CHAPTER-I

INFORMATION OF SELECTED DRUGS AND DIFFERENT TECHNIQUES USED FOR THEIR ASSAY
INTRODUCTION

1.01 Selected Drugs

According to WHO, a drug may be defined as any substance or product that is used or intended to be used for modifying or exploring physiological systems or pathological states for the benefits of the patient.

Pharmaceutical chemistry\(^1\)\(^-\)\(^7\) is a science that makes use of the general laws of chemistry to study drugs i.e., their preparation, chemical nature, composition, structure, influence on an organism and studies the physical and chemical properties of drugs, the methods of quality control and conditions of their storage.

The enormous number of drugs available for the treatment of various diseases has made it necessary to classify them. Hence, a sharp division into the following two classes has been made.

Pharmacodynamic agents

These drugs have certain effects on animal organs, but are not specific remedies for particular diseases. They may be further subdivided into different classes like central nervous system modifiers (depressants or stimulants), adrenergic agents (stimulants or blocking agents), anti-psychotic agents, anti-hypertensives, anti-lipidemics, cholinergic or anti-cholinergic agents, cardiovascular agents, diuretics, anti-inflammatory agents, anti-spasmodics, gastric secretion modifiers, anti-histamines, anti-coagulants etc.

Chemotherapeutic or anti-infective agents

Anti-infective agents treat infection by suppressing or destroying the causative microorganisms – bacteria, mycobacteria, fungi, protozoa, or viruses. Anti-infective agents derived from natural substances are called antibiotics those produced from synthetic substances are called antimicrobials. However,
these two terms are now used interchangeably. An anti-infective agent should be chosen on the basis of its pharmacological properties and spectrum of activity as well as on various host (patient) factors. A combination of drugs should be given only when clinical experience has shown such therapy to be more effective than single-agent therapy in a particular setting. A multiple agent regimen can increase the risk of toxic drug effects and in a few cases result a drug antagonism and subsequent therapeutic ineffectiveness. In the present investigation, the following categories of drugs were selected:

1. GEFITINIB - Anticancer
2. DISODIUM PAMIDRONATE - Anti osteoporotic agent
3. STRONTIUM RANELATE - Anti osteoporotic agent
4. CYPROTERONE ACETATE - Anti androgen
5. CITICOLINE SODIUM - Anti parkinsonian agent

Every country has legislation 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 a book called Pharmacopoeia (e.g., Indian IP, United States USP, United Kingdom BP, European EP, Martindale the Extra Pharmacopoeia MEP, Merck Index MI, Physician desk reference PDR). Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e., with the raw material on whose degree of purity, the quality of medicament depends. The quality of drug is determined after establishing its purity and the quality of pure substance in the bulk drug and its formulations. The source of selected drugs is presented in table 1.01.
### TABLE 1.01

**SOURCE OF SELECTED DRUGS**

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Drugs Selected</th>
<th>I.P9</th>
<th>USP10</th>
<th>B.P11</th>
<th>EP12</th>
<th>MARTINDALE13</th>
<th>MI14</th>
<th>PDR15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GEFITINIB (GTB)</td>
<td>1405</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>727.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>DISODIUM PAMIDRONATE (DSP)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1101.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>STRONTIUM RANELATE (SRL)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1104.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>CYPROTERONE ACETATE (CTA)</td>
<td>1153</td>
<td>589</td>
<td>5907</td>
<td>-</td>
<td>2088.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>CITICOLINE SODIUM (CTS)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2283.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*For all refs. the page nos. of their appearances are indicated but for Merck Index item nos. are indicated.

The drugs are used in various dosage forms in therapy\(^{16-21}\). They are formulated as tablets [Tabs: one or more among diluents (lactose, starch cellulose derivatives, calcium phosphate, mannitol, sorbitol, sucrose, calcium sulphate, dextrose); binders and adhesives (acacia, gelatin, polyvinyl pyrrolidine, alginic acid derivative, tragacanth); disintegrants (clays) etc., lubricants (stearic acid, talc, polyethylene glycol surfactant waxes; flow promoters- silica derivative, corn starch); colours (permitted dyes); flavours (natural or artificial)], Capsules [Caps: certified dyes, opaquing agents, plasticizers, preservatives], parental products [PP: vehicles (water, vegetable and mineral oils, simulated oils, propylene glycol, dioxilamines, dimethyl...
acetamide), stabilizers (antioxidants), buffering agents (citrate, acetate, phosphate), co-solvents, wetting, suspending and emulsifying agents (tween 80, sorbitan oleate, fluronic F-68), preservatives etc., powders [PD:sugars(lactose, sucrose, sorbitol) or of polysaccharides (starch or micro crystalline cellulose)], dusting powders [diluent (talcum)], absorbents [carbonates or oxides of calcium and magnesium], oily or aqueous suspensions, ointments, creams etc.

The drugs are applied in some instances in rather small doses and they are often excipiated as combinations with each other or with other drugs. The assay of the various dosage forms raises several special problems such as skillful sampling and the preparation of sample solutions.

**Sampling**

**Liquids:** They are mixed thoroughly several times by inverting the solution.

**Powders:** They are thoroughly mixed before a portion of the sample is taken for analysis.

**Tablets:** Tablets are mixed thoroughly and twenty tablets are selected at random, reduced to a fine powder and weighed accurately before assay.

**Capsules:** About ten capsules are weighed accurately. They are opened with a razor blade and the contents are emptied into small beaker and mixed thoroughly. In the case of dry filled capsules, the adhering powder to the shells is cleaned with absorbent cotton on the end of a stiff wire.

**Preparation of sample solution**

Some of the problems are associated with the extraction of drugs into the most important solvents and their tendency to be bound to excipients.
Quantitative extraction often causes serious problems, which must be solved separately in each instance. The most difficult problems arise when selective extraction is necessary. The specificity of the extraction is very often insufficient for the success of the subsequent extraction. In these instances separation of components of the extract or its purification by chromatography are widely used. The most convenient means of extracting drugs from tablets is to treat them with a solvent such that the resulting extract can be used directly in the assay method chosen. In general, adequate extraction can be achieved when the finely pulverized tablet is agitated or sometimes boiled with solvent for a period from few minutes to several hours. The test solution can be obtained directly by filtration or centrifugation of the mixture. The solvent is often evaporated to dryness in a stream of nitrogen or air and the residue is dissolved in a solvent more suitable for the subsequent analysis. The binding of the active ingredients to polar excipients such as lactose and starch, decreases the effectiveness of extraction, in some instances. This becomes significantly particular when the dose of drug is very small. The generally used method of decreasing the adsorption losses and increasing the selectivity of the extraction is two-phase extraction. Here, one of the solvents is always water. This solvent dissolves lactose, which is usually the main component of the excipients thereby, affording favourable conditions for the extraction of the drug by other solvent, which is immiscible with water. Starch, which is also critical from the point of view of adsorption losses can be dissolved by treatment with diastase. The organic solvent is usually chloroform, but ethyl acetate, diethyl ether, iso-octane and few others have also been used.

