CHROMATOGRAPHY

Chromo ------ colour

Graph-------- writing

Writing in terms of colour is chromatography. Basically it is separation technique. It is consisting of two phase: mobile phase and stationary phase.

Definition: Chromatography is the separation techniques in which different components of the mixture can be separated by continuous distribution of its components between two phases (mobile phase and stationary phase) where mobile phase moves over stationary phase.

Definition as per USP: Chromatography is the procedure by which solutes are separated by dynamic differential migration in which system consisting of two or more phase, one of which moves continuously in given direction. In this technique different mobility as a reason of different in adsorption, partition coefficient, ionic charges, molecular size, vapour pressure affinity etc.

CLASSIFICATION OF CHROMATOGRAPHY:

Classified on the basis of:

- Instrument which is used
- Nature of stationary and mobile phase used
- Mechanism of separation
- Mode of chromatography

- **Instrument which is used**
- Column chromatography:

-Adsorption column chromatography
-Partition column chromatography

- Paper chromatography:

-Ascending paper chromatography
-Descending paper chromatography
-Two dimensional paper chromatography
-Circular paper chromatography

•Thin layer chromatography:

-Normal TLC

-Two dimensional TLC

-Continuous development TLC

•High performance liquid chromatography (HPLC) OR High pressure liquid chromatography:

-Normal phase HPLC

-Reverse phase HPLC

-Isocratic HPLC

-Gradient HPLC

•Gas chromatography (GC):

-Gas liquid chromatography

-Gas solid chromatography

- **Nature of stationary and mobile phase**
- Gas-solid chromatography
- Gas-liquid chromatography
- Solid-liquid chromatography- column chromatography, thin layer chromatography, HPLC
- Liquid- liquid chromatography- paper partition chromatography, column partition chromatography
- **Mechanism of separation**
- Adsorption chromatography

When a mixture of compounds (adsorbate) dissolved in the mobile phase (eluent) moves through a column of stationary phase (adsorbent), they travel according to the relative affinities towards stationary phase. The compound which has more affinities towards stationary phase travels slower and the compound which has lesser affinity towards stationary phase travel faster. Hence the compounds are separated. No two compounds have the same affinity for a combination of stationary phase, mobile phase and other conditions. Eg. Gas-solid chromatography, thin layer chromatography, column chromatography and HPLC.

•Partition chromatography

When two immiscible liquids are present, a mixture of solutes will be distributed according to their partition co-efficient. When a mixture of compounds are dissolved in mobile phase and passed through a column of stationary phase, the component which is more soluble in the stationary phase travels slower. The component which is more soluble in the mobile phase travels faster. Thus the components are separated because of the differences in their partition co-efficient. No two compounds have the same partition co-efficient for a particular combination of stationary phase, mobile phase and other conditions.

The stationary phase as such cannot be a liquid. Hence a solid support is used over which a thin film or coating of liquid is made which act as a stationary phase.

•Gel permeation chromatography (gel filtration, size exclusion chromatography)

A gel is used to separate the components of a mixture according to their molecular sizes. Different gels are used for different molecular weight ranges. The solvent used can be of aqueous or non-aqueous type. The stationary phase is a porous matrix. The matrix is made up of wide variety of compounds like cross-linked polystyrene, polyvinyl acetate gel, cross linked dextrans (sephadex), polyacrylamide gel, agarose gel. The mobile phases used may be organic solvents or aqueous buffers. The most commonly used detector is differential refractometric detector. For some class of compounds, UV-visible detector, electrochemical detectors are used. The mechanism used in the separation process is because of steric and diffusion effects in the pores of different gels. This technique is used in the separation of proteins, polysaccharides, enzymes and synthetic polymers.

•Ion exchange chromatography

In this type, an ion exchange resin is used. Reversible exchange of ions takes place between similar charged ions and that of ion exchange resin. A cation exchange resin is used for the separation of cations and an anion exchange resin is used to separate a mixture of anions.

