HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

First called “high pressure liquid chromatography”. Since 1970 that technology of producing small silanized silica particles allowed the use of small volume longer columns necessary to give high resolution performance. Today HPLC known as “high performance liquid chromatography”.

Principles:

Classical LC is largely suplimented by the much more power and analytically useful form of HPLC.

Rate of distribution of solute between stationary and mobile phase in traditional chromatography is largely diffusion based. Diffusion in liquids is much slower than gases.

To minimize diffusion and time required for movement of sample in and from interaction point 2 criteria should be met.

1. Packing should be finely divided and have high spherical regularity to allow for optimum homogenicity and packing density.
2. The stationary phase should be in form of this uniform film with no stagnant pools (more rapid transport between two phases, necessary for the high flow rates). This reduces the H at high speed.

Stationary phase:

Original HPLC micro particles were irregularly shaped porous silica gel or alumina particles of 10 µm or less since then spherical particles of that can be packed with more homogenicity and provide more improved efficiency.

The particles are high purity silica low in trace metals and are typically 10 µm in diameter although 3µm diameter is more used for high speed pore size of 60-100 A° although pore size of 300 A° used for larger biomolecules.

HPLC is performed for:

a) Liquid-liquid (partition chromatography)
b) Liquid-solid (absorption chromatography)

- **Liquid stationary phase** is coated on the particles or is chemically bonded. most commonly used particles are “micro porous” or “diffuse particles”.

Majority or surface area is between pores mobile phase moves over surface and diffuse into stagnant mobile phase to interact with stationary phase and diffuse out in moving mobile phase.

Use of small particle size minimize the pathlength hence (shortening) of band broadening

• aggregated spherical particles

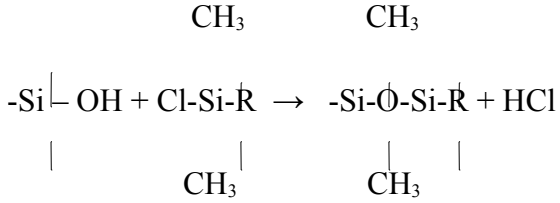
•sponge like

❖ **Silica** (solid stationary phase)

Silica tends to dissolve above PH 8 and cross linked polymeric particles e.g. polystyrene or polymethacrylate are used for separation of basis because these can withstand strongly basic mobile phases with low efficiency.

Silica particles have surface silanol groups –SiOH.

These are produced for the chemical bonding of the stationary phase by silination reaction with chlorosilones



ENDCAPPING:

The middle free SiOH group has been encapped with trimethylsilyl group and render it inert.

ODS: (for reverse phase chromatography)

most common non-polar bonded phase is C_8 and C_{18} , with most popular (ODS, octadecylsilane).

C_8 is intermediate in hydrophobicity.

Phenyl groups are also useful

C_5 are useful for HPLC-MS due to low bleed proal?

Polar phase:

Are used for normal phase chromatography in increasing order of polarity include

Cyano → diol → amino and dimethyle amino

note:

when particles are watered with liquid phase rather than bonded, they are first treated with trimethyle cholosilane to deactivate the silanole group.

packing:

1. **Perfuse packing:**

Made of mixture of small and large pores. (larger= through pores, smaller=diffusive pores).

Smaller, micro porous pores provide sorption capacity. Larger, through pores allow mobile phase to pass directly from the coloum hence increasing the rate of mass transfer in mobile phase.

Since solute spends less time undergoing mass transfer, peaks are narrow.

These packing are larger than micro porous one being 12μm in diameter can be used at high flow rate and for the larger molecules, proteins.

Useful for preparative chromatography.

2. **Non porous packing:**

Either silica or resin, have much smaller particle size 1.5 – 2.5 μm in diameter with thin porous layer. They eliminate the occurrence of stagnant mobile phase that allows much faster rate of mass flow/transfer

Molecules small, large can be separated in few minutes but back pressure is much greater than column of 3-5 μm diameter and is having small loading capacity.

"back pressure is inversely proportional to square of diameter of particle, half reduction in size increase back pressure 4 folds".

Non porous columns are used for separation of complex peptides with in minutes and seconds and in ion chromatography.

Monolithic columns:

Another approach to provide with low pressure drop and high rate mass transfer. These are continuous solid columns of porous silica stationary phase instead of packing material. Like perfusion have bimodal pore structure.

