CHAPTER-1

INTRODUCTION
Section (i): **A brief account on analytical chemistry and its applications to pharmaceutical analysis**

Analytical chemistry seeks ever improved means of measuring the chemical composition of natural and artificially occurring materials in our surroundings and environment as well as within ourselves, in our tissues and body fluids and also in pharmaceutical dosage forms. The techniques of this science involve separation, identification and determination of the relative amounts of the components in simple or complex systems. The applications of analytical chemistry are extended to all the branches of science. Recent advances in analytical chemistry are the significant developments in the field of chemical analysis, which are versatile in nature adopting newer techniques. The present trend includes the employment of spectrophotometry, chromatography, polarography, neutron activation analysis, atomic absorption spectrophotometry, ICP-atomic emission spectrometry etc. Among these, spectrophotometric methods occupy special position due to their ease in application, simplicity and accuracy in the determination of trace amounts of chemical compounds, i.e. drugs, metals and non-metals.

Pharmaceutical chemistry \([1-5]\) is the science that makes use of general law of chemistry to study drugs and their preparation, chemical nature, composition, structure, influence on an organism, the method of quality control and the conditions of their storage .etc. All around the world, a large number of varieties of organic compounds are being synthesized daily and some of them are very useful to cure diseases, pain to get relief from abnormalities of health in humans or animals and these chemical compounds
are known as pharmaceutical compounds or drugs. A pharmaceutical drug also referred as medicine, medication or medicament, can be loosely defined as any chemical substance intended for use in the medical diagnosis, cure treatment or prevention of disease. \(^{[6-7]}\)

Drug is a substance that affects the function of living cells and used in medicine to diagnose, cure, prevent the occurrence of diseases and disorders and prolong the life of patients with incurable conditions. Since 1900, the availability of new and more effective drugs such as antibiotics, which fight bacterial infections, antiviral, which fight viral infections and vaccines, which prevent diseases caused by bacterial and viruses has increased the average humans’ life span from about 60 years to about 75 years.

Pharmaceutical drug analysis provide information on the identity, purity, contents and stability of starting materials, excipients, active pharmaceutical ingredients (APIs) and also the secondary pharmaceutical products i.e. the dosage forms having either single or multi-component formulated product.\(^{[8]}\) The quality of the drug product may deviate from the standard required, but in carrying out an analysis one can confirm that whether the quality of the product is of the required standard or not \(^{[9]}\). The fundamental reasons for the “analysis of drug substances” are perhaps due to the tremendous growth in the progress of medicinal chemistry towards achieving one ultimate objective which is to obtain “better drugs for a better world”\(^{[10]}\). Drug analysis is important in several phases of drug development, such as formulation, stability studies, and dissolution studies and in quality control. The importance of reliable analytical methods for the drug determination in a fast, inexpensive, sensitive and selective way is thus evident. In this context, the objective of this thesis is to present the main advances in the development and validation
of analytical methods for the determination of drugs using spectrophotometric techniques.

**QUALITY CONTROL AND ASSURANCE** \(^{11}\)

Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods, there can be no second quality in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The word quality is derived from the Latin “Qualitas”, which means, incidentally, only the ‘nature’ and ‘inherent characteristics’ of a thing. Quality Control (QC) and Quality Assurance (QA) are integral part of Analytical Research. "The primary aim of the new analytical method is to produce correct results, not by chance, but at all times and the use of quality control methods to achieve and demonstrate this." Two yardsticks are used in QA viz. Accuracy and Precision. QA is thus by choosing an appropriate specific, sensitive and accurate procedure with precision which involves consideration of several other practical aspects such as the speed, economy and the skill required. Such procedures assure both accuracy and precision and should be used by the laboratories aiming QA and QC. In research laboratories, no compromise can be made on QA, as today’s novel analytical method is the trendsetter for tomorrow’s routine analysis. Internal and external quality control measures should be adopted and recorded by each laboratory. Internal quality control measures by using controls, replications and random sample check determine the drifts occurring in the daily tests. External quality control methods measure periodic analysis of unknown samples from reputed QC programmes. The variance index scores are the measure of the performance.
QA & QC methods developed follow standard internal operating procedures directed toward assuring the quality, safety, purity and effectiveness of the drug supply.

In pharmaceutical analysis, a variety of methods have been used for the estimation of the drugs in bulk and in their formulations. The methods for the estimation of drugs are broadly divided into physical, chemical, physicochemical and biological. Among these four methods, physicochemical and physical methods are the most used methods for the analysis of drugs. Physical methods of analysis consider the study of physical properties of substance, i.e. melting, freezing and boiling points, solubility, transparency or degree of turbidity and specific gravity. Physicochemical methods are used to study the physical phenomena that occur as a result of chemical reactions. The most important physicochemical methods are optical and chromatographic methods. The optical methods are refractometry, polarimetry, photometry (colorimetry), spectrophotometry (UV, Visible and IR regions), Flow Injection-spectrofluorimetry and nephelometry. The chromatographic methods are column, paper, thin layer, gas-liquid, HPLC, UPLC and RP-HPLC. Recently hyphenated techniques like HPLC with different detectors, LC-MS, GC-MS, and LC-MS/MS are introduced for the analysis of the drugs for achieving high sensitivity and selectivity with good precision, accuracy and reproducibility.

Analytical method development and validation play important roles in the discovery, development and manufacture of pharmaceuticals. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency and performance of the drug products. The selection of analytical method may be based on one or more of the following design criteria,
accuracy, precision, sensitivity, selectivity, robustness, ruggedness, scale of operation, analysis time, availability of equipment and cost.

