CHAPTER-I

GENERAL INTRODUCTION ON THE UTILITY OF VARIOUS ANALYTICAL METHODOLOGIES IN PHARMACEUTICAL ANALYSIS
Quality control [1-3] pertains to sampling, specifications and testing, organization, documentation and release procedures. It ensures that the necessary and relevant tests are actually carried out before release, sale or supply of any product. In pharmaceutical industry, quality control is not confined to laboratory operations but must be involved in all aspects concerning the quality of the product and the entire manufacturing process.

Without analytical tools the new benchmarks for sample generation and their advantages could not be fully realized in a pharmaceutical industry. Thus, the relationship between sample generation and analysis is a major issue in this industry. Clearly, traditional approaches for analysis are not capable of meeting specialized needs created by dramatic improvements in sample generation. New technologies figure prominently in the success of drug development and directly impact the pharmaceutical analysis activities. As requirements for analysis rapidly adapted to breakthroughs in sample generation, a new scientific and business culture aimed at decreasing costs and accelerating development became entrenched in the pharmaceutical industry. These factors combined to produce more frequent, and perhaps, new demands on analysis. In particular, these demands underscored the importance of analytical instrumentation and the creation of novel strategies for analysis that have paved the way for rapid progress in providing simple analytical procedures for the analysis of compounds in all samples including formulations. As a result analysts have developed large number of instrumental techniques which are extremely sensitive and can yield results rapidly to a high degree of accuracy.
The present trend in pharmaceutical analysis is in the direction of further improvement of instrumental techniques for the analysis of pharmaceutical preparations. The development of simple methods for analyzing the purity is one of the thrust areas of pharmaceutical analysis. The instrumental techniques for the estimation of drugs are classified into physical, chemical, physico-chemical and biological ones.

**Physical methods** involve the study of the physical properties such as solubility, transparency or degree of turbidity, color, density, etc.

**Chemical methods** include the titrimetric, complexometric, gravimetric and voltametric procedures which are based on complex formation and redox reactions etc. used in pharmaceutical analysis.

**Physico-chemical methods** involve the study of the physical phenomena that occurs as a result of chemical reactions. These include spectrophotometric and chromatographic methods.

Among the instrumental techniques available in pharmaceutical analysis, UV-Visible spectrophotometry constitutes the powerful tool for process monitoring and quality control, since this technique is fast, non-destructive and non-invasive with low maintenance cost.

### 1.1. UV-VISIBLE ABSORPTION SPECTROPHOTOMETRY

Absorption spectrophotometry in the ultra-violet and visible regions is considered to be one of the valued techniques for the quantitative analysis in pharmaceutical industry. The basis of spectrophotometric methods is the simple relationship between the color of a substance and its electronic structure. A molecule or an ion exhibits absorption in the visible or ultra-violet
region when the radiation causes an electronic transition in molecules containing one or more chromophoric groups. The color of a molecule may be identified by substituents called auxochromic groups, which displace the absorption maxima towards longer wavelength (bathochromic shift). The color determining factors in many molecules takes place through the introduction of conjugated double bonds by means of electrons donor and electrons acceptor groups. The importance of the colored solution lies in the fact that the radiation absorbed is characteristic of the material responsible for absorption. In addition, a substance that is not colored may often be determined by adding a chromogenic reagent that will convert to an intensively colored species showing strong absorbance in the visible region.

1.1.1. THEORY OF UV-VISIBLE SPECTROSCOPY

Spectroscopic processes rely on the fact that electromagnetic radiation interacts with atoms and molecules in discrete ways to produce characteristic absorption or emission profiles[4]. Electromagnetic radiation is a type of energy that is transmitted through space, taking many forms. Visible light is the most easily recognized, but it also includes X-rays, Ultraviolet rays, Radio waves and Microwave radiation. The visible region constitutes a small part of the electromagnetic spectrum, when compared to other spectral regions. The various types of radiation can be defined in terms of their wave frequency. The interaction of a photon with the electron cloud of a particular molecule promotes an electron from the ground to an excited state (Fig. 1.01, P.4). The difference in the molecular energy levels, $E_2 - E_1$, will correspond exactly to the photon energy.
The interaction between the photon and the electron cloud of matter is specific and discrete, **being quantized and the energies associated with them are related to the type of transition involved.** The wavelength of any absorption is dependent on the difference between the energy levels. Hence, some transitions require less energy and consequently appear at longer wavelengths. If a molecule is only capable of a single electronic transition, it will yield a sharp single spectral line, but molecular spectra are not solely derived from single electronic transitions between the ground and excited states. Quantized transitions do occur between vibrational states within each electronic state and between rotation sublevels. Electronic transitions occur at higher energies (ultraviolet) than vibrational (infrared) or rotational ones (microwave). Hence, the molecular spectra observed in the UV-Visible region are a combination of different transitions[4]. Electronic transitions related to the UV-Visible spectroscopy are only possible when the molecule involved in the absorption process has a chromophore.

