INSTRUMENTAL METHODS OF ANALYSIS

B.PHARM I SEM – 7

UNIT -3 | PART-1

CHROMATOGRAPHY INTRODUCTION

CHROMATOGRAPHY

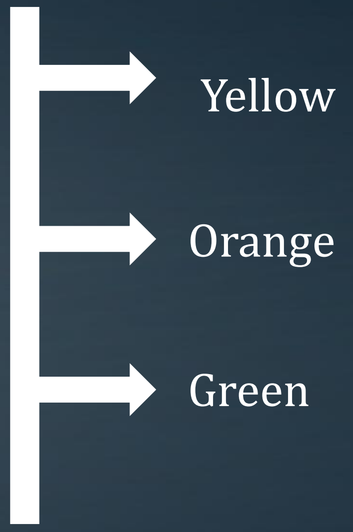
Separation Of Mixture Of Compounds

CHROMA = Color

GRAPHY = Writing

History

- **Michael Tswett** → Russian Botanist (1903)→ Column Chromatography
- **Stationary Phase** → Calcium Carbonate
- **Mobile Phase** → Liquid Hydrocarbon
- **Analyte** → Petroleum Ether Extract Of Plant



Chromatography terms

- **Analytical chromatography** is used to determine the existence and possibly also the concentration of analyte in a sample.
- Preparative chromatography is used to purify sufficient quantities of a substance for further use, rather than analysis.
- A bonded phase is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.

- A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

- A **chromatograph** is equipment that enables a sophisticated separation e.g., gas chromatographic or liquid chromatographic separation.
- **Chromatography** is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.
- The **eluate** is the mobile phase leaving the column.
- The **eluent** is the solvent that will carry the analyte.
- An **eluotropic series** is a list of solvents ranked according to their eluting power.

Chromatogram development technique

- **Frontal analysis**–A large volume of sample mixture is continuously passed through the column. Most weakly retained component of the mixture emerges first.
- **Displacement analysis**–Sample mixture is dissolved in large volume of solvent and applied to the top of the column. Mobile phase containing displacement agent is passed through the column.
- **Elution Analysis**–Most widely used technique. It can be used for quantitative applications.
  - **Isocratic elution** (Solvent composition or strength is not changed during column development)
  - **Gradient elution** (Solvent composition or strength is changed during column development).It is also known as solvent programming.

TECHNIQUE	STATIONARY PHASE	MOBILE PHASE
Column/Adsorption Chromatography	Solid	**Liquid**
Partition Chromatography	Liquid	**Liquid**
Paper Chromatography	Liquid	**Liquid**
Thin Layer Chromatography (TLC)	Liquid/Solid	**Liquid**
Gas – Liquid chromatography (GLC)	Liquid	**Gas**
Gas – Solid Chromatography (GSC)	Solid	**Gas**
Ion Exchange Chromatography	**Solid**	**Liquid**

Classification

- **On the basis of interaction of solute to stationary phase**

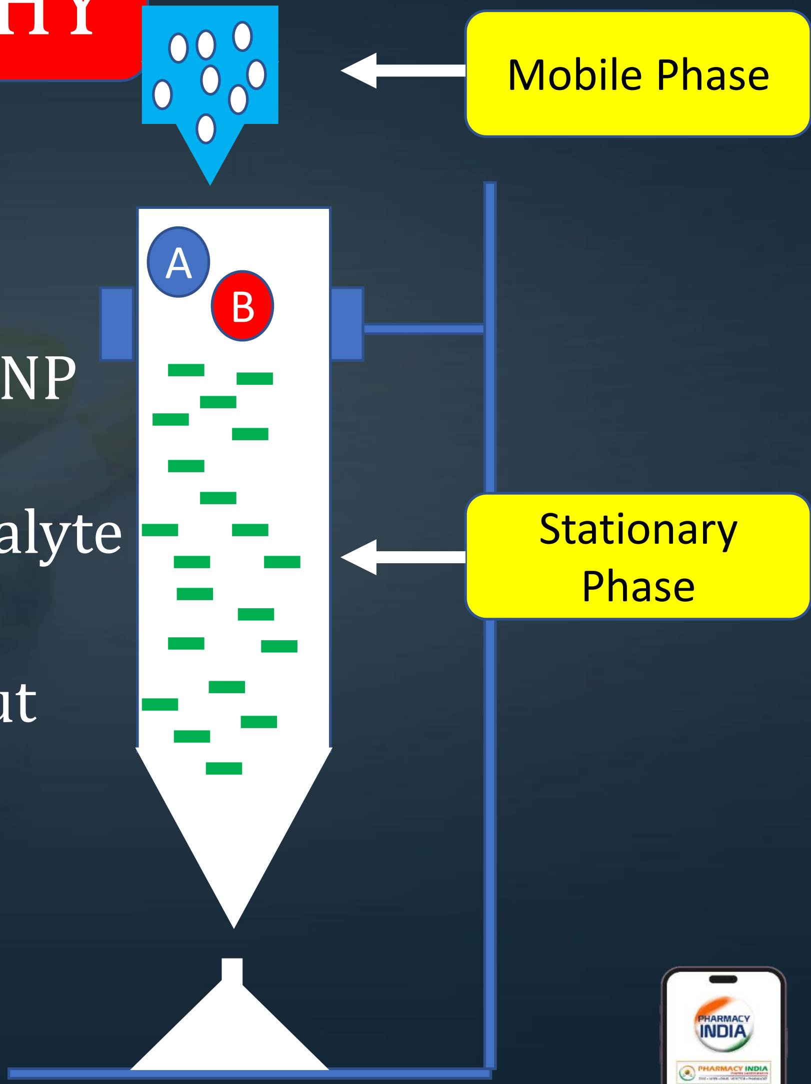
○ Adsorption chromatography
○ Partition chromatography
○ Ion exchange chromatography
○ Molecular exclusion chromatography

- ***On the basis of chromatographic bed shape***
    - Column chromatography
    - Thin layer chromatography
    - Paper chromatography
- ***Techniques by physical state of mobile phase***
    - Gas chromatography
    - Liquid chromatography
    - Affinity chromatography
    - Supercritical fluid chromatography

Types of Chromatography

Technique	Stationary phase	Mobile phase
Column/Adsorption Chromatography	Solid	Liquid
Partition Chromatography	Liquid	Liquid
Paper Chromatography	Liquid	Liquid
Thin Layer Chromatography (TLC)	Liquid/Solid	Liquid
Gas – Liquid chromatography (GLC)	Liquid	Gas
Gas – Solid Chromatography (GSC)	Solid	Gas
Ion Exchange Chromatography	Solid	Liquid

COLUMN CHROMATOGRAPHY



Normal Case

- Mobile Phase → Non-Polar
- Stationary Phase → Polar

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- Stationary Phase → Polar

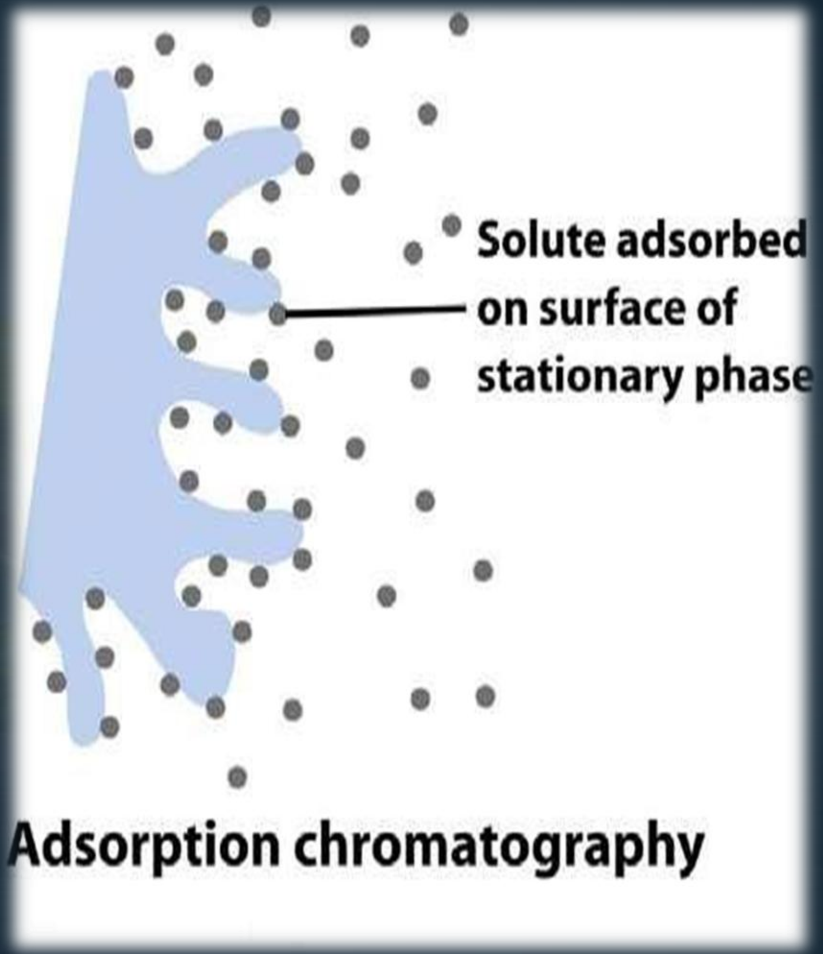
- Non-Polar MP → will take NP analyte
- Polar SP will take polar analyte

- So mobile phase (Non-Polar) will take analyte B & elute out
- After some time, A elute out

We note difference in time between elution which known as Retention Time

Adsorption Chromatography

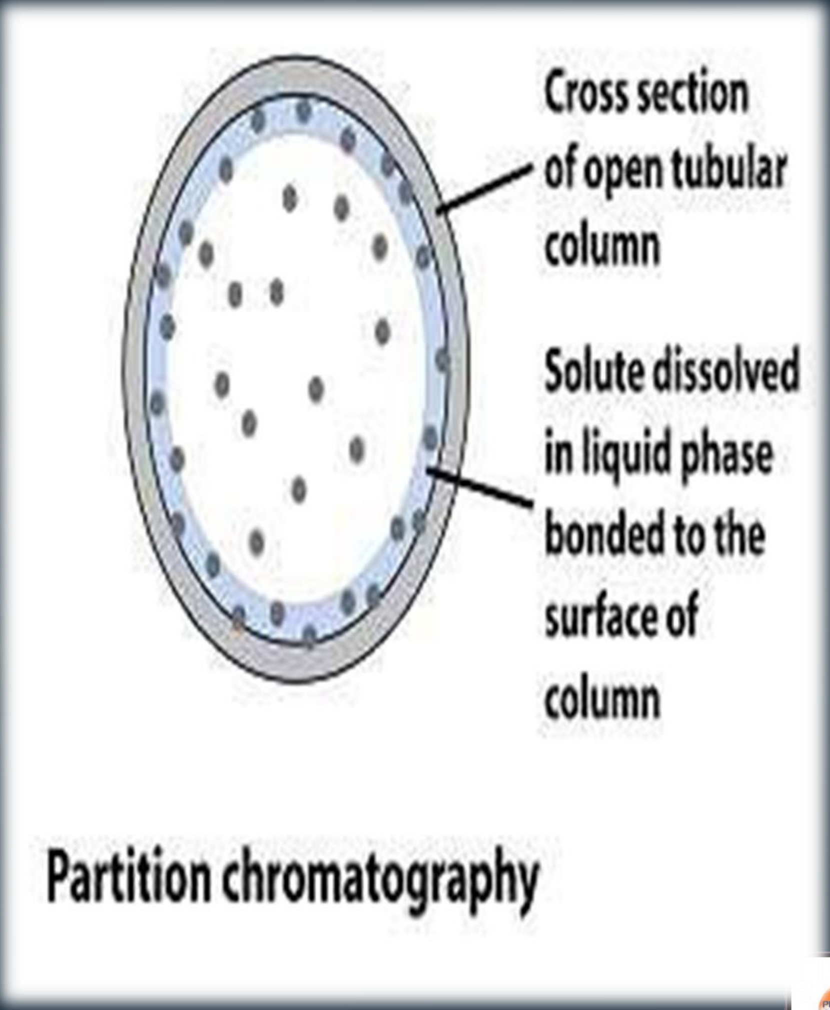
- Principle of separation utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase
- Stationary phase adsorbent filled in a tube (column)
- Mobile phase various solvents (eluents)