Many organic compounds absorb in the ultra-violet region of the spectrum and pre-treatment involves only separation of interferences. Some elements in the periodic table absorb strongly in the visible or UV regions, at least in certain oxidation states and preliminary steps may involve redox reactions as well as separations \[^{[12]}\]. Development of absorption methods by means of inorganic reagents is occasionally possible. Many of the colored complexes formed by metal ions with organic reagents are metal-chelates, offering most impressive variety of spectrophotometric methods, and they are especially useful in the field of trace analysis in biological fluids and in pharmaceutical dosage forms. In some regards, the low aqueous solubility of many of the metal chelate compounds is disadvantageous, but on the other hand, extraction of metals into non-aqueous solvents by means of chelating agents may lead to very powerful analytical methods. Reasonable absorbance values are generally obtained with chloroform solutions whose metal concentrations are in the order of a few $\mu$g mL\(^{-1}\). The solvents used in spectrophotometric methods poses a problem in some regions of spectrum. There is no solvent which is transparent throughout the infrared region. However, in UV or Visible region, most of the organic solvents have UV-cutoff points below 210 nm and water is an excellent solvent in that region, due to transparency throughout the spectrum.

**Analytical methods used in analysis**

Analytical chemistry is divided into two types such as Classical and Instrumental. Modern analytical chemistry is dominated by sophisticated instruments. The roots of analytical chemistry and some of the principles used in modern instruments are from
INTRODUCTION

traditional techniques many of which are still used today. These techniques also tend to form the backbone of most undergraduate chemistry educational labs.

1. Classical methods

a) Qualitative analysis

A qualitative analysis determines the presence or absorbance of particular compounds, but not the mass or concentration.

b) Chemical tests

There are numerous qualitative tests, for example, the acid test for the gold and the Kastle-Meyer test for the presence of blood.

C) Flame test

It is a test where the certain metal ions especially alkali and alkaline earth metals are identified based on the characteristic colors they exhibit in flame.

d) Gravimetric analysis

Chemical analysis is based on weighing a final product is called gravimetric analysis. It involves determining the amount of material present by weighing the sample before and / or after some transformations.

e) Volumetric analysis

It is refers to any procedure in which the volume of reagent needed to react with analyte is measured. In titration, increments of reagent solution, the titrant, are added to analyte until their reaction is complete. Form the quantity of titrant required, we can calculate the quantity of analyte that must have been present. The most common type
titrations are based on acid-base, oxidation-reduction, complex formation and precipitation reactions. In these titrations different types of indicators are used to determine the equivalence point.

2. Instrumental methods

a) Chromatographic analysis

Chromatography is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a mobile phase through a stationary phase, which separate the analyte to be measured from other components in the mixture and allows it to be isolated. There are several different types of chromatographic techniques currently in use such as Paper Chromatography, Thin Layer Chromatography (TLC), Gas Chromatography (GC), Liquid Chromatography (LC), High performance liquid chromatography (HPLC) with different detectors, Ion exchange chromatography (IEC) and Gel permeation or Gel filtration chromatography.

b) Electrochemical analysis

Electro analytical methods measure the potential (volts) and/or current (amps) in an electrochemical cell containing the analyte. Electrochemistry measures the interaction of the material with electric field. These methods can be categorized according to which aspects of the cell are controlled and which are measured. The three main categories are potentiometry (the difference in electrode potentials is measured), coulometry (the cell’s current is measured over time) and voltammetry (the cell’s current is measured while actively altering the cell’s potential).
C) Microscopy

The visualization of single molecules, single cells, biological tissues and nonmaterials is an important activity in analytical science. Microscopy can be categorized into three different fields, optical microscopy, electron microscopy and scanning probe microscopy. Recently, this field is rapidly progressing because of the rapid development of the computer and camera industries.

d) Spectrophotometry and colorimetry

Spectrophotometry involves the use of a spectrophotometer. A spectrophotometer is a photometer which can measure light intensity as a function of color or more specifically, the wavelength of light. There are many kinds of spectrophotometers, among the most important distinctions they used to classify them are the wavelengths they work with, the measurement technique they use, how they acquire a spectrum and the sources of intensity variation they are designed to measure. The use of spectrophotometers is not limited to studies in physics but they are also commonly used in other scientific fields such as chemistry, biochemistry and molecular biology.

Direct spectrophotometric determinations such as colorimetric analysis or UV determination are widely used in pharmaceutical analysis. The estimation of an analyte concentration based on its absorption of ultraviolet or visible radiation is one of the most frequently encountered quantitative analytical methods. One reason for its popularity is that many organic and inorganic compounds have strong absorption bands in the UV or visible radiation. An additional advantage to UV or visible absorption is that in most cases it is relatively easy to adjust experimental and instrumental conditions so that Beer’s law is obeyed. The applications of Beer’s law for the quantitative analysis of
samples in environmental chemistry, clinical chemistry, industrial chemistry, forensic and in pharmaceutical chemistry are numerous. The scale of operation for molecular UV or visible absorption is commonly used for the analysis and is generally better than that of IR absorption. It is routinely used for the analysis of trace analytes in macro and meso samples. The analysis of a sample by molecular absorption spectroscopy is relatively rapid, although additional time may be required when it is necessary to use a chemical reaction to transform a non absorbing analyte into an absorbing form. The cost of UV-Visible instrumentation is relatively less. The selectivity and sensitivity of analyte towards the absorption of light can be increased by converting it into a chromogenic derivative, by adopting a suitable chemical reaction, which also prevents the interferences.

**Classification of functional groups in drugs**[13-16]

A feature of organic drug is the presence of some specific functional groups in their molecules. Knowing the reactions of functional groups, one can easily analyze any organic molecule with a complicated structure.

