# BIO & PHARMA ANALYTICAL TECHNIQUES

## Chapter 4
## Chromatography

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# Chapter Description

- **Aims**
  - Discuss theory, principles and application of analytical techniques used in material characterisation, pre-formulation development, manufacturing process and storage stability.

- **Expected Outcomes**
  - Explain general facts of chromatography including application in other field.
  - Illustrate theory and principle of chromatography: Thin Layer Chromatography (TLC) and High Pressure Liquid.
  - Discuss on the application of both instruments in pharmaceutical.

- **References**

  - Gunzler H. & Williams A. (2002). Handbook of Analytical Techniques. Wiley-VCH, Weinheim, Germany.
  - Mullertz, A., Perrie, Y. and Rades, T. (2016) Analytical Techniques in the Pharmaceutical Sciences (Advances in Delivery Science and Technology). Springer, United States.

# What is CHROMATOGRAPHY

to write

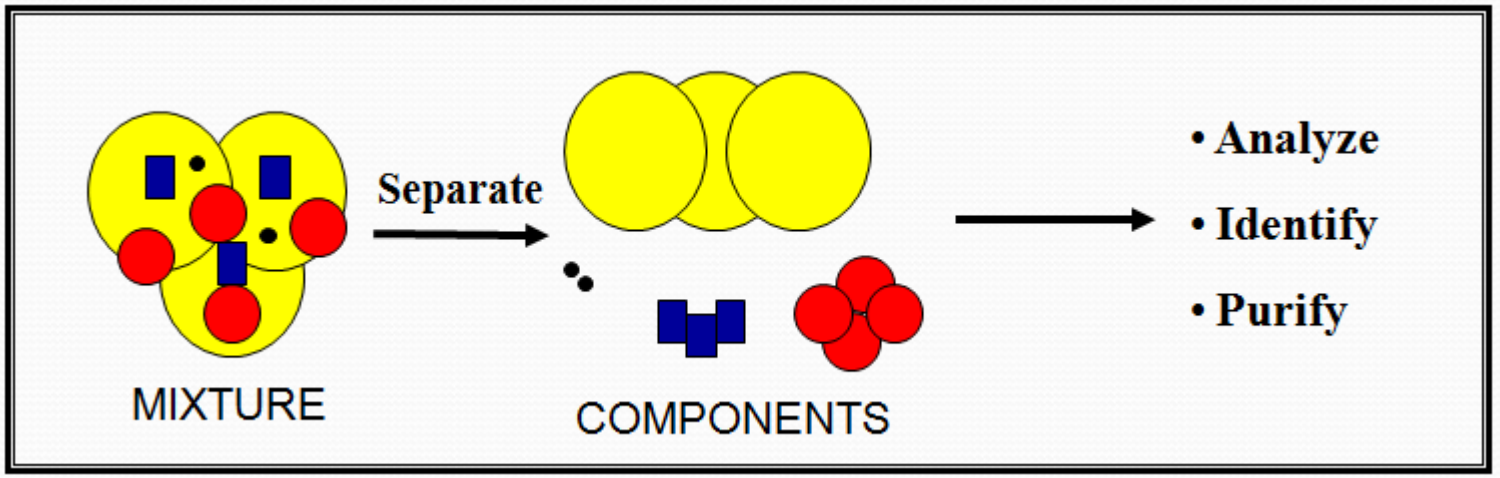
CHROMATOGRAPHY

colour

Laboratory technique for the separation of mixtures

# What is CHROMATOGRAPHY

A technique for separating mixtures into their components in order to **analyze**, **identify**, and **purify** the mixture or components.