•Chiral chromatography

In this type, optical isomers (levo and dextro) can be separated by using chiral stationary phases.

•Modes of chromatography

•Normal phase chromatography: in this stationary phase is polar and mobile phase is non-polar. Not widely used in pharmacy.

•Reverse phase chromatography: in this stationary phase is non-polar and mobile phase is polar. Most widely used in pharmaceutical analysis.

A comparison of normal phase and reverse phase mode

	Normal phase	**Reverse phase**
Stationary phase	Polar	Non-polar
Mobile phase	Non-polar	Polar
Compound eluted first and retained less	Non-polar	Polar
Compound eluted last and retained more	Polar	Non-polar
Examples of stationary phase	Silica gel	ODS (C18), C8, C4- bonded phases

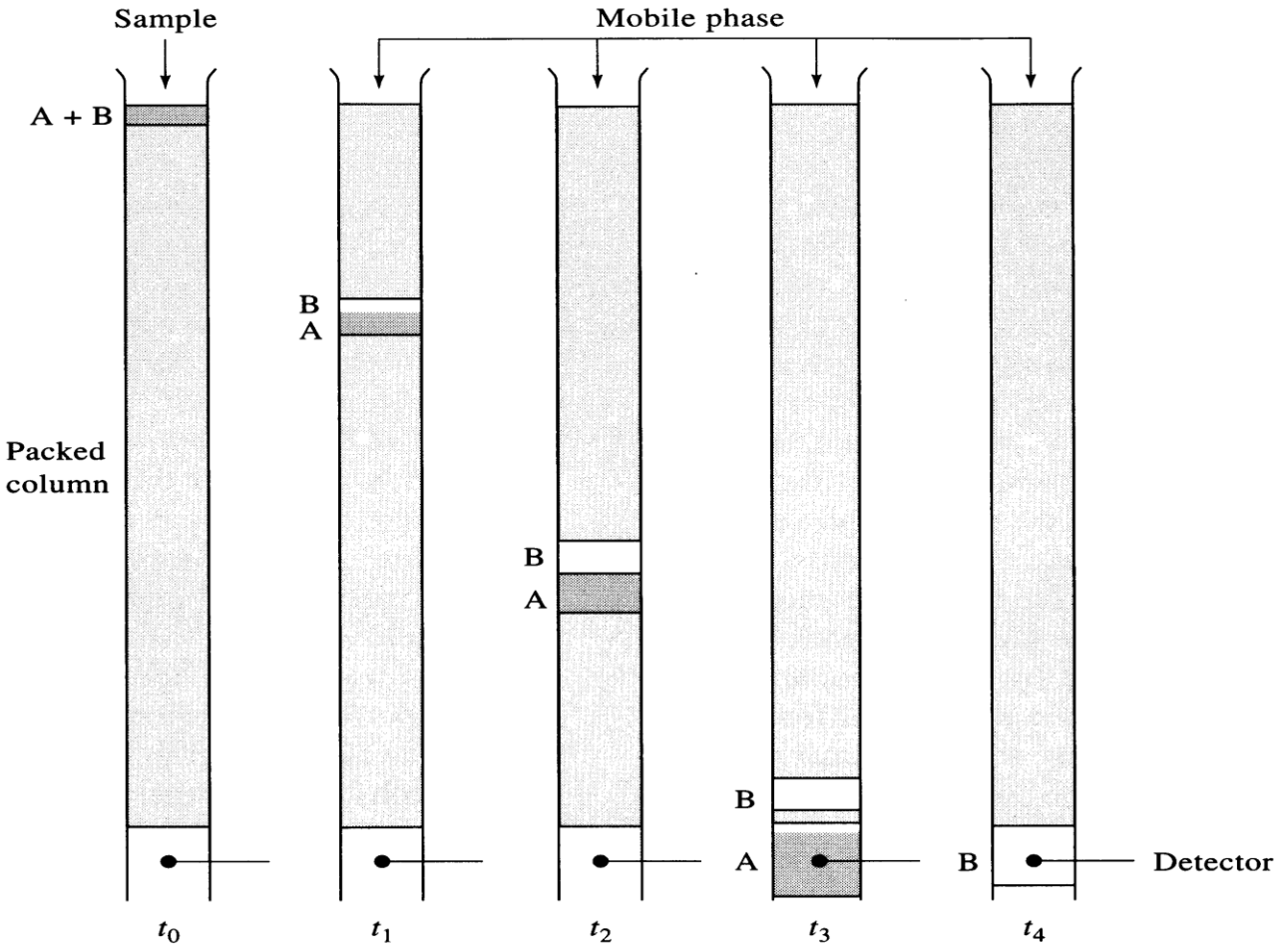
Classification of chromatographic separations