<table>
<thead>
<tr>
<th>Category</th>
<th>Functional group</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acidic nature</td>
<td>- COOH (Carboxylic)</td>
<td>Aspirin, Ibuprofen, Cetirizine</td>
</tr>
<tr>
<td></td>
<td>-SH (Sulpha hydryl)</td>
<td>Captopril, Penicillamine</td>
</tr>
<tr>
<td></td>
<td>(Phenolic Hydroxyl)</td>
<td>Nelfinavir</td>
</tr>
</tbody>
</table>
INTRODUCTION

2. Basic nature

\[ R^1 = H; \quad R^2 = \text{alkyl}; \]

\[ R^1 = R^2 = \text{alkyl groups} \]

(Primary, Secondary and Tertiary amino)

3. Neither acidic nor basic nature

\[ -\text{O-CH}_3 \]  
(Methoxy)

\[ R-O-R^1 \]  
(Ether)

\[ -\text{NH} \]  
(Lactam)

\[ -\text{C≡C-} \]  
(Acetylenic)

(Primary, Secondary and Tertiary amino)
Selection of reagents for the analysis of solutes[^17]

Several papers are being published every year on the reactions and possible applications of new and old reagents for organic analysis. The selection of an appropriate reagent for a particular analytical situation is still a challenging problem. The choice of a particular reagent depends on careful consideration of such factors as the scale and economy of the reaction, the presence of other functional groups that might be adversely affected by the reagent, the deactivation of the reaction centre by steric and electronic effects, the instability or high reactivity of the desired product, the rate of reaction, position of equilibrium (in the case of a reversible reaction) and other related factors.

The selection of a reagent for the determination of a particular compound is made after a literature search for methods that have been used in parallel situations elsewhere or that show reasonable promise for the compound under consideration. If adequate information is not available in this way, then the reagents that act most rapidly and stoichiometrically can be chosen after investigation of the performance of several plausibly selected ones on a pure sample of the compound. Reagent selectivity for a particular functional group is normally the minimum requirement. Specificity of the reagent for a single compound containing that functional group is often desirable, not only to isolate it from other compounds containing the same functional group, but also to eliminate the effects of interfering compounds.

The general objective of treating a compound with a reagent (for purpose of determination of the compound) is to get one or more derivatives having a measurable physical or chemical property that is as completely different as possible from that of any
of the reactants. Some examples of properties in which a specific change may be brought about by the reagent are as follows:

1. The reagent may bring about a change in or destroy an acidic, alkaline, oxidizing or reducing property of the functional group, the amount of change being determined titrimetrically or spectrophotometrically.

2. The reagent forms a product with a solubility product different from that of original sample and this property serves as basis for the gravimetric determination or for the isolation, concentration and purification of the compound for examination by other analytical techniques.

3. The reagent forms a chromophore or reduces the concentration of an already existing chromophore, the change being measured by one of the spectrophotometric techniques—ultraviolet, infrared or visual. Many spot tests for functional groups or spray reagents in TLC depend on the formation of a colored derivative. Similarly a fluorophore may be generated or quenched and the change is measured fluorimetrically.

4. The reagent act on the sample to produce a gas measurable nanometrically or one that can be collected and determined by titrimetric, gravimetric or other types of techniques.

5. The reaction of the reagent with the sample produces a derivative that is less polar and/or more volatile than the original sample and is thus more amenable for determination by high performance liquid or gas liquid chromatography. Many compounds containing polar functional groups show unfavorable properties such as low volatility, tailing, irreversible adsorption on to column packing and thermal
instability. Vast improvements in these respects are easily realized because the polar nature of the compound promotes derivatization with suitable reagents to replace the polar group with a less polar one, giving some times a more sensitive detection response.

6. The reagent forms derivatives with the sample suitable for structural investigation or estimation by the application of NMR and Mass spectroscopic techniques.

7. Enzymatic treatment of the sample may catalyze a specific reaction and structural change which may be monitored by one or more of the several analytical techniques.
Section (ii): SPECTROSCOPY

Spectroscopy is the branch of science that deals with the measurement and interpretation of electromagnetic radiation (EMR) absorbed or emitted when the molecules or the atoms or ions of the sample move from one energy state to another. At ground state, the energy of the molecule is the sum of rotational, vibrational, and electronic energies. EMR is made up of discrete particles or photons. EMR possess both wave and particle characteristics. i.e. it can travel in vacuum also. The different regions of EMR are Visible, Infra-Red, Microwaves, Radio waves, X-rays, Gamma rays, or Cosmic rays. The energy of the radiation depends upon the frequency and the wavelength of the radiation.

Energy of EMR:

\[ E = h \nu \] \hspace{1cm} ............... 1.ii.1

Where; \( E \) = Energy of radiation

\[ h = \text{Plank’s constant} \times 10^{-34} \text{ J.sec}^{-1} \]

\[ \nu = \text{Frequency of radiation} \]

Types of spectroscopy:

1. Based on the study of the spectra obtained that may be at atomic or molecular level:

(i) Atomic spectroscopy: where the changes in the energy takes place at atomic level.

E.g. Atomic Absorption Spectroscopy, Flame Photometry.
(ii) **Molecular Spectroscopy**: where the changes in the energy takes place at molecular level.

E.g. UV Spectroscopy, Colorimetry, Fluorimetry, I.R. Spectroscopy etc.

2. Based on the study of the spectra obtained that may be due to absorption or emission of the EMR:

(i) **Absorption Spectroscopy**: Spectroscopy involving the spectra due to the absorption of the EMR.

E.g. UV- Visible Spectroscopy, I R-Spectroscopy etc.

(ii) **Emission Spectroscopy**: Spectroscopy involving the spectra due to the emission (following the absorption) of the EMR.

E.g. Flame Photometry, Fluorimetry

3. Based on the study carried out at electronic or magnetic levels:

(i) **Electronic Spectroscopy**:

E.g. U.V Spectroscopy, Colorimetry, Fluorimetry

(ii) **Magnetic Spectroscopy**:

E.g. NMR Spectroscopy, ESR Spectroscopy.

