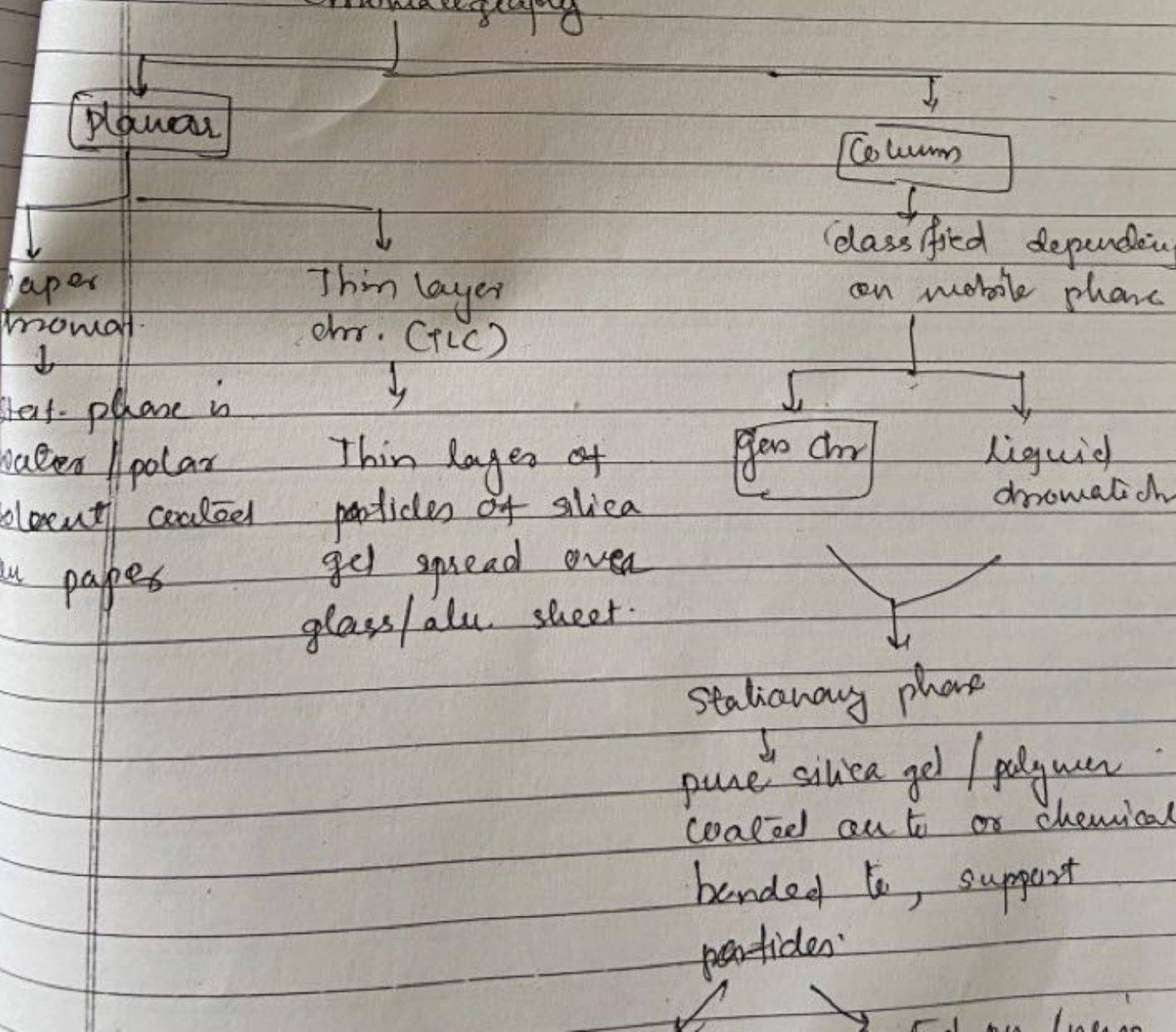
Chromatography

one of the sequential separation tech to isolate the component of interest

PAGE NO.:
DATE: / /

process in ⊆ the component of mixture are separated by differential distribution btw a mobile phase and stationary phase

→ Component ⊆ greater distribution in stationary phase → retained and were slowly



Mobile phase / eluent, ~~exit~~ exit from Column.

↓

passes through a detector and produces signals, plotted as funct^n of.

- time
- distance
- volume.

→ Retention time / Retention Volume.

↓

Interval / volume required for solute to pass from injector, column and to detector.

→ eluting solutes are displayed graphically as peaks → chromatographic peaks.

↓

Described in terms of →
- width,
- height
- area.

→ planner chrom. → separated zones are identified
- by their colour
- or
- visualized through chemical modification that produce coloured spot.

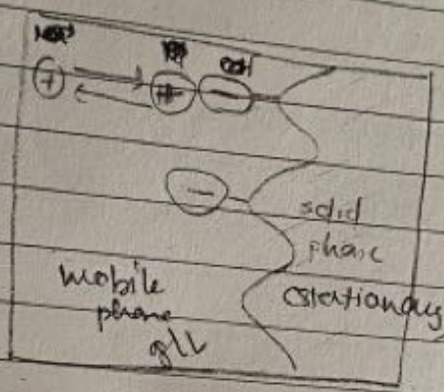
involved in non-specific lipid and lipid
make tumor expressing

* Separation Mechanism :-

PAGE NO.:
DATE: / /

(1) Ion-exchange :-

exchange of ions b/w charged group bound to stationary phase and ions of opposite charge in mobile phase.



