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# CHROMATOGRAPHY – THE ESSENCE OF BIOANALYSIS

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## ABSTRACT

A precise measurement of drug levels both in a pharmaceutical industry's perspective and health care setup is the need of the hour. Pharmaceutical companies spend extravagantly and also untiringly, day in and day out, to delineate a single successful drug moiety from thousands of lead compounds. In this pursuit, they rely on bio-analytical techniques which could help them in separating the most active elements from the crude mixtures. And also, later on in pre-clinical and clinical testing accurate measurement of drug levels in biological tissues using suitable analytical techniques are indispensable. Moreover, a treating physician depends on drug levels especially for those drugs with narrow therapeutic margin. Though the qualitative and quantitative analytical methods existing today are more sophisticated and complex, they actually originated and evolved from the roots of chromatography. Hence, chromatography still prevails as the most significant analytical method in molecular chemistry despite being primitive. This current indisputable status of chromatography is reflected by the fact that majority of the present techniques is based on the principle of chromatography. This review focusses on the various types of chromatography, their working principles and applications.

**KEYWORDS:** chromatography, paper chromatography, thin layer chromatography, high performance liquid chromatography, gas chromatography.

## INTRODUCTION

Chromatography is derived from the Greek words "*chroma"* meaning 'colour' and "*graphien"* meaning 'to write'. The technique was originally developed by the Russian Botanist M. S. Tswett in 1903. It is an analytical technique utilized for the separation, purification and identification of constituents from the mixture. It works on the principle of differential interaction of solutes with two different phases, viz., the stationary phase and the mobile phase. Many modifications were made to the techniques of chromatography to overcome the shortcomings like analysis time and the range of compounds that could be detected. Application of pressure was practised by use of pumps to reduce the time of run. Technologies like spectroscopy and electrochemical methods were added to enhance detection. With these developments and modifications the functional efficiency of chromatographic techniques improved to a great extent and also the range and type of substances that could be analysed.[1]

### Types of chromatography

Chromatography is divided into three broad types such as gas, liquid, and supercritical fluid chromatography. Figure 1 shows the basic classification of chromatography.

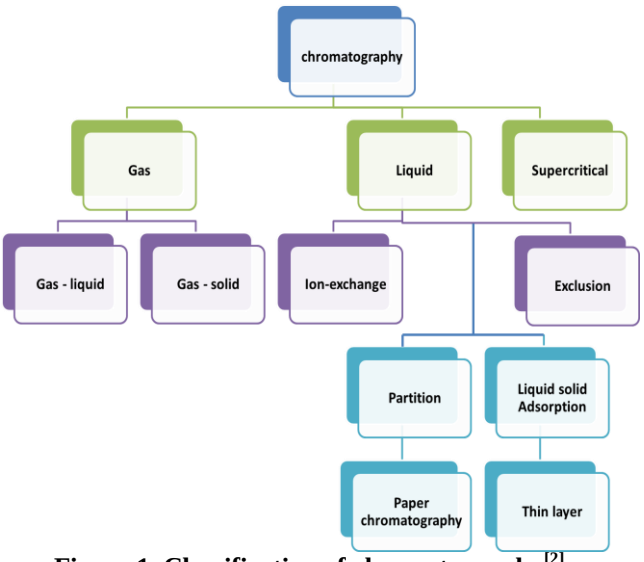


**Figure 1. Classification of chromatography**[2]

Table 1 portrays the different types of chromatography categorized based on various physical and chemical parameters.

**Table 1. Different types of chromatographic techniques**[3]

| Parameters | Types | |
|---|---|---|
| **Physical state of the phase** | Homogenous e.g., LLC | Heterogenous e.g., SLC, SGC, LGC |
| **Separating principle** | Adsorption e.g., SLC | Partition e.g., LLC, LGC |
| **Chemical nature of column (polarity)** | Normal phase | Reverse phase |
| **Shape of stationary phase** | Column e.g., HPLC | Planar e.g., Paper, TLC, HPTLC |
| **Purpose of chromatography** | Preparative | Analytic |
| **Physical and chemical character of stationary phase** | Size exclusion | Ion exchange |

The commonly used chromatographic techniques among those include.

**Planar chromatography**

1. Paper chromatography
2. Thin layer chromatography (TLC)
3. High performance TLC

**Column chromatography**

4. High Performance Liquid Chromatography (HPLC)
5. Ultra-high performance chromatography (UHPC)
6. Gas chromatography (GC)
7. Ion-Exchange chromatography (IEC)
8. Size-exclusion chromatography (SEC)
9. Affinity chromatography (AC)

**Planar chromatography**

**1. Paper chromatography (PC)**

It is a method of separating the mixture of compounds by using specially designed chromatographic paper as stationary phase into individual compounds.

**Principle**

Both partition and adsorption take place in paper chromatography. However, the primary one is partition chromatography wherein the substances are partitioned between two liquid phases, viz., the cellulose layers in filter paper containing moisture and the mobile phase. The movement of mobile phase, due to the capillary action of pores in the paper, separates the compounds in the mixture.

**Components (Figure 2)**

- Stationary phase and papers – Whatman filter paper of different grades, paper impregnated with silica or alumina.
- Mobile phase – pure solvents, mixture of solvents (methanol : water - 4:1).
- Sample applicator.
- Chromatographic chamber.

