Chapter – I

INTRODUCTION
GENERAL INTRODUCTION

Pharmaceutical analysis plays a very vital role in the quality assurance and quality control of bulk drugs and their formulations. Pharmaceutical analysis is a specialized branch of analytical chemistry involves separating, identifying and determining the relative amounts of components in a sample of matter. It is concerned with the chemical characterization of matter both quantitative and qualitative. Pharmaceutical analysis derives its principles from various branches of sciences like physics, microbiology, nuclear science and electronics etc. Qualitative analysis reveals the chemical identity of the sample. Quantitative analysis establishes the relative amount of one or more of these species or analytes in numerical terms [1-2].

Nearly, all physical properties are characteristic of a particular element or compound can be made the basis of a method for its analytical determination. Eg: Spectroscopic technique involves the absorption/ emission of radiant energy in all region of the electromagnetic spectrum. In recent years, several analytical techniques have been evolved that combination of two or more methods into one called “hyphenated” technique Eg: GC-MS, LC-MS etc.

The complete analysis of a substance consists of 4 main steps.

1. Sample preparation / Sampling

2. Dissolution of the sample, conversion of the analyte into a form suitable for measurement.
3. Measurement

4. Calculation and interpretation of the measurement

In the development of organic therapeutic agents, pharmaceutical scientists have explored numerous approaches to find and develop organic compounds that are now available in pharmaceutical formulations suitable for the treatment of diseases and often for the maintenance of human health. It is well to remember that the chemical, physical, conformational and biochemical properties of organic compounds are functions of their structures. Sometimes the activity of the drug is dependent chiefly on its physical and chemical properties, whereas in other instances the arrangement, the position and size of the groups in a given molecule are important and lead to a high degree of specificity. The word quality with reference to formulation is comprehensive and refers to characteristics like the potency, uniformity, purity, pharmacological actions, stability, etc. It is not only the moral responsibility of manufacturers to produce effective, safe and non-toxic forms but also their legal responsibility.

Several distinct problems occur during the analysis of drugs and their formulations, which can be due to interference from excipients, during the assay of drugs and formulated products [Tablets: one or more among diluents-lactose, starch, cellulose derivatives, calcium phosphate, manitol, sorbitol, sucrose, calcium sulphate, dextrose, binders and adhesives-acacia, gelatin. Polyvinyl pyrrolidine, algenic acid derivatives, disintegrants-clays, etc.; lubricants, stearic acid (salt or derivatives), talc, polyethylene glycol, surfactants, waxes; flow promoters-silica derivatives, corn starch; colors (permitted F.D. and C.D. and C dyes

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lakes); flavors (natural and artificial), sweeteners; capsules, certified dyes opaquing agents; plasticizers; preservatives; parenteral products; vehicles- water; vegetable and mineral oils, simulated oils, propylene glycol, dioxalamines, dimethyl acetamide; stabilizers-antioxidants; buffering agents (citrate, acetate, phosphate); co- solvents; wetting suspending and emulsifying agents (Tween 80, sorbitol tristearate, fluronic F-68), preservatives etc., powders, sugars like lactose, sucrose, sorbitol or polysaccharides like starch or microcrystalline cellulose; dusting powders; diluents (talcum), absorbents (carbonates or oxides of calcium and magnesium), difficulties encountered in the determination of minute amounts of degradation products or the analysis of metabolites of drugs in body fluids [3] and the interference from the endogenous compounds.

**Typical Instrumental Techniques**

An important feature of modern pharmaceutical chemistry is the introduction of more refined and sensitive methods of physico-chemical analysis such as spectroscopy [colorimetry, spectrophotometry covering UV, visible and IR regions, fluorimetry, nephelometry or turbidometry, NMR and Mass] and chromatography [GLC, HPLC, TLC] that enable on to assay the quality of drugs more accurately and with the smallest consumption of the analyte [4], reagents and time. The basic trends in pharmaceutical analysis at present include further improvement of physico-chemical methods of analysis of formulations and they are wide spread practical applications in the relevant establishments (pharmaceutics, analytical and quality control laboratories). The modern methods of choice (HPLC, GLC, NMR, and Mass) [5-6] for purity assay involve sophisticated equipment,
which are very costly and pose problems of maintenance. Hence they are not in the reach of most laboratories and small-scale industries. The visible spectrophotometric (or colorimetric) or fluorimetric methods are very simple, cheap; and easy to carry out. The limitations of many colorimetric or fluorimetric methods of analysis lie in the chemical reactions upon which the procedures are based rather than on the instruments available. Many reactions involving formation of color or fluorescent species for a particular drug are quite selective or can be rendered selective through the introduction of masking agents, control of pH, and use of solvent extraction technique, adjustment of oxidation states or by prior removal of interfering substances adopting chromatographic technique. In the present investigation RP HPLC has been utilized for the assay of selected drugs.