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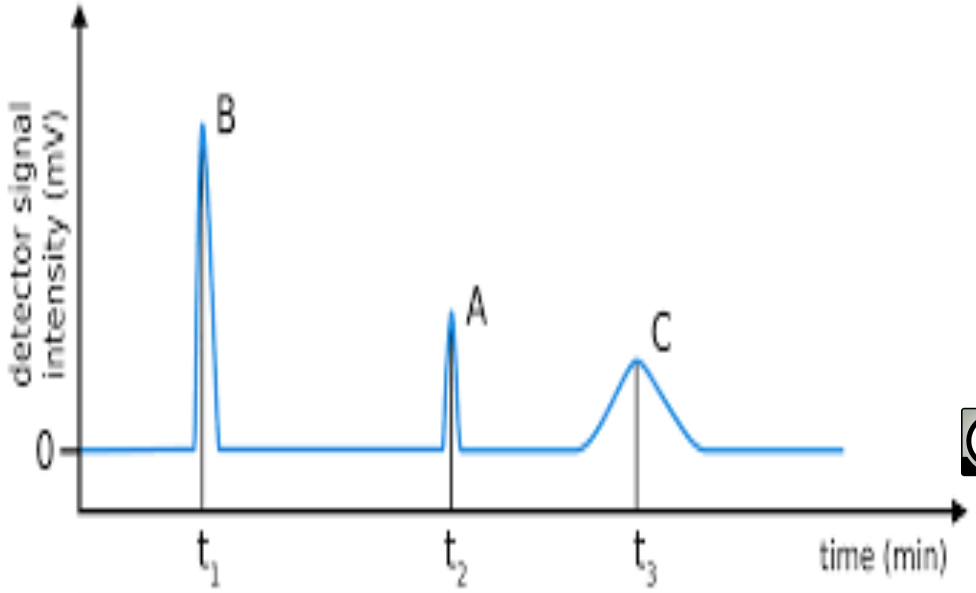
# DEFINITION

- Analyze : To examine the mixture or structure or something especially by separating it into its parts.
- Purify : To make something pure by removing substances that are not wanted out of another substances that contains it.
- Identify : To recognize something and to determine the identity of mixture.

# Chromatogram

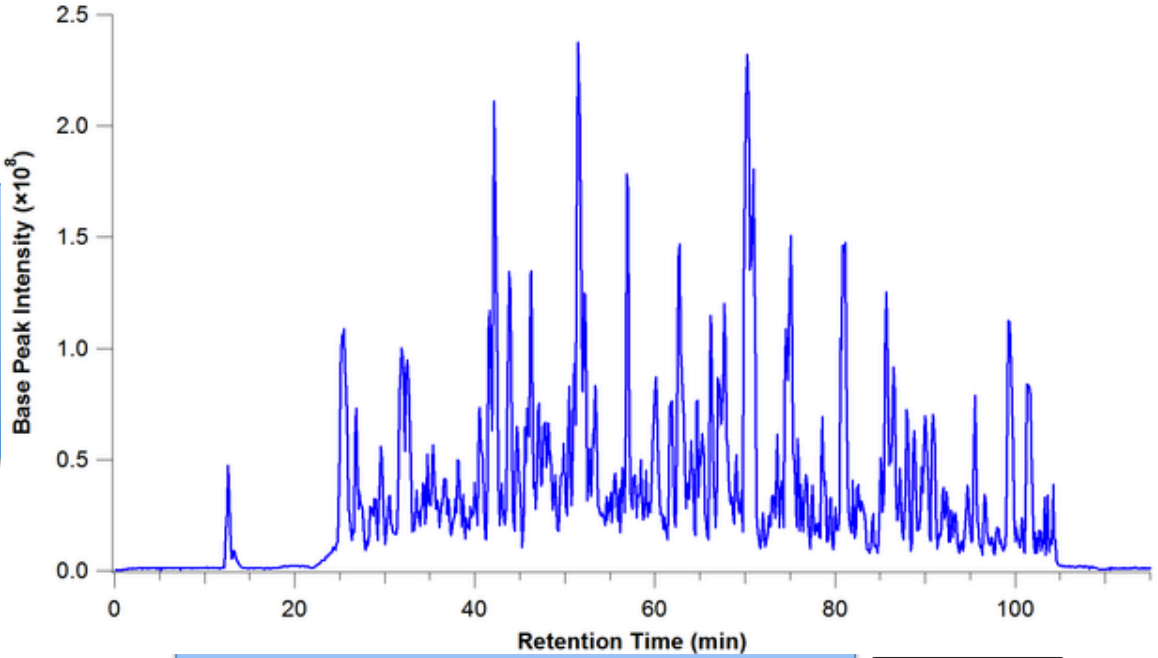





- The **visual output** of the chromatograph. **Different peaks/patterns** on the chromatogram correspond to **different components** of the separated mixture