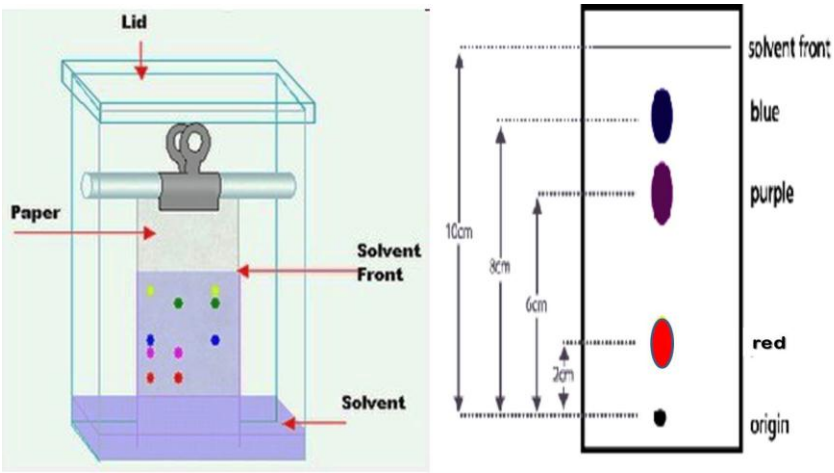


**Figure 2. Paper chromatography**

**Procedure**

The sample mixture is placed on the piece of chromatography paper which is later positioned in a solvent container. Individual components move to a varying degree of distances depending on the differences in their affinity to the adsorbent and the solvent. Polar molecules get adsorbed on the filter paper and taken to smaller distances whereas non-polar molecules travel

farther distances. The extent of movement of components is measured by calculating the "**$R_f$ value**".

$R_f$ value is defined as the distance travelled by the component from application point divided by distance travelled by solvent from application point. **$R_x$ value** is the ratio of distance travelled by the sample and the distance travelled by the standard. $R_x$ value is always closer to one. $R_f$ value is always less than one but $R_x$ can be greater than one. The factors affecting the $R_f$ value are the solvent system and its composition, temperature, pH of the solution, quality of paper and adsorbents and distance through which the solvent runs.

**Detecting agents**

For colourless substances physical and chemical methods are used to detect the spots. Physical methods – iodine chamber method and UV chamber for florescent compounds. Chemical methods – Ferric chloride, Ninhydrin in acetone, Dragendroff's reagents, 3,5 dinitro benzoic acid etc. However, for coloured substances these methods are not necessary.

**Modes of paper chromatography**

Based on the way in which the chromatogram on the paper is developed the following procedures exist.

1. **Ascending chromatography**: Like conventional type the solvent moves in upward direction on the paper. The solvent reservoir is at the bottom of the beaker.
2. **Descending chromatography**: Here the solvent reservoir is at the top. The movement of solvent is assisted by gravity (solvent flows down the paper) besides capillary action.
3. **Ascending-descending mode**: A hybrid of above two techniques where solvent first travels upwards and then downwards on the paper.
4. **Circular/ Radial mode**: Here the solvent travels from the centre towards the periphery of circular chromatography paper. The entire system is kept in a covered Petri dish for the development of chromatogram. The solvent flows onto the paper by means of a centrally placed wick. The sample moves along with the mobile phase to form spots of various compounds as concentric rings.
5. **Two-dimensional development**: The samples are spotted onto one end of the rectangular paper and allowed for first development. The second chromatogram is developed perpendicular to the previous one by immersing it in mobile phase subsequently. Hence, the chromatogram development takes place in two directions at right angles.

**Advantages**

- Simple and easily available equipment
- Better efficacy of separation
- Closely related homologous, isotopes, isomers and very labile and reactive substances can be separated.

**Applications**

Specially used for separation of mixture having polar and non-polar compounds. For separation of pigments, dyes and inks, amino acids. To determine organic and other biochemical compounds in urine. For determination of hormones and drugs. Evaluation of inorganic compounds like salts and complexes.

**2. Thin layer chromatography (TLC)**

Schraiber, in 1939, developed and employed thin layer chromatography for the first time. Modern TLC mainly exists as a complementary technique to other column-based liquid chromatographic methods to provide additional information in separations (multi-modal separation techniques). TLC plays a crucial role in the early stage of drug development when information about the impurities and degradation products in drug substance and drug product is inadequate.[4]

**Principle**

TLC works on the principle of adsorption. However, adsorption and partition or a combination of both is usually present. The elements with more affinity towards stationary phase travel slower and vice versa.

**Components (Figure 3)**

TLC plates – stable and chemically inert plates used as a support for stationary phase (glass, plastic, or aluminium support)

TLC chambers – used for the development of TLC plate, maintenance of uniform environment.

Stationary phase (solid phase) – thin layer (0.25 mm thick) of adsorbent coated on a TLC plates.

Filter paper – prevents the evaporation of solvents.

Mobile phase – comprises of a solvent or solvent..

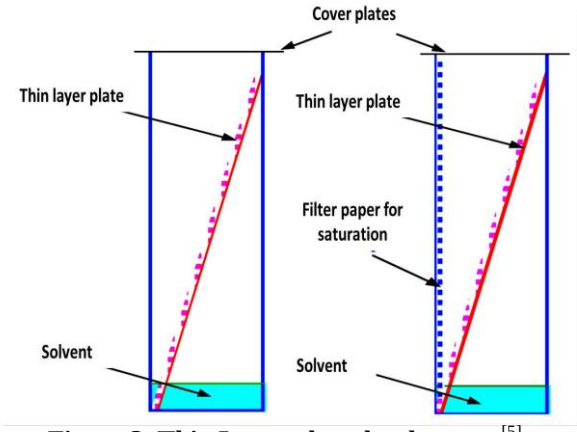


**Figure 3. Thin Layer plate development**[5]

**Procedure**

Near the bottom of the thin layer plate, the sample mixture spots are placed. Solvents are allowed to percolate up the plate by capillary action. The chamber is saturated with solvent vapour so as to prevent the solvent evaporating from the plate surface and also controlling the retention mechanism by surface deactivation. The plate is then placed in the chamber without allowing

dipping of sample spot. A constituent that is strongly adsorbed will move slower. Results are represented by $R_f$ value same as in paper chromatography.

