CHAPTER I
INTRODUCTION

Drugs play an important role in the progress of human civilization by curing diseases. The word “drug” has been derived from the French word “drogue”, which means a “dry herb”. Several authors defined the term drug in different ways. It may be defined as any substance that is employed to control, prevent or cure disease(s) in human beings or animals. The discovery of several new drugs has helped in the successful eradication and/or control of many deadly diseases. Hence, mankind owes much to pharmaceutical chemistry that deals with the chemistry of drugs, medicinal and pharmaceutical formulations [1]. Advances in the field of computational chemistry and other branches of chemometrics have made it possible to design the drugs that exhibit maximum therapeutic effects and minimum adverse effects. An ideal drug when administered to the patient or host, should satisfy the following:

- it should act on a host/recipient with efficiency
- its action should be localized at the site where it is required
- it should not have toxicity and exhibit minimum side effects
- it should not injure host tissues
- should not develop tolerance by the tissues even administered for long period.

Since, it is difficult to observe all the above properties in one drug, search for an ideal drug continues, always. In the past few decades, pharmaceutical chemistry has been widely explored in finding and developing organic
compounds that are now available in various dosage forms for the treatment of diseases and often for the maintenance of better quality of human health.

CLASSIFICATION OF DRUGS

The drugs are classified into two types based on their therapeutic actions, as given below:

1. Chemotherapeutic agents

The term “chemotherapy” was introduced in 1891 by German chemist, Paul Elhrich [2]. Chemotherapeutic agents are the chemicals that are designed to kill the invading organisms without causing harmful effects on the tissues of the patient. They may be sub-divided into various classes viz., antibiotics, antibacterials, antitubercular, antifungal, organometallic compounds, antineoplastics, anthelmintic, antimalarials, antiprotozoals, antiseptics, antileprotics, typanocides, birth control, antiviral drugs, etc.

2. Functional or Pharmacodynamic agents

These are the drugs, which act on various functions of body, but are not specific remedies for particular diseases [2]. Some pharmacodynamic agents include central nervous system modifiers (depressants, stimulants), adrenergic stimulants, blocking agents, cholinergic and anticholinergic agents, antihistamines, cardiovascular agents, diuretics, local anaesthetics, antilipidemics, antispasmodics, gastric secretion modifiers, anticoagulants, haematological agents etc.
ASSAY

The determination of the potency of an active component in the unit quantity of medicinal preparation is known as "assay" [2]. It can be divided into three classes, which include chemical assay, bioassay and immunological assay. Generally, chemical assay is more reliable and precise compared to the biological and immunological assay.

Analytical chemistry [3] has had a big role to play in the quality assurance, control of bulk drugs and their dosage forms. Several analytical techniques have been added to penetrate chemical determinations not only of the active ingredient but also the quantification of related compounds or impurities in incoming chemicals, drug material and formulations.

Official status of selected drugs

All countries have legislation on bulk drugs and their formulations that set standards and obligatory quality indices for them. These regulations are presented in the form of general and specific articles for each drug and are published in the form of a book "pharmacopoeia" (Indian, IP; United Kingdom, BP; United States, USA; European, EP; Japan, JP; Martindale Extra Pharmacopeia; Merck Index etc.,). The official monographs for the drug and their formulations are both descriptive and informative in addition to prescribing standards for product and conditions for its storage.

CHAPTER I
Types of pharmaceutical formulations

Generally, drugs are formulated as capsules, tablets, syrups, liquid orals, creams or ointments, injections in dry or liquid form (parenterals), lotions, dusting powders, aerosols etc. One or more among diluents viz., lactose, sucrose, starch, cellulose derivatives, glucose, mannitol, sorbitol, acacia, gelatin, polyvinyl pyrrolidone, magnesium stearate, propyl paraben, sodium benzoate, alginic acid, permitted flavors and colors may be added to tablets. However, in capsules one or more among diluents, gelatin, plasticizers, preservatives, certified dyes, lactose, starch, talc etc. may be added. Waxes, carbopol, petroleum jelly, preservatives, surfactants, permitted colors and perfumes may be added to creams or ointments. In syrups and liquid orals, sucrose, sorbitol, certified colors, preservatives and flavors may be added. In parenterals, vegetable oils, mineral oils, water, propylene glycol, simulated oils, dioxalamines, dimethyl acetamide may be used as vehicles. In lotions, dusting powders and aerosols, talc, silica derivatives, preservatives, alcohol may be added to lotions. The formulations may contain stabilizers, antioxidants, buffering agents like acetate, phosphate, citrate, co-solvents, wetting, suspending and emulsifying agents etc.

