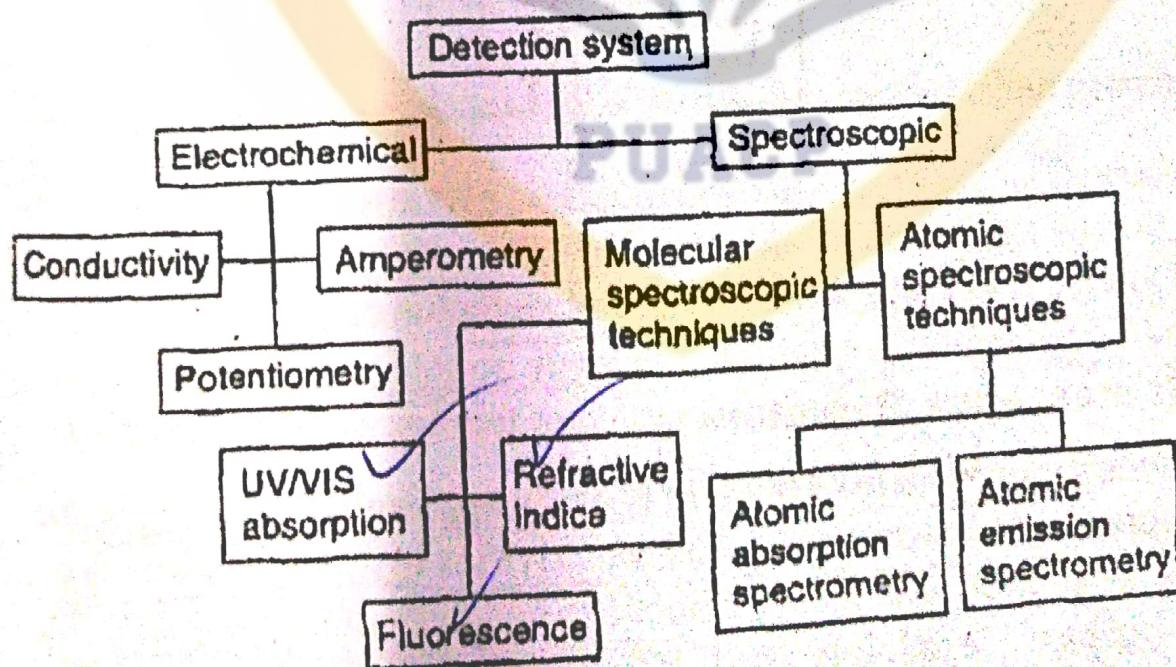


Fig. 21.3. Summary of detection for HPLC.

## Absorbance Detectors

The most frequently used instrument is an ultraviolet visible spectrometer, which has been fitted with a flow cell of very small volume ( $8\mu\text{l}$ ) (T shaped cell, Fig. 21.4) to avoid zone broadening. It is fitted with either filters or monochromators. The simplest filter fitted is fixed at one wavelength usually  $254\text{ nm}$  since most aromatic organic compounds absorb strongly at or near this wavelength. The low-pressure mercury lamps used as light sources have strong emission line at  $280\text{ nm}$  where the aromatic amino acids of protein and peptides absorb or  $214\text{ nm}$  where isolated double bonds such as the carbonyl group absorb. The fixed wavelength detectors have the advantages of low cost and high sensitivity, being able to detect some compounds at the low nanogram range.

