Pharmacognosy

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**Chromatography**

Some materials appear homogenous, but are actually a combination of substances. For example, green plants contain a mixture of different pigments. We can separate these materials by dissolving them in an appropriate liquid and allowing them to move through an absorbent matrix .Chromatography is a method used by scientists for separating organic and inorganic compounds so that they can be analyzed and studied. The word chromatography means "color writing” which is a way that a chemist can test liquid mixtures. While studying the coloring materials in plant life, a Russian botanist invented chromatography in 1903. The ‘chromatographic procedure’ originated by Tswett is one of the most useful techniques of general application. The use of charcoal for the decolorization and clarification of solutions is well known; coloured impurities. A light petroleum extract of green leaves is allowed to percolate slowly through a column of powdered calcium carbonate contained in a vertical glass tube. The pigmented contents of the solution are adsorbed on the substance of the column and undergo separation as percolation proceeds. The more strongly adsorbed pigments, xanthophyll and the chlorophylls, accumulate in distinct, characteristically coloured bands near the top of the column, while the less strongly adsorbed pigments, the carotenes, accumulate lower down.

Chromatography is used in many different ways. It is used to determine unknown substances. The Police and other detectives use chromatography when trying to solve a crime. It is also used to determine the presence of cocaine in urine, alcohol in blood and lead in water. Adsorption chromatography has proved particularly valuable in the isolation and purification of vitamins, hormones, many alkaloids, cardiac glycosides, anthraquinones etc.

 **There are several important terms in chromatography:**

**1) Stationary phase**: also called column, adsorbent, bed, sorbent, opposing force, and retardation force.

**2) Mobile phase:** also called solvent, effluent, and eluent

**3) Chromatographic system:** means the whole conditions of chromatography e.g. stationary phase, mobile phase, temperature, dimension, method of detection…..etc.

**4) Developing:** means separating mechanism (addition of mobile phase that cause separation).

**5) Chromatogram:** the result of the separation procedure.

**Principles of Chromatography**

 The separation of mixture of compound in chromatography to it is components depend on the action of two forces:

1) Mobile force (driving force) that will try to move the components of mixture.

2) Opposing force (stationary or retardation force) that will try to keep components in it is place depending on many factors:

a) Solubility in mobile phase.

b) Adsorption ability of component to be separated.

c) Ionic forces.

\*Any  Chromatography   system is composed of three Components :

* Stationary phase
* Mobile phase
* Mixture to be separated

We can only control stationary and mobile phase as mixtures are the problem we have to deal with. Chromatography is a dynamic process in which the mobile phase moves in definite direction.

**Classification of Chromatography Methods**

Different methods were attempted for classification of chromatography:

**A/ According to mechanism of separation:** The mechanism of separation depends mainly on the nature of the stationary phase. Based on separation mechanisms chromatography can be classified into:

**1/ Adsorption Chromatography:**It is the oldest and most common type of chromatography. The stationary phase is a solid with adsorption power. Mixture components will be adsorbed on the surface of the stationary phase with different powers and that account for separation. Silica gel is the most common stationary phase in adsorption chromatography.

**2/ Partition Chromatography:** The stationary phase is a liquid forming a thin film on inert solid acts as support.  The stationary liquid is usually more polar than the mobile liquid. The two liquids must be immiscible with each other. Cellulose powder and wet silica gel are examples of supports in partition chromatography that carry film of water act as stationary phase. Partition chromatography is preferable over adsorption when dealing with polar compounds

**3/ Ion Exchange Chromatography**: It is used for separation of charged molecules. The stationary phase is an ion exchange resin to which cationic or anionic groups are covalently bonded. Ions of opposite charges (counter ions) in the mobile phase will be attracted to the resin and compete with the components of the mixture for the charged group on the resin. Both the mixture components and the mobile phase must be changed. Mixture of Alkaloids (compounds with positive charges) can be separated on anionic exchanger, while mixture of organic acids (negative charges) can be separated using cationic exchanger. Both types are used for desalination of water

**4/ Molecular Exclusion (Size Exclusion) Chromatography**: Stationary phase has pores of defined diameter. Very large molecules can't enter into the pores and so elute first, large molecule enter with difficulties and so elute second. Small molecules enter all pores and elute as last ones

**5/ Affinity Chromatography**: It uses the affinity of proteins to specific  ligands  such as enzymes. The ligand is attached to suitable polysaccharide polymer such as cellulose - agarose – dextran.