Adsorption chromatography

Partition Chromatography

- Principle : partition of component of sample between sample and liquid/gas stationary phase retard some components of sample more as compared to others. This gives the basis of separation.
- Based on thin film formed on the surface of a solid support by a liquid stationary phase.
- Solute equilibrates between mobile phase and stationary liquid phase.



Partition chromatography

COLUMN CHROMATOGRAPHY

❖ INTRODUCTION

- Column Chromatography was developed by the American chemist D.T Day in 1900, M.S. Tswett, the
- Polish botanist, in 1906 used adsorption columns in his investigations of plant pigments.
- Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids.
- This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid.

- **Principle → adsorption**
- Mixture of components dissolved in the mobile phase is introduced in to the column. Components moves depending upon their relative affinities.
- A compound attracted more strongly by the mobile phase will move rapidly through the column, and elute from, or come off, the column dissolved in the eluent.
- In contrast, a compound more strongly attracted to the stationary phase will move slowly through the column.

COMPONENTS OF COLUMN CHROMATOGRAPHY

- **Stationary phase**

O The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc.,

O Alumina is generally suitable for chromatography of less polar compounds. Silica gel gives good results with compounds containing polar functional groups.

O The particle size of the commercially available grade is in the range 50 – 200 µm.

Mobile phase

O They act as solvent, developer & eluent. The function of a mobile phase are:

1. As developing agent
2. To introduce the mixture into the column – as solvent
3. To developing agent
4. To remove pure components out of the column – as eluent

O Different mobile phases used: (in increasing order of polarity) : Petroleum ether, carbon tetrachloride, cyclohexane, ether, acetone, benzene, toluene, esters, water, etc.

O It can be used in either pure form or as mixture of solvents.

Column characteristics

O The main function of all the columns is to support the stationary phase.

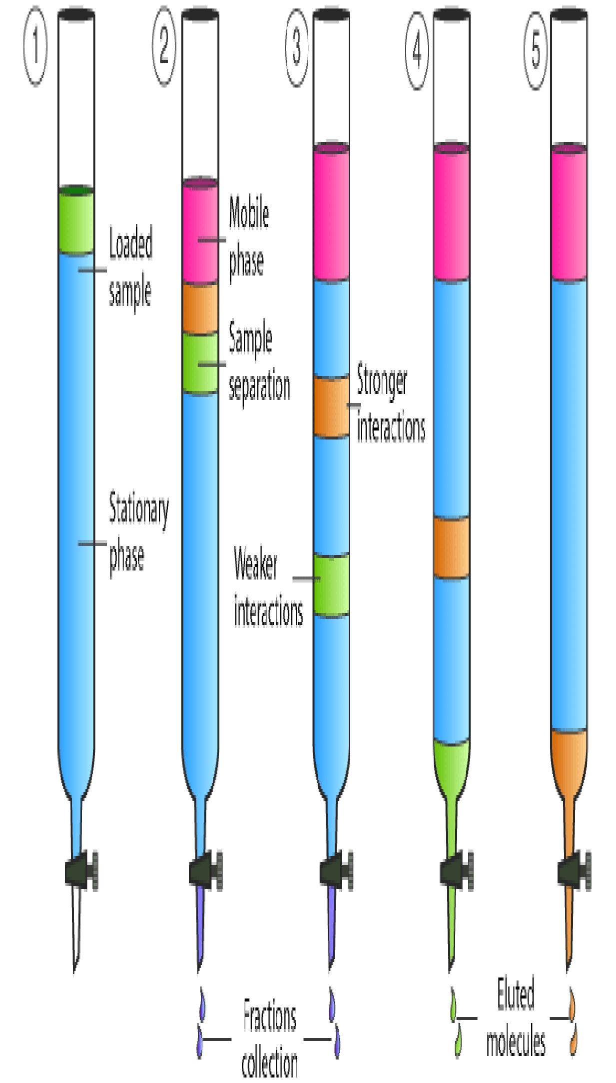
O The material of the column is mostly good quality neutral glass since it shouldn't be affected by solvents.

O An ordinary burette can also be used as column for separation.

O Column dimensions - length & diameter ratio (10:1, 30:1 or 100:1)

of the column for maintenance of the elution procesO Various accessories are attached to the top and bottom s.

O Better separation will be obtained with a long narrow column than short thick column because number of plates will be more.

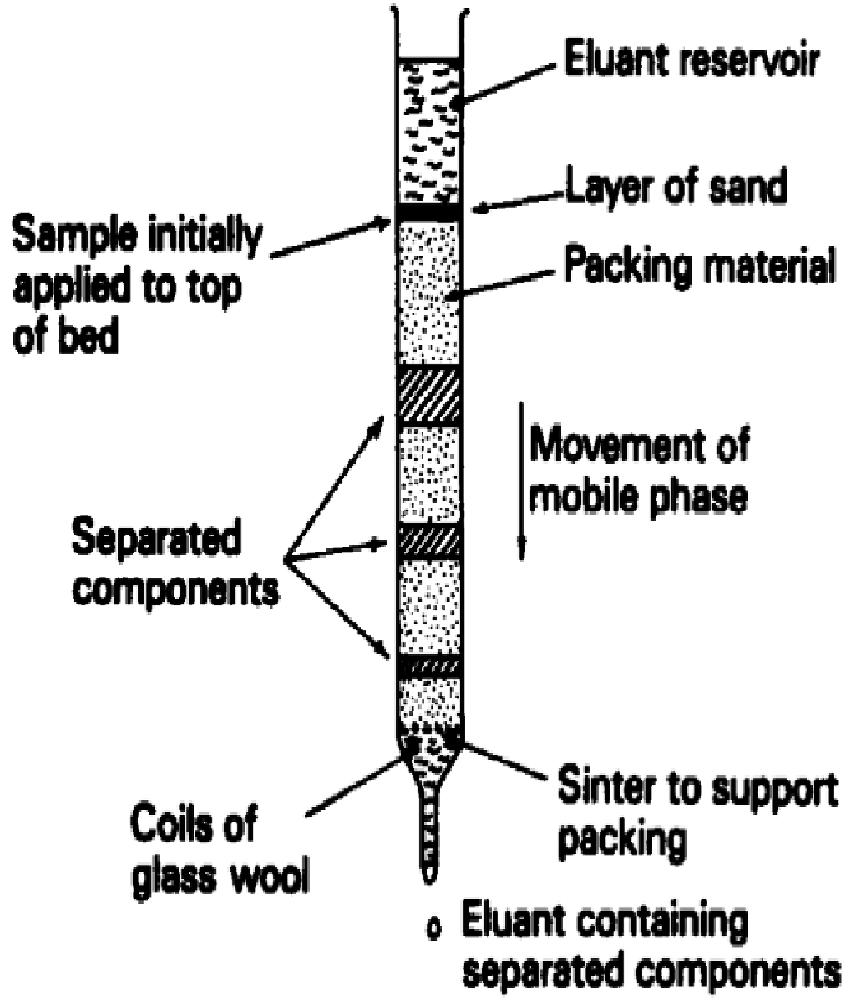


Preparation of the column

O It consists of a glass tube with bottom portion of the column – packed with glass wool/cotton wool or may contain asbestos pad

O Above which adsorbent is packed

O After packing a paper disc kept on the top, so that the adsorbent layer is not disturbed during the introduction of sample or mobile phase.



Packing techniques

O There are two types of preparing the column, they are:

1. Dry packing / dry filling

O Adsorbent is packed in the column in dry form

O Fill the solvent, till equilibrium is reached

O Demerit: Air bubbles are entrapped b/w mobile phase & stationary phasecracks appear in the adsorbent layer.

O After filling tapping can be done to remove void spaces.

2. Wet packing / wet filling

O The material is slurried with solvent and generally added to the column in portions.

O Stationary phase settles uniformly & no crack in the column of adsorbent.

O Solid settle down while the solvent remain upward.

O This solvent is removed then again cotton plug is placed.

DETECTORS	TYPE	SUPPORT GASES	SELECTIVITY	DETECTABILITY
Flame ionization (FID)	Mass flow	Hydrogen and air	Most organic compounds	100pg
Thermal conductivity (TCD)	Concentration	Reference	Universal	1ng
Electron capture	Concentration	Make up	Halides, nitrates, nitriles, peroxides, anhydrides, organometallics	50fg
Nitrogen-phosphorus	Mass flow	Hydrogen and air	Nitrogen, phosphorus	10pg
Flame photometric	Mass flow	Hydrogen and air possibly oxygen	Sulphur, phosphorus, tin, boron, arsenic, germanium, selenium, chromium	100pg
Photoionization	Concentration	Make up	Aliphatics, aromatics, ketones,	2pg

