Chapter

1

High Performance Liquid Chromatography (HPLC)

Learning Objectives

At the end of this chapter one should be able to:

- Introduction to Chromatography
- Classification of Chromatographic Methods
- Separation Techniques in Chromatography
- High Performance Liquid Chromatography (HPLC)
- Principle of HPLC
- Theory of HPLC
- Instrumentation of HPLC
  - Mobile Phase Reservoirs
  - Pumps
  - Mixing Unit, Gradient Controller and Solvent Degassing
  - Injector (Manual or Auto-injectors)
  - Columns (Guard Columns, Pre-columns, Analytical Columns, etc.)
  - Detectors
  - Recorder or Data Systems
- Applications of HPLC

1.1 INTRODUCTION TO CHROMATOGRAPHY

Chromatography is a separation process that is achieved by the distribution of substances between two phases, a stationary and mobile phase. The chromatographic principle was discovered first by a Russian Botanist, Mikhail Tsvet (1906), who used a glass column containing calcium carbonate for separation of chlorophyll pigments from plant by using petroleum ether. The pigments according to their adsorption patterns resolved into various colored zones, he then separated and estimated them.

$$\text{Distribution coefficient} = \frac{\text{Concentration of component A in stationary phase}}{\text{Concentration of component A in mobile phase}}$$

Different affinity of these two components to stationary phase causes the separation.

PRINCIPLE OF CHROMATOGRAPHY

Chromatography is a technique for separating mixtures that depends upon differential affinities of solutes between two immiscible phases. One of the phases is fixed bed of large surface area while other fluid, which flows through or over fixed phase. The fixed phase is called stationary phase and movable phase is called mobile phase. Those solutes distributed preferentially in the mobile phase will move more rapidly through the system than those distributed preferentially in the stationary phase. Thus, solutes will elute in order of their increasing distribution coefficient with respect to the stationary phase. The stronger the forces between the solute molecules and those of stationary phase, greater will be the amount of solute held in the stationary phase under equilibrium conditions and vice versa.

1.2 CLASSIFICATION OF CHROMATOGRAPHIC METHODS

I. Based upon the nature of stationary and mobile phase used

a. Solid-gas chromatography
b. Liquid-gas chromatography
c. Solid-liquid chromatography, e.g. column chromatography, TLC, HPLC
d. Liquid-liquid chromatography, e.g. paper partition chromatography, column partition chromatography.

II. Based on principle of separation

a. Adsorption chromatography, e.g. solid-gas chromatography, TLC, column chromatography, HPLC.
b. Partition chromatography, e.g. liquid chromatography, paper partition chromatography, column partition chromatography (Fig. 1.1).

III. Based on modes of chromatography

a. Normal phase chromatography
b. Reverse phase chromatography

IV. Other types of chromatography

a. Ion-exchange chromatography
b. Gel permeation chromatography (gel filtration)
c. Chiral chromatography (to separate optical isomers)

1.3 SEPARATION TECHNIQUES IN CHROMATOGRAPHY

1. **Elution analysis,** e.g. column chromatography.
   In this method a small volume of mixture to be separated is added on the top of column and mobile phase is allowed to flow through column. As mobile phase moves down the column the mixture is introduced on the column which get separated into zones. Each component of mixture is eluted as a separated component.
2. **Frontal analysis:** In this method the solution of sample mixture is added continuously on the column. No mobile phase (solvent) is used for the development of column.

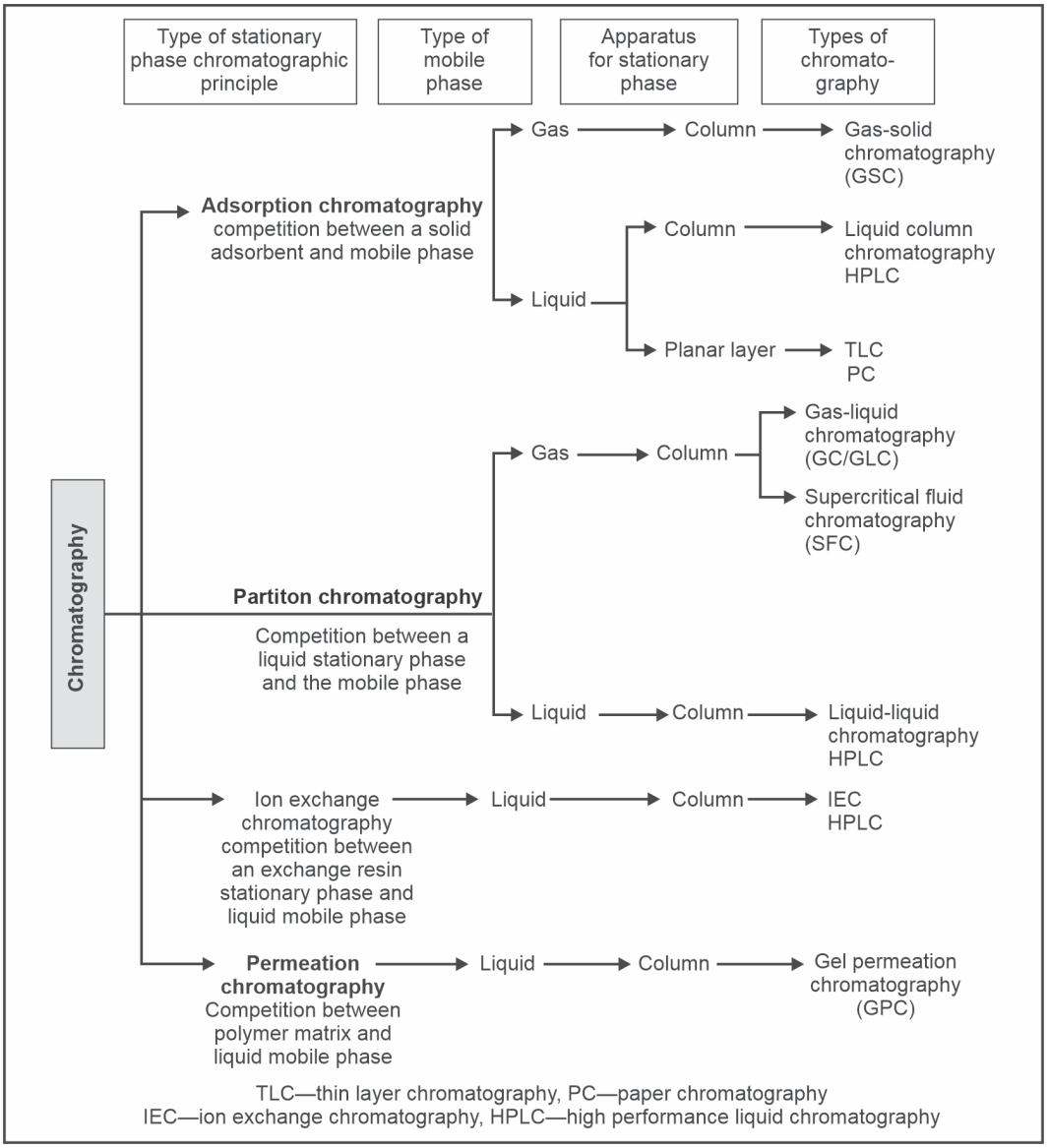


Fig. 1.1: Different forms of chromatography

3. **Displacement analysis:** This method is encountered in adsorption column chromatography.

   In this method the small volume of mixture is added to column and elution is carried out by a solvent containing a solute which has high absorptivity for column material. The absorbed constituents of mixture are displaced by the solute from mobile phase. Each solute in the mixture in turn displaces another solute which is less firmly adsorbed. The least adsorbed constituent is pushed out of the column. The substances used in the mobile phase are called displacer and the technique is called displacement analysis.

For solutes having molecular weight >10,000, exclusion chromatography is often used.

1. For low molecular weight ionic species, ion exchange chromatography is used.
2. Small polar but non-ionic species are best handled by partition makers.
3. Adsorption chromatography is often chosen for separating nonpolar species, structural isomers and compounds such as aliphatic hydrocarbons and aliphatic alcohols.

1.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

INTRODUCTION

Liquid chromatography (LC) is used today to refer to those methods in which the separation takes place within a packed column. The packing material is the stationary phase and may be a solid with adsorptive or exclusion capabilities or an inner support coated with a liquid phase. A liquid mobile phase is used as the eluent. Although thin layer chromatography and paper chromatography use a liquid mobile phase and a solid stationary phase, they differ in that the separation takes place on a planar surface rather than in a column.

High performance liquid chromatography is a technique that has arisen from the application to liquid chromatography of theories. The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by high-pressure pump. Components of this mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column. Today, HPLC is the most widely used analytical separation method. The method is popular because it is non-destructive and may be applied to thermally labile compounds (unlike GC); it is also very sensitive technique since it incorporates a wide choice of detection methods. With the use of post-column derivatization methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as separation methods makes it a valuable separation tool in scientific fields.

HPLC is a method of chemical separation in which stationary phase contained in column and mobile phase in pumped at high pressure from one end. The pressure used here is about 350 atmn (5000 psig = pounds/sq inch). In this method separation of mixture from microgram to gram is done by passing the sample through a column of 5 mm diameter containing a stationary solid bed by means of pressurized flow of liquid mobile phase. The components of the mixture migrate through the column at different rates, due to their solubility or depend upon molecular charge separation of the mixture. This technique involves mass transfer between stationary and mobile phases. HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the

stationary phase. The stationary phase is defined as the immobile packing material in the column.

The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures (Fig. 1.2).

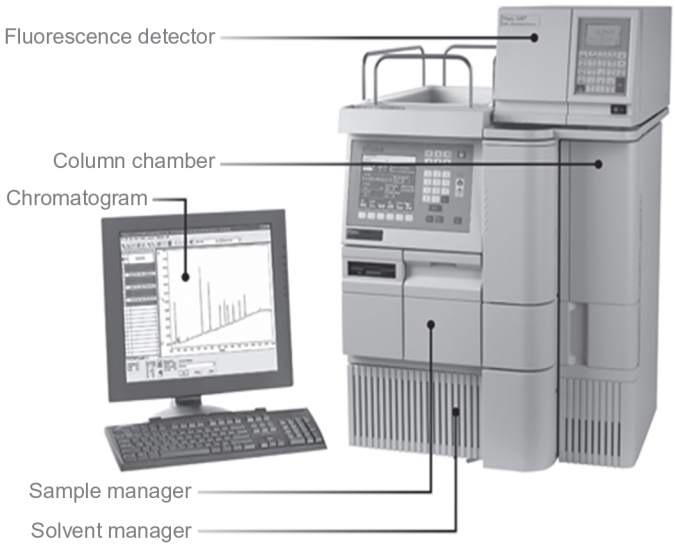


Fig. 1.2: Picture of HPLC

1.5 PRINCIPLE OF HPLC

This method is based on the separation of mixture takes place with a packed columns (i.e. the stationary phase) and liquid mobile phase as the eluent. In this technique, a liquid mobile phase is forced into the columns under high pressure either with **isocratic elution or gradient elution.** Normal flow rates of elute are 2–5 ml per minute but can be up to 10 ml/min depending upon the diameter of the column and applied pressure.

When solvent emerges from the column it passes through the detector which usually measures either refractive index or UV absorption and thereby shows whether the solvent is pure or contains a solute. The area under the peak of the chromatogram depends upon the amount of material present, so that the amount in the mixture can be measured by calibrating the chromatogram with injections with measured quantities of the compound and comparison of the resulting areas with that from the mixture.

TYPES OF HPLC

There are many ways to classify liquid column chromatography. Chromatographic separation of two components depends on the fact that they have different partition or distribution coefficients between the stationary and mobile phases. The variety of

stationary phases used in liquid chromatography (active adsorbent surfaces, polymer loaded glass beds, etc.) gives rise to a variety of separation modes which includes:

- Adsorption chromatography
- Liquid-liquid partition chromatography
- Reversed-phase chromatography
- Ion pair partition chromatography
  a. Normal phase
  b. Reverse phase
  c. Soap chromatography
- Size exclusion chromatography
- Ion exchange chromatography
- Affinity chromatography

If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified as:

1. **In adsorption chromatography,** the stationary phase is an adsorbent (like silica gel or any other silica-based packagings) and the separation is based on repeated adsorption-desorption steps.
2. **In ion exchange chromatography,** the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.
3. **In size exclusion chromatography,** the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the pores of the packing particles and elute later. Mainly for historical reasons, this technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a "gel". Concerning the first type, two modes are defined depending on the relative polarity of the two phases: Normal and reversed-phase chromatography.

In normal phase chromatography, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained. Above mentioned types cover almost 90% of all chromatographic applications. Eluent polarity plays the highest role in all types of HPLC. There are two elution types: Isocratic and gradient. In the first type constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

Initially, pressure is selected as the principal criterion of modern liquid chromatography and thus the name "high pressure liquid chromatography" or HPLC. This is, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact high performance is the result of many factors: Very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, sensitive low volume detectors and of course, good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase; otherwise, pressure is a negative factor not contributing to the improvement in separation. Recognizing this, most experienced chromatographers today, refer to the technique as high performance liquid chromatography still permitting the use of the acronym HPLC.

In adsorption chromatography, molecules which are highly polar give rise to problems of long retention times and peak tailing. This problem can be solved by separating the compounds, by making use of a technique known as reversed-phase chromatography. Here a nonpolar stationary phase is used in conjunction with a polar mobile phase. In this technique, diamond and hydrocarbon polymers coated on pellicular materials have been used as nonpolar supports. The most common method of performing this separation is by making use of a hydrocarbon-bonded surface in conjunction with a polar eluent such as methanol/water mixture (Fig. 1.3). The polar molecules now have little affinity for the hydrophobic support and are eluted relatively quickly by the aqueous mobile phase. Because most polar molecules now undergo elution first, the technique is known as RP chromatography.

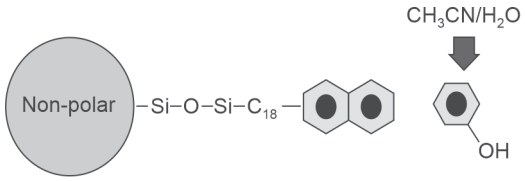


Fig. 1.3: Hydrocarbon-bonded surface in conjunction with a polar eluent such as methanol/water

Retention Mechanism

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing bed, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:

- Hydrophobic (non-specific) interactions are the main ones in reversed-phase separations.
- Dipole-dipole (polar) interactions are dominated in normal phase mode.
- Ionic interactions are responsible for the retention in ion exchange chromatography.

All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface, and the weaker the eluent interaction, the longer analyte will be retained on the surface. SEC (size exclusion chromatography) is a special case. It is the separation of the mixture by the molecular size of its components. In this mode any positive surface interactions should be avoided (eluent molecules should have much

stronger interaction with the surface than analyte molecules). Basic principle of SEC separation is that the bigger the molecule, the less possibility for it to penetrate into the adsorbent pore space, so, the bigger the molecule the less it will be retained.

STATIONARY PHASES (ADSORBENTS)

HPLC separations are based on the surface interactions and depend on the types of the adsorption sites (surface chemistry). Modern HPLC adsorbents are the small rigid porous particles with high surface area. Main adsorbent parameters are:

- **Particle size:** 3 to 10 μm
- **Particle size distribution:** As narrow as possible, usually within 10% of the mean;
- **Pore size:** 70 to 300 Å;
- **Surface area:** 50 to 250 m^2/g
- **Bonding phase density (number of adsorption sites per surface unit):** 1 to 5 per 1 nm^2

The last parameter in the list represents an adsorbent surface chemistry. Depending on the type of the ligand attached to the surface, the adsorbent could be normal phase (–OH, $–NH_2$), or reversed-phase (C_8, C_{18}, phenyl), and even anion (NH_4^+), or cation ($–COO^–$) exchangers.

Mobile Phases

In HPLC type and composition of the mobile phase (eluent) is one of the variables influencing the separation. Despite of the large variety of solvents used in HPLC, there are several common properties:

- Purity
- Detector compatibility
- Solubility of the sample
- Low viscosity
- Chemical inertness
- Reasonable price.

Each mode of HPLC has its own requirements. For normal phase mode solvents are mainly nonpolar, for reversed-phase eluents are usually a mixture of water with some polar organic solvent such as acetonitrile. Size exclusion HPLC has a special reqirements, SEC eluent has to dissolve polymers, but the most important is that SEC eluent has to supress all possible interactions of the sample molecule with the surface of the packing material.

1.6 THEORY OF HPLC

Depending upon the theories involved, HPLC is classified as

1. Liquid-liquid chromatography (LLC) or partition HPLC
2. Liquid-solid chromatography (LSC) or adsorption HPLC
3. Ion exchange chromatography or separation depends upon charge
4. Gel chromatography or size exclusion chromatography or separation depends upon molecular size of the particles.

1. **Liquid-liquid chromatography (LLC) or partition HPLC:** In this method the solid support is coated with liquid stationary phase. The relative distribution of the solutes between two liquid phases determines separation. If the stationary phase is polar and mobile phase is nonpolar it is called normal phase chromatography. If stationary phase is nonpolar and mobile phase is polar it is called reverse phase chromatography (Fig. 1.4a).

   The mechanism can be explained by following diagram

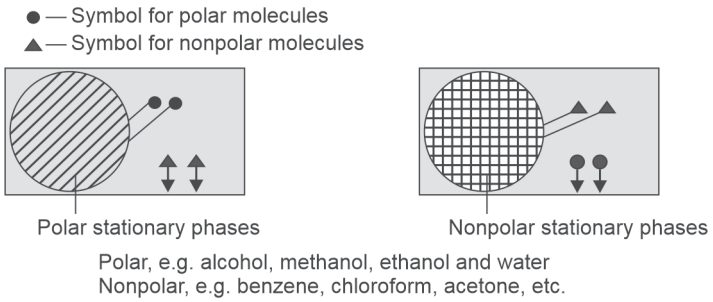


Fig. 1.4a: Polar and nonpolar stationary phases for partition chromatography

2. **Liquid-solid chromatography or adsorption HPLC:** Adsorption chromatography employs large surface area with less particle size. In this method, separation of the components of the mixture is due to affinity of the components for the surface of the stationary phase. Here high affinity components elute later, low affinity components elute first and elute intermediate. Usually polar stationary phase and nonpolar mobile phase are employed in LSC (Fig. 1.4b).
   - **Symbol for polar molecules**
   - **Symbol for nonpolar molecules**

   Polar stationary phase: Silica gel, alumina, porous glass beads, etc.

   Nonpolar mobile phase: Heptane, octane, chloroform, benzene and acetone.

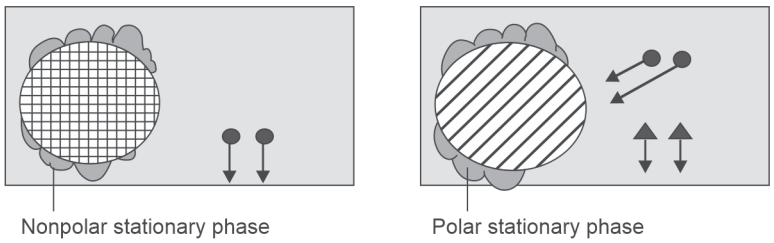


Fig. 1.4b: Polar and non-polar stationary phases for adsorption chromatography

3. **Ion exchange chromatography:** In ion exchange chromatography the stationary phase that exchange cation or anion components with mobile phase are considered. In this method reversible exchange of counter ions takes place between stationary phase ions and mobile phase ions. The separation was achieved due to difference in strength of electrostatic interaction of the solutes with the stationary phase.

Ion exchange packing materials are classified into two groups

a. Cationic exchanger

b. Anionic exchanger.

4. **Gel permeation chromatography or molecular sieves:** It is also called size exclusion chromatography. In this method separation of the mixture takes place, which depends upon the molecular size of solute presents in the solution. The material used as stationary phase contains pores of certain sizes. Molecules, which are too large, are excluded from the pores, while the smaller molecules enter into the pore and larger molecules are eluted first. The smaller molecules while in the pores do not travel as fast and are eluted later.

Chromatogram

If a solute cone is placed at the end of column and its signal is plotted as function of time (or volume), a series of peaks is obtained as shown in Fig. 1.5. A plot of recorder response in arbitrary units' signals verses time (or volume) which is obtained by chromatography is called chromatogram.

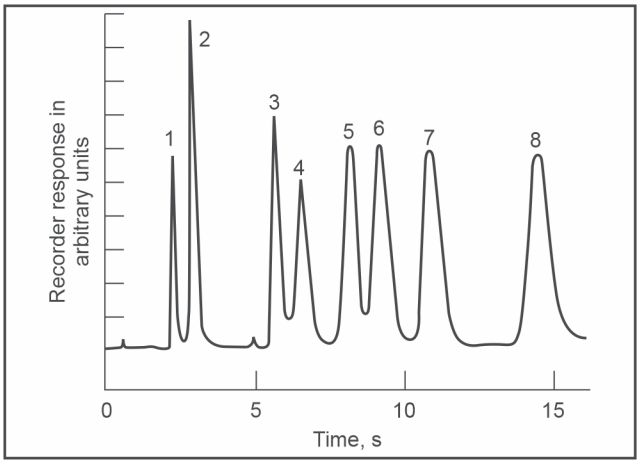


Fig. 1.5: Chromatogram

I. Rate Theory/Solves Efficiency

The separation efficiency depends on the rate of migration of solute through column or plate/paper. This depends on equilibrium established between mobile phase and stationary phase for a sample introduced. Hence, the rate of migration of solutes is most important criteria to be considered for proper resolution of components from mixture. This depends upon:

1. Distribution/partition coefficient
2. Retention factor/capacity factor
3. Selectivity factor

II. Column Efficiency

Capacity of separation of peaks is termed column efficiency. This is studied by equilibrium achieved between mobile phase and stationary phase, i.e. HETP:

Height equivalent to theoretical plate, shape and peak Gaussian shape peak and resolution.