1.02 Typical Instrumental Techniques

The methods of estimation of drugs are divided into physical, chemical, physicochemical and biological ones. Of them, physical and physicochemical methods are used the most. Physical methods of analysis involve the studying of the physical properties of a substance. They include determination of the solubility, transparency or degree of turbidity, colour density or specific gravity
Physicochemical methods\textsuperscript{19-21} are used to study the physical phenomenon that occur as a result of chemical reactions. Among the physicochemical methods are optical (refractometry, polarimetry, emission and fluorescent methods of analysis, photometry including photo colourimetry and spectrophotometry, nephelometry or turbidimetry), electrochemical (potentiometry, amperometry, coulometry, polarography) and chromatography (column, paper, thinlayer\textsuperscript{22}, gas-liquid\textsuperscript{23}, high performance liquid\textsuperscript{24,25}) methods are generally preferable. Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid-base, precipitation and redox reactions. Titration in non-aqueous media and complexometry have been widely used in pharmaceutical analysis when ever the existing amounts are in mg level and the interferences are negligible. The methods (HPLC\textsuperscript{26}, GLC, NMR, and Mass Spectroscopy) of choice for assay involve sophisticated equipment which are very costly and pose problems of maintenance. Hence they are not in the reach of most laboratories and small scale industries which produce bulk drugs and pharmaceutical formulations. However this sophisticated equipment usage eliminate the difficulties encountered in the determination of minute amounts of degradation products., or the analysis of the metabolites of drugs in body fluids.

**Visible Spectrophotometry**

The visible spectrophotometric (or colourimetric) methods\textsuperscript{27-30} which fall in the wavelength region 400-800 nm and fluorimetric methods (may fall in UV & Visible regions ) are very simple, cheap and easy to carry out estimations of drugs in bulk form and their formulations. The limitations of many colourimetric or fluorimetric methods of analysis lie in the chemical reactions upon which the procedures are based rather than the instruments available.
Many of the reactions involve colour or fluorescence of a particular drug are quite selective or can be rendered selective through the introduction of masking agents, control of pH, use of solvent extraction technique, adjustment of oxidation states or by prior removal of interfering ingredients with the aid of chromatographic separation. In the present investigation instrumental technique such as UV and visible spectrophotometry has been utilised for the assay of selected drugs (GTB, DSP, SRL, CTA and CTS).

A survey of literature on selected drugs GTB, Chapter-II; DSP, Chapter-III; SRL, Chapter-IV; CTA, Chapter -V; CTS, Chapter-VI; showed that there are very few or none (GTB, DSP, SRL, CTA and CTS) visible spectrophotometric methods of analysis at the time of commencement of this investigation.

### 1.03 Classification of functional groups in drugs:

A feature of organic drugs is the presence of functional groups (Table 1.02, p.8-9) in their molecules. Knowing the reactions of functional groups\(^{31-33}\), one can easily analyze any organic drug with a complicated structure.

The analytically important functional groups of selected drugs do not seem to have been fully exploited for designing suitable visible spectrophotometric methods for their determinations. The chemical features of selected drug molecules still offer a lot of scope for the development of new visible spectrophotometric methods hopefully with better sensitivity, selectivity, precision and accuracy. The author made some attempts in this direction and succeeded in developing some new methods having advantages of one or more of the above desirable features.
### TABLE 1.02

**FUNCTIONAL GROUPS IMPARTING ACIDIC, BASIC OR NEUTRAL NATURE**

<table>
<thead>
<tr>
<th>Functional groups</th>
<th>Functional groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Functional groups imparting an acidic nature to substance</strong></td>
<td><strong>Functional groups</strong></td>
</tr>
<tr>
<td>Carboxyl</td>
<td>—COOH</td>
</tr>
<tr>
<td>Imide</td>
<td>—CO_NH</td>
</tr>
<tr>
<td>Sulphahydral or Thiol</td>
<td>—SH</td>
</tr>
<tr>
<td>Enol</td>
<td>&gt;C=-(OH)—</td>
</tr>
<tr>
<td>Enediol</td>
<td>—(OH)C=C(OH)—</td>
</tr>
<tr>
<td>Phenolic hydroxyl</td>
<td>OH</td>
</tr>
<tr>
<td>Sulphonic acid</td>
<td>—SO_3H</td>
</tr>
<tr>
<td><strong>2. Functional groups imparting basic properties to a substance</strong></td>
<td><strong>Functional groups</strong></td>
</tr>
<tr>
<td>Primary, secondary and tertiary amino group(R¹=R²=H, R¹=H, R²=alkyl, R¹=R²=alkyl groups) (in aliphatic, alicyclic, aromatic and heterocyclic systems)</td>
<td>R¹ _ R²</td>
</tr>
<tr>
<td>(the tertiary nitrogen is necessary element in molecules of alkaloids, heterocyclic compounds and mono and disubstituted hydrazine derivatives(R²NH-NHR¹))(hy)¹</td>
<td></td>
</tr>
</tbody>
</table>
### 3. Functional groups which exhibit neither acidic nor basic properties

<table>
<thead>
<tr>
<th></th>
<th>Aldehyde</th>
<th>—CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keto</td>
<td>&gt; C=O</td>
</tr>
<tr>
<td></td>
<td>Hydroxy methyl</td>
<td>RCH₂OH</td>
</tr>
<tr>
<td></td>
<td>Nitroso</td>
<td>—N=O</td>
</tr>
<tr>
<td></td>
<td>Nitro</td>
<td>—N=N</td>
</tr>
<tr>
<td>Methoxy</td>
<td>—O-CH₃</td>
<td></td>
</tr>
<tr>
<td>Ether</td>
<td>R-O-R¹</td>
<td></td>
</tr>
<tr>
<td>Ester</td>
<td>—COOR</td>
<td></td>
</tr>
<tr>
<td>Lactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olefinic</td>
<td>&gt;C=C&lt;</td>
<td></td>
</tr>
<tr>
<td>Acetylenic</td>
<td>—C≡C—</td>
<td></td>
</tr>
</tbody>
</table>

### 1.04 Classification of Organic reactions

#### (a) Organic reactions:

An organic reaction may be represented as

Substrate + Attacking reagent → Intermediate → Products

The steps of an organic reaction showing the breaking or making of new bonds of carbon atoms in reactant (substrate) when treated with an attacking reagent lead to the formation of the final products through transitory intermediates. Most of the attacking reagents carry either a positive or negative charge. The positively charged reagents attack the region of high electron density in the substrate molecule, while on the other hand the negatively charged reagents will attack the regions of low electron density in the substrate molecule.
The numerous reactions are classified into four types.

