ADVANCED PHARMACOGNOSY

- A Science of the knowledge of medicinal plants
- Natural plant based medicines vs synthetic drugs
- Search of new molecules with novel biological activities.
- Tremendous support to the use of natural products- less toxic compared to synthetic drugs.
- Bioassay screening, isolation technique and structure elucidation.

Related subjects:

- Botany and phytochemistry (closely related) Pharmacology, Analytical Chemistry, Microbiology, plant tissue culture biotecnology, genetic engineering.

MEANING OF PHARMACOGNOSY

- To acquire knowledge of drugs.
- Naturally occurring substances having a medicinal activity
- Safer for cancer and AIDS
- Seydler (1815) german scientist
- “Analecta pharmacognostica”
- FUTURE OF PHARMACOGNOSY:
- large number of alkaloids, glycosides, Antibiotics isolated-curative agents.
- Artimissinin –antimalarial, taxol-anticancer, forskolin-antihypertensive.

MODERN METHODS OF EXTRACTION

- Process of separation of active constituents by using a suitable liquid solvent
- Liquid-liquid extraction
- Solid – liquid extraction
- Dried herbal material ---- size reduction --- menstrum selected (or) solvent used --- extraction – filtration – concentration --- drying (spray, vaccum, tray, freeze drying)---------packing.

METHODS OF EXTRACTION

Small scale extraction

- Percolation
- Maceration – hot maceration
  - -- Cold maceration

Large scale extraction

- Semi commercial scale of extraction or pilot scale extraction
- Super critical fluid extraction

1. SMALL SCALE EXTRACTION
Very slow, time consuming and inefficient method

MACERATION

- Crude drug immersed in a menstrum or solvent for 3-7 days with shaking. Filtered and concentrated.
- Increase in the yield of active constituents
- Drug is treated several times with solvent.

THE USE OF PLANT REMEDIES IN INDIGENOUS MEDICAL SYSTEMS

- Medicinal plants – sources of medicines and of novel molecules
- Herbal remedies are safer than synthetic drugs
- Digitoxin, digoxin, vinca alkaloids, reserpine are important drugs derived from plants
- Selection based on traditional usage
- Based on chemical composition
- Screening for a specific biological activity
- -anticancer, anti-inflammatory, antihypertensive.

THE PLANT MATERIAL

- Plant identification and authentication – herbarium.
- Selecting plants free from diseases.
- Fresh plant tissues should be used for phytochemical analysis – plunged into boiling alcohol
- Dried at low temperature.

Extraction methods

- SMALL SCALE EXTRACTION PROCESS – Maceration and percolation --- slow and time consuming – inefficient extraction of crude drugs.
- MACERATION—

  Separation of medicinally active portions of the crude drug – immersion of crude drug in a bulk of solvent or menstrum --- 3 to 7 days in a warm place with frequent shaking filtered.
- MULTIPLE SCALE EXTRACTION– increase the yield

  --crude drug – solvent is added – extracted solution removed – stored in the receiver tanks – again recharge with fresh drug.

PERCOLATION

- Continuous flow of the solvent through the bed of the crude drug material to get the extract – powdered drug is treated with sufficient menstrum (wet) – for the saturation of the drug allow the drug material to macerate in the vessel for 24 hrs.
- More menstrum is added from top.
- Collect the menstrum extracted and concentrated.

CONTINOUS HOT PERCOLATION PROCESS OR SOXHLET EXTRACTION

- Hot menstrum is passed over the drug to dissolve the active constituents until the drug is exhausted (soxhlation)
- Small amount of menstrum is required.
- LARGE SCALE EXTRACTION PROCESS

  SUPER CRITICAL EXTRACTION
- Gases, free flowing liquids at critical point of pressure and the temperature, high penetration power and extraction effciency
- Carbon dioxide – bacteriostatic and non explosive.

Methods of Extraction

Maceration

- This method is based on the immersion of the crude drug in a bulk of the solvent or menstrum.
- Solid drug material or powder is taken in a stoppered container with about 750ml of the menstrum and allowed to stand at least for three to seven days in a warm place with occasional shaking.
- The mixture of the crude drug containing solvent is filtered until most of the liquid drains off.

Percolation

- Percolation is a continuous flow of the solvent through the bed of the crude drug material to get the extract. In this process, first the powdered drug is treated with sufficient menstrum to make it uniformly wet.
- Damp material is allowed to stand for about 15mins, and then transferred to a percolator which is generally a‘V’ shaped vessel open at both the ends.
- The drug material is allowed to macerate for24 hrs and the percolation is continued.
- The percolation process is dependent on the flow of solvent through the powdered drug and it yields the products of greater concentration than the maceration process.

Continuous hot percolation (Soxhlet extraction)

- Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extraction for several times (Hot Percolation Process). This process involves extraction followed by evaporation of the solvent.
- The vapours of the solvent are taken to a condenser and the condensed liquid is returned to the drug for continuous extraction (successive solvent extraction process).

SEMI COMMERCIAL SCALE EXTRACTION UNIT

Extraction chamber

- Made up of stainless steel and having outer jacket
- Extraction – condenser – condensed recirculated
- Add solvent, steam is passed through the outer jacket from boiler.
- Extract is removed and concentrated – concentration chamber

Concentration Chamber

- Steam is passed through the jacket – solvent evaporated condensed in to liquid – collected in receiver.