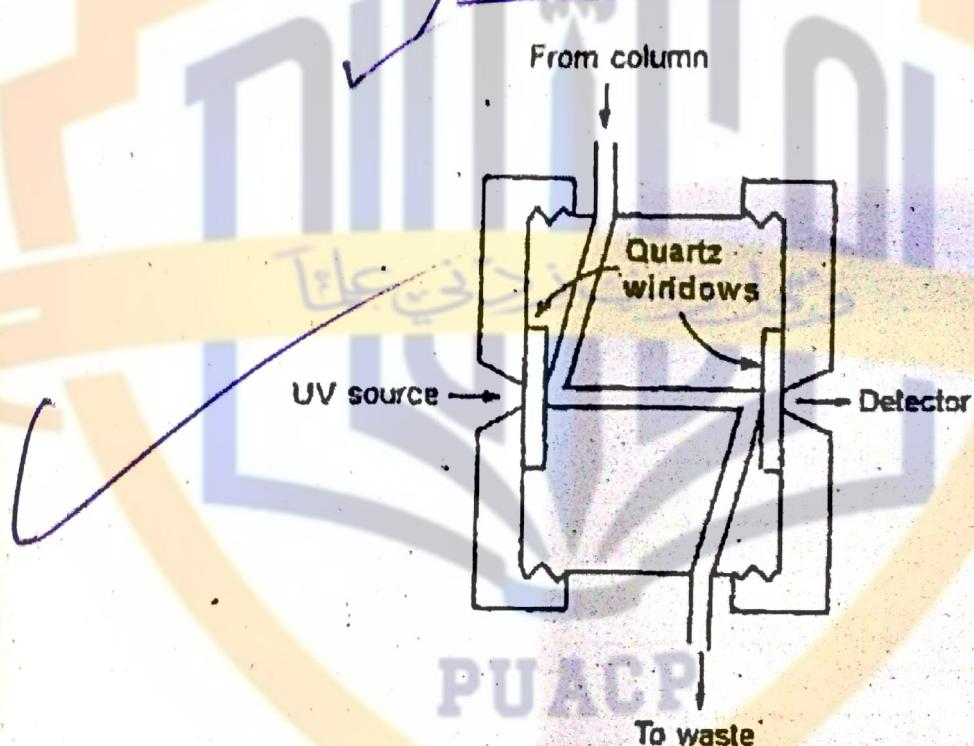


Fig 21.4. Ultraviolet detector cell

## Fluorescence Detectors

A much more sensitive but less broadly applicable detector, is the fluorescence spectrometer. The sensitivities in the picogram range can be attained with those compounds which fluoresce naturally or can be made to fluorescence by derivatization. The less expensive models of these instruments are filter fluorimeters with mercury

excitation source while the more sensitive ones use a prism or grating with Xenon lamp, to provide monochromatic excitation and emission radiation.

### Refractive Index Detectors

The most commonly used detector in HPLC is the differential refractometer which is capable of measuring refractive index change of  $10^{-4}$  to  $10^{-5}$  RI units.

In differential refractive index detector (Fig. 21.5) the solvent passes through one half of the cell on its way to the column; the eluate then flows through the other chamber. A glass plate mounted at an angle separates the two compartments that bending of incident beam occurs if the two solutions differ in refractive index. The resulting displacement of the beam with respect to photo sensitive surface of a detector causes variation in the output signal, which when amplified and recorded, provides the chromatogram.

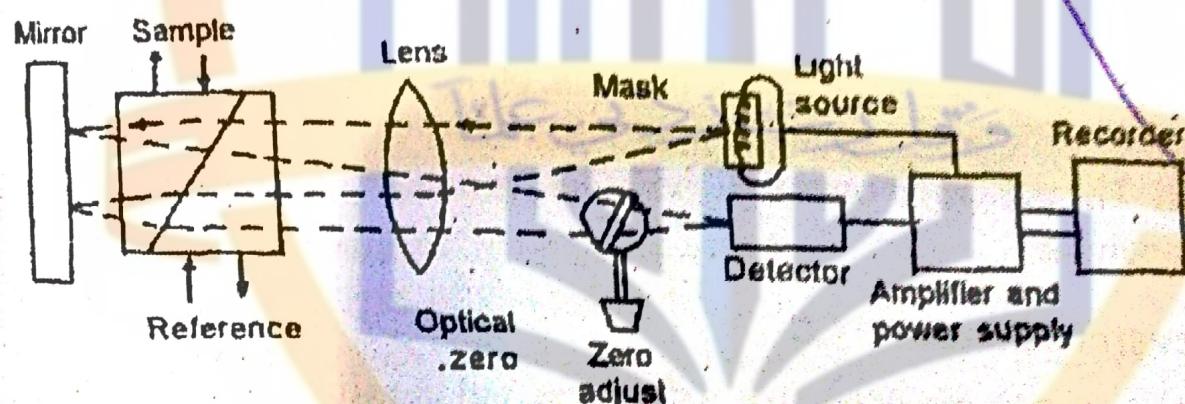


Fig. 21.5. Schematic diagram of differential refractive index detector.

This detector reacts to almost organic and inorganic compounds, but it is not as sensitive as a spectrometer. In addition, changes in ambient temperature causes severe drift change and it cannot be used with gradient elution since in both cases differences in RI are attributable to the solvent and not the solution.

### Electrochemical Detectors

Detectors based on electrochemically measurements such as amperometry, coulometry, polarography or conductometry are used for reading oxidizable or reducible compounds such as

catecholamines. With the development of separator interfaces, which remove part or all of the mobile phase, spectrometric techniques such as mass spectrometry can be used as HPLC detectors.

### 3. The column and the solvent

Confusingly, there are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase.

#### Normal phase HPLC

TLC

This is essentially just the same as the thin layer chromatography or column chromatography. Although it is described as "normal", it isn't the most commonly used form of HPLC.

The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

#### Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol.

In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals

dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.

#### 4. Retention time

The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. For a particular compound, the retention time will vary depending on:

- the pressure used (because that affects the flow rate of the solvent)
- the nature of the stationary phase (not only what material it is made of, but also particle size)
- the exact composition of the solvent
- the temperature of the column

That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds (Qualitative analysis).

