4.1. Theoretical Description of Chromatography

a. System Parameters and Chromatographic Figures of Merit

As discussed in the previous chapter, the solute distributes itself between the two competing phases, namely, stationary and the mobile phase. The distribution equilibrium involved in chromatography can be described as follows, where A solute species,

\[
\begin{array}{c}
\text{A}_{\text{mobile}} \\
\text{A}_{\text{stationary}}
\end{array}
\]

The equilibrium constant K for this reaction, termed as distribution coefficient, defined as,

\[
K = \frac{C_s}{C_m} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (1)
\]

Where, Cs is the molar concentration of the solute in the stationary phase and Cm is the molar concentration of the solute in the mobile phase.

The capacity factor is used to describe the migration rates of solutes on the column. Capacity factor k’ is related to distribution coefficient k as

\[
k'_A = \left(\frac{C_s}{C_m}\right) \times \left(\frac{V_s}{V_m}\right)
\]

Hence, for a solute A, the capacity factor, k’A is defined as,

\[
k'_A = K_A V_s / V_m \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (2)
\]

where, \(K_A\) is the distribution constant for species A, \(V_s\) is the volume of the stationary phase in the column and \(V_m\) is the volume of the mobile phase in the column.

Retention time99, \(t_R\) is the time required to elute the sample component form the column and expressed as, \(t_R = t_0 (1 + k') \quad \ldots \ldots \ldots \ldots (3)\)
where, $k'$ is the capacity factor and $t_o$ is the retention time of the unretained solute, obtained by measuring retention times of the solutes and the unretained solutes, given as,

$$k' = \frac{(t_R - t_o)}{t_o} \quad \text{........... (4)}$$

**b. Theoretical plate number (N) and plate height (H)**

The separation efficiency of a chromatographic column can be expressed in terms of the number of theoretical plates $N$, and the plate height $H$. Consider a single theoretical plate as a narrow layer of height, $H$ (in cm) within a chromatographic column having length $L$ (in cm) where equilibrium conditions continue to exist. If $N$ is the number of theoretical plates or the plate count then,

$$H = \frac{L}{N} \quad \text{........... (5)}$$

$N$ is an important characteristic of a chromatographic system and is a dimensionless quantity. It reflects the number of times the solute molecules partition themselves between the two phases during their passage through the column. A large $N$ value implies high separation efficiency. Value of $N$ towards particular solute molecules can be determined experimentally from the chromatogram using the expression,

$$N = 16 \left[\frac{t_R}{W}\right]^2 \quad \text{........... (6)}$$

where, $t_R$ is the retention time of the analyte peak, $W$ is the width of the peak at its base expressed in the same units as $t_R$ (eg. sec, min or cm). Alternatively, $N$ can also be estimated from the width of the peak measured at a height of one-half of the peak height, $W_{1/2}$ using the expression,

$$N = 5.54 \left[\frac{t_R}{W_{1/2}}\right]^2 \quad \text{........... (7)}$$
c. Resolution

The resolution R of the column provides the quantitative measure of its ability to separate two analytes\(^7^9\). The resolution R, between two peaks is the degree of separation between the two peaks and it shows relation between retention time and the peak width of peak in the chromatogram as,

\[
R = \frac{2 \left[ t_R(2) - t_R(1) \right]}{W_1 + W_2} \quad \text{............... (8)}
\]

Where, \(W_1 = \text{Width of the first peak, } W_2 = \text{Width of the second peak, } t_{R1} \text{ and } t_{R2} \text{ are retention time of first and second peak respectively.}

\[d. \text{ Separation Factor}\]

Separation factor shows the relation between the retention times of peak in chromatograms. Alternatively, separation factor \(\alpha\) is a characteristic of the thermo dynamic difference in partition of two compounds.

The selectivity factor \(\alpha\) of a column for the two species 1 and 2 is defined as\(^7^9\),

\[
\alpha = \frac{k'_2}{k'_1} \quad \text{............... (9)}
\]

where, \(k'_2 > k'_1\). \(k'_2\) is the partition ratio for the more strongly retained species 2 and \(k'_1\) is constant for the less strongly held or more rapidly eluted species 1. Generally, \(\alpha\) is always greater than unity.
e. Symmetry factor

It is defined as the ratio of the distance from the edge to the tailing edge of the peak. The width of the peak at 5% peak height is divided by the distance f, from the peak maximum to the leading edge of the peak. It is denoted in form of the Eq (10),

\[ S = \frac{W_{0.05}}{2f} \]  \hspace{1cm} (10)

where, \( W_{0.05} \) is the peak width at 5% peak height. For a symmetrical peak the tailing factor S is unity and the value of S increases as tailing becomes more pronounced, i.e if S > 1, the peak shows tailing and if S < 1, the peak shows leading front.