Analytical techniques

The amounts of drugs and their formulations are determined by means of physical, chemical, physico-chemical and biological methods. Physical methods of analysis are based on the physical properties of a substance viz., color, solubility, moisture content, melting point, degree of turbidity or
transparency, density or specific gravity, freezing and boiling points while chemical methods of analysis include gravimetric [4] and volumetric [5] procedures, which are based on complex formation, precipitation, acid-base and redox reactions. Physico-chemical methods are used to study the physical phenomena that occur as a result of chemical reactions. Among the physico-chemical methods optical (atomic absorption spectrophotometry [6], spectrofluorimetry [7], polarimetry [8], photometry [9] including photocolorimetry [10], spectrophotometry covering UV region [11], visible region [12-14], IR regions [15] or turbidimetry [16]), electro-chemical (coulometry [17], conductometry [18], polarography [19], voltammetry [20], potentiometry [21], amperometry [22]), chromatography (thin layer [23], HPLC [24,25] LC [26], HPTLC [27], GC [28] and CEC [29]) have been specifically used for the assay of individual components of a mixture. Other analytical techniques including kinetic-spectrophotometry [30], electrophoresis [31], thermal [32] and flow injection [33] methods are also being used for the assay of drugs. In addition, NMR technique [34] is also used for the assay of individual components of a mixture. The combination of mass spectroscopy with gas/liquid chromatography [35,36] is one of the most powerful tools employed in identification and quantification of analyte in bulk or dosage forms. The colorimetric methods are simple and do not involve high cost.

The limitation/success of colorimetric procedure depends on the chemical reactions upon which these procedures are based rather than the sophistication of the instrument. However, colorimetric methods cannot be
extended to all types of drugs, may be due to non-availability of a suitable 
reacting reagent or low detection limit. However, the usage of sophisticated 
instrument like HPLC may eliminate the difficulties encountered in the assay 
of minute amounts of degradation products or the analysis of the metabolites of 
drugs in body fluids.

In general, the analytical method is expected to meet the following 
requirements:

➢ The analysis should take a minimal time

➢ Precision and selectivity of the method should be good

➢ The accuracy of the analysis should fulfill the requirements of 
pharmacopoeia

➢ The cost of analysis should be minimum

These requirements are met by the physico-chemical methods of analysis, a 
merit of which is their universal nature that can be employed for analyzing 
organic compounds with diverse structures. In the present study, 
spectrophotometry and HPLC techniques have been employed as tools for the 
assay of different class of drugs. The existing methods may often need 
improvements to suit laboratory requirements, overcome limitations, improve 
sensitivity etc. Modern methods of analysis (chromatography coupled with 
mass) for purity assay even though afford simplicity, speed, good specificity, 
excellent precision and accuracy, involve sophisticated equipments which are 
not in the reach of most laboratories and small scale industries. Moreover, the
cost of their maintenance is high. In such circumstances, simple spectrophotometric and chromatographic techniques are ideal for the assay of drugs in bulk and pharmaceutical formulations.

SPECTROPHOTOMETRY

Spectrophotometric analysis of drugs and compounds has been used extensively in various fields. While physical methods often necessitate sophisticated and costly instrumentation, spectrophotometric methods need only simple instrumentation besides providing with simple, sensitive, accurate and precise measurements. Spectrophotometric determination of drugs is based on chemical reactions characteristics of various functional groups. The molecule possessing the functional group reacts with suitable reagent(s) to give colored species. The basis of spectrophotometric methods is the simple relationship between the absorption of radiation by a solution and the concentration of the colored product(s) in the solution. Every functional group in a molecule is characterized by the absorption of light in a particular region of the spectrum and this property is used for qualitative and quantitative analysis. In addition to chromophores, a molecule may contain one or more functional groups. These groups may not absorb radiation in visible region but they may affect the behavior of the chromophores that are conjugated with. These are called auxochromes.
**Beer-Lambert law**