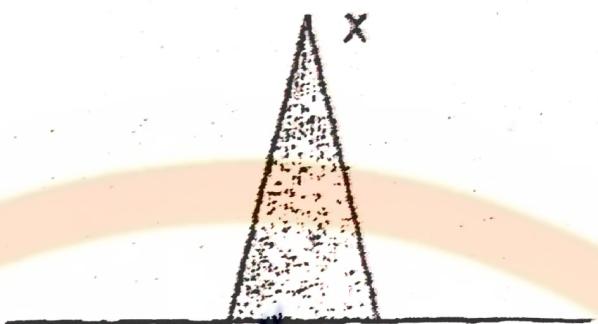
#### 5. Interpreting the output from the detector

The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector. Under controlled conditions (column, flow rate of mobile phase, temperature), the retention times is helpful to identify (qualitative analysis) the compounds present (provided retention time of pure samples of the various compounds under those identical conditions).

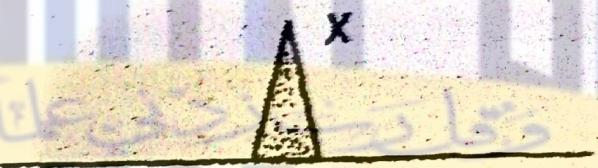
Peaks can also be used as a way of measuring the quantities of the compounds present. Let's suppose that you are interested in a particular compound, X.

If you injected a solution containing a known amount of pure X into the machine, not only could you record its retention time, but you could also relate the amount of X to the peak that was formed.

The area under the peak is proportional to the amount of X which has passed the detector, and this area can be calculated automatically by the computer linked to the display. The area it would measure is shown here (very simplified) diagram.

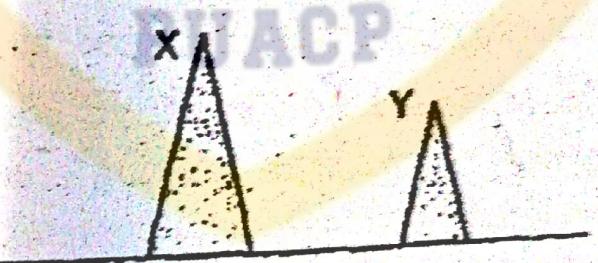


If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:



This means that it is possible to calibrate the machine so that it can be used to find how much of a substance is present - even in very small quantities.

If there are two different substances in the mixture (X and Y), there will be two peaks.



In the diagram, the area under the peak for Y is less than that for X.

## 6. Coupling HPLC to a mass spectrometer

When the detector is showing a peak, it means there is compound. This compound can be identified by connecting HPLC with mass

spectrometer. Some of what is passing through the detector at that time can be diverted to a mass spectrometer. There, it will give a fragmentation pattern which can be compared against a computer database of known patterns. That means that the identification of a huge range of compounds can be found without having to know their retention times.

## Derivatization

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It is used with HPLC for a number of reasons.

- (i) To allow chromatography of compounds which otherwise could not be detected by the instrument currently available e.g. aliphatic amines, alcohols and carboxylic acids.
- (ii) To improve resolution by adding a functional group which enhances the interaction of solutes with the stationary phases of esterification of acids.
- (iii) To improve the sensitivity of the method & formation of fluorescent derivative of amino acid most of the derivatization reactions commonly involve adding a substituted phenyl group to enhance detectability at 254 nm. These include the formation of p-bromophenacyl ester of alcohols, p-nitro benzyl esters of carboxylic acids and p-nitro benzyl oximes of carbonyls.

Dansyl chloride (5-(dimethyl amino)-1-naphthalene sulphonyl chloride) which reacts with primary and secondary amines, amino acids and phenols to give fluorescent compounds, has been widely used for the detection of amino acids protein hydrolyzates. Derivatization may be done before the sample is introduced onto the column (in pre column) or after it has been eluted.

## 7. Chiral HPLC

It is a variant of normal HPLC where the stationary phase is chiral instead of achiral. The enantiomers of the same compound differ in affinity to the stationary phase, thus they exit the column at different times.

The chiral stationary phase can be prepared by attaching a

suitable chiral compound to the surface of an achiral support such as silica gel, which creates a Chiral Stationary Phase (CSP). Many common chiral stationary phases are based on oligosaccharides such as cellulose or cyclodextrin. However, Chiral Stationary Phases are much more expensive than normal stationary phases such as *cyclodextrin*.

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For example, the separation of the enantiomers of zaltoprofen can be done on the Chiralcel OJ-RH column (cellulose tris (4-methylbenzoate) as stationary phase coated on 10 µm silica-gel) with 150 mM sodium perchlorate (pH 2.5)-methanol, 20:80 (v/v), as mobile phase. A chromatogram obtained from analysis performed under these optimum conditions is shown in Fig. 21.6.