Name	Mobile phase	Stationary phase	Method of fixing stationary phase
Gas-solid	Gas	Solid	Held in a tubular column
Gas-liquid	Gas	Liquid	Adsorbed on a porous solid held in a tube or adsorbed on the inner surface of capillary tube
Partition	Liquid	Liquid	Adsorbed on a porous solid held in a tubular column
Adsorption	Liquid	Solid	Held in a tubular column
Paper	Liquid	Liquid	Held in a pores of a thick paper
Thin layer	Liquid	Liquid or solid	Finely divided solid held on a glass plate, liquid may be adsorbed on particles
Gel	Liquid	Liquid	Held in the interstices of polymer solid
Ion exchange	Liquid	Solid	Finely divided ion-exchange resin held in a tubular column

BASIC PRINCIPLE OF CHROMATOGRAPHY:

In chromatography there are two phases:

- Stationary phase
- Mobile phase



Fill the column with stationary phase. Then add mobile phase to remove air gas. Now put the mixture containing A and B on the top of the column. Now pass the mobile phase through column. Because of different adsorption capacity of solute molecule A and B will be distributed between stationary phase and mobile phase.
B will more strongly adsorb on silica layer than A. So after some time A will be coming out first from the column then B.

Definition:

Elution: it is the process of passing mobile phase to stationary phase to separate different component of mixture.

Eluent: it is the fresh mobile phase which is used to separate the mixture.

Eluate: it is the mobile phase which is coming out from column (mobile phase + solute).

THEORIES OF CHROMATOGRAPHY: (FACTORS AFFECTING SEPARATION EFFICIENCY)

- Plate theory (Martin theory)
- Rate theory (Van Deemter theory)
- Random walk theory (Gidding theory)

•Plate theory (Martin theory)

This theory was given by Martin.

Definition:

Plate: it is the small portion of stationary phase within which there is a perfect equilibrium of solute concentration between mobile phase and stationary phase.

HETP (height equivalent to one theoretical plate): it is the linear distance within which there is perfect equilibrium of solute concentration between stationary phase and mobile phase.

More will be the no. of plates i.e. small portion of stationary phase is required to establish perfect equilibrium of solute concentration between stationary phase and mobile phase. Therefore the separation efficiency will be higher.

HETP 1/α n

n α separation 1/α HETP (H)

H=L/n

L= length of stationary phase

n= no. of plates

H=HETP

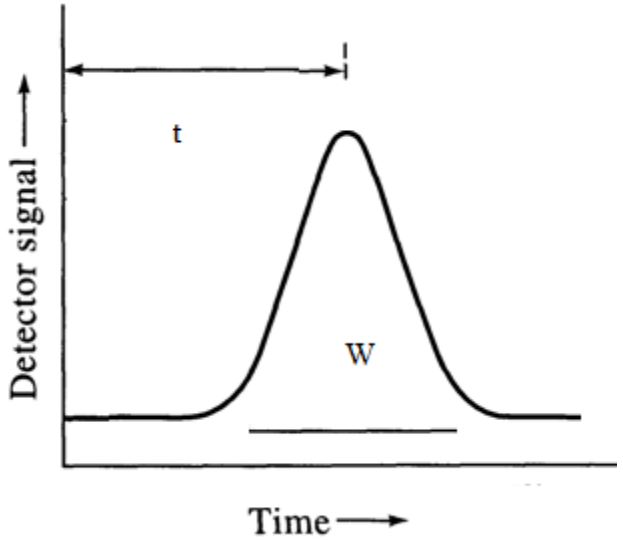
$n=16\ (t/w)^2$

n can be calculated by this formula.

