CHAPTER 1

INTRODUCTION

The pharmaceutical industry represents one of the fastest growing industry sectors in the world today. Although the process of drug discovery has been key for the development of new therapies, increasingly pharmaceutical companies are relying on the development of novel product formulations to maintain market share. Within this, purity estimation and characterization is a key to developing an understanding of the functionality of new products, formulations and delivery systems. Quality control analysis in the pharmaceutical industry involves the determination of multiple parameters for both raw materials and the end products\textsuperscript{1}. Medicines for human use are required to comply with standards, which relate to their quality, safety and quantity of the active ingredients. The evaluation of safety and efficacy and their maintenance in practice is dependent upon the existence of adequate methods for quality control of the product. The standard of purity must, therefore, be strictly defined in such a way as to ensure that successive batches are consistent in composition, irrespective of whether they came from the same or different manufactures.

The multi-components formulations are flooded in Indian pharmaceutical retail market and have gained a lot of importance because of its inherent compliance about the patient’s acceptability and economicity. These combinations are available in the various dosage forms. The quantitative
analysis of such multicomponent formulations is very important for the confirmation about the quality and efficacy. There are different analytical methods have been reported for single drug formulations but due to complexity in the multicomponent formulation, method development is a challenge for the analytical chemist. Official books also do not provide methods of the simultaneous analysis. Most of the methods available for the analysis of active ingredients of such formulations are applicable only after prior separation, which makes it tedious, expensive and time consuming.

The analytical techniques used for estimation of drugs consist of classical and instrumental methods of analysis. In classical methods the various methods used are titrimetry, volumetry gravimetry and different instrumental techniques employed, are spectrophotometry, gas liquid chromatography (GLC), high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC) etc. These methods are based upon the measurement of specific and nonspecific physical properties of the substances.

Simultaneous analysis procedures are being used more frequently for estimation of drugs in multi-component pharmaceutical formulations due to their inherent advantages viz. avoid time consuming extraction and separation. The methods are economical in the sense that use of expensive regents is minimized and are equally accurate and precise. The methods are validated by using parameters as per ICH guidelines.
In recent years, the number of drugs reaching the market steadily declined concomitantly with an increase in the costs of drug development. Therefore, new and innovative ways have to be found to overcome the bottlenecks in drug discovery. Improvements in speed and quality of target finding and validation process are urgently needed and methods have to be developed which allows an early risk assessment. High throughput quantitative analysis can play a significant role in these critical fields of the value chain in drug development.

Quality has been driven by compliance and by reluctance to making changes to the original registration filing. The regulatory climate has lead to an industry reluctant to make improvements. Fortunately there is a revolution going on, driven partly by the FDA and partly by the ICH process, which is encouraging the concepts of quality by design, process knowledge and understanding, with quality based on science and risk management principles. Quality by design recognizes that quality cannot be tested into a product; it has to be built into a product and into the process during development, using the principles of risk management. Process development studies should support the basis for process optimization, process validation and process control requirements. Product quality should be accessed by the product's fitness for use and the process robustness.

1.1 SELECTION PARAMETERS OF AN ANALYTICAL METHOD

Choice of Analytical method depends on, what accuracy is required, how much sample is available what is the concentration range of the analyte,
what components of the sample will cause interference, what are the physical and chemical properties of the sample matrix, how many samples are to be analyzed should be explained. Statistical evaluation, accuracy vs. precision, bias, sensitivity and application of students t test along with other statistical calculations should be done with the help of available analytical data and overviewed. The importance of calibration of instrumental methods and how analytical methods calibration can be carried out must be explained.

Usually involvement of adding one or more increments of a standard solution to sample aliquots of the same size (spiking) is done. Shimadzu HPLC 10 AT vp and UV-Visible Spectrophotometer Pharmaspec 1700 (Shimadzu) were used for HPLC and spectrophotometric estimation of marketed formulations.

Different Instrumental modes and their utility for analytical work as well as techniques in spectrophotometric methods of analysis are discussed. The developed methods are precise, rapid, simple and economical as a new analytical tool for the marketed formulations.

Once the problem is defined the following important factors are considered in choosing the analytical method. These are concentration range, required accuracy and sensitivity, selectivity time requirements and cost of analysis.

**Concentration range:** The ability to match the method to the optimum sample size is usually gained through experience and awareness of the different methods. Sensitivity, as it applied to an analytical method,
corresponds to the minimum concentration or lowest concentration of a substance that is detectable with a specified reliability. It is often expressed numerically as a detection limit or sensitivity. Different analytical methods will provide different sensitivities and the one chosen will depend on the sensitivity that is required to solve a particular problem. Accuracy refers to the correctness of the result achieved by the analytical method.

Selectivity: Selectivity is an indication of the preference that a particular method shows for one substance over another.

Time and cost: Time and cost often go hand in hand usually are a reflection of the equipment, personnel and space required to complete a determination.

A classic analytical problem is the simultaneous determination of two or more compounds in the same sample without previous chemical separation.

1.2 ULTRA-VIOLET AND VISIBLE SPECTROPHOTOMETRY

UV-Visible spectroscopy is one of the instrumental techniques of analysis, which utilizes the measurements of intensity of electromagnetic radiations emitted or absorbed by the analyte. Absorption spectroscopy is one of the most useful and widely used tools available to the analyst for quantitative analysis. The relation between the concentration of the analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy.
UV-absorption spectroscopy deals with absorption of light by a sample in UV region of 200-400 nm, while visible absorption spectroscopy deals with absorption in a region of 400-800 nm. Absorption of UV-visible light generally results from promotion of an electron from bonding to antibonding orbital. Thus the wavelengths of absorption peaks can be correlated with the types of bonds present in the species. There are four such types of transitions observed.

\[ \sigma \rightarrow \sigma^*, \quad \pi \rightarrow \pi^*, \quad \eta \rightarrow \sigma^*, \quad \eta \rightarrow \pi^* \]

Out of these transitions are usually of very high energy and observed only in the vacuum (\( \lambda < 190 \) nm) whereas, \( \eta \rightarrow \sigma^* \) transitions absorption are weak and in most cases occurs at wavelengths too short to be easily measured. Thus these two types of transitions are of little interest in pharmaceutical analysis.

1.2.1 LAWS GOVERNING ABSORPTION SPECTROSCOPY

Two empirical laws have been formulated about the absorption intensity. Lambert's law states that the fraction of the incident light absorbed is independent of the intensity of light source and directly proportional to the path length. Beer's law states that the absorption is proportional to the number of absorbing molecules.

\[ A = \log \frac{I_0}{I} = abc \]

Where, \( A \) = absorbance of the solution at definite wavelength

\( I_0 \) = Intensity of incident beam

\( I \) = Intensity of transmitted beam

\( a \) = absorptivity of the molecule at a definite wavelength
b = path length of beam

c = Concentration of the solution in gms/lit

When concentration is expressed in terms of moles then equation becomes  \( A = EbM \) Where, \( E \) = Molar absorptivity (cm\(^2\)/mol)

b = path length of beam

M = Molarity of the solution

\( E \) (1 cm, 1 %), values are sometimes used instead of molar absorptivity or absorptivity. Particularly in case where the molecular weight is unknown or not clearly defined (e.g. polymer solutions). The relation of \( a \),\( E \) and \( E \) (1 cm, 1 %), is

\[
E \ (1 \text{ cm, 1 } \%) = E \times 10^{\frac{1}{M.W. \times \rho}}
\]

\( P \) = Density of the solution in gm/cc

Beer's law is said to be obeyed over the concentration range investigated, if a plot of substance concentration for a compound yields a straight line passing through the origin. In practical work, deviation from Beer's law are frequently observed. The most important reasons that causes deviation are as follows;

a. The intensity of light should not be excessive

b. Instrumental error's such as changes in chemical equilibrium and pH, presence of complexing agent, competitive metal ion reactions and concentration dependence.

c. Refractive index of the sample

d. Non monochromaticity of radiation

e. Environment such as temperature, pressure and solvent.
1.3 MODERN APPROACHES TO SPECTROPHOTOMETRIC ANALYSIS

Spectrophotometric methods of analysis are further classified into following approaches:

1.3.1 Spectrophotometric multi-wavelength analysis

Absorption spectroscopy is one of the most useful and widely used tools available to the analyte for quantitative analysis. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to simple, rapid, precise, highly accurate and less time consuming. Spectrophotometric multi-component analysis can be applied where the spectra of drugs overlaps. In such cases of overlapping spectra, simultaneous equation can be framed to obtain the concentration of individual component; otherwise multi-component analysis can be applied on any degree of spectral overlap provided that two or more spectra are not similar exactly.