# CHROMATOGRAM

**Y-axis: Response/ Intensity/Signal**

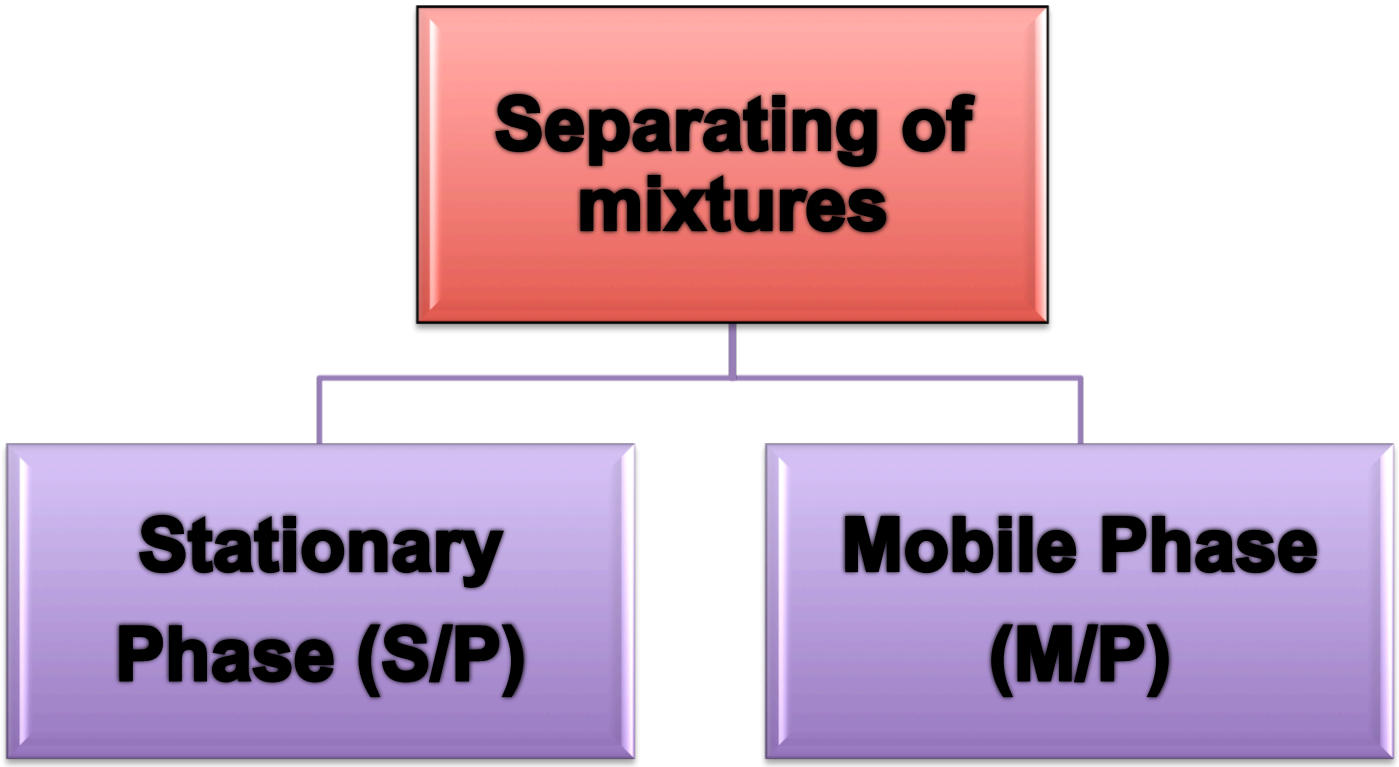



**X-axis: Retention time**



**RETENTION TIME:**
Time takes for a particular analyte to travel through the system (from inlet to detector) under set conditions.