**Absorption of EMR by organic molecules**:

If we pass light from a ultra-violet lamp through an organic sample, some of the light is absorbed. In particular, some of the wavelengths (frequencies) are absorbed and others are virtually unaffected. We can plot the changes in absorption against wavelength
and produce an absorption spectrum. A molecule can only absorb a particular frequency, if there exists within the molecule an energy transition of magnitude \( E = h \nu \).

Although almost all parts of the electromagnetic spectrum are used for studying the matter, in organic chemistry we are mainly concerned with energy absorption from three or four regions - Visible, Infra-Red, and Microwave and Radio frequency absorption which is shown in Fig 1.ii.1.

**Fig 1.ii.1 Electro magnetic spectrum:**

![Electromagnetic Spectrum](image)

**Table: 1.ii.1- Spectroscopic techniques in organic chemistry:**

<table>
<thead>
<tr>
<th>Radiation absorbed</th>
<th>Effect on the molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet-Visible levels</td>
<td>Changes in the electronic energy within the molecules.</td>
</tr>
<tr>
<td>( \lambda: 200 – 380 ) nm and ( 380 – 760 ) nm</td>
<td></td>
</tr>
<tr>
<td>Infra-Red (Mid Infra-Red)</td>
<td>Changes in the vibrational and rotational movements of the molecule</td>
</tr>
<tr>
<td>( \lambda: 2.5 – 25 ) ( \mu )m</td>
<td></td>
</tr>
<tr>
<td>Microwave</td>
<td>Electronic spin resonance and electronic paramagnetic resonance; induces changes in the magnetic properties of the unpaired electrons</td>
</tr>
<tr>
<td>( \lambda: 1 ) mm – 10 cm</td>
<td></td>
</tr>
</tbody>
</table>
Principles of UV-Visible spectrophotometry

Basic principles of spectrophotometry \(^{[18]}\)

Spectrophotometry is one of the advanced analytical methods used in qualitative and quantitative analysis. It is based on the absorption of electromagnetic radiation. Every compound has a unique characteristic relationship with electromagnetic radiation. This is a direct process which does not change composition and properties of the experimental solutions. Spectrophotometry is called colorimetry in the visible region of electromagnetic spectrum. This technique is mainly used for the determination of trace amounts of metals, non-metals and organic compounds and also used in the determination of composition of metal complexes and stability of coordination complexes in solution state.

The measurement of radiation frequency is done experimentally which gives a value for the change of energy involved. From this, we can conclude the set of possible discrete energy levels of the matter. The curve which is obtained by plotting the light transmitted, the height of the ordinate in the plot at a wavelength is directly proportional to the concentration of the absorbing medium.

**Fig 1.1i.2 Energy Transition:**

![Energy Transition Diagram](image-url)
The lowest energy transition is that between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) in the ground state. The absorption of the EMR excites an electron to the LUMO and creates an excited state. The more highly conjugated the system, the smaller the HOMO-LUMO gap, ∆E, and therefore, the lower the frequency, the longer the wavelength (λ) of the absorbed radiation. The colors that our eyes see are typically due to highly conjugated organic molecules. The unit of molecule that is responsible for the absorption is called the chromophore, of which the most common are C=C (π → π*) and C=O (n → π*) systems. By the interaction of UV-visible radiation with matter, only the valence electron absorbs the energy. Thereby the molecule undergoes transition from ground state to excited state. The intensity of absorption depends upon the concentration and path length as given by the Beer-Lambert’s Law.

Fig 1.ii.3 Spectrophotometer common features:

Laws of photometry:

When the light passes through a homogeneous transparent medium, a part of incident light is reflected, a part is absorbed by the medium, and the rest is allowed to
transmit as such. If \( I_0 \) denotes the intensity of incident light, \( I_r \), that of the reflected light, \( I_a \), that of the absorbed light and \( I_t \) that of the transmitted light,

We can write

\[
I_0 = I_a + I_t + I_r \quad \text{………… (1.ii.2)}
\]

In spectrophotometric measurements, comparison cells are used for sample and reference so the \( I_r \) is eliminated. Then

\[
I_0 = I_a + I_t \quad \text{………… (1.ii.3)}
\]

The fraction of incident radiation passing through a medium is governed by two laws, known as Lambert’s law and Beer’s law.

**Lambert’s law**

According to this law, “when a beam of monochromatic light is allowed to pass through a transparent medium, the rate of decrease of intensity with the thickness of medium is directly proportional to the intensity of the incident light.”

This law may be expressed mathematically as follows.

\[
\frac{dI}{dl} \propto I \quad \text{………… (1.ii.4)}
\]

or

\[
\frac{dI}{dl} = kI \quad \text{………… (1.ii.5)}
\]

Where \( I \) denote the intensity of incident light of wavelength \( \lambda \), ‘\( l \)’ denotes the thickness of the medium and ‘\( k \)’ denotes the proportionality factor.
The negative sign indicates that ‘\(I\)’ decreases as ‘\(l\)’ increases. On integrating equation (1.ii.5) and putting \(I = I_o\) when \(l = 0\), we get

\[
\ln \frac{I_o}{I_t} = kl \quad \ldots \ldots \ldots \ldots \quad (1.\text{ii.6})
\]

or

\[
I_t = I_o e^{-kl} \quad \ldots \ldots \ldots \ldots \quad (1.\text{ii.7})
\]

Changing from natural to common logarithms, we get

\[
I_t = I_o 10^{-0.4343 kl} = I_o 10^{-k'l}
\]

Where

\(I_o\) = intensity of the incident light

\(I_t\) = intensity of the transmitted light

\(l\) = thickness of the medium

\(k'\) = absorption coefficient.

