Chapter - I

Introduction
Analytical Chemistry plays an important role since the early days of Chemistry. It provides the methods for identification, separation, quantification and structural characteristics determining the nature of chemical elements and compounds present in the world\textsuperscript{1}. The importance of its study is imperative because it plays a critical role in the understanding of basic science to a variety of the practical applications in industry and medicine.

Also analytical chemistry has become an indispensable subject in the development of nanotechnology, surface characterization instruments, electron and scanning probe microscopy that enable Scientists to visualize atomic structure with chemical characterizations.

The recent developments of computer automation and information technologies have shrunk analytical techniques to chip size (Lab – on – a- chip). Potential advantages of this development are reduction of quantity of chemicals, size of equipment and overall cost of analysis with enhanced speed. It is called as micro Total Analysis System (\textmu TAS). These developments in analytical techniques led to the successful analysis of the complex biological systems and led to the birth of a number of the omics such as Genomics (DNA sequencing, Genetic finger printing, human genome decoding), and Proteomics (peptide sequencing in proteins) etc.

\textmu TAS is considered as a great promise of revolutionary analytical technology that controls and analyses single cells and single molecules. This cutting edge technology has a potential of leading a new revolution in science as integrated circuits did in computer developments.

Modern analytical chemistry plays an increasingly important role in the pharmaceutical industry where aside from quality assurance it is used in discovery of new drug candidates and in clinical applications where understanding the interactions between the drug and the patient are critical\textsuperscript{2-7}.

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical
formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data. Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities.

The quality and safety of a drug is generally assured by monitoring and controlling the assay and impurities effectively. While assay determines the potency of the drug and impurities will determine the safety aspect of the drug. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis. Assay of pharmaceutical products plays an important role in efficacy of the drug in patients. The impurity profile of pharmaceuticals is of increasing importance as drug safety receives more and more attention from the public and from the media.

The wide variety of challenges is encountered while developing the methods for different drugs depending on its nature and properties. Depending on mechanism of action, drugs are formulated into different pharmaceutical dosage forms like Tablets, hard gelatin capsules, soft gelatin capsules, injections etc. Depending on target site of absorption of the drug, drugs are formulated into different dosage forms with different delivery mechanisms like immediate release, delayed release and extended release. Depending on delivery mechanism needed, different kinds of excipients are used in formulations to achieve target release profile of the drug in human body. Depending on the formulation matrix chosen, the complexity of extracting the drug and its impurities from formulations will vary. This along with the importance of achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility and accuracy of results gives an opportunity for researchers to come out with solution to address the challenges in getting the new methods of analysis to be adopted by the pharmaceutical industry and chemical laboratories.

The drugs may be classified according to their chemical structure or therapeutic action, as chemotherapeutic agents and pharmacodynamic agents. Their action and classification is the subject of any postgraduate course in pharmacy or
medicinal chemistry and forms the content of many textbooks\textsuperscript{8-12} and hence needs no reproduction.

Drugs play a vital role in the progress of human civilization by curing diseases. The word drug is derived from the French word drogue, which means a dry herb. In general, a drug may be defined as a substance used in the prevention, diagnosis, treatment or cure of diseases in man or other animals. According to World Health Organization (WHO), a drug may be defined as any substance or product which is used or intended to be used for modifying or exploring physiological systems or pathological states for the benefit (physical, mental as well as economical) of the recipient.

An ideal drug when administered to the ailing individual or host, should satisfy the following requirements. Its action should be localized at the site where it is desired to act, should act on a system with efficiency and safety, should not have any toxicity, may have minimum side effects, should not injure host tissues or physiological processes, should not develop tolerance by the tissues even administered for long duration. Such drugs are rare and hence the search for ideal drug continues.

**Importance of Analytical methods for testing potency and impurities in drugs:**

Safety and efficacy of pharmaceuticals are two fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological-toxicological profile as well as the adverse effects caused by the impurities in bulk and dosage forms. The impurities in drugs often possess unwanted pharmacological or toxicological effects by which any benefit from their administration may be outweighed\textsuperscript{13}. Therefore, it is quite obvious that the products intended for human consumption must be characterized as completely as possible. The quality and safety of a drug is generally assured by monitoring and controlling the impurities effectively. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis. This has become quite clear by the recent research articles on this topic\textsuperscript{14-17}. Control is more important today than ever.
Until the beginning of the 20th century, drug products were produced and sold having no imposed control. Quality was generally not verified. Many products were patent medicines of dubious value. Some were harmful and addictive. In 1937, ethylene glycol was used as a vehicle for an elixir of sulfanilamide, which caused more than 100 deaths.\textsuperscript{18} There upon the Food, Drug and Cosmetic act was revised requiring advance proof of safety and various other controls for new drugs. The impurities to be considered for new drugs are listed in regulatory documents of the Food and Drug Administration (FDA)\textsuperscript{19}, International Conference on the Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)\textsuperscript{20} and United States Pharmacopoeia (USP). Nevertheless, there are many drugs in existence, which have not been studied in such detail. The USP and National Formulary (NF) are the recognized standards for potency and purity of new drugs. These compendia have become official upon adoption of the first food and drug act. They formulate legal standards of quality, purity and strength of new drugs. The good manufacturing practices provide minimum quality standards for production of pharmaceuticals as well as their ingredients.\textsuperscript{21} The ICH, which took place in Yokohama, Japan in 1995, has released new guidelines on impurities in new drug products.\textsuperscript{22}