The fundamental law governing the absorption of all types of electromagnetic radiation is known as the Lambert-Beer, Bouguer-Beer or Beer’s law. This basic law of spectrophotometry is represented by the equation

\[
\log \frac{P_0}{P} = \varepsilon bc = A
\]

where \( P_0 \) = radiant power of the beam striking the sample, \( P \) = radiant power of the beam transmitted by the sample, \( b \) = thickness of the absorbing sample in cm, \( c \) = concentration of the absorbing constituent of the sample expressed in mol/L, \( \varepsilon \) = extinction coefficient. Units of \( \varepsilon \) are l/mol/cm.

Thus, the absorbance (A) of a solution is directly proportional to the concentration of absorbing species when the length of light path is fixed and directly proportional to light path when the concentration is fixed. The plot of absorbance at constant ‘b’ against concentration gives a straight line if Beer’s law is obeyed. This line passes through the origin, since any absorption due to the solvent cancels out in the usual method of making the measurement.

**Theory of solvent extraction**

Solvent extraction, often termed as liquid-liquid extraction, is considered to be the most versatile method amongst the various methods of separations. It is based on the distribution of a solute in a certain ratio between two immiscible solvents, aqueous and organic phase. The technique has proved to be an indispensable in inorganic-analytical and pharmaceutical chemistry [37-39] for quantitative separation and determination.
Ion-association complex extraction has been applied for the assay of several bioactive compounds possessing basic moieties (secondary or tertiary aliphatic amino groups) using a suitable acidic dye as a reagent and a chlorinated solvent as an extractant. The physical forces such as dipole and induced dipole interactions, London dispersion forces, hydrogen bonding and dative bonding interactions lead to the formation of colored molecular complexes, which exist in solutions in equilibrium with their components. The ion-association complex or adduct is a special form of molecular complex resulting from two components and is extractable into organic solvents from aqueous phase at an appropriate pH. Out of the two, one component is a chromogen (dye or drug complex) possessing either cationic or anionic charge and so insoluble in an organic solvent whereas the other is colorless, possessing opposite charge (anionic or cationic) to that of chromogen.

Development of a method

In developing a spectrophotometric method of determination of an analyte, the first step will be the selection of a wavelength of maximum absorption called analytical wavelength. This wavelength can be chosen either from literature or experimentally by recording absorption spectrum. Then, it is important to stabilize the colored species for longer period to make suitable measurements necessary for analysis.

Standardization of reaction conditions

It is necessary to optimize the reaction conditions in order to obtain the colored species of maximum stability and intensity. The absorbance of the
colored species do depend upon the concentration of the reagent, nature of the solvent, pH of the medium, temperature, order of addition of the reagents, time interval for sequential addition of reagents or color development etc. For simple systems having no interaction between variables, the one variable at a time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum reaction conditions. The OVAT approach involves all the variables except one to be held constant, while an univariate study is carried out on variable of interest.

**Calibration curve**

The construction of a calibration curve for a constituent is an important step in spectrophotometric analysis. A good precision and accuracy can only be obtained when a good calibration procedure is followed. Calibration graph is constructed by plotting the values of absorbance *versus* concentration of the analyte. If Beer's law is obeyed, a straight line passing through the origin will be obtained. Linearity range of the method is obtained from the calibration curve. For majority of analytical methods, the analyst employs the calibration equation,

\[ Y = bX + c \]

In calibration, univariate regression analysis is applied, which means that all measurements are dependent upon a single variable, X.

**Correlation coefficient**

The correlation coefficient \( r (x, y) \) is useful to arrive at the relationship between two variables. It is calculated using the equation:
\[
\begin{align*}
    r &= \frac{n \sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{\sqrt{\left[ n \sum_{i=1}^{n} x_i^2 - \left( \sum_{i=1}^{n} x_i \right)^2 \right] \left[ n \sum_{i=1}^{n} y_i^2 - \left( \sum_{i=1}^{n} y_i \right)^2 \right]}}
\end{align*}
\]

where, \(x_i\) and \(y_i\) are the individual values of the variables, \(x\) and \(y\), and \(n\) refers to the number of observations. The value of \(r = 1\) reveals the exact correlation between the true variables while \(r = 0\) indicates the complete independence of the variables. Thus, the value of \(r\) around unity indicates excellent linearity between two variables.