**Factors determining the efficacy**

The efficiency of chromatographic separation is determined by several factors such as adsorbent's selectivity towards the substances being separated and adsorbing power of adsorbent. Some of the commonly employed adsorbents in the decreasing order of their adsorptive power are as follows, alumina ($Al_2O_3$), charcoal (C), florisil [$MgO/SiO_2$ (anhydrous)] and silica gel ($SiO_2$).

**Advantages**

- simple, quick, inexpensive high sample throughput technique
- wide choice of mobile phases
- minimum sample preparation
- several samples can be run simultaneously using a small quantity of mobile phase
- used in analytical laboratories having limited resources.[6]

**Applications**

- It is used for separation of all classes of natural products. E.g., acids, alcohols, amines, macromolecules like amino acids and proteins, etc.
- Extensively used for identification and purification
- To keep a check on the performance of other separation processes
- To evaluate reaction process by assessment of intermediates, reaction course, etc.
- For separation of Inorganic Ions – Used for separating cationic & anionic substances
- Separation of vitamins – Vitamin E, Vitamin $D_3$, vitamin A
- Quantitative analysis[4,7-11]

**3. High performance thin layer chromatography (HPTLC)**

It is an advanced form of TLC with a rapid separation technique, flexible enough to separate a wide variety of samples and requires a short analysis time to analyse the complex or the crude sample clean-up.

**Key characteristics**

- Simultaneous processing of sample and standard - better analytical precision and accuracy less need for internal standard.
- Lesser analysis time and lower cost per analysis; lower maintenance cost.
- Simple sample preparation - handle samples of divergent nature.
- No prior treatment for solvents like filtration and degassing.
- Low mobile phase consumption per sample.
- No interference from previous analysis – fresh stationary and mobile phases for each analysis –no contamination.[6]

**Procedure**

Prepared sample and standards are applied on the pre-washed and pre-conditioned chromatographic layer with the help of applicator machine. Then it is placed in the chromatographic chamber containing mobile phase and allowed to separate. The developed chromatogram is read with the aid of detectors or scanners.

**Preconditioning**

The equilibrium between the solvent vapour and the plate will not be the same as the equilibrium between the solvent and the plate. This is very similar to normal development but the developing solvent is present in a separate reservoir. For equilibrium with the solvent vapour, the plate is placed in the enclosure and allowed for a few minutes. Then the plate is so placed dipping its end into the developing solvent and the separation processed in the usual way. **Figure 4** shows the apparatus that is used for the pre-equilibrium of a thin layer plate and also the differences between the two TLCs.

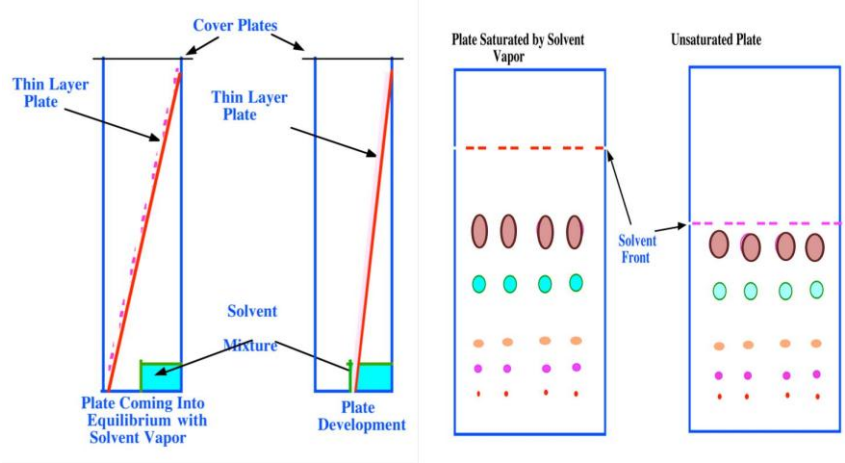


**Figure 4. High performance thin layer chromatography**[5]

As noted in the figure, pre-saturation of the TLC plate leads to increased velocity of the solvent front relative to that of the unsaturated plate and also separated components are much closer to the solvent front in the unsaturated plate.

**Applications**

- An established technique in pharmaceutical industry for determination and purification of many drugs.
- Food and drug analysis - herbal drug quantification, analysis of vitamins, water soluble food dyes, pesticides in fruits, vegetables, and other food stuffs.
- Forensic - finger print analysis, identification of abuse drugs, poisons, adulterations, chemical weapons, and illicit drugs.
- Cosmetology and environmental analysis[8,10]

**Column chromatography**

Column chromatography leads to higher resolution than other purification methods and is thus more efficient in terms of purification of proteins. A glass or metal column is used to pack the stationary phase. The stationary phase is either layered on small discrete particles and packed into the column or smeared as a thin film on the inside wall of the column. After application of the mixture of analytes, the eluent is passed through the column either by use of a pumping system or applied gas pressure. The analytes separate on the basis of their distribution coefficients as the eluent flows through the column. They emerge individually in the eluate as they leave the column. The detector system placed between the column and the fraction collector is used for the monitoring.

