CHAPTER - 1
INTRODUCTION
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1.1 INTRODUCTION IN ANALYTICAL CHEMISTRY:

Analytical chemistry is the science of developing and improving methods for detection and determination of artificial and naturally occurring components in our surroundings and environment as well as within ourselves, in our tissues and body fluids also in Pharmaceutical dosage forms\(^1\). There are two ways of approaching an analytical problem, either qualitatively or quantitatively. Qualitative analysis seeks to establish the presence and identity of a species within a given sample, while quantitative analysis seeks to establish the relative and/or absolute abundance of the species. Furthermore, quantitation of analysis at trace levels in the presence of large amounts of interfering species like additives and excipients in pharmaceutical products must aim for repeatable precision, high accuracy ensuring the closeness to the true values and low values of % RSD for monitoring in regular quality control\(^2\).

An analytical method most often performed in several continuous steps and is sometimes called the analytical chain of events (fig1.1). The chain of events can look very different depending on the application or the intended purpose of analysis, but in general it consists of experimental design and planning, sampling and handling, sample treatment, separation of sample components, detection, evaluation, interpretation of the results, and validation.
Fig. 1.1. A schematic picture showing the analytical chain of events. Each step in the analytical chain is piece of a puzzle.

In developing successful analytical methods including the above mentioned events, the analyst should start from the end by asking relevant questions and making them the foundation of analysis. Analysis of matrices like pharmaceutical dosage forms and biological samples will always be challenging, due to their great diversity and complexity. The selectivity gained by separation together with spectrophotometric measurements is necessary to distinguish between similar molecules like drugs and their degradation products as well as the ability to monitor trace levels of the analytes.

Strategy:

"How ever beautiful the strategy, you should occasionally look at the results."—Winston Churchill

Development of an analytical method usually, and preferably, starts by setting up experimental conditions and criteria for the method. Knowledge about the sample as well as the analytes to quantified is used to choose appropriate sample treatment, separation, detection, evaluation and validation techniques. Strategy includes the planning of the means to validate the method. When dealing with strategic planning of a new method, the important thing is to understand the intended purpose of the method; what is it supposed to monitor? In what levels?
Matrix? Budget and time? Is the purpose of the method to identify or discover compounds in the specific matrix, the criteria for qualitative analysis should be followed. If the purpose instead aims for investigating or monitoring the levels of certain compounds in the intended matrix, a quantitative approach should be performed.

**Tab-1.1**: Consideration regarding the strategy for analytical method development.

<table>
<thead>
<tr>
<th><strong>Event</strong></th>
<th><strong>Examples of consideration</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aim of analysis</td>
<td>Qualitative analysis (identification)</td>
</tr>
<tr>
<td></td>
<td>Quantitative analysis (relative or absolute quantitation) Off-line analysis or Automated</td>
</tr>
<tr>
<td></td>
<td>Sampling, Handling, Storage, Direct treatments</td>
</tr>
<tr>
<td>Sample treatment*</td>
<td>Precipitation(acids, organic modifier, salts)</td>
</tr>
<tr>
<td></td>
<td>Depletion, Ultrafiltration, Derivatization(masking, ionisation, separation).</td>
</tr>
<tr>
<td>Separation</td>
<td>Liquid-Liquid extraction at high alkaline and acid conditions.</td>
</tr>
<tr>
<td>Evaluation</td>
<td>External standard calibration, Internal standard calibration, Standard addition method,</td>
</tr>
<tr>
<td></td>
<td>Derivatization technique.</td>
</tr>
<tr>
<td>Validation/Quality Checks</td>
<td>Sensitivity, Precision, Accuracy, Linearity, robustness, Limit of detection, Limit of quantification, etc.</td>
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</tbody>
</table>
Today’s challenges:

Analysis of pharmaceutical dosage forms will be always be challenging, due to their great diversity and intricacy. Today’s challenges within life science and pharmaceutical analysis lie in the increasing need for tools to discover and monitor upcoming new products and dosage forms. Analyzing complex samples like biological products and biological fluids is a significant challenge even with today’s advanced instrumentation. Rapid, high throughput, sensitive, and selective methods are now a requisite for pharmaceutical analysis. Also the ability to analyze trace mixtures, using an instrumental configuration compatible with sample matrices, emerged as an important feature.

Drug analysis is important in several phases of drug development, such as formulation, stability studies, dissolution studies and quality control. The importance of reliable analytical methods for drug determination in a fast, inexpensive, sensitive and selective way is thus evident. Although there are countless works describing new analytical methods for determination of drugs that act against diseases & metabolic disorders, a review organizing these works in a systematic and complete way is lacking. In this context, the objective of this thesis is to present the main advances in the development of analytical methods for determination of drugs using, spectrophotometric techniques. Quantitative analysis determines the concentration of a specific analyte in a matrix where other compounds are present. Several analytical methods for determination are based on separating substances from one another by utilizing differences in chemical properties. As pharmaceutical dosage forms contain several substances, apart from the analyte of interest, under these conditions, standards and
analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

**Basic criteria for new method development of drug analysis**

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

The overall aim of our research is to develop new methods for quantitative determination of drugs in pharmaceutical dosage forms. The emphasis is to find new methods to estimate the active pharmaceutical ingredients by UV-Visible Spectrophotometric methods and to understand the mechanism behind. Hopefully, based on a firm theoretical basis, more selective, efficient, fast and reproducible analytical methods can be developed.
1.2 QUALITY CONTROL AND ASSURANCE:

The concept of total quality control refers to the process of striving to produce a perfect product by a series of measures requiring an organized effort by the entire company to prevent/eliminate errors in every stage 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 it uses 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 is the trendsetter and novel analytical method 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 measures periodic analysis of unknown samples from reputed QC programmes can be done. The variance index scores are the measure of the performance.
QA & QC develop and follow standard internal operating procedures directed toward assuring the quality, safety, purity and effectiveness of the drug supply.

**Quality of analytical methodologies:**

The nature of the analytical methods may be physical, chemical, microbiological, biological or combination of these types. The analytical methods used for the assessment of potency and purity of drugs may be divided broadly into:

- Biological and microbiological methods
- Physico-chemical methods including instrumental methods.