The Theoretical Plate Model of Chromatography (Fig. 1.6)

The plate models consider that the chromatographic column contains a large number of separate layers, called *theoretical plates* (Fig. 1.7). These are the imaginary hypothetical plates. Separate equilibrations of the sample between the stationary and mobile phase occur in these *plates*. The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.

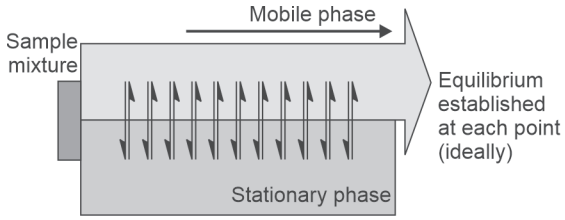


Fig. 1.6: Theoretical plate model of chromatography

Hypothetical zone in which two phases establishes equilibrium with each other.

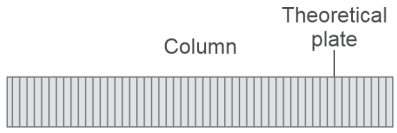


Fig. 1.7: Theoretical plates

Assumptions

- The chromatographic column is treated as a static system in equilibrium
- Each species is in equilibrium between stationary and mobile phases
- Column is of fixed length
- Flow is held constant

It is important to remember that the plates do not really exist. They are a fabrication of the imagination that helps us to understand the processes at work in the column.

They serve as a way of measuring column efficiency, either by

- Stating the number of theoretical plates in a column, N (the more plates the better), or
- Stating the plate height, H; the height equivalent to a theoretical plate (the smaller the better).

Height Equivalents Theoretical Plate (HETP)

Chromatography may be considered similar process to fractional distillation and sequential solvent extraction. In solvent extraction, a solute dissolved in an aqueous vehicle is transferred partially in one step into immiscible solvent. The amount of solute transferred is determined by its partition coefficient. After first SKP, the layers

are separated, fresh solvent is brought in contact with phase and as a result, a new equilibrium is established and more solute is transferred to the nonaqueous phase. Each single equilibration between phases is termed a theoretical plate and length of column ruptured for one equilibration is called height equivalent to a theoretical plate or HETP (Fig. 1.8).

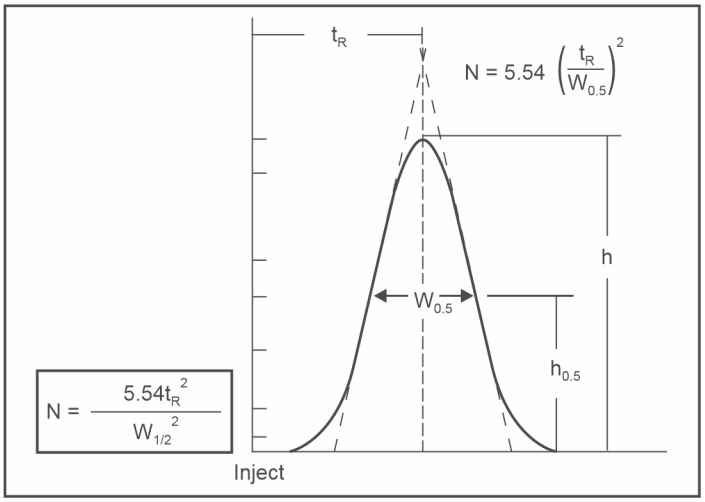
Hence, column efficiency is described by

1. Plate height H
2. Plate counts N

The two are related by the equations

N = L/H
L = Length (usually in cm) of column packing

$$\text{HEPT} = \frac{\text{Column length}}{\text{Theoretical plates}}$$



Where $w_{1/2}$ is the peak width at half-height.

Fig. 1.8: HETP graph

Column efficiencies are expressed in terms of theoretical plates (N). The higher the number of theoretical plates, the higher the separation potential of the column. Theoretical plate numbers vary directly with column length and inversely with column diameter.

Separation is concerned with the relative positions of the band centers.

Resolution describes the overlap of the leading and trailing edges of successive peaks.

1. Chromatograms with poor separation and resolution indicate the presences of two peaks, but are useful neither for quantitation nor for isolation of either substance.
2. In this adequate separation has been achieved, but resolution remains poor because of overlap of trailing edge of peak 1 and the leading edge of peak 2.

3. The separation has remained constant while resolution has been optimized to lessen band overlap, resulting in an ideal chromatogram.

Resolution

Rs Value	Overlap (contamination of each component by other)
Rs = 0.75	No resolution
Rs = 1.00	4% overlap
Rs = 1.25	2% overlap
Rs = 1.5	0.3% overlap

The resolution for a given stationary phase can be improved by lengthening the column, thus increasing the number of plates. Usually resolution >2 is preferable in most of the analysis.

The Rate Theory of Chromatography

The rate theory describes the process of peak dispersion (band spreading) and provides an equation that allows the calculation of the variance per unit length of a column (the height of the theoretical plate, HETP) in terms of the mobile phase velocity and other physical chemical properties of the solute and distribution system.

$H = \sigma^2/L$ (σ standard deviation of the band)

σ^2 = variance.

h = reduced plate height = H/d_P = H/particle diameter.

VAN DEEMTER EQUATION

If we consider the various mechanisms which contribute to band broadening, we arrive at the Van Deemter equation for plate height

$$H = A + B/u + u\,[C_M + C_S],$$

where

u: It is the average velocity of the mobile phase.

A, B, and C are factors which contribute to band broadening

A: Random movement through stationary phase

B: Diffusion in mobile phase

C: Interaction with stationary phase

H: Plate height

u: Average linear velocity

Term A

- Molecules may travel unequal distances
- Independent of u
- Depends on size of stationary particles or coating (TLC)

A-Eddy Diffusion

Mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.

Term B

Longitudinal diffusion

$$B = 2\gamma D_M$$

γ: Impedance factor due to packing
D_M: Molecular diffusion coefficient.

One of the main causes of band spreading is diffusion. The diffusion coefficient measures the ratio at which a substance moves randomly from a region of high concentration to a region of lower concentration.

B-Longitudinal Diffusion

The concentration of analyte is less at the edges of the band than at the centre. Analyte diffuses out from the centre to the edges. This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

Term C

C_s: Stationary phase mass transfer
C_M: Mobile phase mass transfer.

C-*Resistance to Mass Transfer*

The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

Van Deemter Plots (Fig. 1.9)

A plot of plate height *Vs* average linear velocity of mobile phase.

Such plots are of considerable use in determining the optimum mobile phase flow rate.

Most Commonly Used Methods in HPLC

1. Normal Phase Chromatography

Mechanism: Retention by interaction of the stationary phase's polar surface with polar parts of the sample molecules.

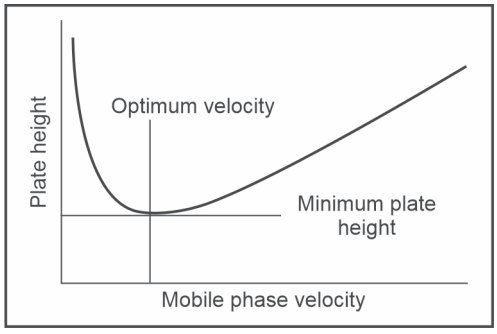


Fig. 1.9: Van Deemter plot

Stationary phase: It is a bonded siloxane with polar functional group like SiO_2, Al_2O_3, $–NH_2$, –CN, $–NO_2$, – Diol.

Mobile phase: Nonpolar solvents like heptane, hexane, cyclohexane, chloroform, ethyl ether, dioxane.

Application: Separation of nonionic, nonpolar to medium polar substances.

Sample elution order: Least polar components are eluted first.

2. Reverse Phase Chromatography

Mechanism: Retention by interaction of the stationary phase's nonpolar hydrocarbon chain with nonpolar parts of sample molecules.

Stationary phase: It is bonded siloxane with nonpolar functional groups like n-octadecyl (C_{18}) or n-octyl (C_8), ethyl, phenyl, $–(CH_2)_n$, -diol, $–(CH_2)_n$, –CN.

Mobile phase: Polar solvents like methanol, acetonitrile, water or buffer (sometimes with additives of THF or dioxane)

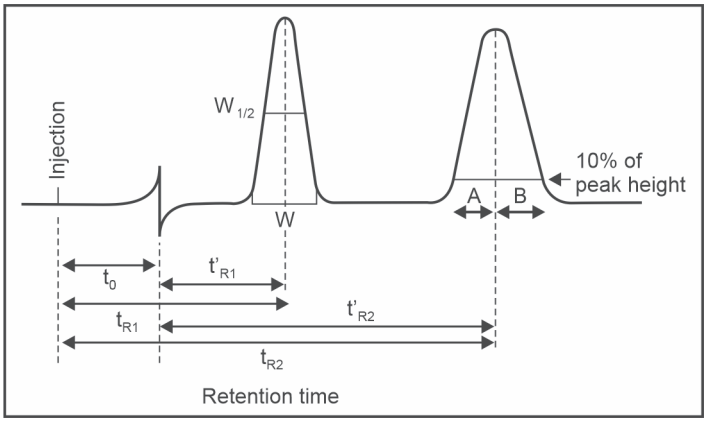
Application: Separation of nonionic and ion forming nonpolar to medium polar substances (carboxylic → acids hydrocarbons).

Sample elution order: Most polar components are eluted first.

FUNDAMENTAL PARAMETERS/SYSTEM SUITABILITY PARAMETERS

Retention Time (Fig. 1.10)

It is the time in between the sample is injected and chromatographic peak is recorded. The total retention time (t_{R1} or t_{R2}) is the time, which is needed by sample component to migrate from column inlet (sample injection) to the column end (detector). The net



Where:
$W_{1/2}$—peak width at half height
W—bandwidth of the peak (intersection point of the inflection tangents with the zero line)
A—peak front at 10% of peak height to peak maximum
B—peak maximum to peak end at 10% of retention times
t_0—dead time of a column retention time of un-retained substance
t_{R1}, t_{R2} …—retention time of components 1, 2 …
t'_{R1}, t'_{R2} …—net retention time of components 1, 2 …

Fig. 1.10: Retention time graph

retention time (t_{R1} or t'_{R2}) is the difference between total retention time and dead time. That is the time the sample component remains in the stationary phase.

Capacity Factor (k')

It is the measure of the position of a sample peak in the chromatogram. It is specific for a given substance. k' depends on the stationary phase, the mobile phase, the temperature, quality of the packing, etc.

$$k' = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0} = \frac{t'_R}{t_0}$$

V_R = Retention volume
V_0 = Retention volume of solvent
t_R = Retention time
t_0 = Retention time of solvent
t'_R = Actual retention time

$$k'_1 = \frac{t_{R_1} - t_0}{t_0} \qquad k'_2 = \frac{t_{R_2} - t_0}{t_0}$$

Relative Retention (α)

Also known as **separation factor** is the ratio between two capacity factors. The relative retention describes the ability of a system of stationary and mobile phase to discriminate between two compounds. Impurities in the mobile phase (e.g. water content) strongly influence the relative retention (Fig. 1.11).

$$\alpha = \frac{k'_2}{k'_1}$$

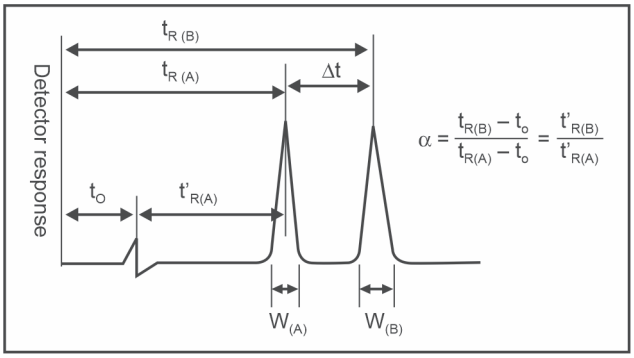


Fig. 1.11: Relative retention graph

Resolution (Rs) (Fig. 1.12)

It is a measure of quality of separation of adjacent bands; obviously overlapping bands have small Rs values. It is calculated from width and retention time of two peaks.

$$Rs = \frac{2(t_2 - t_1)}{W_1 + W_2},$$

where t_1 and t_2 are the retention time of first and second adjacent bands, whereas W_1 and W_2 are their baseline bandwidths. Reliability of calculation is poor if Rs is <2.0.

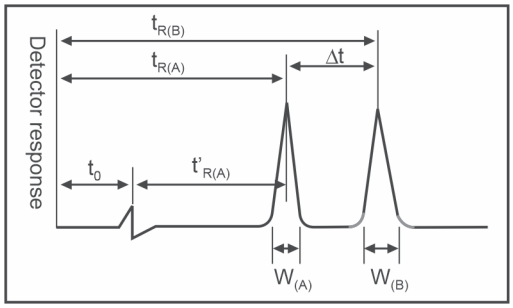


Fig. 1.12: Resolution graph

$$R = \frac{\Delta t}{\frac{W_A + W_B}{2}} = \frac{2\Delta t}{W_A + W_B}$$

$$R = \frac{\Delta t}{W_b} = \sqrt{N_2} - \frac{\alpha - 1}{\alpha}\frac{k'_2}{1 + k'_2}$$

Efficiency | Capacity factor

Selectivity

$$R_s = 0.25\,(\alpha - 1)\,(N^{1/2}[K'/(1+K')],$$

where

α = Selectivity

K′ = Retention

N = Efficiency

To affect resolution

N = Increase column length, decrease particle size, optimize flow rate.

K′ = Increase (weaker solvent), decrease (strong solvent)

α = Change stationary phase, change mobile phase solvent, change buffer pH and temperature.

Reliability of calculation is poor if Rs is <1.0. Resolution can be expressed in terms of three parameters.

α = Separation factor (K_2/K_1)

N = Column plate number

K = Average retention factor for two bands.

Peak width

$W_{1/2}$ = Peak width at half height.

W = Bandwidth of the peak.

Peak symmetry is measured at 10% peak height.

Symmetry parameters

A = Peak front at 10% of peak height to peak maximum.

B = Peak maximum to peak end at 10% of peak height.

Retention time

t_0 = Dead time of the column with retention time of an untreated substances,

t_{R1}, t_{R2} = Retention times of compound 1 and 2,

t_{R1}, t_{R2} = Net retention times of component 1 and 2.

Critical band pair

It is a band pair in chromatogram which has smallest Rs value. While developing the method separation conditions are appropriately/systematically changed to improve upon the critical band pair.

Total retention time

It is the time required by the sample component to migrate from the column inlet to the column end.

Dead volume or non-adsorbed time

Time required by the inert substances to migrate from column inlet to column end without been retained by the stationary phase.

Net retention time

It is the difference between the total retention and the dead volume

$$t'_{R1} = (t_R - t_0)$$

NUMBER OF THEORETICAL PLATES (N)

"N" characterizes the quality of a column packing and mass transfer phenomena. The larger n, the more complicated sample mixtures can be separated with the column.

$$n = 16\left(\frac{t_{R_1}}{W}\right)^2 \quad \text{or} \quad n = 5.54\left(\frac{t_{R_1}}{W_{1/2}}\right)^2$$

The **height equivalent** to a theoretical plate HETP, h, is the length, in which the chromatographic equilibrium between mobile and stationary phase is established. Since a large number of theoretical plates are desired, 'h' should be as small as possible. The value of 'h' is a criterion for the quality of a column values depend on the particle size, the flow velocity, the mobile phase (viscosity) and especially on the quality of the packing

$$h = \frac{L}{n}$$

L = Length of the column

n = Number of theoretical plates

Column efficiency (Fig. 1.13)

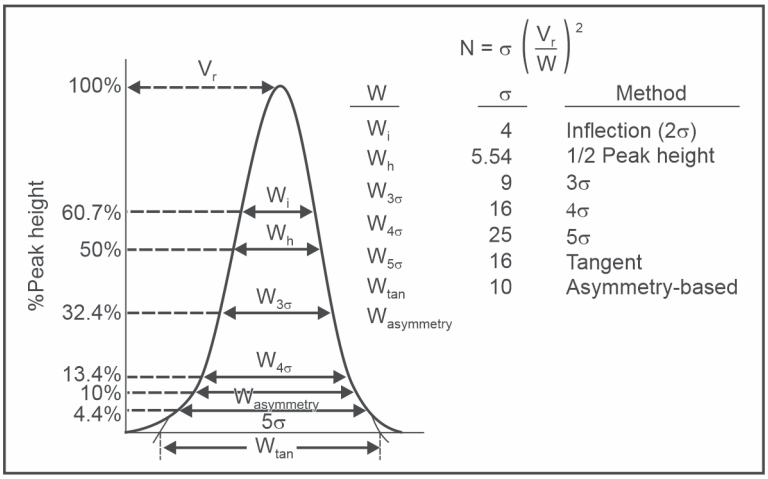


Fig. 1.13: Column efficiency graph

SYMMETRY FACTOR

Symmetry is measured at 10% of peak height, where A is the distance from peak front to peak maximum and B is the distance from peak maximum to peak end. Ideally symmetry should be 1, i.e. A = B (perfectly symmetrical peaks).

$$\text{Symmetry} = \frac{B}{A}$$

System Suitability

Prior to the analysis of samples each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability parameters (Table 1.1).

According to USP, system suitability tests are integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. Parameters such as plate count,

Table 1.1: System suitability parameters and recommendations

Parameters	*Recommendations*
Capacity factor	The peak should be well-resolved from other peaks and the void volume, generally k' >2.0 (generally between 2–20 is acceptable)
Repeatability	RSD </=2% for N >/=5 is desirable
Relative retention	Not essential as long as the resolution is stated.
Resolution (Rs)	Rs of >2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing factor (T)	T of </=2 (0.8–2 is permissible but for best peak it should be ≤1.5)
Theoretical plates	In general, should be >2000

tailing factor, resolution and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of system suitability "sample" that is a mixture of main components and expected by-products.

Methods for Quantitative Analysis by HPLC

The various approaches used for quantitative analysis

- Peak height method
- Peak area method
- Use of internal standards
- Use of external standards

In the **peak area method**, a mixed standard solution containing the known quantities of all the components of the mixture is chromatographed several times. The sample is chromatographed several times and mean peak area for each component is correlated with peak area of the standard and the amount of each component present in the mixture is calculated.

In **external standard method,** the standard is the same substance as that being analyzed in the sample. The external standard must be pure or its composition known through prior analysis and it should be authentic.

By injecting standard solution in different concentration, peak response is plotted *vs* concentration. Unknown samples are analyzed in similar manner and their concentration determined from the calibration curve

$$RF = \frac{\text{Standard peak area (area or height)}}{\text{Concentration of the sample}}$$

$$RF = \frac{\text{Sample peak (area or height)}}{\text{Sample concentration}}$$

Internal standard method a calibration curve is produced by analyzing different concentrations of the pure drug with constant amount of (IS) and from the chromatogram. Ratio (Rs) for each concentration is calculated

$$Rs = \frac{\text{Area of the drug}}{\text{Area of the IS}}$$

Solubility Studies

Perform the solubility experiments to establish the solubility of the API in a number of aqueous and organic solvents like water, buffer, 0.1N sodium hydroxide, methanol, acetonitrile, chloroform, hexane, tetrahydrofuran (THF), etc. covering a range of polarities that are commonly used in the method development. The scientist should check the pH of the water-soluble API to know its acidic or basic nature (i.e. pKa value). The API should have good solubility in the selected diluents (preferably 1 mg/ml). Perform a UV scan in the range of 200–400 nm in the selected solvent to ascertain the spectrophotometric properties of the drug. The solvent is selected as such, which covers the range of polarities that are commonly used in the method development. The typical pharmaceutical compounds are either soluble in water or organic solvent. The

water-soluble API is further differentiated as ionic and non-ionic. The organic soluble API can be classified as polar and nonpolar.

Selection of the Wavelength

The selection of the wavelength is a critical step in the method development. To select the wavelength, prepare the standard solution at the required concentration in the selected solvent and scan it on UV spectrophotometer. Based on the UV scan results, inject the test solution into HPLC system equipped with the photodiode array detector (PDA) and collect the spectra. Select the wavelength, which gives the optimum response for the drug components.

Normal Phase Chromatography (Method Development)

The first choice for developing the HPLC method should be reverse phase chromatography; Normal phase chromatography is preferred if:

a. Sample is dissolved in nonpolar solvent like hexane, chloroform, dichloromethane, etc.
b. The sample is unretained by the reverse phase chromatography.
c. Reverse phase chromatography separation is unable to achieve adequate band spacing.
d. Sample contains positional isomer, stereoisomer, and diastereomers.
e. Normal phase is useful for the compound that decomposes in the aqueous phase.

Normal phase chromatography in HPLC typically encompasses adsorption chromatography on silica and partition chromatography on cyano and amino bonded phase. Unique separations are provided for saccharides, isomers, steroids, and class separation of lipids and polynuclear aromatic hydrocarbons.

Reverse Phase Chromatography

Generally, it is preferred in most of the pharmaceuticals or drugs. Depending upon the nature of the API (acidic/basic/neutral), an initial exploratory run using the gradient elution can be performed by selecting the conditions. After the initial exploratory run, evaluate the chromatogram before proceeding with the next injection and subsequent adjustment should be made to the mobile phase composition, pH of the buffer, column packing, column temperature, flow rate, etc. to get the optimum condition for separations. Each subsequent injection is thus based on the previous condition found. Separation in reversed-phase chromatography depends on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase.

Optimization of the Method Development Parameters

1. Selection and Optimization of Mobile Phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities, i.e. degradants from analyte (API) peak. The selection of mobile phase is done always in combination with selection of column (stationary phase). The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase:

a. Buffer, if any and its strength.
b. pH of the buffer or pH of the mobile phase.
c. Mobile phase composition.

a. Buffer, if any and its strength

It is important to choose the buffers with suitable strength to cope up for the injection load on the column otherwise peak tailing may arise due to changes in ionic form during chromatography. The retention times also depend on the molar strength of the buffer, molar strength is inversely proportional to retention times.