# PHASE IN CHROMATOGRAPHY




Fixed in place either in a column (GC, HPLC) or on a planar surface (TLC)

Carries the analyte through the stationary phase

# Principles of Chromatography

- Physical method of separation that distributes components to separate between 2 phases moves in a definite direction.
- Substances are separated based on their differential distribution between 2 phases
- Substances will move with the mobile phase at different rate depending upon their partition or distribution coefficients.

# Applications of Chromatography

1. Pharmaceutical industry
2. Forensic
3. Research
4. Medicine
5. Food
6. Environment

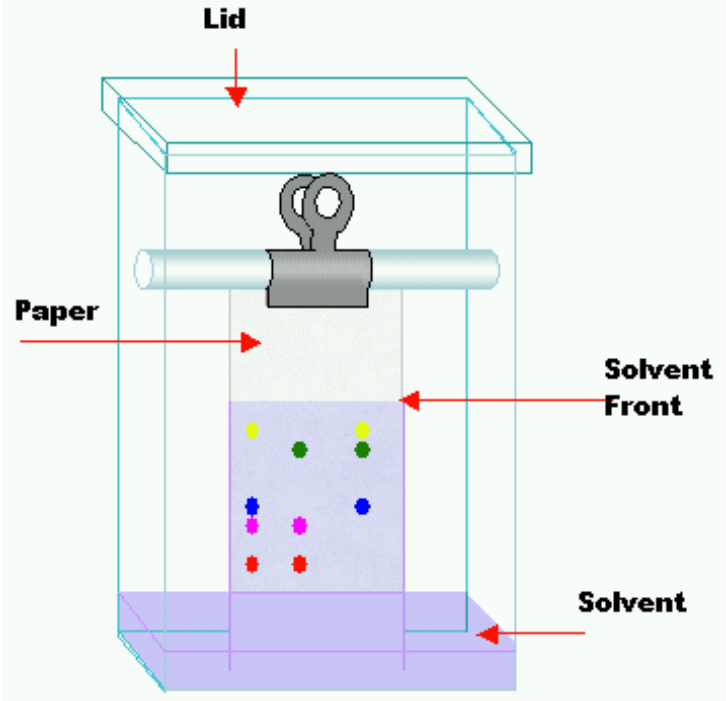
# Types of Chromatography

- Paper chromatography
- Column chromatography
- Thin Layer Chromatography (TLC)
- High Performance Liquid Chromatography (HPLC)
- Gas Chromatography (GC)

# Thin Layer Chromatography (TLC)

# Introduction to TLC

- 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.
- Consists of:
  - A **stationary phase (S/P)** – a plate or strip coated with a form of silica gel/alumina/cellulose
  - A **mobile phase (M/P)** – developing liquid which travels up the stationary phase, carrying the samples with it (volatile organic solvent).
  - Analysis is performed on a flat surface under atmospheric pressure and room temperature.



# PRINCIPLE OF TLC

- 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 component with more affinity towards the S/P travel slower
- The component with lesser affinity towards the S/P travel faster.
- Components of the samples will separate on the S/P according to:

  → How much they adsorb on the S/P *vs* how much they dissolve in the M/P

# Adsorption Chromatography

- Ability of different solutes to be adsorbed on the surface of the stationary phase at different strength.
- S/P: a solid.
- Separation is due to a series of adsorption/desorption steps.