OTHER METHODS

COUNTER CURRENT EXTRACTION

Solution, emulsion, suspension or solid mass is extracted by a liquid phase.

INFUSION – Soak the drug powder in the solvent (room temperature) for a period of time. filter

DECOCTION

Extraction with hot water or cold water is added to the powdered drug – boil carbohydrates, flavanoids, tannins and saponines.

EXTRACTION METHODS FOR SPECIFIC PHYTOCHEMICAL GROUPS

1. Alkaloids – chloroform, dichloro methane or di ethyl ether, ethanol or methanol
2. Carotenoids – red, orange and yellow pigments of the plant. Petroleum ether, ethanol, chloroform
3. Fixed oil, fats and waxes – non polar in nature, expression process
4. Glcosides – acetone, ethanol, methanol, chloroform

TYPE OF EXTRACTS

1. Aqueous extracts – infusion, decoction, maceration
2. Tinctures – ethanol 1 Part of drug with 2 – 10 parts of menstrum
3. Fluid extract – 1 part of drug with 2 parts of fluid extract
4. Thin extract – liquid extract concentrated to honey like consistency

- Thick extracts or viscous extracts (warm) – not fluid at room temperature
- Dry extracts – liquid extracts are dried under mild conditions
- Oil drug extracts or olea medicata – suspending powdered drug material in to non drying oil with mild heat. Eg: rose flowers, mari gold etc…
- Oleo resins – plant gum and resins extracted with ethanol and ethyl acetate

DRYING

- TRAY DRYING – hot air of the desired temperature is circulated through the dryer
- VACCUM DRYER – drugs sensitive to high temperatures
- SPRAY DRYERS – for quick drying (herbal extracts)

STORAGE

store in air tight, moisture proof light proof containers

- QUALITATIVE PHYTOCHEMICAL SCREENING

  The different chemical tests performed for the identification of its chemical composition.

- DRYING BY SPRAY DRYER (MOST SUITABLE METHOD)

1. For free flowing and hygroscopic product
2. The filtered atmosphere air is heated by electrical heater and introduced from the top of spraying chamber till the powder totally dried.

SEPARATION, ISOLATION AND PURIFICATION

- CHROMATOGRAPHY: (for separating plant constituents) ; chromo – color, graphy – separation.
- 1906 – Tswett seperated extracts on column (absorption)
- 1944 – consden, Gordon and martin introduced a partition chromatography using strip of filter paper for the analysis of the amino acid mixtures

DEFINITION

- A group of methods for separating molecular mixtures that depend on the differential affinities of the solute between two immiscible phases.

- STATIONARY PHASE OR FIXED PHASE

  Solid or liquid coated on an inert support material.
- MOBILE PHASE OR MOVABLE PHASE

  Pure liquid or mixture of solution gas or mixture of gas
- ELUTE THE COMPONENT

  Cut out spots, dissolve with suitable solvents – quantitative analysis – UV, NMR, MASS etc…

TYPES OF CHROMATOGRAPHY

TYPE	MOBILE/ STATIONARY PHASE
Absorption or column	Liquid/ solid
Thin layer chromatography	Liquid/ solid
High performance liquid chromatography	Liquid/ solid
Gas liquid chromatography	Gas/ liquid
Paper or partition chromatography	Liquid/ liquid

CHROMATOGRAPHY

Separation of mixtures in to individual components using stationary phase and a mobile phase.

- Thin layer chromatography (TLC)
- High performance thin layer chromatography (HPTLC)
- Gas liquid chromatography (GLC)
- Column chromatography (CC)
- High performance liquid chromatography (HPLC)
- Gel permeation chromatography (GPC)
- Affinity chromatography

PRINCIPLE: Based on the principle of separation
Adsorption and Partition

ADSORPTION	PARTITION
LIQUID/ SOLID	LIQUID/ LIQUID
More affinity – slow Less affinity - fast	More soluble in mobile phase moves fast

Two components will not have same affinity

THIN LAYER CHROMATOGRAPHY

- 1985 , STAHL – Application of TLC
- Principle of separation is adsorption
- 1 or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate
- The compounds move according to their affinities towards the adsorbent and separated
- More affinity – slow , less affinity - less

Stationary phase	**adsorbent : water ratio**
• Silica gel G	1:2
• Silica gel H	1:15
• Silica gel GF	1: 2

- AL oxide , calcium hydroxide,celite, magnesium phosphate, polyamide, cellulose mixtures of two or more

Glass plates:

- 20 cm x 20 cm , 20 cm x 10 gm , 20 cm x 5 cm

  TLC Spreader 20 cm x 10 cm – commercial
- Good quality and with stand temperatures

Preparation and activation of TLC plates:

Preparation of slurry by

- Pouring, dipping, spraying , spreading
- Thickness 0.25 mm – 2mm , TLC spreading

Activation of the TLC Plates

(100-110 c in an oven for 30 min)

APPLICATION OF SAMPLE : The concentration of the sample or standard soln should be minimum 2-5 micro litre of a 1% soln of either standard or sample is spotted using a capillary or micro pipette.