The ratio \(\frac{I_t}{I_o}\) is termed as the transmittance \(T\), and its reciprocal \(\frac{I_o}{I_t}\) is termed as opacity. The ratio \(\log_{10} \frac{I_o}{I_t}\) is termed as absorbance, \(A\).

\[
A = \log \frac{I_o}{I_t} \quad \ldots \ldots \ldots \ldots \quad (1.\text{ii.8})
\]
Beer’s law

This law states that “the intensity of a beam of monochromatic light decreases exponentially with the increase in concentration of the absorbing medium arithmetically.”

This is expressed as

\[- \frac{dl}{dc} = k^{\text{ii}}l \] 

\[I_t = I_o10^{-0.4343k^{\text{ii}}c} = I_o10^{-k^{\text{iii}}c} \]

Where

- \(c\) = concentration of the absorbing medium
- \(k^{\text{iii}}\) = constant

By adding Lambert’s and Beer’s laws, we get

\[I_t = I_o10^{-ael} \] 

\[\log \frac{I_o}{I_t} = a c l \]

Where

- ‘a’ is new proportionality constant.

Since, \(\log \frac{I_o}{I_t} = A\),

We can rewrite the above expression as

\[A = a c l \]
In the above equation ‘a’ is called absorptivity when the concentration ‘c’ is expressed in g L\(^{-1}\). If ‘c’ is expressed in moles L\(^{-1}\) and the thickness of the medium ‘l’ in cm, we write ‘ε’ in the place of ‘a’.

Thus

\[ A = \varepsilon c l \quad \cdots \cdots \cdots \cdots \cdots \quad (1.\text{ii}.14) \]

Where, \(\varepsilon\) is molar absorptivity.

The absorbance of a solution is an additive property. If there are more than one absorbing species present in the solution, the total absorbance is equal to the sum of all the absorbance values of the components present in the solution.

This can be expressed as follows,

\[ A = [\varepsilon c_1 + \varepsilon c_2 + \varepsilon c_3 + \varepsilon c_4 + \cdots + \varepsilon c_n] = \sum_{i=0}^{n} \varepsilon_i c_i \quad \cdots \cdots \cdots \cdots \cdots \quad (1.\text{ii}.15) \]

Where, the path length is kept constant.

**Ringbom’s plot**\(^{[19]}\)

Ringbom introduced a new method of plotting spectrophotometric data in the year 1939. He plotted the percent absorbance (P) against logarithm of concentration (log C).

Absorbance = I - Transmittance (T) and

\[ \% \text{ Absorbance} = 100 - \% \text{ transmittance}. \]

An S-shaped sigmoid curve would be obtained. Ringbom proposed that the accuracy of an analytical determination based on the spectrophotometric method is
greatest when $\Delta P/(\Delta C/C)$ reaches a maximum value, where $\Delta P$ is photometric error and $\Delta C/C$ is relative analysis error.

$$\Delta P/(\Delta C/C) \text{ Can be equated to } \Delta P/2.303.$$ 

The Ringbom plot has two outstanding features.

1. The analysis error is least in the concentration range of determination. This is the concentration range corresponding to the nearly linear portion or the steep slope of the plot.

2. At any concentration, the accuracy of the analysis can be easily evaluated. The relative analysis error is obtained on dividing 230 by the slope of the curve at that point.

**Sensitivity of spectrophotometric methods**

The term sensitivity implies the smallest determinable concentration or the amount of species concerned. In photometry it refers to the slope of the calibration curve plotted between the concentration and absorbance value. Numerically, it is expressed as molar absorptivity at the wavelength of maximum absorbance.

$$\varepsilon = \frac{A}{bc} \quad \cdots \cdots \cdots \cdots \cdots \text{(1. ii. 16)}$$

Where

A = absorbance

b = path length in centimeters,

c = concentration in moles L$^{-1}$. 
The molar absorptivity is expressed in L mole\(^{-1}\) cm\(^{-1}\) in CGS units and in mole\(^{-1}\) nm\(^{-1}\) in SI units.

The methods \[20\] for which \(\varepsilon > 1 \times 10^4\) are considered to be sensitive while others with \(\varepsilon < 1 \times 10^3\) is termed as less sensitive methods. As per the quantum theory, \(\varepsilon\) values never exceed \(1.5 \times 10^5\). In some indirect methods such as amplification methods higher value of \(\varepsilon\) can be obtained.

Sensitivity can also be expressed in terms of specific absorptivity (a) which is related to molar absorptivity \((\varepsilon)\) as

\[
a = \frac{\varepsilon}{\text{At. wt}} \times 1000 \quad \ldots \ldots \ldots \ldots \quad (1.\text{ii}.17)
\]

Sandell’s \[21\] expressed the sensitivity by a quantity \(S\) known as Sandell’s photometric sensitivity.

\[
S = \frac{10^{-3}}{a} \quad \ldots \ldots \ldots \ldots \quad (1.\text{ii}.18)
\]

The units of \(S\) are \(\mu\text{g cm}^{-2}\).

**INSTRUMENTATION:**

1. **Source of Radiation:**

UV-Visible spectrophotometer uses two light sources, a deuterium (D\(_2\)) lamp for ultraviolet light and a tungsten (W) lamp for visible light.

2. **Collimating System:**

It makes the light beam parallel or focused. It includes lenses, mirrors and slits.
3. Monochromator (Filter):

It converts the polychromatic radiation to monochromatic radiation. It consists of filters, prisms and gratings.

4. Sample Compartment:

It is to permit radiation to pass through the sample. These are made of quartz, fused silica glass.

5. Frequency Analyzer:

Which separates out all the individual frequencies generated by the source (the most familiar being the triangular glass prism as used by Isaac Newton for visible light)

6. Detector:

For measuring the intensity of the radiation at each frequency, allowing the measurement of how much energy has been absorbed at each of these frequencies by the sample. e.g. Barrier layer cells, Photo emissive tubes, Photomultiplier tubes etc.