These guidelines have a number of advantages, both for the industry and the regulators. The most critical aspect of the elaboration of the guidelines was the definition of the levels of impurities for identification and qualification. Qualification is the process of acquiring and evaluating data for establishing the biological safety of an individual impurity or a given impurity profile at the levels specified. The level of any impurity present in a new drug substance that has been adequately tested in safety and clinical studies is considered qualified. A rationalized for selecting impurity limits based on safety considerations has to be provided. Analytical procedures should be able to separate all the impurities from each other and the method should be optimized to separate and quantify them in the dosage forms. Such methods are to be validated demonstrating the accuracy, precision, specificity, limit of detection, limit of quantification, linearity range and interferences.

The validation of analytical procedures, i.e., the proof of its suitability for the intended purpose, is an important part of the registration application for a new
drug$^{23-24}$. Additional peak tailing, peak resolution and analyte recoveries are important in case of chromatographic methods.

The ICH has harmonized the validation requirements in two guidelines$^{25-26}$. The first one summarizes and defines the validation characteristics needed for various types of test procedures, the second one extends the previous text to include the experimental data required and some statistical interpretation. These guidelines serve as a basis worldwide both for regulatory authorities and industry and bring the importance of a proper validation to the attention of all those involved in the process of submission of drug master files. The analytical research and development units in the pharmaceutical industry are responsible for preparation and validation of test methods. Every country has legislation$^{27}$ on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles-general and specific-relating to individual drugs, and are published in the form of book called Pharmacopoeia (e.g. Indian, IP$^{28}$, United States, USP$^{29}$, European, EP$^{30}$, United Kingdom, BP$^{31}$, Martindale Extra Pharmacopoeia$^{32}$, Merck Index$^{33}$, etc.).

The monitoring of in-process impurities was an obscure and unidentified field about 20 years ago. Now it has become a major factor in modern pharmaceutical industry. This is mainly because of the pressure for product quality, and the demand for higher standards of process reliability. Toxicological issues have also brought about a greater sensitivity to the significance of impurities at trace levels$^{34}$. New attention has been given to the various classes of toxicants present as impurities in pharmaceutical products. In view of these changes it has become necessary to pay more attention to the origins and pathways of a host of impurities within the process. Frequently, impurities are formed as isomers of the desired reaction products and a critical impurity can often enter with the feed. Analytical identification of the problematic compounds is the first step towards the solution of the problem. Different analytical techniques like preparative HPLC, liquid-liquid extraction, Flash chromatography, Mass spectrometry, High Resolution mass spectrometry, NMR spectroscopy for characterization of impurities. Analytical methods used for Monitoring of the process reactions often lend valuable insight into the types of impurities that may be present. The purity of the final product may often be aided by controlling the purity of the materials used in its synthesis. Wherever possible, the
levels of impurities originating for the starting materials should be limited through appropriate in-process controls in order to avoid the need for their monitoring in the drug substance. The use of chromatographic techniques for monitoring the starting materials, intermediates, and the process reactions is an excellent means for controlling the purity of the final drug and thereby protecting the patient who ultimately receives it.

The best way to characterize the quality of a bulk drug is to determine its purity. There are two possible approaches to reach this goal. The determination of the active ingredient content with a highly accurate and precise specific method or the determination of its impurities. In the early years of drug analysis, when chromatographic techniques were not yet available the characterization of the purity of drugs was based on the determination of the active ingredient content by non-specific titrimetric and photometric methods supported by the determination of physical constants and some limit tests for known impurities based mainly on colour reactions. The deficiencies of this approach are well known. In many cases even highly contaminated drug materials could meet the requirements set in the early editions of different pharmacopoeias. As a consequence of the enormous development of the analytical technology in the last two decades entirely new possibilities have been created for the determination of the purity of drug materials. In principle, it is now possible to replace all non-specific assay methods with highly specific and precise HPLC methods thus greatly improving the value of the determination of the active ingredient content of bulk materials. Nearly all organic impurities are determined by chromatographic or related methods of which Liquid Chromatography (LC) has been the most important for over the last two decades. A thorough literature search has revealed that different methods of estimation of drugs and its impurities based on HPLC, Capillary Electrophoresis (CE), Gas-Liquid Chromatography (GLC), SFC, Thin-Layer Chromatography (TLC) etc. were published. LC has been the main technique used for analysis of impurities in drugs. Most used the reversed-phase mode with UV absorbance detection whenever appropriate, because this provided the best available reliability, analysis time, repeatability and sensitivity. In fact, this technique has set the standard against which others are compared. Recent advances like HPLC coupled with new advances in stationary phases like columns which are having 1.7 μm and 1.3 μm particles has revolutionized the separation science.
Today a majority of the drugs used are of synthetic origin. These are produced in bulk and used for their therapeutic effects in pharmaceutical formulations. These biologically active chemical substances are generally formulated into convenient dosage forms such as tablets, capsules, dry syrups, liquid orals, creams or ointments, parenterals (injections in dry or liquid form), lotions, dusting powders, aerosols, metered dose inhalers (MDI) and dry powder inhalers (DPI) etc.