(1) Substitution (2) Addition (3) Elimination and (4) Rearrangement.

(b) Organic reagents:

Organic reagents fall into two main groups. An electrophilic reagent (cation, dipolar molecule or molecule which has atoms with incomplete octet) is a species having electron deficient atom or centre. The nucleophilic reagent is electron rich. The nucleophilic reactions involve the attack of negative or neutral nucleophile on the positively charged substances resulting in the formation of neutral or positively charged products.

Various reactions may involve the formation of three main intermediates namely, free radicals (from hemolytic fission), carbonium ions (-C+) and carbanion (-C-) from heterolytic fission which then react with the reagents to form the products.

Inductive and mesomeric effects:

The attack by an ion requires polarization in the molecule to be attacked. The inductive effect may be defined as the permanent displacement of electrons forming a covalent bond towards the more electronegative atom or group of atoms. Group of atoms attached to a C – chain will have electron attracting or repelling tendencies, depending on their electronegativity as compared to C-atom to which they are attached. The decreasing order of –I effect and increasing order of +I effect are [NO2-, CN-, F-, Cl-, Br-, I-, OH-, OCH, C6H5, H+] and [-CH3, -C2H5, -CH (CH3)2, -C(CH3)3] respectively. The mesomeric effect refers to the polarity produced in a molecule as a result of interaction between two σ-bonds or a π- bond and a lone pair of electrons. This effect is transmitted along a chain. Especially in conjugated systems, the π-electrons get delocalised as a consequence of mesomeric effect giving a number of resonance structures of the molecule. The +M effect is shown by
groups having lone pairs (e.g., -Cl, -Br, -I, -NH, -OH, -OCH₃). The -M effect is shown by groups such as -COO', -NO₂, -CN, -SO₃H etc and is due to the presence of electronegative atoms like oxygen or nitrogen. It is more prominent in aromatic compounds where an atom or group is directly attached to a benzene ring having conjugated double bonds.

1.05 Selection of reagents for organic analysis

Several papers are being published every year on the reactions and possible applications of new and old reagents for organic analysis. The selection of an appropriate reagent for a particular analytical situation is still a challenging problem. The choice of a particular reagent depends on careful consideration of such factors such as the scale and economics of reaction, the presence of other functional groups that might be adversely affected by reagents the deactivation of the reaction centre by steric and electronic effects, the instability or high reactivity of the desired product, the rate of the reaction, the 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 not enough information is 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 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 the interacting compounds.

The general objective of a reagent is the formation of one or more derivatives having the measurable chemical or physical properties as
completely different as possible from that of any of the reactants. Some examples of more specific reagents go 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 or for the isolation, concentration and purification of a compound for examination by other analytical techniques.

3. The reagent forms a chromophore or reduces the concentration of a chromophore already present, with a change measured by one of the spectrophotometric techniques, ultraviolet, visible or infrared. Many spot tests for functional groups or spraying agents in TLC depend on the formation of coloured derivatives.

4. The reagent acts on the sample to produce a gas measurable manometrically or one that can be collected and determined by titrimetric, gravimetric or other types of finish. The derivatisation phenomenon produces a derivative that is less polar than the original sample, therefore, more amenable to gas and high performance liquid chromatographic analysis.

5. Many compounds containing polar functional groups show unfavourable properties such as low volatility, tailing, irreversible adsorption of many column packings and thermal instability. Vast improvement in these aspects are easily realised because the polar nature of the compound promotes derivatisation with suitable reagents to replace the polar group with a less polar one, giving sometimes a more sensitive detection response.
6&7. The reagent forms a derivative suitable for structural investigation or estimation by NMR and mass spectroscopic measurements.

8. Enzymes selectively catalyse specific reactions.

9. Reagents labeled with radio isotopes to transfer the isotopes to the derivatives of the compound analysed.

In few instances the less reactive functional groups may be converted to more reactive functional groups through some preliminary reaction (e.g. reduction of NO₂ to - NH₂; hydrolysis of acyl substituted functional groups of amines and phenols to free amino and phenolic hydroxyl groups).

1.06 Analytical utility of chromogenic reagents and the chemistry of coloured species formed in the present investigations.

Knowledge of chemical reactions with chromogenic reagents retains its primary importance in analytical chemistry. Speciation 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 categorize the chromogenic reagents (which in turn the chemical reactions) of interest in the proposed methods of analysis of selected drugs (Table 1.03, p.15-17 and in more detailed way in the following text). The analytical application of each chromogenic reagent has been discussed in detail separately. Under proposed methods M₁-M₃₂ refer to the serial number.

In the methods such as (M₆-M₁₁, M₁₆ and M₁₇) the procedures involves two steps. The first step in the analytical procedure of each method (M₆-M₁₁) is the reaction of the drug with excess oxidant (NBS in M₈ and M₉, KMnO₄ in M₆, CAT in M₇, I₂ in M₁₀ and M₁₁) under proposed experimental conditions giving the products along with unreacted oxidant. In methods M₁₆ and M₁₇, the drug is precipitated in the form of an adduct with the excess reagent (PMA in
$M_{16}, I_2$ in $M_{17}$). In method $M_9$, the second step is the reaction of reduced form of oxidant or unreacted oxidant (NBS in $M_9$ with PMAP-SA) with colour producing agent to develop colour. In methods $M_6, M_7$ and $M_8$ the second step is the reaction between the unreacted oxidant and a dye thereby diminishing the intensity of dye colour (FG FCF in $M_6$, GC in $M_7$, CB in $M_8$). In all these methods, the oxidant reacted in the first step, which corresponds to the amount of the drug is calculated by subtracting the unreacted oxidant from the oxidant initially taken. The second step in method $M_{16}$ is the colour development of released precipitant from the adduct through acetone addition followed by treatment with Co II-EDTA. In method $M_{17}$, the second step concerns with the colour development after treating the unreacted precipitant in the filtrate with PMAP-SAc to get coloured charge-transfer complex.

In methods ($M_{13}, M_{14}$), the oxidant (IO$_4^-$ or CAT) converts the other component (brucine or Hae T) of the reagent to furnish highly reactive and less stable in situ formed intermediate, which subsequently couple with the drug to furnish coloured product. In method $M_{15}$, the intermediate (quinone imine) itself exists in stable form and so the oxidant is not required. In the remaining methods, the drug directly involves in different types of reactions for colour production.