Earlier multi component analysis was successfully attempted by using an online computer with an ultraviolet detection system to collect and compare spectral data. Sophistication in analytical instrumentation has resulted in development of spectrophotometers like Shimadzu model 160A, 240A and 1700S. These spectrophotometers have an in-built microprocessor for spectral data processing. The instrument computes accurate results within minimal time. The concentration of each of the component in the mixture is printed through in-built system.
1.3.2 Colorimetry

It is a form of spectroscopy, an analysis that measures how atoms or molecules respond when exposed to electromagnetic radiation of a certain wavelength, and therefore, of a certain energy. In a way, colorimetry is the most familiar kind of spectroscopy, because the wavelengths used are from the visible light region of the electromagnetic spectrum. When the drugs are transparent to the UV region or two components are overlapping and can not be detected simultaneously in the same region then colorimetric analysis can be applied depending upon the chemical structure of the drug. After reviewing the main functional groups present in the drug skeleton, it is reacted with suitable coloring reagent which results in the transformation of drug molecule into a chromogen. This chromogen absorbs in the visible region and so can be now easily detected. An analytical procedure based on comparison of the color developed in a solution of a test material with that in a standard solution and quantitated on the basis of the absorption of light. In a colorimetric test method, reagents are added to a sample and a reaction occurs with the analyte of interest, producing a color. Because the intensity of the color is related to the analytes concentration, the concentration of the analyte is determined by visually measuring the color or electronically measuring the intensity of light at selected wavelengths (i.e., spectrophotometry). Colorimetric can yield a wealth of information on colored solutions. It is a quick and non-destructive method that can identify solutes in a solution and very accurately determine their
concentrations. Computer technology has automated the somewhat tedious calculations required for colorimetric analysis and now allows colorimetric experiments to be performed within the span of a few minutes from start to finish. In short, a colorimetric analysis is straightforward, relatively foolproof, and highly informative.

The light wave of a certain wavelength and intensity is shined at a solution (this is called incident light) in colorimetry. The intensity of the light exiting the sample (transmitted light) is measured on the other side of the sample. By comparing the incident intensity to the transmitted intensity, the absorbance, $A$, can be determined for that wavelength of light. More precisely, $A = -\log (I/I_0)$, where $I$ is the transmitted intensity and $I_0$ is the incident intensity.

A vast majority of the light that has not been transmitted through a translucent sample is absorbed by the sample (a negligible fraction of the energy is lost to scattering). Therefore, a substance that transmits most of the light at a particular wavelength will have a low absorbance at that wavelength. These measurements are repeated at many different wavelengths of light from the visible region of the spectrum.

An absorbance spectrum is created by plotting absorbance versus the light wavelength. Once an absorbance spectrum of a particular substance is available, and the identity of the substance has been established, its concentration in solution can also be measured by colorimetry. This analysis is based on Beer’s Law, which in simple terms relates the color intensity of a solution to its concentration. More precisely, Beer’s Law
states that \( A = elc \), where \( A \) is the absorbance of the sample, \( e \) is a substance- and wavelength-specific coefficient, \( l \) is the length the light travels through the sample, and \( c \) is the sample's concentration.

The approaches available for colorimetric analysis are:

- Oxidation reaction
- Acid Dye reaction
- Oxidation Coupling reaction
- Diazotization and Coupling reaction
- Metal Ligand Complexation reaction
- Reduction of Tetrazolium salt reaction

The chief advantage of colorimetric analysis is that it provides simple means for determining minute quantities of substance.

The FDA currently requires pharmaceutical firms to create enantiomerically pure substances, or that the enantiomer of the drug be thoroughly studied and found to have no adverse side effects. The synthesis of enantiomerically pure substances requires the use of reagents that give enantiomeric excesses (ee) to the various synthetic steps involved in the synthetic procedure. Currently, the enantiomeric excesses of a reaction is typically determined using polarography or chiral HPLC analysis. The ability to screen for enantiomeric excesses or the possibility of quantitative analysis of enantiomeric excesses, using a simple colorimetric method would be a significant advance and simplification over current methods. This new technology \(^5\) describes a rapid and inexpensive color assay which uses a colorimetric method to
detect the enantiomeric excesses and dramatically reduces the time to screen and purify new drugs. Different colors can be seen by the naked eye when the d or l enantiomers of various functional groups are formed. Furthermore, a mathematical analysis can be applied to the UV/VIS spectra obtained for the mixture of enantiomers to determine both the concentrations of the mixture of d and l, as well as the enantiomeric excesses. The method is incredibly simple and could be of general utility for a variety of functional groups. The benefit are reducing screening time of drugs, reduce costs of drug development, expedite steps in FDA approval process, reduce possible human errors in process, does not require experienced technicians. The techniques are faster than current methods, no HPLC required, simple analysis that can be done by basic lab technicians and speedy color reaction

1.4 THE VARIOUS TECHNIQUES USED FOR MULTI-COMPONENT ANALYSIS ARE AS FOLLOWS

1.4.1 Simultaneous Equation Method (Vierordt’s Method)\textsuperscript{6-7}

If a sample contains two absorbing drugs M and N, each of which absorbs at the wavelength maximum of other, it may be possible to determine both drugs by the technique of simultaneous equations (Vierordt’s method).

Concentrations of several compounds present in the same mixture can be determined by solving a set of simultaneous equations even if their spectra overlap. If Beer’s law is followed, these equations are linear.
The calculation of the content of compounds, say M and N, of a binary mixture, a set of two equations with two unknown C_M and C_N is used provided that Beer's law is followed and the two components behave independently of one another. Absorption spectra of individual components and mixture are as shown in Fig. No.1. Where, \( \lambda_1 = \lambda_{\text{max}} \) of component M, \( \lambda_2 = \lambda_{\text{max}} \) of component N and \( \lambda_3 = \text{Isoabsorptivity} \) wavelength i.e. wavelength at which both components have same absorptivities.

Fig. No. 1: Example of selection of analytical wavelength for multicomponent analysis method

The total absorbance of a solution at a given wavelength is equal to the sum of absorbance of individual compounds at that wavelength.

Thus absorbance of the mixture at \( \lambda_1 \) and \( \lambda_2 \) may be expressed as follows:

\[
A^1 = A^1_{\text{M}} + A^1_{\text{N}} \quad \text{(at } \lambda_1) \\
A^1 = a^1_{\text{M}} b C_M + a^1_{\text{N}} b C_N \quad \text{(i)} \\
A^2 = A^2_{\text{M}} + A^2_{\text{N}} \quad \text{(at } \lambda_2) \\
A^2 = a^2_{\text{M}} b C_M + a^2_{\text{N}} b C_N \quad \text{(i)}
\]

Where, \( a = \text{absorptivity} \)
b = path length

c = concentration

The absorptivity can be evaluated from individual standard solutions of M and N. The absorbance of mixture at \( \lambda_1 \) and \( \lambda_2 \) can be experimentally found and thus from these two equations \( C_M \) and \( C_N \) can be readily calculated. Such equations can be solved by:

1. **Using matrices**

From equations (i) and (ii):

\[
A_1 = k_1 MC_M + k_1 NC_N ..............(iii)
\]

\[
A_2 = k_2 MC_M + k_2 NC_N .............(iv)
\]

Where, \( k = a b \)

Let 'A' be a column matrix with 'i' elements, where, 'i' is the number of wavelengths at which measurements are done (here \( i = 2 \)).

Let 'C' be a column matrix with 'j' elements, where 'j' is the number of components (here \( j = 2 \)).

Let 'k' be a matrix with 'i x j' elements so that the number of rows is equal to number of wavelengths and number of columns is equal to number of components (here two rows and two columns). Hence, we have,

\[
A = k.C ............ (v)
\]

\[
\begin{bmatrix}
A_1 \\
A_2
\end{bmatrix} = \begin{bmatrix}
k_1^M & k_1^N \\
k_2^M & k_2^N
\end{bmatrix} \begin{bmatrix}
C_M \\
C_N
\end{bmatrix}
\]

Since \( i = j \), the equation has unique solution

\[
C = k_2 A \\
\]

(vi)
However, in this case it will be faster to solve the equations (iii) and (iv) by means of Cramer’s Rule.

The unknown concentration ‘\( C_j \)’ of component ‘\( j \)’ is found by replacing \( j \) column of matrix ‘\( k1 \)’ by matrix ‘\( A \)’. The determinant of new matrix is divided by determinant of \( k \) matrix.

\[
C_m = \frac{\begin{vmatrix} A_1 & k^1_N \\ A_2 & k^2_N \end{vmatrix}}{\begin{vmatrix} k^1_M & k^1_N \\ k^2_M & k^2_N \end{vmatrix}}
\]

\[
C_m = \frac{A^1xk^2_N - A^2xk^1_N}{k^1_Mxk^2_N - k^2_Mxk^1_N} \quad \text{(vii)}
\]

Similarly,

\[
C_m = \frac{\begin{vmatrix} A_1 & k^1_M \\ A_2 & k^2_M \end{vmatrix}}{\begin{vmatrix} k^1_M & k^1_N \\ k^2_M & k^2_N \end{vmatrix}}
\]

\[
C_m = \frac{A^2xk^1_N - A^1xk^2_N}{k^1_Mxk^2_N - k^2_Mxk^1_N} \quad \text{(viii)}
\]

If path length is kept constant and is equal to 1 cm then all \( k \) values can be replaced by absorptivity values.

\[
C_m = \frac{A^2xa^1_M - A^1xa^1_N}{a^1_Mxa^2_N - a^2_Mxa^1_N} \quad \text{(ix)}
\]

\[
C_n = \frac{A^2xa^1_M - A^1xa^2_M}{a^1_Mxa^2_N - a^2_Mxa^1_N} \quad \text{(x)}
\]
To reduce the random errors during measurements, sometimes instead of carrying out analysis of two components at two wavelengths, it is carried out at 3 or 4 wavelengths. The equations will no longer have a unique solution. But the ‘best’ solution can be found out by the least square criterion; i.e. the best solution is that set of values for which the sum of squares of the deviations is a minimum. It can be found out by multiplying by the transpose of the absorptivity matrix. This gives two equations in two unknowns, such that the solution to these two equations is also the optimum solution to the three (or more) original equations.