Development tank : Glass beakers , specimen jars , TLC Chambers

Mobile phase : according to the chemical constituents or nature of the substances

Petroleum ether, carbon tetra chloride , acetone benzene , toluene , ethyl acetate , chloroform , alcohol, water , pyridine , mixture of acids or bases

- Detecting or visualising agents : colored spots – visually
- Color less – iodine chamber , UV fluorescence
- Nin hydrin in acetone – for amino acids
- Dragendroffs reagent – for alkaloids
- Qualitative analysis by calculating Rf values
- separated compounds are recovered , dissolved in solvent such as ether

Applications of TLC

- For analysis of alcohols , glycosides , iso prenoids , lipids , sugars , and derivatives
- Conc .sulphuric acid can be used as a spraying reagent
- Simple to operate , economical and rapid
- Pre coated plates are commercially available

PAPER CHROMATOGRAPHY

- It is a useful technique because it is relatively quick and requires small quantities of material
- The stationary phase is usually a piece of high quality Whatman's filter paper.
- The mobile phase is a developing solution that travels up the stationary phase carrying the samples with it.
- Components of the sample will separate on the stationary phase versus how much they dissolve in the mobile phase.

Descending paperchromatography

- Suspend the spotted chromatographic sheet in the tank by the use of anti siphoning rods which hold the upper end of the sheet in the solvent trough.
- A sufficient quantity of prescribed mobile phase is introduced through the inlet in to the solvent trough. Close the tank and allow development to proceed for the distance.
- Protect the paper from bright light during development.
- Remove the paper from the tank and allow it to dry in air and visualize the spots.

Ascending paper chromatography

- The mobile phase is saturated in the tank for 24 hours. The spotted
- chromatographic sheet is suspended in to the mobile phase and allowed for development to proceed for the specified distance.
- The paper is protected from the bright light. Remove the paper from the tank and allow it to dry in air and visualize the spots.

Preparing the chamber:

- Choose the developing chamber that can be sealed well. The chamber should be large enough to hold the paper that is to be developed.
- The chamber should be 2cm deep. Seal the chamber tightly and let the chamber stand overnight (24 hours) if possible. The larger the chamber, the longer it should stand.

Preparing the stationary phase

- Cut a square piece of high quality filter paper to fit in to a development chamber.

With a pencil, draw a straight line about 3cm from the bottom edge of the paper.

- (Do not use ink pen or sketch pen for marking)

Spotting the samples

- First each sample should be dissolved in an appropriate solvent to make about a

1% solution (0.01 gm sample/1gm solvent) Less than 1 ml of solution will be needed for the experiment.

- Then the dissolved samples may be spotted to the paper.
- All spots on the chromatogram should be 2 – 2.5 cm away from the edges of the paper and from each other.

Developing chromatograms

- After preparing the chamber and spotting the samples, the paper is ready for development.
- Initially, the chromatogram should be suspended in the chamber without touching the solvent.
- To suspend the chromatogram, fold the top of the paper and thread a piece of string through the paper clip.
- Then tape the string to the outside of the chamber to hold the chromatogram in place.
- Immerse the papers of the bottom edge in to the developing solvent.
- Allow the chromatogram to dry in a well ventilated area.

(CAUTION: Do not look directly in to the lamp)

- Many organic compounds can be seen using this technique.
- Outline the spots with a pencil.

Interpreting the data

- The Rf value for each spot should be calculated. Rf stands for “Ratio of fronts” and is characteristic for any given compound.
- Hence known Rf values can be compared to those to those of unknown substances to aid in identifications.

- Rf = Distance from start to centre of the substance spot
- Distance from start to solvent front

- (NOTE: Rf values often depend on the temperature, solvent, and type of paper
- used in the experiment. The most effective way to identify a compound is to spot
- known substances next to unknown substances on the same chromatogram)

Application

- Preliminary analysis of a plant extracts for the presence of flavanoids, sugars,
- alkaloids, glycosides, carbohydrates, amino acids and proteins.

Column chromatography (ion exchange)

- Column of stationary phase is used either a glass or metallic column (Adsorption)
- solid SP/liquid MP
- Mixture of compounds is dissolved and introduced in the SP in to the column individual components move with different rates depending upon their affinities
- Less affinity moves fast and eluted first

Packing of the column

Dry packing and wet packing(ideal technique)

Advantages: absent of air bubbles , there will not be any crack in the column of adsorbent . The bands eluted will be uniform and ideal for separation.

Detection: uv and flourescence detector

APPLICATIONS: Separation of mixture of compounds like alkaloids, glycosides , amino acids and plant extracts.

Isolation of active constituents.

- Chromatography systems have a stationary phase (which can be solid or liquid) and a mobile phase (usually liquid or gas) In column chromatography both phases are placed in a column container.
- Colum chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out small scale experiments.
- It is a solid liquid technique in which two phases are a solid (stationary phase) and a liquid (moving phase).

- The theory of column chromatography is analogous to that of thin layer chromatography. The most common adsorbents are silica gel and alumina.
- The sample is dissolved in small quantity of solvent (the eluent) and applied to the top of the column.
- The eluent instead of rising by capillary action up, a thin layer flows down through the column filled with adsorbent.
- There is an equilibrium established between the

solute adsorbed on the silica gel or alumina and the eluting solvent flowing down through the column.