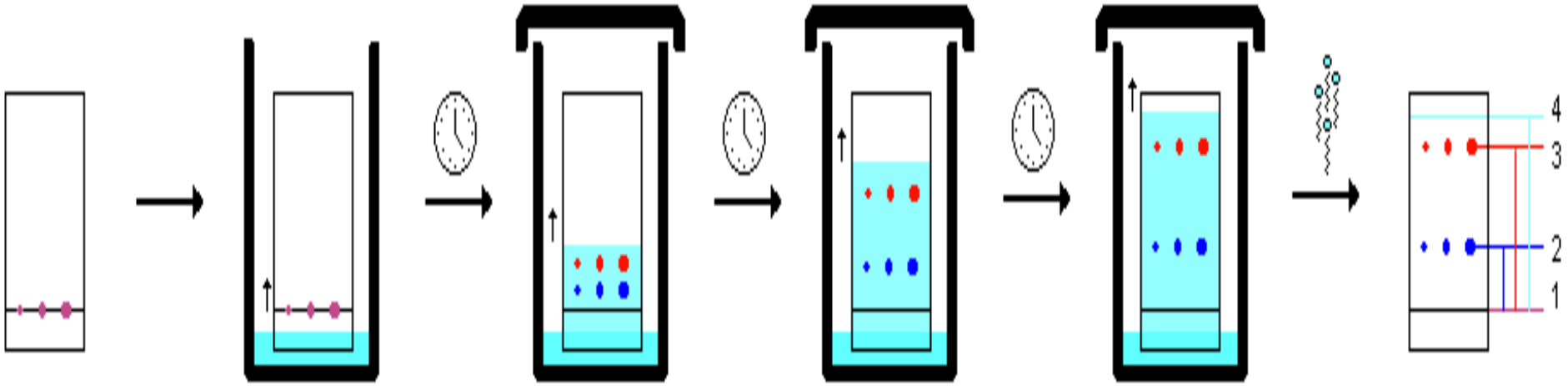
# Partition Chromatography

Separation is based on solute partitioning between 2 liquid phases.

# TLC

- TLC plate (aluminium or glass) – coated by stationary phase (silica gel, alumina or cellulose).
- Coated material: 0.1 – 0.3 mm in thickness
- Fluorescent indicator will make it florescence during the UV light exposure

# Steps Involved in TLC

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# $R_f$ VALUE

**In TLC, the results are represented by $R_f$ value which represent the movement or migration of solute relative to the solvent front. This is indicating position of migrated spots on chromatogram.**

***The $R_f$ value is calculated as:-***

$$R_f \text{ value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

# Calculate the $R_f$ values:

- The $R_f$ value is calculated by measuring the distance the sample zone travels divided by the distance the developing solvent travels

  ✓ Values < 0.1 is considered poor: the spots are too close to origin

  ✓ Values of 0.1 to 0.8 are good and any other spots (impurities) or other actives are resolved form each other

  ✓ > 0.8 = poor; spots may be too broad or distorted

# Factors affecting $R_f$ value

- It depends on following factors:
  - Nature adsorbent
  - Mobile phase
  - Activity
  - Thickness of layer
  - Temperature
  - Equilibrium
  - Loading
  - Dipping zone

# Applications in Pharmaceuticals

1) Separation of mixture of drug of chemical,biological,plant origin.
2) Separation of Carbohydrates, vitamin, antibiotics, proteins, etc.
3) Identification of drug. Ex :Amoxicillin, Levodopa
4) Detection of foreign substances.
5) To detect the decomposition products of drug.
6) To determine how many compounds in the mixture – is it real pure?

# Advantages of TLC

- Low cost
- Short analysis time
- All spots can be visualized
- Adaptable to most pharmaceuticals
- Low cost
- Uses small quantities of solvent
- Requires minimal training
- Reliable and quick
- Minimum amount of equipment is needed

# High Performance Liquid Chromatography (HPLC)

# Introduction

- HPLC is a chromatographic technique that can **separate a mixtures** of compounds
- It is used to **identify, quantify and purify** the individual components of a mixture.
- HPLC is a type of liquid chromatography where the sample is forced through a **column** that is packed with a stationary phase composed of irregularly or spherically shaped particles, a porous monolithic layer, or a porous membrane by a liquid (mobile phase) at high pressure.

# Principle of HPLC

# Adsorption Chromatography

- The principle of separation is **adsorption.**
- Separation of compounds takes place based on the difference in the **affinity** of the compounds towards stationary phase.
- The **lesser the affinity** of the sample particles towards the stationary phase the **faster the time of elution** of the sample.

# Partition Chromatography

- Stationary phase is a **liquid** which is coated on the solid support on the column.
- The mobile phase is also a **liquid.**
- When solute along with the mobile phase is passed over the stationary phase it gets dissolved to the surface of the liquid coated to the solid support.
- The compounds which have more partition co-efficient are eluted slowly (interaction) when compared to the compounds with low partition co-efficient.