Applications

- Separation of mixture of compounds
- Purification process
- Isolation of active constituents
- Estimation of drugs in formulation
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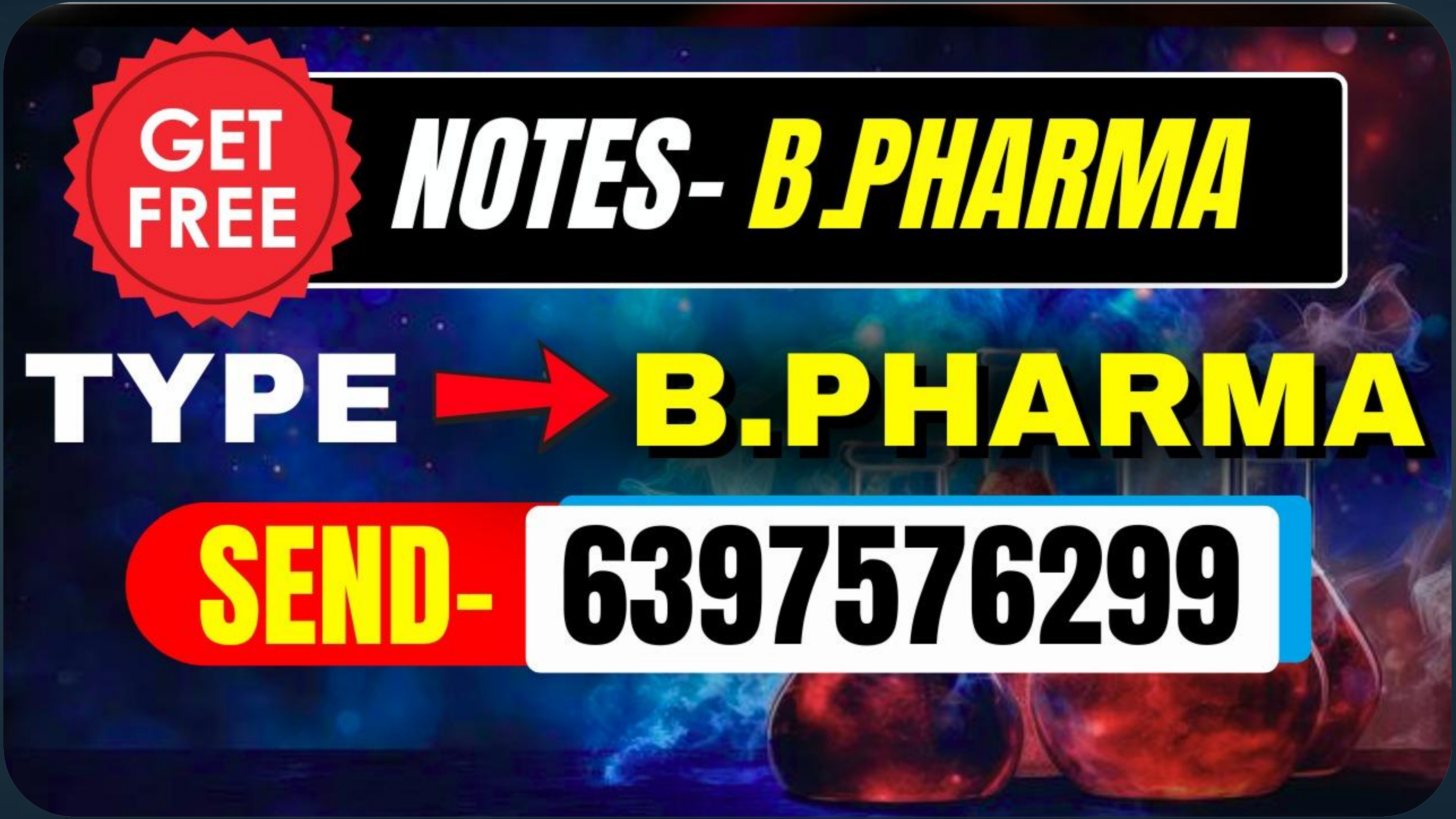
- Determination of primary and secondary glycosides in digitalis leaf.
- Separation of diastereomers



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UNIT-3

PART-2

INSTRUMENTAL METHODS OF ANALYSIS

CHROMATOGRAPHY

- ADSORPTION AND
- PARTITION COLUMN
- CHROMATOGRAPHY

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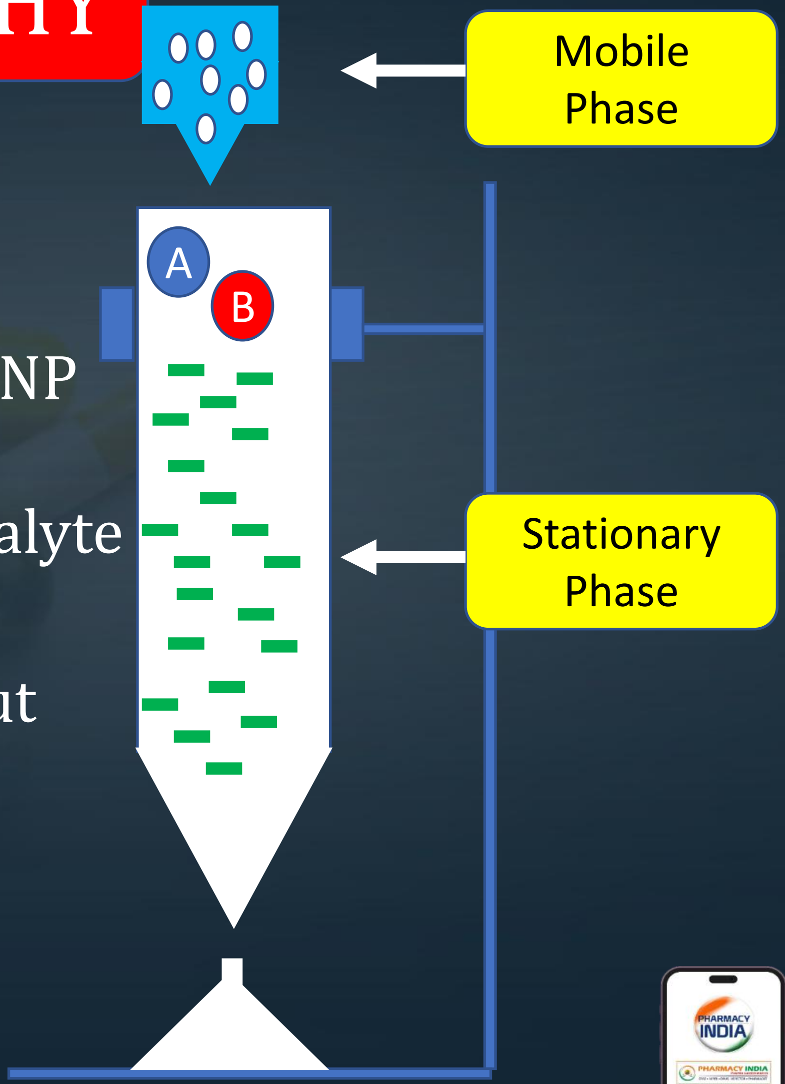

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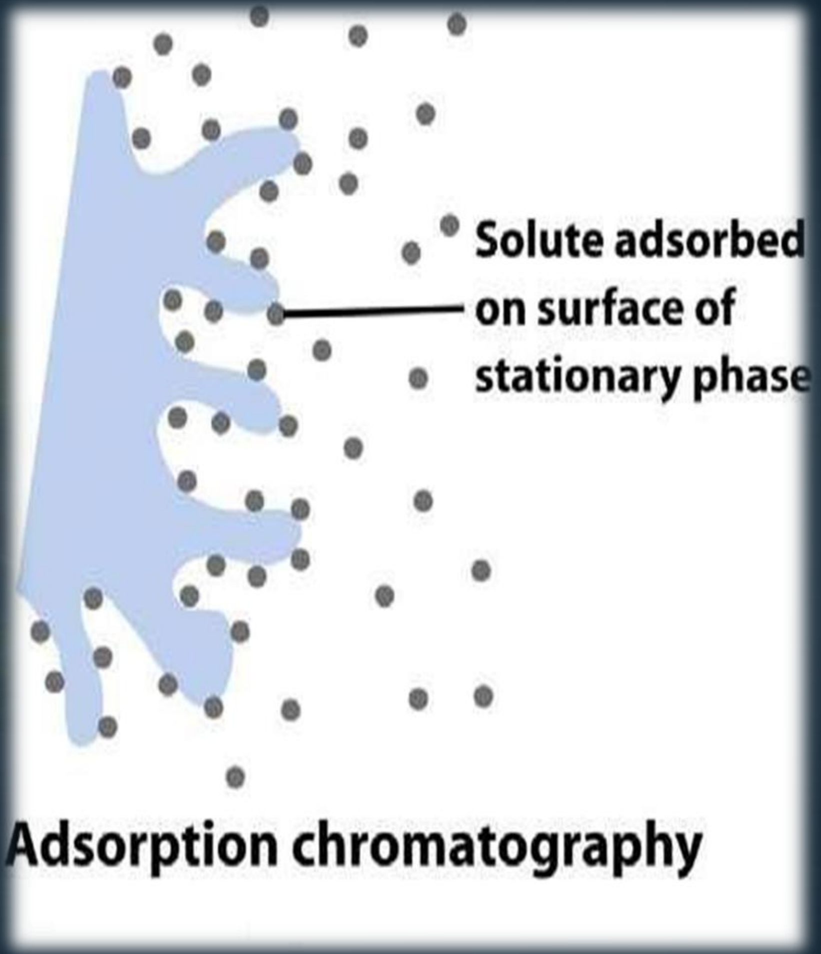

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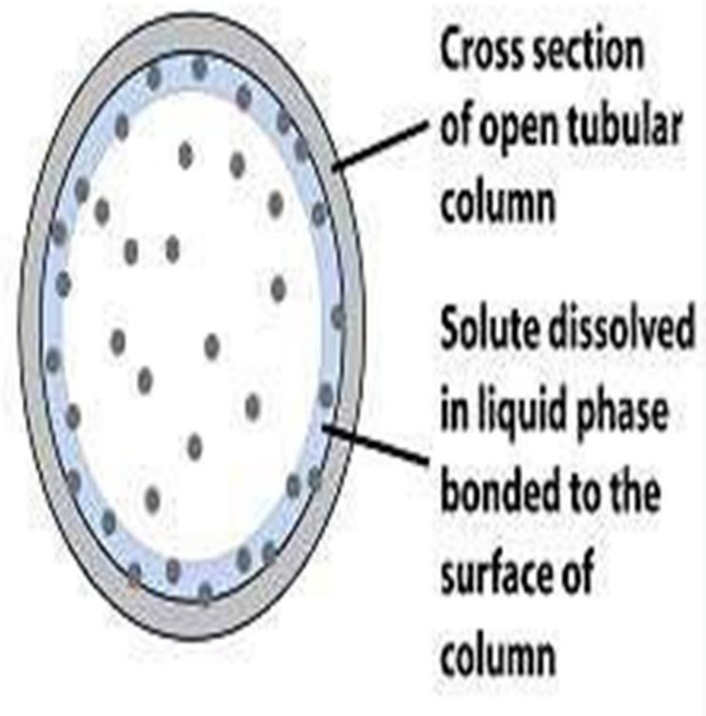