SEPARATION TECHNIQUES – COLUMN CHROMATOGRAPHY

- PACKING OF COLUMN
- Clean the column with acetone, make sure that it is completely dry
- Wash the column with hexane or chloroform depending on whether your starting eluent is hexane or chloroform.
- Saturate the column with a minimum quantity of hexane (100 ml) (if yourstarting eluent is hexane)

- Close the column with aluminium foil.
- Choose your adsorbent – silica gel, alumina, sephadex
- If it is silica gel then decide upon mesh size (60 – 120,100 – 200, 200 –300). Usually 100 – 200 mesh is used.
- Mix the silica gel with hexane and add the silica gel (which is now mixed with hexane) to the column.
- Keep the nozzle of the column open while you load the column.
- Collect the hexane and you can re use the same.
- Load the silica to the desired length.
- Give 4 – 5 washes, allow the silica gel to settle down.
- Check whether the silica gel is packed to the desired height and whether the final level is flat.
- Make sure to have a little amount of solvent (hexane) over the silica gel.

SAMPLE PREPARATION

- The sample must be dry. (without the solvent)
- Take 1 gm of the extract and mix it with a little amount of silica gel and grind to a fine powder in a pestle and mortar.

LOADING OF SAMPLE

- Add the prepared sample over the silica gel column you have packed.
- Above the sample which you have loaded place a small piece of cotton or filter paper to avoid disturbance of the sample when you pour the solvent.
- Keep the nozzle of the column closed when you load the sample.

COLUMN ELUTION

- Start eluting the column with hexane, if your starting eluting solvent is hexane and then increase the polarity of the solvent for e.g.
- Hexane –Chloroform – Ethyl acetate – Methanol.
- There should always be some amount of solvent over the packed column to avoid drying of the column.
- Collect the fractions (20 ml) as they elute from the column. Check the eluted fractions by TLC and pool similar fractions.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

- Separation takes place with a packed column .
- The packing material (stationary phase)may be solid or a liquid coated over an inert material.
- A liquid (mobile phase) is used a eluent , forced through the column under high pressure (Elution)
- Most versatile, safest , dependable ,fstest and sensitive chromatography technique for the quality control of drug components.
- Ex : Drugs like alkaloids, cardiac glycosides , sennosides ,vitamins, antibiotics steroids etc can be analysed by HPLC .

- In microbore (HPLC) chromatography the column used is very narrow (1 mm or less) . Flow rate of the mobile phase is also very less (100µl /min).
- Used for regular analysis of drug
- This method saves the amount of solvent used as the mobile phase.

ISOLATION AND CHARACTERIZATION OF PHYTOCHEMICALS BY HPLC

- “ In 1906 – TSWETT” separated the extracts on column
- “CHROMA”-color “TOGRAPHY”-to write
- “Separation of compounds in a sample by distribution between two phases. One is mobile phase and another is stationary phase.”

ROLE OF EACH COMPARTMENT

- SOLVENT DELIVERY SYSTEM-Pushes the solvent stream through the instrument at constant flow rate.
- AUTO SAMPLER-Introduces the sample into the liquid stream of the instrument .
- COLUMN-A stainless tube packed with silicon beads that separates the compounds from other compounds .
- Ex:Caffeine from tea.

- DETECTOR-An optical sensor that detects the change in the characteristics of the solvent system.
- DATA SYSTEM-Storing ,processing and displaying data.
- PUMP-A high pressure pump force the mobile phase through the column at typical flow rates of 0.1 -2 ml/min .
- The sample to be separated is introduced into the mobile phase by injection device ,manual or automatic prior to the column.

COMMON PROPERTIES OF THE MOBILE PHASE:(ELUENT)

- Purity
- Detection compatibility
- Solubility of the sample
- Low viscosity
- Chemical inertness
- Reasonable price

TYPES OF CHROMATOGRAPHY

S.NO	TYPE	MOBIE / STATIONARYPHASE
1.	Adsorption or column chromatography	Liquid/solid
2.	Thin layer chromatography	Liquid/solid
3.	High performance liquid chromatography	Liquid/solid
4.	Gas- liquid chromatogaphy	Gas/liquid
5.	Partition or Paper chromatography	Liquid/liquid

- **HPLC-**Chemistry based tool for quantifying and analyzing mixtures of chemical compounds.
- **USES-**To find out the amount of chemical compound within a mixture of other chemicals . ex: capsaicin in capsicum fruits
- Ex:caffeine content in the cup of coffee or tea.
- For analyzing the sample should be dissolved in a solvent (like water or alcohol)-LIQUID CHROMATOGRAPHY.

CHROMATOGRAPHY SCALE

- Analytical HPLC-Data using minute quantities of analyte (highly sensitive)
- Semi-preparative HPLC-Data and a small amount of purified analyte(Gram)
- Preparative HPLC-Larger quantities of purified analytes(Kilogram)-high capacity

MOBILE PHASE CHART BASED ON THE DETECTOR

DETECTOR	ANALYTE	SOLVENT REQUIREMENTS
UV-Visible	Any with chromophore	UV grade non absorbing solvents
Flourescence	Flourescent compounds	UV grade absorbing solvents
Refractive index	Compounds with different RI to the mobie phase	Non absorbing solvents
Conductivity	Charged or polar compounds	Mobile phase must be conducting
Electro chemical	Readily oxidized or reduced ends	Mobile phase must be conducting
Mass spectrometer	Broad range of compounds	Must use volatile solvents and buffers

ADVANTAGES

- Very efficient resolution compared to older methods.
- Less dependable on operators skill &reproducibility is greatly improved.
- Automatic and analysis times are generally much shorter.
- Used to add new dimensions to the practical utility of HPLC in plant drug research .