Ideally, the strength of the buffers shall be opted between 0.05 M and 0.20 M. The selection of buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be increased if necessary, to achieve the required separations. But it is to be ensured that the higher buffer strengths shall not result in precipitations/turbidities either in mobile phase or in standard and test solutions while allowed to stand in bench top or in refrigerator. Experiments shall be conducted using different buffers having different strength to obtain the required separations.

b. pH of the buffer or pH of the mobile phase

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Depending on the pKa, drug molecule changes retention, e.g. acids show an increase in retention as the pH is reduced while base show a decrease. Experiments shall be conducted using buffers having different pH to obtain the required separations.

It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns do not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkages are cleaved below pH 2.0, while at pH values above 8.0, silica may dissolve. If a pH outside the range of 2.0 to 8.0 is found to be necessary, packing materials, which can withstand to that ranges shall be chosen.

c. Mobile phase composition

In reverse phase chromatography the separation is mainly controlled by the hydrophobic interactions between drug molecules and the alkyl chains on the column packing material. Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile. Tetrahydrofuran is also used but to a lesser extent.

Experiments shall be conducted with mobile phases having buffer with different pH and different organic phases to check for best separations between the impurities.

2. Selection of Column

The following are the parameters of a chromatographic column which are to be considered while choosing a column for separation of impurities and degradants:

i. Length and diameter of the column
ii. Packing material
iii. Shape of the particles
iv. Size of the particles
v. Percentage of carbon loading
vi. Pore volume

vii. Surface area
viii. End capping

3. Selection of Solvent Delivery System

Chromatography separations with a single eluent (isocratic elution), i.e. all the constituents of the mobile phase are mixed and pumped together as signal eluent is always preferable. However, gradient elution is a powerful tool in achieving separation between closely eluting compounds or compounds widely differing in polarities.

The important feature of the gradient elution which makes it a powerful tool, is that the polarity and ionic strength of the mobile phase can be changed (can be increased or decreased) during the run. Conduct experiments using different mobile phase combinations and different gradient programmers to achieve separations of all the impurities, i.e. degradants from API peak.

In general, while running a gradient two mobile phases having different composite ion is kept in different channels the two mobile phases are then introduced into the column by two different ways. By low pressure gradient, i.e. the mobile phases are mixed at the predetermined ratios and then pumped using a single pump. By high pressure gradient, i.e. the mobile phases are pumped at different flow rates so as to achieve the required composition and then mixed in a chamber and then introduced into the column.

4. Selection of Flow Rate

Flow rate shall be selected based on the following data

- Retention times
- Column back pressure
- Separation of impurities
- Peak symmetries

Preferably the flow rate shall be not more than 2.0 ml/min.

5. Selection of Column Temperature

Always it is preferable to optimize the chromatographic conditions with column temperature as ambient. However, if the peak symmetry could not be achieved by any combination of column and mobile phase then the column temperature above ambient can be adopted. The increase in column temperature generally will result in reduction in peak asymmetry and peak retention. When found necessary the column temperature between 30°C and 80°C shall be adopted. If a column temperature of above 80°C is found to be necessary packing material which can withstand to that temperature shall be chosen.

6. Selection of Diluent for Test Preparation and Extraction Procedure

Diluent for test preparation is selected initially based on solubility of the drug substance. However, finalization of diluent is based on its extraction efficiency peak symmetries and resolution of impurities from API peak and diluent blank injection interference. Select a diluent in which drug substances are soluble and the extraction is complete due to which there is no blank interference in which the peak symmetry and resolution between impurities and API peak is found to be satisfactory.

7. Methods of Extraction

In general methods followed for extraction are—sonication, rotary shaking or both. In some cases where the API is not extracted by above procedures heating can be adapted if the substance is stable and should not precipitate upon cooling to room temperature. Conduct experiments to optimize the extraction of API in presence of excipients at different test concentrations using the diluents chosen based on solubility at different time intervals of sonication or rotary shaking or both and select the test concentration at which the extraction is most efficient.

8. Selection of Test Concentration, Injection Volume

The test concentration is generally chosen based upon the response of API peak at the selected detector wavelength. However, the test concentration shall be finalized after it is proved that API is completely extractable at the selected test concentration. Generally, an injection volume of 10 to 20 ml is recommended for estimation of API. However, if the extractions are found to be difficult, then the test concentration can be kept low and the injection volume column is not overloaded, resolutions between individual impurities from API peak and the peak symmetry are not compromised.

9. Establishment of Stability of Test Preparation

Prepare the test solution and programmed for stability of solution on auto-injector for at least 12 hours. As far as possible select a diluent in which test solution is stable for at least 12 hours. If the solution is found to be unstable by its nature, then incorporate the stability of solution in test method.

10. Establishment of System Suitability

System suitability parameter is to be selected based on the criticality of separation between impurities and API. In general, resolution factor for the closely eluting compounds is selected as system suitability requirements. If the separation of impurities from each other and from API peak is found to be satisfactory there is no need to keep a resolution factor as a system suitability parameter. In such a case, only standard reproducibility and asymmetry of standard peak can be adopted as a system suitability requirement.

1.7 INSTRUMENTATION OF HPLC

HPLC instrumentation (Fig. 1.14) includes:

1. Mobile phase reservoirs
2. Pumps
3. Mixing unit, gradient controller and solvent degassing
4. Injector (manual or auto-injectors)
5. Columns (guard columns, pre-columns, analytical columns, etc.)
6. Detectors
7. Recorder or data system.

The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components

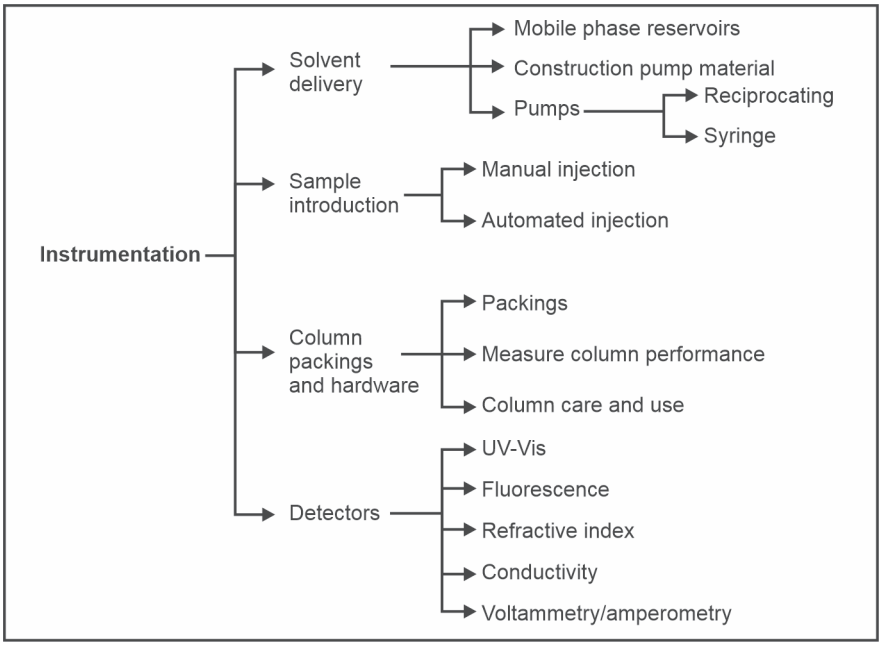


Fig. 1.14: HPLC instrumentation and techniques

occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder. Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, computers, integrators, and other data processing equipment are frequently used (Figs 1.15 to 1.17).

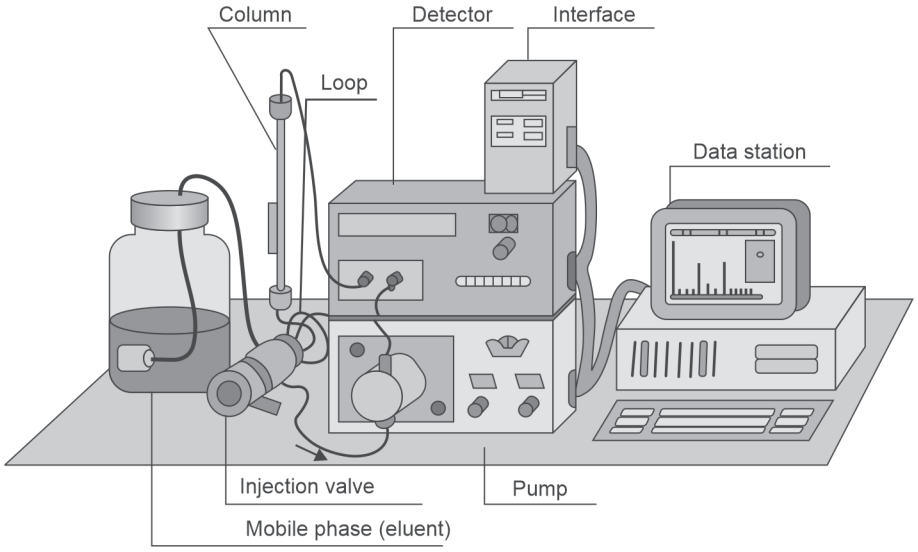


Fig. 1.15: Functional schematic of a modern HPLC instrument

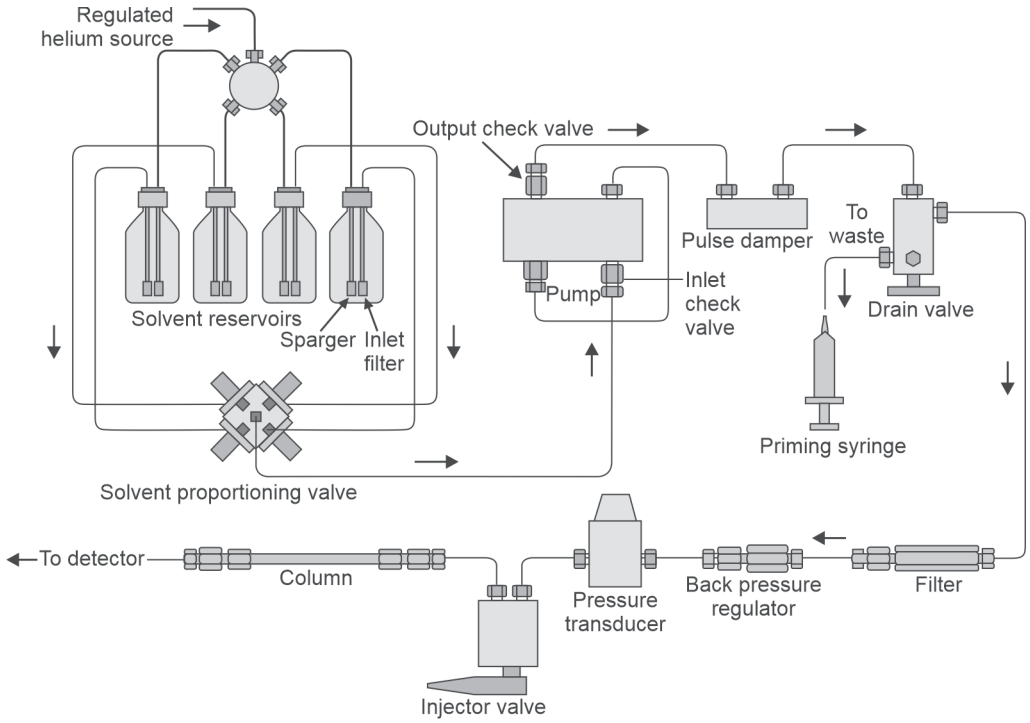


Fig. 1.16: Schematic of an apparatus for HPLC

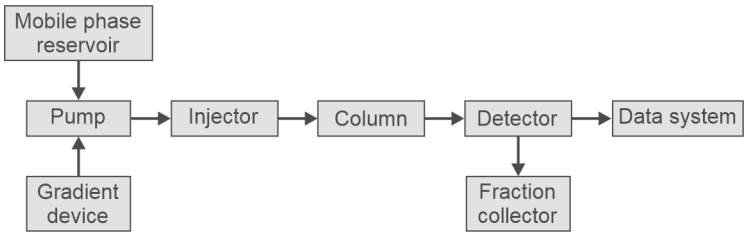


Fig. 1.17: Flow diagram of HPLC

I. MOBILE PHASE RESERVOIRS

The mobile phase supply system consists of number of reservoirs (200 to 1,000 ml in capacity). At least two reservoirs would be necessary and are usually constructed of glass or stainless steel and contain an exit port open to air. Stainless steel, however, is not considered satisfactory for mobile phases buffered to a low pH and containing certain materials that can cause corrosion. Each reservoir is usually fitted with a gas diffuser through which unwanted gases can be bubbled. The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with the special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium was found not to be sufficient for degassing of aqueous solvents. It is useful to apply a vacuum for 5–10 min. and then keep the solvent under a helium atmosphere.

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contains 500 ml or more or solvent. Reservoirs also contain system for sparging in which the dissolved gases are swept out of the solutions by fine bubbles of an inert gas of low solubility. System also contains a means of filtering dust and particulate matter from the solvents (Fig. 1.18).

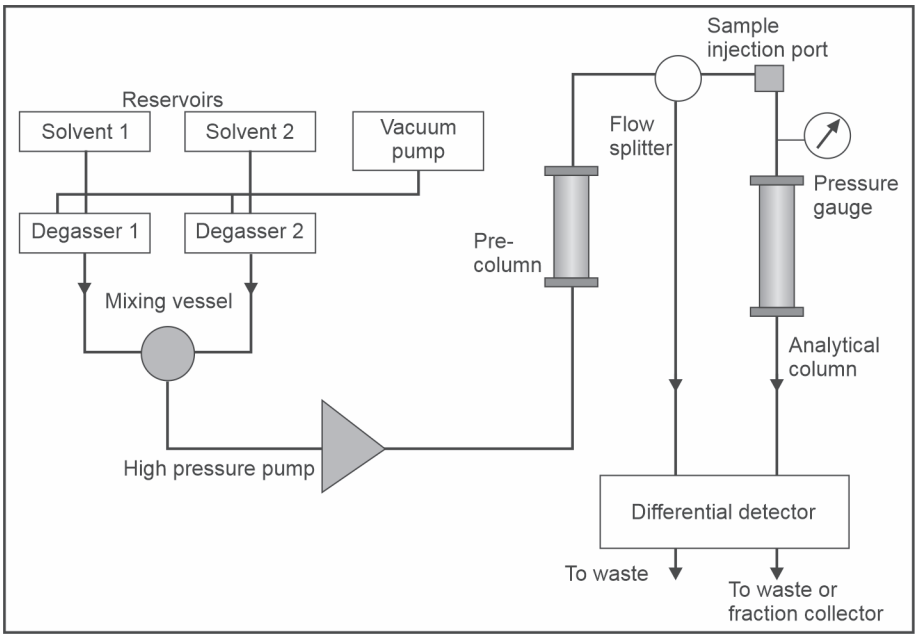


Fig. 1.18: Schematic diagram of binary HPLC system

Mobile phase reservoir: The reservoir that holds the mobile phase is often no more than a glass bottle. Often, the reagent bottle that holds our HPLC solvent can be used as a reservoir. Solvent is delivered from the reservoir to the pump by means of Teflon tubing—called the "inlet line" to the pump. Each HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contains 200 to 1000 ml of a solvent. These solvents are used as the mobile phases and carry the sample through the HPLC system. They must be degassed and filtered to remove the dissolved gases and particulate matter (Figs 1.19 and 1.20).

Requirements for a Solvent Reservoir are Simple

The reservoir and its attachment to the pump should be made of materials that will not contaminate the mobile phase: Teflon, glass, or stainless steel.

The vessel should have some sort of cap to prevent particulate matter from contaminating the mobile phase. If you are using a solvent bottle as a reservoir, the top of the bottle can be wrapped in aluminum foil to keep dust out or the bottle cap can be drilled to allow inserting the inlet line through the cap.

Do not close the bottle too tightly or removal of mobile phase by the pump will create a vacuum. This prevents mobile phase from flowing the pump, creating a "vapor lock" within the pump.

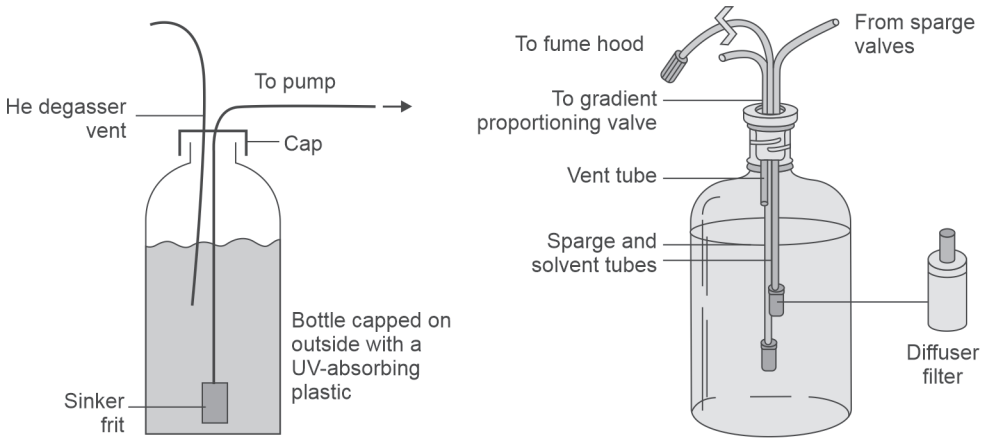


Fig. 1.19: Instead of He degassing, vacuum degassing method can also be used

Fig. 1.20: Vacuum degassing unit

SOLVENTS (Table 1.2 and Fig. 1.21)

Polar Solvents

Water > Methanol > Acetonitrile > Ethanol > Oxydipropionitrile

Nonpolar Solvents

N-Decane > N-Hexane > N-Pentane > Cyclohexane

Polarity of common organic functional groups and solvents

Functional groups		*Organic solvents*
	Nonpolar	
• Aliphatic hydrocarbons	↓	• Hexane
• Olfins		• Carbon tetrachloride
• Aromatic hydrocarbons		• Ether
• Halids		• Benzene
• Sulfides		• Methylene chloride
• Ethers		• THF
• Nitro compounds		• Isopropanol
• Esters, aldehydes, ketones		• Chloroform
• Alcohols, amine		• Ethyl acetate
• Sulfones		• Acetonitrile
• Sulfoxides		• Methanol
• Amides		• Water
• Carboxylic acids	**Polar**	

Table 1.2: HPLC solvents

Solvent	*Formula*	*UV cutoff (nm)*
Acetonitrile	CH_3CN	190
Chloroform	$CHCl_3$	245
Dichloromethane	CH_2Cl_2	235
Ethanol	CH_3CH_2OH	210
Ethyl acetate	$CH_3CO_2CH_2CH_3$	260
Diethyl ether	$(CH_3CH_2)_2O$	220
Heptane	$CH_3(CH_2)_5CH_3$	200
Hexane	$CH_3(CH_2)_4CH_3$	200
Methanol	CH_3OH	205
n-Propanol	$CH_3CH_2CH_2OH$	210
Isopropanol	$CH_3CH(OH)CH_3$	210
Tetrahydrofuran	C_4H_8O	215
Toluene	$C_6H_5(CH_3)$	285
Water	H_2O	None

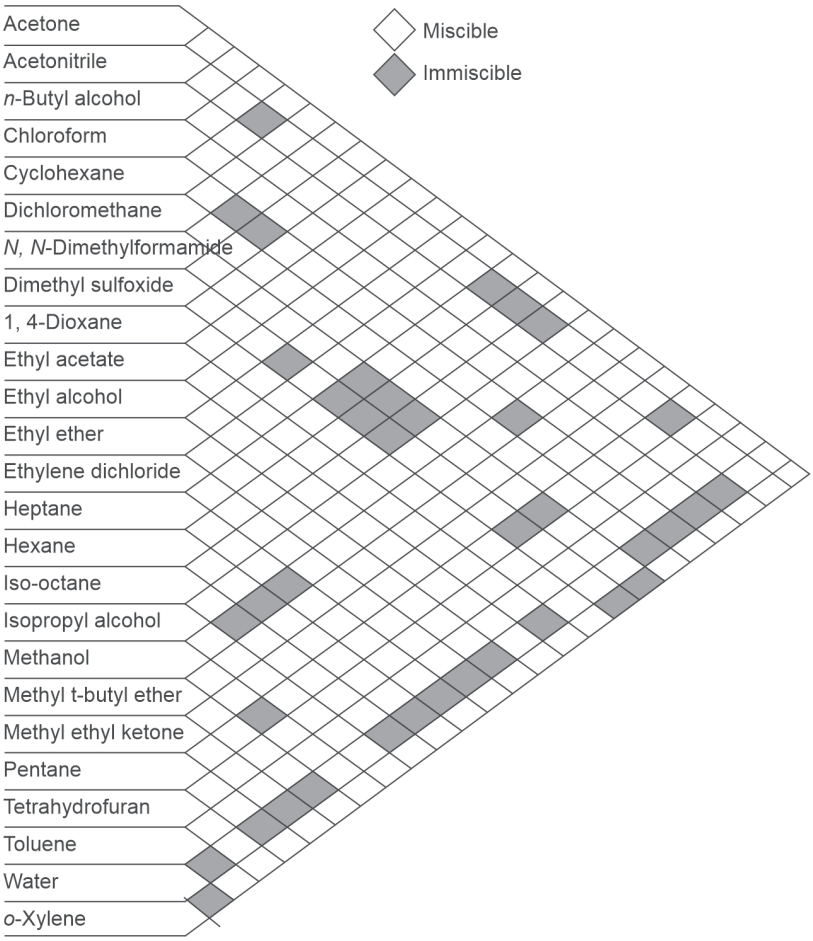


Fig. 1.21: Miscibility of solvents

II. PUMPS

Pumps deliver the solvent (mobile phase) from the solvent reservoir to the injector. The HPLC pump is considered to be one of the most important components in a liquid chromatography system, which has to provide a continuous constant flow of the eluent through the HPLC injector, column, and detector. High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations. The most important advantages are: Higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings that require less pressure. Thus, if the user has only moderate needs and a restricted budget, his money need not be spent on a maximum pressure pump. Flow rate stability is another important pump feature that distinguishes pumps. Very stable flow rates are usually not essential for analytical chromatography.