2. Without using matrices

A way of simplifying the solution of simultaneous equations is by making one of the measurements at isoabsorptivity wavelength. As can be seen from Fig. No. 1, if equation (ii) is replaced by another equation generated at the isoabsorptivity wavelength, we get a new set of simultaneous equations.

\[
\begin{align*}
A^1 &= A^1_M + A^1_N = a^1_M b C_M + a^1_N b C_N \\
A^3 &= A^3_M + A^3_N \\
&= a^3_M b C_M + a^3_N b C_N
\end{align*}
\]\n
\((xii)\)

If \(b = 1\) in all measurements,

\[
A^3 = a^3 (C_M + CN)
\]

\[
A^3/a^3 - C_N = C_M \quad \text{.............. (xiii)}
\]

Substituting this value of \(C_M\) in equation (xi)

\[
A1 = a^1_M b (A^3/a^3 - CN) + a^1_N b C_N \quad \text{.............. (xiv)}
\]

Thus from equation (xiv), we get the value of \(CN\) which can then be put in equation (xiii) to get value of \(C_M\).
The use of isobestic point is of assistance in both qualitative and quantitative analysis. The isobestic point provides a uniquely satisfactory frequency for quantitative determinations of total amount of two species present. It may form a convenient reference point or internal standard to which the absorbance of one or the other of the species may be referred to find the ratio of the two compounds.

1.4.2 Two Wavelength Method

Bicomponent formulations can be analyzed by using this method. It is used to estimate the concentration of a component of interest in a mixture along with interfering components. In order to estimate first component, second component is considered as interfering, or vice versa. Appropriate selection of sampling wavelength is essential to eliminate the interference of interfering component. For each component, the respective absorbance maximum is chosen as the first wavelength. The second wavelength is any wavelength at which the absorbance of interfering component is equal to the absorbance that the interfering component had at the absorbance maximum of the component of interest.

![Fig. No. 2: Example of Selection of Analytical Wavelength for Two Wavelength Method](image)

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The absorbance difference between two points on the mixture spectra is directly proportional to concentration of the component to be determined irrespective of the interfering component as shown in Fig. No 2.

Where, \( X \) = Spectra of pure component, B.

\[ Y = \text{Spectra of pure component, C.} \]

\[ Z = \text{Spectra of unknown mixture containing both B & C.} \]

\( AA_1 \) and \( AA_2 \) = absorbance of mixture at \( \lambda_1 \) and \( \lambda_2 \) respectively.

\( AB_1 \) and \( AB_2 \) = absorbance of component B at \( \lambda_1 \) and \( \lambda_2 \) respectively.

\( AC_1 \) and \( AC_2 \) = absorbance of component C at \( \lambda_1 \) and \( \lambda_2 \) respectively.

According to Beer's law, absorbance of a sample is directly proportional to the concentration

\[ A = k \times C \quad \text{................. (xv)} \]

Also \[ AA_1 = AB_1 + AC_1 \] and \[ AA_2 = AB_2 + AC_2 \]

\[ AA_1 - AA_2 = (AB_1 - AB_2) + (AC_1 - AC_2) \quad \text{................. (xvi)} \]

Now, \( \lambda_2 \) is chosen such that

\[ AC_1 = AC_2 \quad \text{........ (xvii)} \]

\[ AA_1 - AA_2 = AB_1 - AB_2 \quad \text{........ (xviii)} \]

Let 'x' be the concentration of pure component B & 'y' be the concentration of pure component C.

\[ AB_1 = kB_1 x \quad \text{and} \quad AB_2 = kB_2 x \]

\[ AA_1 - AA_2 = AB_1 - AB_2 = (kB_1 - kB_2) x \quad \text{........ (xix)} \]

Where, \( kB_1 \) and \( kB_2 \) are absorption coefficient of component B at \( \lambda_1 \) and \( \lambda_2 \).
Since \((kB_1 - kB_2)\) is constant,

\[
AA_1 - AA_2 = \text{(Constant), } x \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \l
Q-analysis is based on the relationship between absorbance ratio value of a binary mixture and the relative concentration of such a mixture. The ratio of two absorbance determined on the same solution at two different wavelengths is constant. This constant was termed as 'Hufner's Quotient' or 'Q-value, which is Independent of concentration and solution thickness. Hence, it can be used as a tool for qualitative and quantitative assessments for variety of substances.

Fig.No.3: Example of selection of analytical wavelength for Q-Analysis (Absorbance ratio method).

Consider Fig. No. 3, at a₄, total absorbance a₄ of a mixture of X and Y components is equal to the sum of absorbance due to X (a₄ₓ) and Y (a₄ᵧ).

From Beer's law, total absorbance of mixture is:

\[ A₄ = a₆ bCX + a₁ bCY \]  \[\text{(xxi)}\]

Where, Cx and Cy are concentrations of X and Y respectively.

Similarly, \[ A₂ = a₂ bCX + a₃ bCY \]  \[\text{(xxii)}\]

Dividing equation (xxi) by (xxii),

\[ \frac{A₄}{A₂} = \frac{a₆ bCₓ + a₁ bCᵧ}{a₂ bCₓ + a₃ bCᵧ} \]  \[\text{(xxiii)}\]
Let \( b = 1 \), and divide each term by \((Cx + Cy)\) and also replace \([CX/(CX + Cy)]\) by \( F_x \), and \([Cy/(Cx + Cy)]\) by \( F_y \)

\[
\frac{A_4}{A_2} = \frac{a_6 F_x + a_4 F_y}{a_2 F_x + a_3 F_y} \quad (xxiv)
\]

Where, \( F_x \) and \( F_y \) are relative concentrations or fraction of X and Y in mixture. But \( F_x + F_y = 1 \), therefore \( F_y = (1 - F_x) \).

Therefore,

\[
\frac{A_4}{A_2} = \frac{a_6 F_x + a_4 (1-F_x)}{a_2 F_x + a_3 (1-F_x)} \quad (xxv)
\]

Rearrange the terms and substitute \( Q_0 \) for \( A_4/A_2 \) i.e. absorbancy ratio of binary mixture. The ratio is fixed for a specific mixture and the degree of dilution by solvent will not alter this \( Q_0 \) value within the limits of accurate absorptiometric measurements.

\[
Q_0 = \frac{F_x (a_6 - a_4) + a_4}{F_x (a_2 - a_5) + a_5} \quad (xxvi)
\]

Equation (xxvi) can be simplified by taking an isobestic point as a second reference point. Fig. No. 3 indicates that such a point occurs at \( A_3 \). If the derivation is now carried out in a manner similar to derivation of equation (xxiv), the following equation results:

\[
\frac{A_4}{A_3} = \frac{a_6 F_x + a_4 F_y}{a_4 (F_x + F_y)} \quad (xxvii)
\]

As indicated earlier, substitute 1 for \((F_x + F_y)\) and \((1 - F_x)\) for \( F_y \).

\[
\frac{A_4}{A_3} = \frac{a_6 F_x + a_4 (1-F_x)}{a_4} \quad (xxviii)
\]

21
\[
\frac{A_4}{A_3} = \frac{a_6 F_x + a_1 - a_1 F_x}{a_4} \quad (\text{xxix})
\]

\[
\frac{A_4}{A_3} = \frac{a_6 F_x + a_1}{a_4} \quad (\text{xxx})
\]

Therefore,

\[
\frac{A_4}{A_3} = \left(\frac{a_6}{a_4} - \frac{a_1}{a_4}\right) F_x + \frac{a_1}{a_4} \quad (\text{xxxi})
\]

Put, \(A_4/A_3 = Q_0\): absorbance ratio of binary mixture.

\(a_6/a_4 = Q_x\): absorbance ratio of pure X.

\(a_1/a_4 = Q_y\): absorbance ratio of pure Y.

Therefore, equation (xxxi) becomes,

\[Q_0 = (Q_x \cdot Q_y) \cdot F_x + Q_y \quad ............... (\text{xxii})\]

This equation is concentration independent. Analysis of binary mixture therefore does not require careful dilutions and accurate weighing. Relative concentration can be determined very fast.

For absolute concentrations, the initial weight of the sample must be known.

At isoabsorptivity wavelength,

\[A_3 = a_4 b C_x + a_4 b C_y \quad ............... (\text{xxxiii})\]

Assume \(b = 1\), then,

\[A_3 = a_4 (C_x + C_y) \quad ............... (\text{xxxiv})\]

\[A_3/a_4 = (C_x + C_y) \quad ............... (\text{xxxv})\]

By combining equations (xxii) and (xxxv), absolute concentration of X can be determined.

By rearranging equation (xxii),
Since $F_x$ represents fraction of component X in the binary mixture and $A_3/a_4$ represents the total concentration ($C_x + C_y$), the concentration of X can be determined from the following equation:

$$C_x = \frac{Q_0 - Q_y}{Q_x - Q_y} x \frac{A_3}{a_4} \quad \text{(xxxvii)}$$

Similarly, concentration of Y can be calculated as:

$$C_y = \frac{Q_0 - Q_x}{Q_y - Q_x} x \frac{A_1}{a_4} \quad \text{(xxxviii)}$$

or by subtracting the value obtained for $C_x$ from the value obtained for $A_y/a_4$.