Elution of the analytes retained by the stationary phase with the mobile phase can be done by the application of either high pressure or low pressure. In general, if chromatographic procedure carried out at pressures <5, between 6 and 50, and more than 50 bars are considered as low pressure, medium pressure and high pressure (1 bar = 14.5 pounds per square inch) respectively. The flow of eluent through the column should be at a uniform rate.[12]

**Components**

A typical column chromatographic system using a gas or liquid mobile phase consists of the following components (**Figure 5**).

• A stationary phase - as appropriate for the separation of analytes

• A column

1) o Conventional type : filled with the stationary phase
2) o Microbore (tubular) type: stationary phase is coated directly on the inside wall of the column (PLOT - porous layer open tubular column, SCOT - support coated open tubular column, WCOT – wall coated open tubular column)

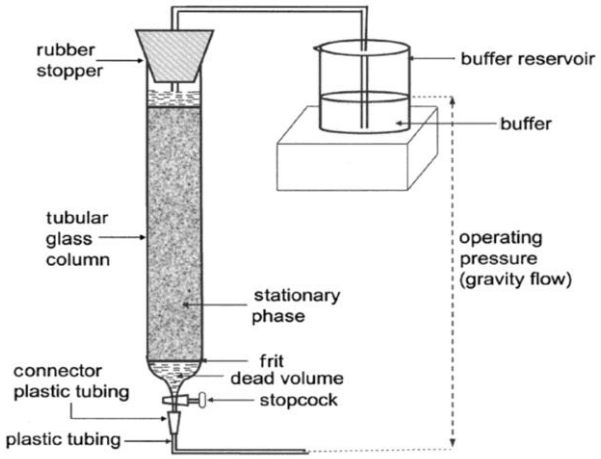


**Figure 5. Components of column chromatography**

- A mobile phase and delivery system - Complement the stationary phase to discriminate between the sample analytes and to deliver a constant rate of flow into the column.
- An injector system - To deliver test samples to the top of the column in a reproducible manner
- A detector and chart recorder – A peak on the chart recorder represents each analyte.
- A fraction collector - For collecting the separated analytes for further biochemical studies.

Column chromatographic techniques can be subdivided on the basis of the analyte development and elution modes.

**Analyte development**

• Zonal development - analytes in the sample are separated on the basis of their distribution coefficients between the stationary and mobile phases.

• Displacement or affinity development - analytes in the sample are separated on the basis of their affinity for the stationary phase.

**Elution**

Isocratic elution - Composition of the mobile phase is constant. E.g., GC and some forms of HPLC

- Gradient elution - Composition of the mobile phase (pH, salt concentration or polarity) is changed continuously or in a stepwise manner to facilitate separation. E.g., HPLC

Two processes occur concurrently in any chromatographic separation to affect the behaviour of each analyte

1) Basic processes - adsorption, partition, ion exchange and molecular exclusion. These characterise the interaction of each analyte with the stationary phase.
2) Secondary processes - diffusion (oppose the separation). These manifest as a broadening and tailing of each analyte band resulting in poor resolution. The analytical challenge is to minimise these secondary processes

**Chromatographic parameters and parts of a chromatogram (Figure 6)**

- Baseline - any part of the chromatogram where only mobile phase is emerging from the column
- Peak maximum - highest point of the peak
- Injection point - position time where the sample is placed on the column
- Dead point - position of the peak-maximum of an unretained solute
- Dead time ($t_o$) - time elapsed between the injection point and the dead point
- Dead volume ($V_o$) - volume of mobile phase passed through the column between the injection point and the dead point; $\mathbf{V_o = Qt_o}$ where Q is the flow rate in ml/min.

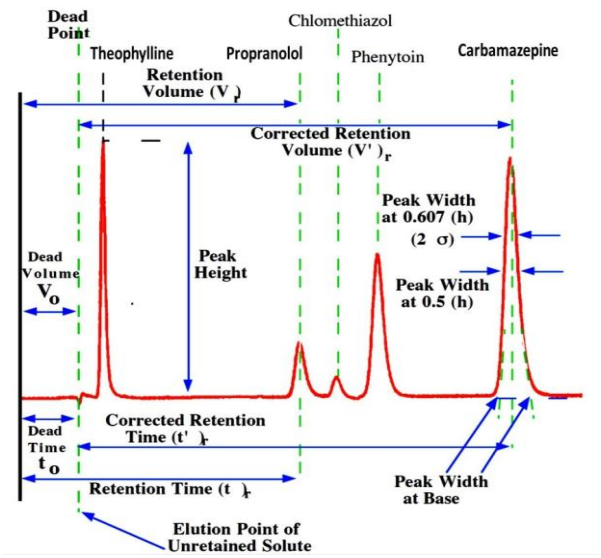


**Figure 6. Parts of a chromatogram**

- Retention time ($t_r$) - time elapsed between the injection point and the peak maximum of solute (It is the time required for 50% of a component to be eluted from a column)

It is determined by the length of the column, L, and the migration velocity of the solute $\mathbf{t_r = L/u_s = L(1+k)/v}$

With $t_0$, the residence time of a non-retained component

$\mathbf{t_0 = L/v}$

$\mathbf{t_r = t_0(1+k) \rightarrow t_r = t_0 + t_0(k) \rightarrow t_r = t_m + t_s}$

$t_s$ - It is the k´-fold dead-time ($t_0.k$)