Chromophores are the basic building blocks of spectra and are associated with molecular structure and the types of transition between
molecular orbitals. Chromophores are characterized by the existence of
electrons liable to absorb a given radiation, the energy of which corresponds
exactly to that required for electron excitation [5]. There are three types of
ground state molecular orbitals: Sigma (\( \sigma \)) bonding, Pi (\( \pi \)) bonding, Non-
bonding (\( n \)), and two types of excited states: Sigma star (\( \sigma^* \)) antibonding,
Pi star (\( \pi^* \)) antibonding, from which transitions are observed in the UV
region (Fig. 1.02, P.6). These four transitions yield different values for \( \Delta E \) and
wavelength [4]. Possible electronic transitions of \( \pi, \sigma, \) and \( n \) electrons are:

**\( \sigma \rightarrow \sigma^* \) Transitions:** An electron in a bonding \( \sigma \) orbital is excited to the
corresponding antibonding orbital. The energy required is large. Maxima
absorption due to \( \sigma \rightarrow \sigma^* \) transitions are not seen in typical UV-Visible
spectra (200–700 nm).

**\( n \rightarrow \sigma^* \) Transitions:** Saturated compounds containing atoms with lone
pairs (non-bonding electrons) are capable of \( n \rightarrow \sigma^* \) transitions. These
transitions usually need less energy than \( \sigma \rightarrow \sigma^* \) transitions. They can be
initiated by light whose wavelength is in the range 150–250 nm. The number
of organic functional groups with \( n \rightarrow \sigma^* \) peaks in the UV region is small.

**\( n \rightarrow \pi^* \) and \( \pi \rightarrow \pi^* \) Transitions:** Most absorption spectroscopy of
organic compounds is based on transitions of \( n \) or \( \pi \) electrons to the \( \pi^* \) excited
state. This is because the absorption peaks for these transitions fall in an
experimentally convenient region of the spectrum (200–700 nm).

These transitions need an unsaturated group in the molecule to provide
the \( \pi \) electrons. Since only \( n \rightarrow \pi^* \) and \( \pi \rightarrow \pi^* \) transitions are possible in the
UV-Visible spectral range, only non-saturated organic compounds or ions,
which contain a chromophoric group, can absorb radiation directly in this spectral region and thus be detected[5].

**Fig.1.02. Transitions between molecular orbitals**

UV-Visible spectroscopic techniques used for quantifying purposes are based on Beer-Lambert law. According to the Beer-Lambert law for a single wavelength and a single component, the following relation is valid:

\[ A = \varepsilon bc \]

Where, \( A \)-Absorbance; \( \varepsilon \)-Molar absorptivity (mol\(^{-1}\).cm\(^{-1}\)); \( b \)-Path length of the cell in which the sample is taken (cm); \( c \)-Concentration of the absorber (mol.dm\(^{-3}\)). Therefore, for a given wavelength and a single component, absorbance is a linear function of the concentration of the component.

The chemical nature and concentration of absorbent dissolved components, together with the physical characteristics and concentration of heterogeneous material are the two phenomena responsible for the shape of the UV-Visible spectrum. Consequently, direct spectroscopy involves two main phenomena: i)the chemical absorption mechanism explained by the Beer-
Lambert law and ii) the scattering effect and its associated diffusion related to the suspended solids and colloids[5].

### 1.1.2. INSTRUMENTATION OF UV-VISIBLE SPECTROPHOTOMETER

The general arrangement of an UV-Visible spectrophotometer is presented in Fig.1.03.P.8 and its usual components are given below.

#### 1.1.2.1. Radiation Source

Two radiation sources are generally used in UV-Visible spectrometers which together cover the range from 200-800 nm. For measurements below 320 nm a deuterium or a hydrogen lamp at low pressure is used for emitting a continuous spectrum. For measurements above 320 nm compact tungsten halogen sources in quartz envelope are often used. This type of source is used in the wavelength range of 350–2500 nm. Tungsten/halogen lamps are very efficient, and their output extends well into the ultraviolet region.

#### 1.1.2.2. Filters/Monochromators

If a tungsten halogen lamp is used to emit radiations below 400 nm, special filters are often included in the optical path, to reduce the stray radiation. Wavelength selectors are needed to guarantee monochromatic radiation, since a narrow bandwidth is required in order to enhance the sensitivity of the absorbance measurements.
1.1.2.3. Sample Container (cuvette)

The sample containers [cuvettes] usually used are made of a material which is transparent to the radiation concerned – silica or quartz for the UV-Visible region and glass or plastic to the visible region [6].

1.1.2.4. Detectors

A variety of detectors are available for UV-Visible measurements. High performance UV-Visible spectrophotometers utilize photomultiplier tube technology from the ultraviolet into the visible region.

![Diagram of a spectrophotometer](image)

**Fig.1.03. Basic construction of a spectrophotometer**

1.1.3. GENERAL METHODOLOGY FOR THE DEVELOPMENT OF NEW UV-VISIBLE SPECTROPHOTOMETRY

The fundamental principle of visible spectrophotometry involves a light with definite interval of wavelength, first allowed to pass through a cell with a colored solution or solvent and later on to the photoelectric cell that converts the radiant energy into electric energy measured by a galvanometer. In ultraviolet (200-380nm) and visible (380-800nm) spectroscopies, absorption of radiation is the result of excitation of bonding (σ,π) and non-bonding (n) electrons.
The methodology for the development of new visible spectrophotometric methods[7] is outlined as given below.