### 1.4.4 Geometric Correction Method

A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in the samples of biological origin. The simplest of this procedure is the three-point geometric procedure, which may be applied if the irrelevant absorption is linear at the three wavelengths selected. This procedure is simply algebraic calculations of what the baseline technique in infrared spectrophotometry does graphically.

### 1.4.5 Absorption factor method (Absorption correction method)

Absorption factor method is further modification of simultaneous equation method. Quantitative determination of one drug is carried out by $E (1\% , 1$
cm) value and quantitation of another drug is carried out by subtraction absorption due to interfering drug using absorption factors.

1.4.6 Orthogonal Polynomial Method

The technique of orthogonal polynomials is another mathematical correction procedure, which involves more complex calculations. The basis of the method is that an absorption spectrum may be represented in terms of orthogonal functions as follows:

$$A(\lambda) = pp_0^\lambda + pp_1^\lambda + pp_2^\lambda + \ldots + pp_n^\lambda$$  \hspace{1cm} (xxxvii)

Where, 'A' denotes the absorbance at wavelength T belonging to a set of n + 1 equally spaced wavelengths at which the orthogonal polynomials, P0, P1, P2, P3 ---- Pn are defined.

The technique of orthogonal polynomials is another mathematical correction procedure, which involves complex calculation than the three-point correction procedure. The basis of the method is that an absorption spectrum may be represented in terms of orthogonal functions.

1.4.7 Difference Spectrophotometry

Difference spectroscopy provides a sensitive method for detecting small changes in the environment of a chromophore or it can be used to demonstrate ionization of a chromophore leading to identification and quantitation of various components in a mixture. The essential feature of a difference spectrophotometric, assay is that the measured value is the difference in absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms, which exhibit different spectral characteristics. The criteria for applying difference spectrophotometry to
the assay of a substance in the presence of other absorbing substances are (i) reproducible changes may be induced in the spectrum of the analyte by addition of one or more reagents (ii) the absorbance of the interfering substances is not altered by the reagents.

Difference spectrophotometry provides a sensitive method for detecting small changes in the environment of a chromophore or it can be used to demonstrate ionization of a chromophore leading to identification and quantitation of various components in mixture. The essential feature of difference spectrophotometric assay is that the measured value is the difference absorbance ($\Delta A$) between two equimolar solutions of the analyte in different chemical forms, which exhibits different spectral characteristics.

1.4.8 Derivative Spectroscopy $^{27-35}$

The application of derivative spectrophotometric method in the simultaneous estimation of drugs is covered along with solvent selection, preparation of sample, selection of wavelength, preparation of drug aliquots, recording of spectra, development of first to fourth order derivatives. The interpretation of data and statistics along with calculating the purity from the concentration is carried out. The main disadvantage of the derivative method is the low reproducibility$^{27}$.

Direct spectrophotometric determination of multicomponent formulation is often complicated by interference from formulation matrix and spectral overlapping. Such interferences can be treated in many ways like solving two simultaneous equations, using absorbance ratios at certain
wavelengths, but still may give erroneous results. Other approaches include pH-induced differential spectrophotometric, least squares\textsuperscript{46} and orthogonal function methods. Also the compensation technique can be used to detect and eliminate unwanted or irrelevant absorption. Derivative spectrophotometry is a useful means of resolving two overlapping spectra and eliminating matrix interferences or interferences due to an indistinct shoulder on side of an absorption band. Here absorbance (or transmittance) of a sample is differentiated with respect to wavelength $\lambda$ to generate first, second or higher order derivatives.

$$[A] = f(\lambda) \quad : \text{Zero order}$$

$$\frac{dA}{d\lambda} = f'(\lambda) \quad : \text{First order}$$

$$\frac{d^2A}{d\lambda^2} = f''(\lambda) \quad : \text{Second order}$$

First derivative spectrum can be obtained with the aid of conventional double beam instrument or a specially designed derivative spectrophotometer. But such spectra can be more easily obtained by dual wavelength measurements. For analysis of binary mixture of X & Y components, the zero crossing technique can be used. The derivative spectra of individual components are generated. Derivatization parameters (order and wavelength interval) are selected such that peaks and valleys of X and Y is dissimilar. At zero crossing wavelength of X, Y shows some derivative value and vice-versa. These derivative values also obey Beer-Lambert's law and hence calibration curve, $\frac{dA}{d\lambda}$ against concentration for each component can be constructed at the zero crossing point of other component as shown in Fig. No. 4.
Fig. No. 4: Example of selection of analytical wavelength for first order derivative zero-crossing method.

If such crossing points are absent, two simultaneous equations can be solved to determine the components. For this the selection of working wavelength to be optimum and component being determined should make a reasonable contribution to the total derivative reading at that wavelength.

The derivative spectra can be used to determine number of peaks and their positions. Even order derivative spectra narrow the bandwidth of composite bands and increase the relative contribution of minor important features of derivative technique include enhanced content, discrimination against background noise and greater selectivity in quantitative analysis. It can be used for detection and determination of impurities in drugs, chemicals and also in food additives, industrial wastes.

In brief derivative spectrophotometry is useful means of resolving two overlapping spectra and eliminating matrix interference due to an indistinct shoulder on side of an absorption bands. It involves conversion of normal spectrum \([A = f (\lambda)]\) to its first \([dA/ d\lambda = f (\lambda)]\), second \([d^2A/ d\lambda^2 = f (\lambda)]\) and higher derivatives spectra where the amplitude in the derivative
spectrum is proportional to the concentration of the analyte provided that Beer's law is obeyed by the fundamental spectrum.

1.4.9 Simultaneous Equation using Area under the Curve Method

The absorptivity values (ε₁ and ε₂) of each of the two drugs were determined at the selected wavelength range in this method. Total area under curve of a mixture at wavelength range is equal to the sum of area under the individual component at that wavelength range. This method is applicable when the λ_max of the two components is reasonably dissimilar, the two components do not interact chemically and both the component must be soluble in same solvent. The methods deviated when overlapping of UV spectra of two drugs significantly and having large difference in labeled strength. The accuracy of the method depends upon nature of solvent, pH of solution, temperature, high electrolyte concentration and the presence of interfering substances.

Consider a binary mixture of components M and N of which the absorption spectra of an individual component is shown in Fig. No. 5.

![Absorption Spectra](image)

Fig. No. 5: Example of selection of analytical wavelength for simultaneous equation using area under the curve method.
AUCM λ1- λ2: area under curve for component M at wavelength range λ1- λ2
AUCM λ3- λ4: area under curve for component M at wavelength range λ3- λ4
AUCN λ1- λ2: area under curve for component N at wavelength range λ1- λ2
AUCN λ3- λ4: area under curve for component N at wavelength range λ3- λ4

Total area under curve of a mixture at a wavelength range is equal to the sum of area under the individual components at that wavelength range. Thus area under the curve of mixture containing M and N components will be,

\[
\text{AUC}_{\lambda 1- \lambda 2} = \text{AUCM}_{\lambda 1- \lambda 2} + \text{AUCN}_{\lambda 1- \lambda 2}
\]
\[
\text{AUC}_{\lambda 3- \lambda 4} = \text{AUCM}_{\lambda 3- \lambda 4} + \text{AUCN}_{\lambda 3- \lambda 4}
\]
\[
\text{AUC}_{\lambda 1- \lambda 2} = \chi_{\lambda 1- \lambda 2} b_{CM} + \chi_{\lambda 1- \lambda 2} b_{CN}
\]
\[
\text{AUC}_{\lambda 3- \lambda 4} = \chi_{\lambda 3- \lambda 4} b_{CM} + \chi_{\lambda 3- \lambda 4} b_{CN}
\]

Where, \( b = 1 \)

\[
\chi_{\lambda 1- \lambda 2} = \text{AUC}_{\lambda 1- \lambda 2} / \text{Conc.g/lit}
\]

\[
\chi_{\lambda 3- \lambda 4} = \text{AUC}_{\lambda 3- \lambda 4} / \text{Conc.g/lit}
\]

By applying the Cramer’s rule and matrix method, concentration of M and N can be calculated as,
\[ CM = \text{AUC}_{\lambda 1} - \lambda 2 \times XN_{\lambda 3} - \lambda 4 \times XN_{\lambda 1} - \lambda 2 / XM_{\lambda 1} - \lambda 2 \times XN_{\lambda 3} - \lambda 4 \times XM_{\lambda 3} - \lambda 4 \times XM_{\lambda 1} - \lambda 2 \]

\[ CN = \text{AUC}_{\lambda 3} - \lambda 4 \times XM_{\lambda 1} - \lambda 2 - \text{AUC}_{\lambda 1} - \lambda 2 \times XM_{\lambda 3} - \lambda 4 / XM_{\lambda 1} - \lambda 2 \times XN_{\lambda 3} - \lambda 4 - XM_{\lambda 3} - \lambda 4 \times XN_{\lambda 1} - \lambda 2 \]

The methods deviated when overlapping of UV spectra of two drugs significantly and large difference in labeled strength, e.g., Valdecoxib 20 mg and paracetamol 500 mg per tablet. The accuracy of the method influence by nature of solvent, pH of solution, temperature, high electrolyte concentration and the presence of interfering substances.