$\mathbf{t_r = t_0 + t_0(k) \rightarrow k = (t_r - t_0)/ t_0}$

- Retention volume ($V_r$) - volume of mobile phase passed through the column between the injection point and the peak maximum of solute; $\mathbf{V_r = Qt_r}$
- Corrected/adjusted retention time ($t'_r$) - time elapsed between the dead point and the peak maximum $\mathbf{[t'_r = t_r - t_o]}$
- Corrected retention volume ($V'_r$) - volume of mobile phase passed through the column between the dead point and the peak maximum. It will also be the retention volume minus the dead volume; $\mathbf{[V'_r = V_r - V_o = Q(t_r - t_o) = Q\ t'_r]}$
- Peak height (h) - distance between the peak maximum and the base line geometrically produced beneath the peak
- Peak width (w) - distance between each side of a peak measure at 0.6065 of the peak height. The peak width measured at this height is equivalent to two standard deviations (2s) of the Gaussian curve and thus has significance when dealing with chromatography theory
- Peak width at half height ($w_{0.5}$) - distance between each side of a peak measured at half the peak height. The peak width measured at half height has no significance with respect to chromatography theory
- Peak width at the base ($w_B$) - distance between the intersections of the tangents drawn to the sides of the peak and the peak base geometrically produced. The peak width at the base is equivalent to four standard deviations (4s) of the Gaussian curve and thus also has significance when dealing with chromatography theory
- Relative retention time - retention time for the analyte divided by that for the standard
- Retention factor (capacity factor) (k) - additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase; $\mathbf{[k = (t_r - t_0)/ t_0 = t'_r/ t_0]}$

This tells that analyte in the stationary phase is k times the mobile phase at any point in the column at any time. It is evident, therefore, that k is related to the distribution coefficient of the analyte, which was defined as the relative concentrations of the analyte between the two phases. Since amount and concentration are related to volume, this can be written as.

$\mathbf{k = t'_r/ t_0 = M_s/M_m = K_d \times V_s/V_m}$

$M_S$ and $M_m$ are the masses of analyte in the stationary and mobile phase, respectively

$V_S$ and $V_m$ are the volumes of analyte in the stationary and mobile phase, respectively

$V_S/V_m$ - volumetric phase ratio (β)

$\mathbf{k = K_d\ \beta}$

- Distribution or partition coefficient ($K_d$) - basis of all forms of chromatography, which explains how a compound distributes between two immiscible phases.

$\mathbf{K_d}$ **= Concentration in phase A/ Concentration in phase B**

Retention factor (k) changes with the change in both the distribution coefficient between the two phases and the volume of the stationary phase and it range from 1 to 10. Retention factors are important as they are independent of the physical dimensions of the column and the flow rate of mobile phase through it. They are also a reflection of the selectivity of the system that in turn is a measure of its inherent ability to discriminate between two analytes.

- Selectivity or Separation factor (α) - relative retention ratio for the two analytes

$\mathbf{\alpha = k_A/k_B = K_{dA}/K_{dB} = t'_{rA}/ t'_{rB}}$

The selectivity factor is influenced by the chemical nature of the stationary and mobile phases.[5,13]

- **Theoretical plate**

An imaginary functional unit of the column where equilibrium has been established between stationary and mobile phases. The length of column containing one theoretical plate makes up the plate height (H). Efficiency of a column is expressed by the number of theoretical plates in the column or HETP (Height Equivalent to a Theoretical Plate). 'N' is no. of plates per column.

If HETP is less, greater the value N, the column is ↑ efficient, the narrower is the analyte peak. If HETP is more, lesser the value N, the column is ↓ efficient.

In chromatography, spatial standard deviation ($\sigma_z$) increases with increasing migration distance. This increase is not proportional, but under-proportional related to the distance. Whereas variance ($\sigma_z^2$), the square of the standard deviation in the space domain is directly proportional to the length of migration (z)

$\sigma_z^2 \alpha z \rightarrow \sigma_z^2 = Hz$

The proportionality constant (H) is known as plate height (height equivalent of a theoretical plate, HETP) and has the dimension of a length.[14]

The measure of the column efficiency is denoted by the plate number 'N' given by L divided by the plate height,

$$N = \frac{L}{H} = \frac{Lx}{\sigma^2}$$

If the position of a peak emerging from the column is such that z = L, width of the peak at its base, w, obtained from tangents drawn to the two steepest parts of the peak, is equal to 4σ (this is a basic property of all Gaussian peaks) hence σ = w/4 therefore

$$N = \frac{L^2}{\sigma^2} = \frac{16L^2}{w^2}$$

If both 'L' and 'w' are measured in units of time rather than length, then $N = \frac{16tr^2}{w^2}$

**Peak broadening**

Factors causing peak broadening are the application of the sample to the column, longitudinal diffusion, multiple pathways (Eddy diffusion) and equilibration time between the two phases **(Figure 7)**.

- Longitudinal diffusion and equilibration time are the factors influenced by the flow rate of the eluent.

Longitudinal diffusion **α** 1/flow rate; equilibration time **α** flow rate

- These two factors together with that of the multiple pathways factor determine the value of the plate height

The precise relationship between the three factors and plate height is expressed by the Van Deemter equation, i.e.,

$$\text{Plate height } (H) = A + \frac{B}{Ux} + CUx$$

$U_x$ - flow rate of the eluent and A, B and C are constants for a particular column and stationary phase relating to multiple paths, longitudinal diffusion and equilibration time respectively.