4.2. Method Validation

Validation is a documented analytical procedure of providing that any process, method, equipment, material, activity, system or analyst performs suitably under a given set of conditions. When extended to an analytical procedure, depending upon the application it establishes whether the method is reproducible when carried out by a different analyst, different laboratories, different regent, different equipment etc. It builds a degree of confidence, not only for the developer but also to the user.\(^88\) Validation of analytical method should follow a well documented procedure beginning with the definition of the scope of the method and its validation criteria including the compounds and matrices, desired detection and quantitation limits and other important performance criteria. For the validation of a method, as defined by the pharmacopoeias of developed world, the analytical performance parameters measured include, precision, accuracy, limit of detection, limit of quantitation, specificity/selectivity, stability, ruggedness and robustness\(^89\).
4.2.1. Linear Regression Least – Squares Fit Data

If in a calibration graph there is a scatter of signals w.r.t the changing parameter, then the best fit for the calibration is obtained by forming an equation for straight line,

\[ Y = mX + b \]  \hspace{1cm} \text{................. (11)}

where, \( m \) is the slope of the straight line and \( b \) is the intercept. \( X \) and \( Y \) signify the changed parameter and the signal respectively. The slope of the best fit line, \( m \) is given by

\[ m = \frac{\sum X_i Y_i - n \bar{X} \bar{Y}}{\sum X_i^2 - n \bar{X}^2} \]  \hspace{1cm} \text{................. (12)}

where, \( n \) is the number of measurements, \( x \) is the mean of \( x_i \) values and \( y \) is equal to the mean of the \( y_i \) values. The intercept of the best fit line is given by,

\[ b = Y - mX \]  \hspace{1cm} \text{................. (13)}

The standard deviation in the slope (\( m \)) and intercept (\( b \)) can be expressed as

\[ S_m = \frac{S_a}{S_x (N - 1)^{1/2}} \]  \hspace{1cm} \text{................. (14)}

\[ S_b = S_m \left[ \frac{\sum X_i^2 - n \bar{X}^2}{u} \right]^{1/2} \]  \hspace{1cm} \text{................. (15)}

where,

\[ S_x = \frac{[\sum X_i^2 - n \bar{X}^2]^{1/2}}{n - 1} \]

The correlation coefficient or the regression factor \( r \), denoting the deviation of the signal from the straight line, should be 1.00 or nearest about it for the success of the calibration range.
The factor $r$ is given by

$$
    r = \frac{\sum X_i \cdot Y_i}{\left( \sum X_i \cdot \sum Y_i \right)^{1/2}} 
$$

(16)

The precision of the method is expressed by the standard deviations in the slope and intercept. The accuracy is found out by spiking known amounts of the analytes and determining the signal. These signals are then related to the slopes and intercepts to find the recoveries. Recoveries determine the accuracy of the method. The standard deviation of the residuals ($S_d$ or $S_{yx}$), which is also called standard error or point error, and is expressed as

$$
    S_{yx} = \frac{\left( \sum Y_i^2 - b \sum Y_i - m \sum X_i Y_i \right)^{1/2}}{n - 2} 
$$

(17)

**4.2.2. Limit of detection and Limit of Quantification (LOD and LOQ)**

Limit of detection is defined as the smallest concentrations which gives a perceptible change in the measured signals. Limit of quantitation is defined as the correct concentration which is measured with acceptable precision and accuracy. Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses. Later, specific calibration curve is studied using samples containing an analyte in the range of detection. The residual standard deviation of a regression line or the standard deviation of $y$-intercepts of regression lines may be used as the standard deviation. The detection limit (LOD) may be expressed as

$$
    LOD = 3.3 \frac{\sigma}{S} 
$$

(18)

where, $\sigma$ = the standard deviation of the response, $S$ = the slope of the calibration curve

This approach is also applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from
samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. Similarly, LOQ can be determined and expressed as \( \text{LOQ} = 10 \frac{\sigma}{S} \) ........................ (19)

where, \( \sigma \) = the standard deviation of the response, \( S \) = the slope of the calibration curve.

4.2.3. Specificity and Selectivity

Specificity/Selectivity is the ability of the method of quantitation to measure the analyte in the presence of possible impurities formed due to endogenous/exogenous materials, concomitants, excipients, forced degradants, etc. During SIM, specificity and selectivity are the most crucial parameters of validation. Peak purity determination is a spectral evaluation technique for detecting the presence of co-eluting impurities in the chromatogram allowing detection of the degradants with the analyte peak.