7. Recorder:

A pen recorder or a computerized data station, with a VDU for initial viewing of the spectrum
8. Applications:

1. Quantitative determination of chromophore concentrations in the solution.

2. Impurity determination by spectrum subtraction.


4. Determination of pKa values of indicators.

5. Determination of molecular weights of amines.


7. Chemical kinetics: The rate of reaction is calculated as the change in absorbance per unit time.

8. Qualitative and quantitative analysis of materials used in dye and pharmaceutical industries.
Analytical Method Validation:

Accuracy\textsuperscript{[23]}

The accuracy of an analytical method is the degree of agreement of results generated by the method to the true value or a conventional true value. Accuracy can be assessed by applying the analytical method to samples or mixtures of sample matrix components to which known amounts of the analyte have been added, above and below the normal levels expected in the samples. Method accuracy is the agreement between the differences in the measured analyte concentrations of the fortified (spiked) and unfortified samples and the known amount of analyte added to the fortified sample. Comparison of the method’s results can be performed by using an established reference method, assuming that the latter is free from systematic errors. Second, accuracy can be measured by analyzing a certified reference material, and comparing the measured value with the true value as supplied with the material. If such reference material is not available, a blank sample matrix can be spiked with a known concentration that should cover the range of concern, including one concentration close to the quantization limit. The expected recovery depends on the sample matrix, on the sample processing procedure, and on the analyte concentration.

Linearity:

The linearity of an analytical method is its ability to elicit test results that directly, or by means of well-defined mathematical transformation, is proportional to the concentration of analyte in samples within a given range. Frequently, the linearity is evaluated graphically in addition or alternatively to mathematical evaluation. The deviations from linearity are minimized by Regression analysis; a statistical technique
which provides the means for objectively obtaining a linear line with minimum deviation between the plot and data. For linear ranges, the deviations should be equally distributed between the positive and negative values. The minimum concentration for the working range is known as the Limit of Detection (LOD) whereas, the maximum concentration is called the Limit of Linearity. It is not always possible to obtain a linear graph due to interferences, signal noise, sample matrix or deviations from Beer’s Law. However, several programs are commercially available for computing the best fit curve for the data set.

**Significant Figures:**

All measurements carry some degree of uncertainty. The degree of uncertainty depends upon both accuracy of the measuring device and skill of its operator. In order to determine the number of significant digits in a measurement or number, the following rules may be applied:

1. If there is no decimal point, the right-most non-zero digit is the least significant digits.
2. In case of numbers that include a decimal point, the right-most non-zero digit is the least significant digit regardless of its value.

Significant digits should not be considered the same as uncertainty in measurement systems. They are used for manipulating experimental data, but not for expressing uncertainty. Significant figures have economic significance simply because the more the significance numbers expected from the measurement, the more expensive will be the equipment and higher the quality of reagents required. Care should therefore, be taken to properly define the optimum number of significant figures when attempting an analysis.
Other Performance Parameters:

Sensitivity:

The sensitivity of an instrument is the change in output signal while a change in the physical parameter is being measured. It is determined with the help of the calibration curves. The sensitivity is constant over the entire range in a linear response system and is found to be changing with respect to the concentration in case of non-linear systems. In case of analytical instruments, the sensitivity is usually expressed as the concentration of analyte required to cause a given instrument response.

Selectivity:

The selectivity of an analytical method is defined as its ability to accurately measure an analyte in the presence of interference that may be expected to be present in the sample matrix.

Range:

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity by using the specified method. The range is normally expressed in the same units as the test results (e.g. percentage, parts per million etc.) obtained by the analytical method.

Limit of Detection (LOD):

The LOD is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified.
LOD = \frac{3.3 \sigma}{S} \quad \ldots \ldots \ldots (1. ii. 19)

Units for limit of detection is \(\mu g \text{ mL}^{-1}\)

Where,

\[\sigma = \text{Standard deviation of the response.}\]

\[S = \text{Slope of the calibration curve.}\]

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

The estimate of \(\sigma\) may be carried out in a variety of ways.

**Limit of Quantification (LOQ):**

\[\text{LOQ} = \frac{10\sigma}{S} \quad \ldots \ldots \ldots (1. ii. 20)\]

Units for limit of Quantification is \(\mu g \text{ mL}^{-1}\)
Section (iii): **TYPES OF REACTIONS AND REAGENTS INVOLVED IN THE PROPOSED SPECTROPHOTOMETRIC METHODS**

Different types of reactions are applied in the present proposed methods for the determination of the following selected pharmaceutical drugs. A brief discussion of these reactions and the reagents used to bring about these reactions are presented here.