**CHROMATOGRAPHY**

The term ‘Chromatography’ covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

Chromatographic methods can be classified most practically according to the stationary and mobile phases, as shown in the Table 1.1
### Table 1.1: Classification of Chromatographic methods

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid</td>
<td>Liquid</td>
<td>Adsorption column, thin-layer, ion exchange, High performance liquid chromatography.</td>
</tr>
<tr>
<td>Liquid</td>
<td>Liquid</td>
<td>Partition, column, thin-layer, HPLC, paper chromatography.</td>
</tr>
<tr>
<td></td>
<td>Gas</td>
<td>Gas – Liquid Chromatography.</td>
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</tbody>
</table>

The importance of Chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by-products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards.

Chromatography is a technique for separation of molecule mixtures that depends on the differential affinities of the solutes between two immiscible phases [7-8]. Out of the two, one phase is in a form of fixed bed of large surface area with small porosity in nature,
spherical or non-spherical in shape [9] whereas the other is a fluid or liquid in nature that moves through or over the surface of stationary bed. In chromatographic terminology, the former one is called as stationary phase while the later is coined as mobile phase. It is very important that the components of mixtures of drugs should be of molecular dimensions, i.e., either in a solution or in gaseous form. The relative affinities of the solutes must be reversible so that the mass transfers occur through the separation. The stationary phase has been coated with inert supporting material, larger surface area, this larger surface area plays role for sorption and desorption of the solutes [10-11].

The chromatographic methods are classified according to the stationary and mobile phases. If the stationary phase is a solid, then it’s called as adsorption chromatography and if it’s a liquid then it is called as partition chromatography. In former mainly electrostatic forces play important role. In the case of gas-solid chromatography, the mobile phase is in gaseous form, in partition chromatography an inert solid materials such as silica, alumina etc., serves as a solid support for thin layer of liquid which is the effective stationary phase, its only plays major role in the interaction with solutes or drug which causes separation and elution. In this the relative solubility of the analytes as determined by their partition coefficients which plays an important role. If the mobile phase is a liquid then it is called as Liquid-Liquid chromatography and the mobile phase is a gas, then it is called as gas-liquid chromatography. In addition to above, other three techniques are commonly adopted are viz., ion-exchange chromatography, size-exclusion chromatography and affinity chromatography.
Techniques

The most common and frequently adopted technique in chromatographic development is elution analysis or elution technique and is carried out by introducing the sample in small volume as possible into the head or inlet of the column or stationary phase and allowing the mobile phase to pass through the column. The elution pattern or order of elution majorly depends upon the relative partition coefficient of the composition of solutes, the separation and elution order can be altered for our requirements by means of changing polarity of solvents used and buffering capacity of the aqueous mobile phase. During the course of HPLC analysis, the percentage composition does not vary throughout the analysis then it is called isocratic pump mode analysis. Otherwise during elution the non-aqueous or solvent ratio or strength of the mobile phase is changed, and then this technique is called as gradient pump mode or gradient mode elution analysis.

Liquid chromatography

The liquid chromatography is usually referred to those methods in which the separation takes place in a packed tube or column under the influence of mobile phases. It can be performed by two classical ways either by column chromatography or High Pressure Liquid Chromatography. The classical procedure is called open column chromatography in which the mobile phase is allowed to flow down through packed column under the influence of gravitational force which operates under low pressure. In the other, the mobile phase is pumped directly into the closely packed column under high pressure using mechanical and pneumatically controlled pumps, this method called as High pressure liquid chromatography.
HPLC INSTRUMENTATION AND THEORY

Liquid chromatography referred as High Pressure Liquid Chromatography but in current scenario the term gradually changed into High performance chromatography, the second one is preferred since it better describes the characteristics of chromatography and avoids the impression of high pressure as prerequisite for performing analysis.