**Limit of detection and limit of quantification**

The limit of detection (LOD) and limit of quantification (LOQ) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (\(S_a\)) which may be related to LOD and LOQ and the slope of the calibration curve, \(b\), by

\[
    \text{LOD} = 3 \times S_a/b
\]

\[
    \text{LOQ} = 10 \times S_a/b
\]

**Sensitivity of a spectrophotometric method**

Sensitivity refers to the slope of a calibration curve, but is frequently used to mean the least determinable concentration or amount of the species to
be determined. The numerical expression of the sensitivity of spectrophotometric methods [40] is the molar absorptivity ($\varepsilon$) at the wavelength of maximum absorbance ($\lambda_{\text{max}}$) of the colored species

$$\varepsilon = \frac{A}{bc}$$

The molar absorptivity is a valuable index in evaluating the relative sensitivity of various spectrophotometric methods. Generally it is greater than $1 \times 10^4$ l/mol/cm and the values of $\varepsilon$ below $1 \times 10^3$ l/mol/cm correspond to less sensitive methods [41].

In order to make a comparison, the sensitivity is often expressed in terms of sensitivity index given by Sandell [40], which represents the number of micro/nanogram of the determinant per mole of a solution having the absorbance of 0.001 for a path length of 1 cm.

**Precision and accuracy**

The purpose of carrying out a determination is to obtain a valid estimate of a "true value". Precision and accuracy together determine the error of an individual determination. They are the most important criterion for evaluating an analytical procedure.

The term precision [42] describes the reproducibility of a result. It can be defined as the agreement between the numerical values of two or more measurements that have been made under identical conditions. One of the most common statistical terms employed is the standard deviation of a
The standard deviation (S) is the square root of the sum of squares of deviations of individual results (x\textsubscript{i}) from the mean (x) divided by one less than the number of results in a set. It is calculated using the following equation:

\[ S = \sqrt{\frac{\sum_{i=1}^{n} (x_i - x)^2}{n - 1}} \]

The square of standard deviation is called the variance (S\textsuperscript{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 to make it a more reliable expression of precision.

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

Accuracy describes how close a measured value is to 'true value' and it is expressed in terms of error. Generally, it refers to the difference between the mean, x, of a set of results and the true or correct value for the quantity measured. According to IUPAC [43], accuracy relates to the difference between a result (or mean) and the true value.

For analytical methods, there are two possible ways of determining the accuracy viz. absolute method and comparative method.

**Absolute method**

Accuracy of a method is checked by taking varying amounts of the analyte and proceeding according to the chosen procedure. The difference
between the mean of an adequate number of results and the amount of analyte actually present, is usually expressed as parts per hundred (%) i.e. % error.

Generally, the analyte in question has to be determined in presence of foreign substances and therefore it is necessary to examine the effects of these in the assay method. This requires testing the influence of a large number of probable compounds in selected samples, each in varying amounts.

**Comparative method**

In order to compare the results of a proposed method with those of an official/reported method, it is necessary to determine the content of the bioactive compound by two or more (proposed and official/reported methods) supposedly “accurate” methods. These methods, which are of essentially different character, can be accepted provided there is no appreciable determinate error.

**Recovery experiments (Standard addition method)**

Recovery studies are conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredient by the proposed methods. A known amount of the drug to be determined is added to each one of the previously analyzed samples and the total amount of the drug is once again determined by the proposed methods. The amount of the added drug is determined by the difference. Satisfactory recovery values will enhance the accuracy of the proposed procedures.
Selectivity of the method

The assay of a method may be affected by matrix and interference effects. Some of the additives and excipients present in pharmaceutical preparations may sometimes interfere in the assay of an analyte and in such instances suitable separation procedure is to be adopted. For this, a known amount of the analyte is determined in presence of variable amounts of additives and excipients. The foreign compound is considered to be interfered in the assay if an error of more than 2% in the absorbance is observed.