1. Macropores: act as flowthrough pores of 2 μm diameter.
2. Mesospores: diameter of 13 nm, can be surface modified with C_{18}. The rod is shrink-wrapped in polyetheretherketone (PEEK) plastic to present wall effects of solution flowing through walls 80% porosity compare 65% in packed column.

Instrumentation:

It is technique for faster, more efficient separation using finer column packing at high pressure with the help of special hardware.

Pressure of 1000-3000 psi required for flow rate of 1-2 ml/min in column of 3-5 mm in diameter and 10-30 cm long.

In some instances pressure up to 6000 psi is used 80-90 % HPLC separation is performed with pressure less than 1200 psi.

HPLC apparatus consist of following parts:

mobile phase supply system:

system consist of pump to provide high pressure and usually contain some means of providing gradient elution.

(i) The solvent reservoir can be filled with range of solvents of different polarities, provided they are miscible or they can be filled with solutions of different PH and are mixed in buffer volume.
(ii) The solvent must be pure and degassed to avoid formation of gass bubbles as they prevent proper check value function or enter the piston chamber and also gives/generate suprious peak when pass through the detectors.
(iii) Typical flow rate is 1-2 ml/min for 4.6 mm column diameter.
(iv) Solvent used must be "HPLC" grade that solvent has been filtered through 0.2 μm. it extends the pump life by preventing deterioration and reducing contamination or plugging of column.

ideal pumps:

i. Ability to generate high pressure.
ii. Pulse free output.
iii. accurate control of flow
iv. corrosion resistant

two groups of pumps:

a. constant pressure.
b. Constant volume

Three types are available:

1. Reciprocating pumps
   (90% of commercial HPLC,produce pulse flow)
2. Displacement pumps
   (Produce flow that are independent of viscosity and back pressure)

3. Pneumatic pumps
   (Cannot do gradient elution and pressure less than 2000 psi
4. Motor driven syringe pump.

Injectors:

The typical injecting system consists of a stain less steel ring with six different ports, one to the column. A moveable Teflon cone with in the ring has three open segments each of which is connected by an external sample loop of known or fixed volume.

In one configuration the cone permits direct flow of effulent into the column, and the loop can be filled with the sample. Then cone is then rotated 30% with the sample loop port of the moving stream which sweeps the sample in to the column.

Sample of a few micro liters can be injected at pressure up to 6000 psi.

Sample can be injected manually by syringe or with the help of auto sampler.

Sample-injecting system:

1. Reciprocating pumps:
The pumps, which are currently used in 90% of commercially available HPLC. It consist of a chamber in which the solvent is pumped by back and forth motion of a motor driven piston.
Two ball check valves, open and close alternately, and control the flow rate of solvent.
Flexible diagram, which is hydraulically pumped is also employed by a reciprocating piston.
Adnatages:
i. Small interval volume (35-400 µl) and high output pressure (10,000 psi)
ii. Adaptability to gradient elution.
iii. Constant flow rates, independent of column back pressure and solvent viscosity.

Disadvantages:

i. It produce pulsed flow.

2. Displacement pumps:
These pumps usually consist of a large syringe like chamber's equipped with a plunger that is activated by a screw driven mechanism powered by stepping motor.
Advantages:
i. Produce flow that tends to be independent of viscosity and back pressure.
ii. Output is pulse free.

Disadvantages:

i. Limited solvent capacity.

ii. Considerable inconvincible when solvents are changed.

3. Pneumatic pumps:

In the simplest pneumatic pumps, the mobile phase is contained in a collapsable container housed in a vessel that can be pressurized by a compressed gas

Advantages:

i. These pumps are inexpensive.
ii. Pulse free flow

Disadvantages:

i. They suffer from limited capacity and pressure output
ii. Flow rate depends upon solvent viscosity and column back pressure.
iii. Not applicable for gradient ellutions
iv. Have limited pressure of about 2,000 psi

Column:

1. Guard column or pre-column:

A small 3-10 cm guard column or pre column is placed b/w injector and analytical column. It contains the same packing as the analytical column.

It is placed for following reasons.

1. It will retain debris and particulate matter that would otherwise foul analytical column affecting columns efficiency and selectivity.
2. It will retain highly sorbed compounds.
3. Guard column extends the life of analytical column it must be replaced or regenerated periodically.

2. Analytical columns:

Straight lengths of stain less steel tubes makes excellent column. Available in different length and diameter depending upon particular application.