ESTIMATION OF CAPSAICIN IN CAPSICUM ANNUM USING HPLC TECHNIQUE(BY:LINDSEY YEOMAN)

- The major pungent principles of" *Capsicum annum*"(Solanaceae) are a mixture of capsaicin , dihydro capsaicin and non dihydro capsaicin.
- Capsaicin is used as a carminative &counter irritant.
- Capsicum contains 1% of pungent principle capsaicin (colourless, crystalline in nature)

Aim: To estimate capsaicin content in powdered capsicum by HPLC.

- **Materials used:**
- Schimadzu SCL-6A model HPLC
- Hamilton syringe
- C-18 column (Bond pack 25*4 mm)
- Chloroform
- Standard * sample capsaicin
- Methanol(HPLC grade)
- UV /Visible detector

Procedure:3 main steps involved in the analysis of capsaicin.

- Extraction of capsaicin & related compounds from capsicum fruits.
- Precipitation of std curve of capsaicin and related compounds
- Calculation and estimation of capsicum from std curve .The conc. Of capsaicin in the test sample.10gm of capsicum fruits with 3 vol. of chloroform on a shaker for 30 min. Separated chloroform layer, evaporate to dryness. Residue is dissolved in methanol (HPLC)grade Store it in refrigerator.

Preparation of STD curve of capsaicin

- Different conc. 50 µg,100µg,150µg,200µg,&400µg/ml are precipitated using methanol(HPLC) grade.
- Flow rate- 1ml/min
- Mobile phase-80%methanol v/v in HPLCgrade
- Column-Reverse phase(c-18 column bond pack 25cm*4mm)
- Inject the samples (10µl) with Hamilton syringe individually. The area and the peak of the curve represents the conc. of the substance. The no. of peaks depends upon the constituents present in the sample.

- The peak of max height –capsaicin – major constituents.
- The std graph is plotted with Con. in µg(x axis) and Peak heights (y axis). A straight line indicats capsaicin.
- Comparision with sample solution ,capsaicin could be estimated

- Different types of bio constituents like cardenolides, tropane alkaloids , flavanoids , steroids , glycosides , lipids etc

Detection and estimation of some herbal constituents

- Flavanol glycosides in gingko biloba leaf extract
- Aloin in tincture of Aloe vera

GAS LIQUID CHROMATOGRAPHY

- James and Martin 1952 / based on Martin and Synge sugges
- Separation of volatile substances by percolating a gas stream over a stationary phase . N and Helium are used as carrier gas.
- Film of liquid spread over an inert solid .

GLC APPARATUS

THE COLUMN: Metal arrow tube in the form of coil with an celite SP Coated with 5-15 %

Silicon oil

THE HEATER: H eat the column 50 – 350 to vaporize the sample.

sample dissolved in ether or hexane is injected in syringe through a rubber septum.

GAS : Inert gas Nor Helium

Separation of the compounds on passing this gas at a control rate

DETECTION DEVICE: M easure the compound based on flame ionisation or electron capture.

Recorded as peaks.

RV-Retention volume (vol of carriergas required to elute a component from the column)

RT-Retention time (time required for the elution of the sample) Peak height x peak width =95% of the peak area

APPLICATIONS OF GLC

- Volatile oils , plant acids , sugars , amino acids etc
- Quantification of drug in formulations
- Assay of the drug substances
- Assay of impurities in the raw material or in drug substances

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY(HPTLC)

HPTLC-Sophisticated and automated form of TLC

- Evaluation of conventional TLC in to a modern instrumental technique started in 1975.
- Modern HPTLC is a powerful, reliable , and cost efficient method for qualitative and quantitative analysis of pharmaceuticals.

Synonyms

- PLANER CHROMATOGRAPHY
- INSTRUMENTAL TLC
- MODERN TLC

- It combines the art of chromatography with quickness at a moderate cost ,shorter time, and better resolution.
- The basic difference between conventional TLC and HPTLC is only in particle and pore size of the sorbents.
- The plates are similar to conventional TLC plates(pre coated) silica gel of very fine is widely used as sorbent in HPTLC.
- The smaller size of the sorbent (4-5µm silica gel with an inert binder to form a 200µm layer)- Greater resolution and sensitivity.
- (7 cm distance is achieved in 4 min)
- The samples are accurately and precisely estimated
- Simultaneous multi sample analysis on a single plate (more than ten ingredients)
- Compared to other methods, this provides rapid analysis of plant

VARIOUS STEPS INVOLVED IN HPTLC STUDY

- Selection of stationary phase and mobile phase
- Sample and standard solution preparation
- Application of a sample and std soln on chroma layer
- Development of the medium
- Detection of spots
- Scanning and documentation of chromato plate

SAMPLE PREPARATION: II

- HPTLC needs a high concentrated solution a very less amount of sample to be applied –The size of the sample spot must not exceed 1mm in diameter .
- For spotting self loading capillaries (small vol. of sample)may be applied to the HPTLC plate surface using platinum irridium tubing fused in to the end of a length of glass tubing.
- Contact spotting ,pre adsorbent or concentrating zones, chemical focusing and programmed multiple development are the other methods for development of plates

DETECTION OF HPTLC PLATES

- HPTLC separation materials is available in the form of pre coated layers supported by glass, plastic sheets or aluminum foil.
- Pre coated layers of aluminium oxide , cellulose , polymide , on exchange materials , reserved phase silica (alkyl bonded) have been commercially available , the vast majority of seperations is carried out on normal phase silica gel, on normal phase silica gel.
- Mainly for economic reasons pre coated HPTLC plates in size of 20x20cm with aluminium or polyester support are usually procured mainly for enonomic reasons.
- These plate were shaped & cut to suit particular analysis by using scissors.
- Pre coated silica gel GF_{254} plates with aluminium supported were used.

