HPTLC

(High performance thin layer chromatography)

Presented by- Anupriya Singh
M.pharm-1st year

- **Content-**

- introduction
- Principle
- Instrumentation
- Difference between TLC and HPTLC
- Steps involving in HPTLC
- Factors influencing separation and resolution of spots
- Applications of HPTLC

- **Introduction-**

- HPTLC (High performance thin layer chromatography) is the automated, sophisticated form and improved method of TLC.
- It is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks.
- It is also known as planer or flat bed chromatography.
- **HPTLC is very popular for many reasons such as-**
- Visual chromatogram,
- Multiple sample handling,
- Enables the most complicated separation,

- Detection limit in nanogram range with UV-absorption detection and in picogram range with fluorimetric detection.
- Large no of theoretical plates in minimum area of plates .
- Analysis time is greatly redused in HPTLC due to shorter migration distant.
- Higher efficiency due to smaller particle size(5 μm).

- **Principle-**
- Same theoretical principle of TLC (Adsorption chromatography) i.e. the principle of separation is adsorption.
- Mobile phase flow by capillary action effect .
- And component move according to their affinities towards the adsorbent.
- The component with higher affinity toward adsorbent travels slowly.
- And the component with lesser affinity towards the stationary phase travels faster.
- Thus the components are separated on a chromatographic plate according to their affinity and seperation also based on their solubility in mobile phase.