An additional pump feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state-of-the-art technology as electronic feedback and multiheaded configurations. Standard HPLC pumps have the following parameters:

- *Flow rate range*: 0.01 to 10 ml/min
- *Pressure range*: From 1 to 5,000 psi
- *Flow rate stability*: Not more than 1% (short-term)
- For SEC flow rate stability should be less than 0.2%
- It is desirable to have an integrated degassing system, either helium purging, or better vacuum degassing.

IDEAL CHARACTERISTICS OF A PUMP

- Noncorrosive and compatible with solvent.
- Provide high pressure to push mobile phase
- Stable flow rate and pressure
- Provide constant flow rate to mobile phase.
- Easy to change for one mobile phase to another.
- Should have reproducible flow rate and independent of column back pressure.
- Should not leak.
- High pressure generated by pump should not lead to an explosion.
- Easy to use, variable solvents usage available
- It should be easy to dismantle and repair
- Pulse elimination system
- In use of both isocratic and gradient mode
- Endurance.

Pump Operation Mode

Isocratic mode: Steady solvent composition during analysis
Gradient mode: Change in the solvent composition during analysis:

Single pump: Low pressure
Multiple pumps: High pressure

Solvent delivery system (Fig. 1.22): The pumps are used to pass mobile, through the column at controlled flow rate. The solvents or mobile phases are used must be passed through the column at a high pressure at about 1000 to 3000 psi. This is because as the particle size of stationary phase is few µm (5–10 µm), the resistance to flow of solvent is high. Hence, such high pressure is recommended.

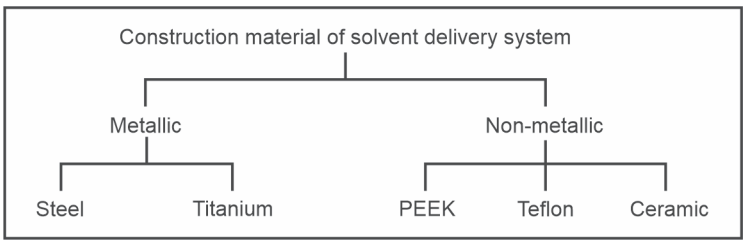


Fig. 1.22: Construction materials of solvent delivery system

TYPE OF PUMP USED IN HPLC

There are different types of pumps available that can provide the necessary pressures and flow rates required by the modern liquid chromatography. They are:

1. Displacement pump/syringe pump
2. Reciprocating piston pump
3. Pneumatic pump

1. Displacement Pump/Syringe Pump (Fig. 1.23)

It consists of a large syringe like chamber equipped with a plunger that is activated by a screw driven mechanism powered by stepping motor.

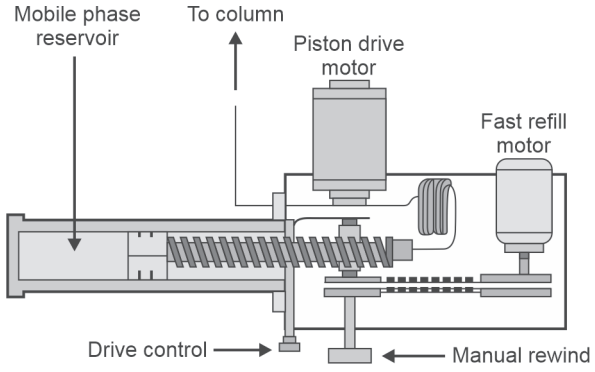


Fig. 1.23: Displacement pump

Working

- Work on the principle of positive solvent pressure.
- Consist of screw or plunger which revolves continuously driven by motor.
- Rotatory motion provides continuous movement of the mobile phase which is propelled by the revolving screw at greater speed and pushes solvent through small needle-like outlet.
- Consist of large syringe like chamber of capacity 250–500 ml.

Advantages

- Flow is pulse free.
- Provide high pressure up to 200–475 atm.
- Independent of column back pressure and viscosity of solvent.
- Simple operation.

Disadvantages

- Limited solvent capacity
- Gradient elution is not easy.

2. Reciprocating Piston Pump (Fig. 1.24)

It consists of a small chamber in which the solvent is pushed back and forth with the help of a motor driven piston or pressure may be transmitted by a diaphragm which is hydraulically, pumped by a reciprocating piston. The piston expels liquid through a one-way valve (check valve). The pumping rate is usually adjusted by controlling the distance the piston retracts, thus limiting the amount of liquid pushed out by each stroke, or by the cam rotating speed. The basic principle of the reciprocating single piston pump is shown below.

Working

- Contains reciprocating piston that moves back and forth in hydraulic chamber.
- By the movement of piston solvent flow into the column under high pressure.
  When piston moves backward inlet valve open while exit valve closes. This result in mobile phase being drawn into the main chamber.

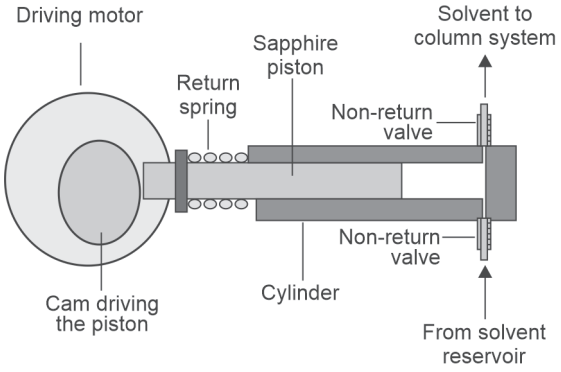


Fig. 1.24: Reciprocating piston pump

- When the piston moves to the front the inlet valve closes and the exit valve opens.
- The reduction in volume in main chamber due to forward motion of piston results in mobile phase moving out of the exit valve under high pressure.

Advantages

- Generate high output pressure (up to 10000 poise).
- Ready adaptability to gradient elusion.
- Provide constant flow rate.
- Pressure generated is so high that any back pressure generated in the column due to higher viscosity of stationary phase can be easily overcome.

Disadvantages

Pulsed flow which must be damped as they produce a baseline noise on the chromatogram.

3. Pneumatic Pump

It operates with constant pressure and use highly compressed gas. The solvents used must be high purity, preferably HPLC grade and filtered through 0.45 µm filters.

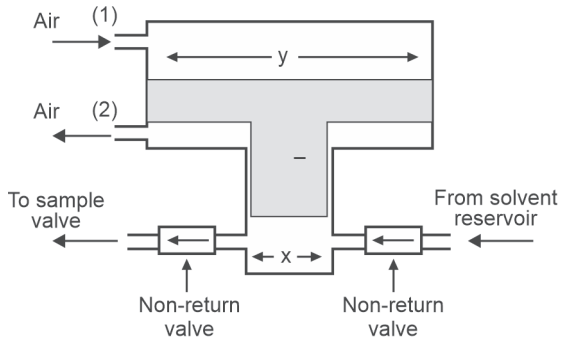


Fig. 1.25: Pneumatic pump

Working

- Pressure from a gas cylinder delivered through a large piston drivers the mobile phase.
- Pressure on the solvent is proportional to the ratio of piston usually 50:1.
- The driving air is applied, piston moves, inlet closes and outlet open pushing mobile phase to the column.
- A lower pressure gas source of 1–10 atm can be used to generate high liquid pressure (1–400 atm).
- About 70 ml of the mobile phase is pumped from every stroke.

Advantages

- Pulse free flow.
- Generates high pressure.

Disadvantages

- Limited volume capacity (70 ml)
- Pressure output and flow rate depend on the viscosity and column back pressure.
- Gradient elusion is not possible.

Mechanical Pump

It operates with constant flow rate and uses a sapphire piston. This type of pump is used in analytical scale.

Pump Operation Guide *(Fig. 1.26)*

- Degassing of solvent
- Do not use when solvent is almost on the bottom
- Rinse with water after using buffer
- Check a miscibility
- Pump priming before use
- Pulse control
- Check the pump pressure

Check valves: These are present to control the flow rate of solvent and back pressure.

Pulse damper: These are used to dampen the pulses observed from the wavy baseline caused by the pump. Important damping methods include:

- ***Triple headed pump*:** Two heads in different stages of filling as the third is pumping.
- ***Tube with an air space or a flexible bellows or tube*:** Where a gas (air space) or a flexible metal vessel takes up some of the solution energy. When pump refills, this energy is released and a smooth pressure pulsation result.
- ***Restrictor*:** In this method, a 25 cm length of 4 mm bore, stainless steel tubing, packed with 20 µm glass beds, is placed between the pump and the column.

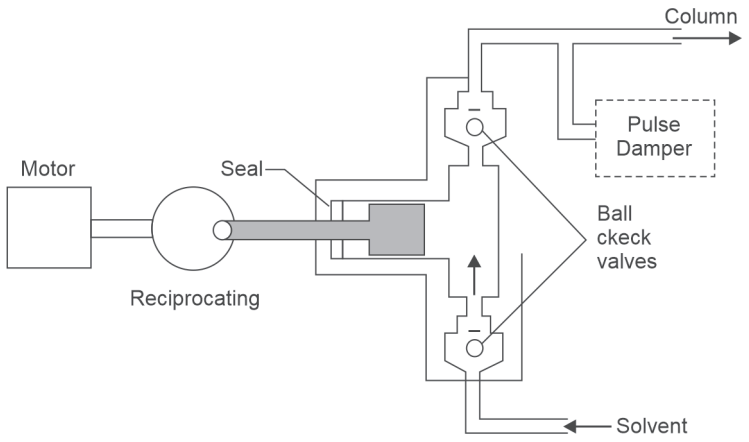


Fig. 1.26: Pump operation

III. MIXING UNIT, GRADIENT CONTROLLER AND SOLVENT DEGASSING

a. Mixing Unit

It is used to mix solvents in different proportions and passed through the column. There are two types of mixing units.

i. Low pressure mixing chamber which uses helium for degassing solvents

ii. High pressure mixing chamber does not require helium 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.

b. Gradient Controller

In an isocratic separation, mobile phase is prepared by using pure solvent or mixture of solvents that is 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 controlled is used when two or more solvent pumps are used for such separation.

c. Solvent Degassing

Several gases are soluble in organic solvent. When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process. These bubbles cause band spreading and also interfere with the performance of the detector. Modern HPLC systems are equipped with on-line degassing units to remove dissolved gases from the mobile phase. These are essentially to reduce the baseline noise and thereby improve the chromatographic performance. The operation of the in-line degasser is based on the selective permeability of gases by polymeric tubing. The polymeric tubing is made up of poly tetrafluoroethylene (Teflon) or polymer V. As shown in Fig. 1.27 solvents are passed through the tubing kept inside the vacuum chamber; the vacuum pulls the dissolved gases from the polymeric tubing and the mobile phase free from dissolved gases enter into the pump. Hence, degassers are to eliminate the air which may otherwise cause variations in analysis. Hence, degassing of solvent is important. By using one of the following techniques degassing can be done:

i. **Vacuum filtration:** It can remove the air bubbles, but it is not always reliable and complete.

ii. **Helium purging:** By passing helium through the solvent, this is very effective but helium is expensive.

iii. **Ultrasonication:** By using ultrasonicator, this converts ultra high frequency to mechanical vibrations. This causes the removal of air bubbles.

Priming the degasser

- Use a syringe when vacuum tubes are empty or for fast solvent change
- Use the pump at 3–5 ml/min when system has been turned off for a period of time (overnight)

Changing solvents

- Make certain that solvents are miscible
- Exchange any inorganic buffer first with pure water and then with the new solvent

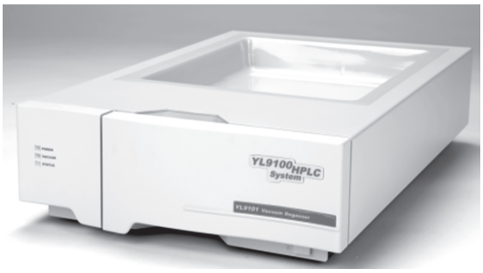
- Use a minimum of 30 ml per channel for solvent changes
- Check each solvent outlet tube for gas bubbles

Maintenance

- Clean the degasser lines by flushing with isopropanol.
- When using buffers, flush with water, then with isopropanol.
- Check for air bubbles in outlet lines.
- Be aware of the possibility of microbial growth in aqueous phases.
- Unused channels should be left in isopropanol.
- May have to exchange the vacuum pump, sensor, solenoid valve, or vacuum chamber.
- No fluctuation, shorten warming up

Specifications

- *Number of channels*: 4 CHs
- *Maximum degassing range*: 10 ml/min



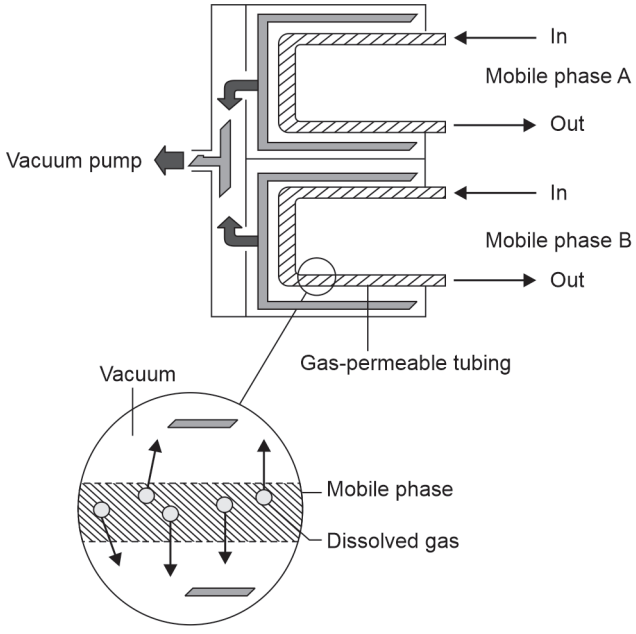


Fig. 1.27: Solvent degassing

- *High capacity of chamber*: 925 µl per channel
- *Excellent compatibility with toxic chemicals*: Degassing membrane with Teflon AF
- *Robust vacuum pump*: 220 million cycles
- *Safety and maintenance*: Vacuum error detection
- *Wide range of line voltage*: 100–240 VAC, ±10%, wide range
- *Line frequency*: 50/60 Hz, ±5%
- *Power consumption*: 20 W

Comparison of Isocratic and Gradient Analysis

Most of the HPLC separations are performed under isocratic conditions in which the same mobile phase is used throughout the elution of the entire sample. Therefore, isocratic analysis is suitable for simple mixtures. While in gradient analysis the strength of the mobile phase is increased with time during sample elution, is preferred for more complex samples containing analytes of diverse polarities.

Advantages of gradient analysis

- It is better suited for complex samples that require quantitation of all peaks or multiple analytes of diverse polarities
- It has better resolution of early and late eluting peaks
- It has better sensitivity of late eluting peaks
- It has higher peak capacity

Disadvantages of gradient analysis

- In gradient analysis binary pump is required
- Method development, implementation, and transfer are more difficult in gradient analysis.

There are several additional parameters in gradient analysis those are not present in isocratic HPLC that need to be optimized. These are initial and final mobile phase composition, gradient time or duration (tG), flow (F), and sometimes gradient curvature (linear, concave, and convex).

IV. INJECTOR (Manual or Auto-injectors)

Sample introduction can be accomplished in various ways. Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. In modern HPLC systems, the sample injection is typically automated (Fig. 1.28).

It must be noted that overloading of sample causes band broadening. Therefore, minimum amount of sample must be introduced. It is convenient to introduce the sample, without depressurizing the system. Sample is injected at the head of the column with minimum disturbance of the column material.

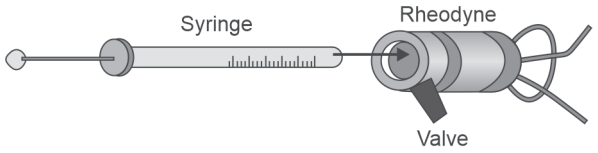


Fig. 1.28: Schematic of injector

a. Septum syringe injectors: Syringe is used to inject the sample through a self-sealing inert septum directly into the mobile phase through a rubber septum. This is not common, since the septum has to withstand high pressure up to 1500 psi. The main drawback of septum injection port is the leaching effect of the mobile phase with the septum resulting in the formation of ghost peaks.

Stop flow (online) in which the flow of mobile phase is stopped for a while and the sample is injected through a valve device using a syringe. The main drawback of this technique is formation of ghost peak.

b. Rheodyne injector (loop valve type): It is the most popular injector. It is a sophisticated modern method with good precision. Sample is introduced in the column without causing interruption to mobile phase flow. It has a fixed volume loop like 20 µl or 50 µl or more. Injector has two modes that is load position when the sample is loaded in the loop and inject mode when the sample is injected. Introduce small sample (0.1–100 µl) without depressurization, micro-syringe/septum system (only <1500 psi).

Sample injection system (operation of sample loop): Sample is loaded at atmospheric pressure into an external loop in the micro-volume sampling valve, and subsequently injected into the mobile phase by suitable rotation of the valve (Fig. 1.29).

Loop type injector → change a loop when injection volume changed and sample loop cleaning is required.

Caution for Injector

- Use of rinsed syringe, remove bubbles
- *Syringe cleaning*: Use of solvent dissolving the sample
- *Additional cleaning*: Solvent in use → acetone → dichloromethane
- Care with solvent for auto-injector needle wash
- Connect tubing as short as possible (injector and column, column and detector)
- Wrong connection of tubing makes dead volume

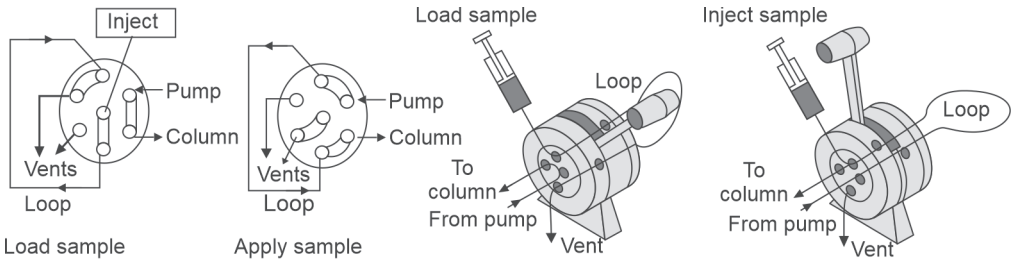


Fig. 1.29: Micro-volume sampling valve operation of a sampling loop

Automatic Injectors

With commercially available automatic sampling devices, large numbers of samples can be routinely analyzed by LC without operator intervention. Such equipment is popular for the analysis of routine samples (e.g. quality control of drugs), particularly when coupled with automatic data-handling systems. Automatic injectors are indispensable in unattended searching (e.g. overnight) for chromatographic parameters such as solvent selectivity, flow rate, and temperature optimization.

Most of the autosamplers have a piston metering syringe type pump to suck the pre-established sample volume into a line and then transfer it to the relatively large loop (~100 ml) in a standard six-port valve. The simplest autosamplers utilize the special vials with pressurization caps. A special plunger with a needle, push the cap down to the vial and displace the sample through the needle into the valve loop. Most of the autosamplers are microprocessor controlled and can serve as a master controller for the whole instrument.

SAMPLE INJECTION LOOP

The injection loop is a critical component of an HPLC and is potentially one of the largest sources of excess HPLC system volume. The sample is injected into the loop while the loop is switched out of the HPLC flow path. After the loop is filled with sample it is switched back into the flow path and the sample is swept onto the head of the HPLC column for later elution or the sample is injected directly into a mass spectrometer as part of a flow injection analysis.

Injection Loop Dimensions

Loop dimensions are important: The most accurate injections are made with a technique known as overfilling. When using the overfill technique a sample injection will be made that is 3 to 5 times larger than the volume of the injection loop. This is done because the core of an injection plug travels faster than the outer perimeter of the injection and if one tries to make, for example, a 100 µl injection onto a 100 µl loop it is likely that the core of that sample injection will overshoot the end of the loop and go to waste. Overfill ensures that the loop is completely filled and will inject a precise amount, the exact volume of the loop. Overfilling is fine if the sample amount is unlimited. However, if you are performing a characterization of a rare species the thought of sending any of that sample to waste is very very scary. Often when the sample is limited an under fill of the sample loop is performed.

Optimizing the loop volume for the injection is important. The loop can be a significant source of HPLC system dead volume. There is no reason to have a 1 ml loop when the injection volume is 10 µl. A large loop volume will delay the peak and possibly adversely affect your chromatography. For a proper under fill, 10 µl of a precious sample can be safely made onto a 25 µl loop. In this latter example the tailored loop volume helps to cut down on surface area, where a dilute sample may adsorb, and also helps to improve the chromatography. In addition, if the HPLC flow rate is low, 25 to 10 µl/min, it is generally better to keep the internal diameter of the injection loop low, preferably below 125 µm to aid in efficient evacuation of the loop.

MAKING SAMPLE INJECTION LOOP

An HPLC loop of any dimension is easy to make. The loop can be constructed of HPLC steel tubing, fused silica, or (our choice) PEEK™ tubing. We use PEEK™ tubing because it is easy to cut to size and flexible and it is easily fitted with "finger tight" nuts and ferrules. PEEK™ tubing is also resistant to most organic solvents and can withstand pressures to 5000 psi. Use the script (form) at the top of this page to calculate the volume for a specific length of tubing in the preparation of an injection loop.

When making partial fill loops where sample conservation is key, we recommend a loop that is 2.5 times the injection volume. To make a 10 µl injection one should make a 25 µl injection loop.