t= retention time

w= base width of peak

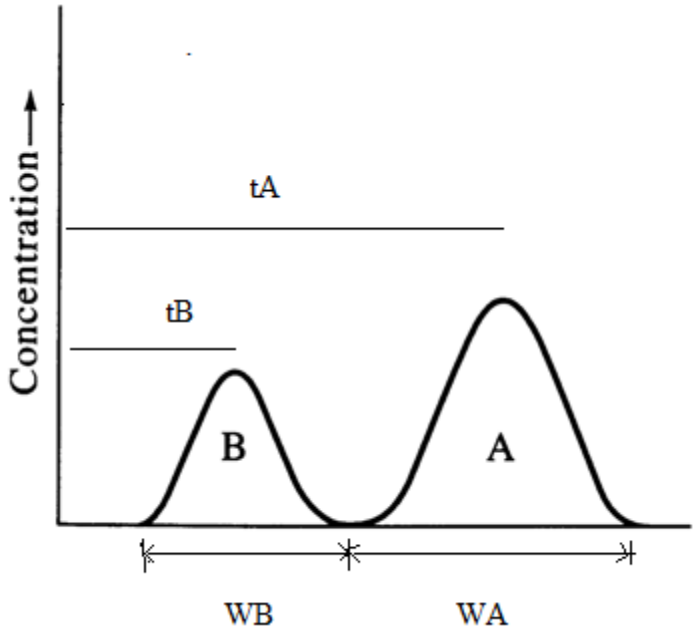
Retention time: it is the time between sample application and maximum response.

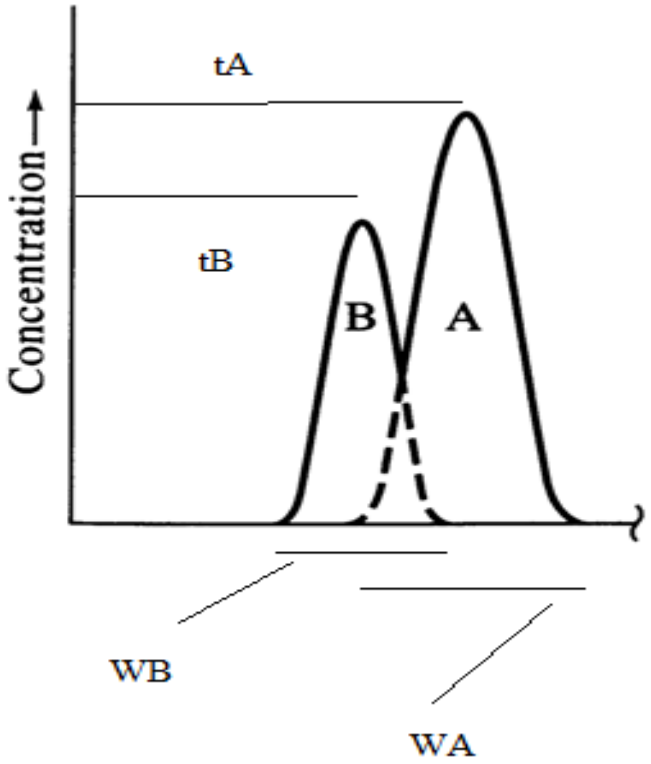


BINARY MIXTURE:

In case of binary mixture, if there is complete baseline separation (no mixing), separatibility factor can be calculated as $\alpha = {}^{tB}/_{tA}$

In position I and II, the retention time for A and B are same. But in I there is complete baseline separation. In case of II there is mixing of peak.