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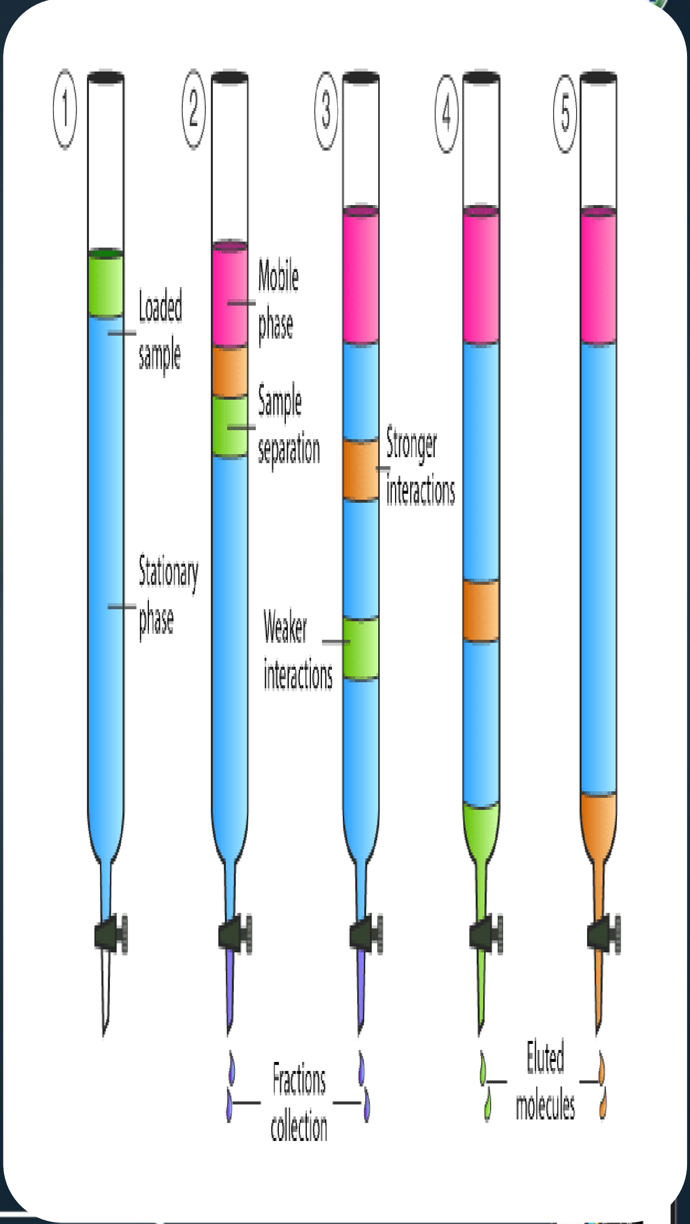

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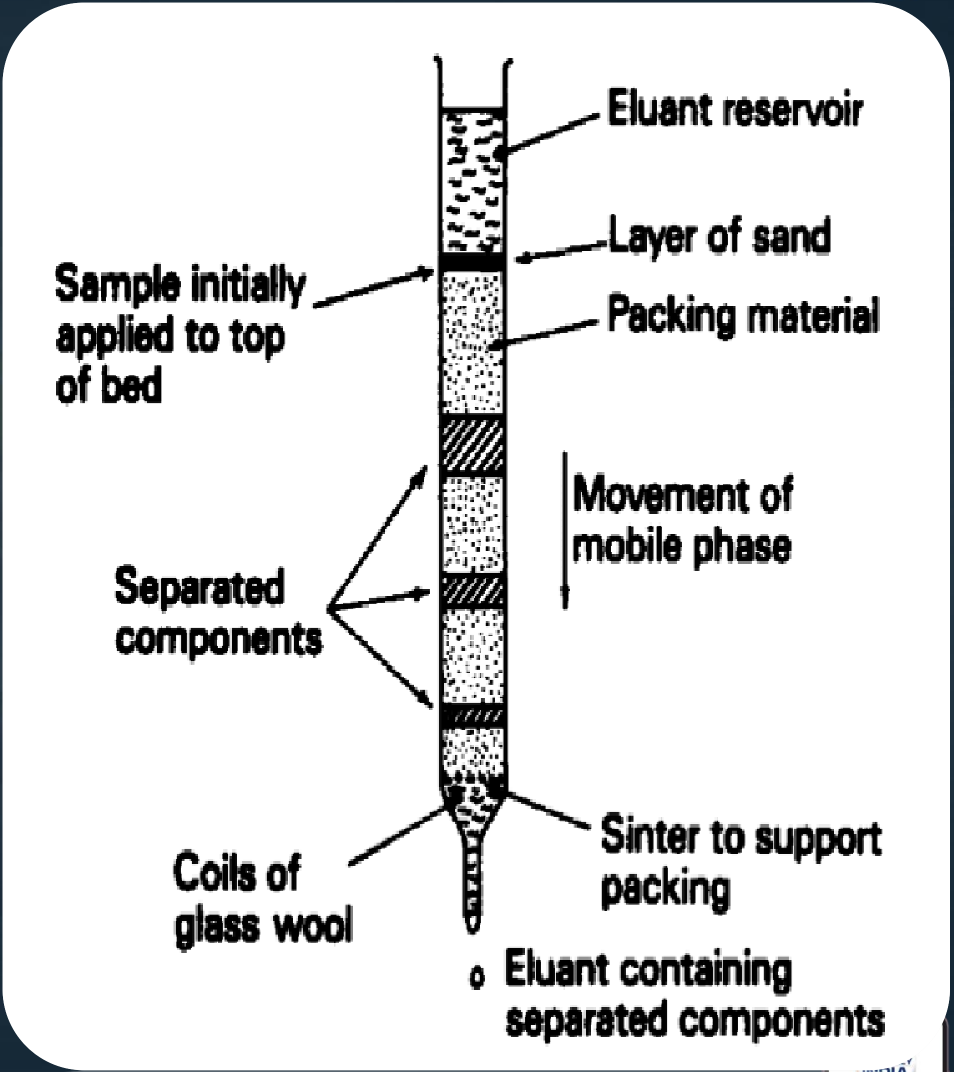

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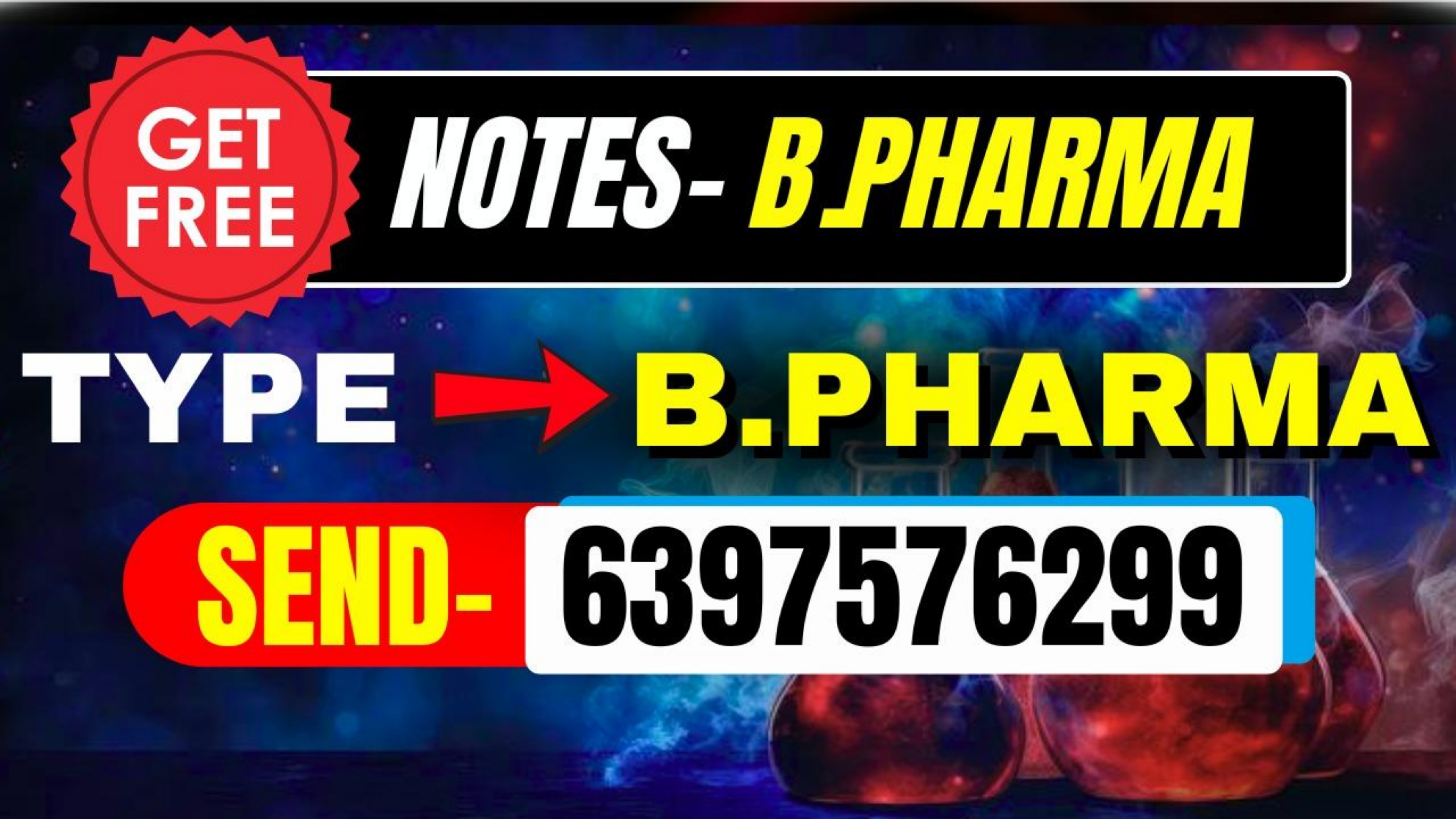
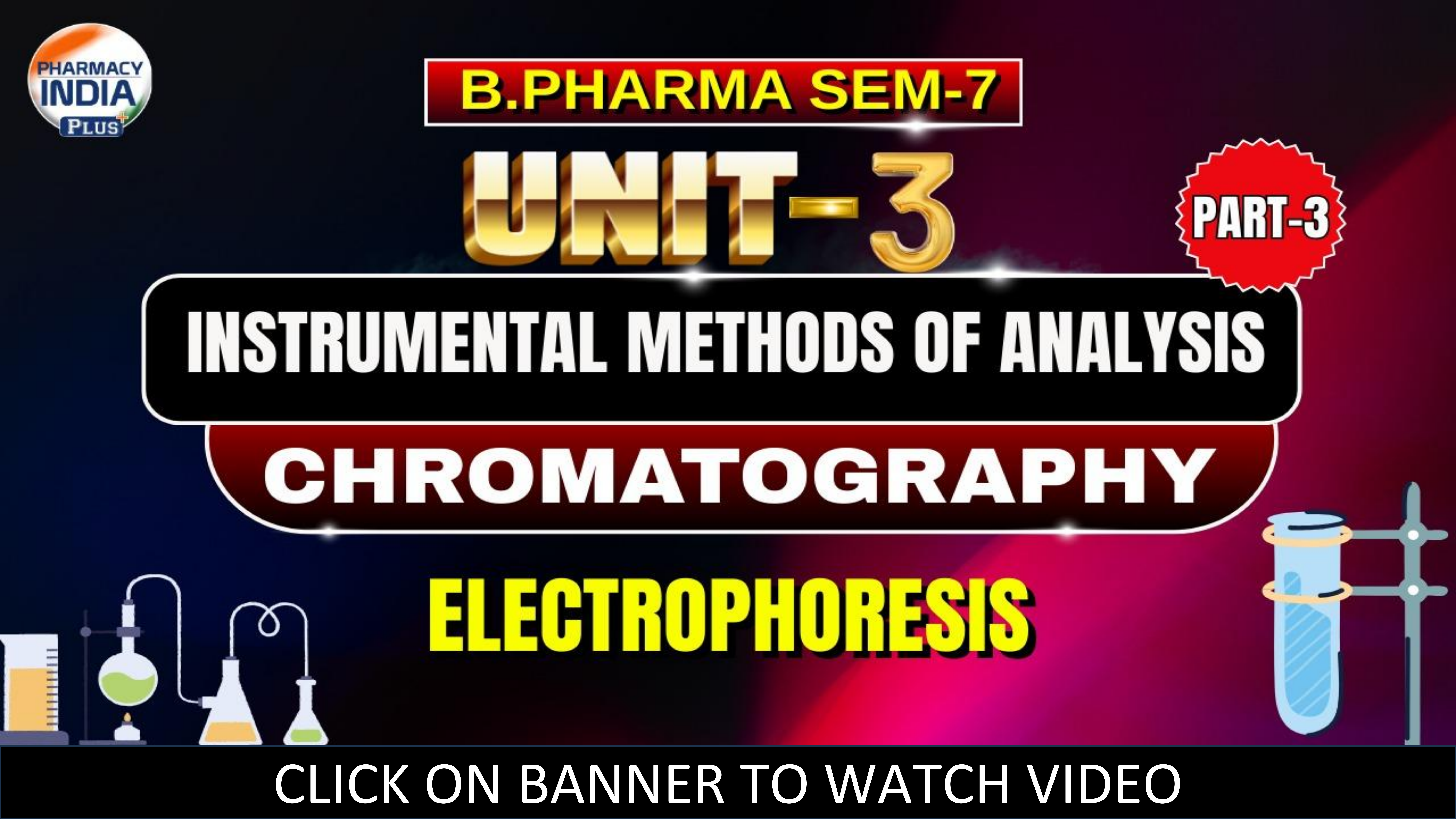
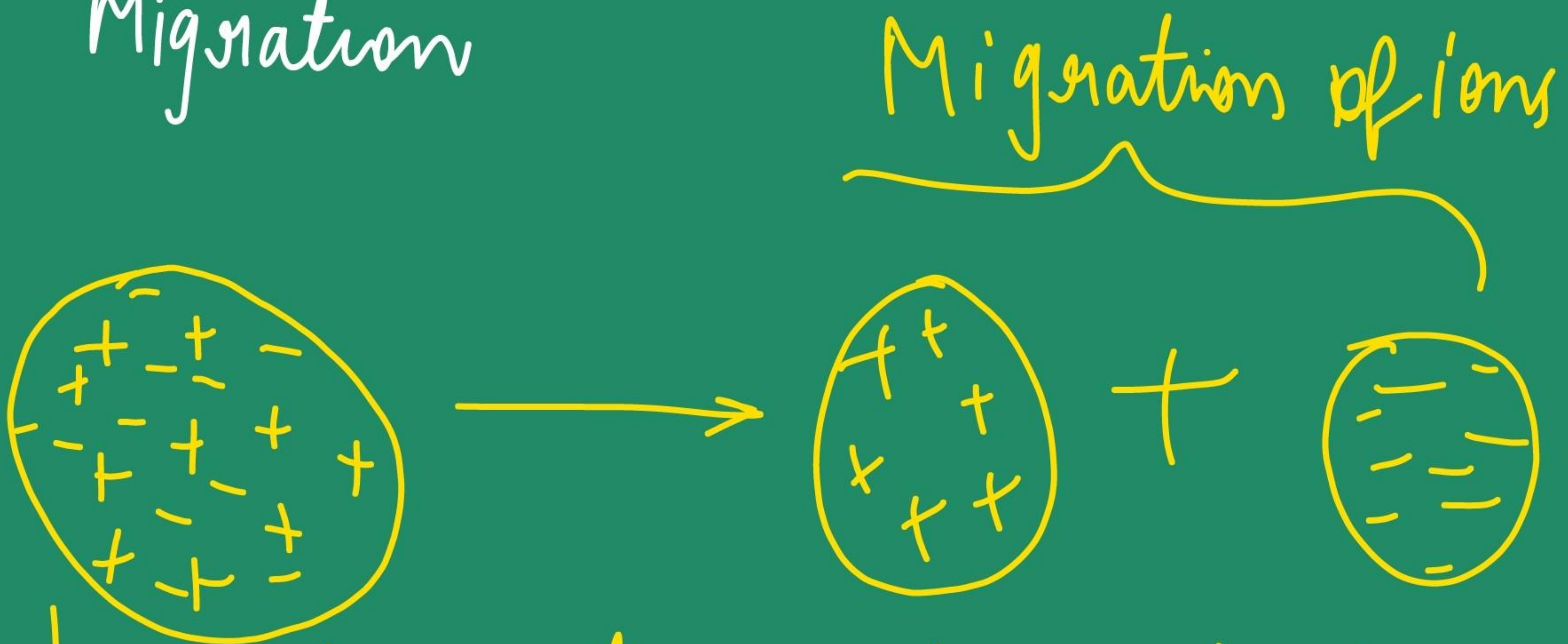