1.1.3.1. SELECTION OF ANALYTICAL WAVELENGTH

In developing a quantitative method for determining an unknown concentration of a given substance by absorption spectrophotometry, the first step will be the selection of analytical wavelength at which absorption measurements are made. The analytical wavelength can be chosen either from literature or experimentally by means of scanning with a spectrophotometer. In order to enhance the sensitivity of the method and signal to noise ratio, the wavelength of maximum absorbance is chosen as analytical wavelength. Absorption spectrum is a graphical representation of the amount of light absorbed by a substance at definite wavelength. To plot a curve, the values of wavelength in the visible region are laid off along the axis of ordinates. A characteristic of an absorption spectrum is a position of the peaks of light absorption by the substance and also the intensity of absorption, which is determined by the absorptivity at definite wavelength. After selection of the analytical wavelength, the chromogenic reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknowns should be on a definite schedule.

1.1.3.2 CHOICE OF SOLVENT

The solvent which is to be used in colorimetric or spectrophotometric determinations must meet certain requirements. It must be a good solvent for the substance under determination. Before using a particular solvent, it must
be ensured that it does not interact with the solute. The solvent must not show significant absorption at the wavelength to be employed in the determination. For inorganic compounds, water normally meets these requirements, but for majority of organic compounds, it is necessary to use an organic solvent. All solvents show absorption at some point in the ultraviolet region and care must be taken to choose a solvent for a particular determination which does not absorb in the requisite wavelength region. Any impurities present in the solvents may affect the absorption at certain wavelength and it is therefore, essential to employ materials of the highest purity.

1.1.3.3. OPTIMIZATION OF ANALYTICAL VARIABLES

The rate of color formation and stability of the spectrophotometric method is affected by the concentration of the reagent in the solution, nature of solvent, temperature, pH of the medium, order of addition of reactants and intervals between additions. For simple systems having no interaction between variables, the one variable at a time (OVAT) strategy appears to be simple and efficient to establish the optimum conditions[7]. The OVAT approach requires all variables but one, to be held constant while a univariate search is carried out on the variable of interest.

1.1.3.4. CALIBRATION

Calibration is one of the most important steps in spectrophotometric analysis. Good precision and accuracy can only be obtained when a good calibration procedure is used. In the spectrophotometric methods, the
concentration of a sample cannot be measured directly, but is determined using another physical measuring quantity “y” (absorbance of a solution). An unambiguous empirical or theoretical relationship can be shown between this quantity and the concentration of analyte. The calibration between $y = g(x)$ is directly useful and yields by inversion of the analytical calculation function. The calibration function can be obtained by positioning an adequate mathematical model through the experimental data. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. In practice, however, many deviations from this ideal calibration line may occur. For the majority of analytical techniques the analyst uses the calibration equation.

$$Y = a + bx$$

In calibration, univariate regression is applied, which means that all observations are dependent upon a single variable $x$.

1.1.3.4.1. THE METHOD OF LEAST SQUARES

Least-squares[8] regression analysis can be used to describe the relationship between response ($y$) and concentration ($x$). The relationship can be represented by the general function.

$$Y = f(x, a, b_1, \ldots, b_m)$$

Where $a, b_1, \ldots, b_m$ are the parameters of the function.

We adopt the convention that the $x$ values relate to be controlled on independent variable (e.g. the concentration of a standard) and the $y$ values to the dependent variable (the response measurements). This means that the $x$ values have no error on the condition that the errors made in preparing the
standards are significantly smaller than the measuring error (which is usually the case in analytical problems). The values of the unknown parameters $a, b_1, \ldots, b_m$ must be estimated in such a way that the model fits the experimental data points $(X_i, Y_i)$ as well as possible. The true relationship between $x$ and $y$ is considered to be given by a straight line. The relation between each observation pair $(X_i, Y_i)$ can be represented as

$$Y_i = \alpha + \beta X_i + e_i$$

The signal $y_i$ is composed of deterministic component predicted by linear model and a random component $e_i$. One must now find the estimates of $a$ and $b$ of the two values $\alpha$ and $\beta$. This can be done by calculating values $a$ and $b$ for which $e_i^2$ is minimal. The component $e_i$ represents the differences between the observed $y_i$ values and the predicted $y_i$ values by the model. The $e_i, a$ and $b$ are called the residuals, intercept and slope respectively.

$$b = \frac{n \sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n \sum_{i=1}^{n} x_i^2 - \left( \sum_{i=1}^{n} x_i \right)^2}$$

$$a = \frac{\sum_{i=1}^{n} y_i \sum_{i=1}^{n} x_i^2 - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i y_i}{n \sum_{i=1}^{n} x_i^2 - \left( \sum_{i=1}^{n} x_i \right)^2}$$

### 1.1.3.4.2. STANDARD ERROR ON ESTIMATION

The standard error on estimation ($S_e$) is a measure of the difference between experimental and computed values of the dependent variable. It can be represented by the following equation,
\[ S_e = \sqrt{\frac{\sum_{i=1}^{n} (Y_i - y_i)^2}{n(n-2)}} \]

\( Y_i \) and \( y_i \) are the observed and predicted values, respectively. Standard deviations on slopes (\( S_b \)) and intercepts (\( S_a \)) are quoted less frequently, even though they are used to evaluate proportional differences between or among methods as well as to compute the independent variables such as concentration etc. It is important to understand how uncertainties in the slope are influenced by the controllable properties of the data set such as the number and range of data points, and also how properties of data sets can be designed to optimize the confidence in such data.