Column chromatography existing or applied across the world is rely on gravitational force or works under low pressure. This leads to greater time consumption for band broadening by diffusion phenomena. In this case, the draw back is that the faster flow rate can’t be applied, because at higher flow rate the back pressure will build up, which indirectly damages the matrix structure of the stationary phases, there by the resolution between the components gets impaired. In the past decade, tremendous development has been occurring in column chromatographic techniques, which results in availability of various types of stationary phases which can withstand these pressures and of pumping systems. These developments lead to different types of separation techniques resulted in faster analysis by using HPLC, hence it is emerged as most popular, powerful and versatile form of all chromatographic techniques.
Instrumentation:

Figure 1.1: Schematic Diagram of High Performance Liquid Chromatography
Figure 1.2: Typical High Performance Liquid Chromatography
The basic HPLC system consists of the following parts:

1. Solvent or mobile phase reservoir

2. High pressure pump

3. Injector

4. Column

5. Detector

6. Data recording and interpretation unit

1. **Solvent or Mobile phase reservoir**

   The solvents or buffers or mixture of solvents and buffers in the form of homogenous mixture are stored in solvent reservoir and are allowed to enter the mixing chamber through mechanical pump via flowing tube reservoirs mainly made up of glass bottled with properly covered.

2. **High pressure pump**

   Different types of pumps are used in high pressure liquid chromatography which can be classified as direct gas pressure pump, pneumatic intensifier pump, reciprocating pump and syringe pumps. Now a day only two types of pumps are employed in HPLC equipments, one is mechanical controlled which delivers mobile phase at a constant flow rate and another one is pneumatic controlled which provides and works at constant pressure.
In mechanical pumps, the most frequently used is the reciprocating piston type in which a motor or mechanical driven which drives sapphire plunger into small liquid end chamber to force out the mobile phase. In the mean time the check valves control the flow of the solvent into and out of the liquid end which prevents back flow of liquid. Because of the continuous flow by means of pulses the plunger moves constantly in and out, during these movements there is a possibility of pressure fluctuation which may cause upset of base line during analysis. In case of pneumatic pumps may be either the gas displacement type which uses direct pressure from a highly compressed gas to force solvent out of a tube or the pneumatic amplifier type which compressed gas at a lower pressure impinges on the large end of a piston to force the smaller end to deliver the liquid. The amplification of pressure is proportional to the ratio of the areas of the two ends of the piston.

3. Injector

Injector is mainly used to inject the sample components either in the form of liquid or gaseous form into the column compartment which allows mixing up with mobile phase and gets detected. There are two types of injectors, one is manual injector using Injection valves and which is called Rheodyne injector and another one is fully automated electronically controlled device.

The manual injector operates by loading sample loop by means of glass or plastic syringes while the mobile phase is pumped into the column.
In case of automated injector, devices control automatically number of injections, injection volumes and time gap between injections which ensures the reproducibility of injections. In auto injector the volume of injections can be modified at any time depends upon requirements.