Electrophoresis

Electro
Electric field
Cation (+ve)
Anion (-ve)

Phoresis
Migration



It is a Chromatographic Technique, by which a mixture of charge molecule is separated on the basis of their size & charge.

It is based on the migration of charged molecule.

It is widely used for the separation of larger biomolecules such as DNA, RNA, Proteins, Nucleic acids etc.

Principle

It consists of two opposite charges (+/-) they are connected through a conducting medium i.e., Electrolyte.

Ions will start migrating according to their speed (Mobility) based on size & no. of charges.

$$\boxed{V = mE}$$

V = Velocity
m = Mobility
E = Electric field strength.

Factors affecting Mobility :-

$$\text{Mobility } (u) = \frac{Q}{6\pi r \eta}$$

Where,

r = radius

η = viscosity of solution.

1. Nature of ions

$$\text{Size of Ion} \propto \frac{1}{\text{Migration of ions}}$$; (High charge $\propto$ Migration of ions)

$\boxed{\text{Shape}}$.

2. Electric field

$$\left(\text{Current} \propto \text{Rate of Migration} \right) ; \left(\text{Resistance} \propto \frac{1}{\text{Migration of current}} \right)$$

3. Environmental Characteristics

- → Optimum pH range is required for a better resolution.
- → Mobility increases with Temperature.
- → An inert supporting medium is required for electrophoresis.

Applications

→ It is used for the DNA sequencing.

→ Used as an anti-biotic, Chiral Analysis.

→ Useful for the analysis of carbohydrates.

Advantages

→ Reproducible.

→ Gives a good separation for Proteins.

Disadvantages

1. It is expensive
2. Analysis of Macromolecules is difficult.

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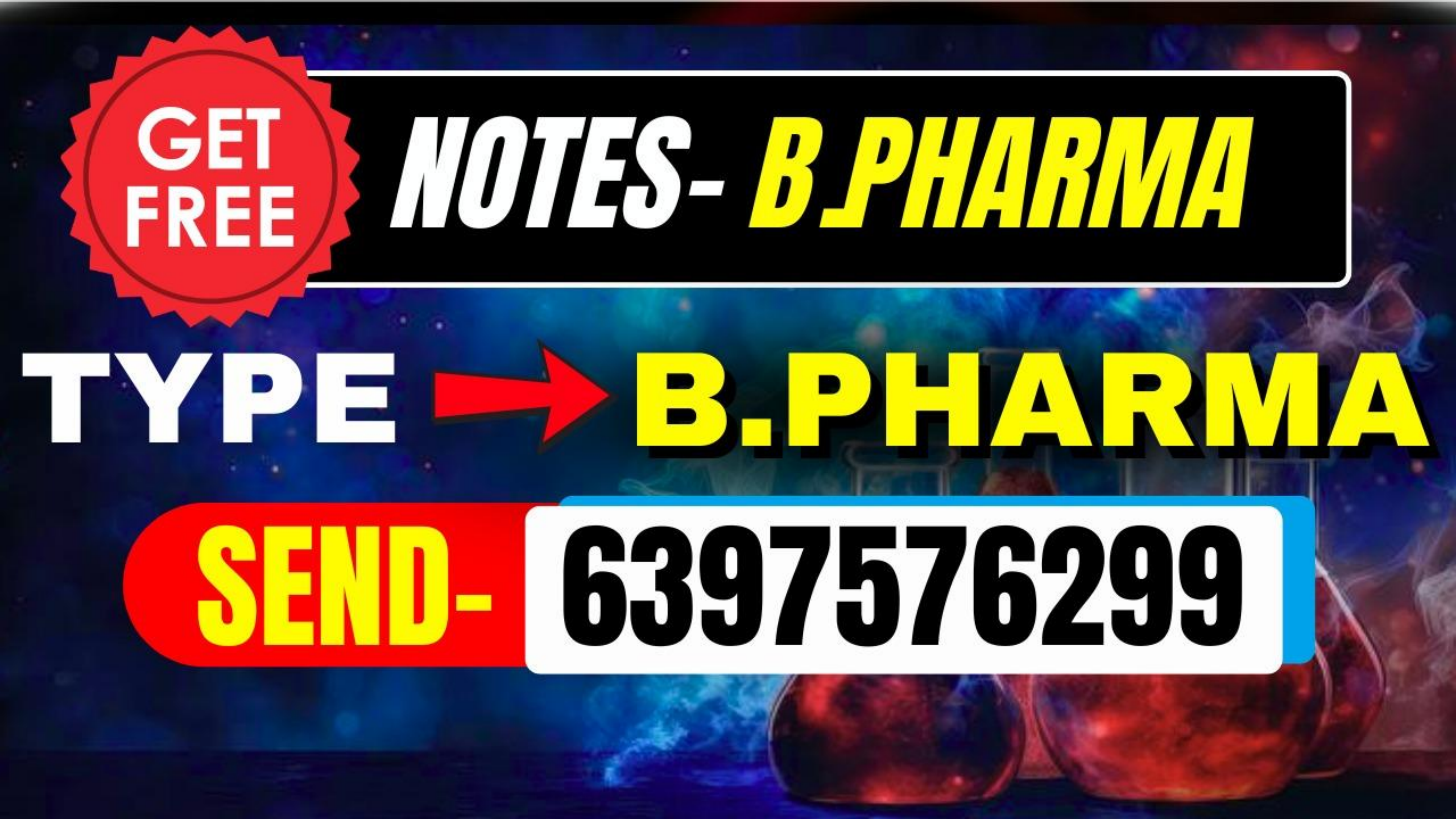
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UNIT-3

PART-4

INSTRUMENTAL METHODS OF ANALYSIS

CHROMATOGRAPHY

- PAPER ELECTROPHORESIS
- GEL ELECTROPHORESIS
- CAPILLARY ELECTROPHORESIS

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SEM-7

Instrumental method of Analysis

Topic:
- ✓ Paper Electrophoresis
- ✓ Gel Electrophoresis
- ✓ Capillary Electrophoresis

Imp

Short + long

Electropharesis
- Principle
- Factors.
- Applications

Adv & dis

Paper Electrophoresis

→ In this type of Electrophoresis, a filter Paper is used.