V. COLUMNS

Column is referred to as heart of HPLC separation process (Fig. 1.30). Stable high performance column is essential requisite for rugged and reproducible method. For high efficiency of separation large number of theoretical plates is necessary per unit length of the column. Column separates mixed compounds into individual components. Columns are made up of stainless steel or heavy glass to withstand the pressure. The columns are usually long (10–30 cm) narrow tubes. Columns contain stationary phase at particle diameters of 25 µm or less. The interior of column should be smooth and uniform. Column end fittings are designed to have a zero-void volume. The internal diameter of the columns is usually 4 or 4.6 mm; this is considered the best compromise among sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), larger diameter columns may be needed. Packing of the column tubing with the small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment (Tables 1.3 and 1.4).

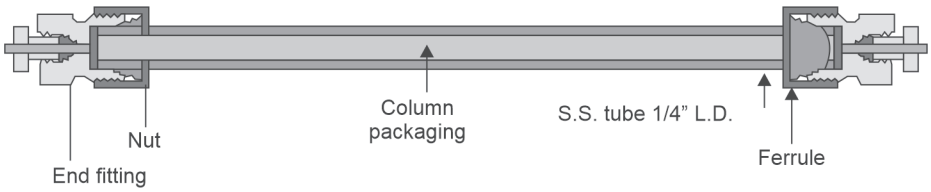


Fig. 1.30: HPLC column

In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new, and to retain the chromatogram. If questionable results are obtained later the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

Table 1.3: Column dimensions

Type	*Inner diameter (cm)*	*Length (cm)*	*Particle size (µm)*
Analytical	0.3–0.46	3–25	3–10
Semi-micro	0.1–0.21	10–25	3–8
Semipreparative	0.8–1.0	10–25	5–10
Preparative	2.0–5.0	10–25	10–20

Table 1.4: Packing material

	Silica	*Alumina*	*Polymer*		*Carbon*
			Styrene-divinylbenzene	*Methacrylate*	
Organic solvent	+++	+++	++	++	+++
pH range	+	++	+++	++	+++
Swelling/shrinking	+++	+++	+	+	+++
Pressure capability	+++	+++	++	+	++
Surface chemistries	+++	+	++	+++	o
Efficiency	+++	++	+	+	+++

Particle Size

The resolution and back pressure will be higher if particle size is smaller.

Size	*Use*
5 µm	High resolution, optimum pressure, analytical use in general
3 µm	Short analysis time
10 µm	QA, preparative
>10 µm	Preparative

Column Selection

The column is selected on various column efficiency parameters said above. The column having high column efficiency is required. Primarily the column may be selected based on 3 criteria necessary:

1. The attainable resolution.
2. The speed of analysis.
3. The load capacity of the column.

If rapid analysis and good resolution is desired, it is advisable to use a column packed with PLB (porous layer beads)

1. High resolution can be achieved most readily by using the minimum sample size and a long analysis time. Sufficient pressure should be available to enhance the resolution by lengthening the column if necessary (which would extend the analysis time). To obtain a sufficient number of plates, the stationary phase should have the smallest particle size possible.

2. If high-speed operation is desired, the shortest possible column should be employed. Optimization here process at the expense of resolution. The optimum speed of analysis is obtained at 'K' values of about 2 (K is capacity factor and generally a value of 2–20 for K is acceptable). In HPLC, it is possible to carry out analysis in second. However, where this may be appropriate remains an open question.
3. To achieve adequate load capacity, the column cross section must be enlarged to accommodate sufficient stationary phase The analysis time is always longer than for comparable analytical separations because long columns are unavoidable.

Optimization can be accomplished only along any one straight line at a time and in one direction. A system that combines high resolution with high load capacity and rapid analysis has not yet been described.

Other factors are: "If the operating pressure of a column is limited simple separations will be achieved very rapidly on short columns packed with very small particles. In contrast difficult separations will take longer and will require long columns packed with larger particles".

Particle diameter	*Column length (cm)*	*Analysis time*	*Peak capacity*
10.5	148	3.6 hrs	115
7.7	58.4	58.4 min	84
4.6	12.6	8.2 min	51
<4.6	3.1	74.7 sec	32

Column Diameter

Short columns packed with small particles should have relatively large diameters, long, columns packed with larger particles will have small diameter.

Column	*N (Plates)*	*L (Meters)*
100 irregular silica (dry packed)	750	20
50 irregular silica (dry packed)	900	8
Porosil A (35–75)	1000	3.5
20 irregular silica (pressure slurry packed)	1200	1
50 Corosil-11 (dry packed)	1200	4

- Silica of small particles diameter is not commercially available and pressure slurry packing is good but costly pieces of equipment are required. Dry packing is easy.
  - Corosil-11 (pellicular adsorbent) is expensive.
  - A column of alumina provides lower efficiency compared to silica.
- Water content of adsorbent plays an important role in packing of adsorbents. Some water must be added to the adsorbent to increase linear capacity to a usable value and to maximize separation efficiency. Added water also decreases the build up of static charges during dry packing, there by favoring denser, more efficient columns.

In case of porosil—an optimum water content of 8–15 gm of water/100 gm of adsorbent is added.

CLASSIFICATION OF COLUMN (BASED ON APPLICATIONS)

1. **Analytical LC columns:** These are employed to obtained qualitative or quantitative information about the sample. This is a sensitive method. It includes the conventional LC where separated solutes are not isolated or very less amount of the solute is use, i.e. up to 100 gm.

   Columns: They are packed with the stationary phase with the particle size of 3–20 µm, has small volume of sample injection, i.e. <10 ml usually 50–150 ml. Due to small particle size, the packing is expensive. These have very good column efficiency and internal diameter <8 mm. The internal diameter is 4–5 mm and length is 10–30 cm. The column load should be as light as possible.
2. **Preparative LC:** This is a scale up of an analytical separation, i.e. to isolate, enrich or purify the sample quantities in the range of 10–1000 mg from complex mixtures. This is of three types:
   A. *Micros preparative (semipreparative)*: To purify the sample of about <100 mg. The columns tend to be slightly larger versions of analytical columns, using the same packing.
   B. *Preparative*: Sample size is 0.1–1000 gm. The columns are 2–5 cm in diameter and 25 cm (length), packing of 15–1000 µm (diameter).
   C. *Macropreparative (large scale preparative separations)*: The sample size is <0.1 kg. The columns are 20–30 cm in diameter and 60 cm long, flow rates up to 10000 ml/min. Due to high output, simple pumps are used, low pressure drop required. All the preparative columns have internal diameter of <8 mm (Fig. 1.31).

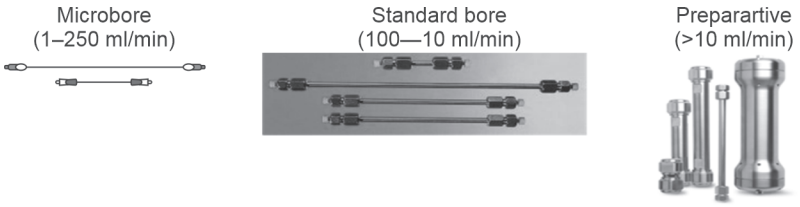


Fig. 1.31: Different columns

Microbore columns are used with diameter up to 2 mm (inner diameter). Narrow column diameter and small size of the packing material require low flow rate. Reliable separation (<10 L/min) is difficult unless syringe pumps are used (Table 1.5).

Standard bore is most commonly used for pumping system for both analytical and semipreparative work (2–12 mm inner diameter) columns.

Preparative column: High flow rate requires special design pump. Upper range for flow rate is usually around 50 ml/min.

Guard Columns (Pre-columns)

Guard columns are placed anterior to the separating column and serve as a protective factor that prolongs the life and usefulness of the column. They are dependable column designed to filter or remove particles that clog the separation column. Guard column has very small quantity of adsorbent and improves the life of the analytical column. It also acts as a pre-filter to remove particulate matter if any, and other material and thus prevents contamination of the analytical column. Guard column has the same material

Table 1.5: A comparison of analytical and preparative LC

Analytical LC	*Basic of comparison*	*Preparative LC*
To obtain qualitative or quantitative information about sample	Purpose	To isolate, enrich or purify sample components
Maximum peak capacity # of components separated or measured/unit time)	Practical objective	Maximum throughput (amount of material purified/unit time)
Up to 10 mg	Typical sample size	Micro-preparative: <100 mg Preparative: 0.1–100 gm Macropreparative: >0.1 kg.
As light as possible typical range 10–10 to 10–3 gm of sample/gm of packing	Column load	As heavy as possible; typical sample/gm of packing
Matched to sophistication of detection technique. Base line separation is typical	Degree of separation	Matched to requirements for level of purity and recovery often, moderate resolution is sufficient
Small volume (<5–15 ml). Small particle (3–20), expensive packing. Excess separation power relative to need. International diameter <8 mm	Typical column characteristics	Volume scaled to sample size, mill-liter to multiliter particles (20–100) economic packing. Separation power matched to requirements. Internal diameter <8 mm
Required, often with high sensitivity and wide linear dynamic range	Detector	Desirable to assay fraction on line or off line. Extra range at low sensitivity
Usually sample is discarded along with mobile phase	Disposition of sample	Sample fractions are collected; mobile phase may be recycled

that of analytical column. Guard column does not contribute to any separation. Guard column serves to saturate the mobile phase with the stationary phase, so that losses of this solvent from the analytical column can be minimized.

Bonded Phase Column

Here the molecules, comprising the stationary phase, i.e. the surface of the silica particles, are covalently bonded to a silica-based support particle. The most popular bonded phase, siloxanes are formed by heating the silica particles in dilute acid for the day so as to generate the reactive Silonal group. These bonded phases are stable between the pH range 2–9 and up to temperature of 80°C. Bonded phase is made with a linear C_{18} hydrocarbon, also known as ODS (octadecyl silane) bonded phase. Used in pharmaceutical analysis or separation of less polar components.

Advantages

- Can withstand high pressure exerted by mobile phase.
- Life of column is more.
- No bleeding effects

Disadvantages

- Very expensive
- Manually cannot be fabricated

Reversed-phase column (Table 1.6)

- u-Bondapak C_{18}, CN, phenyl
- Resolve C_8, C_{18}, CN, phenyl
- Lichrosorb RP_{18}, RP_8
- Delta-Pak C_{18}, C_8
- Symmetry C_{18}, C_8
- Symmetry shield RP_8
- Hypersil ODS, phenyl CPS
- Zorbax ODS, C_8, CN, phenyl N, phenyl

C_{18} column
↓
Silica (SiO_2)
↓
Acid treatment
↓
Free silanol (Si-OH)
↓
Chemical bonding of C_{18}
↓
End-capping by TMS
↓
Element analysis

Table 1.6: Normal phase *vs* reverse phase

	Normal phase	*Reverse phase*
Packing polarity	Polar	Nonpolar
Solvent polarity	Nonpolar~medium	Medium~polar
Sample elution order	Nonpolar first	Polar first
Effect of increasing solvent polarity	Increased RT of polar components	Increased RT of nonpolar components

Analytical Columns

Analytical column is the most important part of HPLC technique is made up of polyethylene and PEEK (polyether ether ketone), most widely used are stainless steel, which can withstand high pressure. Latest ones are PEEK column. It is used for analytical separation, i.e. to isolate or purify sample in the range of 10–100 mg form complex mixture (Tables 1.7 and 1.8).

Column length	Varies from 25 to 100 cm
Column diameter	Ranges from 2 to 50 mm
Particle size	From 1 to 20 µm
Particle nature	Spherical uniform sized; porous materials are used.
Surface area	1 gm of stationary phase provides surface area ranging from 100 to 800 sqm with an average of 400 sqm

Table 1.7: Restoration of HPLC columns performance

Silica column flush with the following	*Silica bases reserved phase column flush with the following*
50 ml of hexane	50 ml of hot (40–60°C) distilled water
50 ml of methylene chloride	50 ml of methanol
50 ml of 2-propanol	50 ml of acetonitrile
50 ml of methanol	50 ml of tetrahydrofuran
25 ml of methylene chloride	25 ml of methanol
25 ml of mobile phase	25 ml of mobile phase

Table 1.8: Useful separations guide

S. No.	*Application*	*Column*	*Detector*	*Conditions*
1.	Water-soluble vitamins	C_{18}	UV (254 nm)	8% acetonitrile/water
2.	Fat-soluble vitamins	C_{18}	UV (280 nm)	80% acetonitrile/water
3.	Steroids	C_{18}	UV (230 nm)	60% methanol/water
4.	Triglycerides	C_8	UV (220 nm)	60% acetonitrile/water
5.	Monosaccharides	CX-Ca	UV (195 nm), RI, CAD	Water (80°C)
6.	Polysaccharides	TSKpw	UV (254,280 nm)	Water (<20% acetonitrile)
7.	Amino acids	C_{18}	UV (254 nm)	10% THF/acetonitrile
8.	Anticonvulsants	C_{18}	UV (220 nm)	40% methanol/water
9.	Tricyclic antidepressants	C_{18}	UV (254 nm)	55% acetonitrile/water
10.	Aspirin	C_{18}	UV (254 nm)	10% acetonitrile/water, acetic acid, pH 2.5

Recently, manufacturers have been providing high speed; high performance micro-columns have small dimensions of size:

Length 3–7.5 cm
Internal diameter 1–4.6 mm
Particle size 3–5 µm

Preparative columns are of three types (Figs 1.32 and 133)

1. Micropreparative or semipreparative column:
   - Modified version of analytical column
   - Uses same packaging and meant for purifying sample less than 100 mg.
2. Preparative column:
   - Inner diameter: 25 mm
   - Stationary phase diameter: 15–100 µm
3. Macropreparative column:
   - Column length: 20–30 cm
   - Inner diameter: 600 mm

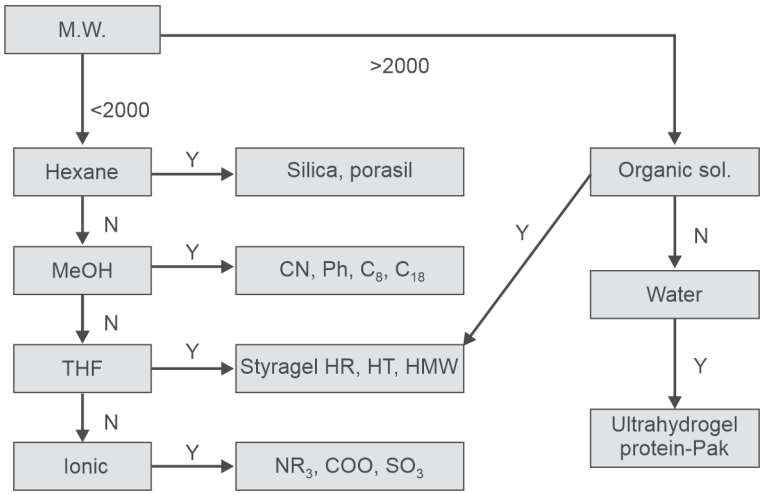


Fig. 1.32: Column selection guide

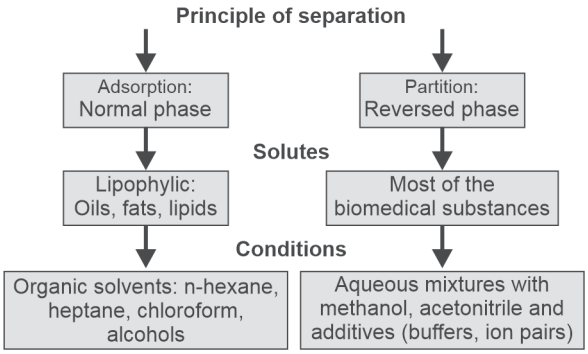


Fig. 1.33: Packed column

GENERAL FACTORS INCREASING RESOLUTION (Table 1.9)

1. Increase column length
2. Decrease column diameter
3. Decrease flow rate
4. Pack column uniformly
5. Uniform stationary phase (packing material)
6. Decrease sample size
7. Proper stationary phase
8. Proper mobile phase
9. Proper pressure
10. Gradient elution

Table 1.9: Some commonly used HPLC stationary phases

Stationary phase	*Applications*
ODS silica gel	The most commonly used phase, ODS silica gel can even be applied to the analysis of peptides, where wide-pore packings are used to improve access of these bulky molecules to the internal surface of the packings.
Octyl silane and butyl silane silica gels	Useful alternatives to ODS phases.
Phenyl silane silica gel	Useful for slightly more selective analyses of compounds containing large numbers of aromatic rings, e.g. propranolol and naproxen, where some additional interactions can occur with the phenyl groups on the stationary phase.
Silica gel	Used in the separation of different classes of lipids and in the analysis of surfactants, which tend to form micelles under the conditions used for reverse phase chromatography
Aminopropyl silica gel	A moderately polar phase often used for the analysis of sugars and surfactants
Cyanopropyl silica gel	A moderately polar phase applicable to the analysis of surfactants
Strong cation exchanger (SCX)	Usually based on ion pairing of the analyte with sulfonic acid groups on the surface of the stationary phase. Useful for analysis of very polar compounds such as aminoglycosides and other charged sugar molecules and polar bases such as catecholamines
Strong anion exchanger (SAX)	Useful for the separation of polar compounds with anionic groups such as nucleotides and anionic drug metabolites such as sulphates or glucuronides

VI. DETECTORS

A chromatographic detector is a device which measures the number of separated components in the column effluent. The output of the detector is an electrical signal that is proportional to some property of mobile phase or the solute. Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~10 ml) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission, or change in refractive index (depending on the type of detector used) from the sample components passing through the cell are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into an integrator or computer to provide retention time and peak area data.

The most commonly used detector in LC is the ultraviolet absorption detector. A variable wavelength detector of this type, capable of monitoring from 190 to 460–600 nm, will be found suitable for the detection of the majority samples. Other detectors in common use include: Refractive index (RI), fluorescence (FL), electrochemical (EC) and mass spectrometric (MS). The RI detector is universal but also the less sensitive one. FL and EC detectors are quite sensitive (up to 10–15 mole) but also quite selective. The MS detector is the most powerful one but it still the most complicated and most expensive.

The ideal detector should have the following

1. It should have high sensitivity and fast response, i.e. larger detector signal for small amount of solute. High signal to noise ratio (S/N ratio) and low drift and noise level and a wide linear response to solute present
2. It should have good stability and reproducibility
3. It should respond to all components of the mixture in a wide range of mobile phases
4. It should not respond to mobile phase
5. It should not constitute to zone spreading
6. It should have stable flow rate, temperature and pressure
7. It should have a temperature range from room temperature to perhaps 400°C
8. High reliability and ease of use
9. Nondestructive of sample cheap
10. Operational simplicity and reliability.

Optical detector

1. UV/visible detector
2. Fluorescence detector
3. Refractive index detector
4. Evaporative light scattering detector (ELSD detector).

Electrochemical detector

1. Conductivity detector
2. Electrochemical detector

A number of detectors used in LC are also suitable in case of HPLC and in fact based on the applications; the detectors can be classified into two types:

a. Bulk property detectors, which compare an overall change in a physical property of the mobile phase with and without an eluting solute. These types of detectors tend to be relatively insensitive and require temperature control, e.g. refractive index detector and conductivity detectors.
b. Solute property detectors, which response to a physical property of the solute which is not exhibited by the pure mobile phase. Such types of detectors are about 1000 times more sensitive, giving a detectable signal for a few nanograms of sample, e.g. UV/visible detector, electrochemical detector, fluorescence detector.

DETECTORS USED IN HPLC

A brief account of the principal types of detectors used in HPLC is given below.

1. DIFFERENTIAL REFRACTROMETER (REFRACTIVE INDEX DETECTORS)

- The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow cell.
- It responds to any solute whose refractive index is significantly different from that of the mobile phase.

- It is based on two principles: Deflection (deflection type refractometer) and reflection (reflection type refractometer)
- Differential refractometer monitors the difference in refractive index between the mobile phase (reference) and the column eluent.
- It responds to any solute whose refractive index is significantly different from that of the mobile phase.
- Although these detectors have widely been used in HPLC, they suffer from several disadvantages. For example:
  - They lack in high sensitivity.
  - They are not much suitable for gradient elution.
  - They need strict temperature control to be operated at their high sensitivity.
  - Used for samples that cannot be analyzed using UV/visible, conductivity detector.

Differential refractometers operate on one of two principles (Fig. 1.34)

- The deflection type measures the deflection of a beam of monochromatic light by a double prism.
- Eluent passes through half of the prism; pure mobile phase passes through, or fills, the other half.
- An optical mask confines a beam of light from an incandescent tungsten lamp to the face of the sample and the reference compartments.
- The beam, collimated by a lens, passes through the compartments and is reflected back by the mirror through the compartments again.
- The beam is then focused on a beam-splitter before passing into twin photo detectors.
- The reference and sample compartments are separated by a diagonal glass divider.
- If the refractive index of mobile phase is changed due to presence of a solute, the beam from the sample compartment is slightly deflected.
- As the beam changes location on the detector, an out-of-balance signal is generated that is proportional to the concentration of the solute.

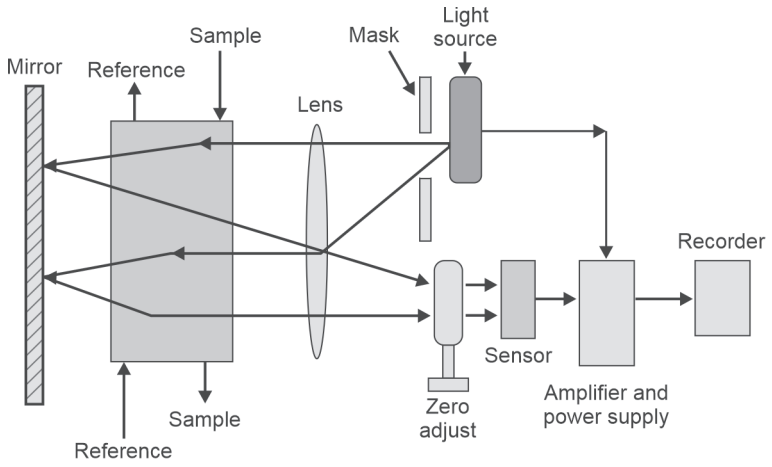


Fig. 1.34: The refractive index detector

- An optical flat, which deflects the beam from side to side, is used to adjust for a zero output signal when the mobile phase is in both prism compartments.
- The cell volume is 15–25 µl.