Types

↓

Cation exchange

↓

Solid phase contain -vely charge functional group

strong → SO_3^-

weak → COO^-

↓

Bind + positively charged ions in mobile phase

Anion exchange

↓

solid phase contain +ve charge groups

strong → quaternary amines

$N-R_1$ (with R_2, R_3)

- triethyl aminoethyl

weak → 1°, 2°, 3° amino

↓

Diethyl amino ethyl

↓

Bind - positively charged ion in mobile ph

⇒ Separation

① Analyte are charged and bound to ion-exchange stationary phase

↓

Solutes are eluted by solution containing competing counter ions

ex → Na^+ for cation exchange

Cl^- for anion ,,

② change in pH

↓

↓ the charge of ~~bound~~ bound ions or ion-exchange surface

↓

eluted.

⇒ factors affecting performance.

① type of stationary phase ionic group

② charge density

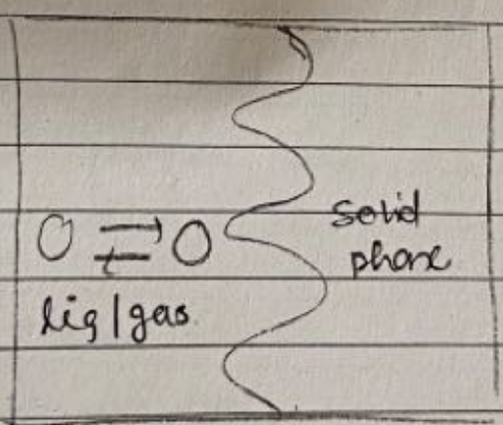
③ stat. phase matrix

④ type and conc. of ions in mobile phase

⑤ mobile phase pH.

1) Analysis of AA and Hb
2) Analysis of small i organic / organic ions
3) Water purification
↓
mixed bed resin (both cation and anion exchanger
↓
H^+ and OH^- ions displaces salt ions from water

4) Adsorption :-
basis of separation is the difference b/w adsorption and desorption of solutes at the surface of solid particles



forces → electrostatic
H-bonding
vander-waals
↓
physical phase forces that control separation

→ For polar stationary phase, elution tracks the polarity of component in mixture
↳ less polar elute first
more polar elute late

Absorbant med

↓ Nonpolar
↓
charcoal,
polystyrene - divinyl benzene
(PS - DVB)

↓ Acid polar
↓
silica gel
(silanol group)
SiOH
↓
Adsorb basic substance

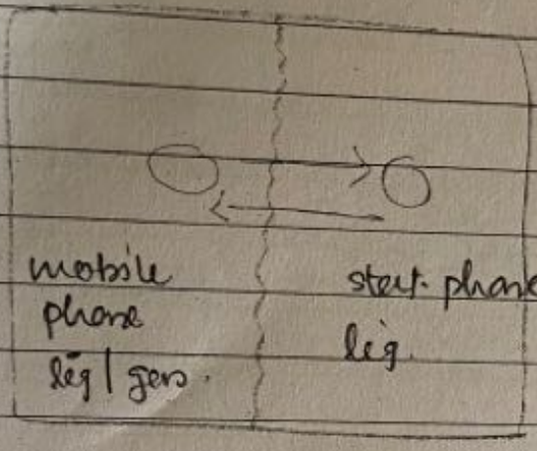
↓ Basic polar
↓
Alumina
florisil
↓
adsorb acidic substance

⇒ Hydrophilic interact^{n} LC (HILIC)
- Type of adsorpt^{n} chromatography
- Separate highly polar compounds that form H-bond c stationary phase (polar surface)
  - ↳ hydroxyl rich compound
  - ↳ Amide " "
  - ↳ Chemodified silica.

Limitations -
Biological fluids are highly polar and include salts that interact c polar stationary phase.
↓
Specimen need to be extracted or modified by addition of less polar ~~stationary phase~~ solvent eg. Acetonitrile. (organic solvent)
↓
to prevent binding of analyte to stationary phase.

Partition chromatography :-

Basis → Differential distribution of solutes b/w two immiscible liquids
(& can not form homogenous mixture)



(N) phase { s = polar, m = nonpolar

Revers phas { s = non polar, m = relatively polar

→ one of immiscible liquid serves as stationary phase → liquid is absorbed / ~~on~~chemically bonded on surface of support particle. Or inner wall of capillary.