4. Columns

The column is the heart of a High pressure liquid chromatography system and its importance needs to elaborate. HPLC columns are either polymers or silica based. Silica based packings are the most popular and often used systems. The chemical natures of these packings vary with the stationary phase; their physical characteristics are similar [12]. The average particle diameter of the packings is between 3 to 10 μm with a narrow size distribution [13]. These are produced by grinding and sizing of the irregularly shaped, bulk manufactured silica; the HPLC columns packings should be based on spherical silica particles having good mechanical strength and a narrow size distribution. The bulk particles are usually precipitated from silica solutions in the presence of formaldehyde and urea at a pH of 2 [14]. The columns of smaller particles permit faster separation than columns of larger particle size. Higher strength particles provide columns that exhibit lower back pressures and long lifetime [15]. Small molecules are usually chromatographed on particles having 5-12 nm pore size while large molecules are usually separated with particles having a pore size of 12 to 30 nm, e.g., proteins are usually separated on 30 nm pore size particles [16]. The silica surface contains various kinds of SiOH (silanol) groups and those particles heated at high temperatures, e.g., 800°C, are devoid of such groups and hence cannot be used
Individual silanols exist in three general types. The SiOH groups on a fully hydroxylated surface occur mainly in the hydrogen-bonded form [17]. The second type contains hydroxylated and free silanol and the third type contains very few silanol sites. Free silanols are undesirable for the separation of the basic molecules. The addition of appropriate alkali can eliminate problems of secondary interactions between the basic compounds and acidic silanols (e.g., triethylamine). The secondary reaction between the acidic solutes can also be prevented by the addition of small amount of carboxylic acid into the mobile phase [18]. A more detailed discussion of silanol related problems and their correction is given in reference [19]. Most bonded phase silica packings are made surface-reacted organosilanes. Various alkyl and alkyl substituted silica are made with this reaction. A few packings use a polymerized surface layer that results from the reaction of di or tri functional silanes with silica particles. The stability of the bonded phase is important in HPLC reproducibility. Long chain alkyl bonded phase packings (e.g, C8 or C18) generally are more stable than monomeric phases (e.g, diols). End capping is often used to more completely bond (silanize) packings and consists of a subsequent reaction with trimethylchlorosilane or hexamethyldisilazane, to increase the cover support and to minimize unwanted reaction with free silanols [20, 21]. Reversed phase separations can be made using polymer (polystyrene) particles, which are spherical and porous. Such polymeric(nonsilica) particles can withstand solutions with pH 2 to 13, where silica columns degrade gradually, normally reverse phase separations can be made with C8, C18, C3, C4 Phenyl, phenyl ethyl, cyano, amino, polystyrene packings while normal phase separations must utilize cyano, diol, amino and pure silica packings. Column specifications should specify particle size, length, internal diameter etc. Normal columns are
3-25 cm in length and internal diameter of most analytical columns is 0.4-0.5 cm. Micro bore column (0.1-0.2 cm i.d) are used for interfacing with detectors such as mass spectrometer [22]. The column plate number N is the single most important characteristic of a column and defines the ability of the column to produce sharp, narrow peaks and to achieve good resolution for band pairs with small $\alpha$ values. N depends on the particle size and is usually expressed per meter length of the column. The values of N for a 25 cm column should be approximately 10000. The shape of the peaks produced by the column is equally important. The peak symmetry should be between 0.9 to 1.1. Retention time reproducibility or consistency in RRT is the criterion for a good column. Knox and Parcher [23] report that the most efficient chromatography can be expected if the injected solution is introduced centrally at the top or inlet of the column.

Knox and Laird [24] have enunciated the relationship between column length and diameter and particle size as

$$\frac{D_c - 60dp}{LdP} \geq 16(1.8/v + 0.06) \quad (\text{Eqn.1.1})$$

Where $D_c$ is the column diameter and $L$ is the column length. The pressure drops for columns packed with spherical particles can be approximated by

$$P = \frac{3500L \eta}{t_0dP^2} \quad (\text{Eqn.1.2})$$
Where \( P \) is the pressure in psi, \( L \) is the column length in cm, \( \eta \) is the mobile phase viscosity in centipoises, \( t \) is the column dead time in sec and \( dp \) is the particle diameter in micrometers.

5. Detector:

The most widely used detector in HPLC is the UV-absorption or spectrophotometer. In this detector the changes in UV-absorption when the solution passes through a flow cell is measured [25-26]. UV detectors are concentration sensitive and have the advantage that they don’t destroy the solute. UV detection can utilizes the fixed emission line of a mercury line (254 nm) to allow the detection of molecules with some absorption at this wavelength. The continuous emission of energy by deuterium lamp can be utilized in conjunction with a monochromator to provide a variable wavelength detector. The variable wavelength facility is extremely useful for getting better sensitivity in difficult analysis as solutes can be monitored at their wavelength of maximum absorption [26-30]. The stability of current detector is such that the concentrations in the eluent of about one part in \( 10^9 \) of molecules with high molar absorption coefficients can be measured. This corresponds to a few nanograms of injected sample. Double beam UV detectors are available which can record the spectrum if the flow is stopped; while the solute passes through cell. Not all molecules possess sufficiently strong UV chromophore for satisfactory UV absorption. Bile acids, lipids, sugars etc., are examples of such compounds. Today such molecules are detected by the process of derivatisation when a chromophoric group is attached to the solute either at
The minimum weight of solute can be detected with this detector with a reasonable peak maximum absorbance, can be estimated from Beer’s law and various experimental conditions [31].

\[
W_m = \frac{1000 \, M \, (k'+1) \, (S/N) \, (N_0) \, L^{0.5}}{\varepsilon \, L_c \, d_c^2 \, N^{0.5}}
\] (Eqn.1.3)

Where, \(W_m\) is the minimum detectable mass in ug, \(M\) is the molecular weight, \(k'\) is the capacity factor, \(S/N\) is the required signal/noise ratio (usually > 2), \(N_0\) is the detector baseline noise in absorbance units, \(L\) is the column length (cm), \(L_c\) is the length (path) of the detector flow cell (cm), \(d_c\) is the internal diameter of the column in cm, \(N\) is the plate number.