The biological and microbiological methods are tedious, cumbersome and also expensive; are replaced by physicochemical methods. The classical physicochemical methods like gravimetry and titrimetry are either non-specific or not sensitive enough for monitoring the very low concentrations of drugs in biological fluids. An important development in pharmaceutical chemistry is the introduction of more refined and sensitive instrumental methods of analysis\(^8\) such as Spectroscopy (UV-Visible Spectrophotometry, IR, NMR, MS, fluorimetry, nephelometry & turbidimetry), Chromatography (TLC, GLC, and HPLC) that enable one to perform the assay of drugs and formulations more accurately and with the smallest possible consumption of the analyte, reagents and time. 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, at least in certain oxidation states and preliminary steps may involve redox reactions as well as separations. Development of absorption by means of inorganic reagents is occasionally possible. The colored complexes formed by metal ions with organic reagents, many of them were metal-chelates, offer 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 nonaqueous 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 on the order of a few µg/mL. 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/Visible region most of the organic solvents have UV-cutoff points are below 210 nm and water is an excellent solvent in that region, due to transparency throughout the spectrum.

Direct spectrophotometric determinations such as colorimetric analysis or ultra-violet determination is widely used in pharmaceutical analysis. The estimation of an analyte's 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/Visible radiation. An additional advantage to UV/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, forensic and in pharmaceutical chemistry are numerous. The scale of operation for molecular UV/Visible absorption is routinely used for the analysis 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 nonabsorbing analyte into an absorbing form. The cost of UV/Vis 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**

A feature of organic drug is the presence of functional groups in their molecules. Knowing the reactions of functional groups, one can easily analyse any organic molecule with a complicated structure.
<table>
<thead>
<tr>
<th>Category</th>
<th>Functional groups</th>
</tr>
</thead>
</table>
| (1) Functional groups imparting an acidic nature to substance | Carboxyl $-\text{COOH}$  
Sulphahydryl or Thiol $-\text{SH}$  
Imide $-\text{CO} \quad \text{NH} \quad -\text{CO}$  
Enol $\backslash\text{C} = \text{C(OH)}$  
Enediol $-\text{C(OH)} = \text{C(OH)}$  
Phenolic Hydroxyl  
Sulphonic acid $-\text{SO}_3\text{H}$ |
| (2) Functional groups imparting basic properties to a substance. | Primary, secondary and tertiary amino  
(R$^1 = R^2 = H$;  
R$^1 = H$, R$^2 = \text{alkyl}$;  
R$^1 = R^2 = \text{alkyl groups}$)  
Aldehyde $-\text{CHO}$  
Keto $R - \text{C} - R^1$ |
<p>| (3) Functional groups which exhibit neither acidic nor basic properties. |</p>
<table>
<thead>
<tr>
<th>chemical</th>
<th>molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy methyl</td>
<td>R – CH₂ – OH</td>
</tr>
<tr>
<td>Nitroso</td>
<td>–N=O</td>
</tr>
<tr>
<td>Nitro</td>
<td>–NO₂</td>
</tr>
<tr>
<td>Methoxy</td>
<td>–O – CH₃</td>
</tr>
<tr>
<td>Ether</td>
<td>R – O – R¹</td>
</tr>
<tr>
<td>Ester</td>
<td>–COOR</td>
</tr>
<tr>
<td>Lactone</td>
<td><img src="image" alt="chemical structure" /></td>
</tr>
<tr>
<td>Lactam</td>
<td><img src="image" alt="chemical structure" /></td>
</tr>
<tr>
<td>Olefinic</td>
<td><img src="image" alt="chemical structure" /></td>
</tr>
<tr>
<td>Acetylenic</td>
<td><img src="image" alt="chemical structure" /></td>
</tr>
</tbody>
</table>
Classification of organic reactions:

An organic reaction may be presented by the general schemes:

\[
\text{Substrate + attacking reagent} \quad \rightarrow \quad \text{intermediate} \quad \rightarrow \quad \text{Products}
\]

The steps of an organic reaction show the breaking of an existing bond or making of new bonds of carbon atom in the reactant (called substrate) leading to the formation of the final products through intermediates which in some cases have only a transitory existence. More often than not, the attacking reagents carry positive or negative charge. The positively charged reagents attack the regions of high electron density in the substrate molecules while the negatively charged reagents attack the regions of low electron density.

Organic reactions are generally classified into four types; substitution (at saturated or unsaturated carbon atom) or displacement reactions which may be initiated by attack of electrophiles, nucleophiles or free radicals. Elimination reactions involve loss of atoms or groups of atoms from a molecule and generate unsaturated centres in the product. Addition reactions (characteristic of unsaturated compound i.e. existence of a π bond) are distinguished on the basis of the attacking reagent (nucleophile, electrophile or a free radical).

Rearrangement reactions involve the migration of an atom or groups of atoms (carbanions, carbonium ions or free radicals) from one site to another within the same molecule.

Classification of organic reagents:

Organic reagents fall into two main groups: An electrophilic reagent (a cation, a dipolar molecule or a molecule which has atoms with incomplete
octet) is a species having an electron deficient atom or centre and may be of two types – positive electrophiles \((H^+, Br^+, Cl^+, NO_2^+, NH_4^+, H_3O^+, R_3O^+),\)

\[
\text{Ar-N}^+ \equiv \text{N etc.) and neutral electrophiles (BF}_3, \text{ AlCl}_3, \text{ FeCl}_3, \text{ SO}_3
\]

\[
\begin{align*}
\text{R—C—Cl etc)} \\
\mid \\
\text{O}
\end{align*}
\]

An electrophilic reagent is most likely to attack a molecule at the point of high electron density. Nucleophilic reagents are electron rich and can be classified into negatively charged species.

\[
(\text{:X}^-, \text{H:O}^-, \text{NH}_2, \text{C ≡ N, R}_3\text{C ≡}, \text{COO}, \text{HSO}_3, \text{CH(COOC}_2\text{H}_5)_2, \text{CH}_3\text{COCH}_2 \text{ etc.) and neutral species (H—O—H, R—S—H, R—O—R, :NH}_3, \text{R—N—H, etc.}).
\]

The attack of negatively charged or neutral nucleophiles on the positively charged substrates results in the formation of neutral or positively charged products. A nucleophile reagent is most likely to attack a molecule at the point of low electron density.

The often transitory intermediates formed during the course of the various organic reactions may be free radicals (from homolytic fission) or carbocations \((\text{C—C}^+)\) and carbanions \((-\text{C :}^-)\) (from heterolytic fission) which then undergo further change to form the products.