* Reverse phase partition chromatography:
- stationary phase = non polar
- mobile phase = relatively polar (water / organic solvents)
- hydrophobic molecules (analytes) are preferentially retained

↓

most widely used tech for solid phase extract and liquid chromatography

Example of stationary phase:
1. silica ~~&~~ bonded octadecyl (C18), octyl (C8) phenyl, butyl ; ~~OS-PO3 & SiO2~~

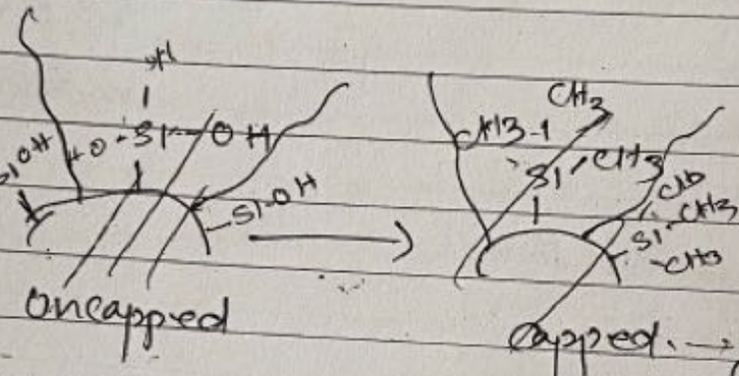
→ Retention characteristic of silica-based stationary phase depends on

① nature of bonded phase.

② Amt of " . (% of carbon load)

③ surface area and pore size of particles

④ quantity of accessible silinol (Si·OH) group
s depends on surface treatment e.g. end-cap



Uncapped

Capped

Si-OH groups replaced by trimethyl silyl group

Si land

Si-OH → Si(CH3)3 trimethyl silyl

* Separation :

• Solution is applied in relatively polar solvent (water) or water & low conc. of organic solvent methanol / Acetonitrile

↓ the polarity of mobile phase ↑

• Partitioning is favoured by adjustment of pH - ing pH

↓

organic acid are in uncharged form (ion suppression)

* Phase collapse :-

when stationary phase is highly aqueous and polar mobile phase

↓

long-alkyl-chain phase

+

highly aqueous and polar mobile phase

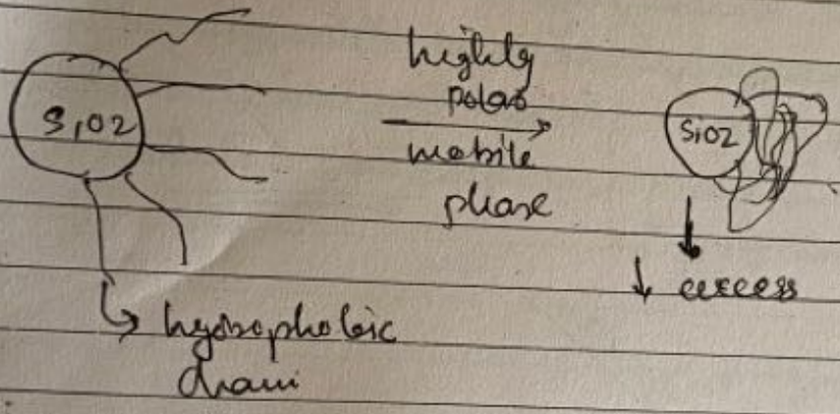
result in self-association of long aliphatic/ hydrophobic chain

↓

Folding down on to surface of stationary phase

↓

Loss ass access to solvent



→ Prevention of phase collapse.

(i) By use of balanced hydrophobic - hydrophilic phases for extract^{n} reduces problem of phase collapse ∵ loading of aqueous solution

(ii) pre wetting of stat. phase ∵ less polar solvent eg. methanol before sampling

* Elution :-

relies on competition of molecules in mobile phase ∵ molecules bound to stationary phase.

↓

elution is hastened by changing solvent strength ∵ linear / stepwise gradient → elution series

or

by changing pH to ↑ polarity of solutes

Isocratic → separation ē single solvent or solvent mixture of constant composition.

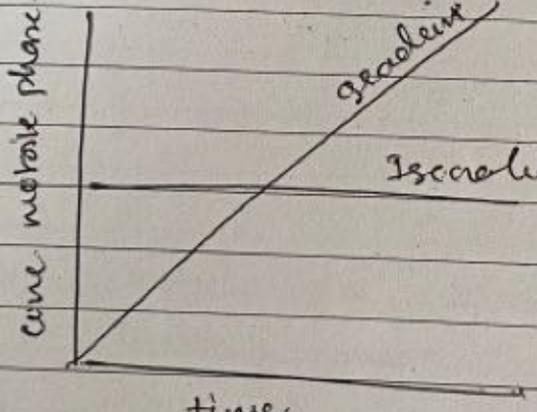
→ elution series in xverse phase chromatography

Pentane > cyclohexane > Acetonitrile > Ethanol > methanol > water

↓

- more hydrophobic = stronger eluents
- For normal phase chromatography → elutropic series is reverse

→ elution is carried by
① by changing solvent strength by introducing 2 or more solvent system ē different composition and polarity → gradient elution
② By changing pH



* Hydrophobic interaction chromatography:
- applied mainly for protein separation / purification