1.1.3.4.3. SENSITIVITY

Sensitivity is often described in terms of the molar absorptivity (\( \varepsilon \)) which is expressed in L mol\(^{-1}\) cm\(^{-1}\). The awareness of the sensitivity is very important in the spectrophotometric determination of pharmaceutical compounds which depends on the monochromaticity of the radiation. The numerical expression [9-11] for the molar absorptivity (\( \varepsilon \)) at the wavelength of maximum absorbance (\( \lambda_{\text{max}} \)) of the colored species is,

\[
\text{Molar absorptivity (}\varepsilon\text{) = } \frac{A}{c \lambda}
\]

The molar absorptivity diminishes as the bandwidth increases. Savvin [12] suggested a relation between sensitivity and molar absorptivity. He suggested the following criteria for describing the sensitivity.

Low sensitivity, \( \varepsilon < 2 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1} \)

Moderate sensitivity, \( \varepsilon = 2 - 6 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1} \)

High sensitivity, \( \varepsilon > 6 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1} \)
Sandell’s sensitivity\cite{13,14} is the concentration of the analyte (\(\mu g.mL^{-1}\)) which will give an absorbance of 0.001 in a cell of path length 1.0 cm and is expressed as \(\mu g.cm^{-2}\).

### 1.1.3.4.4. DETECTION LIMIT

Detection limit is the smallest concentration of a solution of an element that can be detected with 95\% certainty\cite{15,16}. This is the quantity of the element that gives a reading equal to twice the standard deviation of a series of ten determinations taken with solutions of concentration which are close to the level of the blank. Several approaches for determining the detection limit are possible, depending on whether the procedure is instrumental or non-instrumental. Based on the standard deviation of the reagent blank and the slope of the calibration curve of the analyte, the detection limit (DL) may be expressed as,

\[
DL = \frac{(3.3 \sigma)}{S}
\]

Where \(\sigma\) = standard deviation of the reagent blank; \(S\) = slope of the calibration curve.

The slope \(S\) may be estimated from calibration curve of the analyte. The estimate of \(\sigma\) may be measured based on the standard deviation of the reagent blank.

### 1.1.3.4.5. QUANTITATION LIMIT

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte with those of blank samples and by establishing the minimum level at which the analyte can be quantified.
with acceptable accuracy and precision[17,18]. Based on the standard deviation of the reagent blank samples and the slope of the calibration curve of the analyte, the quantitation limit \( (Q_L) \) may be expressed as,

\[
Q_L = \frac{(10 \sigma)}{S}
\]

where \( \sigma \) = standard deviation of the reagent blank; \( S \) = slope of the calibration curve.

The slope \( S \) may be estimated from calibration curve of the analyte. The estimate of \( \sigma \) may be measured based on the standard deviation of the reagent blank.

### 1.1.3.4.6. PRECISION AND ACCURACY

The purpose of carrying out determination is to obtain a valid estimate of a ‘true’ value. When one considers the criteria according to which an analytical procedure is selected; precision and accuracy are usually the first parameters to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important critical for judging analytical procedures by their results.

**A) Precision:** Precision[19] refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term “set” is defined as referring to a number \( (n) \) of independent replicate & measurements of some property. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean divided by one less than the number of results in the set. The standard deviation \( S \), is given by
\[ S = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2} \]

Standard deviation has the same units as the property being measured. The square of standard deviation is called Variance \((S^2)\). Relative standard deviation is the standard deviation expressed as a fraction of the, mean, i.e. \(S/x\). It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

\[ \% \text{ Relative standard deviation} = S \times \frac{100}{x} \]

**B) Accuracy:** Accuracy[19] normally refers to the difference between the mean \(x\) of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

**Absolute method:** The test for accuracy of the method can be carried out by taking amounts of the constituents and proceeding according to specified instructions. The difference between the means of an adequate number of results and amount of constituent actually present, usually expressed as parts hundred (\%) is termed as % error.

The constituent in question will be determined in the presence of other substances, and it will therefore be necessary to know the effect of these upon the determination. This will require testing the influence of a large number of probable compounds in the chosen samples, each with varying amounts. In a
few instances, the accuracy of the method controlled by separations (usually solvent extraction or chromatography technique) is involved.

**Comparative method:** In the analysis of pharmaceutical formulations, the content of the constituent sought (expressed as percent recovery) has been determined by the comparison of the values obtained from a set of results with either (i) the true value or (ii) other sets of data which makes it possible to determine whether the analytical procedure has been accurate and / or precise, or if it is superior to another method.

There are two common methods for comparing results [20,21]: Student’s t-test and the variance ratio test (F-test).