Deflection type refractometer

- Measure the deflection of a beam of a monochromatic light by double prism.
- Eluent passes through one half of prism and pure mobile phase to other half known as reference compartment.
- Reference and sample compartment are separated by diagonal glass divider.
- Auto zero is used to set, out put signal to zero when mobile phase is in both the compartments.
- Tungsten lamp provides beam of light collimated through lens and passes through Eluent and reference compartment.
- Reflected by the mirror through the same compartment again.
- The beam of light is focused on a beam splitter before passing into the photo detector.
- Refractive index of the mobile phase is changed due to the presence of solute, the beam from the sample compartment is deflected which produces the change signal that is proportional to the concentration of solute.

Advantages

- Wide range of linearity. One cell covers the entire refractive index range.
- The reflection type refractometer measures the change in percentage of reflected light at a glass-liquid interface as the refractive index of the liquid changes.
- The instrument design is based on Fresnel's law of reflection, which states that amount of light reflected at a glass-liquid interface varies with the angle of incidence and the refractive index of the liquid.
- In the optical path (Fig. 1.34) two collimated beams from the projector (light source, masks and lens) illuminate the reference and sample cells.
- The cells are formed with a Teflon gasket, which is clamped between the cell prism and a stainless-steel reflecting back plate.
- As the light beam is transmitted through the cell interface, it passes through the flowing liquid film and impinges on the surface of the reflecting back plate.
- This diffuse, reflected light appears as two spots of light that are imaged by lenses onto dual photo detectors.
- Since the ratio of reflected light to transmitted light is a function of the refractive index of the two liquids, the illumination of the cell back plate is a direct measure of the refractive index of the liquid in each chamber.
- The cell volume is 3 µl.
- This type of refractometer has a relatively limited range.
- Two different prisms must be used to cover the useful refractive index range.
- RI range: 1.00 ~ 1.75 RIU
- Noise: $< \pm 1.3 \times 10^{-8}$ RIU
- Linear dynamic range: 80×10^{-5} RIU
- Drift range: 0.8×10^{-7} RIU/h

- Flow rate range: 0.2 ~3.0 ml/min (analytical)
- Narrobore and semi-prep also available
- Cell pressure: 6 kg/cm^2(84 psi).

Disadvantages

- Lack of sensitivity compared to selective solute property detector
- Baseline instability
- Possibility of both positive and negative peaks in the chromatogram.

Reflection type refractometer

- Measure change in % of reflected light at glass liquid interface as the reflective index of liquid changes.
- Based on the Fresnel's law of reflection which states
- The amount of liquid reflected at a glass-liquid interface varies with the angle of incidence and the refractive index of the liquid

Working: Two collimated beams from the projector (light source and lens) illuminate the reference and sample cell.

- Cells are formed of Teflon gasket, which is clamped between the cell prism and a stainless steel reflecting back plate.
- As the light of beam is transmitted through the cell interfaces, it passes through the liquid film and impinges on the surface of the reflecting back plate.
- Diffused, reflected light appears as two spots and passes through the lens and detected by photodetector.
- The ratio of the reflected light to transmitted light is function of refractive index of the two liquids, the illumination of the cell back plate is direct measure of the refractive index of the liquid in each chamber.

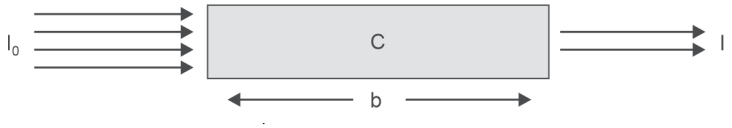
LASER-BASED DETECTORS

- Detectors that utilize lasers have been developing and some laser-based detectors are available commercially, but they are not widely used in routine chromatographic practice. Lasers offer several beneficial properties relevant to the chromatographer.
- In most cases, they offer improved sensitivity and selectivity than other detectors.
- In other cases, improved signal-to-noise ratios (S/N) and deletion of background disturbances are observed.
- Lasers are capable of better resolution than most liquid chromatographic needs, and they are well suited for pulsed-detection.
- On the other hand, they are not only costly but the instrumentation can be complex.
- High energies of lasers can cause thermal distortions, and sensitivity can decrease due to scattering at the optical sections of the system.

Laser-based Refractive Index Detector

- Differences in RIs are accomplished by using interferometry.
- A monochromatic laser measures changes in RI for a sample contained in a Fabry-Perot interferometer.

- A photoamplifier senses the light when the interferometer is scanned.
- A computer calculates the likely position of maximum interference and converts this data into a signal that represents a change in RI (Fig. 1.35).



$$\text{Log}\frac{I_0}{I} = A = abc$$

Fig. 1.35: Detector flow cell

2. ULTRAVIOLET (UV)-VISIBLE ABSORBANCE DETECTORS

Principles: UV detectors (Fig. 1.36) are the most commonly used detector in HPLC. They measure the ability of a sample to absorb light. This can be accomplished at one or several wavelengths. A variable wavelength UV detector, capable of monitoring from 190 to 460–600 nm will be found suitable for the detection of the majority of samples. The fraction of light transmitted through the detector cell is related to the solute concentration according to Beer's law. When the light of specific wavelength passes through the cell, some parts are absorbed, others are transmitted. Specific sample has high absorbance to specific wavelength. Amount of absorbed light (A) is proportional to the concentration of sample.

$$A = \varepsilon bc \text{ (Lambert-Beer's law)}$$

A: Absorbance
ε: Mol absorbance factor
b: Cell path length
c: Concentration of sample

The mobile phase from the column is passed through a small flow cell held in the radiation beam of the UV-visible spectrophotometers. Selective in nature, detect only those solutes that absorb UV-visible radiation, e.g. alkenes, aromatic compounds and compound having multiple bonds between C and O, N or S.

Characteristics: Specific, concentration sensitive, good stability, gradient capability.

Special: UV-Vis spectral capability (diode array technology).

- These detectors are selective, thus detect only those solutes that absorb UV visible radiations, e.g. alkenes, aromatic compounds having multiple bonds between C and O, N or S.
- This is 1000 times more sensitive than RI detectors
- Low pressure mercury lamp, deuterium lamp or a tungsten filament with intermediate filters can also act as a source
- Combination of separation and analysis, e.g. GC-MS, HPLC-UV-Vis is very powerful
- Long-life D2 lamp
- Advanced bandwidth: 5.5 nm
- Excellent sensitivity: $<\pm 0.5 \times 10^{-5}$ AU at 254 nm

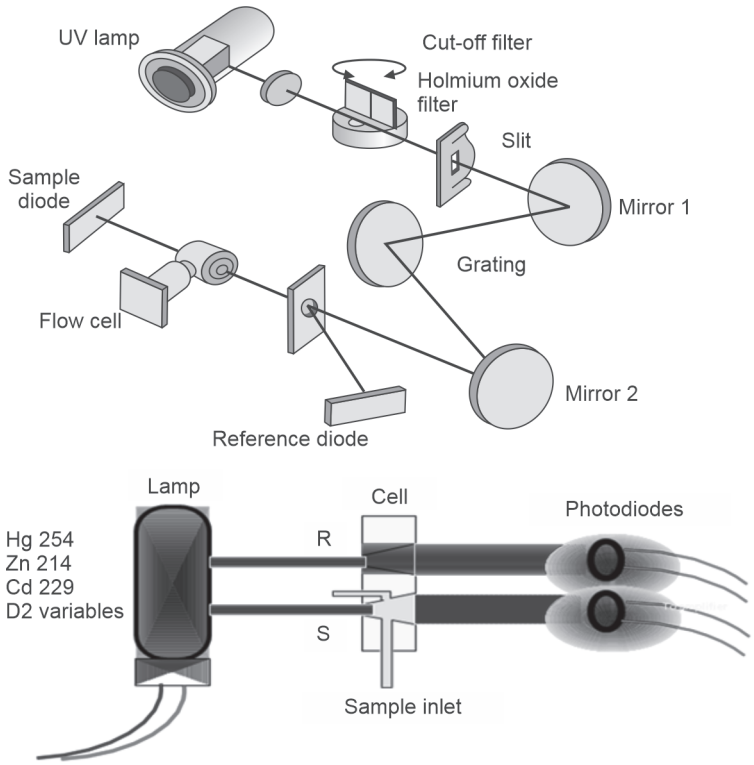


Fig. 1.36: UV detector

- Integrated cell and slit assembly
- Greater cooling of the lamp's thermal environment
- Tested flow cell guarantees up to 1,500 psi (103 bar)
- Optimal cell volume and path length for analytical column
- Method development and QC/QA
- Leak sensor

Advantages

a. With the aid of the computer, the UV spectra of the element from the column can be continuously recorded for postoperative processing in number of ways. It is possible to optimize the output of the detector for each peak, at the wavelength of its maximum absorption.
b. The possibility of ascertaining the peak homogenicity and integrity the peak homogenicity is established by extracting the spectra at different points on the peak.

Disadvantages

a. Absorbance detection is generally not as sensitive as fluorescence detector, electrochemical detector or mass spectrometry using single ion monitoring.
b. Unlike a differential refractometer, absorbance detectors are not universal detectors.

Molecules that do not contain a chromophore cannot be detected, e.g. aliphatic hydrocarbons.

Two types of absorbance detectors are available:

a. **Fixed wavelength detector** measures at one wavelength, usually operate at 254, 280 nm, etc. Other wavelengths are 214, 229, 254, 265, 280, 313, 340, 405, 436, 546 nm. In use with some fixed wavelength depending on lamp and filter.
b. Variable wavelength/multi-wavelength detector measures at one wavelength at a time, but can detect over a wide range of wavelengths. Operates between 190 and 900 nm.

   Diode array measures a spectrum of wavelengths simultaneously.

   UV detectors have a sensitivity to approximately 10^{-8} or 10^{-9} gm/ml.

A. THE FIXED WAVELENGTH DETECTOR

HPLC detectors which do not allow changing the wavelength of the radiation called fixed wavelength detectors. The fixed wavelength UV detector is shown in Fig. 1.37.

- The detector consists of a small cylindrical cell (2.0 to 10.0 ml in volume) through which flows the eluent from the column.
- UV light from an appropriate UV lamp passes through the cell and falls on a UV photoelectric cell.
- In the fixed wavelength detector the wavelength of the light depends on the type of lamp that is used.
- There are a number of lamps available that provide wavelengths ranging from about 210–280 nm.
- The lamps that are commercially available at the time of writing this book are as follows:

Lamp type	*Emission wavelengths*
Mercury vapor lamp	253.7 nm
Zinc vapor lamp	2123.9 nm and 307.6 nm
Cadmium vapor lamp	228.8, 326.1, 340.3, and 346.6 nm

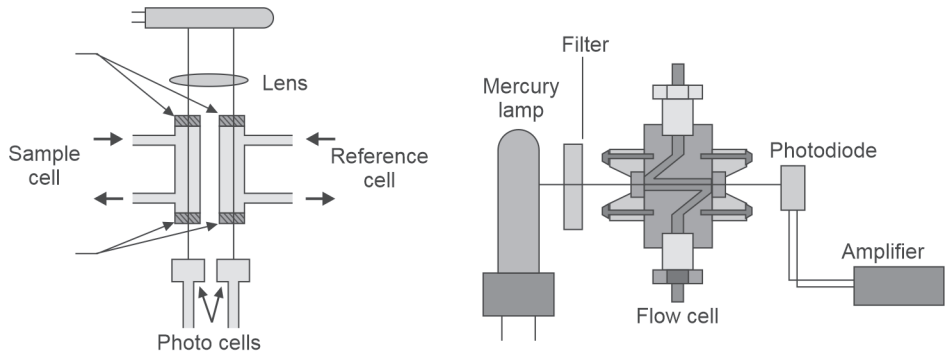


Fig. 1.37: The fixed wavelength UV detector

- The mercury vapor lamp is the most popular as it has an emission wavelength that allows the detector to sense a wide range of solute types.
- Low pressure mercury lamp emits very intense light at 253.7 nm.
- By filtering out all other emitted wavelengths, utilize only 254 nm line to provide stable, highly sensitive detectors capable of measuring sub-nanogram quantities of any components which contain aromatic ring.
- The 254 nm was chosen since the most intense line of mercury lamp is 254 nm, and most of UV absorbing compounds have some absorbance at 254 nm.
- The detector usually contains both a sample and reference cell and the output from the reference cell is compared to that from the sample cell.
- The difference is fed to a nonlinear amplifier that converts the signal to one that is linearly related to concentration of solute in the sample cell.
- The fixed wavelength detector is the least expensive and, as the majority of the light is emitted at a specific wavelength(s) it has a high intensity, and thus, a higher intrinsic sensitivity than the multi-wavelength UV detectors.
- However, the multi-wavelength detector can often compensate for the lower sensitivity by choosing a wavelength that has the highest extinction coefficient for the solutes of interest.
- By the use of very small sensing cells and electronic systems with very small-time constants, the fixed wavelength detector can be designed to give a very fast response at high sensitivity and very low dispersion and for this reason it can be used for high speed separations.

B. THE MULTI-WAVELENGTH DETECTORS

- Detectors which allow the selection of the operating wavelength called variable wavelength detectors.
- Multi-wavelength UV detectors utilize a single (perhaps more accurately a narrow range) of wavelengths to detect the solute.
- Most multi-wavelength UV detectors can also provide a UV spectrum of the eluted solute if appropriately arranged.
- There are two types of multi-wavelength detectors, the dispersion detector that monitors the eluent at one wavelength only and the diode array detector that monitors the eluted solute over a range of wavelengths simultaneously (Table 1.10).

Table 1.10: UV Cut-off of common solvents

Solvent	*UV cutoff*	*Solvent*	*UV cutoff*
Water	180	n-Heptane	197
Methanol	210	Cyclohexane	200
n-Propanol	205	Carbon tetrachloride	265
Acetonitrile	190	Chloroform	245
THF	230	Benzene	280
Acetone	330	Toluene	285
Methyl acetate	260	Methylene chloride	232
Ethyl acetate	260	Tetrachloroethylene	280
Nitromethane	380	1, 2-Dichloroethane	225

- The former passes the light from a broad emission light source through a monochromator, selects a specific wavelength and allows it to pass through the detecting cell.
- The second also uses a broad emission light source, but all the light is allowed to pass through the sensing cell and subsequently the light is dispersed by means of a holographic grating and the dispersed light allowed falling on an array of diodes.
- Wavelength calibration is done automatically using a holmium filter.

1. The Multi-wavelength Dispersive Detector

- A diagram of the multi-wavelength dispersive detector is shown in Fig 1.38.
- Sensitivity for any absorptive component by selecting an appropriate wavelength individual sample component have high absorptivity at different wavelengths and thus, operation at a single wavelength would reduce the system's sensitivity.
- Depending on the sophistication of the detector, wavelength change is done manually or programmed on a time basis into the memory of the system.
- Light from a broad wavelength source such as a deuterium or xenon discharge lamp is collimated by two curved mirrors onto a holographic diffraction grating.
- The dispersed light is focused by means of a curved mirror, onto a plane mirror and light of specific wavelength selected by appropriately positioning the angle of the plane mirror.
- Light of the selected wavelength is then focused by means of a lens through the flow cell and consequently, through the column eluent.
- The exit beam from the cell is focused by another lens onto a photo cell which gives a response that is some function of the intensity of the transmitted light.
- The detector is usually fitted with a scanning facility that, by arresting the flow of mobile phase, allows the spectrum of the solute contained in the cell to be obtained.

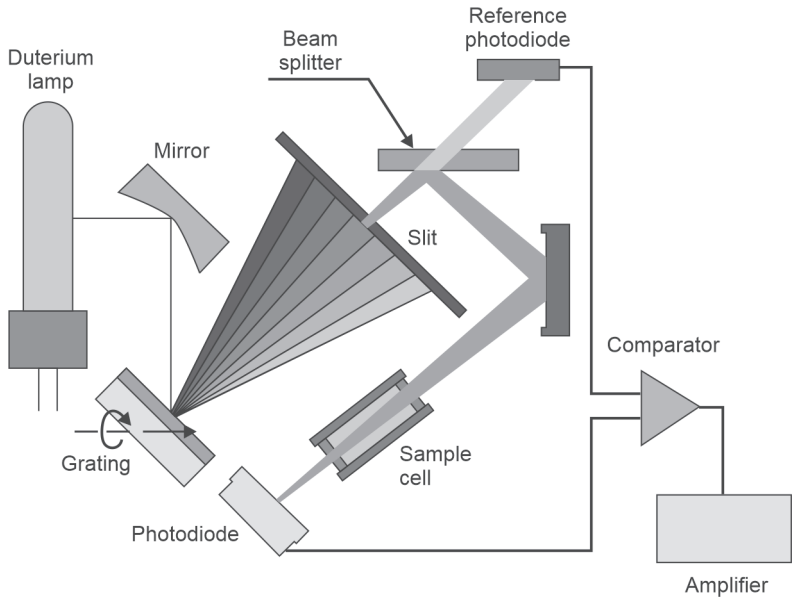


Fig. 1.38: Variable wavelength detector

- Due to the limited information provided by UV spectra and the similarity between many spectra of widely different types of compound, UV spectra are not very reliable for solute identification.
- The technique is useful, however, for determining the homogeneity of a peak by obtaining spectra from a sample on both sides of the peak.
- The technique is to normalize both spectra, then either subtract one, from the other, and show that the difference is close to zero or take the ratio and show it is constant throughout the peak.
- A more common use of the multi-wavelength detector is to select a wavelength that is characteristically absorbed by a particular component or components of a mixture.
- This can be done to either enhance the sensitivity of the detector to those particular solutes, or render the detector more specific and consequently, not give a significant response to other substances in the mixture.
- The multi-wavelength dispersive detector is probably the most useful type of UV detector providing adequate sensitivity, versatility and a linear response.
- It is however somewhat bulky, due to the need for a relatively large internal 'optical bench', has mechanically operated wavelength selection and requires a stop/flow procedure to obtain spectra "on-the-fly".
- The diode array detector has the same advantages but none of the disadvantages, though, as one might expect, is somewhat more expensive.

2. Photodiode Array (PDA) Detector

- Diode array UV-Vis detector allows online measurement of spectra.
- Highest sensitivity
- Scanning wavelength ranges 190–950 nm at same time.
- Wavelength resolution: Up to 1 nm.
- Wavelength calibrated with holmium oxide filter.
- The diode array detector, although offering detection over a range of UV wavelength, functions in an entirely different way from that of the dispersive instrument.
- A diagram of a diode array detector is shown in Figs 1.39 and 1.40.

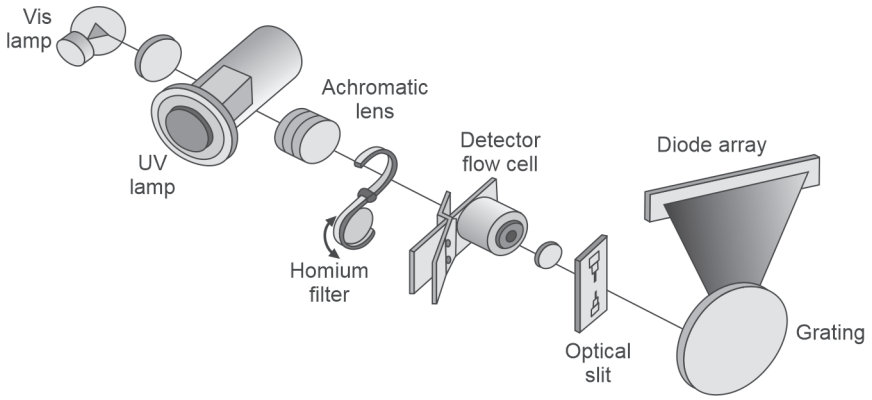


Fig. 1.39: The diode array detector

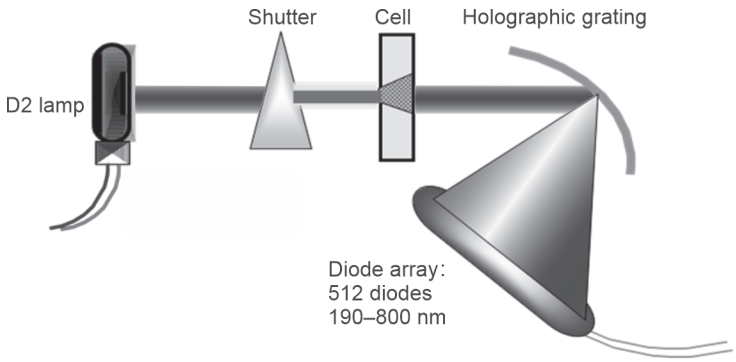


Fig. 1.40: The diode array detector

- Light from a broad emission source such as a deuterium lamp is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating (Fig. 1.40).
- In this way the sample is subjected to light of all wavelengths generated by the lamp.
- The dispersed light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on a hard disc.
- At the end of the run, the output from any diode can be selected and a chromatogram produced employing the UV wavelength that was falling on that particular diode. Most instruments will permit the monitoring of a least one diode in real time so that the chromatogram can be followed as the separation develops.
- This system is ideal in that by noting the time of a particular peak, a spectrum of the solute can be obtained by recalling from memory the output of all the diodes at that particular time.
- This gives directly the spectrum of the solute, i.e. a curve relating adsorption against wavelength.
- Photodiode array detectors can be used to measure and detect samples over the entire UV to visible (UV-visible) spectrum.
- They are highly beneficial tools in identification and analysis of sample compounds.
- To detect over an entire spectrum, the detector must proceed in one of two ways.
- The first is to scan across the entire spectral region, which may be accomplished by a scanning monochromator spectrometer.
- A standard scanning monochromator spectrometer uses a tungsten or deuterium lamp that emits a continuous light source.
- The light is then directed across a grating or prism which reflects the light through an exit slit to the sample cell.
- The sample is then detected by a photomultiplier tube.
- The wavelength of the light can be adjusted by rotating the grating or prism, but only one region can be scanned at a time.
- Subsequently, data points are obtained at different times, which may hinder efficiency and accuracy.