**Table 1.iii.1 Different types of the proposed methods.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the drug determined</th>
<th>Method</th>
<th>Reagent(s) used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atazanavir (AZV)</td>
<td>AZV-BTB</td>
<td>Bromothymol blue (BTB); Phthalate buffer</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>AZV-BPB</td>
<td>Bromophenol blue (BPB); Phthalate buffer</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>AZV-BCG</td>
<td>Bromocresol green (BCG); Phthalate buffer</td>
</tr>
<tr>
<td>4</td>
<td>Emtricitabine (EMT)</td>
<td>EMT-PDAB</td>
<td>P-Dimethylaminobenzaldehyde (PDAB); H$_2$SO$_4$</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>EMT-MBTH</td>
<td>3-Methyl 2-benzothiazolinone hydrazone (MBTH); FeCl$_3$</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>EMT-HMBA</td>
<td>4-Hydroxy-3-methoxybenzaldehyde (HMBA); H$_2$SO$_4$</td>
</tr>
<tr>
<td>7</td>
<td>Eplerenone (EPL)</td>
<td>EPL-BTB</td>
<td>Bromothymol blue (BTB); Phthalate buffer</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>EPL-BPB</td>
<td>Bromophenol blue (BPB); Phthalate buffer</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>EPL-BCG</td>
<td>Bromocresol green (BCG); Phthalate buffer</td>
</tr>
<tr>
<td>10</td>
<td>Olopatadine (OLO)</td>
<td>OLO-BTB</td>
<td>Bromothymol blue (BTB); Phthalate buffer</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>OLO-BPB</td>
<td>Bromophenol blue (BPB); Phthalate buffer</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>OLO-BCG</td>
<td>Bromocresol green (BCG); Phthalate buffer</td>
</tr>
<tr>
<td>13</td>
<td>Pemetrexed disodium (PMD)</td>
<td>PMD-PDAC</td>
<td>P-Dimethylaminocinnamaldehyde (PDAC); H$_2$SO$_4$</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>PMD-HMBA</td>
<td>4-Hydroxy-3-methoxybenzaldehyde (HMBA); H$_2$SO$_4$</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>PMD-MBTH</td>
<td>3-Methyl2-benzothiazolinone hydrazone (MBTH); ceric ammonium sulphate</td>
</tr>
</tbody>
</table>
INTRODUCTION

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Zolmitriptan (ZOL)</td>
<td>ZOL-PDAC</td>
<td>P-Dimethylaminocinnamaldehyde (PDAC); H$_2$SO$_4$</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>ZOL-PDAB</td>
<td>P-Dimethylaminobenzaldehyde (PDAB); H$_2$SO$_4$</td>
</tr>
<tr>
<td>18</td>
<td>ZOL-NQS</td>
<td></td>
<td>1,2-Napthoquinone-4Sulphonic acid (NQS); NaOH</td>
</tr>
</tbody>
</table>

**Oxidative coupling reactions:**

Oxidative coupling procedure involving the use of 3-methyl-2-benzothiazolinone hydrazone (MBTH) in the presence of an appropriate oxidant under acidic, neutral or alkaline conditions to form highly colored products were explored for the determination of some of the mentioned active pharmaceutical ingredients possessing amenable structural features.

**MBTH – Oxidant: (EMT and PEM)**

The reported applications of 3-Methyl-2-benzothiazolinone hydrazone (MBTH) include the detection and determination of phenols$^{[24-26]}$, formaldehyde$^{[27]}$ and other aldehydes$^{[28,29]}$, polyhydroxy compounds$^{[30,31]}$, aromatic amines including heterocyclic amines such as antipyrine and amidopyrine,$^{[32]}$ iminohetero aromatic compounds, indoles, carbazoles and phenothiazines$^{[33]}$. MBTH was also used for the indirect determination of compounds such as glycerides$^{[34]}$ and glycosamine glycans$^{[35]}$ etc., which yield one of the above mentioned groups on preliminary treatment with certain reagent.

The reaction of MBTH with phenols or amines was found to be based mainly on the nature of the oxidant and acidic, alkaline or neutral conditions employed. Friested.et al$^{[36]}$ initially carried out, the coupling under alkaline conditions with ferricyanide, but a modification, to use ceric ammonium sulphate in acidic medium was successful and led to an increased sensitivity.$^{[25]}$ Later, Umeda$^{[37]}$ carried out the reaction using a basic
solvent (triethanolamine) with phenols. El-Kommos \cite{25} suggested ceric ammonium sulphate as an oxidant under acidic conditions for the determination of 17 pharmaceutical phenols. Ferric chloride has been mostly used for the determination of aromatic and heteroaromatic amines by Sawicki.et al \cite{33} (in neutral conditions) and by Pays.et. al \cite{32} (in acidic conditions). Other oxidants such as periodate (acidic conditions), ammonium persulphate (alkaline conditions) and potassium dichromate (acidic conditions) were used for the determination of ethylenic compounds and primary alcohols \cite{38} (after oxidation with ruthenium textraoxide) and phenidone \cite{39}. Recently, Sastry and Sastry reviewed various aspects of MBTH chemistry in pharmaceutical analysis \cite{40}.

Under the reaction conditions, on oxidation MBTH loses two electrons and one proton, forming an electrophilic intermediate which has been postulated to be the active coupling species.\cite{41-45} This intermediate undergoes electrophilic substitution with the drug to form the colored product. It also reacts with phenol or amine by electrophilic attack on the most nucleophilic site on the aromatic ring of the phenol or amine (i.e. Para-position to phenolic hydroxyl or amino group, if it is either free or substituted with electron withdrawing substituent like -Cl, -COOH etc.; where Para-position is substituted with electron releasing groups, coupling proceeds through ortho-position to phenolic hydroxyl or amino group) and the oxidant to form the colored species.

The absorption maximum and molar absorptivity values of the colored species are influenced by the nature (inductive, mesomeric or steric effects) of other substituents present in the compound besides the desired substitutes, the oxidizing agent used, the solvent employed and the pH of the medium. Some of the antiviral and anticancer agents under investigation (Emtricitabine and Pemetrexed disodium) possess structural features
that are likely to enable them to react with MBTH. Attempts were made by the present
author to study the suitability of MBTH reagent for the estimation of these two drugs.
The effect of the concentration of the regent (MBTH with different oxidants) pH,
temperature, time and order of addition of reagents with respect to maximum sensitivity,
minimum blank and obedience to Beer’s law have been investigated and the results are
recorded in chapter 4 of EMT and chapter 7 of PEM in the thesis.