Sample (protein) containing hydrophobic (core) and hydrophilic (surface) regions

↓

Salt in buffer ↓ solvation of sample solutes causing hydrophobic regions to expose.
e.g. Ammo. sulfate (anti-chaotropic agent)
causing salting out

water molecule by weakening ex → SDS, urea,

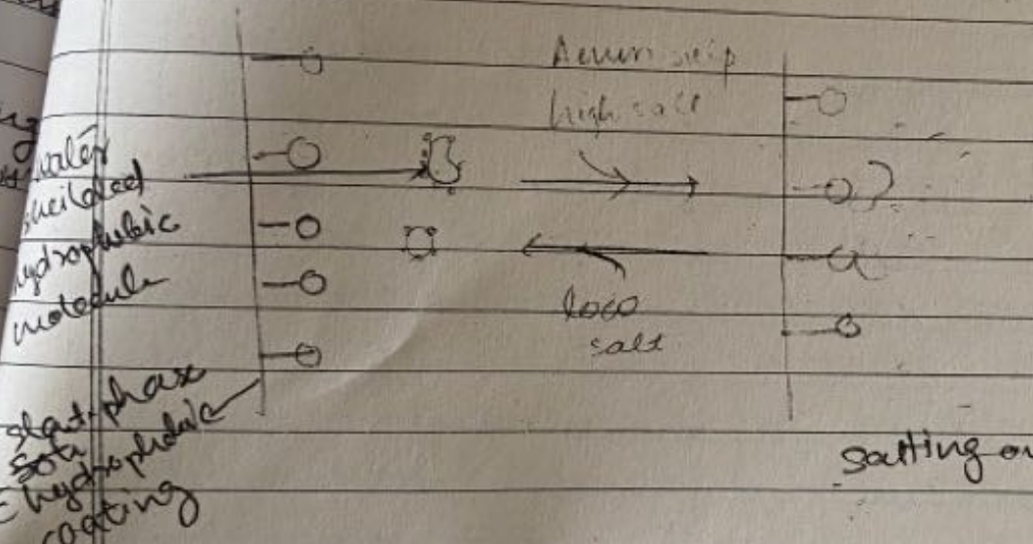
disrupt hydrogen bonding network ↓ stability of protein hydrophobic effect

PAGE NO.:
DATE: / /

molecules :-
exposed hydrophobic region absorbed by solid phase

↓

elution done by reducing salt concentration rather than adding organic solvent



(4) Chiral separation

specific stereoisomers / enantiomers / mirror-image molecules e.g. L-AA

↓

having same physical property

↓

can not be separated by ordinary chromatography

↓

separation by use of stationary phase that has enantiomeric components that will react w molecule in stereospecific manner

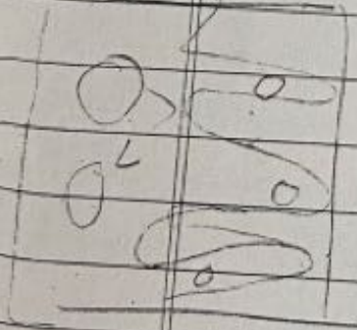
or → chiral specific phase (CSP)

use → for drug analysis → L-cetrizine, Relaxine, Levo flexacin

(5) Size exclusion chromatography.

gel exclusion / gel permeation / steric exclusion / molecular exclusion / molecular sieve chromatography

principle



stationary phase contain porous particles w inert surface designed to have minimum Adsorption of component

↓

molecules are separated based on size.

→ Material of stationary phase

↳ polyacrylamide
Agarose
porous glass.

} → Beads of these materials are porous and. pore size that allows small molecules to enter

PAGE NO.:
DATE: / /

↓

larger molecules remain in mobile phase and rapidly eluted.

→ By calibrating size exclusion column ē known molecular size, molecular size of other particles e.g. protein K can be estimated

Disadvantage

① low resolution tech.

② requires substantial proportional dif in molecular weight.

ex →

Advantage → Allows separation of molecules ↓ physiological condition.

useful for intact complex e.g. lipoprotein, Ag-Ab complex

* Other size exclusion separation

Ls ultrafiltration → ① preparative tech to separate small molecules e.g. drugs / AA from ~~from~~ ē passes through membr. from protein bound ē remain in retentate fraction

ex- phenytoin, thyroxin conc. (protein bound)

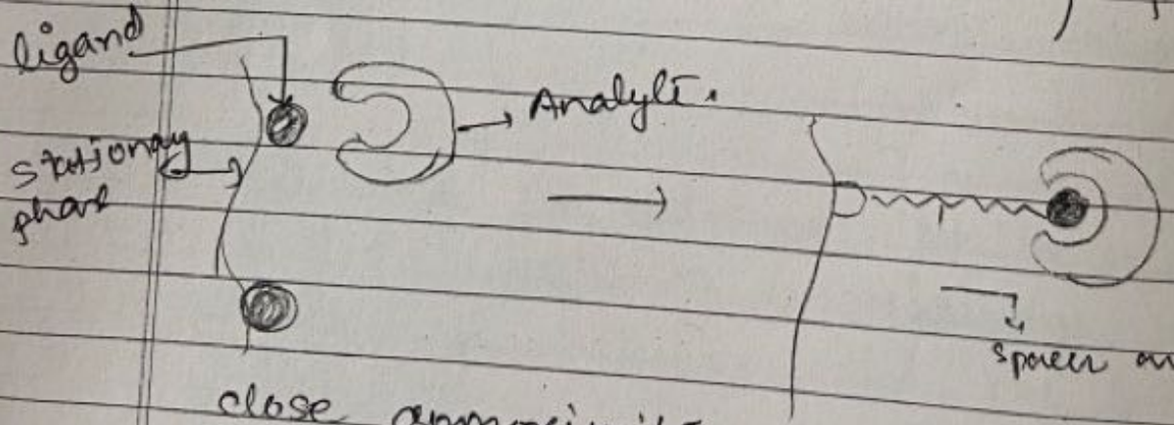
② concentrating solution protein in dilute sample e.g. urine → desalting

⑥ Affinity chromatography :-

→ Specific molecular interact^{n}
- Ab-Ag interact^{n}
- enzyme - substrate
- (B) - ligemel.
- lectin - sugar.