These formulations deliver the drug substances in a stable, non-toxic and acceptable form, ensuring its bioavailability and therapeutic activity. In tablets one or more among the diluents such as lactose monohydrate, microcrystalline cellulose, hydroxy propyl cellulose, sodium starch glycolate, magnesium stearate, crospovidone, calcium phosphate, mannitol, sorbitol, sucrose, aerosil, acacia, gelatin, alginic acid, tragacanth, sodium stearyl fumarate, talc, waxes, methyl paraben, propyl paraben, meglumine, sodium benzoate, permitted flavors and colors are added. In capsules one or more among the excipients, certified dyes, gelatin, plasticizers, starch, lactose, talc, preservatives are added. In dry syrups and liquid orals, sucrose, sorbitol, preservatives, certified colors and flavors are added. In creams and ointments, waxes, carbopol, petroleum jelly, surfactants, preservatives, permitted colors and perfumes are added. In parenterals, water, vegetable oils, mineral oils, simulated oils, propylene glycol, dioxalamines, dimethyl acetamide are used as vehicles. Any one or more among stabilizers, anti-oxidants, buffering agents like citrate, acetate, phosphate, co-solvents, wetting, suspending and emulsifying agents like tween-80, sorbitol oleate and preservatives are added. In lotions, dusting powders and aerosols, talc, silica derivatives, alcohol, preservatives are added.

In view of wide variety of excipients used in formulating drugs for administration to patients, drug substances can undergo transformation by interacting with one or more components of the formulation. For example, drugs which contain primary amine as function group in their structure can undergo Millard reaction with reducing sugars like lactose. Drug substances can also react with trace impurities like formic acid, acetic acid, formaldehyde present in cellulose derivatives and peroxides present in excipients like povidone, cross povidone. Formulated drugs can degrade due to acidic or basic environments created due to the formulation matrix. Drugs can degrade due to exposure to temperature, humidity and light during manufacturing, transportation and storage during its shelf life. Due to this it is essential to know the
degradation pathways of the drugs in acidic, basic, neutral, oxidation conditions and their susceptibility to temperature and humidity to formulate them in a manner in which they are stabilized and retains its quality throughout their shelf life. As most drugs contains functional groups which can participate in reactions in some way or the other, it is essential that the analytical methods developed for estimation of the purity and impurities are capable enough to separate all the desired and undesired components and devoid of any interferences from the formulation matrix.

When analytical methods are able to precisely and accurately quantify without missing any impurities, without underestimation or over estimation, and detect all possible impurities and degradants those can form during stability studies with adequate sensitivity and exactly reflect the quality of drug substances and drug products (formulated products of drugs), those methods are called stability indicating methods.

In view of the foregoing discussion assaying and stability testing in pharmaceutical analysis occupies an important role to meet the requirement of statutory certification of drugs and their formulations by the industry. The complexity of problems encountered in pharmaceutical analysis coupled with the importance of achieving high selectivity, speed, cost, simplicity, sensitivity, precision and accuracy, new methods of analysis are being quickly absorbed by the pharmaceutical industry and chemical laboratories depending upon the facilities available.

Among the several instrumental techniques (HPLC GC, CE (Capillary electrophoresis), Fluorimetry, NMR, mass spectroscopy, spectrophotometry covering IR, Raman, UV and visible regions) available for the assay of drugs, usually visible spectrophotometric technique is simple and less expensive. The selectivity and sensitivity of the visible spectrophotometric method depends only on the nature of chemical reactions involved in color development and not on the sophistication of the equipment. Spectrophotometric analytical procedures are not generally stability indicating. Most widely used methods are based on HPLC, GC. Capillary electrophoresis and super critical fluid chromatography are slowly gaining ground in recent years.

A stability-indicating assay method should accurately measure the active ingredients, without interference from degradation products, process impurities,
excipients, or other potential impurities. If an industry uses a non-stability indicating analytical procedure for release testing, then an analytical procedure capable of qualitatively and quantitatively monitoring the impurities, including degradation products, should complement it. Analytical procedures for stability studies of assay should be stability indicating. As a result of stability testing a re-test period for the active substance or a shelf life for the pharmaceutical product can be established, and storage conditions can be recommended.

The ICH (International conference on Harmonization) guideline QIA on Stability Testing of New Drug Substances and Products\textsuperscript{38-39} emphasizes that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability indicating testing methods. It is also mentioned that forced decomposition studies (stress testing) at temperatures in \(10^0\text{C}\) increments above the accelerated temperatures, extremes of pH, under oxidative and photolytic conditions should be carried out on the drug substance and drug product so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures.

Formulations containing various drugs and combinations of drugs for potentiating or complementing one another in therapy are available in market. Pharmaceutical equivalents containing identical amounts of the same active ingredient(s) in the same dosage form and assay and impurities of drugs, HPLC is a versatile tool for the qualitative and quantitative analysis of drugs and pharmaceuticals and has become indispensable. HPLC technique has been regarded as the best among various instrumental techniques in spite of its cost and maintenance problems.