**1.5 High performance liquid chromatography**

The analytical technique most frequently used in quality control analyses of pharmaceutical products is high-performance liquid chromatography (HPLC). It is a useful tool to analyze samples of complex nature, like ointments and creams, because it provides not only the separation and determination, but also eliminates most interference problems. This technique is based on the same method of separation as classical column chromatography, i.e., adsorption, partition, ion exchange and gel permeation but it differ from column chromatography, in that mobile phase is pumped through the packed column under high pressure. The technique is most widely used for all the analytical separation technique due to its sensitivity, its ready adaptability to accumulate quantitative determinations, its suitability for separating nonvolatile species or thermally fragile ones. In normal HPLC, polar solids such as silica gel;
alumina (Al$_2$O$_3$) or porous glass beads and non-polar mobile phase such as heptane, octane or chloroform are used but if the opposite case holds, it is called as reversed phase HPLC. The technique is used for the separation and determination of organic and inorganic solutes in any samples especially biological, pharmaceutical, food, environmental, industrial, etc. In a liquid chromatographic process a liquid permeates through a porous solid stationary phase and elutes the solutes into a flow-through detector.

High performance liquid chromatography replaced numerous spectroscopic methods and gas chromatography in the quantitative and qualitative analysis in testing the marketing of drugs and their control in the last ten years. In the first period of HPLC application it was thought that it would become a complementary method of gas chromatography, however, today it has nearly completely replaced gas chromatography in pharmaceutical analysis. The application of the liquid mobile phase with the possibility of transformation of mobilized polarity during chromatography and all other modifications of mobile phase depending upon the characteristics of substance which are being tested is a great advantage in the process of separation in comparison to other methods. The greater choice of stationary phase is the next factor which enables realization of good separation. The separation line is connected to specific and sensitive detector systems, spectrofluorimeter, diode detector, electrochemical detector as other hyphenated systems HPLC-MS and HPLC-NMR, are the basic elements on which is based such wide
and effective application of the HPLC method. The purpose high performance liquid chromatography (HPLC) analysis of any drugs is to confirm the identity of a drug and provide quantitative results and also to monitor the progress of the therapy of a disease. It may also be used to further enhance the understanding of the normal and disease process in the human body through biomedical and therapeutically research during investigation before of the drugs registration. The analyses of drugs and metabolites in biological fluids, particularly plasma, serum or urine is one of the most demanding but one of the most common uses of high performance of liquid chromatography. Blood, plasma or serum contains numerous endogenous compounds often present in concentrations much greater than those of analyte. Analyte concentrations are often low, and in the case of drugs, the endogenous compounds are sometimes structurally very similar to the drug to be measured. The binding of drugs to the plasma protein also may occur which decreases the amount of free compound that is measured. To undertake the analyses of drugs and metabolites in body fluids the analyst is facet with several problems. The first problem is due to the complex nature of the body fluid, the drugs must be isolated by an extraction technique, which ideally should provide a relatively clean extract, and the separation system must be capable of resolving the drugs of interest from co extractives. All mentioned when we are using high performance liquid chromatography require good selections of detectors, good stationary phase, eluents and adequate program during separation. UV/VIS detector is the most versatile detector
used in high performance liquid chromatography, it is not always ideal
since it is lack of specificity means high resolution of the analyte that may
be required. UV detection is preferred since it offers excellent linearity
and rapid quantitative analyses can be performed against a single
standard of the drug being determined. Diode array and rapid scanning
detector are useful for peak identification and monitoring peak purity but
they are somewhat less sensitive than single wavelength detectors. In
liquid chromatography some components may have a poor UV
chromophores if UV detection is being used or be completely retained on
the liquid chromatography column. Fluorescence and electrochemical
detector are not only considerably more sensitive toward appropriate
analytes but also more selective than UV detectors for many compounds.
If at all possible fluorescence detectors are sensitive, stable, selective
and easy to operate. The selectivity shows itself in the lack of frontal
components observed in plasma extract whereas electrochemical
detection is nearly always associated with a major frontal peak than tails
considerably. To date, the most sensitive method has been the reductive
electrochemical detection and giving the excellent results in the
investigation on some classes of drugs. Several high performance liquid
chromatography oxidative electrochemical methods have been developed
for the analyses of drugs and metabolites in body fluids. Mass
spectrometer as specific detector with all variation of ionization and
interface (thermo spray, moving belt etc.) or liquid chromatography-
tandem mass spectrometry. NMR as selective and specific detector in
high performance liquid chromatography is also in use. The development of a non-aqueous eluents for ion-exchange separation on silica has provided an excellent system which, when used in conjunction with an electrochemical detector, permits the analyses of an extensive range of especially basic drugs and metabolites. New packing materials such as polymeric, base deactivated silica's, pyrolysed carbon and the internal surface packing should offer the improved stability and higher efficiencies for certain classes of the compounds such as basic drugs. Microbore columns should become more accepted since they offer not only improved sensitivity but also a lower solvent consumption and consequently the reduced needs to dispose of noxious solvents. Many analyses of basic drugs are still performed by the same method of the ion-exchange chromatography on unmodified silica columns with an eluents buffered to about pH 9. Neutral or weakly acidic drugs for instance barbiturates can be chromatographed on a reversed phase system whilst acidic drugs for example paracetamol, cannabis are separated either by ion suppression or ion-pair chromatography on a reversed-phase packing material. In micelar liquid chromatography micelar mobile phases in reversed-phase instead of conventional hydro organic mobile phase is used. In micelar liquid chromatography complex electrostatic hydrophobic and steric interactions exist between the solute and both stationary and mobile phases. These enable the effective separation of samples of different nature. The main advantages of the use of a micelar solution in reversed-phase liquid chromatography are to
avoid toxicity and biodegradability of the solvent. The easy dissolution of analytical samples, which enables the determination of drugs in physiological fluids without previous separation.

The three critical components for HPLC method are: sample preparation, HPLC analysis and standardization (calculations). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives a chance to critically evaluate the method performance in each component and streamline the final method optimization. The stationary phase is usually in the form of small-diameter (5-10 mm) uniform particles, packed into a cylindrical column. The typical column is constructed from a rigid material (such as stainless steel or plastic) and is generally 5-30 cm long and the internal diameter is in the range of 1-9 mm. A high pressure pump is required to force the mobile phase through the column at typical flow rates of 0.1-2 ml/min. The sample to be separated is introduced into the mobile phase by injection device, manual or automatic, prior to the column. The detector usually contains low volume cell through which the mobile phase passes carrying the sample components.

**1.6 High performance thin layer chromatography (HPTLC)**

The principle is based on plane chromatography. The mobile phase normally is driven by capillary action. The prominent advantages of this technique includes possibilities of separating of up to 70 samples and standard simultaneously on a single plate leading to high throughout, low cost analogs and the ability to construct calibration curves from standard
chromatography under the same condition as the sample. Analyzing a sample by use of multiple separation steps and static post chromatographic detection procedures with various universal and specific visualization regents that are possible because all the sample components are stored on the layer without the chance of loss.

1.7 Gas chromatography (GC)

Gas Chromatography is one of the most extensively used separation technique in which separation is accomplished by partitioning solute between a mobile gas phase and stationary phase, either liquid or solid. It provides a quick way of determining the components in a mixture, including the presence of impurities, and in many cases, prima facie evidence of the identity of compound. The chief requirement is same degrees of stability at the temperature necessary to maintain the substance in gas state. GC is a powerful tool for the separation and quantitation of complex-organic, metal-organic, biochemical and pharmaceutical systems.

1.8 Infrared spectrophotometry (IR)

Near infrared spectroscopy (NIRS) meets many requirements, so it has grown substantially in use as a quality control technique in the pharmaceutical industry. In fact, the NIRS technique provides a number of attractive advantages, namely:

(a) It allows direct recording of spectra for solid with little or no sample pre-treatment.
(b) It allows chemical and physical information about samples (e.g. viscosity, moisture content, polymorphism) to be derived from spectra

(c) It affords multiparameter determinations from a single spectrum

(d) It can be used with various spectral recording modules compatible with virtually any type of working site and operating procedure

On the other hand, the NIR spectroscopy has two major disadvantages, namely:

(a) NIR spectra exhibit strong band overlap, which requires the use of multivariate chemometric techniques in both qualitative and quantitative analytical applications

(b) The low sensitivity of the technique restricts its scope to major components and a few minor components at most.

The use of multicomponent analytical methods in general and NIR spectroscopy in particular can substantially improve the analytical control of production processes by shortening analysis times and improving quality as a result. While the vast majority of pharmaceutical preparations contain a single active principle, some include two or more at concentrations frequently spanning wide concentration ranges. The joint determination of all the active principles in a multicomponent formulation is made especially difficult by the frequent fact that some species are present at concentrations near the determination limit of the technique. As HPLC technique is expensive, labour-intensive and time-consuming, and produces chemical waste. This has aroused interest in the development
of simple, reliable alternative methods providing accurate, precise results with an increased throughput and less human manpower.