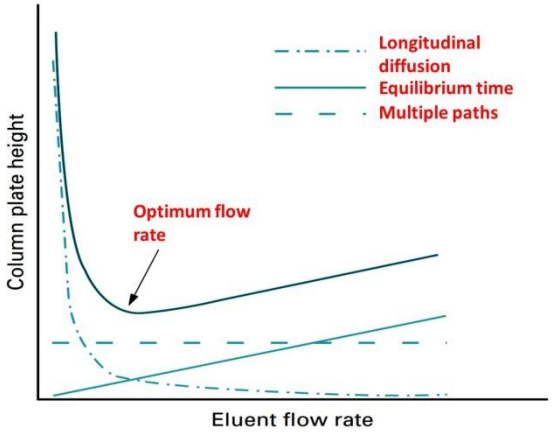


**Figure 7. Example of a van Deemter plot.**

**Resolution**

Resolution is the measure of true separation of two consecutive peaks on a chromatogram. It is the measure of both column and solvent efficiencies.

Resolution ($R_S$) is defined as the ratio of the difference in retention time ($\Delta t_R$) between the two peaks ($t_{RA}$ and $t_{RB}$) to the mean ($w_{av}$) of their base widths ($w_A$ and $w_B$).

$Rs = 2\Delta t_R / (W_A + W_B)$

**Liquid chromatography**

It is a basic column chromatography in which liquid act as a mobile phase and inert solid substances like silica gel, alumina or cellulose supported in a column act as a stationary phase. It is vital that the stationary phase is saturated with solvent while setting up a liquid chromatography column because any air present will interrupt the smooth flow and will result in inefficient or incomplete separation.[15]

A more elaborate variation on liquid chromatography is high performance liquid chromatography (HPLC).

**4. High performance liquid chromatography (HPLC)**

HPLC is a superior form of liquid chromatography and is one of the most used analytical techniques. High pressure should be applied to have an eluent flow through the column due to the physical properties of HPLC columns. Because of this earlier the method was termed as high pressure chromatography.[4,8]

**Principle**

The main principle of separation is adsorption

Factors determining the Resolving power ($R_s$) are

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{1+k_{av}}\right)$$

- number of theoretical plates (N) in the column
- column selectivity (α)
- column retentivity (k)

**Chromatographic analysis**

- The number of peaks obtained determines the number of components in a sample
- The amount of a given component in a sample is determined by the area under the peaks.
- The retention time aids in the identity of components

Small particle size stationary phase which increases the separation efficiency forms the basis for HPLC. This is because the solute can equilibrate more rapidly between the two phases. Larger particle size stationary phase forms the basis of low-pressure liquid chromatography in which flow of the eluent through the column is either gravity-fed or pumped by a peristaltic pump (low pressure pump). It is cheaper to run but lacks the high resolution. As the size of the particles of stationary phase decreases, surface area increases and indirectly number of plates increases and hence increased resolution. But resistance to the flow of the mobile phase increases with these small particle sizes due to increased capillary action. This resistance creates a backpressure which reduces the flow rate and it becomes harder to drain the column under gravity. To overcome this high pressure system has to be applied to maintain the flow of solvent.[15]

**Components of HPLC system (Figure 8)**

**Mobile phases**

Up to four different eluents can be handled by HPLC instruments. High purity eluents should be used. In isocratic elution, single eluent or two or more eluents premixed in fixed proportions may be made with a single pump whereas in gradient elution separate pumps are used to deliver two eluents in proportions predetermined by a gradient programmer. Eluent, which is strong in normal phase HPLC, is weak in reversed phase HPLC and the opposite is also true.

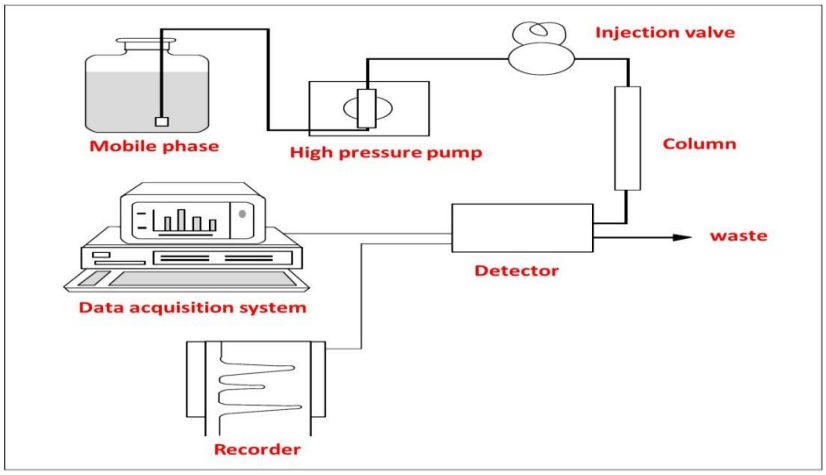


**Figure 8. Schematic representation of an HPLC system.**

**Buffer systems**

**Composition**

Elution buffer composition should not influence the resolution.

**Column elution**

Suitable eluent kept in a buffer reservoir is passed through the column at a stable uniform rate for separation of sample containing the components. The sample is uniformly applied to the top of the stationary phase bed.[12]

**Filters and degassers**

The membrane filter is necessary to remove dust particles. Degassing by warming, by stirring vigorously with a magnetic stirrer, by applying a vacuum, by ultra-sonication, and by bubbling helium gas through the eluent reservoir.