Keeping in view the above discussion, this study has examined the present state of development of such analytical methods for some of the widely used drugs. Hence, the study has proposed to develop stability indicating methods for assay and impurities for four most widely used drug products made up of drugs, namely, Evacetrapib (Cardiovascular disease), Niguldipine (Calcium channel blocker), Glisoxepide (anti-diabetic drug) and Lomitapide (anti-lower cholesterol). The above drugs are selected for research to which there is wide scope for the development of
new analytical methods for their assay and impurities determination by HPLC by exploiting their characteristics, physical and chemical properties.

**Selection of reagents for organic analysis**

Several papers are being published every year on the reaction and possible applications of new and old organic reagents in organic analysis (inclusive of drugs). 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 of economies of the reaction, the presence of other functional groups besides the chosen one that might be adversely affected by the reagents, the deactivation of the reaction center by steric and electronic effects, the instability or high reactivity of the desired product, the rate of the reaction, position of equilibrium as in the case of reversible reaction and other related factors. The objective is to get the best yields possible. The selection of a reagent for the determination of a particular compound is made after a literature survey for methods that have been under consideration. If much information is not found in this way, the reagent that acts most rapidly and stoichiometrically or at least giving reproducible results can be chosen after investigation of the performance of plausibly selected ones on a pure sample of the compound sought. Reagent selectivity for a particular functional group (in selected drug) is normally the minimum requirement specificity for a single compound containing the 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 reagent is the formation of one or more derivatives having measurable chemical or physical properties as completely different as possible from that of any of the reagents. Some examples of more specific reagents are as follows.

1. The reagent forms or destroys 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 the original sample and this property is the basis for gravimetric determination for
the isolation, concentration and purification of a compound examination by other analytical techniques.

3. The reagent generates a chromophore or reduces the concentration of the chromophore already present with change measured by one of the spectrophotometric techniques, ultraviolet, infrared. Many spot tests for the functional groups or spraying agents in TLC depend on the formation of colored derivatives. Similarly, a fluorophore may be produced or quenched and the change measured fluorimetrically.

4. The reagents act on the sample to produce a gas measurable manometrically one that can be collected and determined by titrimetric, gravimetric or other type of finish.

5. The derivatisation phenomena produces a derivative that is less polar than the original sample, therefore, more amenable to gas and high performance liquid chromatographic analysis. Many compounds containing polar functional groups show unfavorable properties such as low volatility, tailing irreversible adsorption on many column packages and thermal instability. Vast improvement in these respects, are easily realized because the polar nature of the compound promotes derivatisation with suitable chromogenic reagents to replace the polar group with a less polar one, giving sometimes a more sensitive detection response.

6. The reagent produces derivatives suitable for structural investigation or estimation by NMR and mass spectroscopic measurements.

7. Enzymes selectively catalyze specific reaction.

8. Reagents labeled with radioisotopes transfer the isotopes to the derivatives of the compound analyzed.

In few instances the less reactive functional group may be converted to more reactive functional group through preliminary reaction (e.g., reduction of –NO₂ to –NH₂ hydrolysis of acyl substituted functional group of amine or phenol to free amino or phenolic hydroxyl group respectively).
Features of Chemical Reactions and Reagents of Interest

Knowledge of chemical reactions retains its primary importance in analytical chemistry in spite of and in many cases because of the already impressively large and continually growing body of instrumental and nondestructive methods of analysis. Speculations in complex mixtures of various kinds require the most intimate knowledge of the entire panorama of chemical transformations and the best reagents to employ for bringing these about. Direct attention is given to categorizing and describing the major features of chemical reactions and reagents of interest in the proposed methods of analysis of selected drugs.

Dyes as Analytical Reagents

In the present investigation dyes have been used either freely or in combination with an oxidant in the assay of selected drugs. Dye may be defined as a colored substance which when applied to the fiber gives it a permanent color resistant to the action of light, water and soap. Because of their commercial importance a very large number of dyes have been placed in the market. The color index sponsored jointly by the “society of dyers and colorists” lists about 4500 different dyes and pigments. They have assigned names according to the method of application and given a color index number according to their structures. Each manufacturer however usually labels his products with registered trademark. The dyes are categorized according to common parent structure. The chemical categories of dyes are given in Table 1.1.
## Table 1.1