**1.9 Validation of methods**

Validation by definition is an act of providing that any process, method, equipment, material, activity, system or analyst performs as expected under given set of conditions. When extended to an analytical procedure, depending upon the application it means that a method works reproducibility when carried out by a same or different person, in same or different laboratories, using different reagent, different equipment etc. It will ensure commitment to quality of products and services. It builds a degree of confidence not only for the developer but also to the user.

Validation of analytical method should follow a well documented procedure beginning with the definition of the scope of the method and its validation criteria and including the compounds and matrices, desired detection and quantitation limits and any other important performance criteria. The scope of method should include different equipment and locations where the method will be run. The methods were validated in terms of linearity, accuracy, precision, specificity and reproducibility of sample applications. Analytical method validation has been performed according to ICH guidelines.

**1.9.1 Analytical method validation - The regulatory perspective**

The FDA guidelines on validation of analytical procedures were published on 1st March, 1999. The contents of this guideline were prepared under the auspices of the Technical Requirements for Registration of
Pharmaceuticals for Human Use. According to section 501 of the Federal Food, Drugs and Cosmetics assays and specifications in monographs of the USP and the NF constitute standards. As a result every analytical method should be validated according to the current pharmacopoeial standards.

Among the pharmacopoeias, USP XXII 1225 (1995) carries a section which describes requirements of validation of compendial methods. The British Pharmacopoeia includes the definition of method validation in 15 latest edition, but the term is completely missing from the Indian Pharmacopoeia (1996). The validation aspects are well covered by ISO (5-11) documents.

1.9.2 Need for Analytical Method Validation

Validation of analytical methods is the cornerstone of process validation. Validation is required for:

1. In the absence of a proven measurement system there is no way of judging whether or not the process has done what it purports to do.
2. Assay validation is an integral part of quality control system, also the current good manufacturing practice regulations requires that the test methods used to assess the compliance of pharmaceutical products with established specifications must meet proper standards of accuracy and reliability.
3. All regulatory bodies require the validation of analytical methodology as a part of their procedure for reviewing New Drug Applications (NDAs).
This is an essential aspect of drug registration filing e.g. method used for impurity determination, for testing the stability of a new drug.

4. It reduces the cost associated with process sampling, retesting and thus eliminating product rejection.

5. To avoid the differences that exists when a method is developed in the analytical development laboratory and then transferred to quality control laboratory.

6. Limited degree of revalidation is required by another laboratory, which is supposed to use the validated method.

7. Assay validation plays an important role in universal control cycle, which involves setting standards, appraising conformance to standards and taking appropriate action when standards are not met.

8. Reproducible and accurate analytical results are pre-requisite throughout pharmaceutical development and manufacturing.

1.9.3 Method validation - The benefits

1. Validation will ensure commitment to quality of products and services.

2. The biggest advantage is that it builds a degree of confidence not only for the developer but also to the user. Although the validation exercise may appear costly and time consuming, it eliminates frustrating repetitions and leads to better time management in the end.

3. When the method is used in the developer's laboratory, a small adjustment can be made to make the method work, but the flexibility to change it is lost once the method is used for official product testing or transferred to other laboratories or submitted to regulatory agencies.
Changes may require formal approval before they can be implemented for official testing. Adequate validation is the best way to minimize method problems.

4. The method validation absorbs the shock of changes in conditions such as reagent supplier or grade, analytical set up, analyst, environmental variations and pays for more than invested on the process. Ruggedness studies has been carried out for different parameters i.e. days and analysts. The results shall be compared with the method. Ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc and provides an indication of its reliability during normal usage. Robustness can be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method. The term ruggedness is frequently used as a synonym. Several definitions for robustness or ruggedness exist which are, however, all closely related. The one nowadays most widely applied in the pharmaceutical world is the one given by the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH). The latter definition will not be applied since detailed
guidelines exist for the estimation of the reproducibility and the intermediate precision\textsuperscript{71-72}. The ICH guidelines\textsuperscript{61} also recommend that ‘one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g. resolution tests) is established to ensure that the validity of the analytical procedure is maintained whenever used’. The assessment of the robustness of a method is not required yet by the ICH guidelines, but it can be expected that in the near future it will become obligatory. Robustness testing is nowadays best known and most widely applied in the pharmaceutical world because of the strict regulations in that domain set by regulatory authorities which require extensively validated methods. Therefore, most definitions and existing methodologies, e.g. those from the ICH, can be found in that field, as one can observe from the above. However, this has no implications for robustness testing of analytical methods in other domains and this guideline is therefore, not restricted to pharmaceutical methods.

1.9.4 Situating robustness in method development and validation:
Robustness tests were originally introduced to avoid problems in interlaboratory studies and to identify the potentially responsible factors\textsuperscript{62}. This means that a robustness test was performed at a late stage in the method validation since interlaboratory studies are performed in the final stage. Thus the robustness test was considered a part of method validation related to the precision (reproducibility) determination of the method\textsuperscript{63, 73-76}. However, performing a robustness test late in the validation procedure involves the risk that when a method is found not to
be robust, it should be redeveloped and optimized. At this stage much effort and money have already been spent in the optimization and validation, and therefore, one wants to avoid this. Therefore, the performance of a robustness test has been shifting to earlier points of time in the life of the method. The Dutch Pharmacists Guidelines, the ICH Guidelines\textsuperscript{76} as well as some authors working in bio-analysis\textsuperscript{77} consider robustness a method validation topic performed during the development and optimization phase of a method, while others \textsuperscript{78} consider it as belonging to the development of the analytical procedure. Therefore, the robustness test can be viewed as a part of method validation that is performed at the end of method development or at the beginning of the validation procedure. The exact position has relatively little influence on how it is performed.

\textbf{1.9.5 Objectives of a robustness evaluation}

The robustness test examines the potential sources of variability in one or a number of responses of the method. In the first instance, the quantitative aspects (content determinations, recoveries) of the method are evaluated. However, besides these responses also those for which system suitability test (SST) limits can be defined (e.g. resolution, tailing factors, capacity factors, column efficiency in a chromatographic method) can be evaluated. To examine potential sources of variability, a number of factors are selected from the operating procedure and examined in an interval that slightly exceeds the variations which can be expected when a method is transferred from one instrument to another or from one
laboratory to another. These factors are then examined in an experimental design and the effect of the factors on the response(s) of the method is evaluated. In this way the factors that could impair the method performance are discovered. The analyst then knows that such factors must be more strictly controlled during the execution of the method. Another aim of a ruggedness/robustness test may be to predict reproducibility or intermediate precision estimates. In this guideline this kind of ruggedness testing is not considered.

The information gained from the robustness test can be used to define SST limits. This allows determining SST limits based on experimental evidence and not arbitrarily on the experience of the analyst.

1.9.6 Validation of analytical procedures

The ICH has adopted the following terms as defining how the quality of an assay is controlled.

A. The analytical procedure: It provides an exact description of how the analysis is carried out. It should describe in detail the steps necessary to perform each analytical test. The full method should describe:

(i) The quality and source of the reference standard for the compound being analysed.

(ii) The procedures used for preparing solutions of the reference standard.

(iii) The quality of any reagents or solvents used in the assay and their method of preparation.
(iv) The procedures and settings used for the operation of any equipment required in the assay.

(v) The methodology used for calibration of the assay and methodology used for the processing of the sample prior to analysis.

In fact it is difficult to be comprehensive in this short account, since the description of a fully validated method is a lengthy document.

B. Precision: The ICH guidelines define precision as an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements." There is no absolute guideline for how good precision should be for the active ingredient in a formulation but, in general, a precision of $\leq 1.0\%$ is desirable. The precision achievable depends on the nature of the sample being analysed. The RSDs achievable in the analysis of trace impurities in a bulk drug or drugs in biological fluids may be considerably greater than $\pm 1.0\%$ because of the increased likelihood of losses when very low concentrations of analyte are being extracted and analyzed. The precision of the assay of a particular sample, in the first instance, is generally obtained by repeating the assay procedure a minimum of five times starting from five separate aliquots of sample (e.g. five weights of tablet powder or five volumes of elixir) giving a total of 25 measurements. Repetition of the sample extraction gives a measure of
any variation in recovery during extraction from the formulation matrix. One difficulty in defining the precision of an assay is in indicating which steps in the assay should be examined. Initially an assay will be characterized in detail but thereafter, in re-determining precision (e.g. in order to establish repeatability and intermediate precision), certain elements in the assay may be taken for granted. For example, the same standard calibration solution may be used for several days provided its stability to storage has been established or a limited number samples will be extracted for assay provided it has been established that the recovery of the sample upon extraction does not vary greatly. According to the ICH guidelines, precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

C. Repeatability: It expresses the precision obtained under the same operating conditions over a short interval of time. Repeatability can also be termed intra-assay precision. It is likely that the assay would be repeated by the same person using a single instrument. Intermediate precision: It expresses within-laboratory variation of precision when the analysis is carried out by different analysts, on different days and with different equipment. Obviously a laboratory will want to cut down the possibility for such variations being large and thus it will standardize on particular items of equipment, particular methods of data handling and make sure that all their analysts are trained to the same standard.
D. Reproducibility: It expresses the precision between laboratories. Such a trial would be carried out when a method was being transferred from one part of a company to another. The data obtained during such method transfer does not usually form part of the marketing dossier submitted in order to obtain a product license.