Evaluation of precision and accuracy by comparison of two procedures [44]

The accuracy of a method is often evaluated by comparing the results of the method under investigation with the results obtained from a known accurate method called reference method.

Student t-test

This is used to compare the means of two related (paired) samples analyzed by reference and proposed methods. It gives answer to the correctness of the null hypothesis with a certain confidence at 90% or 95% or 99%. If the number of pairs (n) are smaller than 30, the condition of normality of x or at least the normality of the difference (d_i) is required. If this is the case the quantity t can be calculated using the equation:

\[ t = \frac{d}{S_d / \sqrt{n}} \]

The calculated "t" values are compared with the tabulated value for a given number of replicates at the desired confidence level. If the calculated t values
are smaller than the tabulated t values, one can conclude that the two methods are not significantly different at a given confidence level.

**F-test**

This test is meant to test the significance of the difference in variances of reference and proposed methods. Suppose that one carries out 'n₁' replicate measurements using the proposed method and 'n₂' replicate measurements using the reference method. If null hypothesis is true, then the estimates $S_T^2$ (variance of proposed method) and $S_R^2$ (variance of reference method) do not differ very much and their ratio should not differ much from unity. In fact, one uses the ratio of the variance: \[ F = \frac{S_T^2}{S_R^2} \]

It is conventional to calculate the F-ratio by dividing the larger variance by the smaller in order to obtain a value equal or larger than unity. If the calculated F-values are smaller than the tabulated F-values, it could be concluded that the procedures are not significantly different in precision at a given confidence level.

**Chemistry of the colored species**

The chemistry of the colored species formed in each method is ascertained either through probability with the existing experimental evidences or through analogy with the reported methods.
CHROMATOGRAPHY

Tswett, a botanical scientist first discovered chromatographic technique in 1903. Chromatography involves a sample (or sample extract) being dissolved in a mobile phase. Then, the mobile phase is forced through an immobile and immiscible stationary phase. Generally, the phases are chosen such that components of the sample will have differing solubilities in each phase. A component, which is quite soluble in the stationary phase, will take longer time to travel through it than a component, which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

High performance liquid chromatography (HPLC)

The modern form of column chromatography has been called high pressure, high performance, high-resolution and high speed liquid chromatography. In HPLC, separations are achieved by partition, size exclusion, ion exchange and adsorption depending on the stationary phase. The partition mode is the most widely used for routine purposes. In this, the solute components distribute themselves between the mobile and stationary phase. This distribution depends on the relative phase solubility of the components. Due to difference in solubilities, components spend varying amounts of time in mobile phase and thus elute from the column separately. The extent of interaction with the stationary phase governs the degree of retention. The partition mode is divided into two types: ‘normal’ phase and
'reverse' phase. Normal phase HPLC method uses a polar stationary phase and a nonpolar mobile phase and generally used when the analyte is polar while reverse phase HPLC consists of a non-polar stationary phase and a moderately polar mobile phase.

The essential components in HPLC include a pump, injector, column, detector and recorder. The heart of the system is considered to be the column. In addition, components such as solvent reservoirs, inline fitters, pressure gauge, and integrators may be required. The chromatographic process begins by injecting the solute on the top of the column. Separation of components occurs as mobile phase and solute are pumped through the column. Eventually, each component of the solute elutes from the column.

**Modes of chromatography**

Modes of chromatography are defined based on the nature of interactions between the solute and stationary phase, which may arise from hydrogen bonding, van der Waals forces, electrostatic forces or hydrophobic forces or based on the size of the particles (e.g. Size exclusion chromatography).

Different modes of chromatography include Normal Phase Chromatography, Reversed Phase Chromatography, Reversed Phase–ion pair Chromatography, Ion Chromatography, Ion-Exchange Chromatography, Affinity Chromatography, Size Exclusion Chromatography etc.
HPLC detectors

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. Detector for HPLC is the component that emits a response due to eluting sample compound and subsequently signals a peak on the chromatogram. Different detectors that are used with HPLC include refractive index detectors, UV detectors, fluorescent detectors, radio chemical detectors, electrochemical detectors etc.