- stationary phase → immobilizat^{n} of one component of interact^{n} pair.
↓
Capture the other component in mobile phase.

→ Orientation & accessibility of capture molecule - are imp. elements for function
↓
spacer arm is used to provide distance from surface of stationary phase



close approximity of to stationary phase prevents the binding

→ using spacer arm. ↑ accessibility of analyte & ligand

↓

Non bound components are eluted and removed by washing

↓

specifically bound components are eluted by adding competing ligands / changing pH / ionic strength

* Uses

① purifying Ab and other proteins

② Lectin affinity chromatography
↳ assessing change in oligosacch. structure on glycoprotein binding

③. glycated Hb → Immobilized phenylboronic acid used to separate HbA1c from Hb.

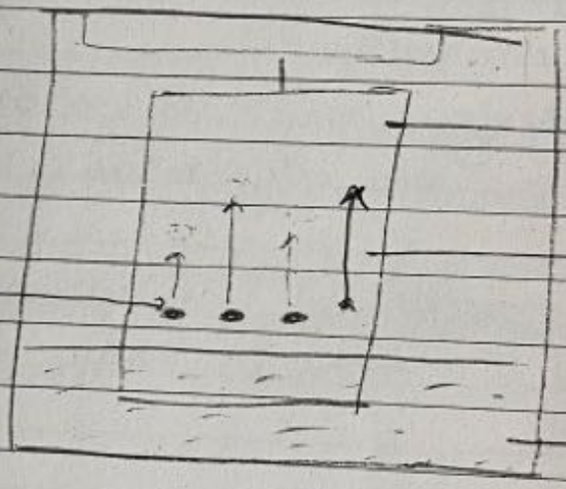
DATE: / /

Resolution :-

* planer chromatography:
  - paper chromatography – polar / polar solvent
  - TLC → Silica gel
    microparticulate cellulose.
    Alumina.

Stationary phase

spread over glass plate / plastic sheet.



Sample is applied as small spot / band.

↓

plate is placed in closed container ē lower edge ē in solvent / mobile phase.

↓

mobile phase travel ti desired distance by capillary action

↓

separated components are identified by ① UV illumination
② spraying colour generating reagents

PAGE NO.:
DATE: / /

• application → qualitative analysis
(1) Analysis of amniotic fluid to L-S ratio
(2) Urine analysis for screening test for drugs / AA

• Advantage
(1) simple
(2) lesser equipments
(3). allow 2D separation by using diff. solvent in 2nd. direct

• Disadvantage
(1) lack of automation
(2) No precise quantification.

* Liquid chromatography:-

Distribution of solutes b/w liquid mobile phase and stationary phase.

HPLC → stationary phase → very small diameter particles
↓
mc used form of LC.