### Chemical Categories of Dyes

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Category of the dye</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nitro dyes</td>
<td>Naphthol yellow S</td>
</tr>
<tr>
<td>2</td>
<td>Nitroso dyes</td>
<td>Fast green O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tropaeoline OO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tropaeoline OOO*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naphthol blue black</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naphthalene blue 12 BR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Congo red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erichrome Black T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tartrazine</td>
</tr>
<tr>
<td>3.</td>
<td>Thiazoles</td>
<td>Primuline</td>
</tr>
<tr>
<td>4.</td>
<td>Diphenyl methanes</td>
<td>Auramtne</td>
</tr>
<tr>
<td>5.</td>
<td>Triphenyl methane and analogous Dyes</td>
<td>Fast green FCF*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromo phenol blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromo cresol green</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromothymol violet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eriglauacine A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rosaaaniline</td>
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<tr>
<td></td>
<td></td>
<td>Pyronine G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eosine</td>
</tr>
<tr>
<td>6.</td>
<td>Acridines</td>
<td>Acridine orange NO</td>
</tr>
<tr>
<td>7.</td>
<td>Phenazines</td>
<td>Azocarminine G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lissammine blue BF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wool fast blue BL</td>
</tr>
<tr>
<td>8.</td>
<td>Phenoxazines</td>
<td>Celestine blue*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galloxyanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cresyl fast violet acetate</td>
</tr>
<tr>
<td>9.</td>
<td>Thiazines</td>
<td>Methylene blue</td>
</tr>
<tr>
<td>10.</td>
<td>Benzoquinones and Naphthaquinones</td>
<td>Naphthaizarin</td>
</tr>
<tr>
<td>11.</td>
<td>Anthraquinones</td>
<td>Alizarin red S*</td>
</tr>
<tr>
<td>12.</td>
<td>Indigoids</td>
<td>Indigotin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cibascarlet G</td>
</tr>
<tr>
<td>13.</td>
<td>Solubilized vat dyes</td>
<td>Indigosol O</td>
</tr>
<tr>
<td>14.</td>
<td>Sulfur dyes</td>
<td>Sulfur black T</td>
</tr>
<tr>
<td>15.</td>
<td>Sulfurised vat dyes</td>
<td>Hydron blue R</td>
</tr>
<tr>
<td>16.</td>
<td>Phthalocyanines</td>
<td>Monastrial fast blue BS</td>
</tr>
<tr>
<td>17.</td>
<td>Cyanines</td>
<td>Kryptocyanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Astraphloxine FF</td>
</tr>
<tr>
<td>18.</td>
<td>Miscellaneous dyes</td>
<td>Quinoline yellow</td>
</tr>
</tbody>
</table>
Recently, chemists extended their dyes study to correlate visual color with structural features of molecule in 1976, Witt pointed out that two types of groups are usually present in highly colored compounds (Chromophores) and color intensifying groups (auxochromes).

Table: 1.2

List of Important Chromophores and Auxochromes

<table>
<thead>
<tr>
<th>Chromophores</th>
<th>Auxochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acidic</td>
</tr>
<tr>
<td>-N=N-</td>
<td>Phenolic -OH</td>
</tr>
<tr>
<td>-N=O</td>
<td></td>
</tr>
<tr>
<td>( \text{C} = \text{O} )</td>
<td></td>
</tr>
<tr>
<td>( \text{N} = \text{O} )</td>
<td></td>
</tr>
<tr>
<td>C=C, C=N, C=S</td>
<td>-COOH</td>
</tr>
<tr>
<td>( \text{SO}_3\text{H} )</td>
<td></td>
</tr>
</tbody>
</table>

Subsequently it was suggested that the entire conjugate system is responsible for color and that either nitro or amino group shifts the absorption to longer wavelengths. The more the extension of conjugation the greater will be the number of molecular orbital’s present and energy levels are spaced more closely. Hence less energy required for electronic transitions Table 1.3 and the absorption is shifted to longer wavelengths (bathochromic shift). The interaction of auxochromes with the conjugated system not only extends the conjugation but also leads to large dipole moments and large transition dipole moments resulting with high dyes intensity absorption. Derivatisaion such as acylation of an amino or a hydroxyl group merely decreases the availability of an unshared pair of electrons for interaction with conjugated systems.
## Table: 1.3

**Electronic Transition in Absorbing Species**

<table>
<thead>
<tr>
<th>Transition in energy level involving sigma, pi and n electrons</th>
<th>Involving ‘d’ &amp; ‘f’ electrons</th>
<th>Charge – transfer spectral absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma - \sigma^<em>$, n- $\sigma^</em>$, n-$\pi^<em>$, $\pi$-$\pi^</em>$ (imp). $\sigma^<em>$ and $\pi^</em>$ are anti bonding orbital while ‘n’ involves non-bonding orbital having an energy in between bonding and anti bonding orbitals. The polarized force between solvent and species lower the energy levels of excited and unexcited states. As (n-sigm* or sigma-sigma* require much higher energies, they are seen in a vacuum – UV and are harder to observe)</td>
<td>Transition metals involve electronic transition among different energy levels of d-orbitals $t_{2g}$ ($d_{xy}, d_{yz}, d_{zx}$) and $e_{g}$ ($d_{x^2-y^2}, d_{z^2}$) are split by delta in presence of ligands. I’ &lt; BR’ &lt; ClF &lt; OH &lt; Oxalate&lt; H₂O &lt; SCN &lt; NH₃ &lt; en &lt; NO₂ &lt; CN (crystal field theory)</td>
<td>In charge transfer complexes, components should be both electron donor and electron acceptor which in turn involves transfer of electron acceptor which in turn involves transfer of electrons to give absorption radiation (longer wavelength)</td>
</tr>
</tbody>
</table>

## Information relating to the dyes used in the present investigation

Dyes are used as analytical reagents in two different ways depending upon the type of their involvement.

A. Colored anionic or cationic form, which involves in ion association complex formation with oppositely charged ion of the drug.