Column-packing materials

Classic HPLC column packing are based on silica (SiO_2 x H_2O). Silica based packings are very hard and mechanically strong. They can easily withstand the high pressures encountered in HPLC without measurable compression or breakage. Silica consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing inter connecting pores. Thus, a wide range of commercial products is available with surface areas ranging from 100 to 800 m^2/g and particle sizes from 3 to 50 μm.

The silanols on the surface of the silica can be reacted with different agents to prepare bonded phases. The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non polar organic eluents. Silica can be drastically altered by reaction with organo chloro silanes or organo alkoxy silanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon chain to silica produces a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is
octadecyl-silica (ODS-Silica), which contains C\textsubscript{18} chains, but materials with C\textsubscript{2}, C\textsubscript{6}, C\textsubscript{8} and C\textsubscript{22} chains are also available.

Reversed phase chromatography is carried out using a polar mobile phase such as methanol, acetonitrile, water, buffers etc., over a nonpolar stationary phase. Ranges of stationary phases (C\textsubscript{18} C\textsubscript{8}, -NH\textsubscript{2}, -CN, phenyl etc.) are available and very selective separations can be achieved. The pH of the mobile phase can be adjusted to suppress the ionization of the drug and thereby to increase the retention on the column.

**Distribution of analytes between phases**

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases;

\[
A_{\text{mobile}} \Leftrightarrow A_{\text{stationary}}
\]

The equilibrium constant, \(K\), also called as partition coefficient is defined as the molar concentration of an analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase.

The time between sample injection and an analyte peak reaching the detector at the end of the column is termed as the retention time \((t_R)\). Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called \(t_M\). In gas chromatography, \(t_M\) is usually taken as the time needed for methane to travel through the column.
The retention factor, $k'$, describes the migration rate of an analyte on a column. Retention factor, also known as the capacity factor is defined as

$$k'_A = \frac{(t_R - t_M)}{t_M}$$

The values of $t_R$ and $t_M$ are easily obtained from a chromatogram. When an analyte’s retention factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideal retention factor for an analyte generally lies between 1 and 5.

The selectivity factor, $\alpha$, which describes the separation of two species (A and B) on the column can be evaluated using the equation,

$$\alpha = \frac{k'_B}{k'_A}$$

When calculating the selectivity factor, the species A elutes faster than species B. Ideal selectivity factor value will be always greater than one.
Peak asymmetry (Asymmetry factor)

Few chromatographic peaks are perfectly Gaussian (symmetrical) while others exhibit a degree of asymmetry due to tailing or fronting. The degree of asymmetry is calculated as follows:

\[ A_s = \frac{B}{A} \] and the tailing factor, \[ T_F = \frac{(A+B)}{2A} \]. For Gaussian peak, \( A = B \) and both \( A_s \) and \( T_F \) are exactly 1.

Band broadening and column efficiency

In order to obtain optimal separations, sharp and symmetrical chromatographic peaks must be obtained. This means that band broadening must be limited. It is also beneficial to measure the efficiency of the column.

The theoretical plate model of chromatography

The plate model supposes that the chromatographic column contains a large number of separate layers, called theoretical plates. Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". It is important to remember that the plates do not really exist; they are a figment of the imagination that helps us to understand the processes at work in the column. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, \( N \) (the more plates the better), or by stating the plate height; the height equivalent to a theoretical plate (HETP; the smaller the better). If the length of the column is \( L \), then the HETP (H) is

\[ \text{HETP} = \frac{L}{N} \]
The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution;

\[ N = 16 \left( \frac{t_R}{W} \right)^2 \]

where \( W \) is the peak width.

**Resolution**

Another measure of how well species have been separated is provided by measurement of the resolution. The resolution of two species, A and B, is defined as \( R = \Delta t_R / W_{AV} \). For quantitative analysis, a resolution > 1.5 is highly desirable.