APPLICATION OF SAMPLE

- The suitable sample volume may be applied either spotwise with devices with a controlable delivery speed or they are sprayed on the form of narrow bands.
- Sample application in the form of narrow bands provides the indent resolution.
- The “Linomat” allows sample application In narrow bands by a spray on technique under an inert nitrogen gas blanket.
- All the sample solution were applied on thin band of 6 mm width containing a sample of 5µl for the sample solution in to a suitable track on the plate by using “Camag Linomat”IV- A microprocessor controlled and programmable applicator.

SELECTION OF DEVELOPING MEDIUM:IV

The quantitative/qualitative analysis can be done only if an ideal mobile phase on developing medium, that can resolve themaximum no . of compounds present

CHROMATOGRAPHIC DEVELOPMENT: V

- Ascending , decending ,two dimensional , horizontal, multimodal, forced flow planer chromatography are most common methods.
- Rectangular glass chamber, two trough chamber are used commonly for development.(the chamber saturated with mobile phase)
- The linear development method is most familiar technique in HPTLC . The plates are placed vertically in solvent system in a suitable container . The solvent is usually fed by capillary action and chromatogram can be developed.
- Circular development , multiple development were used

- After proper development the plates were removed from the chamber and air dried with the help of hand dried to effect faster removal of the mobile phase.
- For ex: sample ppn of Senna-2 gm of Senna leaf powder +15ml of methanol triturated well, filtered – adjust the filterate vol. to 25ml with methanol- used for the analysis of Sennoside A.
- Mobile phase: n-propanol:Ethyl acetate:water:G. Acetic acid (40:40:29:1)-100ml.
- After the sample application in narrow bands the plate are dried and kept in a saturated chamber for the development.

CHROMATOGRAM EVALUATION (SCANNING): VI

- For densitometric measurements of a thin layer chromatogram its separation tracks are scanned with a light beam in the form diffusely reflected light measured by photosensor
- By measuring the absorbance or fluorescence most often at 366 nm- the resultant peaks are printed in the detector .
- The areas included in those peaks are measured and related to amount of material in the spot.

STANDARDIZATION AND STANDARDIZED EXTRACTS

- Herbal preparations are made from different parts of plants and formulated in to herbal dosage forms including tablets, capsules,powders, cream etc.
- Herbal drugs consist of active principles and active constituents
- In order to obtain a uniformity of the active principles in the formulation it is necessary to standardize the herbal raw materials
- Most commonly used method is HPTLC.

- ADVANTAGE: Standardized extracts have increased therapeutic efficacies than the whole herb or non standardized extracts.
- DISADVANTAGE: The active components in many herbal products are not known or not well understood. These are standardized by “Marker” compound –may not posses the biological activity

FINGER PRINT ANALYSIS

- For herbal analysis HPTLC is the most suitable technique
- Finger print analysis is essentially analytical data that compares a std extract with sample.
- The analytical data is produced by which phytoconstituents present is mostly unknown.
- So the finger print is a set of data points from the standard which are to be compared with data points obtained from the samples under exactly identical conditions.

CHROMATOGRAPHIC CONDITIONS OF CURCUMIN:

- MOBILE PHASE: Pour 10 ml of mobile phase consisting of Chloroform- Ethanol-Glacial acetic acid(95:5:1) into a Camag turn trough chamber and allow to stand for 15 min for chamber , saturation . Develop the plates in the mobilephase up to 8 cm long by ascending technique.

CHROMATOGRAPHY : Apply sample and std solution on to plates as 8 mm bands by means of Camag linomat IV.

- Pour mobile phase in to a Camag twin trough chamber and leave for 15 minutes for saturation.
- Develop the HPTLC plates up to 8 cm long by ascending technique
- Remove the developed plates from the chamber and dry
- Observe the plates under UV light scanning at 366nm.
- Find out the Rf values of each end, calculate the amount const from peak area &peak height.Record the spectrum of curcumin

VALIDATION METHODS

Validation of HPTLC methods has 2 kinds

- **Qualitative Methods**: Focused on Rf values, sequences and colour of zones , visual or densitomatric evaluation of chromatogram is preferably done based on image of the HPTLC plate.
- **Quantitative Methods**: Determine the amount of known, well separated substances. Peak areas/heights of analyte are calculated against calibration curves of sample and std generated from the plates

The following Parameters should be considered for the validation of HPTLC Method:

PRECISION: (PRECISION ON THE PLATE):

- It refers to multiple application of the same test solution on one plate
- It describes homogeneity of chromatography across the plate including application, development and evaluation.

REPEATABILITY: (INTRA-ASSAY PRECISION)

- It refers to the analysis of the same test solution on the different plate on the same day.

INTERMEDIATE PRECISION: (WITH IN LABORATORY PRECISION)

- Analysis of sample in the same laboratory on different days, if possible by different analytes using diff equipments it assess environmental affects as well as human factors.

REPRODUCIBILITY: (INTER LABORATORY PRECISION):

- It refers to the analysis of sample in different laboratories reproducibility applies only to collaborative studies.

SPECIFICITY

- For quantitative determination of wave length unique for the absorption of the target compound.