Refractive index Detector (RI) functioning by measuring the change in the refractive index as the eluent passes through the flow cell. The sample and reference cell have to be thermo stated, if better sensitivity required. This detector is at least three times as sensitive as the UV-visible detector, but variations in ambience produce several fluctuations. On the other hand a fluorimetric detector is extremely sensitive and works on the principle that the fluorescent energy emitted at a longer wavelength, known as emission wavelength, is proportional to the concentration. First, however the solute has to be excited with the excitation wavelength energy, this wavelength is always lower than the emission wavelength, but solute has to possess initial fluorescence [32-33], it can react with
compounds, which are fluorescent derivatizing agents, as in UV detectors to produce fluorescence. Electrochemical detectors also very sensitive, but its usage is restricted to only oxidation and reductions. The system works on the principal of polarography [34-36]. A serious drawback of this detector is that the electrodes get contaminated and poisoned by absorption of the oxidized or reduced compounds. Partially to obliterate this defect a moving electrode has been used [37] in HPLC to detect solutes containing halogens [38]. The column effluent is volatilized in an oven purged with nitrogen gas and the halogenated solutes like chlorinated insecticides, polychlorinated biphenyls, DDT in milk etc., are detected in favorable cases upto very less concentration level. A potentially useful and selective range of detection systems involving reactions of the solutes after they elute from the column. Several such post-column reaction detectors include the well known ninhydrin reaction of amino acids [39], the 2, 4-dinitriphenyl hydrazine reactions of carbonyl compounds [40] and cerium detector for carbohydrate analysis [41]. In GC, column effluent or solvents vaporizes and solute pyrolysed in a hydrogen /air atmosphere and then detected by flame ionization detector [42-44]. The idea of LC-MS combination, though expensive is undoubtedly attractive. Various methods of stream splitting, transport and solute ionization have been reported [45-48], the limitation of this detector buffer solution being non volatile can’t be used. Various other detectors, though commercially not popular have been highlighted, they include a microwave plasma detector [49], infrared detector [50], flame photometric detector [51], a phosphorus/ sulfur detector [52], a dual UV fluorescence detector [53] and radiometric detection system [54-55].
6. Data Recording and Interpretation

Once the detection is completed the detected signals is converted into electronic signals and then amplified by means of amplifier and recorded as a chromatogram in the form of data points. Then these are manually or by aide of software it’s interpreted as per requirement to convert as a presentable format.

THEORY OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The basis of all forms of chromatography is the partition or distribution coefficient which describes the way in which a compound distributes itself between two immiscible phases.

If a compound distributing itself between equal volumes of two immiscible solvents A and B, the value for this coefficient is a constant at a given temperature and is given by expression:

\[
\frac{\text{Concentration in Solvent A}}{\text{Concentration in Solvent B}} = K_d \quad \text{(Eqn.1.4)}
\]

The distribution of a compound can be described not only in terms of its distribution between two solvents, but also by its distribution between any two phases such as a solid / liquid or gas/liquid phases. The term effective distribution coefficient is explained as the amount of substance present in one phase divided by the total amount present in the other
transfer of the solute to and from the stationary phase. For a qualitative evaluation of a chromatogram a quantity retardation factor \((R_f)\) can be defined

\[
R_f = \frac{V_mC_m}{V_mC_m + V_sC_s}
\]

(Eqn. 1.5)

Where \(V_m\) is the volume of the mobile phase and \(V_s\) is the effect of the stationary phase. The variables \(C_m\) and \(C_s\) denote the concentration of the solute in the mobile and stationary phases at any time of equilibration. Dividing each term in Eqn 2.4 by \(C_m\) becomes

\[
R_f = \frac{V_m}{V_m + KV_s}
\]

(Eqn. 1.6)

Where \(K\) is the partition coefficient equal to \(C_s/C_m\) and is an equilibrium coefficient which denotes the differential affinity of the solute for the two phases. A component with a large partition coefficient will have a small value for \(R_f\) and long elution. By dividing each in Eqn 2.5 by \(V_m\), an alternative results

\[
R_f = \frac{1}{1 + K'}
\]

(Eqn. 1.7)
Where \( K' \) is the capacity factor

\[
K' = \frac{K \times V_s}{V_m}
\]  
(Eqn.1.8)

Since the volumes of the stationary and mobile phases are constant for any chromatography experiment, \( K' \) is directly proportional to the partition coefficient.