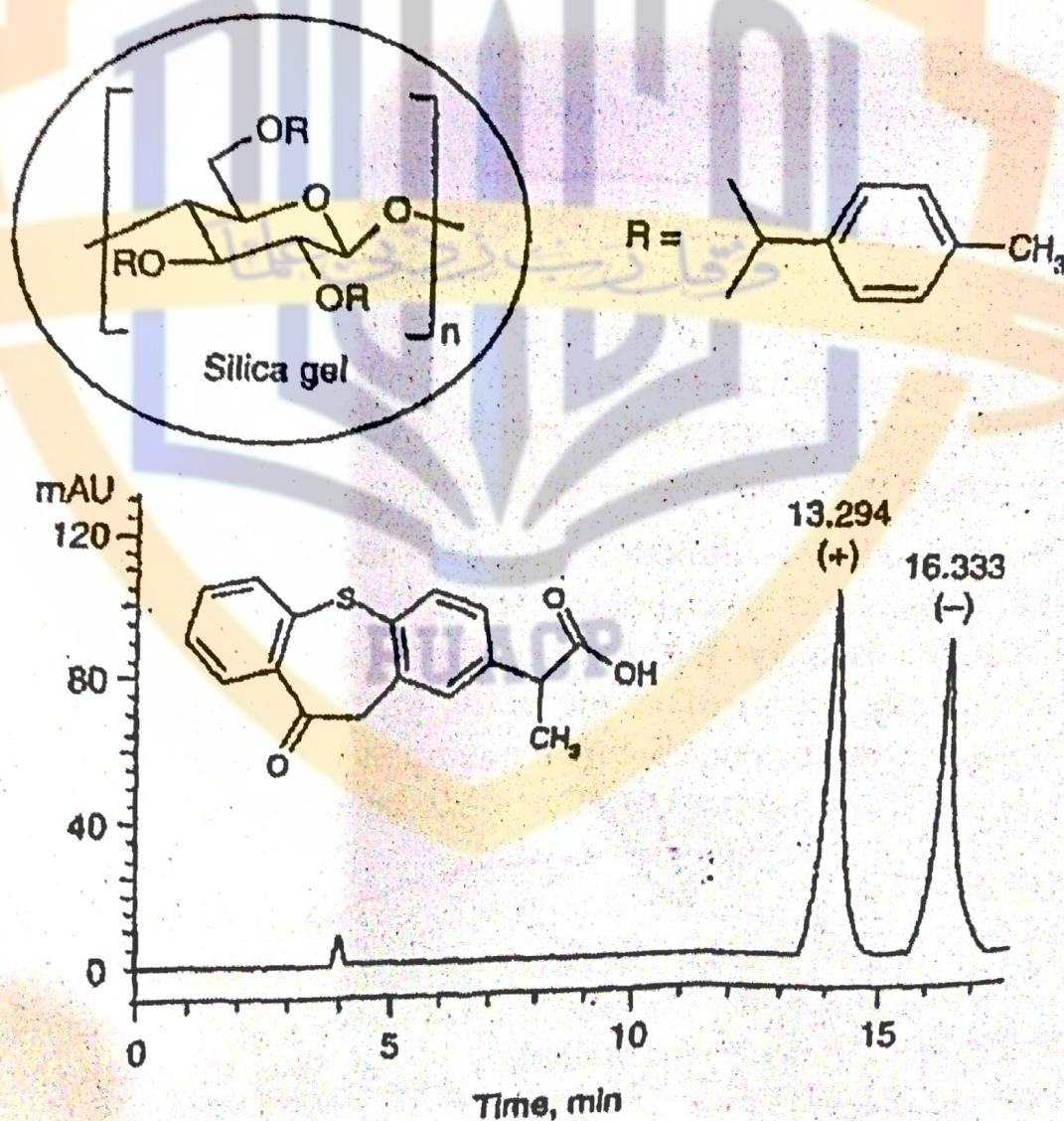


Fig. 21.6. Chromatogram obtained from analysis of the enantiomers of zaltoprofen on a Chiralcel OJ-RH column.

## Application of HPLC

It is used in the quantitative analysis of number of drugs and its metabolites. The recent Indian Pharmacopoeia enlists the number of compounds/drugs assayed by HPLC. Some are listed here:

- (i) acyclovir —
- (ii) valproic acid
- (iii) amitriptyline
- (iv) amiloride
- (v) verapamil —
- (vi) atenolol —
- (vii) warfarin
- (viii) triameterene
- (ix) captopril —
- (x) nifedipine
- (xi) digoxin —
- (xii) amlodipine
- (xiii) furosemide
- (xiv) promethazine
- (xv) famotidine
- (xvi) piroxicam
- (xvii) phenytoin —

Use with  
technology.

Zaltoperzon C by chiral HPLC

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