**Ion association complex formation reactions: (AZV, EPL and OLO)**

Ion-association is a chemical reaction where by ions of opposite electrical charge
come together in solution to form a distinct chemical entity. Ions of opposite charge are
naturally attracted to each other by the electrostatic force. This is described by coulomb’s
law.

\[
F = \frac{q_1 q_2}{\varepsilon r^2}
\]

F is the force of attraction, \(q_1\) and \(q_2\) are the magnitudes of the electrical charges,
and ‘\(\varepsilon\)’ is the dielectric constant of the medium and ‘\(r\)’ is the distance between the
ions\[^{[46]}\]. Sulphonaphthalein dyes are commonly used as anionic dyes to form ion-pair
complexes with the nitrogenous compound present in positively charged protonated
forms. Ion-pair complexation reaction has been applied for the spectrophotometric
determination of many organic compounds\[^{[47-52]}\].

The term molecular complex is used to describe a variety of types of association
products of two or more molecules. In recent years, extensive attention has been given to
a large group of complexes formed by weak interaction of certain classes of organic
compounds functioning as electron donors (bases), with others which act as electron
acceptors (acids)\textsuperscript{[53-55]}. The forces which lead to the formation of molecular complex include physical forces such as dipole and induced dipole interactions, London dispersion forces, hydrogen bonding and dative bonding interactions. The donor-acceptor complexes (whose composition can be represented by integral ratio of the components) are, in many instances, so unstable that they cannot be isolated in the pure state at ordinary temperatures but exist only in solutions in equilibrium with their components. They can, however, usually be detected readily because of differences in physical properties (e.g. Absorption spectra, solubility in organic solvents) from those of the pure components.

The ion-association complex or adduct (commonly known as ion pair, if two ions are involved, colored, neutral and extractable into organic solvents) is a special form of molecular complex resulting from two components extractable into organic solvents from aqueous phase at suitable pH. One component is a chromogen (dye or metal complex) possessing charge (cationic or anionic in nature) and so it is insoluble in organic solvents. The second component is colorless, possessing opposite charge to that of chromogen.

Acid dyes such as bromothymol blue, bromophenol blue, bromocresol green, mordant black-III, solochrome black-T, metanil yellow dye, bromocresol red, eosin, methyl orange, phenol red, alizarin violet 3B, alizarin brillaint violet R, and fast green FCF have been used arbitrarily by various workers for the determination of components exhibiting basic properties e.g. amines, quaternary ammonium compounds and heterocyclic compounds\textsuperscript{[56-62]}. Some basic dyes (parent moiety - xanthene, triphenyl methane, azine, oxazine and thiazone) and metal complexes have been tried by some workers for the determination of components exhibiting acidic properties \textsuperscript{[63-65]}. 


In the present investigations, it was found that atazanavir (AZV), eplerenone (EPL) and olopatadine (OLO) form ion-association complexes with the bromothymol blue, bromophenol blue, and bromocresol green, in the presence of phthalate buffer (pH 2.4). The formed respective colored ion-association complexes are extracted into chloroform and the analytical results obtained in these methods are reported in chapters-3, 5 and 6.

Condensation reactions using aromatic aldehydes like PDAC, PDAB and HMBA in acidic media (EMT, PEM and ZOL)

PDAB, PDAC and HMBA are used as analytical reagents\[66-68\] for the detection and spectrophotometric determination of aromatic and aliphatic primary and secondary amines. PDAC and PDAB has demonstrated a wide usefulness as a chromogenic reagent for spectrophotometric analysis of several compounds such as urea, thiourea and their N-alkyl/aryl derivatives\[69\], aceclofenac\[70\], sodium diclofenac and oxyphenbutazone\[71\], glafenine and metoclopramide\[72\]. PDAC and PDAB have also been applied for microplate-based assay of p-aminohippuric acid\[73\] in plasma and urine samples, and for rapid testing of benzodiazepines\[74\], nimesulide\[75\], satranidazole\[76\] and sympathomimetic amines in pharmaceuticals. The use of modified dimethylaminocinnamaldehyde has also been described for the analysis of flavanols in wines.\[77\] The majority of the methods mentioned above involve a reaction in acid medium with heating to produce coloured compounds (ranging from orange to red or pink).

In the present study, PDAB, PDAC and HMBA form orange red or red colored chromogens with zolmitriptan, emtricitabine and pemetrexed Disodium. The reaction between PDAB or PDAC with secondary amino group of zolmitriptan is assumed to take
place via condensation of amino group with the carbonyl group of the reagent to produce Schiff’s bases or enamines [78-82]. The primary amino group of emtricitabine and pemetrexed disodium is involved in the condensation process with PDAB, HMBA and PDAC to form Schiff’s base complexes between these drugs in acidic medium.

The attempts were made by the author to study the suitability of the reagents for the estimation of these three drugs. The effect of the concentration of the regents, temperature, time and order of addition of reagents with respect to maximum sensitivity, minimum blank and obedience to Beer’s law have been investigated and the results are recorded in Chapter-4, Chapter-7 and Chapter-8 which are to a large extent based on reactions suggested in the literature.

**Reaction of 1, 2-naphthoquinone-4-sulfonic acid (NQS) with zolmitriptan (ZOL):**

1, 2-Napthaquinone-4-sulphonic acid (NQS) is a reagent widely applied for analytical determinations of amines and amino acids by using UV-Visible spectrophotometric method. NQS is readily able to react in basic medium and at moderate temperatures with both primary and secondary amino groups to form colored derivatives, which were determined by spectrophotometrically. The reagent (NQS) was first used as a colorimetric reagent by Folin [83] for the determination of amino acids in an alkaline solution to form highly colored complexes. Later on, this reagent was employed for the spectrophotometric determination of many amino drugs [84-110].

In the present investigation, the author utilized the reagent, NQS to estimate the drug zolmitriptan in bulk and in its pharmaceutical formulations and the results are reported in Chapter-8.
REFERENCES:


[52]. Higuchi T., Roubal E., Division of analytical chemistry, ACS Abstract papers, April-1965, 149 Th meeting, 28B.