**Inductive and Mesomeric effects:** The electron cloud in a \(\sigma\)-bond between two unlike atoms is not uniform and is slightly displaced towards the more
electronegative of the two atoms. This causes a permanent state of bond polarization, where the more electronegative atom has a slight negative charge($\delta^-$) and the other atom has a slight positive charge($\delta^+$).

If the electronegative atom is then joined to a chain of atoms, usually carbon, the positive charge is relayed to the other atoms in the chain. This is the electron withdrawing inductive effect, also known as the -$I$ effect. Some groups, such as the alkyl group, are less electron withdrawing than hydrogen and are therefore considered as electron releasing. This is electron releasing character is indicated by the +$I$ effect.

As the induced change in polarity is less than the original polarity, the inductive effect rapidly dies out, and is significant only over a short distance. The inductive effect is permanent but feeble, as it involves the shift of strongly held $\sigma$-bond electrons, and other stronger factors may overshadow this effect.

The attack by an ion requires polarization in the molecule to be attacked. An inductive effect is the polarization of a chemical bond caused by the polarization of an adjacent bond. An inductive effect may be defined as a permanent displacement of electrons forming a covalent bond towards a more electronegative atom or group of atoms. Groups of atoms attached to a carbon chain will have electron attracting or repelling tendencies, depending upon their electro negativity as compared to the carbon atom to which they are attached. The decreasing order of -$I$ effect and the increasing order of +$I$ effect shown by different functional groups are [NO$_2$, CN, F, Br, I, OH, OCH$_3$, C$_6$H$_5$, H] and (-CH$_3$,-C$_2$H$_5$,-CH(CH$_3$)$_2$,-C(CH$_3$)$_3$] respectively.
The mesomeric effect or resonance effect in chemistry is a property of substituents or functional groups in a chemical compound. The effect is used in a qualitative way and describes the electron withdrawing or releasing properties of substituents based on relevant resonance structures and is symbolized by the letter M. The mesomeric effect is negative (-M) when the substituent is an electron withdrawing group and the effect is positive (+M) when based on resonance the substituent is an electron releasing group.

The net electron flow from or to the substituent is determined also by the inductive effect. It's important to note that the mesomeric effect as a result of p-orbital overlap (resonance) has absolutely no effect on this inductive effect, as the inductive effect is purely to do with the electro negativity of the atoms and their structural chemistry (which atoms are connected to which).

The mesomeric effect refers to the polarity produced in a molecule as a result of interaction between two $\pi$ bonds or a $\pi$ bond and a lone pair of electrons. This effect is transmitted along a chain, especially in conjugated systems, in which the electrons get delocalized, making possible a number of resonance or mesomeric structures for the molecule.

The +M effect is shown by groups having lone pairs of electrons (e.g. -Cl : , -Br : , -I : , NH$_2$, -OH, -OCH$_3$ etc.) The -M effect is shown by groups such as, $\stackrel{\circ}{\boxed{\text{O}}}$, -NO$_2$, $\text{-C} \equiv \equiv \text{N}$, -SO$_3$H etc. and is due to the presence of an electronegative atom like oxygen or nitrogen functioning as an electron sinks.

It is more prominent in aromatic compounds where an atom or group is directly attached to a benzene ring having conjugated double bonds.
**Selection of reagents for organic analysis**:

Several papers are being published every year on the reactions and possible applications of new and old organic 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 economies 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 a 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 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 to determination by high performance liquid or gas liquid chromatography. Many compounds containing polar functional groups show unfavourable properties such as low volatility, tailing, irreversible adsorption on to many column packings 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 technique.

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.

8. Reagents labelled with radio isotopes may transfer the isotopic label to the derivative of the compound under investigation.

**Selection of drugs for analysis:**

The survey of literature showed that, very few visible spectrophotometric and HPLC methods for the analysis of selected drugs were reported at the time of the commencement of this investigation. A detailed account of all analytical methods existing for the drug is made to avoid duplication of the method developed. Details about the structure of
the drugs and their physicochemical properties are also collected to find out the stability and homogeneity of the sample solutions. The choice of these analytes results from the presence of analytically potential functional groups, aromatic ring resonance of the selected drugs along with simplicity of the spectra and their direct relationship to molecular functional groups results in easy & robust calibrations. The author has made successful attempt in exploiting these features in development of new analytical method.

A wide variety of biologically active agents ranging from simple ions [eg: lithium] and low molecular weight organic molecules [Caffeine & Hydrocortisone] to high molecular weight polypeptides [growth hormonones] can be considered as drugs. The emergence of many basic concepts of pharmacology and chemistry provide a theoretical frame work for learning about vast range of drugs. A mixed classification also has been given in some literature for the sake of convenience. This includes some of the following widely used therapeutic agents, taken up for present investigation.

**Anti bacterial agents:**

An antibacterial is a compound or substance that kills or slows down the growth of bacteria\(^\text{18}\). The term is often used synonymously with the term antibiotic(s); today, however, with increased knowledge of the causative agents of various infectious diseases, antibiotic(s) has come to denote a broader range of antimicrobial compounds, including anti-fungal and other compounds\(^\text{19}\).
Teicoplanin is actually a mixture of several compounds, five major (named teicoplanin A2-1 through A2-5) and four minor (named teicoplanin Rs-1 through Rs-4).  

Teicoplanin is an antibiotic used in the prophylaxis and treatment of serious infections caused by Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus and enterococcus faecalis. It is a glycopeptide antibiotic extracted from Actinoplanes teichomyceticus, with a similar spectrum of activity to vancomycin. Its mechanism of action is to inhibit bacterial cell wall synthesis.

**Antiretroviral:**

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. When several such drugs, typically three or four, are taken in combination, the approach is known as Highly Active Antiretroviral Therapy, or HAART. The American National Institutes of Health and other organizations recommend offering antiretroviral treatment to all patients with AIDS. Because of the complexity of selecting and following a regimen, the severity of the side-effects and the importance of compliance to prevent viral resistance, such organizations emphasize the importance of involving patients in therapy choices, and recommend analyzing the risks and the potential benefits to patients with low viral loads.

There are different classes of antiretroviral drugs that act at different stages of the HIV life-cycle.
Antiretroviral (ARV) drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits.

- Nucleoside and nucleotide reverse transcriptase inhibitors (NRTI) inhibit reverse transcription by being incorporated into the newly synthesized viral DNA strand as a faulty nucleotide. This causes a chemical reaction resulting in DNA chain termination.: Emtricitabine, Tenofovir.

- Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virons.: Atazanavir.

  **Atazanavir** is used in the treatment of HIV infection. AIDS is caused by infection with HIV. This virus invades cells of the immune system, particularly the white blood cells known as CD4 T-helper lymphocytes\(^{22}\). These cells normally work to activate other cells in the immune system to fight infection. Atazanavir is a protease inhibitor. Protease inhibitors prevent the protease enzyme from working. HIV protease acts like a chemical scissors. It cuts the raw material for HIV into specific pieces needed to build a new virus. Protease inhibitors "gum up" these scissors.

  **Emtricitabine** is used in the treatment of HIV infection. Since HIV kills CD4 T-helper cells, the body cannot fight the virus or subsequent infections. Once the virus is inside the CD4 T-cell it
multiplies. When viruses multiply the genetic material is copied (this involves the conversion of RNA to DNA)\textsuperscript{23}.

\textbf{Tenofovir} is an inhibitor of the human immunodeficiency virus (HIV) protease. HIV protease is an enzyme that is essential for viral growth. It is responsible for the post-translation modification of core protein into structural protein\textsuperscript{24}. The drug Tenofovir is effective in reducing HIV RNA levels and increasing CD4\textsuperscript{+} lymphocytes. The drug is used in combination with other antiretroviral medications as part of a potent antiretroviral regimen.

\textbf{Antimetabolite} is a chemical that inhibits the use of a metabolite, which is another chemical that is part of normal metabolism\textsuperscript{25}. Such substances are often similar in structure to the metabolite that they interfere with, such as the antifolates that interfere with the use of folic acid. The presence of antimetabolites can have toxic effects on cells, such as halting cell growth and cell division, so these compounds are used as chemotherapy for cancer\textsuperscript{26}.

\textbf{Pemetrexed Disodium} is used in mesothelioma, a rare cancer of the lungs often related to exposure to asbestos non-small cell lung cancer, a type of lung cancer. It is a folate analog metabolic inhibitor indicated for non-squamous non-small cell lung cancer, initial treatment in combination with cisplatin, nonsquamous non-small cell lung cancer\textsuperscript{27} as a single-agent after prior chemotherapy and in mesothelioma in combination with cisplatin.
Capecitabine is an orally-administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is a prodrug, that is enzymatically converted to 5-fluorouracil in the tumor, where it inhibits DNA synthesis and slows growth of tumor tissue. The activation of Capecitabine follows a pathway with three enzymatic steps and two intermediary metabolites, 5'-deoxy-5-fluorocytidine (5'-DFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR), to form 5-fluorouracil.

These methods are applied to drug determination during routine analysis in various pharmaceutical dosage forms. The author has made some humble attempts, hoping to fill this gap and succeeded in developing analytical methods using spectrophotometric methods.

Types of reactions and reagents involved in the proposed spectrophotometric methods:

Different types of reactions are involved in the proposed methods. A brief discussion of these reactions and the reagents used to bring about these reactions is presented here.

Oxidative coupling reactions:

Oxidative coupling procedure involving the use of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 4-aminophenazine (4-AP) in the presence of an appropriate oxidant under acidic, neutral or alkaline conditions to form highly coloured products were explored for the determination of some of the mentioned active pharmaceutical ingredients possessing amenable structural feature.
3-Methyl-2-benzothiazolinone hydrazone the reported applications of MBTH include the detection and determination of phenols²⁹-³¹, formaldehyde³² and other aldehydes³³,³⁴, polyhydroxy compounds³⁵,³⁶, aromatic amines including heterocyclic amines such as antipyrine and amidopyrine³⁷, iminohetero aromatic compounds, indoles, carbazoles and phenothiazines³⁸. MBTH was also used for the indirect determination of compounds such as glycerides³⁹ and glycosamine glycans⁴⁰ 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. Friestet al.⁴¹ initially carried out, the coupling under alkaline conditions with ferricyanide, but a modification, to use Cerric ammonium sulphate in acidic medium was successful and led to an increased sensitivity³⁰. Later Umeda⁴² carried out the reaction using a basic solvent (triethanolamine) with phenols. El-Kommos⁴⁰ suggested Cerric 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.⁴⁸. [in neutral conditions] and Pays et al.⁴⁷. [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 alcohols⁴³ [after oxidation with ruthenium
textraoxide) and phenidone. Recently, Sastry and Sastry reviewed various aspects of MBTH chemistry in pharmaceutical analysis.

Under the reaction conditions, MBTH loses two electrons and one proton on oxidation, forming the electrophilic intermediate which has been postulated to be the active coupling species. The intermediate 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 with drawing 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 coloured species.

The \( \lambda_{\text{max}} \) and the \( \varepsilon_{\text{max}} \) values of the coloured 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. In a few instances the oxidative coupling products (conjugated chromophore, electron donor) may form a complex with oxidised or reduced form of the oxidant [eg. Cr (VI) or Cr (III)] possessing in completely filled 3d orbitals.

Since some of the antiviral agents under investigation possess (EMT and PEM) structural features that are likely to enable them to react with MBTH. The attempts were made by the 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 IV [EMT] and chapter VI [PEM] of the thesis.

4-APA – OXIDANT: (CAPA)

In 1940 the highly sensitive colour reaction of phenol with 4-Aminophenazone was introduced into analytical practice by Emmerson46. 4-Aminophenazone on condensation with phenols in presence of an alkaline oxidizing agent forms a red coloured antipyrine dye47 which has been used extensively for determination of trace amounts of phenols48-53. The sensitivity is greatly increased, with little hypsochromic shift in the Amax by extracting the reaction products with organic solvents. The colours of the aqueous reaction mixtures are generally found to be less stable (40-50 mins) when compared to organic solvent extracts (3 hr or more).