**Pumping system**

Special pumps are available for the transport of eluent and are one of the most important features of HPLC systems. The function of the pump is to force a mobile phase through the liquid chromatograph at a specific flow rate (mL/min). Pressures in the range of 6000-9000 psi (400 to 600 bar) can be attained by these pumps. Standard flow rates in HPLC are in the range of 1 to 2 mL/min. Good pumping system should have main features like pressure capability of at least 50 MPa, no pulses (i.e. cyclical variations in pressure).[13,16]

**Sample introducer**

The most common method of sample introduction is by use of a loop injector. It consists of a small volume metal loop that can be filled with the sample [5-to 20-microliters (μL)]. By changing the position of the valve,

the eluent is channelled through the loop and the sample is flushed onto the column. HPLC injectors can be manual ones but in case of high number of samples an auto sampler is more convenient to apply.[5]

**Columns**

The column is often called the "heart of chromatography" in separation process, and the availability of stable, high performance stationary phases and columns is critical to the development of reproducible and robust methods. Silica is the most commonly used column packing material for its reliable strength and rigidity, relative inertness and ability to be modified chemically. Many changes have been made to improve the morphological and physico-chemical characteristics of these silica materials. Fully porous silica microspheres [octadecyl-silica (ODS-silica), which contains C18 coating] are the most commonly used because they offer many important benefits in HPLC columns such as good efficiency, high sample loading, durability and wide commercial availability.

**Types of columns in HPLC depending on the use**

•Analytical [internal diameter (i.d.) 1.0 – 4.6-mm; lengths 15 – 250 mm]
•Preparative [i.d. > 4.6 mm; lengths 50 – 250 mm]
•Capillary [i.d. 0.1 – 1.0 mm; various lengths]
Tubing materials
•Stainless steel (the most popular; gives high pressure capabilities)
•Glass (mostly for biomolecules)
•PEEK polymer (biocompatible and chemically inert to most solvents)

Reduced eluent consumption, ideal for interfacing with a mass spectrometer due to the slower flow rates and increased sensitivity due to the higher concentration of analytes that can be used are the advantages of open tubular columns over conventional.[13]

Newer columns are made of stainless steel with highly polished interior walls, plastic material, glass-lined inner surfaces and outer column surfaces made from the rigid polymer [polyether ether ketone (abbreviated as PEEK)], soft polymeric material for the outer surface and by compressing the column radially.

HPLC is divided into normal phase (NP) HPLC and reversed phase (RP) HPLC methods based on the polarity of the stationary and the mobile phase.

The introduction of column ovens has significantly improved method development by ensuring a more reliable and reproducible process. The development of eluent mixing systems in HPLC has meant that efficient mixing of solvents is now possible for isocratic and gradient elution.[4]

Guard column – maintains the resolving power by preventing the impurities entering into the analytical column. It is a short (12 cm) column having same internal diameter and contains packed material similar to analytical column and often installed between the injector and the column.

**Applications**

HPLC is optimum for the separation of chemical and biological compounds that are non-volatile and thermally unstable, viz.,

- Pharmaceuticals like aspirin, ibuprofen, or acetaminophen.
- Sodium chloride, potassium phosphate and other salts.
- Proteins like egg white or blood protein.
- Organic chemicals like polymers (e.g., polystyrene, polyethylene).
- Motor oil and other hydrocarbons.
- Many natural products such as ginseng, herbal medicines, plant extracts.
- Thermally unstable compounds such as trinitrotoluene (TNT), enzymes.

HPLC instruments are everywhere in drug research and development, pharmaceutical manufacturing, quality assurance, diagnostics, toxicology, research and other laboratories.[16]

**6. Gas Chromatography (GC)**

Gas Chromatography is a highly sophisticated analytical procedure in which the mobile phase is gaseous.

**Types**

• **Gas-solid chromatography** (stationary phase is solid) – acts by the principle of adsorption
- Not in use because of limited number of stationary phases
• **Gas-liquid chromatography** (stationary phase is liquid that is fixed or immobilized on a certain support material) – acts by the principle of partition and adsorption.

**Components**

- Supply of carrier gas in a high pressure cylinder with attendant pressure regulators and flow meters. Carrier gas: He (common), $N_2$, $H_2$ and argon- methane (He is preferred due to high thermal conductivity. $N_2$ is preferable when large consumption of carrier gas is employed.)

A flow rate of 25 to 150 mL/min is preferred for the packed column whereas a flow rate of 1 to 25 mL/min for the open tubular column.

Pressure regulators: Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 mL/min), capillary restrictors, and a pressure gauge (1-4 atm). Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.[17]

- **Sample injection system**

Sample in liquid form is injected using microsyringe. The injector must be kept at some high temperature to change into vapour phase for easy analysis. The sample is directly injected into heated port which has a temperature higher than that of the oven. The volume to be injected for a packed column and capillary column are 1-20 µL and $10^{-3}$ µL, respectively.

- The separation column - The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape. Copper is useful up to $250^0$C. Several sizes of columns are used depending upon the requirements.

Column: 2-50 m coiled stainless steel/glass/Teflon
Types of columns depending on its use:
1. Analytical column 1-1.5 meters length, 3-6 mm diameter
2. Preparative column 3-6 meters length, 6-9mm diameter
Types of columns depending on its nature:

- Packed column - adsorption and partition
- Open tubular or Capillary or Golay column
- WCOT (wall coated open tubular) - partition
- SCOT (sorbent coated open tubular) - partition
- PCOT (porous layer open tubular) - adsorption
- Detector
- Recorder
- Separate thermostat compartments for columns and detectors (Oven: 0-400 °C)

**Procedure**

Components of the mixture which are vaporized in heat bar attach strongly or weakly to the stationary phase in the column. Mobile phase takes away the weakly bound component first to the outlet of the column. Thus, the components will be separated according to the boiling point, molecular weight, polarity, ability to for hydrogen bonding with the stationary phase.[18] The partial pressure of the solute in the gas and the sensitivity of the detector to the sample determines the magnitude of the output. The output is recorded as the amount of solute leaving the column as a function of time nothing but chromatogram **(Figure 9)**.