**B/ According to the mobile phase**

In this regard chromatography is classified into:

**1/ Liquid Chromatography (LC)**: The mobile phase is liquid. In case of separation by adsorption the stationary phase is solid so it is called: Liquid-Solid Chromatography (LSC). If separation occurs through partition the stationary phase is liquid so it is called: Liquid -Liquid    Chromatography (LLC).

**2/ Gas Chromatography (GC)**: Where the mobile phase is inert gas nitrogen or helium. Again if the stationary phase is solid it is called: Gas–Solid Chromatography (GSC). When stationary phase is liquid it called:  Gas-Liquid Chromatography (GLC).

**C- According to the technique applied** (methods of  holding  the stationary  phase)

**1- Planar or Plane Chromatography:** In this type of chromatography the stationary phase is used in the form of layer. Plane chromatography is further classified into:

* + - a- Thin Layer Chromatography (TLC): The stationary phase in the form of fine powder is spread on glass or plastic or aluminum sheets.
		- b- Paper Chromatography (PC): A specific type of papers is used as stationary phase in the form of sheets.

**2- Columnar or Column Chromatography (**CC): The stationary phase is held in to a tube made of glass or metal.

**D- According to purpose of use**

Chromatography can be used for analytical work and also to obtain pure materials from mixtures.

**1- Analytical Chromatography:**

**Qualitative Chromatography:** In this case Chromatography   can be used to:

1- Confirm the absence or probable presence of certain constituent in the sample under investigation:

2- Give an idea about the complexity of the mixture   and the least number  of  compounds  present.

3- Check purity and identity of any compound.

4- Monitor both column chromatography and organic chemical reactions.

**2- Preparative and Industrial scale Chromatography**: This was the first and is the main application of chromatography. The technique was developed primarily for this purpose. Chromatography is used to obtain reasonable quantities of pure compounds from mixtures.

**Different types of chromatography**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Technique** | **Stationary phase** | **Mobile phase** | **Basis of separation** | **Notes** |
| Paper chromatography | Liquid | liquid | polarity of molecules | compound spotted directly on a cellulose paper |
| Thin layer chromatography (TLC) | solid (silica or alumina) | liquid | polarity of molecules | glass is coated with thin layer of silica on which is spotted the compound |
| column chromatography | solid (silica or alumina) | liquid | polarity of molecules | glass column is packed with slurry of silica |
| Size exclusion chromatography | solid (microporous beads of silica) | liquid | size of molecules | small molecules get trapped in the pores of the stationary phase, while large molecules flow through the gaps between the beads and have very small retention times. So larger molecules come out first. In this type of chromatography there isn’t any interaction, physical or chemical, between the analyte and the stationary phase. |
| Ion-exchange chromatography | solid (cationic or anionic resin) | liquid | ionic charge of the molecules | molecules possessing the opposite charge as the resin will bind tightly to the resin, and molecules having the same charge as the resin will flow through the column and elute out first. |
| Affinity chromatography | solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and antibodies) | liquid | binding affinity of the analyte molecule to the molecule immobilized on the stationary phase | if the molecule is a substrate for the enzyme, it will bind tightly to the enzyme and the unbound analytes will pass through in the mobile phase, and elute out of the column, leaving the substrate bound to the enzyme, which can then be detached from the stationary phase and eluted out of the column with an appropriate solvent. |
| Gas chromatography | liquid or solid support | gas (inert gas like argon or helium) | boiling point of the molecules | samples are volatilized and the molecule with lowest boiling point comes out of the column first. The molecule with the highest boiling point comes out of the column last. |

**Column Chromatography**

Column chromatography is a separation technique in which the stationary bed is placed within a tube.

The stationary phase consists of very small particles or particles coated with a liquid in which case the solid acts as a support placed in a column. The particles of the stationary phase may be solid may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column).