The antipyrine dye formation reaction has been utilised for the estimated of Phenol54-58, resorcinol59,60, catecho61, hydroquinone60, 1-naphthol59-62, 2-naphtho59, cresol60-64, thymo59, p-cresol69, salicylamide 59, guaiaco51, tyrosine65,66, piroxicam68, pyrazolone derivatives58 primaquine71 and muzolimine70. The antipyrine dyes formed with catechol and resorcinol67 are sufficiently acidic and are therefore not extractable into chloroform from the aqueous phase. Rosenblatt et al. took advantage of these properties to determine small amounts of guaiacol in the presence of catecho51. Miyamoto et al.
estimated phenolic compounds with 4-Aminophenazone and hydrogen peroxide in the presence of peroxidise enzyme at pH 7.25. Emerson\textsuperscript{47} postulated that the treatment of phenol, enol or amine with 4-Aminophenazone and an oxidant under neutral or alkaline conditions forms an oxidative coupling product (antipyrine dye). Emerson and Beegle\textsuperscript{48} observed that some compounds containing the keto-enol system in their ring structures undergo oxidative coupling reaction with 4-Aminophenazone in the presence of an oxidant. Systematic investigations revealed that 4-APA in combination with sodium periodate produces stable and high intense colored oxidative product suitable for the assay of Capecitabine. The coloured product formation based on analogy in chapter VII.

**Ion association complex formation reactions:** \((ATZ_A, ATZ_B, EMT_C, TEF_A \text{ and } TEF_B)\)

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{72-74}. 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, coloured, 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 colourless, possessing opposite charge (anionic or cationic) to that of chromogen.

Acid dyes such as Mordant Black-III, Solochrome Black-T, Metanil Yellow dye, Bromophenol blue, Bromocresol green, Bromocresol red, Eosin, Methyl orange, Phenol red, Thymol blue, Alizarin violet 3B, Alizarin brilliaint violet R, Fast Green FCF and organe-II have been used arbitrarily by various workers for the determination of components exhibiting basic properties e.g. Amines, quaternary ammonium compounds and heterocyclic compounds. Some basic dyes (parent moiety - xanthene, triphenyl methane, azine, oxazine and thiazine) and metal complexes (e.g. ferrous orthophenanthroline complex) have been tried by some workers for the determination of components exhibiting acidic properties.

In the present investigations, it was found that ATZ, a widely used antiviral agents forms an association complex with the Mordant Black-
III (Chapter III, Method A) and Solochrome Black-T (Chapter III, Method B), EMT with Mordant Black-III (Chapter IV, Method C) and TEN with Metanil Yellow (Chapter V, Method A) and Solochrome Black-T dye (Chapter V, Method B).

**Charge transfer molecular complex formation using Ferric chloride and 1, 10-phenanthroline [TEPA] / 2,2'-bipyridyl [TEPb and TENc].**

Charge transfer transitions between a metal ion and complexing ligand are very intense. In such transitions, a redox reaction occurs between the metal ion like Iron and the ligand (1, 10-phenanthroline/2,2'-Bipyridyl). Metal complexes of this type are intensely colored due to their high absorption and are well suited for the detection by visible spectrophotometry. Iron (Fe) exhibits variable valency and exists as ferrous (Fe II) and ferric (Fe III) salts. The former one (Fe II) acts as a reductant and also involves in complex formation with certain types of compounds such as 1,10-phenanthroline, bipyridyl, triazine, hexacyanoferrate III, [Fe(CN)6] giving colored species of high tinctorial value. The latter one (Fe III) functions as an oxidant and also has a tendency to give high intense colored species with potassium thiocyanate, phenolic compounds, oxamic ester and few other bifunctional substrates possessing complexing ability to give four or five membered cyclic compounds. There are several reports with the usage of reagent combination such as FeCl3/1,10-phenanthroline, triazine or hexacyanoferrate (III) for the estimation of certain bioactive compounds,
which have a tendency to be oxidized by Fe(III) and the resulting reduced form Fe(II) was estimated by any one of the reagents mentioned above.

Phenanthroline and bipyridyl are particularly well suited for complex formation, since the nitrogen unshared electron pairs are directed so that both nitrogen atoms can participate in bonding to a metal ion such as Iron. The Fe (II) binds three molecules of phenanthroline and bipyridyl to form ferroin (Tris-(1,10-phenanthroline)iron(II) and (Tris-(2,2'-bipyridyl) iron(II)).

In the present investigation, the above mentioned chromogenic reagents are used to estimate Teicoplanin (Chapter II) and Tenofovir (Chapter V).

**Oxidant mixture Iron (III) as an oxidant & Potassium ferricyanide [TEPc]:**

Iron (Fe) exhibits variable valency and exists as ferrous (Fe II) and ferric (Fe III) salts. The former one (Fe II) acts as a reductant and also involves in complex formation with potassium ferricyanide to form a prussian blue complex. The (Fe III) functions as an oxidant and also has a tendency to give high intense colored species with potassium thiocyanate, phenolic compounds, oxamic ester and few other bifunctional substrates possessing complexing ability to give four or five membered cyclic compounds.

In the present investigation, the above mentioned chromogenic reagent is used to estimate Teicoplanin (Chapter II).
Condensation Reactions using PDAC & PDAB in acidic media
[EMTa & PEMc]:

PDAC is a useful analytical reagent\textsuperscript{91} which has been utilized for the detection and spectrophotometric determination of aromatic and aliphatic primary and secondary amines. PDAC and PDAB has demonstrated a wide usefulness as a chormogenic reagent for spectrophotometric analysis of several compounds such as urea, thiourea and their N-alkyl/aryl derivatives\textsuperscript{92}, aceclofenac\textsuperscript{93}, sodium diclofenac\textsuperscript{94}, oxyphenbutazone\textsuperscript{94}, glafenine and metoclopramide\textsuperscript{95}. PDAC and PDAB has also been used for microplate-based assay of p-aminohippuric acid\textsuperscript{96} in plasma and urine samples, and for rapid testing of benzodiazepines\textsuperscript{97}, Nimesulide\textsuperscript{99}, Satranidazole\textsuperscript{100} and Sympathomimetic amines in pharmaceuticals. The use of modified dimethylaminocinnamaldehyde has also been described for analysis of flavanols in wines\textsuperscript{98}. The majority of the methods mentioned above involve a reaction in acid medium with heating to produce colored compounds [ranging from orange to red or pink]. In our study an orange red colored chromogen is produced with Emtricitabine and Pemetrexed Disodium. The reaction between PDAB/PDAC with primary amino group [in PEM and EMT] is assumed to take place via condensation amino group with the carbonyl group of the reagent to produce an iminum salt\textsuperscript{93,94}. The probable mechanism for this reaction is shown in scheme (Chapter IV and Chapter VI)- which is to a large extent based on reactions suggested in the literature.
1, 2-naphthoquinone-4-sulfonic acid (NQS) or Folin's Reagent\textsuperscript{101} [PEM\textsubscript{A} and CAP\textsubscript{B}]:

NQS was previously reported to be a sensitive chromogenic reagent for several primary and secondary amines, amides and phenols. Drugs like prazosin, procaine HCl, antipyrine, novalgin and phenolic compounds were analyzed by this reagent.