The time which elapses from the time of the chromatogram to the elution maximum of the solute are called retention time \( t_R \), a function of the length of the column and the rate of travel of the solution. The rate of travel is defined as

\[
\text{Rate} = u \cdot R_i
\]  
(Eqn.1.9)

Where \( u \) is the linear velocity of the mobile phase, usually expressed as cm/sec

Thus

\[
\text{Length} \quad \frac{L}{\text{Rate}} = \frac{1}{u} \quad \frac{1}{(1+K')} = \frac{t_0 (1+K')}{(1+K')}
\]  
(Eqn.2.0)

Where \( t_0 \) is the time for the elution of a solute that is not retained by the chromatogram. From this a convenient expression for the determination of capacity factor experimentally can be formulated as
The optimum values of $K'$ should be between 1 and 10, another parameter used to describe the retardation of the solute is the retention volume $V_r$, which is equal to the volume of the mobile phase required to elute the solute or analyte. Therefore retention volume is equal to the product of the retention time and the flow rate of the mobile phase, i.e., $t_x F$ or to $(1+K') F$. Since to $F$ is equal to the volume of mobile phase in the system ($V_m$ or void volume) the retention volume can be expressed as

$$V_R = V_m (1+K') = V_m + k V_s$$

(Eqn.2.2)

The most important factor influencing retention volume is partition coefficient. A large partition coefficient means a long retention time, since the solute spends more time in the stationary phase. Retention times and volumes are not thermodynamic parameters and may change with the operating parameters such as flow rates, temperature, columns etc., Hence these quantities are measured usually with reference to another peak in the chromatogram. In these cases the parameters are called Relative Retention Time (RRT) and relative retention volume (RRV). Elution pattern of an ideal chromatogram is a curve whose shape is called as Gaussian peaks. Normal statistical distribution can be employed to describe these parameters, i.e., the standard deviation $\sigma$ and the variance $\sigma^2$. The peak width at any time can be expressed in multiple of standard deviation. The inflection points are
located at one standard deviation on either side of the mean level which is 60.7% of the overall height of the peak. The width at this point is therefore $2\sigma$. If tangents to the peak are drawn through inflection points and are extended to the baseline, the width at the base $W_b$ is $4\sigma$. The width at one-half the height is $2.354\sigma (W_H)$.

Two further characteristics of the peak are the height and area. The area is equal to the integral of the equation representing the curve from the point where it leaves the baseline to the point where it returns and is proportional to the amount or concentration of the solute. The height is measured at the maximum and therefore corresponds to the largest concentration of the zone.

Two parameters commonly used for estimating the effectiveness of a chromatographic system are $N$, the number of theoretical plates and $H$, the height equivalent to the theoretical plates or $L/N$, where $L$ is the length of the column. Since width and standard deviation of a peak can vary under different experimental conditions, a better indication for the sharpness of a peak is its relative standard deviation or RSD, $\sigma/t_R$. In practice, $N$ is defined in terms of the reciprocal of RSD by the expression $N= (t/\sigma)^2$. If one substitutes the relationships given earlier, i.e., $W_b= 4\sigma$, $W_H= 2.354\sigma$, the final equation will be

$$N=16(t/W_b)^2 \text{ and } N=5.545(t/W_H)^2$$
(Eqn.2.3)

The inter-relationship of retention and peak shape becomes more prominent when more than one peak is present. This becomes prominent when a mixture of solutes is present
and separation is desired and it's denoted by resolution $R$, describes the overlap of the leading and the trailing edges of the two peaks. If a complete separation is to be achieved, the parameters of the experimental variables are to be selected, so that the bound centers or peak maxima elute at different retention times. This happens when the partition coefficients of the two solutes are sufficiently different, so that one is absorbed more strongly than the other. Hence, 'α' the separation factor or selectivity factor, can be defined as $K_1/K_2$ which is the ratio of the partition coefficient of the solute producing the second band to that of the solute producing the first band. Since $K'$ capacity factor, is directly proportional to $u$, the separation factor may also be stated as the ratio of the respective $K'$ values, i.e., $K'_2/K'_1$.

Then the separation factor $\alpha$ can be stated in terms of the adjusted retention times or volumes as

$$\frac{(tr)_2-to}{(tr)_2-to} = \frac{(V_R)_2-Vm}{(V_R)_1-Vm}$$

(Eqn.2.4)

The separation factor is also termed as resolution, the general formulae to calculate the resolution is given by equation

$$Rs = \frac{2(tr_2.tr_1)}{w_1+w_2}$$

(Eqn.2.5)
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