**Student t-test:** Student t-test [20,21] is used to compare the means of two related (paired) samples analyzed by reference and test methods. It gives answer to the correctness of the null hypothesis with a certain confidence such as 95% or 99%. If the number of pairs (n) are small than 30, the condition 'normality of x is required or at least the normality of the difference (di). If this is the case the quantity has a student t-distribution with (n - 1) degrees of freedom, where di = X_R (Reference method) – X_T (Test method) and s_d is the standard deviation

\[ t = \frac{\bar{d}_i}{s_d / \sqrt{n}} \]

These methods of test require knowledge of what is known as the number of degrees of freedom. In statistical terms this is the number of independent values necessary to determine the statistical quantity. Thus a sample of ‘n’ values has ‘n’ degrees of freedom, whilst Σ (x−X)^2 is considered to
have n-1 degrees of freedom, as for any defined value of x only n-1 values can be freely assigned, the nth being automatically defined from other values.

**F-test:** By the F-test [21,22] we can test the significance of the difference in variances of reference and test methods. Let us suppose that one carried out \( n_1 \) replicate measurements by test methods and \( n_2 \) replicate measurements by using reference method. If the null hypothesis is true then the estimates \( S_T^2 \) (variance of the test method) and \( S_R^2 \) (variance of reference method) do not differ much and their ratio should not differ much from unity. In fact, one uses the ratio of variances

\[
F = \frac{S_T^2}{S_R^2}
\]

It is conventional to calculate the F-ratio by dividing the larger variance by the smallest variance in order to obtain a value equal or larger than unity. If the calculated F-value is smaller than F-value from the table, one can conclude that the procedures are not significantly different in precision at given confidence level.
1.2: INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [HPLC]

The simple liquid chromatographic method developed in the late 1960’s had evolved into high performance and high speed chromatography as it has become one of the most versatile tools in a wide variety of fields, finding application in the pharmaceutical sciences[23-25], chemical[26-28], cosmetic[29-31], biotechnological[32-34], biomedical and clinical fields[35-37] and has many advantages over the classical column chromatography in fast separation and resolution of analytes with increased precision and accuracy.

1.2.1. THEORY OF RP-HPLC

The separation of compounds in RP-HPLC is a complex process and several mechanisms for the retention of analytes in RP-HPLC have been proposed, each having distinct advantages. The retention of an analyte in RP-HPLC is a function of its distribution between a liquid polar mobile phase and a non-polar stationary phase. When the layer of bonded alkyl chains is exposed to the contents of a mobile phase, it adsorbs some of the contents of the mobile phase and swells to a degree, forming a complex, thick solid-liquid interface[38].

Analyte retention was initially proposed to be due to interactions between a solute and the mobile phase[39,40]. The retention of solutes increases with an increase in molecular connectivity of a molecule and therefore, more hydrophobic entities, which occupy larger surface areas, have longer retention times. The solvophobic tendency results in the interaction of
solutes with the stationary phase and hydrophobic molecules therefore tend to interact more with the stationary phase compared to more hydrophilic compounds. The size of the molecule and the surface tension of the mobile phase are therefore important factors in determining the mechanism of retention [40].

Later studies have provided evidence of the importance of the stationary phase in the retention process [41-43] that include both the characteristics of the stationary and mobile phases. Experimental data revealed that the density and length of the alkyl chains in a stationary phase are important components in determining the retention mechanism for small non-polar solutes [44]. The stationary phase was found to be an important determinant in the retention mechanism.

The mechanism of retention can be described by two methods [45]:

i. **Partitioning of an analyte between a stationary and mobile phase.**

ii. **Adsorption of an analyte onto the surface of a non-polar adsorbent** [46].

i. **Partitioning** [47] implies that a solute becomes fully embedded in a stationary phase. In the partitioning model [45], the stationary phase can be considered an amorphous bulk fluid medium and the transfer of solutes between the stationary medium is driven by the relative chemical affinity of a solute for the mobile and stationary phases. Equation given below depicts the analyte distribution/partition coefficient used to describe the partitioning theory [46].

\[ K = \frac{C_s}{C_m} \]
Where, $C_s =$ Concentration of analyte in the stationary phase; $C_m =$ Concentration of analyte in the mobile phase

The partitioning of solutes in RP-HPLC can be considered to occur in three steps \textit{viz.}, a) the creation of a solute-sized cavity in the stationary phase, b) the transfer of the solute from the mobile phase leaving a cavity and c) is finally closed by the mobile phase in the third step [45]. The contribution of adsorption to the retention process was initially underestimated, but adsorption is now recognized to play a vital role in the retention of analytes in RP-HPLC[45,46].

\textbf{ii. Adsorption} describes an accumulation of one component in close proximity to an adsorbent surface, under the influence of surface forces. When a liquid binary solution is used, the accumulation of an analyte onto a stationary phase surface occurs with the simultaneous displacement of solvent from the surface region near the adsorbent, into the bulk solution. At equilibrium, a certain amount of analyte will remain accumulated on the adsorbent surface at a higher concentration than that present in the bulk solution. The organic modifier used in a mobile phase can also be adsorbed at the stationary phase interface thereby forming a thick layer suggesting that a modified model may be necessary to explain the retention process.