In case of mixing, we have to consider resolution factor Rs that can be determined by equation.

Rs= change in retention time/average of base width

$$\frac{tB - tA}{(wA + wB)/2}$$

$$\frac{2(tB - tA)}{wA + WB}$$

•Rate theory (Van Deemter theory)

Chromatographic peaks are generally broadened by three kinetically controlled processes: eddy diffusion, longitudinal diffusion and non-equilibrium mass transfer. The magnitudes of these effects are determined by such controllable variables as flow rate, particle size of packing, diffusion rates and thickness of stationary phase. The Van Deemter equation provides relationship between flow rate and plate height.

Van Deemter's equation:

$H = A + B/\mu + C\mu$

H=HETP

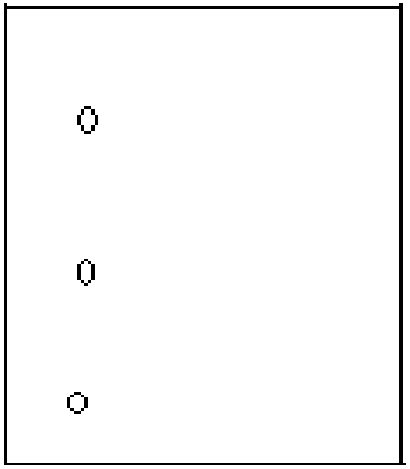
A=Eddy diffusion factor

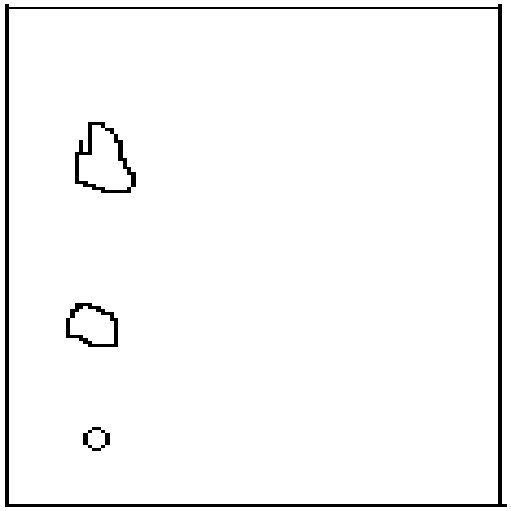
B= Longitudinal diffusion factor

C= Mass transfer

μ = Rate of mobile phase

Band Broadning:





Separation efficiency
Is higher

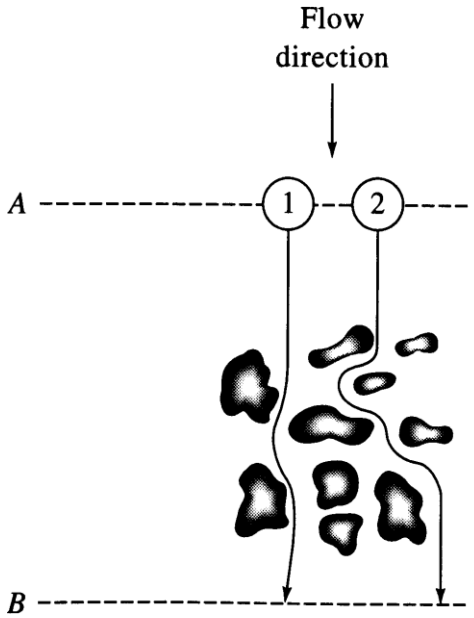
Separation efficiency
is lower

In chromatography, if mixture is placed in form of narrow band, then solute molecules should be separated in narrow band. This is called efficient separation. In some cases due to some reasons solute molecules separated in broad zone that is known as band broadning or zone broadning.