NQS (folin's) reagent has been used as a chromogenic agent for a number of compounds containing a free amino group. Aqueous solutions fade slowly on exposure to light but may be stabilized by hydrochloric acid.

In the present investigation, the above mentioned NQS reagent is used to estimate Pemetrexed Disodium (Chapter VI) & Capecitabine (Chapter VII).

**Systematic study in UV-Visible Spectrophotometry\textsuperscript{102, 103}:**

All molecules can absorb radiation in the UV-Visible region because they contain electrons, both shared and unshared, which can be excited to higher energy levels. The wavelength at which absorption occurs depends upon how firmly the electrons are bound in the molecule. The electrons in a single covalent bond are tightly bound, and radiation of high energy or short wavelength, is required for their excitation, which shows no absorption above 160 nm. This means the electron in a sigma-bonding orbital is excited to sigma-antibonding orbital.

Many years ago, the association between unsaturation and absorption of light was recognized by organic chemists and the term
"chromophore" was introduced to describe the role of C=C, C=O, and N=N in shifting the absorption of light towards visible region. Most applications of UV-Visible spectrophotometry to organic compounds are based on n-\(\pi^*\) or \(\pi-\pi^*\) transitions and hence require the presence of chromophores in the molecule. These transitions occur in the region of the spectrum about 380-760 nm which is convenient to use experimentally.

A characteristic of the absorption spectrum is the position of the peaks of light absorption by the substance and also by the intensity of absorption. The intensity of the color is measured by spectrophotometer in the visible region.

**Development of a method:**

The first step in developing the spectrophotometric method is the choice of the absorption band at which absorbance measurements are made. A few analytes have specific absorbances that are sufficiently high to allow them to be quantified by direct measurement. However, in the majority of cases the analyte must be reacted with a spectrophotometric reagent (chromogenic compound) to form a highly absorbing derivative. Not all chromogenic compounds are suitable for quantitative measurements, and the choice of reagent is principally influenced by the chemistry of the species to be determined. When selecting or developing a procedure, the following factors should be investigated, either by direct experiment or by reference to the literature.
1. **Color development:**

   The colored derivative must be sufficiently stable to allow a reliable measurement to be made. The absorbance should be stable for at least 30 minutes after preparation of the derivative, and should not be affected by minor variations in pH, ionic strength or temperature.

2. **Reagent stability:**

   The storage requirements and lifetimes of the reagents must be established. If, for example, a reagent must be stored in the dark or at low temperature, the necessary storage facilities and procedures must be used. Each reagent must be replaced at the end of its life, and a new calibration should be prepared for each new batch of reagent.

3. **Reagent selectivity:**

   The degree of selectivity of a complexing agent should be understood. The effect of other species likely to be present must be considered, and also the effect of an excess of the reagent. If the reaction goes to completion, and the product and reagent absorb at different wavelengths, it is often satisfactory, and very convenient, to use an excess of the reagent.

4. **Conformity to the Beer-Lambert’s law (and the value of the molar absorptivity & Sandell’s Sensitivity)**

   The product of the reaction should obey the Beer-Lambert law over a wide range of concentrations, thus producing a linear calibration graph.
Molar absorptivity, is a measurement of how strongly a chemical species absorbs light at a given wavelength. It is an intrinsic property of the species; the actual absorbance, ‘A’ of a sample is dependent on the pathlength ‘l’ and the concentration ‘c’ of the species via the Beer-Lambert’s law, $A = εcl$.

The SI units for $ε$ are m²/mol, but in practice, they are usually taken as $M^{-1} \text{cm}^{-1}$ or $L \text{ mol}^{-1} \text{ cm}^{-1}$. In older literature, $\text{cm}^2 \text{ mol}^{-1}$ is sometimes used with corresponding values 1000 times larger. These units may look different, but it is just a matter of expressing volume in $\text{cm}^3$ or in $L$.

Different disciplines have different conventions as to whether absorbance is Naperian or decadic, i.e. defined with respect to the transmission via natural or common logarithm. The molar absorption coefficient is usually decadic, but when ambiguity exists it is best to qualify it as such.

The molar extinction coefficient should not be confused with the different definition of "extinction coefficient" used more commonly in physics, namely the imaginary part of the complex index of refraction (which is unitless). In fact, they have a straightforward but nontrivial relationship; see Mathematical descriptions of opacity.

In biochemistry, the extinction coefficient of a protein at 280 nm depends almost exclusively on the number of aromatic residues, particularly tryptophan, and can be predicted from the sequence of amino acids. If the extinction coefficient is known, it can be used to determine the concentration of a protein in solution.
When there is more than one absorbing species in a solution, the overall absorbance is the sum of the absorbances for each individual species (X, Y etc.):

\[ A = (\varepsilon_X c_X + \varepsilon_Y c_Y + \cdots) l, \]

The composition of a mixture of N components can be found by measuring the absorbance at N wavelengths (the values of \( \varepsilon \) for each compound at these wavelengths must also be known). The wavelengths chosen are usually the wavelengths of maximum absorption (absorbance maxima) for the individual components. None of the wavelengths must be an isosbestic point for a pair of species. For N components with concentrations \( c_i \) and wavelengths \( \lambda_i \), absorbances \( A(\lambda_i) \) are obtained:

\[ A(\lambda_i) = l \sum_{j=1}^{N} \varepsilon_j (\lambda_i) c_j. \]

This set of simultaneous equations can be solved to find concentrations of each absorbing species.

The molar extinction coefficient \( \varepsilon \) (if expressed in units of \( \text{L mol}^{-1} \text{ cm}^{-1} \)) is directly related to the Absorption cross section \( \sigma \) (in units of \( \text{cm}^2 \)) via the Avogadro constant:

\[ \sigma = 1000 \ln(10) \frac{\varepsilon}{N_A} = 3.82 \times 10^{-21} \varepsilon. \]

The molar absorptivity is also closely related to the mass attenuation coefficient, by the equation

\( \text{(Mass attenuation coefficient)} \times \text{(Molar mass)} = \text{(Molar absorptivity)}. \)
5. Interfering substances:

One of the major problems lies in the extent of interference from other constituents of a sample. A variety of techniques are available to minimize this interference, ranging from the use of masking agents, or pH control, to a whole diversity of computer based mathematical solutions. If no solution is available using these techniques, the interfering substances must be removed during sample preparation, for example using chromatography or solvent extraction.