\textbf{1.2.2. INSTRUMENTATION OF HPLC}

The basic instrument HPLC \textit{[Fig.1.04,P.23]} consists of a high-pressure pump, an injector system, a stationary phase embedded in a stainless steel column and a detector.
HIGH PRESSURE PUMP: It is an important part needed to deliver a constant flow of the mobile phase with a decisive pressure. Most pumps are able to deliver a constant pressure range of 600bar. A dual Piston reciprocating pump is utilized due to its pulse-free flow. In this system as one shaft phase is filling the valve and another phase is pumping the mobile phase. A damping device is required to smoothen the flow and also to avoid excessive noise at high level of sensitivity causing high base line noise preventing small quantities of substances to be detected.

INJECTOR SYSTEM: The sample solution is introduced into the flowing mobile phase at or near the head of the column using an injection system which can operate at high pressure. They contain Fixed- loop and variable volume devices which are operated manually or by an auto-sampler. Manual partial filling of loops may lead to poorer injection volume precision. The sample is introduced into the loop when the valve is in the load position. At this stage the eluent flows from the pump to the column through another passage. When the valve is switched to inject, the loop is redirected to flow into the column conveying the sample into its destination.

COLUMN: The heart of the HPLC system is the column where the actual separation of components takes place. The columns used in HPLC are made of highly polished stainless steel usually having a column length of 10cm to 30cm and an internal diameter of 4.5mm to 5mm. The most widely used stationary phase is Silica (SiO$_2$) as it has excellent chromatographic
performance. Due to the strong adsorptive characteristics of unbounded silica, columns packed with such silica are rarely used for analytical work.

**Fig.1.04. Basic instrumentation of HPLC**

The stationary phase consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing interconnecting pores. The pore size and the amount of silanol groups are controlled in the manufacturing process. Usually used in the separation of polar compounds, Silica can be modified to the reversed stationary phase. This is done by a controlled reaction of organochlorosilanes with the silanol groups or the use of organoalkoxysilanes which modify the surface of the silica. The linkage of these hydrocarbons to the surface impacts a non polarity and enhances partitioning, thus resulting in the separation of compounds. The most popular stationary phase material used is the (ODS) Octadexyl-silica is C$_{18}$. Others include, Octyl (C$_{8}$), Phenyl (C$_{6}$H$_{5}$), Cyanopropyl ((CH$_{2}$)$_{3}$-CN) and aminopropyl ((CH$_{2}$)$_{3}$-NH$_{2}$).
DETECTORS: Main types of detectors which are frequently used in high performance liquid chromatography [HPLC] are the electrochemical detectors, Fluorescent detector, Refractive index detector, Mass spectrometers, Radioactivity detectors and the Ultra-Violet visible detectors. Among these, the most widely used is the Ultra- Violet Visible detectors.

Electrochemical detectors: Used in analysis of compounds that undergoes oxidation or reduction reactions. They measure the difference in electrical potential when the sample passes between the electrodes [Fig.1.05,P.24].

Fig.1.05. Electrochemical detector

Refractive index detectors: They measure the ability of sample molecules to bend or refract light. This property is called refractive index. Detection occurs when light is bent due to samples eluting from the column and this is read as a disparity between the two channels [Fig.1.06,P.24].

Fig.1.06.Refractive index detector
**Fluorescent detectors:** They measure the ability of a compound to absorb and then re-emit light at given wavelengths. Each compound has a characteristic fluorescence [Fig.1.07,P.25].

![Fluorescent detector](image)

**Fig.1.07. Fluorescent detector**

**Ultraviolet (UV) detector:** The UV detector is well known and widely employed in all LC applications. Ultraviolet (UV) detector has good selectivity, linearity, versatility and reliability for separation of analytes. The different types of UV detectors used in HPLC are the **diode array detector**, **fixed wavelength detector** and the **multi-length wavelength detector**.

Diode array detector uses deuterium or xenon lamp which emits light over the UV spectrum range. Light rays from a deuterium lamp move parallel by an achromatic lens to focus it through a detector cell on a holographic grating and the sample is exposed to it at all wavelengths produced by the lamp. The array consists of many diodes and the output from a single diode is sampled by a data system which is stored on a hard disc. The output from the diode is selected and a chromatogram is produced by UV wavelength falling on the diode [Fig.1.08,P.26].
Fig.1.08. Photodiode array detector

1.2.3. CRITERIA OF HPLC METHOD DEVELOPMENT

Method development[52-53], involves considerable trial and error procedures. It starts with literature survey of the molecule in which we find the nature of the molecule, its pKa, solubility, molecular weight etc. The various steps involved in HPLC Method Development is represented in the Table.1.01, P.26

Table.1.01. Steps Involved in HPLC Method Development
1.2.3.1. Optimization studies

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that a near symmetrical peak detects all the compounds, a good separation and a reasonable run time. It includes the study of various experimental parameters that directly influence the resolution of the analyte. The following parameters given below are extensively studied under optimization studies in developing a new HPLC method.