Only such procedures in the methods (based on the type of reactions mentioned above) whose selectivity ($\lambda_{max}$), sensitivity ($\epsilon_{max}$) and Beer’s law limits appear to be better over others are preferred for further investigations (Chapter II-VI)
## Table 1.03
### List of Proposed and Reported Visible Spectrophotometric Methods

<table>
<thead>
<tr>
<th>Types of reaction(s)</th>
<th>Reagent</th>
<th>Method</th>
<th>Drug responded</th>
<th>Optical characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ion-association complex formation</strong></td>
<td>TPoo0</td>
<td>M₁</td>
<td>GTB</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 480</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.63x10⁴</td>
</tr>
<tr>
<td><strong>(Acid dyes)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beer's law limits (µg. ml⁻¹) 2-10</td>
</tr>
<tr>
<td>-do-</td>
<td>AG</td>
<td>M₂</td>
<td>GTB</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 525</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.29x10⁴</td>
</tr>
<tr>
<td>Basic dyes</td>
<td>MB</td>
<td>M₃</td>
<td>DSP</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 650</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 1.43x10⁴</td>
</tr>
<tr>
<td>-do-</td>
<td>SFN-O</td>
<td>M₄</td>
<td>SRL</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 520</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 1.39x10⁴</td>
</tr>
<tr>
<td>Redox</td>
<td>FC</td>
<td>M₅</td>
<td>SRL</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 740</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 7.76x10³</td>
</tr>
<tr>
<td>-do-</td>
<td>KMnO₄/</td>
<td>M₆</td>
<td>SRL</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 630</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 3.21x10⁴</td>
</tr>
<tr>
<td>FGFCF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beer's law limits (µg. ml⁻¹) 2-10</td>
</tr>
<tr>
<td>-do-</td>
<td>CAT/GC</td>
<td>M₇</td>
<td>SRL</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 540</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.53x10⁴</td>
</tr>
<tr>
<td>Redox / Charge transfer complex</td>
<td>NBS/CB</td>
<td>M₈</td>
<td>SRL</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 520</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 1.38x10⁴</td>
</tr>
<tr>
<td>-do-</td>
<td>NBS/PMAP-SA</td>
<td>M₉</td>
<td>SRL</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 520</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.76x10⁴</td>
</tr>
<tr>
<td>Redox</td>
<td>I₂-OH⁻</td>
<td>M₁₀</td>
<td>CTA</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 540</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.48x10⁴</td>
</tr>
<tr>
<td>/WFBBBL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beer's law limits (µg. ml⁻¹) 2-10</td>
</tr>
<tr>
<td>Redox / Charge transfer complex</td>
<td>I₂-OH⁻ /</td>
<td>M₁₁</td>
<td>CTA</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 620</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.13x10⁴</td>
</tr>
<tr>
<td>H⁺/PMAP- INH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beer's law limits (µg. ml⁻¹) 2-10</td>
</tr>
<tr>
<td>Redox / Charge transfer complex</td>
<td>BTZ</td>
<td>M₁₂</td>
<td>CTA</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm) 525</td>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (1.mole⁻¹ cm⁻¹) 2.53x10⁴</td>
</tr>
<tr>
<td>-do-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beer's law limits (µg. ml⁻¹) 2-10</td>
</tr>
</tbody>
</table>
Table 1.03. Contd.

<table>
<thead>
<tr>
<th>Process</th>
<th>Reaction</th>
<th>M</th>
<th>Method</th>
<th>pH</th>
<th>T (°C)</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oxidative coupling</em></td>
<td>Brucine –IO₄⁻</td>
<td>M₁₃</td>
<td>GTB</td>
<td>520</td>
<td>8.80 x 10⁵</td>
<td>5-25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DSP</td>
<td>520</td>
<td>5.90 x 10⁵</td>
<td>5-25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTS</td>
<td>520</td>
<td>5.25 x 10³</td>
<td>8-40</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>Hae T-CAT</td>
<td>M₁₄</td>
<td>SRL</td>
<td>550</td>
<td>1.53 x 10⁴</td>
<td>4-20</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>DCQC</td>
<td>M₁₅</td>
<td>DSP</td>
<td>620</td>
<td>1.08 x 10⁴</td>
<td>2-10</td>
</tr>
<tr>
<td><strong>Precipitation / Reduction</strong></td>
<td>PMA/Co(II)-EDTA</td>
<td>M₁₆</td>
<td>GTB</td>
<td>840</td>
<td>1.35 x 10⁴</td>
<td>4-20</td>
</tr>
<tr>
<td><strong>Precipitation/charge transfer</strong></td>
<td>I₂/PMAP-SAc</td>
<td>M₁₇</td>
<td>GTB</td>
<td>520</td>
<td>1.36 x 10⁴</td>
<td>4-20</td>
</tr>
<tr>
<td><strong>Precipitation / Complex formation</strong></td>
<td>AM/PTC</td>
<td>M₁₈</td>
<td>GTB</td>
<td>480</td>
<td>1.02 x 10⁴</td>
<td>6-30</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>TA/PMAP-Cr(VI)</td>
<td>M₁₉</td>
<td>GTB</td>
<td>560</td>
<td>9.18 x 10³</td>
<td>4-24</td>
</tr>
<tr>
<td><strong>Complex formation</strong></td>
<td>CTC</td>
<td>M₂₀</td>
<td>GTB</td>
<td>620</td>
<td>5.46 x 10³</td>
<td>10-50</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>DDQ</td>
<td>M₂₁</td>
<td>GTB</td>
<td>470</td>
<td>5.52 x 10³</td>
<td>10-50</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>DHQ</td>
<td>M₂₂</td>
<td>GTB</td>
<td>540</td>
<td>3.17 x 10³</td>
<td>20-100</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>TQ</td>
<td>M₂₃</td>
<td>GTB</td>
<td>560</td>
<td>2.88 x 10³</td>
<td>20-100</td>
</tr>
<tr>
<td><strong>Condensation</strong></td>
<td>VN</td>
<td>M₂₄</td>
<td>DSP</td>
<td>560</td>
<td>1.37 x 10⁴</td>
<td>2-10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTA</td>
<td>560</td>
<td>1.70 x 10⁴</td>
<td>2.5-12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTS</td>
<td>560</td>
<td>1.43 x 10⁴</td>
<td>4-20</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>PDAB</td>
<td>M₂₅</td>
<td>DSP</td>
<td>480</td>
<td>6.18 x 10³</td>
<td>4-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTA</td>
<td>480</td>
<td>1.10 x 10⁴</td>
<td>4-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTS</td>
<td>480</td>
<td>9.89 x 10³</td>
<td>6-30</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>PDAC</td>
<td>M₂₆</td>
<td>CTS</td>
<td>490</td>
<td>8.95 x 10³</td>
<td>6-30</td>
</tr>
<tr>
<td><em>-do-</em></td>
<td>NH/AA</td>
<td>M₂₇</td>
<td>DSP</td>
<td>560</td>
<td>4.64 x 10³</td>
<td>5-25</td>
</tr>
<tr>
<td>-do-</td>
<td>NQS</td>
<td>M_{28}</td>
<td>GTB</td>
<td>480</td>
<td>8.24 \times 10^3</td>
<td>5-25</td>
</tr>
<tr>
<td>-------</td>
<td>-----</td>
<td>--------</td>
<td>-----</td>
<td>-----</td>
<td>-----------------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DSP</td>
<td>480</td>
<td>6.19 \times 10^3</td>
<td>4-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTS</td>
<td>480</td>
<td>5.51 \times 10^3</td>
<td>8-40</td>
</tr>
<tr>
<td>Internal salt formation</td>
<td>CiA/Ac_{2}O</td>
<td>M_{29}</td>
<td>GTB</td>
<td>580</td>
<td>1.42 \times 10^4</td>
<td>4-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTS</td>
<td>580</td>
<td>6.37 \times 10^3</td>
<td>8-40</td>
</tr>
<tr>
<td>Charge transfer complex</td>
<td>TQ-CH_{3}CHO</td>
<td>M_{30}</td>
<td>GTB</td>
<td>660</td>
<td>9.14 \times 10^3</td>
<td>5-25</td>
</tr>
<tr>
<td>-do-</td>
<td>SNP-CH_{3}CHO</td>
<td>M_{31}</td>
<td>GTB</td>
<td>560</td>
<td>4.13 \times 10^3</td>
<td>10-50</td>
</tr>
<tr>
<td>--do-</td>
<td>SNP-ACETONE</td>
<td>M_{32}</td>
<td>DSP</td>
<td>550</td>
<td>7.57 \times 10^3</td>
<td>4-20</td>
</tr>
<tr>
<td>Ion association complex formation</td>
<td>BCG, BPB</td>
<td>-</td>
<td>GTB</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Condensation/complex formation</td>
<td>2,4 DNPH</td>
<td>-</td>
<td>CTA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Condensation/complex formation</td>
<td>NH_{2}OH/Ferric per chlorate</td>
<td>-</td>
<td>CTA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Dyes as analytical reagents:

In the present investigation, dyes have been used either freely or in combination with an oxidant in the estimations of selected drugs (Table 1.03, p.15-17). Dye may be defined as a coloured substance which when applied to the fibre, gives it a permanent colour, resistant to the action of light, water and soap. Because of their commercial importance, a very large number of dyes have been synthesized and many of them have been placed in the market. The latest colour index, sponsored jointly by the "society of dyers and colourists" (Great Britain) and "The American association of textile chemists and colourists" lists about 4500 different dyes and pigments. They have assigned names according to the method of application and given a colour index number according to their structures. Each manufacturer, however, usually labels his products with registered trademark. The dyes are categorized according to common parent structures. The chemical categories of dyes are given in Table 1.04, p.19.
### TABLE-1.04
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>3</td>
<td>Azo dyes</td>
<td>Tropaeoline OO, Tropaeoline OOO*, Naphthol blue black, Naphthol blue 12 BR, Congo red, Erichrome Black T, Tartrazine, Palatine fast blue GGN</td>
</tr>
<tr>
<td>4</td>
<td>Thiazoles</td>
<td>Primuline</td>
</tr>
<tr>
<td>5</td>
<td>Diphenyl methanes</td>
<td>Auramine O</td>
</tr>
<tr>
<td>6</td>
<td>Triphenyl methane and analogous dyes</td>
<td>Fast green FCF*, Bromo cresol green Bromo phenol blue, Chrome violet, Erioglaucine A, Rosaniline</td>
</tr>
<tr>
<td>7</td>
<td>Xanthines</td>
<td>Pyronine G, Eosine</td>
</tr>
<tr>
<td>8</td>
<td>Acridines</td>
<td>Acridine orange NO</td>
</tr>
<tr>
<td>9</td>
<td>Phenazines</td>
<td>Azocarmine G*, Lissamin blue BF, Woolfast blue BL*, Safranin – O*</td>
</tr>
<tr>
<td>10</td>
<td>Phenoxazines</td>
<td>Celestine blue <em>, Galloycyanine</em>, Cresyl fast violet acetate</td>
</tr>
<tr>
<td>11</td>
<td>Thiazines</td>
<td>Methylene blue*</td>
</tr>
<tr>
<td>12</td>
<td>Benzoquinones and Naphthaquinones</td>
<td>Naphthazarin</td>
</tr>
<tr>
<td>13</td>
<td>Anthraquinones</td>
<td>Alizarin red S, Suprachen violet 3B, Duranol blue B</td>
</tr>
<tr>
<td>14</td>
<td>Indigoids</td>
<td>Indigotin, Cibascarlet G</td>
</tr>
<tr>
<td>15</td>
<td>Solubilized vat dyes</td>
<td>Indigosol O</td>
</tr>
<tr>
<td>16</td>
<td>Sulfur dyes</td>
<td>Sulfur Black T</td>
</tr>
<tr>
<td>17</td>
<td>Sulfurised vat dyes</td>
<td>Hydron Blue R</td>
</tr>
<tr>
<td>18</td>
<td>Phthalocyanines</td>
<td>Monastrial fast blue BS</td>
</tr>
<tr>
<td>19</td>
<td>Cyanines</td>
<td>Kryptocyanine, Astraphloxine FF</td>
</tr>
<tr>
<td>20</td>
<td>Miscellaneous dyes</td>
<td>Quinoline yellow</td>
</tr>
</tbody>
</table>

*Dyes used in the present investigation.*
Until recently, chemists extended their study to correlate visual colour with structural features of molecule. In 1976, Witt pointed out that two types of groups are usually present in highly coloured compounds, unsaturated groups which he called ‘chromophores’ and groups that intensify the colour, which he called ‘auxochromes’ (Table 1.05, p.21).