LINEARITY

- Linear working can be reached by diluting the sample amounts will be below 250 ng .for absorption measurements

ACCURACY AND RECOVERY:

- Recovery is calculated as quotient of measured amount and added amount of analyte.

CHROMATOGRAPHIC CONDITIONS OF CURCUMIN

Stationary phase : pre coated silica gel GF254.

Moblie phase: chloroform –ethanol – glacial acetic acid.

(95:5:1).

Developing mode : ascending.

Developing chamber: twin trough chamber(camag).

Saturation time: 15 minutes .

Lamp used: deu terium.

Scanning wave length: 366nm .

Measurement mode: absorption/reflection.

Volume applied: 2-4ml (STD).

Volume applied: 2-5ml,(sample).

Band width: 8mm.

SELECTION OF MOBILE PHASE

- If the chemical nature of analyte (sample) is known, mobile phase is as follows.
- **Alkaloidal drugs:** Toluene: Ethyl acetate: Diethyl amine. (7:2:1).
- **Flavonoiddrugs**:Ethylacetate:Aceticacid:Formicacid: Water (100:11:11:26)
- **Essential oils**: Toluene: Ethyl acetate: Chloroform (95:5.85:15).
- **Saponins**:1-butanol: Water: Acetic acid(5:4:1).
- Chloroform: Methanol: Water (7:4:1).
- **Cardiac glycosides:** Dichloromethane; Methanol: F0rmamide(8:2:1)
- **Amino acid:** Butanol: Acetic acid: Water (4:5:1).

SELECTION OF STATIONARY PHASE

- Silica gel is widely accepted as adsorbent it is cheap and highly recommended.
- Results obtained on pre coated plate from different manufacturers are not always comparable.
- Initial steps of method development plates do not have to be pre washed.
- Silica gel stationary phase should be charged if there is no migration observed.

HPTLC METHODS USED FOR DETECTION/ ESTIMATION:

18 BETA-Glycyrrhetinic acid

- Plant product :liquorice formulation
- Stationary phase :silica gel
- Mobile phase :Ethylacetate –methanol – ammonia(10:3:1)
- Quantification :uv absorbance 260nm

Panaxadiol and panaxatriol

- Source :marketed formulation of ginseng
- S.P :silica
- M.P :chloroform-ether(1:1)
- Detection:spraying with 10%sulphuric acid in methonal ,heating at 105ºc,10 min
- Quantification :UV absorbance :544 and 52 nm

Flavonol glycosides

- Source : Gingko biloba leaf extract
- S.P :silica gel
- M.P :chloroform :ethanol:acetic acid:water
  (11:4:2:1:2)
- Detection :spraying with 8%ALCL3 in ethanol
- Quantification :uv absorbance 370 nm in densitometer

Carvone

- Source : extract of *cuminum cyminum*
- S.P : silica gel
- M.P :chloroform – acetone (100:2)
- Detection : BY dipping in anisaldehyde sulphuric acid reagent ,heating at 80ºc for about 10 min
- Quantification : uv absorbance in densitometer at 410nm

Cholestrol

- Source : bear gall bladder powder
- S.P :silica gel
- M.P :ethyl acetate –acetone –petroleum ether(2;1:11)
- Detection : spraying with 10% sulphuric acid in alcohol ,heating at 100ºc for 5 min
- Qualification : uv absorbance in densitometry at 400nm

Aloin

- Source :Tincture of aloe vera
- S.P :SILICA gel
- M.P :ethyl acetate - formic acid – water(17:2:3)
- Quantification : uv absorbance in densitometry at 350nm

HPTLC IS BEING USED IN THE FOLLOWINGS

- PHARMACEUTICAL industry
- Food analysis and other regulatory laboratories
- Natural products ,botanicals and herbal cosmetics
- Environmental analysis eg: pesticides in drinking water
- Clinical analysis eg :therapeutic drug monitoring, metabolism disorder
-

Advantages

- HPTLC is cost effective
- Sample clean up is simple or not required at all the stationary phase is used only once
- Densitometric scanning of individual or all fractions can be carried out easily since all the fractions remain stored on the plate
- The analytical profiles for cardenoloids ,tropane alkaloids, flavonoids steroidal compounds, anthracene aglycones, lipids have been developed by using HPTLC technique

- HPTLC is rapidly gaining importance in biochemistry of natural products and in analysis of biofluids in the field of pharmacokinetics
- HPTLC is used for finger print patterns of herbal formulations, quantifications of active ingredients and also in detection of adulteration
- HPTLC is a major advancement of TLC principle requiring shorter time and better resolution

APPLICATIONS

- Most safest , fastest and sensitive method
- Morphine , papaverine, codiene , emetine , anti biotics , steroids , vitamins etc are analysed by HPLC
- Drug levels in biological fluids

AFFINITY CHROMATOGRAPHY

- For the separation of proteins , peptides , enzymes , antigens and anti biotics , nuclic acids , ribosomes etc
- The adsorbent used is one of the biological substances (which may be termed as receptor) having a specific affinity for other substances
- Adsorbent is attached with a SP placed in a column

- When a mixture pass through the column selective separation occurs according to its affinity (reversible adsorbtion process)

APPLICATIONS:

- Separation and purification of en zymes
- isolation of antibiotics etc

DROPLET COUNTER CURRENT CHROMATOGRAPHY(DCCC)