Spectral impurity indicates a distortion of the analyte spectrum by the presence of background absorbance from solvents and matrix compounds along with the impurity. Peak impurity refers to a distortion of the analyte spectrum by an additional component which partially or completely co-elutes with the major compound. Specificity of the method is generally established by determining the peak purity using a PDA detector. The resolution factor for the drug and nearest resolving peak is determined. Peak purity and resolution of all the degradation products’ peaks from the drug peak is also determined.
Fig.4.1: Elucidation of sampling the chromatographic peak at upslope, apex and downslope for peak purity determination.

Taking into consideration of both types of impurities, namely spectral and peak impurities, comparison of spectra is the most common method for peak purity determination. Peak purity analysis software allows the analyst to sample spectra at equidistant points across an HPLC peak. A spectrum is sampled upslope, at the apex and downslope of the eluted peak, as shown in the Fig.4.1. The visual comparison and purity parameters obtained by the software clearly indicate the difference between the pure and impure peaks.89

4.2.4. Stability

The stability of drug solutions can be studied on the basis of room temperature and refrigerated stock solution stability. Room temperature stability is carried out to assess the stability of solution left on the bench while preparing the stock dilution, also known as bench-top stock solution stability. Generally, drug solution at its target concentration is injected in replicate at 0, 3, and 6 h period and peak areas are determined along with the mean, standard deviation and variance.

Calculations: For room temperature stability,

\[ \text{Percent comparison} = \frac{\text{Mean response at 0,3,6 h}}{\text{Mean response at 0 h}} \times 100 \ldots \ (20) \]
Refrigerated stock solution stability is carried out to assess the stability of store stock in refrigerate over a period of time 7, 14, & 21 days during which it can be used.

**Calculations:**

*Corrected Response = Comparison Response x Correction Factor  
Correction Factor = Corrected conc of stability standard stock solution  
Corrected conc of comparison standard stock solution ........(21)

Percent Comparison Response = Mean Response of Stability Samples/Mean Corrected Response of Comparison Sample. The acceptance criterion is that the percent comparison response must be between 85 % & 115 %.

**4.3. Kinetics of Degradation**

The application of chemical kinetics to accelerated stress testing is a crucial concept to study the rates of drug degradation. By applying well-established kinetic concepts, it is not only possible to numerically summarize the role of each variable contributing to the kinetics of degradation, but also provide an insight into the mechanism of degradation. Consider a drug D reacting to form a product P. This process is described by the following scheme,

\[ D \rightarrow P \]

Most drugs degrade by reactions that involve a bimolecular reaction in which drug D collides with a reactant A to produce one or more products. This is illustrated by the following equation:

\[ D + A \rightarrow P \]
P will be formed if D and A collide with sufficient energy (and an appropriate orientation) to result in a molecular rearrangement to form P. In this simple case, the rate of loss of D, \(-d[D]/dt\), is said to be proportional to the activity (or, more simply, the concentration) of both D and A, as indicated by Eq. (21).

\[-\frac{d[D]}{dt} \propto [D][A] \]

(22)

When the proportionality constant is included, the following equation is obtained:

\[-\frac{d[D]}{dt} = k[D][A] \]

(23)

where, \(k\) is the proportionality constant, referred to as the rate constant. If \(k\) is large, the reaction is fast. If \([A] \gg [D]\), then even though some of A is consumed during the reaction, only D will be lost. Hence,

\[-\frac{d[D]}{dt} = (k[A])[D] = k_{obs}[D] \]

(24)

where, \(k_{obs}\) is said to be the observed rate constant, a pseudo-first-order constant. Especially in aqueous solutions, the degradation kinetics of drugs is often simplified to pseudo-first-order conditions.

Further, on integration, \(\ln[Ct/Co] = k_1t\)

(24)

A fractional life is the length of the time it takes for a product of a drug substance to decrease to a level indicated by a fraction. The half life \(t_{50}\) of a substance is the length of time it takes to decrease the content of active compound to 50% of its value.