→ Filter Paper having adsorbing capacity & Pore size must be Uniform and it behaves as a supporting medium.

→ This whole Phenomena takes Place under the influence of an electric field.

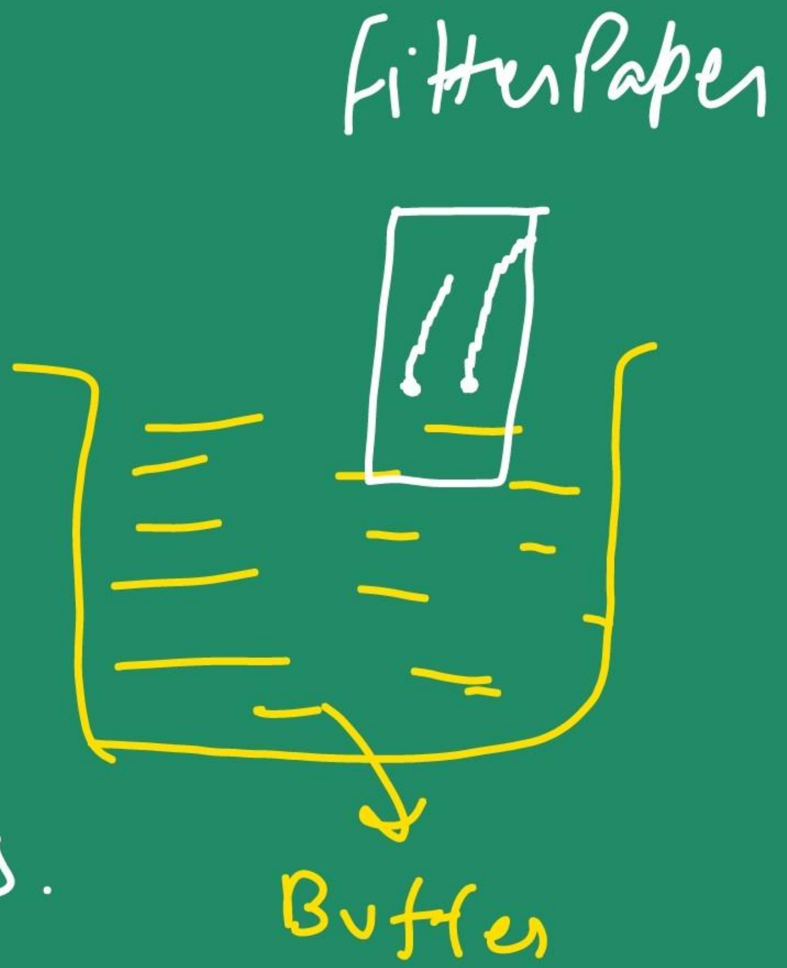
$$\text{Filter}\ \underbrace{\text{Paper} + \text{Electric}}\ \text{field} = \overset{\text{Paper.}}{\text{Electro}}\ \text{Phoresis}$$

→ The strip of filter Paper is moistured with buffer.

→ The sample is then spotted at the centre of the filter Paper, High Voltage is applied.

→ The spots will start migration according to their sizes.

→ After Electrophoresis, The separate compounds may be detected easily (Staining Technique).



#. Applications

1. It is useful for serum analysis.
2. Muscle Protein.
3. Egg Proteins etc. are analysed.

Gel Electrophoresis

In this Technique, Gels are used as a supporting medium for the separation of DNA, RNA or Protein molecules.

Most commonly used Electrophoresis in biotechnology laboratories.

Principle

When a Potential difference is applied between Electrodes of any Electrophoretic tank containing aerosel gel and biomolecules are loaded. then they get separated a/c to their molecular size.

Applications

1. To the determination of size of any DNA molecule.
2. Analysis of PCR Products.
3. For the estimation of quality & quantity of DNA.
4. Used in Forensic science department.

Capillary Electrophoresis

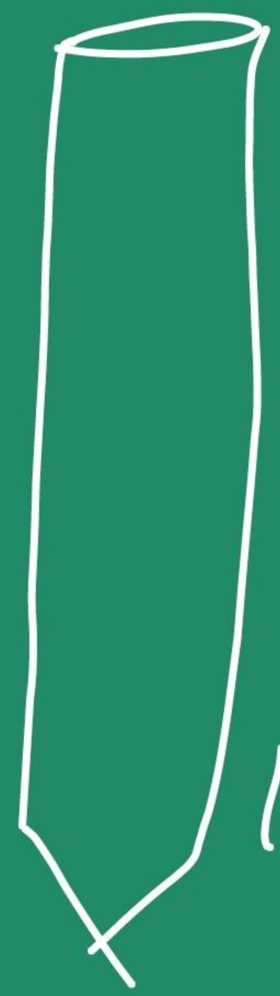
Capillary is a type of narrow tube which is employed for the separation of sample based on their size as well as charge ratio.

→ It is useful for the separation of charged & un-charged molecules.

→ The sample is introduced into the capillary (narrow tube) as a small plug.

Advantages

- → Higher separating Efficiency.
- → Short analysis time.
- → less waste generation.



Disadvantages

- → Due to narrow size of tube (less diameter) resolution is not always proper.

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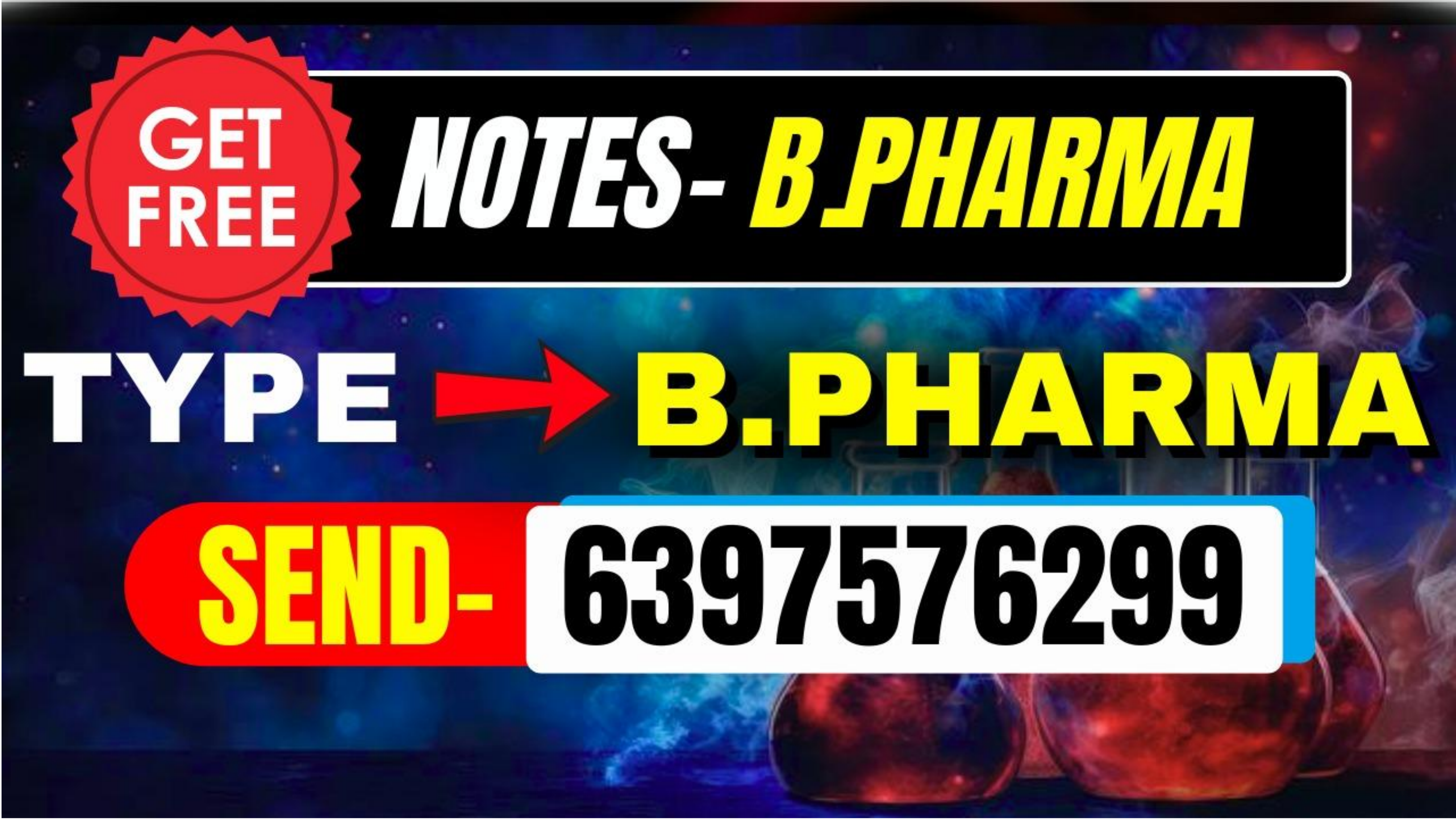
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UNIT-3

PART-5

INSTRUMENTAL METHODS OF ANALYSIS

CHROMATOGRAPHY

PAPER CHROMATOGRAPHY

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UNIT -3

PAPER CHROMATOGRAPHY

INTRODUCTION OF THE PAPER Chromatography:

- Paper Chromatography (PC) was first introduced by German scientist Christian Friedrich Schonbein (1865).
- PC is considered to be the simplest and most widely used of the chromatographic techniques because of its applicability to isolation, identification and quantitative determination of organic and inorganic compounds.
- Analysis of unknown substances it is carried out mainly by the flow of solvents on specially designed filterpaper.

❖ Two types

- **Paper adsorption chromatography:** Paper impregnated with silica or alumina acts as adsorbent (stationary phase) and solvent as mobile phase.
- **Paper partition chromatography :** Moisture / Water present in the pores of cellulose fibers present

❖ PRINCIPLE OF SEPARATION

- The principle of separation is mainly partition rather than adsorption.
- Cellulose layers in filter paper contain moisture which acts as stationary phase & organic solvents/buffers are used as mobile phase.

❖ Practical requirements

1. Stationary phase & papers used
2. Application of sample
3. Mobile phase
4. Development technique
5. Detecting or Visualizing agents

Stationary phase & papers used

- Whatman filter papers of different grades like No.1, No.2, No.3, No.4, No.20, No.40, No.42 etc are used. In general this paper contains 98-99% of α-cellulose, 0.3 – 1% β –cellulose.
- **Factors that governs the choice of paper:**
  - O Nature of Sample and solvents used.
  - O Based on Quantitative or Qualitative analysis.
  - O Based on thickness of the paper.

- Modified Papers – acid or base washed filter paper, glass fiber type paper.
  Hydrophilic Papers – Papers modified with methanol, formamide, glycol, glycerol etc.
- Hydrophobic papers – acetylation of groups leads to hydrophobic nature, hence can be used for reverse phase chromatography.
  Impregnation of silica, alumna, or ion exchange resins can also be made.