Instrumentation of HPTLC-

Applicator

Autometic developing chamber

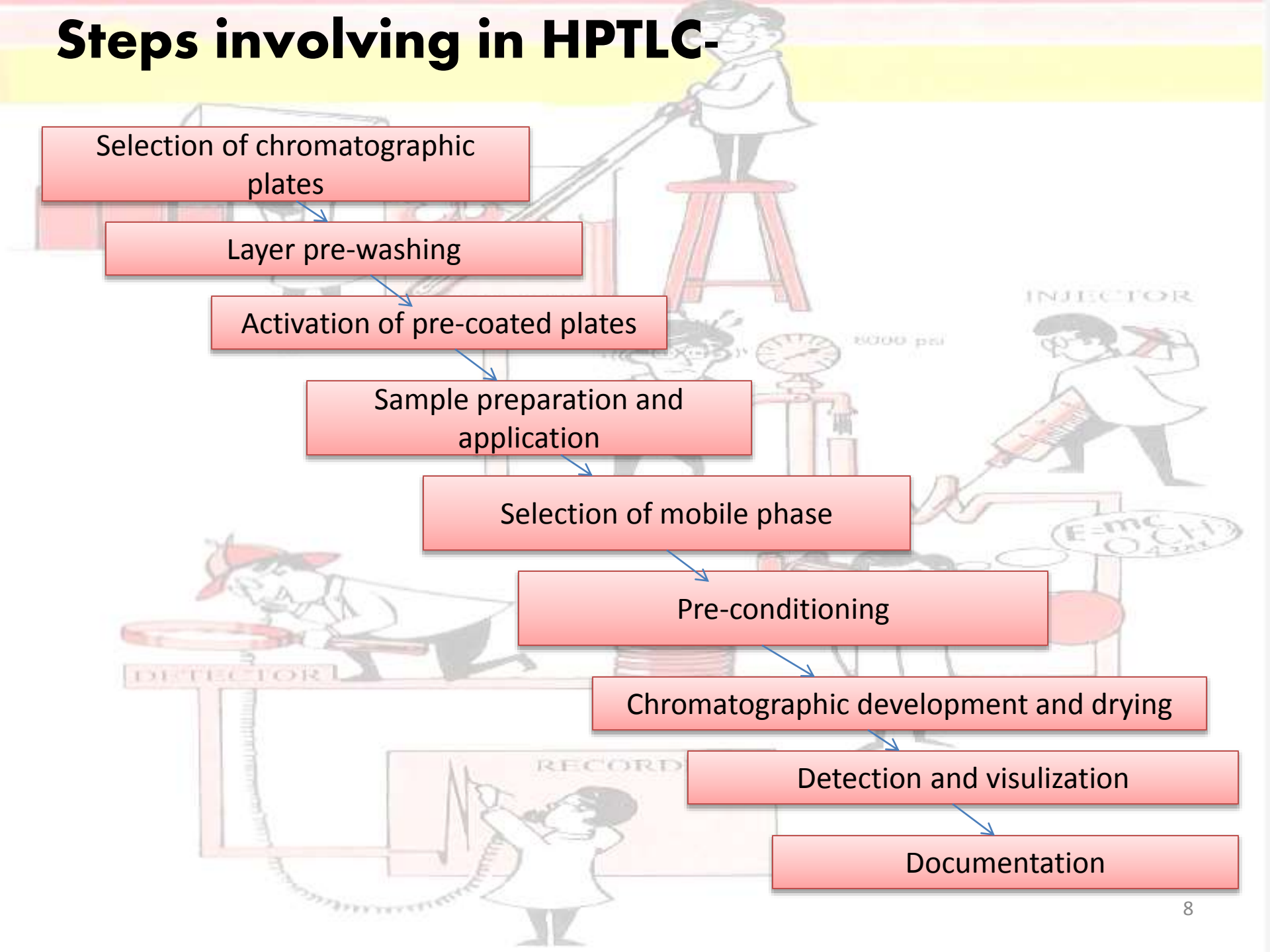
Digital camera for photo documentation

Scanner

Difference between TLC and HPTLC-

parameters	TLC	HPTLC
•Chromatographic plate used	Hand made	Pre-coated
•Sorbent layer thickness	250μm	100-200μm
•Pre-washing of plates	Not followed	must
•Application of sample	Manual	automatic
•Shape	spot	Spot/band
•Sample volume	1-10μl	0.2-5μl
•Efficiency	Low	High
•Analysis time	Slow	Greatly reduced
•Development chamber	More amount of solvent	Less amount of solvent
•Spots size	2-4mm	0.5-1mm
•Scanning	Not possible	Use of UV/ Visible/ Fluorescence scanner (densitometer)

Steps involving in HPTLC-



1. Selection of chromatographic plates-

- Hand made plates which are made up of cellulose and other materials which are not used much now a day.

- **Pre-coated plates-** The plates with different support materials and sorbent layers with different format and thickness are used for qualitative and quantitative analysis.
- **Support materials used in plates-** Glass
  Polyester/polyethylyne
  Aluminium
- **Sorbents used in plates-**Silica gel 60F, Aluminium oxide, Cellulose, silica gel chemically modified –a)amino group(NH2), b) CN group
- Smaller particle size of silica helps in greater resolution and sensitivity.

2. Layer pre-washing-

- It is purification step.
- The main purpose of the pre-washing is to remove impurities which include water vapours and other volatile substances from the atmosphere when they get exposed in the lab environment.
- In case of silica 60F(most widely used sorbent) the major disadvantage of this sorbent is that it contain iron as impurity.
- This iron is removed by using Methanol : water (9:1), this is the major advantage of the step of pre-washing.
- **Some common methods are-** Asending
  Dipping
  Continuous
- **Solvents used for pre-washing-**

  Methanol

  Chloroform:Methanol (1:1)

  Choloroform: Methanol: Ammonia (90:10:1)

3.Activation of pre coated plates-

- Freshly opened box of HPTLC plates doesn't need activation.
- If plates exposed to high humidity or kept in hand for longer time then activation is required and it's activation results by removing moisture.
- The plates are activated by placing in an oven at 110-120^0 C for 30 min, this step will removes water that has been physically absorbed on surface at solvent layer.
- Activation at higher temp and for longer time is avoided which leads to very active layer and there is risk of sample being decomposed.

4.Sample preparation and application-

Sample preparation-

- It’s important to prepare proper sample for successful separation.
- Sample and reference substances should be dissolved in the same solvent to ensure comparable distribution at starting zones.
- It needs a high concentrated solution,as very less amount of sample need to be applied.
- After that dry the plates and store in dust free atmosphere.
- Solvents used are-
    - Methanol,
    - Chloroform: Methanol (1:1),
    - Ethyl acetate: Methanol (1:1),
    - Chloroform: Methanol: Ammonia (90:!0:1),

Sample application-

- Usual concentration range is 0.1-1µg / µl,above this causes poor separation and volume recommended for HPTLC-0.5-5µl .
- The size of sample spot applied must not exceed 1mm in diameter.
- Problem from overloading can be overcome by applying the sample as band.
- **Some applicators used for application of sample-**