- Liquid /liquid extraction
- For separating water soluble constituents , plant proteins and nucleic acids

ELECTROPHORESIS

- SARGENT (1969) Introduced
- Applicable to compounds which carry a charge amino acids , alkaloids , amines , organic acids and proteins
- Certain classes of neutral compounds (sugars ,phenols)can be made to move in an electric field by converting them in to metal complexes (by use of sodium borate)

GEL PERMEATION CHROMATOGRAPHY

- (GEL FILTRATION OR SIZE EXCLUSION CHROMATOGRAPHY , MOLECULAR SEIVES)
- The separation occurs not on the basis of adsorption or partition but the effective size of solutes present in the solution.
- Cross linked polymers with a large no of forces of fairly uniform siz e– very large sized molecules of MP excluded

- Other molecules with low molecular size enter freely in to different pores during the elution , the largest molecules in MP elute first followed by the molecules decreasing in sizes
- SP soft gels or semi rigid or rigid gels
- Soft gels are used with primarily aq mobile phases and the process is called as GEL FILTRATION .

- Semi rigid gels used with non aqueous mobile phases like acetone , pyridine , chloroform etc called as GEL PERMIATION.

APPLICATIONS:

- Employed for the separation of large bio molecules like proteins and poly peptides .
- It is also used for de salting protein solutions , for determining molecular size .
- studies of plasma binding of drugs etc.

APPLICATIONS OF SEPARATION AND PURIFICATION METHODS

- PC,TLC,GLC,HPLC- Using one or other , or a combination can be used both on micro and macro scale.
- Choice of technique depends upon the solublity and volatilities of the compounds to be separated .
- PC - water soluble plant constituents like carbohydrates , amino acids
- TLC - lipid soluble compounds lipids , steroids , carotenoids , and chlorophylls.

- GLC – V olatile compounds , fatty acids mono sesqui terpinoids,hydro carbons and sulphur compounds .

- HPLC – Less volatile constituents

- C C – purified compounds in gram amounts .

METHODS OF IDENTIFICATION

- After the separation and purification of compounds or plant constituents determination of particular substances can be carried out after obtaining a single spot in several TLC and PC.
- The class of compounds is usually clear from its color tests solubility RF Values UV, IR , NMR, MS .
- Comparing the data with those in he literature.

APPLICATIONS OF SPECTROSCOPIC METHODS IN THE IDENTIFICATION OF THE PLANT CONSTITUENTS

Ultra violet and visible spectrophotometer

- Analytical method based on the measurement of light absorption by substances in the wave length region from 190 -900 nm.
- Absorbtion in the uv – visible region arises from electronic transition within the molecule.

- **Single beam instrument –** light passes through a mono chromator and then through the sample and in to detector
- **Dual beam instrument** – measures the ratio of the intensity of beam coming through the sample cell and does not pass through the sample .
- Eg- lobeline (249nm) reserpine (268 nm) morphine (286 nm) colchicine (360 nm) vanillin (301 nm) – uv region

INFRA RED SPECTROSCOPY

- IR is the study of the reflected , absorbed or transmitted radient energy of electro magnetic spectrum ranging from wave length 0.8 – 500 nm expressed in wave number

  Near (IR – 12500-4000 cm) mid (IR 4000 – 400 cm) far (IR 400 – 20 cm)
- IR Spectro photo meters can be single or double beam instruments.

APPLICATIONS

- Identification of drugs, functional groups of bio molecules thus adding in their structural elucidation
- The quantitative analysis of antibiotics , alkaloids , quinine , strychnine and steroidal sapogenins

FLUORESCENCE ANALYSIS

The organic molecules adsorb light usually over a specific range of wave length and many of them re-emit radiations (LUMINESCENCE) The re emmision of absorbed light (FLOURESCENCE)

Fluorescence characters of some herbals drugs

Cinchona – purple blue

Quassia – whitish blue

Ipomoea- deep purple violet

Gentian – whitish blue

Rhubarb – violet

Nuclear magnetic resonance spectroscopy

- Absorptin of radio frequency by substances held in a magnetic field. Adsorbtion results from inter action of radiation with magnetic movement of nuclei in the sample
- It occurs at different frequencies for nuclei with chemically different environment within a molecule
- important method for the elucidation of molecular structures for the determination of the impurities and minor components

MASS SPECTROSCOPY

- It is mainly concerned with the electron ionisation and subsequent fragmentation of molecules for the determination of mass to charge ratio and relative abundances of ions, a possible structure of the original molecule can be suggested
- Determination of molecular weight of compounds

APPLICATIONS OF PHYTO CHEMICAL TECHNIQUES (PPBEGS)

PLANT PHYSIOLOGY: For determining the structures , bio synthetic origins and mode of action of natural growth hormones.

PLANT PATHOLOGY : Important to the pathologist for the chemical characterization of phytotoxins (products of microbial synthesis produced in higher plants)

- PLANT ECOLOGY : S econdary plant constituents are significant in plant ecology research areas such as plant animal and plant – plant interactions.
- PALAEO BOTANY : Phytochemical technique includes the identification of partially degraded chlorophyll pigments in lignite deposits – 50 million years old, carbohydrates in palacozoic plants 250 – 400 million years old.

- PLANT GENETICS: For identifying anthrocyanin, flavones and carotenoid pigments occuring in different color genotypes of garden plants.

- PLANT SYSTEMATICS: Preliminary screening of plants and in the more detailed analysis of individual components.