Subsequently, it was suggested that the entire conjugated system is responsible for colour and that either nitro or amino group shift the absorption to longer wavelengths. The more the extension of conjugation, the greater will be the number of molecular orbitals present and the energy levels are spaced more closely. Hence less energy is required for electronic transitions (Table 1.06, p.22) 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 with resulting high intensity absorption. Derivatizations such as acylation of an amino or a hydroxyl group merely decreases the availability of an unshared pair of electrons for interaction with the conjugated systems.
<table>
<thead>
<tr>
<th>Chromophores</th>
<th>Auxochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Chemical Structures" /></td>
<td><strong>Acidic</strong></td>
</tr>
<tr>
<td>-OH in aromatic nucleus</td>
<td>-NH₂, -NHR, -NR₂</td>
</tr>
<tr>
<td>-COOH</td>
<td>-NR₂</td>
</tr>
<tr>
<td>-SO₃H</td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
TABLE 1.06
ELECTRONIC TRANSITIONS IN ABSORBING SPECIES

<table>
<thead>
<tr>
<th>Transitions in energy levels involving σ, π and n electrons</th>
<th>Involving ‘d’ &amp; ‘f’ electrons</th>
<th>Charge – transfer spectral absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>σ - σ⁺, n- σ⁺, n- π⁺, π - π⁺ (imp). σ⁺ and π⁺ are anti bonding orbitals while ‘n’ involves non-bonding orbital having an energy in between bonding and anti bonding orbitals. The polarization force between solvent and species lower the energy levels of excited and unexcited states. As (n- σ⁺ or σ - σ⁺) require much higher energies, they are seen in a vacuum – UV and are harder to observe.</td>
<td>Transition metals involve electronic transitions among different energy levels of d – orbitals t₂g (dₓᵧ, dᵧz, dₓz) and e₈ (dₓ² - y², dₓz) are split by Δ in presence of ligands. I⁻ &lt; Br⁻ &lt; Cl⁻ &lt; F⁻ &lt; OH⁻ &lt; oxalate²⁻ &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 electrons to give absorption radiation (longer wavelength)</td>
</tr>
</tbody>
</table>
Dye stuffs are used as analytical reagents in two different ways depending upon the types of their involvement.

(a) Coloured anionic or cationic form, which involves in ion-association complex formation with oppositely charged ion of drug through electrostatic forces of attraction.

(b) Variations in $\lambda_{\text{max}}$ and $C_{\text{max}}$ values of the dye on treatment with an oxidizing, reducing or complex forming agent lead to the development visible spectrophotometric determination of analytes (direct; reducing agent; indirect; initial oxidation of analyte with an oxidant followed by estimation of unreacted oxidant with a dye; oxidant reacted with analyte is oxidant initially taken minus oxidant unreacted). In the present investigations, dyes have been used in both the ways, directly (Table 1.07, p.25-27) and in combination with an oxidant (Table 1.08, p.28-29) for the estimation of selected drugs (Table 1.03, p.15-17, Chapters II-V).

Molecular complexes formation

The term molecular complex is used to describe a variety of association products of two or more molecules. Recently, 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{40-44}. Four classes of molecular complexes are well known: (a) those in which molecules are trapped in cages in the host lattice (clathrate compounds); (b) those which are held together by hydrogen bonding; (c) those which are 'polarization bonded'; (d) those in which molecules of low ionisation potential are associated with molecules of high electron affinity to form electron-donor-acceptor (EDA) complexes, recognized by the modification of the physico-chemical properties of the component molecules. Most characteristic is the presence of a characteristic charge-transfer band in the visible-UV spectrum of the complex, not found in the
spectrum of either component. The most general interpretation includes all complexes in which one partner is a potential Lewis base (electron-donor) and the other is a potential Lewis acid (electron-acceptor). This broad definition can include polarization-bonded complexes at the weak end of the interaction energy scale and complexes of transition metal ions or boron trifluoride adducts at the strong end of the scale. Mulliken\textsuperscript{45} has classified electron donors and electron acceptors according to the functional type and structural type. This classification is acceptable only for those complexes between increvalent or sacrificial electron donors and sacrificial electron acceptors. Increvalent donors are lone-pair (n) electrons for which electrons are donated from an essentially non-bonding orbital. Sacrificial σ or π electron donors or sacrificial σ* or π* electron acceptors are molecules in which electrons are donated from bonding σ or π molecular orbitals or accepted by antibonding σ* or π* molecular orbitals respectively, with the consequent weakening of intermolecular bonding of the electron donor or acceptor. Interactions between increvalent electron donor and increvalent electron acceptors lead to much stronger complexes which are generally better regarded as covalently bonded molecules.

The forces which lead to the formation of molecular complex include 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 temperature but exist in solutions in equilibrium with their components. They can be detected readily because of differences in physical properties (eg. absorption spectra, solubility in organic solvents) from those of the pure components.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Dye name / C.I. No.</th>
<th>Chemical category</th>
<th>Chemical name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tropaeolin 000 (TP 000)/14600</td>
<td>Azodye</td>
<td>Benzene sulphonic acid, 4-(4-hydroxy-1-napththalenyl)azo] - monosodium salt</td>
<td><img src="image1.png" alt="Structure 1" /></td>
</tr>
<tr>
<td>2</td>
<td>Azocarmine G (AG)/50085</td>
<td>Phenazine dye</td>
<td>Benzo[a]phenazinium, 5-(4-sulphophenyl) amino]- inner salt, monosodium salt</td>
<td><img src="image2.png" alt="Structure 2" /></td>
</tr>
<tr>
<td>Table 1.07 contd.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Benzofulphenazinium, 7-phenyl-5,9-bis(phenylamino)-4,10-disulpho, inner salt, monosodium salt.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="" alt="Chemical Structure" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3,7-bis (dimethylamino) phenazothionium chloride.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="" alt="Chemical Structure" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenazine dye</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wool fast blue BL (WFB BL)/50315</strong></td>
</tr>
<tr>
<td><img src="" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><strong>Thiazines</strong></td>
</tr>
<tr>
<td><img src="" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Methylene blue (MB)/52015</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
### TABLE 1.08
CHEMICAL FEATURES OF DYES USED IN OXIDANT/DYE COMBINATIONS

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Dye name / CI No.</th>
<th>Chemical category</th>
<th>Structure</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Celestine Blue</td>
<td>Oxazine</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>Phenoxazin-5-i um, 1- (aminocarbonyl) -7 - (diethylamine) -3,4 - dihydroxy chloride</td>
</tr>
<tr>
<td>2</td>
<td>Galloccyanin</td>
<td>Phenoxazine</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>Phenoxazin-5-i um, 1-carboxy-3,4-dihydroxy, chloride</td>
</tr>
<tr>
<td>3</td>
<td>FG FCF</td>
<td>Triphenyl methane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Benzenemethanaminium, N-ethyl-N-[4-[[4-ethyl[(sulphaphenyl)methyl]amino]phenyl (4-hydroxy-2-sulphophenyl)methylene]-2,5-cyclohexadien-1-ylciden]-3-sulpho-, inner salt, disodium salt. (C.I. 42053)
Dyes in ion-association complex formation: (Methods M₁ to M₄)

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 chromogen (dye or metal complex) possessing charge (cationic or anionic in nature) and so insoluble in organic solvents. The other is colourless, possessing opposite charge (anionic or cationic) to that of chromogen.