B. Variations in $\lambda_{max}$ and $\varepsilon_{max}$ value of the dye on treatment with an oxidizing, reducing or complex forming agent lead to the development of visible spectrophotometric determinations of analysts (direct: reducing agent; Indirect: Initial oxidation of analyte with an oxidant followed by estimation of un reacted oxidant with a dye, oxidant reacted with analyte is oxidant initially taken minus oxidant un reacted). Information relating to the dyes used for estimation of selected drugs are presented in the Table 1.1.
Oxidation followed by complex formation

Fe (III)-1, 10-Phenanthraline (M₁) Fe (III) 2, 2'‐Bipyridine (M₂) and Fe (III) K₃Fe (CN)₆

Ferric salts (ferric chloride) play a prominent role in the colorimetric determination of organic compounds. Many phenols, hydroxamic acid esters and more complicate compounds containing the phenolic OH groups in their molecule react with ferric salt in an aqueous, water alcoholic or chloroform media to give intense coloration characteristic of each particular phenol. The color dyes to the strongly ionized complex percolates of trivalent iron, which is formed according to the equation⁸¹.

\[
\text{FeCl}_3 + \text{ArOH} \rightarrow 6\text{H}^+ + [\text{Fe(OAr)}_6]^3
\]

The color intensity and stability of the complex increases when the –COOH group is adjacent to phenolic hydroxyl (eg. Salicylic acid). Addition of acid, glycerol, alcohol and sometimes excess ferric chloride decreases the degree of phenolate dissociation (Hence the concentration of color decreases), and the color of the solution vanishes. Alkalizing also destroys the color by binding the iron into hydroxide.

The amides and esters of fatty acids are characterized by oximes with which the -NHOH group is substituted for the -NH₂ group. The substitution takes place during boiling with solutions of hydroxylamine salts.

\[
\begin{align*}
\text{R COO C}_2\text{H}_5 + \text{NH}_2\text{OH} & \rightarrow \text{R CO NHOH} + \text{C}_2\text{H}_5\text{OH} \\
\text{R CO NH}_2 + \text{NH}_2\text{OH} & \rightarrow \text{NH}_3 + \text{R CO NHOH}
\end{align*}
\]

Hydroximic acids that are formed in this reaction can easily be detected since they react with anion of trivalent iron to give intensely colored complex salts. The nitro group is strong electron acceptor produces a clear –I effect (Inductive) in an organic molecule. Nitro methane is a pseudo acid, and displays tautomerism.

\[
\text{CH}_3\text{NO}_2 \rightarrow \text{CH}_2=\text{NOOH} \text{ (iso nitro group)}
\]

The sodium salt of nitro methane reacts with FeCl₃ to give a complex iron salt, which is intensely colored. This reaction is characteristic of primary and secondary
nitro compounds. FeCl₃ reacts with sodium acetate to give first ferric acetate (by the usual exchange reaction), which is immediately hydrolyzed to give a complex compound, chlorides of used ferric hexa acetate (brown color).

\[
[\text{Fe (OH)}_3 (\text{CH}_3 \text{COO})_6]^+ \text{Cl}^-
\]

Polyhydroxy alcohols or oxy acids react with FeCl₃ to give stable complexes. Oxy acids react with FeCl₃ to give complex salts of iron, which simultaneously oxidize the oxy acids.

Ferric chloride can also oxidize phenols. It oxidizes hydroquinone to quinine, which then gives quinhydrone. Naphthols are oxidized by FeCl₃ to give sparingly soluble dinapthols, in which two naphthalene rings are combined.

\[
3 \text{C}_{10} \text{H}_7 \text{OH} + 2\text{FeCl}_3 \rightarrow \text{HOC}_{10}\text{H}_6\text{C}_{10}\text{H}_6\text{OH} + 2\text{FeCl}_2 + 2\text{HCl}
\]

Other phenols also form phenolates of iron with partial oxidation.

Acting as an oxidant a Ferric salt is converted into ferrous salt. They can easily be detected by the usual reagent for divalent iron, potassium ferricyanide (given below), 1, 10-phenanthroline bispyridyl or triazine.

1, 10-phenanthroline forms a complex of low functional value with Fe (III) which in turn functions as a better oxidant than Fe (III) itself. The reduction product is tris complex of Fe (II), well known as Ferroin. Based on complexing tendency and oxidizing properties. Ferric salts were suggested in the estimation of several drugs.

2,2’-bipyridine forms a complex of low functional value with Fe(III) which in turn functions as a better oxidant than Fe (II) itself. The reduction product is tries complex of Fe (II). Based on its complexing tendency and oxidizing properties, ferric salt was suggested in the estimation of several drugs.

In the present investigation the drug was treated with excess ferric salt under specified experimental conditions. Acting as an oxidant, ferric salt converts to ferrous salt, which corresponds to the drug investigation.
In the present investigation, 1, 10-phenanthroline (M₁), 2, 2’bipyridine (M₂) and potassium fericyanide (M₃) method were used for estimation of Evacetrapib, Niguldipine, Glisoxepide and Lomitapide.