Illustration of column chromatography with labeled terms

|  |  |
| --- | --- |
| Term | Definition |
| Mobile phase or carrier | solvent moving through the column |
| Stationary phase or adsorbent | substance that stays fixed inside the column |
| Eluent | fluid entering the column |
| Eluate | fluid exiting the column (that is collected in flasks) |
| Elution | the process of washing out a compound through a column using a suitable solvent |
| Analyte | mixture whose individual components have to be separated and analyzed |

**Principle of separation of different components:**

Differential affinities (strength of adhesion) of the various components of the analyte towards the stationary and mobile phase results in the differential separation of the components. Affinity, in turn, is dictated by two properties of the molecule: ‘Adsorption’ and ‘Solubility’.

We can define adsorption as the property of how well a component of the mixture sticks to the stationary phase, while solubility is the property of how well a component of the mixture dissolves in the mobile phase.

* Higher the adsorption to the stationary phase, the slower the molecule will move through the column.
* Higher the solubility in the mobile phase, the faster the molecule will move through the column.

So, the interplay between the above two factors determines the differential rates at which the different components of the analyte will move through the column. Adsorption and solubility of a molecule can be manipulated by choosing the appropriate stationary phase and mobile phase.

Suppose we have a mixture of two molecules A and B, where ‘A’ is a protein and ‘B’ is a lipid. Our column is packed with silica, which is polar in nature; our mobile phase is hexane, which is non-polar in nature. What do you think will happen when we load this mixture of A and B onto this column?

**INSTRUMENT:**

The instrument used consists of vertical glass tube, where the adsorbent is packed. A small plug of glass wool or sintered glass disc at the bottom of the tube supports the column.

**MECHANISM OF SEPARATION IN COLUMN CHROMATOGRAPHY**

There are two mechanisms of separation in column chromatography:

 (1) **Adsorption mechanism**: in which the stationary phase is solid and the mobile phase is liquid.

 (2) **Partition mechanism**: in this case the silica gel is exposed to water; water will surround the silica gel, here the silica gel acts as a support for water. Silica gel mixed with water then packed inside the column, then the column is eluted with the mobile phase which is water immiscible e.g. chloroform, n-butanol. Passing of the mobile phase through the column is known as ELUTION.

**There are different types of ELUTION:**

A) **Simple Elution**: one solvent or a mixture of the solvents is used from the beginning to the end of the procedure.

B) **fractional or step wise Elution**: by changing the mobile phase, during the procedure we can use another solvent for example more polar that will lead to further separation of the sample.

C) **Gradual Elution:** in this method we are not going to change the solvent completely but we are going to use a mixture of two solvents e.g. Ethyl acetate +5% Ethanol and then we can gradually increase the polarity of the mobile phase.

**Packing of column**

 There are two methods of packing:

1. WET METHOD.

2. DRY METHOD.

The wet method is preferred over the dry method.

**Wet packing method**

1. Place the small amount of the mobile phase in the column and allow the mobile phase to pass through the lower end of the column to remove the air bubbles that may be present in the column and which may be disturb the separation procedure.

2. Prepare the slurry by mixing Ethyl acetate and silica gel.

3. Pour the slurry in the column and allow setting down and removing the excess of the mobile phase. The mobile phase should always be above the silica gel to prevent the entry of air.

4. Place a small quantity of pure sand above the stationary phase to prevent the disruption of the stationary phase.

5. Place the sample (mix. of dyes).

6. Open the lower end to start separation.

7. Add Ethyl acetate from the upper end to reconstitute the Ethyl acetate removed from the lower end. (Inlet = outlet).

8. Collect the separated compounds after detection.

**DETECTION METHODS**

1. By using Ultra violet lamp.

2. Physical method.

3. Chemical fractional collector.

**Dry packing method**

* In this method the adsorbent is introduced as a dry powder with aid of vibration or packed with piston after that put a circular filter paper cut at a size equal to the circumference and placed at the top of the powder surface
* Introduce the sample in powder form
* Introduce another round filter paper
* The solvent is added on the surface of the filter paper and the tap is opened and the elution is continued
* After that the fractions is collected
* The disadvantage of this method is that any defect will be formed cannot be corrected