It is useful to relate the resolution to the number of plates in the column, the selectivity factor and the retention factors of the two solutes;

\[ R = \frac{\sqrt{N}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k_2'}{1 + k_2' V} \right) \]

An increase in \( N \), the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening which may not be desirable. It is often found that by controlling the capacity factor, \( k' \), separations can be greatly improved. This can be achieved by changing the temperature (in Gas Chromatography) or the composition of the mobile phase (in Liquid Chromatography).

**Validation of chromatographic methods**

The objective of the method of validation process is to provide evidence that the method does what it is intended to. For chromatographic methods used in analytical applications, there is more consistency in validation practice with
key analytical parameters including recovery, response function, sensitivity, precision and accuracy.

**Recovery**

The absolute recovery of a method is measured as the response of a spiked matrix standard expressed as a percentage of the response of pure standard, which has not been subjected to sample pre-treatment. It is best established by comparing the responses of extracted samples at low, medium and high concentrations in replicates of at least 5-6 with those of non-extracted standards, which represent 100% recovery.

\[
\text{Absolute recovery} = \frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of analyte of pure standard (unprocessed)}} \times 100
\]

If an internal standard is employed its recovery should be determined independently at the concentration level used in the method.

**Response function**

In chromatographic methods of analysis, peak area or peak height may be used as the response function to define the linear relationship with concentration known as the calibration model. It is essential to verify the calibration model selected to ensure that it adequately describes the relationship between response function (y) and concentration (x). A plot of studentised residual (raw residual/standard error) *versus* log concentration will then show how well the model describes the data.
**Sensitivity**

The method is said to be sensitive if small changes in concentration causes larger changes in response function. The sensitivity of an analyte method is determined from the slope of the calibration line. Limits of detection (LOD) can be established at a signal-to-noise ratio (S/N) of 3 while limits of quantification (LOQ) can be established at a S/N value of 10.

**Precision**

It is expressed as the percentage coefficient of variation (% CV) or relative standard deviation (RSD) of the replicate measurements.

\[
\% \text{ CV} = \frac{\text{Standard deviation} \times 100}{\text{mean}}
\]

Precision can be considered as having a within assay batch component or reproducibility which defines the ability to repeat the same methodology with the same analyst, and the same reagents in a short interval of time, e.g. within a day (intra-assay precision).

The ability to repeat the same methodology under different conditions, e.g. change of analyte, reagent or equipment or analysis carried on subsequent occasions, e.g. across several weeks or months (inter-assay precision).

**Accuracy**

The accuracy of the analytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value. Accuracy is best expressed as percentage bias, which is calculated from the expression,

\[
\% \text{ Bias} = \frac{\text{(Measured value - True value)} \times 100}{\text{True value}}
\]
Since the true value is not known for real samples, an approximate value is obtained based on spiking drug-free matrix to a nominal concentration. The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured nominal concentrations of the analytes in the spiked drug-free matrix samples.

DRUGS SELECTED FOR THE PRESENT STUDY

The ever increasing use of various classes of drugs makes their determination a matter of foremost importance in biological and pharmaceutical sciences. The following drugs of pharmaceutical importance have been selected in the present study:

- Trazodone hydrochloride [*Lifecare Laboratories Pvt. Ltd., India*]
- Oxcarbazepine [*Himedia Chemicals, India*]
- Cefadroxil [*Himedia Chemicals, India*]
- Nortriptyline hydrochloride [*Wallace Pharmaceuticals Ltd., India*]
- Tegaserod maleate [*Cipla Ltd., India*]
- Ezetimibe [*Merind Pharmaceuticals Ltd., India*] and
- Pentoxifylline [*Sun pharmaceuticals Ltd., India*]