  Selection of applicator to be used depends on-

  -Sample volume

  -No. of sample to be applied

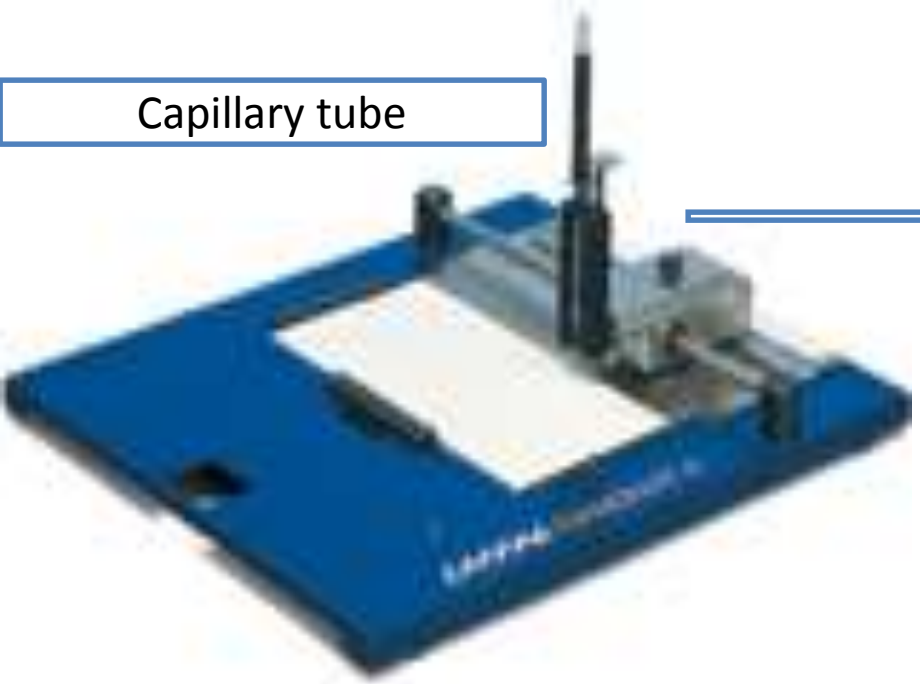
a) Capillary tubes

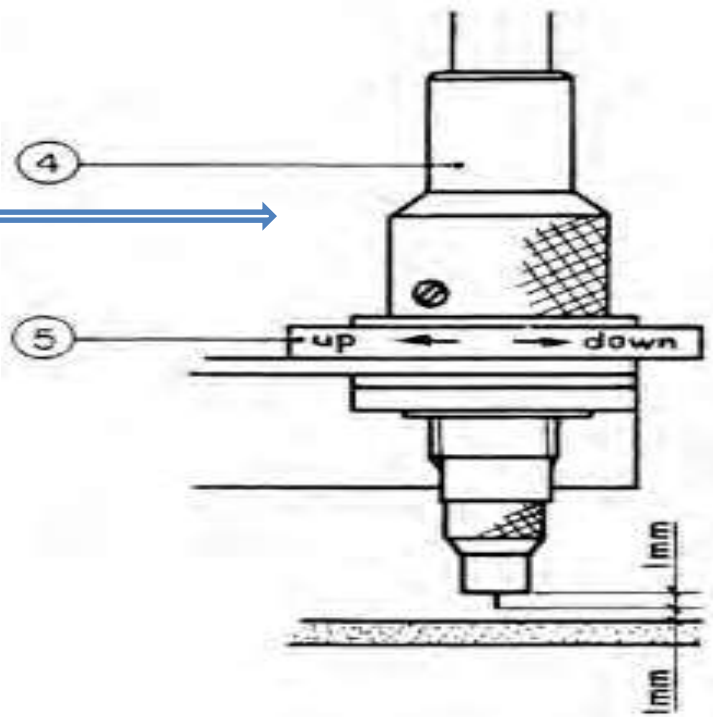
b) Micro bulb pipettes.

c) Micro syringes.

d)Automatic sample applicator.

Capillary tube





Micro syringes

Autometic sample applicator

- The major criteria is that they shouldn’t damage the surface while applying sample.
- The sample are completely transfer to the layer.
- Advantage of application of sample as bands are-

  Better seperation due to rectengular shape.

  Response of densitometer is higher in case of band because of uniform distribution of sample in band.

5.Selection of mobile phase-

- Chemical properties of analytes and sorbent layer factors should be considered while selection of mobile phase.
- Various components of Mobile Phase should be measured separately and then placed in mixing vessel.
- The less amount of mobile phase is required then TLC .
- This prevents contamination of solvents and also error arising from volumes expansion or contraction on mixing.
- Multi component mobile phase once used not recommonded for further use due to diffirent evaporation and adsorption by layer.