Usually internal diameter is 3.9 or 4.6 mm of 5µm should give a plate count on the order of 60,000 to 90,000 plates /meter, at the rate of I ml/ min.

Fast chromatography can be performed with wide bore, short column stationary phase but narrow columns give better resolution.

Temperature control is not necessary for liquid solid chromatography but it is significant in liquid-liquid chromatography. Temperature is also significant while using refractrometric detectors as they are sensitive to temperature.

Detectors: (eyes)

1. UV-Vis absorbance spectrophotometer
2. Fluorescence
3. Refractive index
4. Electrochemical
5. Conductivity

6. Mass spectrometry
7. FTIR
8. Light scattering
9. Others (under developed)

Some are explained below which are widely used.

1. Refreactrometer detector:

Advantages:

Often called universal detector.

They detect change in refractive index of the elute due to presence of the sample.

Disadvantage:

Cannot be used effectively due to change in baseline and impractical for gradient elution.

It is highly temperature sensitive, "detection limit" is $10^{-5} – 10^{-6}$.

Applications:

Organic acids, sugars, fungal metabolites, oligosaccharides can be used as sample.

2. UV-Vis detectors:

Advantages:

Has much better sensitivity of 10^{-8} g/ml.

Not temperature sensitive, simple, reliable, inexpensive, compatible with gradient elution and non-destructive.

Disadvantage:

Non universal, used for organic compounds only. Having chromophoric group (vitamins, carotinoids, phytonutrients.

Types:

UV-Visible detectors are available in 3 forms.

1. Fixed wavelength
2. Variable wavelength (monochromatic light → more expensive)
3. Diod array

- Variable wavelength UV detector uses a monochromator (slit or grating) to select one wavelength of light to pass through sample.
- A photo diod array detector passes all wavelengths of light. Through the sample cell then focus each wavelength on a single sensor element.

4. Fluorescence detectors:

Advantages:

Can give better selectivity over UV-Vis detector because only fewer compounds fluoresce. Then absorb, highly sensitive (10^{-15}=femtomole), low background, highly selective (two distinct wavelengths instead of one in abs. detection)

Disadvantage:

Perceived difficulty of its use, more instrumental variable to account for during optimaization, change in fluorescence can occur with PH or viscosity.

Sample system: vitamin E, drugs (aflatoxins), aminoacids, carbamate pesticides.

Recorder/ data collector:

Many recording devices are available:

- Strip chart recorder (retention time/ peak area or peak height)
- Integrator
- Computer controlled data collections

HPLC method development:

1. HPLC is used either in liquid-solid absorption chromatography
   And normally non polar materials are best separated using absorption.AC is sensitive to the steric effects are preferred for the separation of isomers having different steric configuration. Most common absorption phases are alumina and silica particles.
2. The liquid-liquid partition chromatography is most common, either normal or reverse phase. As solute is distributed depending upon solubility coefficient and solute/solvent polarity.
   Liquid-liquid partition chromatography are quite sensitive to small molecular weight differences and so are preferred for the separation of members of homogenous series.
   In general highly polar materials are best separated using partition chromatography.
   (a) Normal phase chromatography:
   Stationary phase is polar, a non polar mobile phase is used, such as an n-hexane, methylene chloride or chloroform.
   A stationary phase is bonded siloxane with a polar functional group (polarity order cyano < diol < amino < dimethyleamino). These phases retain place compounds in preference to non polar compounds.
   (b) Reverse phase chromatography:
   A relatively non polar stationary phase is used with a polar mobile phase such as methanol, acetonitrile, tetrahydrofurane, water or usually mixture of water with one of the organic solvents. The organic solvent is called “modifier”.

Applications:

1. Widely applicable to nurses field of study; both academic, industrial and biomedical.
2. Great for separation of non volatiles, amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, pesticides, pharmaceuticals, terpenoids, pigments, antibiotics, sterioids, vitamins and various other organic and inorganic substances.
3. Generally, if a compound can be solubilized in common solvents such as water, alcohol, acetonitrile, acetone…. Then HPLC can probably be used.
4. One of the most widely used analytical separation techniques.
5. Uses a liquid mobile phase to separate components in a mixture.
6. Use high or low pressure to push solvent through a separate column.
7. Popular because:

- Sensitive
- Accurate, quantitative methods can be used.
- Great for separation of non volatile compounds, heat labile compounds and semi volatile compounds.
- Non destructive