E. Accuracy: Methods may be precise without being accurate. The determination of accuracy in the assay of an unformulated drug substance is relatively straightforward. The simplest method is to compare the substance being analyzed with a reference standard analyzed by the same procedure. The reference standard is a highly characterized form of the drug which has been subjected to extensive analysis including a test for elemental composition. The methods for determining the accuracy of an assay of a formulated drug are less straightforward. The analytical procedure may be applied to a drug formulation prepared on a small scale so that the amount of drug in the formulation is more precisely controlled than in a bulk process; a placebo formulation spiked with a known amount of drug or the formulated drug spiked with a known amount of drug. The accuracy of the method may also be assessed by comparison of the method with a previously established reference method such as a pharmacopoeial method. Accuracy should be reported as percent recovery in relation to the known amount of analyte added to the sample or as the difference between the known amount and the amount determined by analysis. In general, at least five determinations, at 80, 100 and 120% of the label claim for drug
in the formulated product, should be carried out in order to determine accuracy.

Accuracy of the method is certain on the basis of recovery studies performed by the standard addition method. The formula used for calculating recovery of pure drug is as follows:

\[
\text{Percentage recovery} = \frac{T - A}{S} \times 100
\]

Where,

- \( T \) = Total amount of drug estimated
- \( A \) = Amount contributed by formulation
- \( S \) = Amount of pure drug added.

Precision of analytical method is expressed as SD and RSD of series of measurement by replicate estimation of drug.

The stability indicating ability of the method has been investigated by deliberately degrading the sample preparation. The stress conditions applied are acidic (0.1 M HCl), alkalis (0.1M NaOH) and mild oxidizing condition (3% \( \text{H}_2\text{O}_2 \)) for 24 hr at 50\(^o\) C. Also heat (60\(^o\)C) and U.V. exposure for 24 hr will be carried out on the sample.

The linearity of the method was investigated by serially diluting the stock solutions of drugs and measured values.

**1.9.7 Strategies for method validation**

Validation of analytical method should follow a well documented procedure banning with the definition of the scope of the method and its validation criteria and including the compounds and matrices, desired detection and quantitation into and any other important performance criteria. The scope of the method should include different equipments and
locations where the method will be run. An instrument is used to validate a method and its performance (using standards). Operators should be adequately trained and/or in the use of the instrument and any material used to determine validation parameters should be checked.

1. Develop a validation protocol or operating procedure for execution of the method.

2. Define purpose of the method and performance criteria.


4. Qualify/validate materials.

5. Perform pre-validation experiments.

6. Adjust performance criteria if necessary.

7. Perform full internal (and external) validation experiments.

8. Develop SOP’s for executing the method.

9. Define criteria for revalidation.

10. Define type and frequency of analytical quality control checks.

11. Statistical evaluation of the analytical results.


1.9.8 Documentation for Validation

Documentation for validation consists of a protocol, test data and report. Validation must have a written approved protocol prior to their initiation. It allows an analyst to select characteristics required for testing. If an alternate acceptance criterion is appropriate, the analyst should add it to the form. Writing a validation report may be simplified by documenting results of testing and evaluations in a validation report form. This form
should include supporting chromatograms, graphs or other pertinent data and must be received and approved. A method is considered validated when it meets the acceptance criteria of a validation protocol or when there is adequate justification for it.

**ANALYTICAL METHOD PROTOCOL**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Characteristic Required</th>
<th>Proposed use acceptance criteria</th>
<th>Alternate acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>Yes No</td>
<td>R² ≥ 0.99, similar response ratios</td>
<td></td>
</tr>
<tr>
<td>Precision-System</td>
<td>Yes no</td>
<td>RSD 2%</td>
<td></td>
</tr>
<tr>
<td>Precision-Method</td>
<td>Yes no</td>
<td>RSD&lt; 2%</td>
<td></td>
</tr>
<tr>
<td>Precision-Repeat/Reproducibility</td>
<td>Yes no</td>
<td>% R &amp; R&lt;20%</td>
<td></td>
</tr>
<tr>
<td>Accuracy</td>
<td>Yes no</td>
<td>FDA 98-02%, EPA 50-50%</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>Yes no</td>
<td>No interference</td>
<td></td>
</tr>
<tr>
<td>Detection limit</td>
<td>Yes no</td>
<td>&gt; 2 times baseline</td>
<td></td>
</tr>
</tbody>
</table>

**Analytical method protocol**

Instructions: Describe the characteristics that must be determined for method validation by circling "yes" or "no" in the characteristic required column. If the acceptance criteria differ from that in proposed use acceptance criteria column, enter the new acceptance criteria in the acceptance criteria column.
Quantification Limit
Yes no Signal-to-noise Ratio=10:1

Range Yes no Concentrations Where data can be reliably determined

Reason for Revalidation (if applicable):

Reviewed By Date Approved By Date

Analytical method datasheet and final report

Method Name: Method Number:
Method Revision: Validation Number:
Equipment: Date:
Analysis:

Location of Data:

1. Linearity File Name:

Std. Concentration:
Replicate 1
Replicate 2
Replicate 3
Replicate 4
Replicate 5
Replicate 6
Mean
Standard Dev.
Rel. Std. Dev.

Regression equation = Coefficient of determination \([r^2]\)
1.10 EXPERIMENTAL PARAMETERS FOR VALIDATION OF METHOD

1.10.1 Linearity

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration/content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. An alternate way of evaluating the data is to plot response ratio versus concentration. If an equivalent response is obtained at each concentration the data points will form a straight line with a zero slope.

A. Test procedure: A range of standards containing at least five different concentrations of analyte, which are approximately evenly spaced and span 50% (lowest conc.) to 150% (highest conc.) of the expected working range. At least six replicates per concentration must be used. Standards should be prepared in the same matrix used in the samples if possible.

B. Documentation: Record the results on a datasheet. Calculate the mean, standard deviation and relative standard deviation for each concentration. Plot concentration (X-axis) versus mean response for each concentration (Y-axis) for graph no. 1. Calculate the regression equation and coefficient of determination ($r^2$). Plot concentration (X-axis) versus response ratio (response /concentration) (Y-axis) for graph No. 2. If the
intercept in graph no.1 is significantly different from zero, then first subtract the intercept value from each response and then divide the response by its concentration.

C. Acceptance criteria: The curve in graph no. 1 should be linear with an r value of at least 0.99 (as close as possible to 1.000). All values for the response ratio curve in graph no. 2 should fall within a narrow horizontal zone.

Linearity is expressed in terms of the variance around the slope of the line. The slope of the regression line provides the mathematical relationship between the test results and the analyte concentration. The y-intercept is an estimation of any potential bias in the assay method.

1.10.2 Range: The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The following minimum specified ranges should be considered:

- For the assay of an active substance or a finished product: normally from 80-120% of the test concentration.
- For the determination of an impurity; from reporting level of an impurity to 120% of the specification.
For content uniformity, covering minimum range is from 70-130% of the test concentration, unless a wider more range based on the nature of the dosage form (e.g. metered dose inhalers is justified).

For dissolution testing: ± 20% over the specified range.

If assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

1.10.3 Precision: The precision of an analytical method is the amount/degree of scatter in the results obtained from multiple analysis of a homogenous sample. It determines closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels:

A. Repeatability

The precision of a method when reported by the same analyst, same test method and under same set of laboratory conditions (reagent, equipment, etc) within a short interval of time. It is also known as intra-assay precision.

B. Intermediate Precision

Intermediate Precision is the precision obtained when the assay is performed by multiple analysts using multiple instruments, on multiple days in one laboratory [i.e. within laboratory variations due to random events such as different days, analysts, equipments etc] It is not necessary to study the effects individually. An experimental design should
be employed so that the effects (if any) of the individual variables can be monitored.

**C. Reproducibility**

The measure of test methods variability when carried out by different analysts in different laboratories using different equipments, reagents, and laboratory settings and on different days. It is assessed by means of an inter-laboratory crossover studies. It focuses more on measuring bias in results than on determining differences in precision alone. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias.

(i) Precision (for the method): Test procedure: One representative composite sample is prepared. The sample should contain 20 times the amount of analyte needed for one assay. Use the midpoint concentration of the expected working range for the analyte. Prepare six replicates from this sample and analyze.

(ii) Documentation: Record the results on the datasheet, as well as the calculations for the mean, standard deviation and relative standard deviation. Acceptance criteria: RSD < 2%.

(iii) Precision (for the system): Test procedure: Six replicates of a standard solution, which is in the middle of the expected operating range, should be analyzed.

Documentation: Record the peak height and area or response ratios on the datasheet. Calculate the mean, standard deviation and RSD.
Acceptance criteria: RSD < 2%. Precision (for repeatability and reproducibility) Repeatability (intra-assay precision) and reproducibility (variation under different conditions) can be evaluated by the R and R studies using two operators for analyzing the variation of measurements.

(iv) Test Procedure: Prepare one set of nine different concentrations of samples that are evenly spaced over the expected range. Each concentration should be approximately 10 times the amount of analyte needed for one assay. Each operator should analyze all nine samples twice in any order using the same equipment. They may analyze sample on different days, if the samples are stable.