6.Pre-conditioning (chamber saturation)-

- Un- saturated chamber causes high Rf values.
- Saturated chamber by lining with filter paper for 30min prior to development-uniform distribution of solvent vapours-less solvent for the sample to travel-lower RF values
- For low polarity mobile phase there is no need of saturation.However saturation is needed for highly polar mobile phase.
- Chamber saturation influence separation profile.

7.Chromatographic development and drying-

- Plates are spotted with sample and air dried and placed in the developing chambers.
- The different methods used for development of chambers are like-

  Ascending

  Descending

  Horizontal

- Autometic multiple development,Circular, anti-circular device and multiple developments are some other methods.
- After development, remove the plate and mobile phase is removed from the plate to avoid contamination of lab atmosphere.
- Dry in vacuum desiccators with protection from heat and light.

Autometic multiple development

Autometic multiple development

Twin trough chamber

Horizontal development

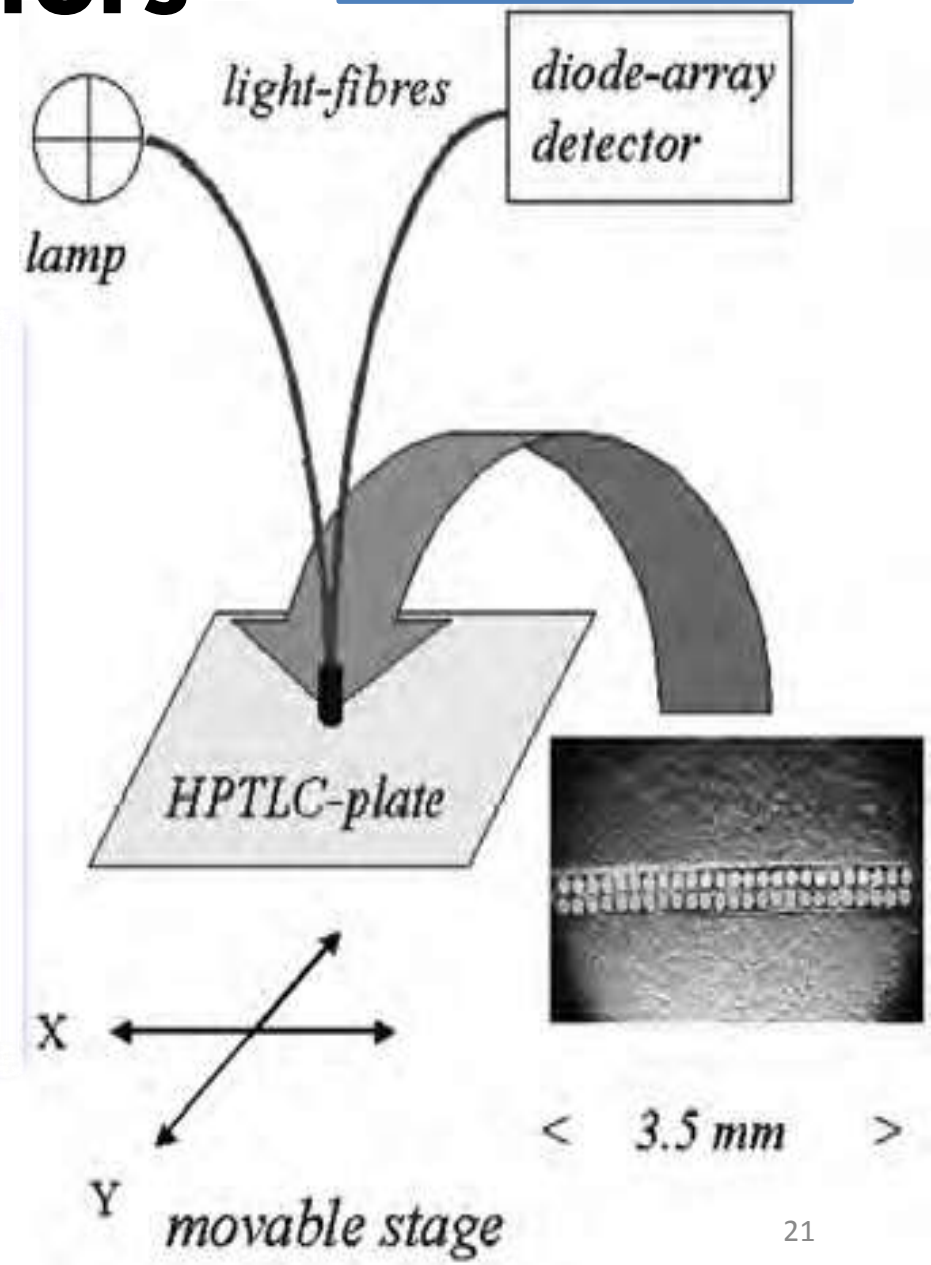
8.Detection and visulization-

- Detection under UV light is first choice.
- Non destructive and spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length).
- Spots of non fluorescent compounds can be seen fluorescent stationary phase is used - silica gel Gf.
- Non UV absorbing compounds like ethambutol, dicylomine dipping the plates in 0.1% iodine solution .