**Calculation of effective concentration range:**

One approach to this problem was proposed by Ayres\textsuperscript{102}, based on an earlier proposal by Ringbolm. Rather than plotting absorbance against known concentrations, $T$ is plotted against the logarithm (base 10) of the concentration. The linear region of maximum slope indicates the range of concentration that will give maximum sensitivity and linearity for the determination. This method assumes constant instrumental error across the whole transmission range, which is in fact an invalid assumption.

A more rigorous method for estimating the region of precision for a selected method is given by Youmans & Brown\textsuperscript{103}, where statistical data from a series of measurements is used to generate an absorbance versus error curve.
1.3 VALIDATION:

Having established the methodology, and the range over which the calibration is applicable, the final task in any good analytical process must be validation of the procedure. Essentially three processes are available to the user, which may be selected as required.

1. Check using standard reference materials (SRM):

Standard reference materials are used in complex matrices e.g. biological fluids, foods, sea water, etc, in which all the analytes of interest have been determined. The quoted concentrations are validated by various statistical processes, and are usually expressed as a definitive figure with an attached ± tolerance. This is usually the preferred procedure, but it is often limited by the availability of a suitable material.

2. Check using values achieved by an unrelated technique:

The analyte in question is determined by another technique, the fundamental principle of which is totally different to UV-Visible spectrophotometry. Chromatography (HPLC or TLC) is often the chosen technique. This procedure relies upon the essential validation of the alternative technique.

3. Addition of known amounts of the analyte of interest at the start of the procedure and then determination of the actual amounts at the end:

These ‘recoveries’ are usually expressed as a percentage of the original concentration added, and will, of course, reflect interference on the determination by the matrix. Ideally recoveries of about 100% should be achieved.
4. Establishment of optimum conditions of the method:

The basis of most Spectrophotometric methods, is usually

i) Complex formation reaction.

ii) An oxidation-reduction process

iii) A catalytic effect.

In each type of reaction, the yield of colored species whose absorbance is measured and thus the sensitivity of the method, rate of color formation and stability is effected by the concentration of the reagent in the solution, the nature of the solvent, the temperature, the pH of the medium, order of addition of reactants and waiting periods. It is necessary to establish the optimum conditions in the procedure to be developed through control experiments by varying one among the above parameters and keeping others constant at a time and measuring the absorbances at $\lambda_{\text{max}}$. The range of different parameters within which attainment of high absorption at $\lambda_{\text{max}}$ coupled with maximum intensity and stability of the colored species is achieved, are known as the optimum conditions.

1.4 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 total 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 types 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:**

\[ E = h\nu \]

Where; \( E \) = Energy of radiation

\( h \) = Plank's constant \((6.625 \times 10^{-34} \text{ J/sec})\)

\( \nu \) = Frequency of radiation

**Types of spectroscopy:**

1. Based on the study of the spectra obtained that may 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. U.V 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. U.V. Visible Spectroscopy, IR. 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 a light from a ultra-violet lamp through a sample of an organic molecule, 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 electro magnetic 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.
Table: 1.2- 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 with in 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>

**Spectrophotometers:**

These are little more expensive than colorimeters. They can be used for a wide wavelength region i.e. 200nm to 800nm. The accuracy of the instrument is very high since they employ gratings, monochromators and photomultiplier tubes. Amplifiers, recorders or plotters for hard copy to support them, the recent ones are the microprocessor or computer based for easy data manipulation. They are designed to read % transmittance or absorbance, record the absorption spectrum using a plotter or recorder, are of double beam type where we can use sample and reference solution at a time.

Wavelength accuracy of such instruments is ± 1.0 nm. These instruments are hence more accurate and reliable than the other types.
The spectrophotometers used to record the UV/Visible, IR; NMR spectra vary enormously, and yet have certain essential features in common:

1. A source of radiation of appropriate frequency range (a UV or IR lamp, a radio frequency transmitter, etc.)
2. A sample holder to permit efficient irradiation of the sample.
3. A frequency analyser which separates out all the individual frequencies generated by the source (the most familiar being the triangular glass prism as used by Issac Newton for visible light)
4. A 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.
5. A recorder or a computerized data station, with a VDU for initial viewing of the spectrum.

Figure 1.2: Spectrophotometer common feature:
UV-Visible Spectroscopy:

Figure 1.3: UV-Vis spectrophotometer:

*Ultra-Violet (UV) Spectroscopy* is concerned with the study of absorption of UV-radiation, which ranges from 190nm – 380nm.

*Visible Spectroscopy* is concerned with the absorption of visible radiations whose wavelengths range from 380nm to 760nm.

Figure 1.4: Wavelength range:
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, $\Delta E$, and therefore, the lower the frequency, the longer the wavelength, $\lambda$. 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 ($\pi \rightarrow \pi^*$) and C=O ($n \rightarrow \pi^*$) systems.

The absorption of UV and Visible spectroscopy, 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.
Law of light absorption- Beer-Lambert's law:

These are the two empirical laws, which govern the absorption of light by molecules. Beer's law relates the absorption to the concentration of absorbing solute, and Lambert's law relates the total absorption of optical path length. They are most conveniently used as the Beer-Lambert's Law.

According to the **Beer-Lambert's law:**

"The intensity of absorption is directly proportional to the concentration of the sample and the path length of the sample."

**Mathematical equation for Beer-Lambert's law:**

\[ A = \varepsilon Cl \]

Where;

- \( A \) = Absorbance or Optical Density
- \( \varepsilon \) = Molecular Extinction Coefficient
- \( C \) = Concentration of the drug (m mol/lit)
- \( l \) = Path Length (usually 10mm or 1cm)
Deviation from Beer's law:

1. Dilute solutions (true deviations):
Applicable for dilute solutions only. The index of refraction for the absorbed radiation is changed at high concentration and hence, Beer's law is not obeyed.

2. Instrumental deviation:
- Stray radiation reaching the detector
- Sensitivity changes in detector employed
- Fluctuation of radiation source
- Defect in detector amplification system

3. Chemical deviation:
The absorbing species in the solution may undergo ionization, dissociation or even may react with the solvent. These processes may produce two or more species in the solution with varying absorptivity values.