# HPLC

**HPLC is a separation technique that involves:**

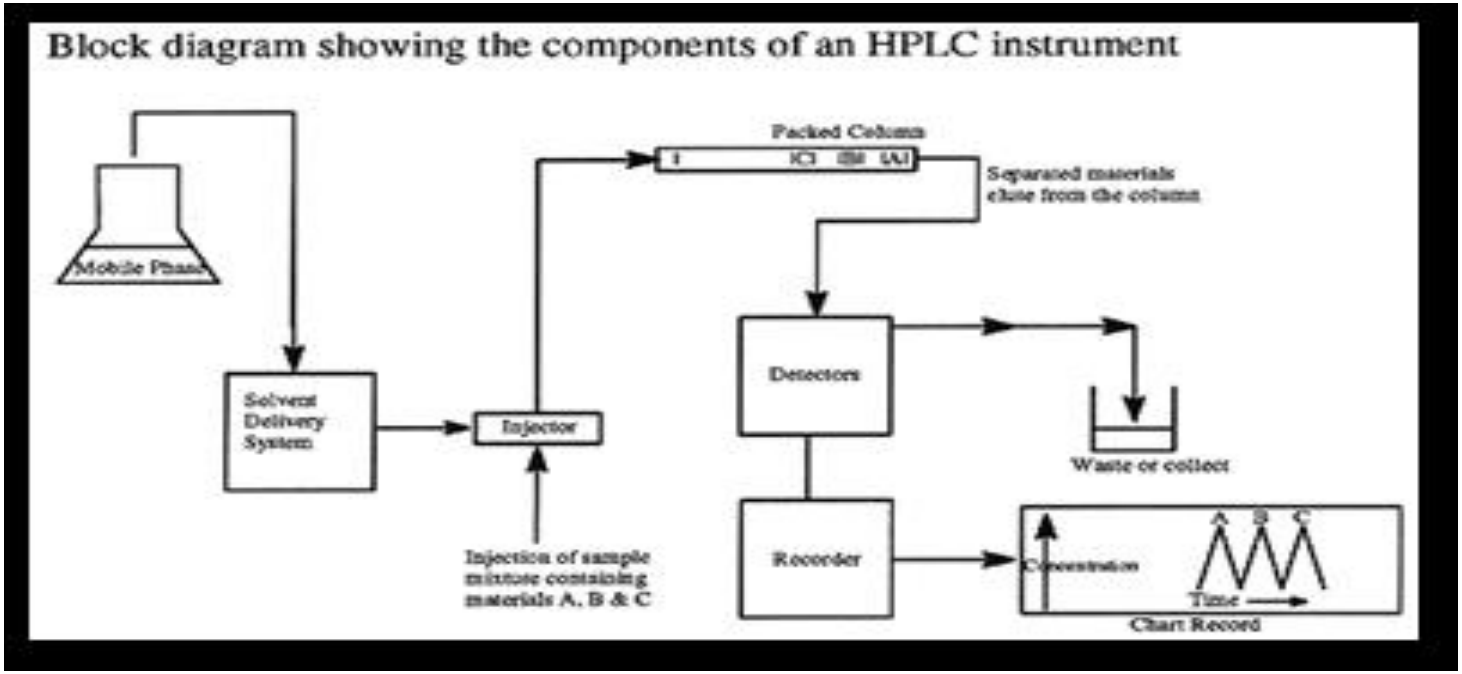
- the **injection of a small volume of liquid sample** into a tube packed with tiny particles (3 to 5 micron (μm) in diameter called the **stationary phase)** where individual components of the sample are moved down the packed tube (**column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump.**
- These **components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.**
- These separated components are detected at the exit of this tube (**column) by a flow-through device (detector) that measures their amount. An output from this detector is called a "liquid chromatogram".**

# Types of HPLC

| | Stationary phase | Mobile phase |
|---|---|---|
| Normal Phase Chromatography | Polar (hydrophilic) | Non-polar (hydrophobic) |
| Reverse Phase Chromatography | Non-polar (hydrophobic) | Polar (hydrophilic) |

- Most common used

# Schematic diagram of HPLC



# INSTRUMENTATION OF HPLC

1. Solvent storage bottle
2. Gradient controller and mixing unit
3. De-gassing of solvents
4. Pump
5. Pressure gauge
6. Pre-column
7. Injector
8. Column
9. Detector
10. Recorder