REAGENTS/COMPOUNDS USED IN THE PRESENT INVESTIGATIONS

The selection of an analytical reagent depends on careful consideration of factors such as the scale and economics of the reaction, the presence of other functional groups that might be adversely affected by the reagents, the instability, high reactivity, rate of the reaction etc. The reagent is expected to
form a chromophore with the analyte and hence, could be followed by spectrophotometry. Selection of a reagent for a particular drug is made after a thorough literature survey or that shows reasonable promise for the assay of drug under consideration. The list of compounds/reagents employed in the present study is shown below:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromo cresol purple</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Bromo phenol blue</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Methyl Orange</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>3-methylbenzothiazolin-2-one hydrazone</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>1, 10- Phenanthroline</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>2,2'- Bipyridyl</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Sulphanilic acid</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>p-Nitroaniline</td>
<td>Merck, India</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
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<tr>
<td>Sodium citrate</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
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<tr>
<td>Potassium chloride</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Potassium hydrogen pthalate</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Glucose</td>
<td>s.d. fine-CHEM Ltd., Mumbai, India</td>
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</table>
Talc s.d. fine-CHEM Ltd., Mumbai, India
Lactose s.d. fine-CHEM Ltd., Mumbai, India
Starch s.d. fine-CHEM Ltd., Mumbai, India
Magnesium stearate s.d. fine-CHEM Ltd., Mumbai, India
Sodium alginate s.d. fine-CHEM Ltd., Mumbai, India
Gum acacia s.d. fine-CHEM Ltd., Mumbai, India
Sodium metabisulphite s.d. fine-CHEM Ltd., Mumbai, India
Dextrose s.d. fine-CHEM Ltd., Mumbai, India
Phosphoric acid (AR) s.d. fine-CHEM Ltd., Mumbai, India
Hydrochloric acid (AR) s.d. fine-CHEM Ltd., Mumbai, India
Sulphuric acid (AR) s.d. fine-CHEM Ltd., Mumbai, India
Acetone (HPLC grade) Ranbaxy Fine chemicals Ltd., Delhi, India
Acetonitrile (HPLC grade) Ranbaxy Fine chemicals Ltd., Delhi, India
Chloroform (HPLC grade) Ranbaxy Fine chemicals Ltd., Delhi, India
Methanol (HPLC grade) Ranbaxy Fine chemicals Ltd., Delhi, India
Water (HPLC grade) Ranbaxy Fine chemicals Ltd., Delhi, India
Amoxycillin Cipla Ltd., Mumbai, India
Ceterizine hydrochloride Dr.Reddy’s laboratory, India
Cefadroxil Cipla Ltd., Mumbai, India
Ibuprofen Cipla Ltd., Mumbai, India
Nimesulide Cipla Ltd., Mumbai, India
Piroxicam Sun Pharmaceuticals Ltd., Mumbai, India
SCOPE OF THE PRESENT WORK

With ever growing demand on the drug specificity to fight against new illness, the newer drugs are being pushed into market at such a great pace that it has become difficult to keep abreast of their merits and demerits. Moreover, the increasing emphasis on the fatality or toxicity of drugs has placed heavy burden on drug analysis or the assay of drugs at trace levels in seized materials, complex matrices or in body fluids besides pharmaceutical products. Nevertheless a strict control on the quality of the drugs and their therapeutic actions is very important. For this purpose, pharmaceutical industries or drug regulatory authorities require new, sensitive and simple methods of analysis for routine quality control.

The survey of existing analytical methods reveals that not much attention was paid for the development of high performance liquid chromatographic and spectrophotometric methods for the assay of the selected drugs. Hence, the investigator has made some attempts in this direction and succeeded in developing accurate, precise and sensitive spectrophotometric and HPLC methods for the assay of various class of selected drugs.
INSTRUMENTATION

➢ The absorption spectra were recorded on a double beam CARY 50-BIO UV-visible spectrophotometer (Varian, Australia) with 1cm matched quartz cells, equipped with 150 W Xenon lamp and a slit width of 5 nm.

➢ The pH measurements were made on a Schott Gerate pH meter CG 804.

➢ Chromatograms (HPLC) were noted down using a Shimadzu Corporation system (Analytical Instruments division, Kyoto, Japan) consisting of a LC10AT solvent pump, SPD10AVP detector and a data station with win chrome software version 3.1. The separation was performed on a CLC C_{18} column (5μ, 25cm x 4.6 mm i.d.). A CLC ODS (4 cm x 4.6 mm i.d.) was used as a guard column to protect analytical column. Hamilton 702 μR injector with a 25 μl loop was used for the injection of the samples.
REFERENCES


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7. H.E. Abdellatef, M.M. El-Henawee, H.M. El-Sayed, M.M. Ayad,


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CHAPTER I