Detectors-

Diode –array detectors

UV cabinet



- **Detector consists of following-**

Lamp selector

Entrance lens slit

Monochromator entrance slit

Grating

Mirror

Slit aperture disc

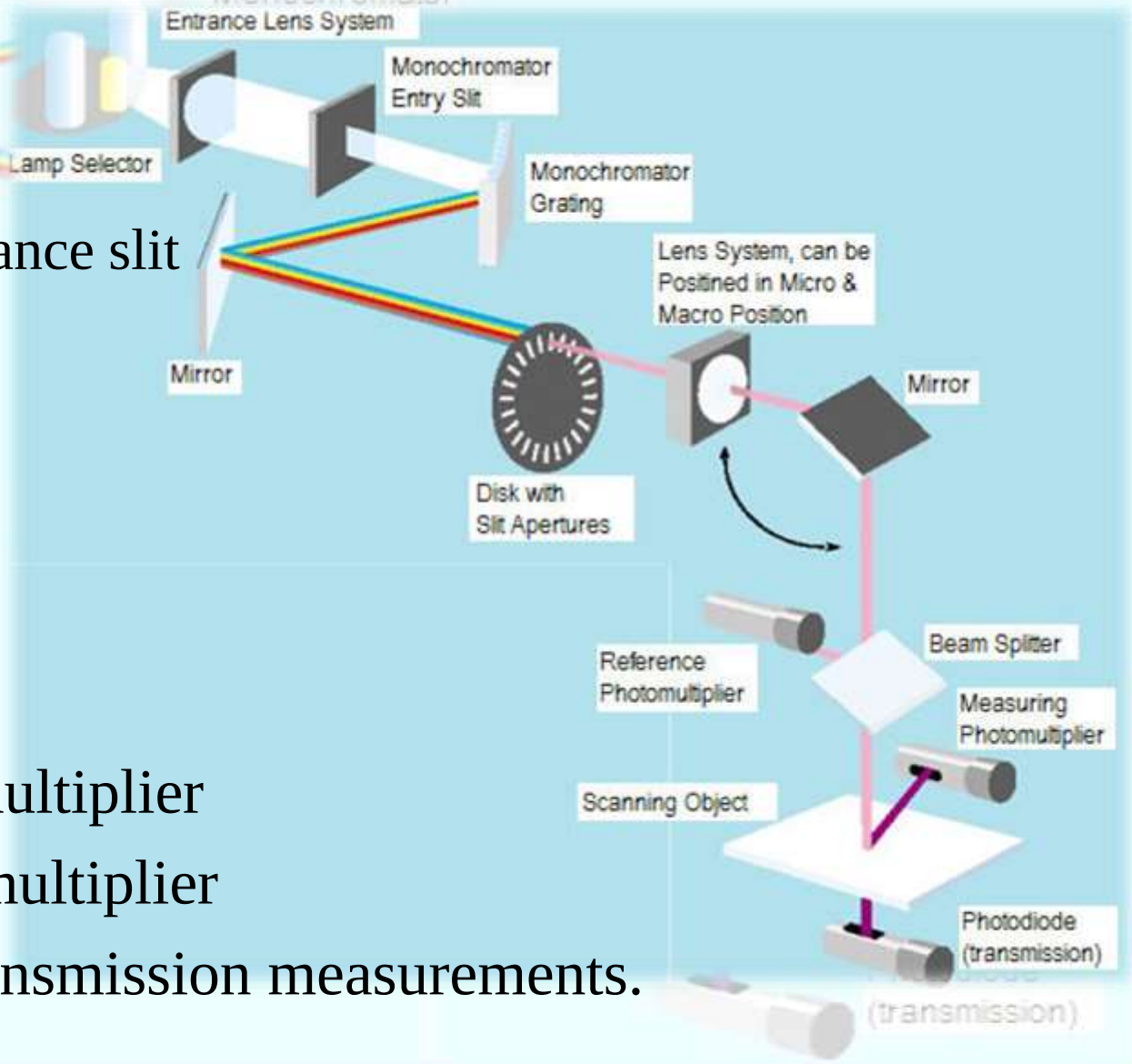
Mirror

Beam splitter

Reference photo multiplier

Measuring photo multiplier

Photo diode for transmission measurements.