- The second method involves monitoring the entire UV-Vis region simultaneously.
- **Optimum wavelength resolution:** 1,024 diodes
- Wide range from 190 to 950 nm
- $<\pm 1.0 \times 10^{-5}$ AU at 254 nm, 2 sec rise time
- $<\pm 2.0 \times 10^{-5}$ AU at 254 nm, 1 sec rise time
- **Fast data acquisition:** 50 Hz
- Greatest drift level
- $<2.0 \times 10^{-4}$ AU/h at 254 nm, baseline correction
- $<1.0 \times 10^{-3}$ AU/h at 254 nm

3. THE FLUORESCENCE DETECTOR (FLUORIMETRIC DETECTOR)

- Very sensitive, but very selective.
- It is possible to detect even a presence of a single analyte molecule in the flow cell.
- Fluorescence occurs when compounds having specific functional groups are excited by shorter wavelength energy and emit higher wavelength radiation.
- Many compounds (e.g. aflatoxins, riboflavins, polynuclear aromatics, porphyrins, etc.) are capable of absorbing radiation of a particular wavelength (usually in the UV region) and upon excitation, emit radiation of a characteristic (usually longer) wavelength. This phenomenon is called fluorescence.
- Fluorescence detectors for HPLC are similar in design to the fluorometers and spectrofluorometers.
- Fluorescence is observed by a photoelectric detector located at 90° to the excitation beam.
- Fluorescence is often collected at right angle to excitation beam.
- Only one sixth of fluorescence is collected. If concave mirror is placed around the sample cell abut 75% of the emission is collected.
- With all sample cells, scattered radiation from the excitation source is selectively removed with cut off or band pass filters placed before photomultiplier tube.
- In the Fig. 1.41 xenon deuterium radiation source is used and employed a granting monochromator to characterize the fluorescent radiation.
- The fluorescence detector is one of the most sensitive LC detectors and for this reason is often used for trace analysis. Unfortunately, although the detector is very sensitive, its response is only linear over a relatively limited concentration range.
- In fact, the response of the detector can only be assumed to be linear over a concentration range of two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce which is a serious disadvantage to this type of detector.
- It follows, that in many instances fluorescent derivatives must be synthesized to render the substances of interest detectable.
- A diagram of the fluorescence detector is shown in Fig. 1.42.
- In its simplest form, light from a fixed wavelength UV lamp passes through a cell, through which the column eluent flows and acts as the excitation source.
- Any fluorescent light that is emitted is sensed by a photoelectric cell positioned normal to the direction of exciting UV light.

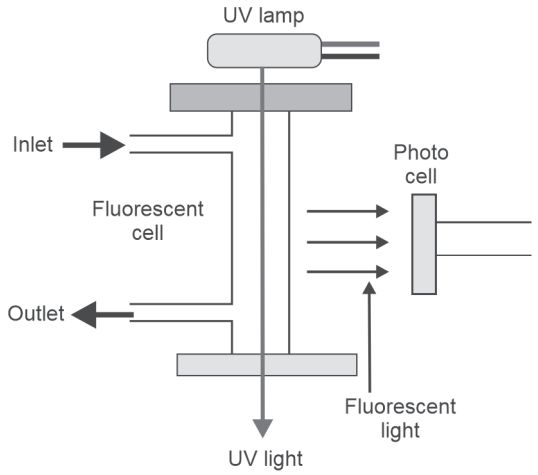


Fig. 1.41: Fluorescence detector

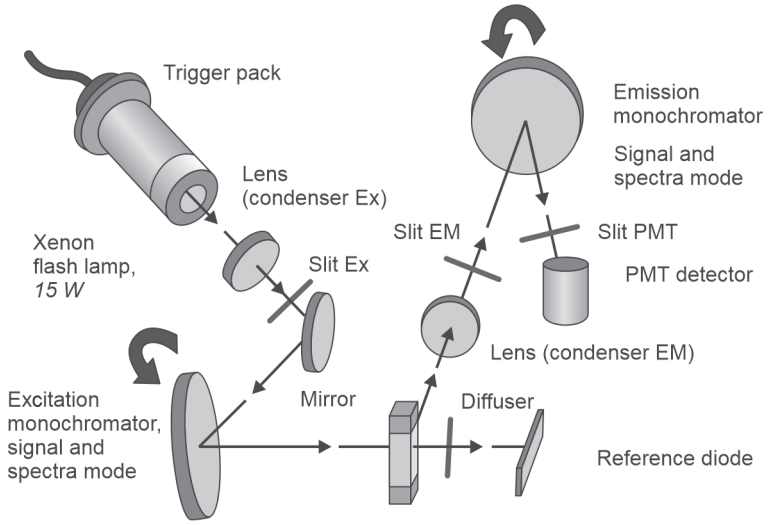


Fig. 1.42: 8 µl flow cell, auto-recognition

- The photo cell senses fluorescent light of all wavelengths but the wavelength of the excitation light can only be changed by use of an alternative lamp.
- This simple type of fluorescence detector was the first to be developed, it is relatively inexpensive and for certain compounds can be extremely sensitive.
- A more elaborate form of fluorescence detector uses a monochromator to select the excitation wavelength and a second monochromator to select the wavelength of the fluorescent light.
- This instrument gives the maximum versatility and allows the maximum sensitivity to be realized for any type of solute.
- The system can also provide fluorescence spectra by arresting the flow of mobile phase when the solute resides in the detecting cell and scanning the fluorescent light.

- Fluorescence has been shown to be extremely useful as a detection process and detectors based on fluorescent measurement have provided some of the highest sensitivities available in LC.
- When a molecule adsorbs light, a transition to a higher electronic state takes place and this absorption is highly specific for the molecules concerned; radiation of a specific wavelength or energy is only absorbed by a particular molecular structure.
- If electrons are raised to an upper excited single state, due to absorption of light energy, and the excess energy is not immediately dissipated by collision with other molecules or by other means, light will be emitted at a lower frequency as the electron returns to its ground state and the substance is said to fluoresce.
- As some energy is always lost before emission occurs then, in contrast to Raman scattering, the wavelength of the fluorescent light is always greater than the incident light.
- Detection techniques based on fluorescence affords greater sensitivity to sample concentration, but less sensitivity to instrument instability (e.g. sensor temperature and pressure).
- This is due to the fluorescent light being measured against a very low light background (i.e. against a very low noise level). This is opposite to light absorption measurements where the signal is superimposed on a strong background signal carrying a high noise level.
- Unfortunately, relatively few compounds fluoresce in a practical range of wavelengths. However, some compounds, including products from foods, drugs, dye intermediates, etc. do exhibit fluorescence and can be monitored by fluorescent means.
- In addition, many substances can be made to fluoresce by forming appropriate derivatives.
- Has sensitivity limit of 10^{-9} to 10^{-11} gm/ml.

Laser-induced Fluorescence

- Offers increased sensitivity but Rayleigh light scattering at optical sections and in the eluents can present a problem to certain detectors.
- Detection can proceed through one- or two-photon excited fluorescence (OPEF or TPEF).
- TPEF results in absorption of a pair of photons that occupy a certain molecular energy level; it is feasible due to availability of high-powered lasers and absence of noise levels often seen in OPEF.

There are several detector types

A. Flowing droplet cell: 4 µl of eluent is kept between the end of an HPLC column and a solid probe. A laser is emitted perpendicular to this flow channel. The fluorescence is measured using an amplifier. Rayleigh scattering of the radiation source is mostly avoided.

B. Laminar flow cell: Effluent is confined in the cell by one-way, laminar flow. The cell utilizes a sub-microliter flow-through cuvette whose windows are 5 mm from the stream which minimizes scattering. The laser beam and effluent are perpendicular to the optical component.

C. Optical fiber cell: A capillary tube cell is connected to an optical fiber, which conducts light rays to the detector flow. The fluorescence collection optics is placed at a 30° angle in relation to the capillary in order to minimize scattering.

4. THE ELECTRICAL CONDUCTIVITY DETECTORS

- The electrical conductivity detector can only detect those substances that ionize and consequently, are frequently used in the analysis of inorganic acids, bases and salts.
- It has also found particular use in the detection of organic acids and bases that are frequently required in environmental studies and in biotechnology applications.
- The sensor is the simplest of all the detectors consisting of only two electrodes situated in a suitable flow cell.
- An example of an electrical conductivity sensing cell is shown in Fig. 1.43.

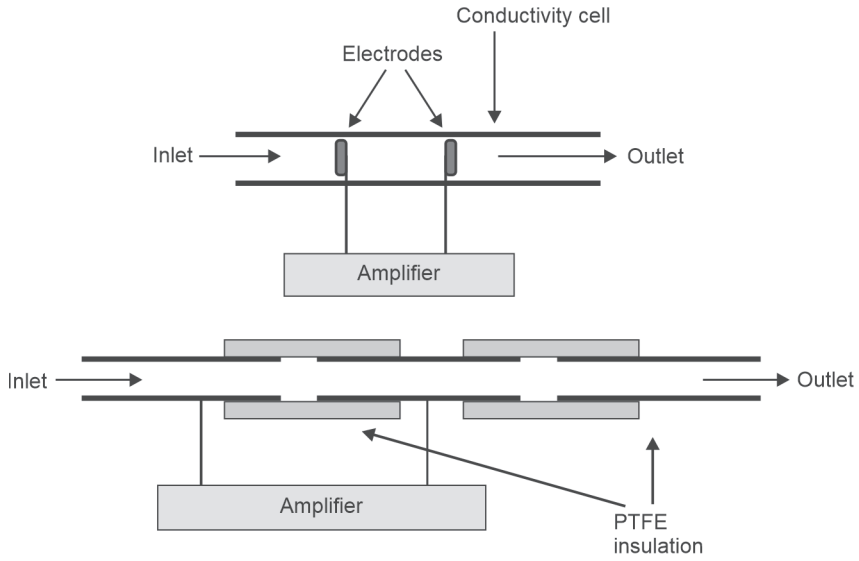


Fig. 1.43: An electrical conductivity detector sensing cell

- It consists of two electrodes situated in a suitable flow cell as depicted in the upper diagram.
- The electrodes are arranged to constitute one arm of a Wheatstone bridge.
- When ions enter the detector cell, the electrical resistance changes and the out of balance signal is fed to a suitable amplifier.
- The output from the amplifier is either digitized, and the binary number sent to a computer for storage, or the output is passed directly to a potentiometer recorder.
- The detector actually measures the electrical resistance between the electrodes which by suitable non-linear amplification, can be made to provide an output that is linearly related to solute concentration.

- It is essential that an AC voltage is used across the electrodes to measure the cell impedance to avoid electrode polarization.
- The frequency of the AC potential across the electrodes is usually around 10 kHz.

A more practical system shown in the lower part of the diagram consists of short lengths of stainless steel tube insulated from each other by a PTFE connecting sleeves. For convenience, the first tube (that connected to the column) is usually grounded (earthed). The resistance between the inlet tube and the center tube is continuously monitored which will constitute the resistance across the tiny gap between the tubes contained in the first PTFE sleeve. The volume of eluent in this gap can be extremely small and thus, the peak dispersion can also be made very small. The resistance of the solution situated between the tubes is inversely proportional to the electric conductivity of the solution which, in turn, is related to the ion concentration in mobile phase (Fig. 1.44).

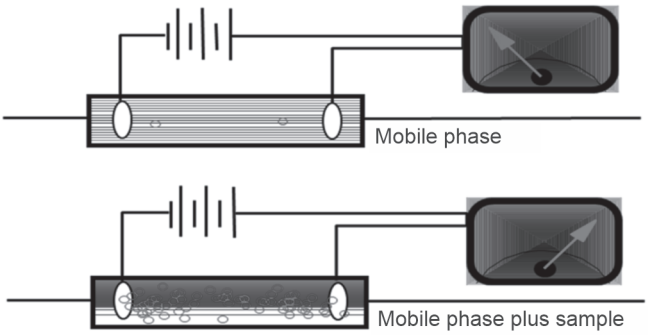


Fig. 1.44: Conductivity detector

5. THE ELECTROCHEMICAL DETECTOR (AMPEROMETRIC DETECTORS)

- It is based on the measurements of the current resulting from an oxidation/reduction reaction of the analyte at a suitable electrode.
- Measure the electrochemical reaction (oxidation/reduction) between a working electrode and the sample.
- The level of the current is directly proportional to the analyte concentration.
- Amperometric or coulometric detectors are commonly known as electrochemical detectors which measure the current associated with the oxidation or reduction of the solute.
- When current is passed through solution at each electrode electron exchange takes place and is given as follows:

  At cathode—substance electrons, that is, reduction

  At cathode—substance electrons, that is, oxidation

- The electrochemical detector responds to substances that are either oxidizable or reducible and the electrical output is an electron flow generated by a reaction that takes place at the surface of the electrodes.
- If the reaction proceeds to completion (exhausting all the reactant) the current becomes zero and the total charge generated will be proportional to the total mass of material that has been reacted. This process is called coulometric detection.

- If, however, the mobile phase is flowing past the electrodes, the reacting solute will be continuously replaced as the peak passes through the detector.
- All the time there is solute present between the electrodes, a current will be maintained, albeit varying in magnitude. Until relatively recently, this procedure was that most common employed in electrochemical detection and is called amperometric detection.
- The electrochemical detector requires three electrodes, the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any changes in the background conductivity of the mobile phase).
- The processes taking place at the electrode surface can be very complex; nevertheless, the dominant reaction can be broadly described as follows.
- At the actual electrode surface the reaction is extremely rapid and proceeds almost to completion. This results in the layer close to the electrode being virtually depleted of reactant. As a consequence, a concentration gradient is established between the electrode surface and the bulk of the solution.
- This concentration gradient causes solute to diffuse into the depleted zone at a rate proportional to the solute concentration in the bulk of the mobile phase.
- Thus, the current generated at the electrode surface will be determined by the rate at which the solute reaches the electrode and consequently, as the process is diffusion controlled, will depend on solute concentration and the magnitude of solute diffusivity (Figs 1.45 and 1.46).

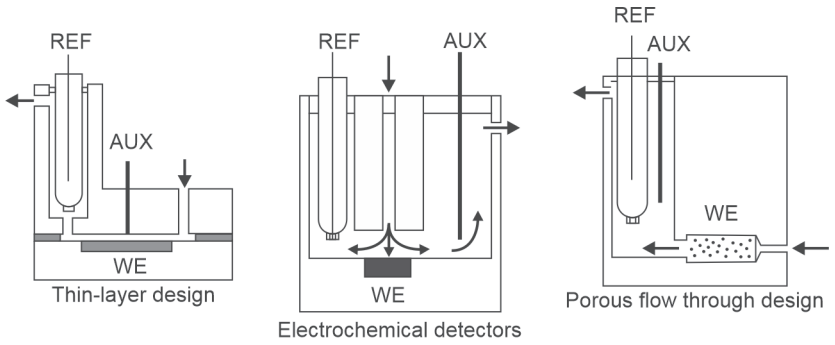
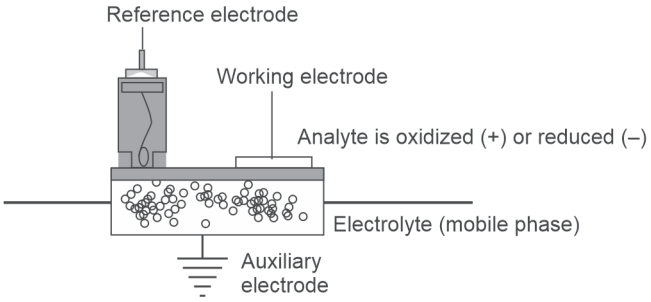


Fig. 1.45: Electrochemical detectors



As compounds are oxidized or reduced, a current proportional to concentration is produced.

Fig. 1.46: Electrochemical reaction

- Has sensitivity of 10^{-12} to 10^{-13} gm/ml.
- **Gold** for carbohydrates.
- **Platinum** for chlorite, sulfate, hydrazine, etc.
- **Carbon** for phenols, amines.
- **Silver** for chloride, bromide, cyanide.

6. NUCLEAR MAGNETIC RESONANCE (NMR) DETECTORS

- Certain nuclei with odd-numbered masses, including H and ^{13}C, spin about an axis in a random fashion.
- However, when placed between poles of a strong magnet, the spins are aligned either parallel or anti-parallel to the magnetic field, with the parallel orientation favored since it is slightly lower in energy.
- The nuclei are then irradiated with electromagnetic radiation which is absorbed and places the parallel nuclei into a higher energy state; consequently, they are now in "resonance" with the radiation.
- Each H or C will produce different spectra depending on their location and adjacent molecules, or elements in the compound, because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

7. LIGHT-SCATTERING (LS) DETECTORS/ELSD

- When a source emits a parallel beam of light which strikes particles in solution, some light is reflected, absorbed, transmitted, or scattered.
- For universal detection, Better sensitive than RID
- Universal detector to analyze components which do not have UV absorption cannot be separated with an isocratic solvent and must use a gradient elution, which cannot be used with a refractive index detector. Only mobile phases without any buffers should be used
- **Light source:** LED (light emitting diode)
- **Detectection:** PMT (photomultiplier)
- **Temp range:** Ambient ~70°C
- **Gas flow:** Less than 1 L/min (for nebulizer), less than 2 L/min (for additional rotatory drying gas)
- **Gas flow controls:** Through built-in flow controller for the additional drying gas
- EPC (electronic pressure control) for the nebulization gas
- **Outputs:** Analog Output 0 ~ 5 Volt
- **Inputs:** Autozero, start
- Instrument Control: Keypad

Two forms of LS detection may be used to measure the two latter occurrences

A. Nephelometry: This is defined as the measurement of light scattered by a particulate solution. This method enables the detection of the portion of light scattered at a multitude of angles. The sensitivity depends on the absence of

background light or scatters since the detection occurs at a black or null background.

B. Turbidimetry: This is defined as the measure of the reduction of light transmitted due to particles in solution. It measures the light scatter as a decrease in the light that is transmitted through the particulate solution. Therefore, it quantifies the residual light transmitted. Sensitivity of this method depends on the sensitivity of the machine employed, which can range from a simple spectrophotometer to a sophisticated discrete analyzer. Thus, the measurement of a decrease in transmitted light from a large signal of transmitted light is limited to the photometric accuracy and limitations of the instrument employed.

Laser Light Scattering

- Considered the most "mature" of the laser-based techniques, this process involves the detection of Rayleigh scattering.
- This approach utilizes a low-angle laser light scattering (LALLS) photometer and a gel permeation chromatograph (GPC). GPC, otherwise known as size-exclusion chromatography (SEC), separates compounds based on molecular weight.
- The LALLS focuses a laser beam on the sample placed between two quartz windows separated by a Teflon spacer that reduces background noise.
- The scattered light can be measured at scattering angles as little as 2 degrees.
- In series, the GPC/LALLS can provide online molecular weight distributions; consequently, this technique is well-suited for polymer characterization.

8. TRANSPORT DETECTORS

- A transport detector consists of a carrier such as a metal chain, wire or disc that passes continually through the column eluent extracting a sample of the mobile phase containing the solute as a thin film adhering to its surface.
- The mobile phase is eliminated by evaporation leaving the solute as a coating on the carrier.
- The carrier is then scanned by an appropriate sensing technique to monitor the residual solute.
- For example, an FID could be used to sense the pyrolysis products of the solute by heating the carrier and most of the pyrolysis products containing carbon would be detected.
- The system is obviously restricted to those solutes that are in volatile and, in addition, the solvents used for the mobile phase must be volatile and extremely pure.
- The former condition is usually met in LC; otherwise the analysis would probably be carried out by GC. The latter condition is normally easy to achieve as there is a wide choice of solvents readily available for LC.
- The system appears to be ideal but the early models had some disadvantages.
- The instruments were bulky, expensive and some incorporated a ^{90}strontium source (for the argon detector) all of which were unpopular.
- In addition, as a result of the basic design, the anticipated high sensitivity was not realized and the apparatus was clumsy and difficult to operate.
- However, as it was a universal detector and was unaffected by the solvents used, it was readily accepted by the soap and cosmetic industry (Table 1.11).

Table 1.11: The principle and sensitivity of various detectors

S. No.	*Detector*	*Principle*	*Type (min. mass detected)*	*Sensitivity*	*Remarks*
1	Refractive index	A measure of change in the refractive index of the column eluate passing through the flow cell.	Bulk property	1–5 µg	Universal detector
2	Evaporative light scattering	A measure of light scattered from the solute particles remaining after nebulization and evaporation of the mobile phase.	Bulk property and mobile phase modification	1–100 ng	Universal detector
3	Conductivity	A measure of change in electric current of the column eluate with a constant voltage applied between the electrodes.	Sample specific	10–50 ng	Specific to charged moiety in solutions
4	UV-Vis	A measure of absorption of light by the column eluate in the ultraviolet and visible region.	Sample specific	0.5–1.0 ng	Specific to chromophoric compounds
5	Electrochemical	Detects the compounds displaying oxidation reduction reactions and measures the electric current generated by these reactions.	Sample specific	50–500 pg	Specific to compound displaying oxidation and reduction reaction.
6	Fluorescence	A measure of light emitted immediately by a compound of the column eluate that has absorbed light or other electromagnetic radiation of different wavelength.	Sample specific	10–100 pg	Specific to compound exhibiting fluorescence property
7	Mass spectrometer	Ionization of the compounds from the column eluate.	Hyphenated technique	10–100 fg	Universal detector

VII. RECORDER OR DATA SYSTEMS

Recorders are used to record the response obtained from the detector after amplification. They record the baseline and all the peaks obtained, with respect to time. Retention time for all the peaks can be calculated. Integrators are improved versions of recorder with data processing capabilities. They can record the individual peaks with retention time height and width of peak, peak area, etc.