Band broadning (B.B.) α H
B.B. 1/ α separation efficiency
B.B. 1/ α n

A=Eddy diffusion factor

Zone broadning from eddy diffusion arises from the multitude of pathways by which a molecule can find its way through a packed column. As shown in figure the length of these pathways differ, thus the residence times in the column for molecules of the same species are also variable. Solute molecules then reach the end of the column over a time interval that tends to broaden the elution band. The quantity A can be related to particle size, geometry and tightness of packing of stationary phase. A is independent of flow rate.

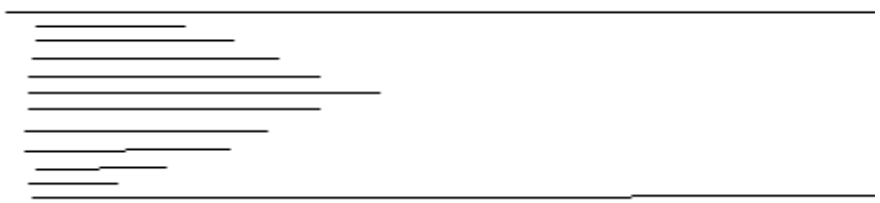


$A=2\lambda d_R$

d_R= average particle diameter

λ=packing factor (range of particle sizes and how they are packed)

Band broadning due to A is minimized by careful packing of a column with small spherical particles possessing limited range of sizes. Particular care is needed to avoid open channels.



In chromatography the rate of mobile phase should be adjusted, so that it should be streamline or laminar.

A solution at periphery (near the column wall) will travel with low velocity due to the resistance of column wall.

The rate of mobile phase will increase as we go from periphery to centre and it is found maximum at centre, because the resistance is minimum at the centre.

This factor is known as eddy diffusion factor.
Because of this factor solute molecule do not move uniformly. Therefore there is decrease in separation efficiency.

B= Longitudinal diffusion factor

It arises from the tendency of molecules to migrate from the concentrated center part of a band toward more dilute regions on either side. This can occur in both mobile phase and stationary phase. It causes further band broadning. It is most important where mobile phase is a gas, because diffusion rates in the gas phase are several orders of magnitude greater than those in liquids. The amount of diffusion increases with time. Thus the extent of broadning increases as the flow rate decreases.

$B=2\psi D_M$

Ψ=obstruction factor

D_M=temperature

B can be reduced by decreasing the temperature and increasing velocity of flow.

This B is due to time. It is also called diffusion of solute molecule within the column.

More will be the time spent by molecule in column, more will be the longitudinal diffusion. i.e. more will be the B.B.

This B can be minimize by increasing the rate of mobile phase.

H $1/\alpha$ μ

Time $1/\alpha$ separation efficiency

C= Mass transfer

Chromatographic bands are broadened because the flow of the mobile phase is ordinarily so rapid that true equilibrium between phases cannot be realized. For example, at the front of the zone, where the mobile phase encounters fresh stationary phase, equilibrium is not instantly achieved and solute is therefore carried somewhat farther down the column than would be expected under true equilibrium conditions.

Similarly, at the end of the zone, solutes in the stationary phase encounter fresh mobile phases; again, the rate of transfer of solute molecule is not instantaneous. Thus the tail of the zone is more drawn out than it would be if time existed for equilibrium. The net effect is a broadning at both ends of solute band.

The effect of non equilibrium mass transfer become smaller as the flow rate is decreased because more time is available for equilibrium to be approached. The layers of immobilized liquid on a stationary phase should be as thin as possible.

All the molecules of stationary phase are not perfectly even. It has some uneven surface, so solute molecules will be distributed within this uneven surface.