9.Scanning and documentation-

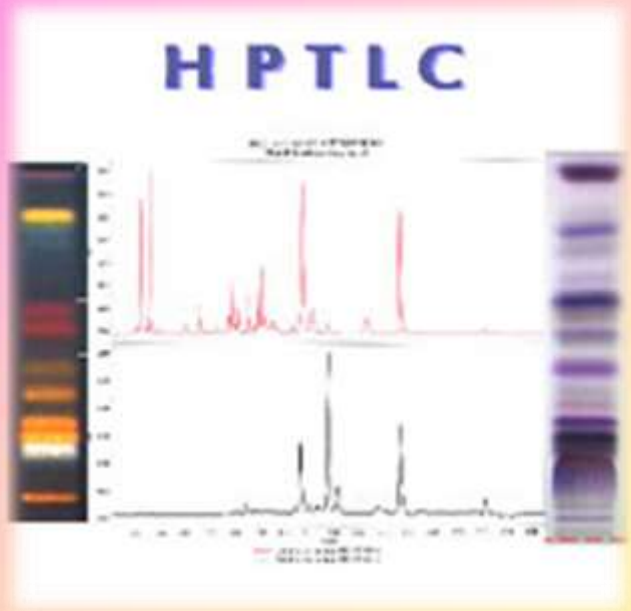
Scanning-

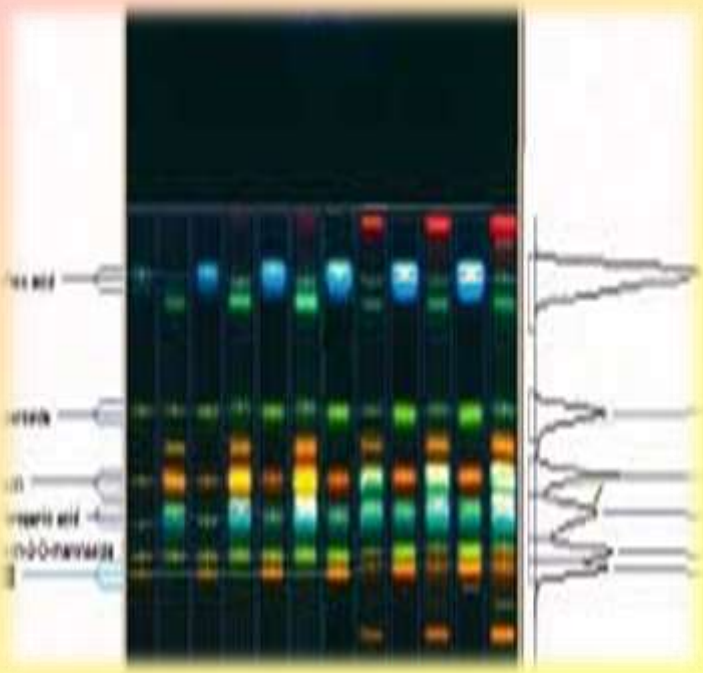
- The scanner converts band into peak and peak hieght or area is related to the concentration of substance on spot/band.
- The peak height and area under spot are measured by instrument and recorded .

Scanner

Documentation-

- Documentation is important because labeling every single chromatogram can avoid mistake in respect of order of application.
- Type of plate, chamber system, composition of mobile phase, running time and detection method should be recorded.





Factor affecting HPTLC

- Types of stationary phase.
- Mobile phase
- Layer thickness
- Temperature
- Mode of development
- Amount of sample
- Dipping zone, etc.

Applications of HPTLC

- **Pharmaceutical industry-** Quality control,identity purity test etc.
- **Food Analysis-** : Quality control , additives , pesticides ,stability testing etc.
- **Clinical Applications-** Metabolism studies , drug screening ,stability testing etc
- **Industrial Applications-** Process development and optimization etc.
- **Forensic-** Poisoning investigations
- **Biomedical Analysis-** Seperation of gangliosides
- **Environment Analysis-**Pesticides in drinking water etc.
- **Cosmetics-** Hydrocortisone & cinchocaine in lanolin ointment etc.
- **Natural products ,plant ingredients-** Glycosides in herbal drugs,Piperine in piper longum etc.
- **Finger print** Analysis-Finger prints for identification of liquorice, ginseng etc.
- **Analysis of drugs in blood-**

Thank You…