Since the detector signal is electronic, use of modern data acquisition techniques can aid in the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing

operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimization and deconvolution (i.e. resolution) of overlapping peaks. Finally, software safeguards can be designed to reduce accidental misuse of the system. For example, the controller can be set to limit the rate of solvent switching. This acts to extend column life by reducing thermal and chemical shocks. In general, these stand-alone, user programmable systems are becoming less expensive and increasingly practical (Table 1.12).

Table 1.12: HPLC separations guide

S. No.	*Detector*	*Conditions*	*Application*
		Column C_{18}	
1	UV (192), CAD	50%MeOH/H_2O	Phosphate pesticides
2	UV (192), RI, CAD	50% MeOH/H_2O	Carbamate pesticides
3	UV (192 nm)	35% AN/H_2O, PO_4. pH 2.5	Prostaglandins
4	UV (220 nm)	40% MeOH/H_2O	Anticonvulsants
5	UV (220nm)	80% AN/H_2O	Chlorinated pesticides
6	UV (230 nm)	60% MeOH/H_2O	Steroids
7	Fl (230/418)	8% AN/PO_4, pH 2.6 DMSO: MeOH: AN: H_2O (3 : 25 : 30 : 40)	OPA amino acids
8	UV (254 nm)	8% AN/H_2O	Water-soluble vitamins
9	UV (254 nm)	8% MeOH/H_2O, PO_4, pH 5.5	Nucleosides
10	UV (254 nm)	15–80% AN/H_2O	Bromophenol acids
11	UV (254 nm)	20% AN/H_2O, TBA, PO_4, pH 2.6	Nucleotides
12	UV (254 nm)	10% AN	PTH amino acids
13	UV (254 nm)	55% AN/H_2O	Tricyclic antidepressants
14	UV (254 nm)	10% AN/H_2O, AcOH, pH 2.5	Aspirin, tylenol
15	UV (270 nm)	7% AN/H_2O, PO_4, pH 4.0	Theophylline
16	UV (270 nm)	6% MeOH/H_2O	Catecholamines
17	UV (280 nm)	80% AN/H_2O	Fat-soluble vitamins
		Column C_8	
1UV (220 nm)		60% AN/H_2O	Triglycerides
2UV (254 nm)		>30% n-BuOH/0.1% TFA, H_2O	Peptides (<99 amino acids)

Abbreviations:
AN: Acetonitrile; AcOH: Acetic acid; MeOH: Methanol; BuOH: Butanol; H_2O: Water; CAD: Charged aerosol detector; DMSO: Dimethyl sulfoxide; PO_4, pH 2.6: Phosphate buffer; RI: Refractive index detector; TFA: Trifluoroacetic acid; TBA: Tertiary butylamine; UV: Ultraviolet detector.

Other more advanced features can also be applied to a chromatographic system. These features include computer controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors. These added features are not found on many systems, but they do exist, and can save much time and effort for the chromatographer.

1.8 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, environmental applications, forensic applications, biochemical separations, biotechnology, food analysis, etc. 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 standard. Under identical conditions, the retention time of the standard and the sample are same. If there is a deviation, then they are not of the same compound.
   - *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 peak obtained, the percentage purity can also be known.
   - *Presence of impurities*: This can be seen by the presence of additional peaks compared with a reference standard or reference material. The percentage of impurities may also be calculated from peak areas.
2. **Quantitative analysis:** The quantity of a component can be determined by several methods like:
   1. *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

      $$A1/A2 = \alpha\ W1/W2,$$

      where A1 and A2 are peak area of sample and standard

      W1 and W2 are weight or concentration of sample and standard

      α is the response factor
   2. *Calibration curve method*: In calibration curve method, series of standards are used to determine their peak areas. A calibration curve of peak area versus 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 minimized.
   3. *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.
   4. *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 above methods. Marketed formulations which contain several drugs can be determined quantitatively for each component.

5. *Isolation and identification of drugs* or metabolites in urine, plasma, serum, etc. can be carried out.
6. *Isolation and identification of mixture* of components of natural or synthetic origin.
7. *Biopharmaceutical and pharmacokinetic studies.*
8. *Stability studies.*
9. *Purification* of some compounds of natural or synthetic origin on preparative scale.

Other Applications of HPLC

A. Analytical Applications

1. *Analysis of carbamazepine in plasma in HPLC*

Plasma levels of anticoagulant drugs should be continuously monitored because of their narrow therapeutic range and toxic effects. Hence, the measurement of serum concentration of carbamazepine is useful adjuvant to therapy with carbamazepine. The extract is first prepared by shaking 20 ml of ether with one ml of plasma then 2.5 gm of ammonium biphosphate is added and contents are shaken. Then the mixture is centrifuged and organic solvent is evaporated. The residue is dissolved in specified amount of chloroform and specified volume of this sample is subjected into the column of liquid chromatogram.

2. *Application of HPLC to analysis of clinically important porphyrins*

The porphyrins are a group of metabolic diseases characterized by the abnormal production and excretion of porphyrins. The clinical diagnosis of porphyria is based on the qualitative and quantitative determination of the pattern of excreted porphyrins which is shown to be characteristic of the type of porphyrin.

3. *For protein identification and characterization*

The human genome initiative that took a stronghold on biotechnology companies in the early 1990s through the first few years of the twenty-first century. The expanding role of HPLC in drug discovery spawned a completely new field that had analytical chemistry as its cornerstone. Specifically, high-resolution capillary and nano-column HPLC coupled with tandem mass spectrometry became one of the tools of choice for characterizing proteins and identifying potential therapeutic protein targets. In essence, the mandate of the proteomics field since its inception has been to identify differences at the protein level, in cells, tissues, plasma, and so on, between a disease state and control ("normal"). The basic premise is that proteins will be either up- or down-regulated (i.e. over- or under-expressed) in the disease state relative to "normal" state, and these differences can be identified and quantified by mass spectrometry. There have been several analytical advances made in the field of proteomics since its inception, far too numerous to capture in this review.

One noteworthy advance in proteomics is the technique of multidimensional protein identification technology (MUDPIT), developed by Yates and co-workers, which has been used widely in place of the more laborious, less automated method of 2D-polyacrylamide electrophoresis. MUDPIT is a column chromatography

method whereby ion exchange chromatography is used in the first dimension of chromatography to simplify the complexity of the complex mixture of peptides by separating them based on charge followed by reversed-phase HPLC for the higher-resolution separation based on molecular weight and hydrophobicity. An equally important development in the field of proteomics has been isotope-coded affinity tags (ICAT) technology, a method whereby isotopic labeling of peptides containing cysteine residues is performed so as to facilitate peptide quantitation and identification of putative biological targets.

The focus of proteomics has turned to identifying potential biomarkers of disease. A biomarker, by definition, is (a) a molecular indicator for a specific biological property or (b) a feature or facet that can be used to measure the progress of disease or the effects of treatment. As an example, a biomarker for type II diabetes is higher fasting blood glucose levels relative to age-matched controls. Another, more definitive biomarker of type II diabetes is elevated HbA1C levels. For many diseases, however, the relevant biomarkers are less well understood. This is especially true in the fields of oncology and inflammation research. Biomarker research is a particularly intense area of focus for many pharmaceutical companies, with new departments being formed for the purpose of identifying both preclinical and clinical biomarkers to facilitate their drug discovery and development programs. Like the field of proteomics, the field of biomarker research is far too vast to warrant its review here. A very nice review article by the late Wayne Colburn, a pioneer in diabetes biomarker research, describes this maturing field.

4. *In support of assay development and screening*

The overwhelming majority of biological assays have been developed in microtiter plate format (typically 96-well, 384-well, 1536-well) and with parallel detection methods such as fluorescence polarization. The vast majority of druggable targets, including enzymes, ligand-gated ion channels, and G protein-coupled receptors, are all amenable to screening in high-throughput microtiter plate format. In general, serial-based chromatographic methods, such as HPLC and HPLC/MS, are unable to compete with the high-throughput screening technologies. However, a small number of targets, such as those involved in mediating protein-protein interactions, are not well-suited to HTS methodologies. For this class of targets, HPLC coupled with mass spectrometry has proved to be a very reliable, albeit lower throughput, alternative. The technique that has been used most widely for directly assessing protein-small molecule and protein-protein interactions is affinity chromatography-mass spectrometry. Kassel et al presented one of the first papers coupling affinity chromatography with mass spectrometry. In their work, a two-dimensional LC-LC/LC-MS method was developed to assess protein-ligand binding. Affinity chromatography was used in the first dimension of separation, followed by reversed-phase chromatography coupled with mass spectrometry for the identification of binders.

5. *To support compound characterization*

Combinatorial chemistry paved the way for high-throughput, parallel organic synthesis techniques, now mainstream in the pharmaceutical and biotechnology industries for lead generation activities. The ability to synthesize compound libraries rapidly using automated solution-phase and solid-phase parallel synthesis has led to a dramatic increase in the number of compounds now available for high-throughput

screening. The unprecedented rate by which compound libraries are now being generated has forced the analytical community to implement high-throughput methods for their analysis and characterization. As early as 1994, groups adopted high-speed, spatially addressable automated parallel solid-phase and solution-phase synthesis of discrete.

Both solution-phase and solid-phase parallel syntheses permit the production of large numbers as well as large quantities of these discrete compounds, eliminating the need for extensive decoding of mixtures and re-synthesis following identification of "active" compounds in high-throughput screening of combinatorial libraries. Importantly, parallel synthesis is performed readily in microtiter plate format amenable to direct biological screening, as was touched upon earlier. The relative ease of automation of parallel synthesis led to a tremendous in flux of compounds for lead discovery and lead optimization. Almost all of the analytical characterization tools (e.g. HPLC, NMR, FTIR, and LC/MS) are serial-based techniques, and parallel synthesis is inherently parallel. Consequently, this led rapidly to a new bottleneck in the discovery process (i.e. the analysis and purification of compound libraries). Parallel synthesis suffers from some of the same shortcomings of split and mix synthesis (e.g. the expected compound may not be pure, or even synthesized in sufficient quantities). The analytical community was faced with the decision of how to analyze these parallel synthesis libraries.

6. *Purity assessment of compound libraries*

The issue of compound purity has received a great deal of attention over the last several years as more and more chemists have adopted high-throughput organic synthetic protocols but are unwilling to compromise the quality of the molecules submitted for biological evaluation. The general consensus target purity of a compound library compound before it is to be archived or screened for biological activity is between 90% and 95% pure. This purity criterion is more stringent than in the past, where 85–90% (based on UV detection) was considered acceptable. This may be attributed primarily to a shift toward smaller, focused (or biased) libraries than larger, diverse collections of compounds. The majority of mass spectrometry manufacturers now offer software packages that aid in the automatic determination of purity. UV chromatograms are typically used, rather than the total ion current chromatogram, to assess purity. This is because the total ion current chromatogram is a measure of a compound's "ionizability," which is well known to vary dramatically from one compound to the next.

Orthogonal detection methods, such as chemiluminescence nitrogen detection (CLND) and ELSD, have been proposed to be more universal detection methods than UV and hence are being used with increasing frequency to assess reaction yields and purity. CLND, as indicated from its name, measures the amount of nitrogen in a sample. In this method, a compound is transferred to a high 4-minute HPLC/MS separation of a solution-phase parallel synthesis library. The gradient profile for fast HPLC/MS was 10–90% acetonitrile in H_2O in 4 minutes with a 1-minute equilibration time. A 1-minute, total cycle time chromatographic separation of the same crude product. Temperature oxygen reaction chamber set to 1000°C whereby the compound undergoes rapid decomposition to form nitrous oxide (NO). The liberated NO reacts with ozone (O_3) to form metastable NO_2, which is selectively detected by release of a photon.

7. *ADME applications*

The continuing quest for novel and safer drugs has led to the introduction of myriad new technologies within the pharmaceutical industry. Notably, advances in genomics, high-throughput screening, combinatorial chemistry, parallel synthesis, automation, and miniaturization have enabled large numbers of potent (active) and selective compounds to be identified at early stages of drug discovery. However, the fact that a compound is active and selective does not necessarily make it an attractive drug development candidate. To convert these "actives" into qualified clinical candidates have proved challenging. It has been reported that a significant number of compounds nominated for clinical development fail due to poor pharmacokinetics and toxicological properties (63% of all preclinical compounds).

In order to identify chemotypes and lead compounds that have good pharmacokinetic and safety profiles (e.g. no hERG liability), it has been recognized that studies that assess absorption, distribution, metabolism, and elimination (ADME) should be initiated as early as possible in the discovery process. The shift from late-stage optimization of ADME properties to a strategy of identifying potential liabilities early in the discovery process has taken hold within the pharmaceutical community, adding the dimension of structure-ADME relationships in parallel to structure-activity relationships as an integral part of the iterative drug discovery process. Because of the large number of hits that are now routinely identified from screening compound collections and gene family compound libraries, the industry has recognized the need for high-throughput ADME assays. Fortunately, many ADME assays can be run in a high-throughput fashion, due principally to the widespread incorporation of liquid chromatography/mass spectrometry (LC-MS) and liquid chromatography/tandem mass spectrometry LC-MS/MS-MS. LC/MS and LC-MS/MS-MS have become the preferred techniques for ADME analyses due principally to enhanced sensitivity, selectivity, and ease of automation relative to traditional analytical methods. The selectivity advantages of LC/MS have made possible the ability to analyze endogenous and nonfluorescent probe substrates in cytochrome inhibition assays, enabled rapid permeability assessment (e.g. Caco-2 assay), provided faster methods for assessing lipophilicity and solubility of drug leads, and provided much more facile assessment of liver metabolism.

8. *Role in preformulation*

Discovery formulation support and early preclinical development support can be provided by the preformulation group as a part of the drug development process. The functions of this unit is to help discovery in physicochemical characterization of new drug molecules by providing information on solubility, stability, pKa, and LogP/LogD as well as formulation support for PK animal studies to recommend a final candidate for selection to development. After candidate selection, the early preformulation unit provides a major source of information to formulation and analytical scientists regarding the properties of the recommended drug molecules. The development preformulation support provides the additional testing of prototype formulation and excipient compatibility samples as well as guidance for salt form selection and polymorphs screening. The major role of this unit is to bridge discovery and development stages. HPLC coupled with not only UV but also other alternative detectors, is the predominate tool for analyzing drug substances with

high speed and efficiency, which is required in the preformulation stage of drug development.

9. *Role of HPLC in process development*

HPLC plays a significant role in the analytical aspect of process development. It is the most commonly used tool to determine the purity of the active pharmaceutical ingredient and to track impurity generation and yield during the process. There is a plethora of options available in terms of separation mode, stationary phase, and mobile phase to cover most of the wide range of diverse physiochemical properties associated with the active pharmaceutical ingredients, raw materials, intermediates, and impurities. This chapter has presented some of these options and how they can be applied. Procedures to develop HPLC methods and to ensure that the methods are precise and accurate have also been presented. Finally, how these HPLC methods fulfill regulatory requirements and how they are successfully transferred to manufacturing sites have been outlined.

10. *Role of HPLC during formulation development*

HPLC is currently playing a major role in the implementation of Process Analytical Technology (PAT) in the pharmaceutical industry. During the development of PAT models, the results obtained through HPLC analysis can be utilized as reference values. Fast and accurate HPLC methods must be developed. Several examples where HPLC is used as a reference method for NIR and Raman analysis are cited. HPLC has been historically been an important off-line analytical tool in the pharmaceutical industry laboratory for measuring product quality; however, it can also play a valuable role in online process monitoring as well. Online liquid chromatography can be used for process monitoring, automation, and control and can be considered a PAT application. Online HPLC has been used for both small- and large-molecule processes for process monitoring to increase process knowledge and as an enabling technology for process automation and control to increase process efficiency and reduce process variability. One particular example was the design and implementation of online HPLC analyzers for closed-loop control of chromatographic purification processes in the production of Humulin, a biosynthetic insulin.

B. Non-analytical Application of HPLC

1. Determination of partition-coefficient.
2. Measurement of activity coefficient at infinite dilution.
3. Measurement of heat of solution and heat of vaporization.
4. Measurement of partial molar excess free energy and entropy of solution.
5. Study of complex formation and determination of stability constant.
6. Determination of second visual coefficient.
7. Determination of rates of reaction.
8. Determination of gaseous diffusion coefficient.
9. Measurement of interfacial resistance.
10. Determination of surface area.

HPLC Applications

- AD/HD drugs
- Amines
- Amino acids
- Analgesics
- Anesthetics
- Anti-inflammatories
- Antibiotics, antimicrobials
- Anticholesterolemic
- Anticholinergics
- Anticoagulants
- Anticonvulsants
- Antidepressants
- Antiglaucoma drugs
- Anthelmintic drugs
- Antihistamines
- Antihypertensives
- Antimalarials
- Antineoplastic
- Antiobesity drugs
- Antioxidants
- Antiparkinsonians
- Antipsychotics
- Antiretrovirals
- Antirheumatics
- Antitussives
- Antiulcer compounds
- Anxiolytics, sedatives
- Bronchodilators
- Carbohydrates
- Catalysts
- Decongestants
- Diuretics
- Drugs of abuse
- ED drugs
- Fatty acids, FAMEs
- Flavors, fragrances
- Glycols, diols
- Hormones
- Hydrocarbons
- Immunosuppressants
- Muscle relaxants
- Mycotoxins
- Nucleic acids
- Organic acids
- Organic bases
- Organic chemicals
- Peptides
- Pesticides and herbicides
- Phenols
- Preservatives
- Proteins
- Semivolatiles
- Steroids
- Surfactants
- Vitamin

HPLC can also be Used in Many Research Fields

1. To study metabolic pathways and reproductive mechanisms in basic biochemical research.
2. To follow cell growth and reproduction in physiology.

3. In chemical industries it is important in quality control.
4. It is used for measuring levels of certain compounds such as amino acids, nucleic acids, and proteins in physiological samples.
5. It is used for spotting metabolic and genetic defects.
6. It is used to analyze protein and peptides.
7. It is used for the separation of alkaloids and vitamins especially the water-soluble ones.
8. It is used for the analysis of antibiotics such as penicillins, streptomycins, polypeptide antibiotics.
9. It is used for measuring the levels of active drugs, synthetic by-products, or degradation products in pharmaceutical dosage forms.
10. It is used to measuring levels of hazardous compounds such as pesticides or insecticides.
11. It is used to monitor environmental samples.
12. It can also be used to purify compounds from mixtures.
13. It is used for separating polymers and determining the molecular weight distribution of the polymers in a mixture.
14. It is used in quality control.
15. It is used in synthetic reactions.
16. HPLC in chemical research: In organic, inorganic and analytical chemistry as well as in biochemistry we need to analyze polar nonvolatile compounds quickly, sensitively and accurately. Hence, we make use of HPLC (Figs 1.47 and 1.48).

Advantages of HPLC

1. It is fastest and precise quantitative analytical technique
2. Simplicity, specificity and wide range of sensitivity
3. Analysis of drugs in both dosage from and biological fluids can be done
4. Separation efficiency is 5–10 times greater than other methods
5. It is most widely used method for purity assay
6. Automated operation
7. High-sensitivity detection
8. Quantitative sample recovery

Limitations of HPLC

- Only one compound can be analyzed at a time
- Compound identification may be limited unless HPLC is interfaced with mass spectrometry
- Resolution can be difficult to attain with complex samples
- Requires training in order to optimize separations
- Time analysis can be long
- Sample preparation is often required

PDA detector Refractive index detector

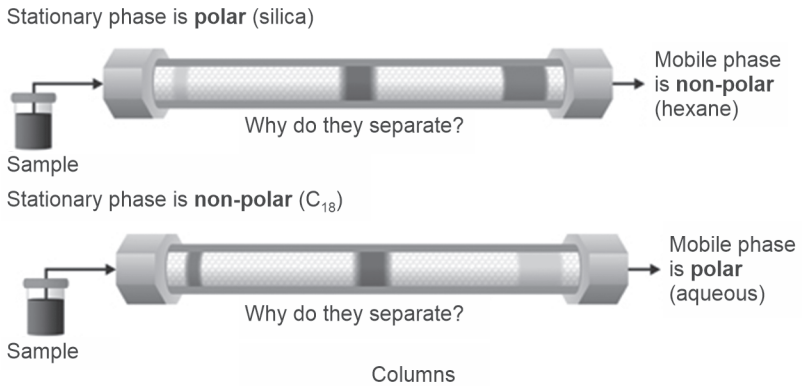


Fig. 1.47: Different stationary phases

- No universal detector
- Less separation efficiency than capillary GC

Related Techniques

- Mass spectrometry (MS) provides structural identification and information of molecular weight.
- Nuclear magnetic resonance (NMR) provides detailed information of molecular structure.
- Infrared spectroscopy (IR) provides functional groups information.

Auto-injector

Vacuum degassing

Single piston pump

Double piston pump

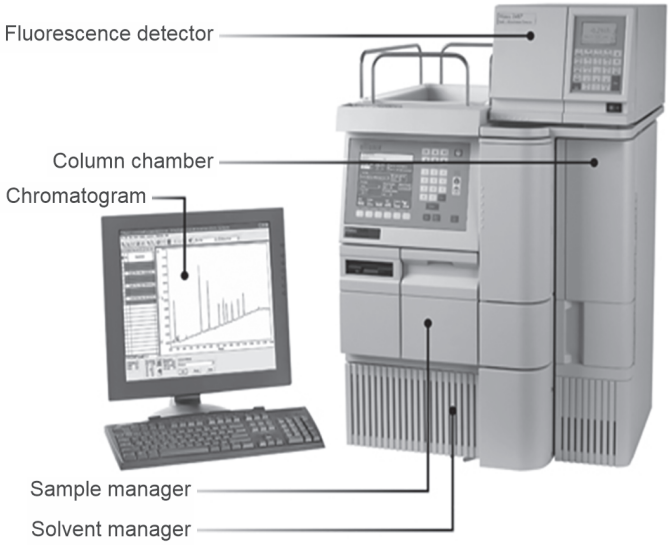


Fig. 1.48: Complete HPLC system