(v) Documentation: Record results and perform calculations on the datasheet. Determine repeatability reproducibility and R & R. Acceptance criteria: An R and R reading of 10% or less is excellent, 11% to 20% adequate, 21% to 30% marginally acceptable, greater than 30% is unacceptable.

1.10.4. Accuracy

Accuracy is the measure of exactness of an analytical method, or the doseness of agreement between the value that is accepted as either a conventional true value or an accepted reference value and the value found. Sometimes termed as 'trueness'. It is measured as the percent of analyte recovered by assay by spiking samples in a blind study. In a true sense, accuracy is linked to "recovery". Four approaches to determine accuracy. First approach is by analyzing a sample of known concentration and by comparison of results with an analyte of known purity (e.g.
reference standard). Second approach is comparison of the test results from the new method with results from an existing alternate method that is known to be accurate. The third approach is the most widely used recovery study and it is performed by spiking analyte in blank matrices or the mixture of excipients involved. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte.

(i) Test procedure: Prepare a range of samples containing at least five different concentrations of analyte, which are approximately evenly spaced and span 50% (lowest cone.) to 150% (highest cone.) of the expected working range. Use at least six replicates per concentration.

(ii) Documentation: For each sample, report the theoretical value, assay value and % recovery. Calculate the mean, standard deviation and RSD and % recovery for all samples. The data should be reported as the % recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals. Record the results on the datasheet. When it is impossible to prepare known placebos, use a low concentration of known standard.

(iii) Acceptance criteria: For non-regulated products the percent recovery should be within 90%-110% of the theoretical value. For the U.S. pharmaceutical industry 98% to 102%. The % recovery should be within 50%-150% of the theoretical value for methods used in EPA products.
Lower percent recoveries may be acceptable based on the needs of the method.

1.10.5 Specificity and Selectivity

Specificity is the ability of the method to measure unequivocally the analyte of interest in the presence of all other components, expected to be present in the sample matrix. It is a measure of the degree of interference from other active ingredients, excipients, synthetic precursors, impurities, metabolites and degradation products. Thus, if an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively, the method is called selective. If the method determines or measures it quantitatively the compound of interest in the sample matrix without separation, it is said to be specific.

Measuring a method’s specificity is extremely important during the validation of non-chromatographic methods because they do not contain a separation step that ensures non-interference from excipients. They rely on intrinsic differences in chemical or physical properties to ensure their ability to accurately determine the concentration of analyte in complex sample mixtures.

(i) Test procedure: Spike experiments using known related impurities, degradation products or excipients. The spiked sample can be analyzed along side the unspiked sample to demonstrate lack of interference. Determine any possible bias in an assay method by the measurement of any difference in test results between the drug substances spiked in to a
placebo matrix and a sample not containing the excipients. Assay bias is evaluated by calculating the percent agreement.

Percentage agreement = (Tp/Ta) 100

Where, Tp = test result in the presence of potentially interfering species
Ta = test result in the absence of potentially interfering species.

Discrimination of the analyte when impurities or degradation products are unidentified or unavailable: Analyze samples containing the impurities or degradation products and compare the results with those obtained from a pharmacopoeial method or other validated analytical procedure or an alternative purity assay like DSC or HPLC. The alternative assay is considered to be the reference and the degree of agreement between the two sets of test results is a measure of the specificity. The bias is obtained by using the formula:

Percentage agreement = (Tp/To). 100

Where, Tp = test result in the presence of potentially interfering species.
To = test results obtained using the alternative purity assay. This includes samples stored under relevant stress conditions such as heat (50°C), light (600 FC), acid (0.1 N HCl), base (0.1 N NaOH), oxidant I (3% H2O2) and humidity (85%).

(ii) Documentation: Print chromatograms or data to show a method's resolution.

(iii) Acceptance criteria: A 100% agreement would be taken to indicate the absence of any bias in the analytical procedure caused by the presence of the potentially interfering species. Agreement values >100%
indicate the existence of a positive assay bias, while agreement values <100% indicate negative assay bias.

1.10.6 Limit of Detection (LOD)

The lowest concentration of an analyte in a sample, which can be detected but not necessarily quantified with a given limit of confidence using the specified experimental procedure. It is the lowest analyte concentration that produces a response detectable above the noise level of the system typically three times the noise level. It is a limit test where concentrations below this may not be detected while concentrations above this limit are certainly detected in analysis.

1.10.7 Limit of Quantitation (LOQ)

The smallest amount of an analyte in a sample matrix that can be quantified with acceptable precision and accuracy. Both quantities are expressed in units of concentration ng/ml, pg/ml, and mg/ml. There are several approaches for determining the detection limit and quantitation limit.

(i) Based on visual evaluation- Visual evaluation may be used for non-instrumental methods but may also be used for instrumental methods. Visual non-instrumental methods include LOD and LOQ determined by TLC or titration.

(ii) Lowest concentration for which RSD is < 5.0% - The method involves choosing the LOQ as the lowest concentration for which the RSD is < 5.0%. The LOD value is taken as 0.3 x LOQ.
(iii) Based on signal-to-noise ratio- This approach can be applied to analytical procedures, which exhibit baseline noise. Determination of signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishes a minimum concentration at which the analyte can be reliably detected. LOD value is equal to three times this value (i.e. 3:1 signal-to-noise ratio). The LOQ concentration is 10 times this value (i.e. 10:1 signal-to-noise ratio).

(iv) 95% confidence interval of a best fit line, Oppenheimer approach- It involves the use of the 95% confidence interval (CI) lines surrounding a best fit regression line. A horizontal line is drawn from the y-intercept of the upper 95% confidence interval line to the lower 95% confidence interval line. A vertical line is then drawn from the lower 95% confidence interval line to the X-axis, yielding an x-intercept. The x-intercept represents the LOD concentration. The LOQ value is 3.3 x LOD.

(v) Based on the standard deviation of the response and the slope- Analyze a number of low concentration samples. Each sample is assayed repetitively, and the standard deviation (SD) of the analyte response is calculated. The SD values are then averaged to deduce the mean standard deviation (MSD).

\[
\text{Detection Limit (DL)} = \frac{3.3 \times \text{SD}}{S}
\]

\[
\text{Quantitation Limit (QL)} = \frac{10 \times \text{SD}}{S}
\]
Where, SD = standard deviation of the response, S = slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte.

The estimate of SD may be carried out in a variety of ways.

Based on the standard deviation of the blank- Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the SD of these responses.

Based on the calibration curve- A specific calibration curve should be studied using samples containing an analyte in the lower range. The residual standard deviation of a regression line or the SD of y-intercepts of regression lines may be used as the SD.

(vi) Plot of standard deviation versus concentration- The y-intercept (So) of this plot represents the virtual SD at zero concentration. The LOD value is three times the value of S0. The LOQ value is 10 times the value of S0. This technique is a new convention and is currently being promoted by the ICH.

(vii) Documentation: Print the chromatogram or record the lowest quantifiable concentration on the datasheet. Provide data that demonstrates the accuracy and precision required in the acceptance criteria. Acceptance criteria: The limit of quantitation for chromatographic methods has been described as the concentration that gives a signal-to-noise ratio of 10:1 and is less than or equal to 10
percent precision or that gives a ratio of 20:1 and is less than or equal to five percent precision.

1.11 Computer Applications

Analytical method development involves generation of huge data, station and treatment of data. Handling of such huge data is a difficult task here is a high chance that error may be included at the stage of data handling; therefore, this necessitated the use of computer in data storage and data in analytical method development. At the same time, apart from this, retrieval and transfer of data becomes easier by use of computer. For storage of data, suitable database may be designed. The data from the data documents may be directly transferred for mathematical is to obtain concentration and various related statistical parameters. Preparation of the data is an important part in analytical method development. Graphical representations of data reveal the complicated ships in a simple form e.g. recovery studies, limit of detection and limit of quantitation. Hypothesis testing is an important statistical treatment frequently in analytical method development. Use of statistical softwares simplifies the task e.g. UNISTAT, SPSS etc. Recently advanced mathematical treatments such as multivariate analysis, principle component analysis and canonical correlations partial least square technique are used in the development of analytical with the help of advanced statistical software. Effectiveness of the computer system has been criticized in many cases. Programming, hardware flaws, calibration errors or electronically data that were directly
caused by the problems in the computer system is a matter of concern. Therefore as per GLP, validation and auditing of systems used in data collections, statistical analysis and related is critical.

1.12 Conclusion
The multi-drug therapy is an ancient phenomenon to combat interrelated symptoms of diseased status of human beings. Since it ensure timely and complete medication for disorder and it has patient compliance, as it reduces the number of formulations to be taken at a time. Therefore, the pharmaceutical formulations with combinations of drugs have shown an increasing trend to counteract other symptoms specific to one drug and formulation, and hence analytical chemist will have to accept the challenge of developing reliable methods for analysis of drugs in such formulation.

Simultaneous analysis procedures are now being used more frequently for estimation of drugs in multi-component pharmaceutical formulations due to their inherent advantages viz. avoid time consuming extraction and separation, economical in the sense that use of expensive reagents is minimized are equally accurate and precise. For the estimation of multi-component formulation, the instrumental techniques, which are commonly employed, are spectrophotometry, GLC, high performance thin layer chromatography (HPTLC), HPLC etc. The validation of methods has to validate by using same parameters as per ICH guidelines.


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