   It can be corrected by the use of buffers, choosing suitable solvent, and by selecting appropriate narrow band of wavelength for measurements.

1.5 INSTRUMENTATION:

1. Source of Radiation:
UV-Visible spectrophotometer uses two light sources, a deuterium (D₂) 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 efficient radiation of the sample. These are made of quartz, fused silica glass.

5. Frequency Analyser:  
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:

- Quantitative determination of chromophore concentrations in the solution.
- Impurity determination by spectrum subtraction.
- Structural elucidation of organic compounds (saturation and unsaturation, and the extent of aromatic).
- Determination of pKa values of indicators.
- Determination of molecular weights of amines.
- Identification of cis-trans isomer: trans isomer absorbs less energy than cis isomer.
- Chemical kinetics: The rate of reaction is calculated as the change in absorbance per unit time.
- Qualitative and quantitative analysis of materials used in dye and pharmaceutical industries.
- UV-Visible spectrophotometer used in quality control department.

9. Limitations:
- UV and Visible light have high energies which may decompose the organic compounds.

**Transmittance, Absorbance and the Beer-Lambert's Law:**

We define **transmittance** as the ratio of the amount of light transmitted to the amount of light that initially fell on the surface.

\[
\text{Transmittance} = \frac{P}{P_0} = \frac{\text{intensity of transmitted light}}{\text{intensity of incident light}}
\]

**Absorbance** is defined as the negative logarithm of the transmittance, and will be noted that absorbance & transmittance bear an inverse relationship.

\[
\text{Absorbance} = -\log T = -\log \frac{P}{P_0}
\]
Beer-Lambert Law

Absorbance $\propto$ path length ($l$) $\cdot$ concentration

$$A = \varepsilon \cdot l \cdot c$$

Where

- $A$ is a dimensionless number.
- $\varepsilon$- is the proportionality constant, is called the molar extinction coefficient or molar absorptivity. It is a constant for a given substance, provided the temperature and wavelength are constant. It has units of liter/mol $\cdot$ cm.
- $l$ and $c$ have the usual units of length (cm) and concentration (mol/liter).

The quantitative measurement of light absorption as a function of wavelength can establish both the identity and the concentration of a substance in solution. The spectrophotometer is an instrument that separates electromagnetic radiation into its component wavelengths and selectively measures the intensity of radiation after passing through a sample.
VALIDITY OF THE BEER-LAMBERT'S LAW\textsuperscript{106}:

Beer's law is a limiting case applicable only to dilute solutions and samples. Apparent deviations may be summarized as follows:

1. At concentration $> 0.01\text{M}$, refractive index changes and the perturbing effect of solute molecules or ions on the charge distribution of their neighbours both affect the value of $\varepsilon$.

2. Solutes involved in chemical equilibria i.e., dissociation, association or complex formation or interaction with solvent molecules may show marked spectral changes with concentration.

3. Negative deviation occurs if the radiation used is polychromatic as in case of filter-photometers.

4. Stray-light passing through the optical system is the most common cause of negative deviation from Beer's law.

The best way to minimise the chemical deviations from Beer's law is by adequate buffering of the pH, adding a large excess of complexing agent, ionic strength adjustment and so forth. The absorptivity at a given wavelength may vary from instrument to instrument. Therefore always run a standard.

Analytical Method Validation:

Accuracy\textsuperscript{107}:

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 difference 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 quantitation 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 analytes 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 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 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:

- If there is no decimal point, the right-most non-zero digit is the least significant digits.
- 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:\(^{107}\):

**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) 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 anlayte in a sample that can be detected but not necessarily quantified.
**Student's t-test**: The Student's t-distribution was first introduced by W.S. Gossett in 1908 under the pen name Student. It is useful for:

- Establishing confidence limits (error bias) for the mean estimated from smaller sample sizes.
- Testing the statistical significance of a non-zero mean;
- Testing the statistical significance of the difference between means from two independent samples.

When using a t-test of significance, it is assumed that the observations come from a population which follows a normal distribution. The t-distribution is essentially a corrected version of the normal distribution in which the population variance is unknown and hence is estimated by the sample standard deviation.

**Independent one-sample t-test**:

In testing the null hypothesis that the population means is equal to a specified value \( \mu_0 \), one uses the statistic

\[
t = \frac{\bar{x} - \mu_0}{s/\sqrt{n}},
\]

where:
- \( s \) = sample standard deviation
- \( n \) = sample size.

Degrees of freedom used in this test is \( n-1 \)

\( \bar{x} \) is the average of the population

This is sometimes known as a sigma test.
Independent two-sample t-test\textsuperscript{109}: This test is only used when both

- the two sample sizes (that is, the \( n \) or number of participants of each group) are equal;
- It can be assumed that the two distributions have the same variance. The \( t \) statistic to test whether the means are different can be calculated as follows:

\[
t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_1X_2} \cdot \sqrt{\frac{2}{n}}}
\]

where \( S_{X_1X_2} = \sqrt{\frac{S_{X_1}^2 + S_{X_2}^2}{2}} \).

\( \bar{X}_1 \) group one average population.

\( \bar{X}_2 \) group two average population.

Here \( S_{X_1X_2} \) is the grand standard deviation (or pooled standard deviation), 1 = group one, 2 = group two. For significance testing, the degrees of freedom for this test is \( 2n - 2 \) where \( n \) = number of participants in each group. This is sometimes known as Welch's test.

F-test\textsuperscript{108}:

It is also known as Variance-ratio test. The \( F \)-test is used to test for differences among sample variance. Like the Student's \( t \), one calculates an \( F \) and compares this to a table value.

\[
F = \frac{s_1^2}{s_2^2}; \text{ The variance are arranged so that } F > 1. \text{ That is; } s_1^2 \text{ greater than } s_2^2
\]
We use the $F$-test as the Student's $t$ test, for testing the significant differences in the variances; invokes the null hypothesis that states that the two variances we are comparing are from the same population. (i.e., they are not statistically different). If the calculated $F$ is greater than the table value, then the null hypothesis is not correct. Else, the two could have come from the same population of measurements.

**CHEMISTRY OF THE COLOURED SPECIES FORMED:**

The chemistry behind the chromogen formation in each method is ascertained either through probability with the existing experimental evidence or the probable mechanism for this reaction, which is to a large extent based on reactions suggested in the literature.
REFERENCES


6. Rashmin, "Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals", in Latest Reviews, 2008, 6(4), 1.