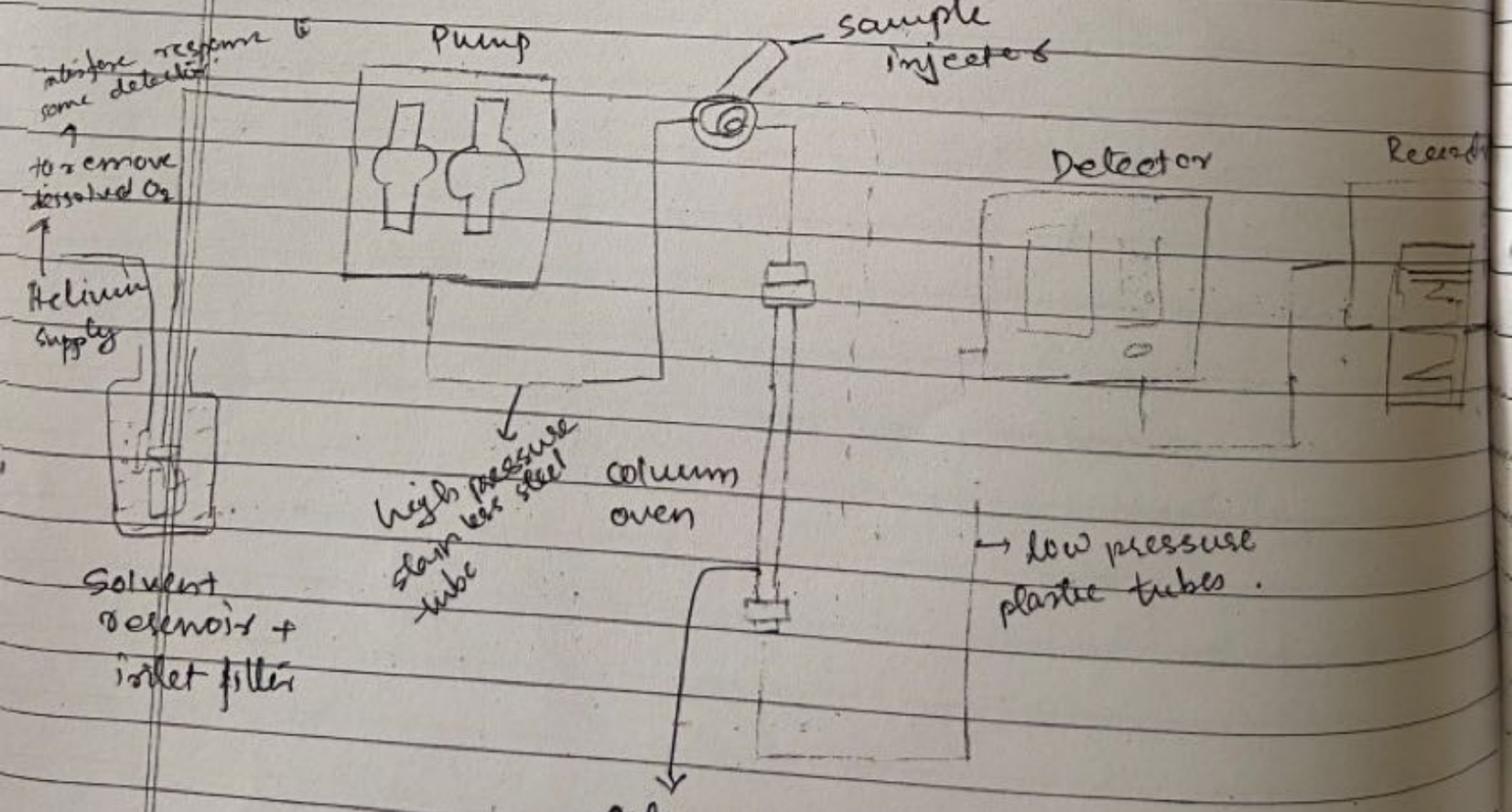
↓

Pressure drop ∝ (Diameter)^2

↓

high pressure required to pump liquid through column.

⇒ Instrumentation :-



PAGE NO.:
DATE: / /

① Solvent reservoir
- simple glass bottles ī
① feed line
② inline filter → tu remove particles.
③ Helium supply → tu remove dissolved O_2.
④
- Degassing → tu remove bubble / O_2 by applying vacuum / gas exchange device.

② Solvent delivery system include ① valve ② gradient mixing chamber ③ pressure sensor ④ pumps

↳ provide controlled flow and delivery of solvent

↓ peristaltic / Diaphragm valve
↓ low pressure tu

↓ piston / syringe type
↓ HPLC → pressure limit 2 400 bar - 1000 bar ~~6000 psi - 15000~~
↓ Reciprocating piston pump
↓ 2 pumps working alternately

Ultra high performance / ultra (UPLC)

→ switching valves → change tu diff. solvent at prescribe time. or mixing ≥ 2 solvent tu create solvent gradient.

improper mixing → poor result.

≥ 2 reservoir bcoz single pump

≥ 2 pumps ī different reservoir.

③ Sample injector -

↳ Introduce sample into column.

• most used type = Fixed loop injector

↓

Switch into and out of the flow path

① Loading mode

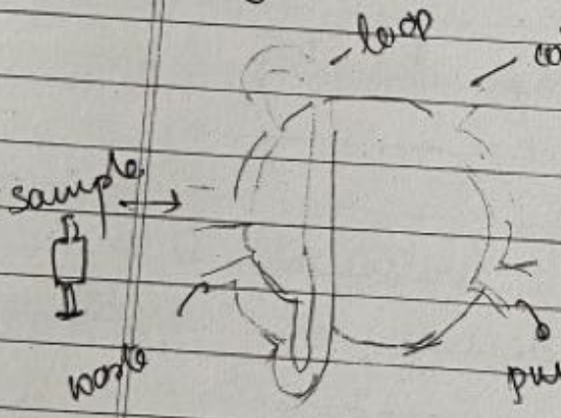
↓

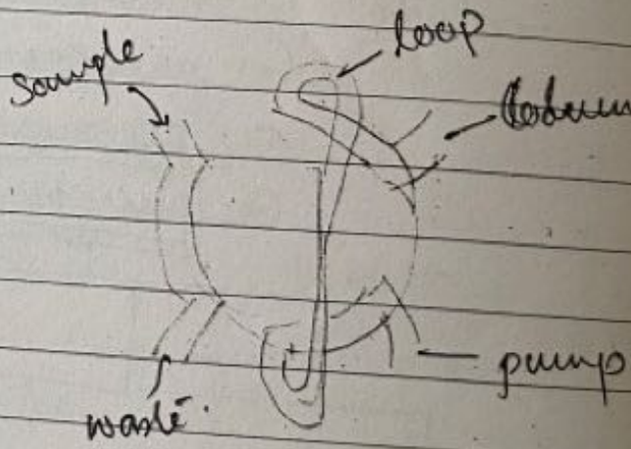
Sample is carried into loop through syringe.

② inject mode

↓

loop is switch into flow path → sample is carried downstream into column





→ characteristics of injecting system

① reproducibility

② Amt of carry over

③ range of injected volume.

⇒ Automated injectors → multiple aliquots inject°.

→ mixing sample and reagent prior to inject°.