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\textsuperscript{43-45}. The structure of the species formed may depend upon the experimental conditions (concentration of the components, pH of the aqueous phase). The colour can be altered or intensified upon acidification or reextraction into a buffer. The presence of hydrophilic substituents such as $-\text{OH}$ or $-\text{COOH}$ often prevents extraction of the complex into the organic phase.

The selectivity of the reaction may increase by using appropriate organic solvent as an extractant, which then depends upon parameters such as the polarities of the amine and of the dye. Several acidic dyes belonging to different chemical classes have been used for the assay of basic drugs\textsuperscript{46-48}. According to the same principle, basic dyes\textsuperscript{49} can be used for the assay of acidic drugs.

Preliminary investigations were carried out using acidic dyes (AG\textsuperscript{50}, TPoo\textsuperscript{51}) and basic dyes (MB, SFN-O) by extraction spectrophotometric technique in the assay of selected drugs (acidic dyes for basic drugs and basic dyes for acidic drugs). The chemical features of the dyes used in the ion-association complex formation are given in table 1.07, p.25-27.

Based on the preliminary investigations it was noticed that three drugs (GTB, DSP and SRL) have responded to acidic or basic dyes respectively by
extraction spectrophotometric technique. Two acidic dyes and two basic dyes were preferred TPoo, AG, MB, SFN-O as they were found to be superior (Table 1.03, p.15-17 and Chapters II, III & IV) over the others.

**Dyes used in oxidant / dye combinations: (Methods $M_6$, $M_7$, $M_8$)**

Variations in $\lambda_{\text{max}}$ and $C_{\text{max}}$ values of the dye on treatment with an oxidizing, reducing or complex forming agent lead to the development of visible spectrophotometric determinations of analytes. In these methods analytes are oxidized with an excess of oxidant and unreacted oxidant is estimated with a dye. The chemical features of the dyes used in oxidant / dye combinations are given in table 1.08, p.28-29. The coloured species formation is shown under the analytical utility reviews of the respective oxidants FG FCF, GC and CB ($M_6$, $M_7$, and $M_8$).

**Nature of oxidizing agents in redox reactions : (Methods $M_5$ to $M_{22}$)**

As one of the major categories of chemical reactions, redox reactions have wide application in analytical chemistry. Oxidation and reduction usually occur simultaneously in a reaction, one substance being in the process of oxidizing the other. Many oxidations, which are quantitative (stoichiometric or reproducible yields under specified experimental conditions), can be used as basis for volumetric determinations. A standard solution of an oxidizing agent is used for the titrations. The oxidizing agents used in visible spectrophotometric determination of selected drugs in the present investigation are N-bromosuccinimide (NBS, $M_8$ & $M_9$), sodium metaperiodate ($\text{IO}_4^-$, $M_{13}$), potassium permanganate ($\text{KMnO}_4$, $M_9$), chloramine-T (CAT, $M_7$ & $M_{14}$), and Folin ciocalteu reagent (FC, $M_5$). In some instances, the reaction between analyte and oxidant is slow, in which case a back or indirect procedure has been-suggested. An excess of standard oxidant is added to the analyte solution and is allowed to react for a given time. The excess oxidant present is determined either by titrimetry or colourimetry. The reacted oxidant (blank-
test) corresponds to the amount of analyte present. The titrimetric methods are suitable for the determination of analyte at mg level and not up to µg level. The most serious limitation of oxidimetry is lack of specificity and it cannot be used with complex mixtures unless separation by physical methods such as differential solubility, adsorption or micro distillation is possible. In the visible spectrophotometry (colourimetry), both the oxidant and the analyte being used are present at low concentrations and the reaction rate is 1/1000 to 1/100000th of the rate at the concentration commonly used. The magnifying of the time scale confers some selectivity in oxidants and makes it possible to oxidize certain compounds specifically in the presence of other more stable compounds. Useful differences in reaction rate however will exist only between compounds in different structural classes (basic moiety, functional groups present or both differ), but not between the compounds in the same classes. Selectivity can also be attained by using different oxidants and by varying the experimental conditions, but the suitability of an oxidant depends upon the associating ingredients. The mentioned oxidants are selective, as they react only with certain functional groups under controlled experimental conditions. In addition, the selection of an oxidant in the oxidative coupling (or charge-transfer complex formation) reactions involving the organic compound (analyte) possessing reactive functional groups such as aromatic amine, phenol, thiol or enol and a reagent (PMAP) is very important. It is possible to determine either the organic compound or oxidant by careful control of the experimental conditions. The extent of oxidation of the reagent (PMAP) and the stability of the highly reactive in situ formed intermediate, which reacts with analyte from each one, depends upon the experimental conditions (pH, nature and concentration of oxidizing agent, order of addition, temperature maintenance and time during each addition).

A survey of comparison of published oxidation potentials of organic compounds produces little guidance for the prediction of behavior of reactants because the potentials are specified for a particular reaction that are often
applicable to the situation at hand. The selection of an oxidant is usually based on the experimental data related to selectivities and rates of reaction. It is essential to know the nature and importance of each oxidant chosen in the present investigation.

N-Bromosuccinimide in all reactions is irreversibly reduced to succinimide. It is a highly selective oxidant. NBS in combination with CB or PMAP-SA has been used in the methods M3 and M5, respectively. Sodium metaperiodate has been extensively applied as a selective oxidant for the analysis of poly hydroxy compounds in particular. Under mild conditions, it cleaves the carbon-carbon bonds carrying adjacent hydroxyls, aminols etc. After completion of oxidation, the estimation involves in either the periodate consumed, the iodate formed or the aldehyde produced. Sodium metaperiodate in combination with Brucine (M13) has been preferred for the estimation of GTB and DSP. Potassium permanganate is a strong oxidizing agent. Under specified experimental conditions, the reaction in acidic medium is a complex reduction of Mn (VII) to Mn (II). The unreacted permanganate is estimated with FG FCF (method M6).