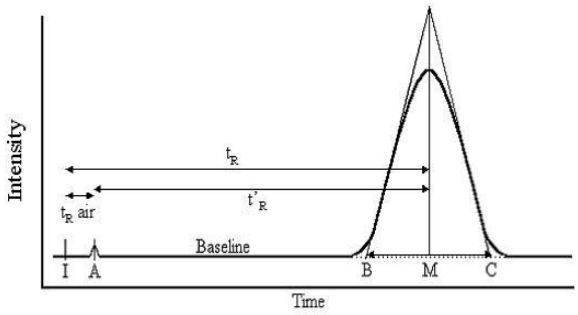


**Figure 9. Schematic representation of an HPLC system.**

**Chromatographic parameters**

The components will appear at the end of the column at different times, $t_R$ , thus more than one peak will be recorded with different retention times. The degree of separation can be controlled by changing the oven temperature, mobile phase, stationary phase or column length.

- The ratio of the distance between two peak maxima to the mean value of the peak width gives the resolution between two peaks, i.e.,

$$\mathbf{Rs = 2(t_{Ra} - t_{Rb}) / (W_a + W_b)}$$

- **Corrected Retention Time ($t'_R$):** The time between the appearance of the air peak and the solute peak ($t_R$-$t_{Rair}$)
- Some detectors do not produce air peak then corrected retention time is calculated from the column flow rate.
- **Retention Volume, $V_R$:** The volume of the carrier gas which succeed to separate the component.

$$\mathbf{V_R = t_R . F}$$

F = average volumetric flow rate in mL/min
F is estimated by measuring flow rate exiting the column using soap bubble meter

**Chromatographic Analysis**

- The number of peaks obtained determines the number of components in a sample
- The amount of a given component in a sample is determined by the area under the peaks.
- The retention time aids in the identity of components

**Detectors in Gas chromatography**

For a reasonable GC analysis the detector needs to be sensitive ($10^{-8}$-$10^{-15}$ g solute/s), operate at high temperature (0-400°C), stable and reproducible, linear response, wide dynamic range, fast response, simple (reliable), non-destructive, uniform response to all analytes. The detectors used are thermal conductivity detector (TCD), atomic emission detector, thermionic detector, electron capture detector, flame photometric detector and flame ionization detector.[19]

**Advantages**

- Complex mixtures can be resolved into its components owing to its very high resolution power
- Very high sensitivity with TCD
- It is a micro method, even a small sample size is sufficient
- Fast analysis is possible, gas as moving phase- rapid equilibrium
- Relatively good precision & accuracy
- Qualitative and quantitative analysis

**Applications**

- Quality management - $R_t$ & $R_V$ are used for the identification & separation

- Purity control, Environmental and pharmaceutical analysis - chromatogram of the standard & that of the sample are compared
- Quantitative analysis of main and trace components - It is necessary to measure the peak area or peak height of each component[19]

**Other chromatographic techniques**

❖ **Ion-Exchange chromatography (IEC)**

Process that allows the separation of ions and polar molecules based on their charge Stationary phase: cation- or anion exchangers.

- Zeolites (sodium-aluminum-silicate) (cation exchanger)
- Cation exchanger resins, - anion exchanger resins

Mobile phase: aqueous solution of ionic compounds (e.g., phosphate, formate, etc.)

Principle: Different affinity of the ions of the mixture to the ion-exchanger

Ion-exchange: $RH + K^+A \rightarrow RK + H^+A$

The geometrical size and electrical charge of the ions determines the selectivity.

**Applications**

- Analysis of mixtures of amino acids (amino acid analyzers)
- Separation of charged compounds like anions, cations, amino acids, peptides, and proteins.

❖ **Size-exclusion chromatography (SEC)**

SEC is also known as gel permeation chromatography (GPC) or gel filtration chromatography and separates molecules according to their size by their ability to penetrate a sieve-like structure-the stationary phase. Molecules that are larger than the average pore size exclude first with no retention time.

**There are two modes,**

- non-aqueous SEC [sometimes termed as Gel Permeation Chromatography (GPC)]
- aqueous SEC [sometimes referred to as Gel Filtration Chromatography (GFC)]

**Applications**

- Preparative separations of macromolecules of biological origin
- Purification of synthetic organic polymers
- Determination of tertiary and quaternary structure of purified proteins

❖ **Affinity chromatography (AC)**

It is a novel kind of chromatography which utilizes high specific interactions (non-covalent interaction between an analyte and affinity ligand covalently attached (immobilized) to the stationary phase. This is the most selective type.

e.g., Antigen by Antibody, Enzyme by Inhibitor /Substrate /Cofactor/coenzyme

**Applications**

- Purification of proteins (based on interaction between a protein of interest, and a ligand immobilized on a stationary phase substrate or product analogue)
- Separation of biomolecules based on the relative affinity for the metals like Zn, Cu, Fe, etc. (Immobilized Metal Affinity Chromatography (IMAC).

❖ **Supercritical fliud chromatography (SFC)**

SFC is a hybrid of a gas chromatography and liquid chromatography

- Mobile phase is carbon dioxide
- To separate thermally labile molecules
- Separation of chiral compounds

❖ **Chiral column chromatography**

- Chiral stationary phase
- For separation of enantiomers or racemic mixture[15,16]

## CONCLUSION

Though there are numerous types of analytical techniques as discussed above, the selection of the most appropriate method for a particular pharmaceutical is to be individualized. The search for the most specific or the so-called “gold standard” technique for a pharmaceutical analyte begins even in the very early phases of drug development. Hence, development of novel bio-analytical techniques (including novel chromatographic techniques) along with further advancements in the existing practices is vital for the making of a successful drug.

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