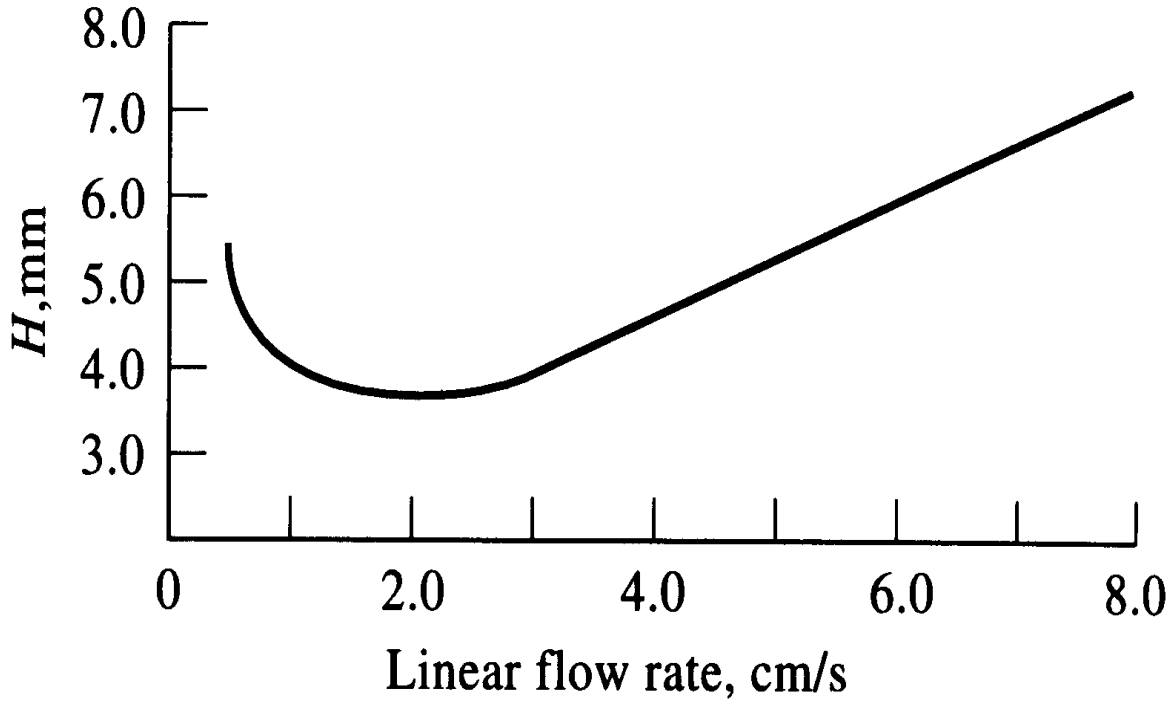
Because of this mass transfer there are chances of band broadning. This B.B. can be reduced by decreasing the rate of mobile phase.

$C \alpha \mu$

According to Van Deemter equation, for higher separation efficiency H should be minimum.

To minimize this H; A, B, and C should be minimum. But B is inversely proportional to μ and C is directly proportional to μ.

So rate of mobile phase should be optimum.



•Random walk theory (Gidding theory)

This theory was given by Gidding.

This theory gives the relationship between random walk of the molecule and separation efficiency.

If all the factors are perfect, even though there is band broadning due to the random walk of the molecule because all the molecules will not travel at the same speed.

This random walk is expressed by σ.

$\sigma = (LH)^{1/2}$

L=Length of the column

Definition:

σ: it is the linear distance which covers 68% of the solute concentration on both the side of mean.

CONCLUSION:

•Plate theory: the no. of plate should be minimum (H should be minimum).

•Rate of mobile phase should be optimum.

•Length of the column should be minimum.

These factors are known as intracolumn factors

•Extra column factors:

Factor which effect the rate of separation outside the column.

1) Sample volume: should be minimum.

B.B. $\alpha\ \upsilon^2$

2) Detector volume: should be minimum.

B.B. $\alpha\ Vd^2$

3) Particle size: should be minimum, as we decrease the particle size surface area will be increased. i.e. the no. of plates will be increase. Therefore separation efficiency will be increase.

4) Temperature: should be constant throughout the separation because it will affect partition co-efficient and solubility.