**Oxidation/Reduction reaction**

**FC Reagent: Folin-Ciocaltaeu Reagent**

Reduction of the heteropoly acid complexes by organic reagents was utilized as the basis for the determination of several organic compounds, particularly phenols⁵²-⁵⁴, amines and enols⁵⁵-⁵⁶. The wavelength of maximum absorption and stability and reproducibility of the reaction depends upon pH, composition of heteropoly acidic complex, nature and concentration of the reducing agent, temperature and time⁵⁷ it may be said generally, that more the number of hetero acids in the complex, and the more venerable it is to reduction under certain critical conditions. Among the various hetero poly acids, phosphor molybdotungstic acid, the well-known Folin ciocaltaeu reagent was preferred by a number of workers for the determination of drugs containing not only phenolic or amino groups but also certain other drugs that do not contain the groups⁵⁸.

The color formation by Folin ciocaltaeu (FC) reagent⁵⁹ with organic compounds may explain in the manner based a the analogy with the reports on earlier workers⁶⁰-⁶³.

**Diazo Coupling Reactions⁶⁴**

The diazonium salts derived from p-amino benzoic acid⁶⁵, p-nitroaniline⁶⁶, p-sulphanilic acid (Pauli reagent)⁷²-⁷⁶, 2-aminobenzothiazole⁷⁷-⁷⁸, 3-phenyl 1-5-nitrosamine 1,2,4 thiadiazole⁷⁹-⁸⁰, 4-amino 6-chloro1,3-benzenedisulphona-mide⁸¹, benzocaine⁸², dapsone⁸³ and sulphanilamide⁸⁴ are commonly used reagents for direct coupling procedures. The aryl amine coupling reagents are converted to their diazonium salts with HCl and sodium nitrite. The reaction is usually carried out in an ice bath, the excess nitrate is removed by reaction with sulphamic acid or ammonium sulphamate, and the pH is adjusted for the coupling reaction. The reagent is used immediately since most diazonium salts are not stable.

The most common source of interference in analysis using the direct coupling procedure is impurities in the sample, which also couple with the diazonium salt and
exhibit some absorbance at the wavelength chosen for the analysis. Interference of this type can only be avoided by employing a separation step prior to color development.

Several compounds of pharmaceutical importance are analyzed by direct coupling with a diazonium salt. They include, levartenol\textsuperscript{85}, 8-hydroxy quinoline\textsuperscript{86}, isonazide, 3-amino-1H-1, 2, 4-triazole\textsuperscript{87}, estradioldipropionate\textsuperscript{88-89}, tyrosine\textsuperscript{90}, thiamine\textsuperscript{91}, salbutamol\textsuperscript{92}, nyidrin\textsuperscript{93}, pholedrin\textsuperscript{94} and terbutaline\textsuperscript{95}.

**Diazotization of the Analyte and Coupling**

The second group of diazo coupling reactions are those in which the analyte is converted to a diazonium salt and then coupled to a substrate. Procedures of this type are encountered much more frequently than direct coupling procedures in pharmaceutical analysis. The most common substrates are (1-naphthyl) ethylenediamine dihydrochloride (Bratton-Marshall Reagent)\textsuperscript{96} and 2-naphthol. Bratton-Marshall Reagent is preferred for quantitative work because the products are usually soluble and have high molar absorption. 2-naphthol often forms insoluble coupling products and is more frequently used for qualitative identification tests\textsuperscript{97}. When the substance being analyzed is diazotized, optimization of the reaction conditions and time becomes especially important because the diazonium salts are generally unstable and any loss through decomposition or side reactions will decrease the sensitivity and precision of the analysis. The reaction of aromatic amines with nitrous acid to form diazonium salts is very general and is carried out regardless of other ring substituents. The mechanism of the reaction\textsuperscript{98-99} is outlined in the following scheme.

\[
\text{H}^+ + \text{HONO} \rightarrow \text{H}_2\text{O} - \text{NO}^+ \rightarrow \text{NO}_x + \text{NO}_2
\]

\[
\text{NO}_x + \text{ArNH}_2 \rightarrow \text{X}^- + \text{Ar} \text{N}^+\text{H}_2 \text{N}=\text{O} \rightarrow \text{H}^+ + \text{Ar}-\text{N}=\text{O} \rightarrow \\
\text{H}^+ + \text{Ar}-\text{N}=\text{N}=\text{OH} \rightarrow \text{Ar-N}=\equiv\text{N} + \text{H}_2\text{O}
\]

Formation of diazonium salt is usually fast enough that any convenient $\text{pH}$ between 0 and 3 can be used. In case where the reaction is unusually slow or where the diazonium salt is unusually labile, it might be well to optimize the $\text{pH}$ in order to increase the reaction rate and minimize the effects of decomposition. The reaction rate is also increased if the $\text{pH}$ is adjusted with hydrochloric acid rather than sulphuric
acid, since NOCl is a better nitrosating agent than NOHSO₄. Adding NaBr or KBr can increase the rate even further, presumably due to the formation of NOBr, which is a better nitrosating than NOCl¹⁰⁰.

The coupling reaction requires a polar solvent to accommodate the ionic intermediates and water and ethanol are most frequently used. Careful control of solvent pH is very important in achieving rapid, quantitative reaction coupling to amine substrates should be carried out between pH 5 and 9, and phenols between pH 9 and 10. The exact pH must be determined experimentally in each case.