→ Refrigerat° of sample → Limited stability

PAGE NO.:
DATE: / /

Column heater / cooler:

→ to generate reproducible retention time

In LC → Constant temp is maintained

~~Thorough~~ by.

① Air column chamber

② water jacket

③ temp. controlled blanket

④ heating / cooling ~~blocks~~ blocks.

↑ Column temp → ↓ solvent viscosity
→ ↑ rate of transfer
→ allows higher flow rate → ↓ analysis time.

↓

However, ~~temp~~ temp

Thermal stability of sample - impose upper limit of temp.

↓

preparative work for extra in cold room / refrigerated cabinet.

⑤ Column:- ② material ① Dimension

Dimension → length, Diameter

length ↓ 50 mm – 250 mm

Diameter ↓ 0.3 mm to 5 mm.

Column end Fitting

↓ have zero dead volume

↓ Frit

- to retain support particle
- connect to column & injector & detector without leak

Material

packed bed of small particles.
- Diameter = 1.8 to 10 μm.
- backpressure α $\frac{1}{(\text{particle diameter})^2}$
- smaller particles → higher separation efficiency.
  → ↑ resistance to solvent flow.

Monolithic rod.
single cylinder
silica / polymer
porous structure.

- Types
① bonded phase packing
- $-CH_2-CH_2-CH_3$ → reverse phase chr (C18, C8 type)
- $-NH_3^+$ / NH_3^+ – anion exchange
- $-COO^-$ – cation exchange.
- OH – Normal phase chromatography.

② Polymeric Packing: → graphite carbons
→ mixed copolymers – PS-DVB

③ Chiral Packing – separate enantiomers

④ Restricted access packing:

DATE: / /

Detectors

is dissolved analyte eluted from column and passes through flow cell

↓

generate signals → detected through detectors.

Types

Detectors

↙ ↘

① Individual | linked in series.

post column / pre column reactors are inserted before column or after column e.g. ninhydrin.

↓

React^n of AA and generate product

↓

more stronger and specific signal.

Types

① Spectrophotometer & spectrophotometer diode assay:- measurement of absorbance at UV region (190-400nm) and visible light (400-700 nm) wavelength.

(i) Fixed wavelength UV photometer detectors

↳ mc 254 nm resonance → by mercury arc lamp.

OR

A Phosphor placed b/w lamp and fluro cell

↓

excitation at 254 nm

emitted fluorescence = 280 nm used as light source

↓

fixed

Dual wavelength photometer.

(2) Variable λ detector :-

* ↑ λ is selected from given λ range.

↓

Detector is tuned to operate at absorbance maximum for given analyte / set of analyte

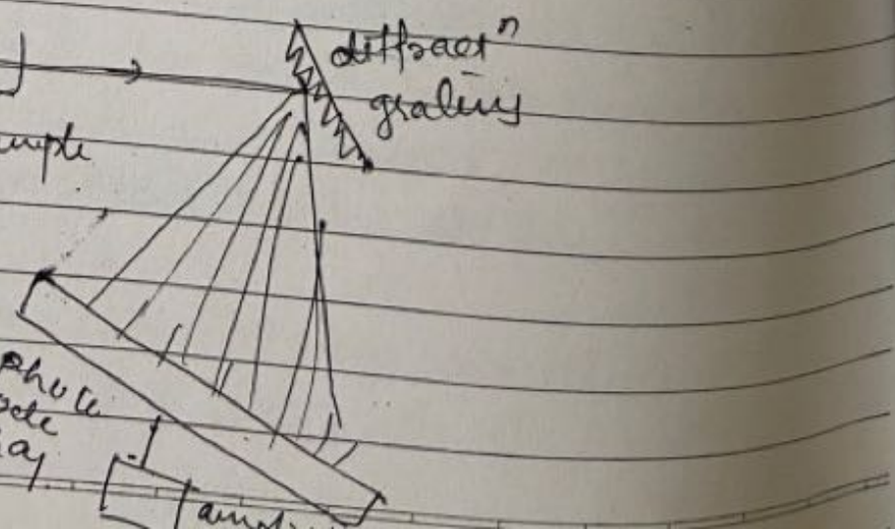
Adv → use of lower λ (<190 nm)

↳ many compounds (eg. cholesterol) absorb at lower λ.

~~(2) lower λ : can not be absorbed by mobile phase.~~

⇒ Diode Array.

yeild spectral data over entire λ range of 190 to 600 nm. in about 10 ms.



Centrifugation :-

Process of using centrifugal force to separate the lighter portion of solution, mixture or suspension from the heavier portion.

① Remove cellular elements from blood to provide cell free plasma / serum for analysis.

② Concentrate cellular elements for microscopic examination

③ Remove chemically precipitated protein from specimen → DNA isolation

④ Separate protein bound or Ab bound ligand from free ligand in immunochemical assay ↑

⑤ Separate lipid components (chylomicron) from plasma/serum

Types

① Horizontal head

- ex: ~~capillary centrifuge~~
- tube remain horizontal in rotor - fixed.

② Fixed angle head.

- tubes remain fixed in one angle.
- ↓ movements of rotor

↓

↓ resistance and heat.

polychromatic light from sample

↓

Dispersed by diffract° grating

↓

spectrum is Directed to photodiode array

↓

~~Intd~~ Intensity of light at multiple λ in spectrum is measure.

use → helpful in identificat° of drugs in urine and serum.