It is noted that a-fractional life is given by, \(\ln[a] = k_1t_a\)

(25)
The most common of the a-lives is the half life and the $t_{90}$ (the point where 90% of the original concentration of the drug is remaining) is given by the following equations,

$$k_1t_{1/2} = -0.693 \hspace{1cm} \text{……………….}(26)$$

$$k_1t_{90} = -0.1059 \hspace{1cm} \text{……………….} \hspace{0.5cm} (27)$$

If we assume the degradation of drug D is a result of the direct reaction with a species A, the rate is proportional to the concentration of "activated complex," also known as the "transition state," $X^\ddagger$, formed between D and A:

$$\begin{align*}
D + A & \stackrel{k'}{\rightleftharpoons} X^\ddagger \rightarrow P \\
-\frac{d[D]}{dt} = & \frac{d[P]}{dt} = k'[X^\ddagger] \\
& = k''[D][A]f_Df_A/f_{X^\ddagger} \\
& = k[D][A] \hspace{1cm} \text{……………….} \hspace{0.5cm} (28)
\end{align*}$$

In Eq. (28), $[X^\ddagger]$ is the concentration of $X^\ddagger$ and $f_D, f_A,$ and $f_{X^\ddagger}$ are the activity coefficients of D, A, and $X^\ddagger$, respectively. The rate constant, $k$, can be described by the following equation, based on transition-state theory:

![Free-energy diagram showing reactants proceeding to products through a transition state or activated complex](image)

Fig.4.2: Free-energy diagram showing reactants proceeding to products through a transition state or activated complex
\[
  k = \frac{\kappa T}{h} \exp\left(\frac{-\Delta G^\ddagger}{RT}\right) - \frac{\kappa T}{h} \exp\left(\frac{\Delta S^\ddagger}{R}\right) \exp\left(\frac{-\Delta H^\ddagger}{RT}\right) \quad \text{(30)}
\]

where \(\Delta G^\ddagger\), \(\Delta S^\ddagger\), and \(\Delta H^\ddagger\) are the free energy, entropy, and enthalpy of activation, respectively. \(\Delta G^\ddagger\) is the difference in free energy between the reactant state and the activated complex, as shown in Fig.4.2. The term \(\kappa\) is the Boltzmann constant, \(h\) is the Planck constant, and \(T\) is the temperature in degrees kelvin. In descriptive terms, Eq. (30) essentially suggests that for chemical reaction to occur molecules must first collide and additionally it must collide with overall free energy of rearrangement of the molecules to occur. The term \(\kappa \frac{T}{h}\) represents a universal collision number. The term \(e^{-\Delta G^\ddagger / RT}\) represents the fraction of molecules colliding with sufficient energy to overcome the free-energy barrier to reaction. This free-energy barrier is made up of both an enthalpic term \((\Delta H^\ddagger)\) and an entropic term \((\Delta S^\ddagger)\). Eyring\(^90\) developed the theory of absolute reaction rates by introducing the concept of the formation and breakdown of an activated complex. It is illustrated in Fig.4.2, and represented by,

\[
  k = \frac{\kappa T}{h} \frac{Q^\ddagger_{\Delta} Q^\ddagger_{\alpha \beta}}{Q^\alpha_{\Delta} Q^\beta_{\Delta}} \exp\left(\frac{-E_0}{RT}\right) \quad \text{(31)}
\]

where, \(Q_{\Delta}\), \(Q_{\alpha \beta}\), and \(Q^\ddagger\) are the partition functions of \(\Delta\), \(\alpha \beta\), and the activated complex, respectively, and \(E_0\) is the energy required for the formation of 1 mol of the activated complex at \(0^\circ\text{K}\).

Obtaining reliable drug degradation data requires the development and validation of a stability-indicating assay. A kinetic model is selected to describe the degradation curve, and a rate constant is calculated by fitting the observed degradation curve to a suitable rate equation according to the assumed model. This section describes the selection of the kinetic model(s) and the calculation of a rate constant\(^91\).
Let us consider the rate of degradation in a solution state. When a drug substance, D, degrades via a certain mechanism in which reactants A, B, ... participate, the degradation rate generally depends on the concentrations of the various reactants A, B, ... and D according to Eq. (32), assuming that all the reactants are involved directly or indirectly in the rate-controlling step.

\[
\frac{-d[D]}{dt} = k[D]^n[A]^m[B]^m ... \\
\text{......... (32)}
\]

When the concentrations of A, B, ... are maintained constant, i.e., when the change in their concentrations during the reaction is negligible owing to their presence at much higher concentrations than drug D, or when these species are components that are maintained constant through the use of buffers, such as hydronium ion, the degradation rate is often described by,

\[
\frac{-d[D]}{dt} = k'[D]^n \\
\text{............ (33)}
\]

When \( n \) equals 0, 1, or 2, the reaction is said to be a pseudo-zero-, pseudo-first-, or pseudo-second-order reaction respectively. If the concentration of an additional reactant other than drug D is not constant during the reaction, the reaction order becomes \( n + 1 \). Complex degradation pathways can be represented by the combinations of zero-, first-, and second-order reactions.