(data collection)

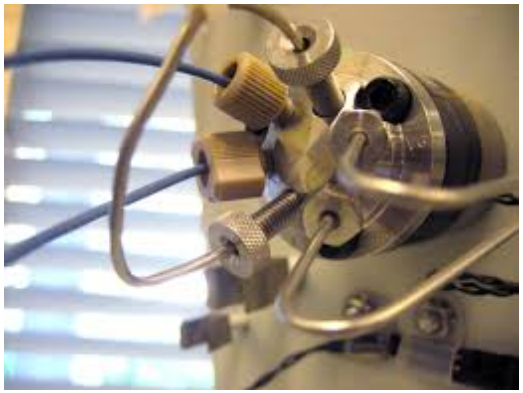
# PUMP

- The role of the pump is **to force a liquid** (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL /min).
- Normal **flow rates** in HPLC are in the 1-to 2-mL/min range.
- Typical pumps can reach pressures in the range of 6000-9000 psi.
- During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient).

# Injectors

Injectors are used to provide **constant volume injection of sample** into the mobile phase stream. Inertness and reproducibility of injection are necessary to maintain high level of accuracy.

- Divide:
  - Manual Injector
  - Auto injector – automatic operation



# Mobile Phase

- Mobile phase serves to transport the sample to the system.
- Essential criteria of mobile phase are inertness to the sample components.
- Pure solvents or buffer combinations are commonly used.
- The mobile phase should be free of particulate impurities and degassed before use.

# Column

- A column is a stainless steel tube packed with stationary phase.
- It is a vital component and should be maintained properly as per supplier instructions for getting reproducibility and separation efficiency run after run.

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# Detectors

- A detector gives specific response for the components separated by the column and also provides the required sensitivity.
- It has to be independent of any changes in mobile phase composition.
- Majority of the applications require UV-VIS detection, though detectors based on other detection technique are also popular these days.

# Detectors

- Absorbance (UV/Vis or PDA)
- Refractive index (detects the change in turbidity)
- Fluorescence (if the analyte is fluorescent)
- Electrochemical (measures current flowing through a pair of electrodes, on which a potential difference is imposed, due to oxidation or reduction of solute)
- Conductivity (for ions)
- Light scattering
- Mass spectrometry (HPLC-MS)

# Selection of Detectors

| Detectors | Type of compounds can be detected |
|---|---|
| UV-Vis & PDA | Compounds with chromophores, such as aromatic rings or multiple alternating double bonds. |
| RF | Fluorescent compounds, usually with fused rings or highly conjugated planar system. |
| CDD | Charged compounds, such as inorganic ions and organic acid. |
| ECD | For easily oxidized compounds like quinones or amines. |

# APPLICATIONS OF HPLC

## Pharmaceuticals:

- Tablet dissolution of pharmaceutical dosages
- Shelf life determinations of pharmaceutical products
- Identification of counterfeit drug products
- Pharmaceutical quality
- control

## Environmental:

- Phenols in drinking water
- Estrogens in coastal waters – the sewage source
- Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria

## Forensics:

- Determination of cocaine and metabolites in meconium
- Simultaneous quantification of psychotherapeutic drugs in human plasma

## Food:

- Ensuring soft drink consistency and quality
- Analysis of vicinal diketones in beer
- Sugar analysis in fruit juices

# Advantages of HPLC

- Separations fast and efficient (high resolution power)
- Continuous monitoring of the column effluent
- It can be applied to the separation and analysis of very complex mixtures
- Accurate quantitative measurements
- Repetitive and reproducible analysis using the same column
- Both aqueous and non aqueous samples can be analysed with little or no sample pretreatment
- A variety of solvents and column packing are available, providing a high degree of selectivity for specific analyses.
- It provides a means for determination of multiple components in a single analysis.

# Conclusion of The Chapter

1. Chromatography is really important in pharmaceutical industry especially in quality control department.
2. Moreover, it is also play a role in forensic science and research area.
3. TLC is the most simplest technique in chromatography while HPLC gives the more accurate results as compared to TLC.

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# Any Question?

## Please refer to:


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