⇒ Problem ~ photometric detection.

↓

Out gassing → as solvent exits from high pressure to low pressure flow

↓

solvent ~~in~~ interfering gases ~~that are~~ liquified in high pressure converted to gases

↓

Interfere on detection dt bubbles as noise

- Proper degassing needed.
- ~~Some~~ maintainance of some backpressure across detector to minimise bubble format°.

(2) Fluorometer :

- detect fluorescing compounds
- more sensitive
- pre column / post colum. reaction to bring compound e.g. fluorophore.

ex → AA tagged by dansyl or fluorescamine tags
↓
fl^b HPLC separation &
⇒ fluorometric detect^n.

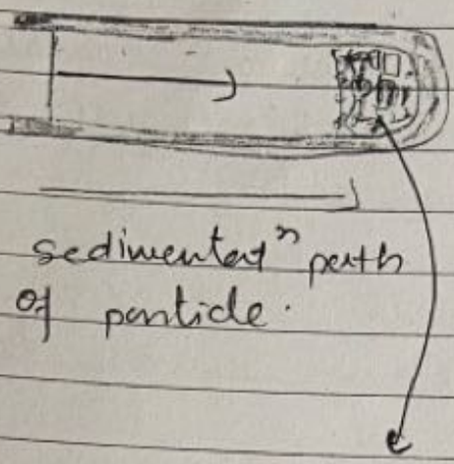
(3) Electrochemical detectors.
↓
Amperometric electrochemical detectors.
↓
analyte enter flow cell, oxidised or reduced at surface of electrode at constant potential
↓
produce current α analyte conc.

use → Urinary catecholamines
homovanillic
Ascorbic acid
} HPLC & CE detect^n

(4) MS → Combined techs
↙ ↘
GC-MS LC-MS.
- sensitive, specific

DATE: / /

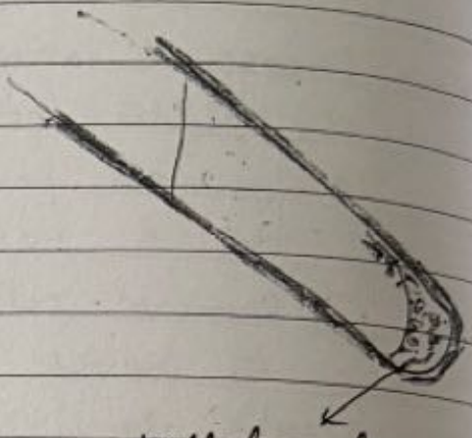
Horizontal head



sedimentation path of particle.

pallet deposited at bottom ē horizontal surface. → good recovery of sample

Fixed angle head



pallets deposited at angle.

③ Swinging buckets

Tubes are attached to rotor by hinge pins.

↓

Bucket swings out in horizontal position.

↓

pallet deposited ē horizontal surface

↳ good recovery.

↑ resistance ← ↓ ↑ heat

⑤ Ultra centrifugation :-

② high rpm centrifuges

① cooling

② vacuum → ↓ ~~heat~~ air → ↓ resistance & heat

⑥ Axial Units :-

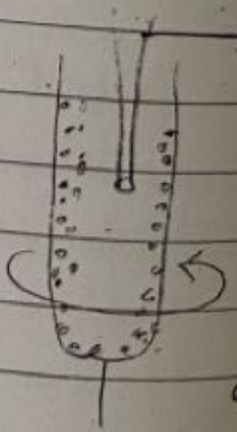
Tubes rotates at its axis

↓.

RBC seperate ti periphery.

↓

plasma separated in center



→ can be directly seperated

↓

applied in fully automat.

ed angle head

deposited
angle.

ins.

Principle :-

Relative centrifugal force (RCF)

↓

Separate two phase in centrifuge.

$RCF = 1.118 \times 10^{-5} \times r \times rpm^2$.

where, 1.118×10^{-5} : constant

r = radius in cm from center of rotation to bottom of tube

rpm = rotation per minute.

- Time required to sediment particles depends on (1) rotor speed
  (2) Radius
  (3) path travelled by sedimented particles.

↓

length of time is calculated, so running a other rotor of diff. size is equilent to running a original rotor

$$\text{alternate rotor time} = \frac{\text{time} \times \text{RCF (original)}}{\text{RCF (alternate)}}.$$

Time includes - time to reach operating speed and do not include deceleration time., sedimentation still occur but less efficiently.

DATE: / /

* Operation of centrifuge :-

- Recommended tubes should be used
  - ↳ withstand RCF
  - ↳ made up of polypropylene
  - ↳ tapered end.
  - ↳ should be size to fit into rotor

- Properly balanced rotors ⇒
  - ↳ weight of racks, tubes and fluid content should be similar on opp. side
  - ↓
  - diff. should not > 1% of acceptable limit by manufacturer.

- Automatic balancing centrifuges are now available

- Sample should be centrifuged before aerosol being produced
- No practice of using wooden applicator to remove clot from cap → hemolysis.

- Cleanliness → to ↓ possible spread of infections → Hepatitis

- Breakage → carefully cleaned.

- Speed of centrifuge → checked every 6 months.
  - ↳ Not differ > 5% from stated speed.