❖ Preparation of paper

- Cut the paper into desired shape and size depending upon work to be carried out.
- The starting line is marked on the paper with an ordinary pencil 5cm from the bottom edge.
- On the starting line marks are made 2cm apart from each other.

❖ Preparation of solution

- Choice of suitable solvent for making solution is very important. Pure solutions can be applied direct on the paper but solids are always dissolved in small quantity of a suitable solvent.
- Biological tissues are treated with suitable solvents and their extracts obtained. Proteins can be precipitated with alcohol and salts can be removed by treatment with ion exchange resin.

- **Application of samples**
- The sample to be applied is dissolved in the mobile phase and applied as a small spot on the origin line, using capillary tube or micropipette.
- Very low concentration is used to avoid larger zone.
- The spot is dried on the filter paper and is placed in developing chamber.

- **Choice of solvent**
- uitable polarity may be app The commonly employed solvents are the polar solvents, but the choice depends on the nature of the substance to be separated.
- If pure solvents do not give satisfactory separation, a mixture of solvents of slied.

Mobile phase

- Pure solvents, buffer solutions or mixture of solvents
- Examples-

❖ **Hydrophilic mobile phase**

- Isopropanol : ammonia : water (9 : 1 : 2) 9:1:2
- Methanol : water (4:1)
- N-butanol : glacial acetic acid : water (4:1:5)

❖ **Hydrophobic mobile phases**

- dimethyl ether: cyclohexane
- kerosene : 70% isopropanol

- **Chromatographic chamber**
  The chromatographic chambers are made up of many materials like glass, plastic or stainless steel.
- Glass tanks are preferred most. They are available in various dimensional sizes depending upon paper length and development type.
- The chamber atmosphere should be saturated with solvent vapor.

Development techniques

- Paper is flexible when compared to glass plate used in TLC; several types of development are possible which increases the ease of operation.
- The paper is dipped in solvent in such a manner that the spots will not dip completely into the solvent.
- The solvent will rise up and it is allowed to run 2/3rd of paper height for better and efficient result.

- **Ascending development (go up) : Like conventional** type**, the solvent flows against gravity.**

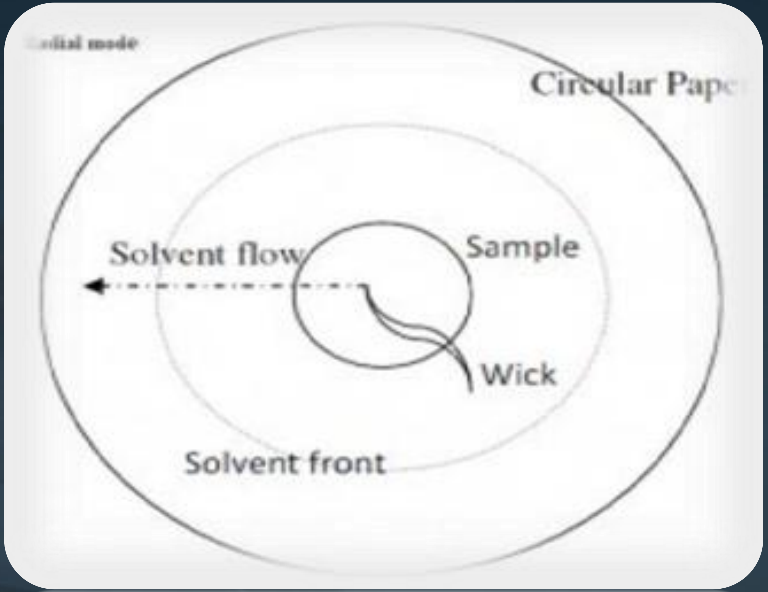
The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom.

- **Decending development (a downward slope)**

- This is carried out in a special chamber where the solvent holder is at the top. The spot is kept at the top and the solvent flows down the paper.
- In this method solvent moves from top to bottom so it is called descending chromatography.
- Advantage is that, development is faster.

❖ Ascending – decending development

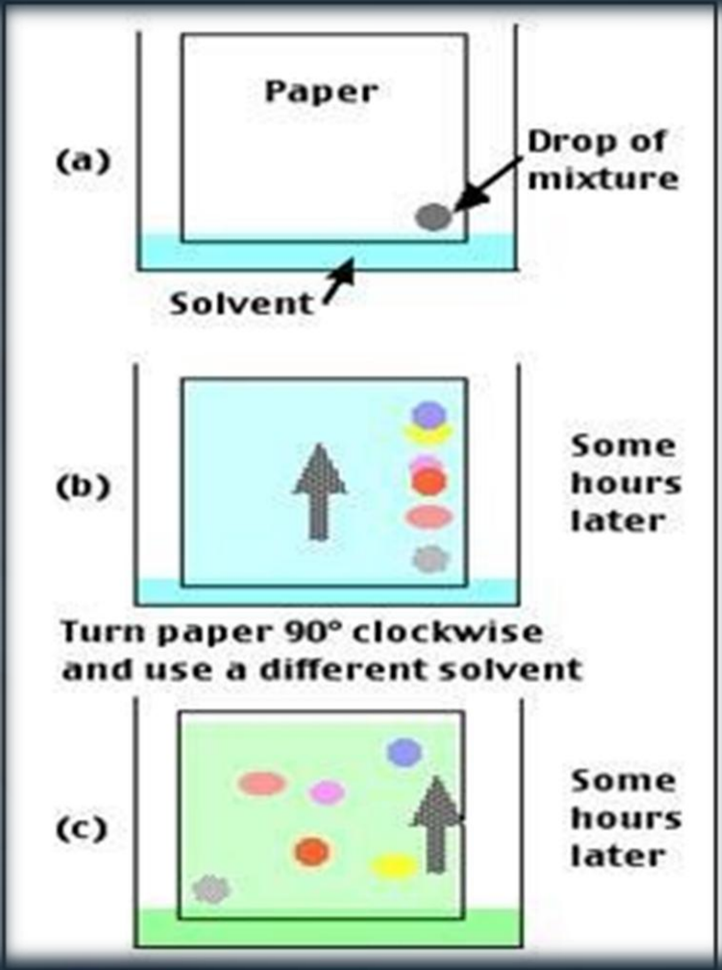
- ➢ A hybrid of above two techniques is called ascending-descending chromatography.
- ➢ Only length of separation increased, first ascending takes place followed by descending.
- ➢ **Circular/ radial development :** Spot is kept at the centre of a circular paper. The solvent flows through a wick at the centre & spreads in all directions uniformly.



Circular development

❖ Two dimentional development

- ➢ In this method the paper is developed in one direction and after development, the paper is developed in the second direction allowing more compounds to be separated into individual spots.
- ➢ In the second direction, either same solvent/different solvent system can be used for development.



Two dimentional development

❖ Drying of chromatogram

- After the solvent has moved a certain distance for certain time the chromatogram is taken out from the tank & position of the solvent front is marked with a pencil.
- They are dried by cold or hot air depending on volatility of solvent.
- A simple hair dryer is a convenient device to dry chromatograms.

❖ Detecting and visualizing agents

- ➢ If the substance are colored they are visually detected easily.
- ➢ But for colorless substance, Physical and chemical methods are used to detect the spot.
- ➢ Non-specific methods (Physical methods) E.g. iodine chamber method, UV chamber for fluorescent compounds – at 254 or at 365nm

- **Specific method/ chemical method/ spraying method→**
  - Ferric chloride → Phenolic compounds and tannins
  - Ninhydrin with acetone → Amino acid
  - Dragendroff's reagent → alkaloids
  - 3, 5 – dinitro benzoic acid → cardiac glycosides

- **Rf value (retardation factor)**
  In paper chromatography the results are represented by Rf value which represent the movement or migration of solute relative to the solvent front.
- The Rf value ranges from 0 to 1. But ideal values are from 0.3 to 0.8.
- Rf value is constant for every compound in a particular combination of stationary and mobile phase. When the Rf value of a sample and reference compound is same, the compound is identified.

$$\text{Rf value} = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent front}}$$

❖ Applications

- Separation of mixtures of drugs
- Separation of carbohydrates, vitamins, antibiotics, proteins, etc.
- Identification of drugs
- Identification of impurities
- Analysis of metabolites of drugs in blood, urine.

❖ THIN LAYER CHROMATOGRAPHY

✓ INTRODUCTION

- TLC is one of the simplest, fastest, easiest and least expensive of several chromatographic techniques used in qualitative and quantitative analysis to separate organic compounds and to test the purity of compounds.

- **TLC is a form of liquid chromatography consisting of:**
  - A mobile phase (developing solvent).
  - A stationary phase (a plate or strip coated with a form of silica gel).
  - can be defined as a method of separation or identification of a mixture of compAnalysis is performed on a flat surface under atmospheric pressure and room temperature.

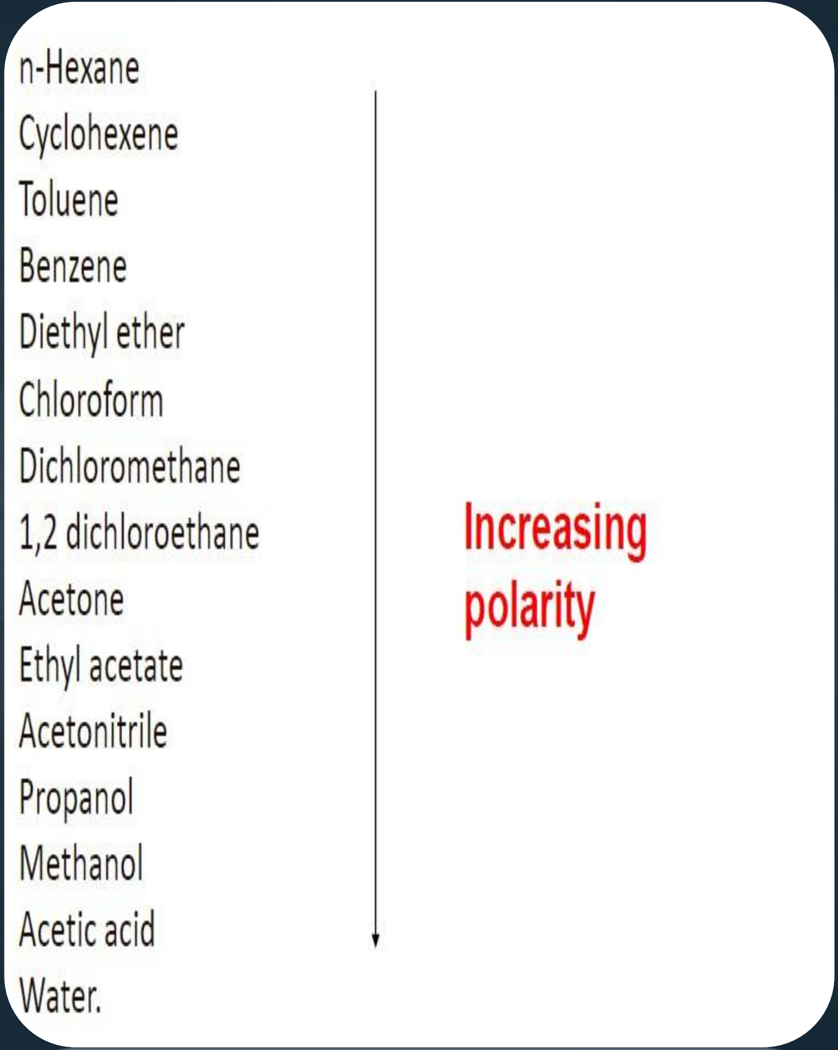
- **Thin Layer Chromatography**→ onents into individual components by using finely divided adsorbent solid / (liquid) spread over a glass plate and liquid as a mobile phase.
- **Synonyms** → Drop, strip, spread layer, surface chromatography and open column chromatography.
- Separation of the adsorbed substances by the mobile phase.
- •Recovery of the separated substances by a continuous flow of the mobile phase (elution).