A. SELECTION OF MOBILE PHASE: If the sample contains ionic or ionizable compounds, then a buffered mobile phase[48,49] is used to ensure reproducible results. Under unfavorable circumstances, pH changes as little as 0.1pH units and can have a significant effect on the separation. Buffer works best at the pKa of its acid. At this pH, the concentration of the acidic form and the basic form of the buffering species is equal, and the buffering capacity is maximum. Phosphate has two pKa values in the range of interest for silica-based chromatography. One at pH-2.0 and the other at pH-7.0. The pKa of the acidic buffer is 4.75. Citrate has three-pKa value: 3.08, 4.77 and 6.40. Between citrate and phosphate buffers, the entire pH range useful for silica chromatography can be covered. Acetonitrile is the preferred organic modifier in reverse-phase chromatography and these mobile phases have higher column efficiency. The retention changes by roughly 10% for every 1% change in the concentration of organic modifier (acetonitrile).
B. SELECTION OF pH: pH is another factor in the resolution that will affect the selectivity of the separation in reversed-phase HPLC. Selecting the proper buffer pH[50,51] is necessary to reproduce separation of ionizable compounds by Reversed-Phase HPLC. Selecting an improper pH for ionizable analytes often leads to asymmetric peaks that are broad, tail, split, or shoulder. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low Relative Standard Deviation (RSD) between injections, and reproducible retention times. Sample retention increases when the analyte is more hydrophobic. Thus when an acid (HA) or base (B) is ionized (converted from the unionized free acid or base) it becomes more hydrophilic (less hydrophobic, more soluble in aqueous phase) and less interacting with column binding sites, as a result the ionized analyte is less retained on the column. When the pH = pKa for the analyte, it is half ionized, i.e. the concentrations of the ionized and unionized species are equal. As mostly all of the pH caused changes in the retention occur within ± 1.5 pH units of the pKa value, it is best to adjust the mobile phase to pH values at least ± 1.5 pH units of above or below the pKa to ensure practically 100% unionization of analyte for retention purposes. Generally at low pH peak tailing is minimized and method ruggedness is maximized. On the other hand, operating in the intermediate pH offers an advantage in increased analyte retention and selectivity.

C.SELECTION OF BUFFER: In Reverse-Phase liquid chromatography mobile phase pH values are usually between 2.0 and 7.5. Buffers are needed in reverse-phase conditions when the sample solution is outside this pH range.
Analytes ionizable under Reverse-Phase conditions often have amine or acid functional groups with pKa between 1.0 and 11.0. A correctly chosen buffer pH will ensure that the ionizable functional group is in a single form, whether ionic or neutral. If the sample solution is at pH damaging to the column, the buffer will quickly bring the pH of the injected solution to a less harmful pH. As pH is lowered analyte retention time shortens and peak shapes sharpen as the buffer protonates the acidic silanols on the silica surface. Any buffer with a pKa less than 7.0 is suitable, but mostly phosphate buffer of pH 3.0 is found to be best for all the LC separations.

D. SELECTION OF COLUMN: The HPLC column is the heart for all LC separations that performs the separation process. The column chosen for separation should possess the features of selectivity, efficiency and reproducibility to provide good separation. Commonly used reversed phase columns are C\textsubscript{18} (Octadecyl silane,), C\textsubscript{8} (Octyl silane,) Phenyl and Cyano. They are chemically different bonded phases and demonstrate significant changes in the selectivity using the same mobile phase. The selection of column can be streamlined by starting with shorter columns (150,100 or even 50mm long). By selecting a shorter column with an appropriate phase the run time can be minimized so that an elution order and an optimum mobile phase can be quickly determined. Many laboratories use 4.6mm ID column as standard as it is more advantageous when considered with the use of 4mm ID column as an alternative. This requires only 75% of the solvent flow than that of 4.6mm column. Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, an Octyl phase (C\textsubscript{8}) can
save time over a Octadecyl (C\textsubscript{18}) as it doesn’t retain analytes as strongly as the C\textsubscript{18} phase. For normal phase applications Cyano phases are the most versatile. C\textsubscript{18}(250x4.6mm) columns are frequently used in many pharmaceutical laboratories as these columns are able to resolve a wide variety of compounds due to their selectivity and high plate counts.

**E. SELECTION OF TEMPERATURE:** Temperature variations over the course of a day have quite significant effect on HPLC separations. This can even occur in air conditioned rooms. While temperature[50] is a variable that can affect the selectivity, its effect is relatively small. Also retention time generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. An increase of 1°C will decrease the retention time by 1 to 2%. Because of possible temperature fluctuations during method development and validation, it is recommended that the column be thermostated to control the temperature.

**F. SELECTION OF FLOW RATE:** Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased, there is a slight increase in the resolution, resulting in a corresponding increase in the run time and also decrease the column back pressure.
1.2.4. METHOD VALIDATION

When once any analytical method has been developed, it is important to validate it according to recognized protocols to ensure that the specific test is suitable for its intended use. Owing to the importance of method validation in the field of analytical chemistry, a number of guidance documents on this subject have been issued by various International Organizations and Conferences (ICH) [54-60] to ensure patient safety.