The spectrophotometric measurement of the coupling products of diazotized sulphanamides with Bratton-Marshall Reagent has been studied in detail¹⁰¹-¹⁰². The absorption maximum is near 545 nm for coupled sulphanamides and maximum absorption intensity is achieved in the pH range 1 to 2. A few examples of the utility of this reaction for the analysis of drugs containing an aromatic amino group include bendroflumethiazide, sulphodaxime¹⁰³, and sulphamerazine¹⁰⁴-¹⁰⁸, sulphamethazine¹⁰⁹, and procainamide¹¹⁰-¹¹¹.

A number of drugs have also been determined by first converting them to a diazotisable species (either by hydrolysis or reduction) which is then coupled in the usual manner. Chloramphenicol contains a nitro group, which can be reduced to an amine with a suitable reducing agent, (metallic zinc/acid, stannous chloride or sodium dithionate). Reduction to the amine followed by the Bratton Marshall reaction has been used for the analysis of this drug¹¹²-¹¹⁴. Using it to diazotize an aromatic amine, coupling the diazotized amine with a substrate and measuring the product spectrophotometrically can determine nitrous acid. The Griess method for nitrite employs sulphanilic acid as the source of coupling agent and α-naphthyl amine as the substrate¹¹⁵. This reaction has been used for the analysis of pharmaceutical preparations including gelatin capsules and can also be used for nitrate after a reduction step. A typical procedure will consist of an alkaline hydrolysis, followed by acidification, diazotization of coupling agent and finally coupling with the substrate¹¹⁶. Isosorbide dinitrate has been determined using sulphanilic acid and α-naphthyl amine (Griess method), and also using p-nitroaniline as the coupling agent and aniline as the substrate¹¹⁷. The later combination has also been used to analyze pentaerythritol tetranitrate¹¹⁸.
In the present investigation diazotization methods M1, M2 have been used for the determination of NGD.

**Dyes in Ion Association Complex Formation:**

The term molecular complex is used to describe a variety of association products of two or more molecules. In recent years, extensive attention has been given to a large number of complexes formed by weak interaction of certain classes’ of organic compounds functioning as electron donors (bases), while others act as electron acceptors, (acids)\(^{119-121}\). The forces, which lead to the formation of molecular complex, include forces such as dipole and induced dipole interactions, London dispersion forces, hydrogen bonding and dative bonding interactions. The donor-acceptor complexes (where composition can be represented by integral ratios 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 the physical properties (e.g. absorption spectra, solubility in organic solvents) from those of the pure components. The ion association complex or adduct 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 chromate (dye or metal complex) possessing charge (cationic or anionic nature) and so insoluble in organic solvents. The other is colorless, possessing opposite charge (anionic or cationic) to that of chromate.

The ion association complex extraction has been applied to the estimation of numerous compounds, possessing basic moieties (secondary or tertiary aliphatic amino groups) by using an acid dye as a reagent and a chlorinated solvent as an extractant. The structure of the species formed may depend upon the experimental conditions\(^{122-123}\) (Concentration of the components, pH of the aqueous phase). The color can be altered or intensified upon acidification or re extracted into a buffer. The presence of hydrophilic substituents such as –OH or –COOH often prevents extraction of the complex into organic solvent as an extractant, which then depends upon parameters such as the polarities of the amine and of the dye. Relatively poor amines such as aryl amines, diamines and many alkaloids react with bromothymol blue poorly or not at all with bromocresol purple. Contrary wise, this second dye
behaves like the first one with weakly polar amines such as amine derivatives of biphenyl methane or phenothiazone.

Therefore, compounds may be determined in binary mixtures, the less polar amine is estimated with bromophenol blue, that the sum of the two amines is given by bromocresol purple. Mixtures of diphenyl hydramine and ephedrine were also analyzed\textsuperscript{124}.

According to the same principle, acid dyes can be colorimetrically determined with basic dyes. P-toluene-sulfonic acid, camphosulfonic acid and bromocamphosulfonic acid were estimated with fuchsin\textsuperscript{125}, chlorotoluenesulfonic acid with acridine orange, rhodamine S or chrysoidine\textsuperscript{126} and phenylbutazone, which develops a slightly acidic reaction, with Gentian violet\textsuperscript{127}.

The same principle has been applied for the determination of secondary and tertiary aliphatic amines with tropaeolinOO and of tertiary aliphatic amine with erythrosine\textsuperscript{128}. Several dyes belonging to different chemical classes have been used for the determination of basic drugs\textsuperscript{129-134}. Few dyes have been utilized for the development of new extractive spectrophotometric methods for the determination of selected drugs in the present work. The acidic dyes have been summarized in Table1.1.

**PRESENT STUDY:**

The Spectrophotometer and HPLC techniques are most widely using techniques in drug and pharmaceutical industry. It is very important to develop better sensitive methods with simplification to use for routine pharmaceutical analysis. In this present study the author is focused on development of some of new spectrophotometric and HPLC methods for four durg products named by Niguldipine (Calciumchannel blocker) Evacetrapib (Cardiovascular disease), Glisoxepide (anti diabetic) Lomitapide (anti lower cholesterol) with better sensitivity and accuracy.
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