❖ Principle

- It is based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed
- The components with more affinity towards stationary phase travels slower.
- Components with less affinity towards stationary phase travel faster.
- Components of TLC.
- Stationary phase → Silica gel G, Silica gel H, Alumina, Kieselghur

❖ Mobile phase

- ➢ A mixture of an organic solvent and water with the addition of acid, base or complexing agent to optimize the solubility of the components of a mixture can be used.
- ➢ For example, good separations of polar or ionic solutes can be achieved with a mixture of water and n-butanol.
- ➢ If the stationary phase is hydrophobic, various mixtures of benzene, cyclohexane and chloroform provide satisfactory mobile phases.



n-Hexane
Cyclohexene
Toluene
Benzene
Diethyl ether
Chloroform
Dichloromethane
1,2 dichloroethane
Acetone
Ethyl acetate
Acetonitrile
Propanol
Methanol
Acetic acid
Water.

- ❖ Chromatogram plates
- ➢ Glass plates or flexible plates are commonly used for adsorbent.
- ➢ The standard sizes are 20 x 5 cm, 20 x 10 cm or 20 x 20 cm.
- ➢ The surface should be flat without irregularities.
- ➢ The standard film thickness is 0.25mm.
- ❖ Methods of application of adsorbents
- ➢ **Pouring** →The adsorbent of finely divided and homogeneous particle size is made into slurry and is poured on a plate and allowed to flow over it so that it is evenly covered.

- **Spraying** → Slurry is diluted further for the operation of sprayer. But this technique is not used now days as it is difficult to get uniform layer.
- **Dipping** → This technique is used for small plates by dipping the two plates at a time, back to back in slurry of adsorbent in chloroform or other volatile solvents. Exact thickness of layer is not known and evenness of layer may not be good.
- **Spreading** → All the above methods fail to give thin and uniform layers. Modern methods utilize the spreading devices for preparation of uniform thin layers on glass plates. Commercial spreaders are of two types (a) Moving spreader, (b) Moving plate type. It gives layer thickness from 0.2 to 2.0 mm.

❖ Activation of plates

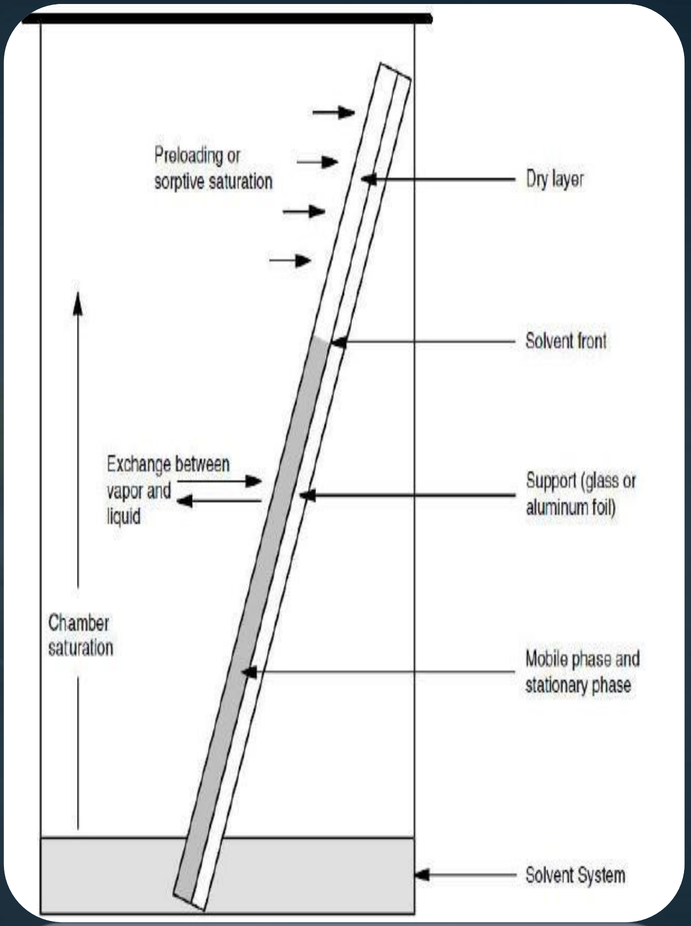
- After spreading plates are allowed to dry in air and further dried and activated by heating at about 1000c for 30 mins.
- By removing the liquids associated with layer completely, the adsorbent layer is activated.

❖ Application of sample

- Sample solution in a non-polar solvent is applied.
- The concentration of a sample or standard solution has to be minimum of a 1% solution of either standard or test sample is spotted using a. capillary tube or micropipette
- The area of application should be kept as small as possible for sharper and greater resolution.
- Sample should be applied 2cm above from bottom.

TLC Column development

- Place the prepared TLC plate in the developing beaker, cover the beaker with the watch glass, and leave it undisturbed on your bench top.
- The solvent will rise up the TLC plate by capillary action. Make sure the solvent does not cover the spot.
- Allow the plate to develop until the solvent is about half a centimeter below the top of the plate.
- Remove the plate from the beaker and immediately mark the solvent front with a pencil.



Detection or visualization of spots

- If there are any colored spots, circle them lightly with a pencil.
- Most samples are not colored and need to be visualized with a UV lamp.
- Hold a UV lamp over the plate and circle any spots you see.

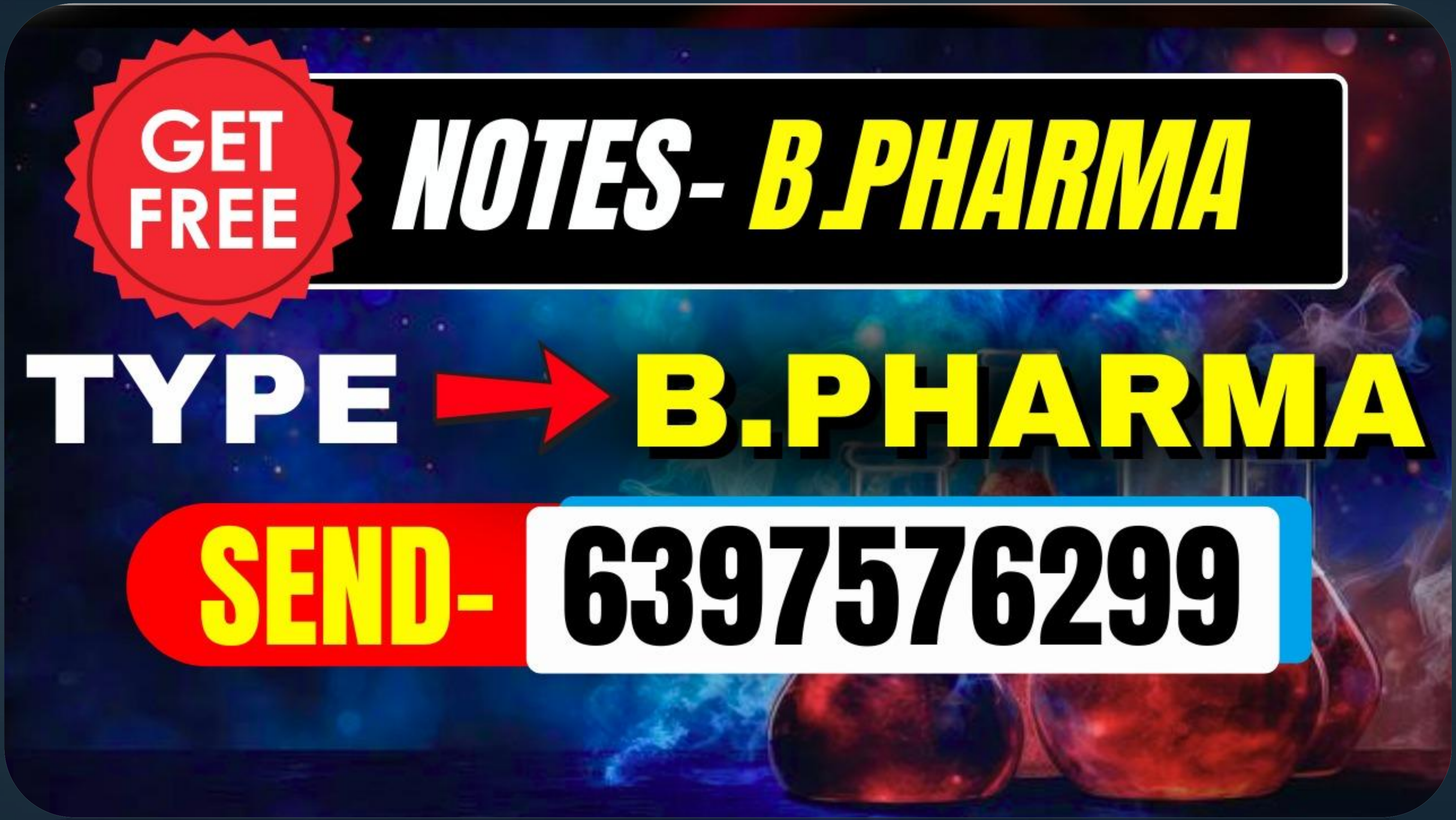
Reagents for visualization of TLC chromatograms

Reagents	Application
Iodine vapour	Organic or unsaturated compounds
Phosphomolybdic acid	General organic
Fluorescein/ bromine	General organic
Sulphuric acid	General organic
Ninhydrin	Amino acid
2,4 – dinitrophenylhydrazine	Aldehyde and ketones
Aniline phthalate	Sugar
Antimony trichloride	Steroids, essential oils
Mercuric nitrate (yellow brown)	Alkaloids
Bromothymol blue (light green)	Lipids
Diphenyl carbazone	Barbiturates
Acids	Bromocresol green

Application of TLC

- It is used for separation of all classes of natural products and is established as an analytical tool in modern pharmacopoeias. E.g. Acids, alcohols, glycols, alkaloids, amines, macromolecules like amino acids, proteins and peptides, and antibiotics
- for checking the purity of samples
- as a purification process

- examination of reaction
- for identifying organic compounds
- Extensively used as an identification test and test for purity.
- As a Check on process – checking of distillation fractions and for checking the progress of molecular distillation.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