Validation of an analytical method is a process that establishes the performance characteristics of a developed analytical method and ensures that it meets its intended purpose and analytical application. Method validation includes an assessment of the adequacy of the analytical procedure by means of statistical testing, including linear regression analysis, and relative standard deviation determination in order to demonstrate the validity of the method. During validation, an analytical method is tested for reliability, accuracy and preciseness of the intended purpose of that method. Typical analytical parameters used in assay validation include:

- **Linearity**
- **Precision**
- **Accuracy**
- **Ruggedness**
- **Limit of detection**
- **Limit of quantification**
A) Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A calibration curve is the relationship between instrument response and known concentrations of analyte. The matrix based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration response relationship using appropriate weighting and statistical tests for goodness of fit.

B) Precision: The precision of an analytical method is a measure of the degree of scatter or agreement among individual data values, when the method is applied to multiple samples. In addition, precision is a means of evaluation of how close the data values are to each other for a number of measurements taken using the same analytical conditions. The ICH guidelines define precision on three levels, viz., repeatability, intermediate precision and reproducibility. The ICH[55] recommends that the standard deviation, relative standard deviation or coefficient of variation and a confidence interval be reported for each type of precision that is investigated.

Intra-day Precision (Repeatability): Repeatability refers to the precision of an analytical method used under the same operating conditions by the same analyst and measured over a short period of time[55]. The percent relative standard deviation (% RSD) of a group of samples is used to assess the
precision of a method and the permitted % RSD depends on the intended use of the analytical method. For an assay method, the limit of the % RSD would be 2%, whereas that for determining impurities at trace levels is 10% [56].

**Inter-day Precision (Intermediate Precision):** The intermediate precision of an analytical method evaluates the reliability of the method in a different environment other than that used during development of the method to ensure that the method will provide the same results once method development has been completed. The ICH defines intermediate precision as the long-term variability of a measurement process, expressing within laboratory variation, analysis on different days, different analysts, and/or equipment. Intermediate precision can be determined by comparing the results of an analytical procedure run over a period of days to weeks. Intermediate precision was determined for three (3) concentrations, injected in triplicate (n = 3) on three (3) consecutive days.

**Reproducibility:** The reproducibility of an analytical method is a measurement of the precision of a method that is used in more than one laboratory, i.e. precision between laboratories. The assessment of reproducibility serves to show that a method that has been developed can be transferred between laboratories. Inter-laboratory trials must be considered in the standardization of a procedure such as those intended for inclusion in an official compendial publication[55].
C) **Accuracy:** The accuracy of an analytical method is defined as the closeness of a measured value to the true value for that sample. There are several ways of determining the accuracy of an analytical method including comparison to a reference standard, recovery of spiked analyte, and the standard addition of an analyte to a sample. For drug products, it is recommended that accuracy be performed at 80, 100 and 120% of the label claim. The accuracy of a method may be determined by recovery which is expressed as the amount/weight of a compound of interest reported as a percentage of the theoretical amount present in a medium and where full recovery of the analytes is desirable. Percent bias (% Bias) is used to determine the extent of deviation of a result for a sample from the true value for that sample. The closer the recovery to 100%, lower the % Bias, the more the accuracy of the analytical method. Accuracy of a method is determined at three (3) concentrations and analyzing each solution in replicates of five (n = 5) and the % Bias was calculated by interpolation of the data from a calibration curve.

iv) **Specificity:** Specificity is defined as the ability of an analytical procedure to accurately and quantitatively measure the concentration of an analyte in the presence of all sample materials, including extraneous components from which it must be well resolved. The determination of the specificity of an analytical method is considered one of the most important steps in the development and validation of that method. The chromatographic procedure that is developed must resolve the peak of the compound(s) of
interest from any possible excipients or contaminants that may be present in a dosage form, during analysis of that dosage form.

V) Limits of Quantitation (LOQ) and Detection (LOD): The limit of quantitation (LOQ) of an individual analytical procedure is defined as the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The limit of detection (LOD) is defined as the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value, under the defined experimental conditions. The ICH [55] recommended three different techniques that can be used to determine the LOQ and LOD of a given method. a) based on visual evaluation of analytical data. b) by the analysis of samples with known concentrations at minimal level of analyte that is quantified with acceptable accuracy and precision c) the signal-to-noise ratio can be used to determine the LOQ and LOD of a method[55]. The relationships of LOQ and LOD are shown in the equation respectively.

\[ \text{LOQ} = \frac{10\sigma}{S} \quad \text{LOD} = \frac{3\sigma}{S} \]

\( \sigma = \text{standard deviation of the response} \)
\( S = \text{slope of the calibration curve} \)

The residual standard deviation of the y-intercept may be used as the standard deviation in these equations.
AIMS & OBJECTIVES

The main aim of this research work is to develop and validate procedures to meet most of the demands of analytical chemists namely selectivity, simplicity, reliability and cost of analysis for the selected drugs in pure and pharmaceutical dosage forms using UV–Visible spectrophotometric and HPLC techniques as analytical tools.

The specific objectives of this research are;

- To exploit the possibility of using various organic compounds as analytical reagents for the analysis of selected drugs in pure and pharmaceutical dosage forms. Since, the analytically functional groups of the selected drugs have not been exploited completely for developing and designing simple, sensitive easily accessible UV-Visible spectrophotometric methods.

- To develop and validate HPLC assay procedures for the selected drugs in pure and pharmaceutical dosage forms.