4.3.1. General Kinetic Parameters

Temperature is one of the primary factors affecting drug stability, because most reactions proceed faster at elevated temperatures. The rate constant/temperature relationship has traditionally been described by the Arrhenius equation\(^91\),

\[
k = A \exp \left( \frac{-E_a}{RT} \right) \\
\text{........ (34)}
\]
where, $E_a$ is the activation energy, $A$ is the frequency factor, $R$ is the gas constant and $T$ is the absolute temperature ($^0K$). The frequency factor $A$ in the Arrhenius equation corresponds to the product of the universal collision and entropy terms in Eq. (30) and the $E_a$ term is related to the enthalpy term in Eq. (30).

A plot of the logarithm of $k$ against the reciprocal of absolute temperature generally yields a linear relationship, known as the Arrhenius plots. The frequency factor and activation energy are regarded as independent of temperature and the activation energy, $E_a$, is used as a measure of the temperature dependence of the rate constant. Arrhenius plots have been traditionally used to describe the temperature dependency for various chemical reactions. A prerequisite for the application of Eq. (34) and Eq. (30) is that the degradation mechanism does not change in the temperature range of interest. $E_a$ values of about 10-30 kcal/mol (40-130 kJ/mol) are generally observed in the degradation of drug substances. The terms $E_a$ and $\Delta H^\ddagger$ are a measure of how sensitive the degradation rate of a drug is to changes in temperature.

### 4.3.2. pH and pH-Rate Profiles

The effect of pH on degradation rates of drug substances in aqueous solutions has been studied extensively.\textsuperscript{91,92} Degradation rates of drug substances are generally affected by pH because most degradation pathways are catalyzed by hydronium and/or hydroxide ion, as water is a critical reactant. If the critical path in a reaction involves a proton transfer or abstraction step, other acids and bases present in solution (usually buffer species) can affect the reaction rate. A reaction in which hydronium ion, hydroxide ion, and water catalysis are observed can be described by,

$$k_{obs} = k_{\text{H}^+a_{\text{H}^+}} + k_{\text{H}_2\text{O}} + k_{\text{OH}^-a_{\text{OH}^-}} \quad \text{......... (35)}$$
where, \( k_{\text{obs}} \) is the sum of the specific rate constants and activities for each parallel pathway, and \( a_{H^+} \) and \( a_{OH^-} \) are the activities of hydronium and hydroxide ion, respectively. This equation is for the case when the drug itself is neutral in the pH range of study, i.e., where ionization of the drug does not have to be taken into account.

The contributions of the first and second terms in Eq. (35) are larger than that of the third term, the pH-rate profile shown in Fig 4.3. If the second and third terms are dominant, then the profile illustrated in panel 2 is observed. If the first and third terms are dominant, then a V-type pH-rate profile (panel 3) occurs. If all terms contribute significantly, the U-shaped pH-rate profile shown in panel 4 is observed.

![Fig.4.3: pH-rate profiles for drug degradation](image)

Alternatively, one can deduce semilogarithmic plots describing the effect of pH on the degradation of the drug. Here, the plot of the percentage of the drug remaining after degradation against time at varying pH can be obtained.

Generally, drug substances capable of undergoing ionization yield more complex pH-rate profiles. For example, each ionic and nonionic form of the drug could be subject to hydronium ion, hydroxide ion, and water catalysis. When this happens, the expression for \( k_{\text{obs}} \) may contain more than three terms. For example, apparent degradation rate constants for a drug substance that
is a weak base will depend on the ionization constant, $K_a$ of the conjugate acid of the weak base and the concentration of hydronium ion and other species, as described by,

$$k_{ab} = k_{H^+)H_2O}\frac{a_{H^+}}{K_a+a_{H^+}} + k_{H^+)OH}\frac{a_{H^+}}{K_a+a_{H^+}} + k_{H^+)H_2O}\frac{K_a}{K_a+a_{H^+}} a_{OH} + k_{OH}\frac{a_{OH}}{K_a+a_{H^+}} \quad \text{……… (36)}$$

where, $k_{H^+}$ and $k_{OH}$ are hydronium ion- and hydroxide ion-catalyzed rate constants for ionized and non-ionized drug respectively, and $k_{H_2O}$ and $k_{H_2O}^\prime$ are the $H_2O$-catalyzed rate constants for ionized and non-ionized drug respectively.