Chloramine-T, the sodium salt of p-toulene sulphochloramide slowly liberates hypochlorite on contact with water. CAT is more stable than NaOCl solution and may be either alkaline or acidic. Chloramine-T in combination with GC or Haematoxylin has been used in methods, M7 and M14, respectively. N-Bromosuccinimide (NBS) like CAT possesses a positive halogen that, on hydrolysis, combines with negative hydroxyl ion from water to form hypobromous acid. The reagent acts as a mild oxidizing agent. The analytical utility reviews on the mentioned oxidants used in the present investigations have been given separately. The selected drugs estimated by using oxidant/other reagent combinations52-54 are presented in table 1.03, p.15-17.
**NBS as an oxidant: (Methods M₈ and M₉)**

N-bromosuccinimide (NBS) contains unstable bound bromine and is used for brominations and dehalogenation in organic chemistry\(^{55,56}\). The solution of the reagent is useful for oxidative titrations\(^{57-59}\) and for titrations based on addition reactions\(^{60,61}\). The reagent behaves as a mild oxidizing agent and converts primary and secondary alcohols to the corresponding aldehydes and ketones\(^{62}\). It has been used as a quantitative oxidizing agent for hydrazines\(^{63,64}\), for thiourea and some of its derivatives\(^{65}\) (e.g. isoniazid) and for the ene-diol group in ascorbic acid\(^{66}\). The reagent has also been the basis for the determination of carbohydrates such as glucose, galactose and xylose\(^{67}\).

The reagent is highly-selective oxidant and after all the analyte has been oxidized, a slight excess gives a blue colour with potassium iodide – starch or decolourizes methyl red, either of which can be used for the end point detection. By the titrimetric procedure, analyte can be estimated only at milligram level. After completion of the reaction with analyte, the unreacted NBS can also be determined using visible spectrophotometric determination technique in which estimation at µg level is possible. The reacted NBS (NBS originally added – NBS unreacted) corresponds to the analyte. Two reagents (CB\(^{68}\), PMAP – SA\(^{69}\)) have been suggested in the literature for the colourimetric determination of NBS and in turn few drugs.

In the present investigation the author has proposed two methods (Methods M₈ & M₉) using above two reagents, for the assay of SRL. The details of investigations are furnished in table 1.03, p. 15-17 and separately in Chapter IV.

**Potassium permanganate as an oxidant: (Method M₉)**

Oxidation with potassium permanganate (MnO₄\(^{-}\)) takes place in acidic, alkaline and neutral solutions. Alkaline solutions are normally preferred for quantitative oxidations because of the speed of reaction is enhanced in this
medium. However in alkaline solution at higher temperatures spontaneous partial reduction of permanganate to manganate can cause difficulties. There are many reagents that add two -OH groups to a double bond, alkaline permanganate give syn addition from the less hindered side of the double bond. Permanganate is a strong oxidizing agent and can oxidize the glycols that are the products of the reaction\textsuperscript{70}. In acid and neutral media it always does so, hence it is not feasible to prepare glycols in this manner. They can be prepared with alkaline permanganate under mild conditions. Moreover alkaline permanganate cleaves glycols giving carboxylic acids rather than aldehydes. Double bonds can be cleaved by many oxidizing agents, the most common of which are neutral or acid permanganate. Depending upon the groups that are attached to the olefin, the products formed are generally 2 moles of carbonyl compound, 2 moles of carboxylic acid or 1 mole of each of them. The mechanism of oxidation probably involves in most cases the initial formation of glycol or cyclic ester and the further oxidation to cleave the C-C bond. Because of the availability of more selective chromogenic reagents, the usage of the non specific permanganate oxidation has been decreased for organic analysis. In some cases however with appropriate control of conditions, with use of appropriate blanks and checks with known standards and with assurance of the absence of substances (if necessary separating them by appropriate preliminary treatment) that can be attacked other than the compound determined, oxidation with permanganate can still supply useful analytical information. Compounds like carboxylic acids\textsuperscript{71}, esters of malic, citric and tartaric acids\textsuperscript{72}, propylene glycol\textsuperscript{73}, methanol\textsuperscript{74}, unsaturated compounds\textsuperscript{75}, terminal methylene groups\textsuperscript{76}, thiourea\textsuperscript{77}, and hydrazobenzene\textsuperscript{78} were determined either by the direct titration with permanganate or determination of excess permanganate. Very low concentrations of many oxidizing agents of the order of 1 to 10 micronormal, can be determined colourimetrically by using dye as a reagent.
Gordan79 reported an analytical method for the determination of 1 to 10 microgram amounts of many organic compounds (e.g. sorbic acid, citric acid) using permanganate and Fast green FCF.

In the present investigation the author has proposed a method (Method M6) using the above reagent, for the assay of SRL. The details of the investigation are given in table.1.03, p. 15-17, chapter IV.

Metol, as one of the reagents: (Methods M9, M11, M17 & M19)

Research work carried out by Sastry et al in foods and drugs laboratories over the past few years lead to the moulding of a very simple molecule like metol (P-N-methyl amino phenol sulphate, PMAP) into the versatile chromogenic reagent, capable of reacting with different functional groups under different conditions, enabling the estimation of several pharmacodynamic agents belonging to different classes80.

PMAP is a bifunctional substrate, when treated with an oxidizing agent it undergoes oxidation with two-electron transfer to yield the very unstable and highly reactive P-N-methyl benzoquinone monoimine (PMBQMI). The extent of oxidation of PMAP and the stability of the quinoneimine

![Chemical structure](image)
formed depend upon the experimental conditions (pH, concentration of the oxidizing agent and its redox potential). The selection of an appropriate oxidant in the colourimetric estimation of different compounds with PMAP, depends upon its reactivity towards the compounds and PMAP and also on the behavior of the reduced form. The reaction of the drug molecule with the PMBQMI formed in situ from PMAP and oxidizing agent results in the formation of a charge transfer complex. The charge-transfer interactions depend upon the electron affinity of the acceptor (PMBQMI), ionization potential of the donor (aromatic primary amines) and the polarity of the solvent. In the case of oxidant / PMAP – sulphanilamide (SA) system the formation of charge-transfer complex (given below) involving two moles of PMBQMI and one mole of sulphanilamide may be represented as $\pi \rightarrow \pi^*$ electron transfer and hydrogen bond formation between quinoneimine oxygen and amine hydrogen of sulphanilamide.

In one reported indirect method (M9), the drug was allowed to react with excess of the oxidant (NBS) and excess oxidant was then determined either directly or after release of iodine from potassium iodide with PMAP-sulphanilamide$^{81}$.

In the present investigations, NBS/PMAP-SA, $I_2$-$OH$/$H^+$/PMAP-INH or $I_2$ / PMAP – SAc and TA/PMAP-Cr(VI) have been used as reagents in the determination of GTB, SRL and CTA (Method M9, M11 or M17&M19) and the details of the investigations have been incorporated in chapters II , IV &V.

**Chloramine – T as one of the reagents: (Methods M7 & M14)**

There has been a growing importance in the role of chloramine – T (CAT) in recent years, as an analytical reagent in the determination of organic compounds$^{82}$. CAT was basically developed for its disinfectant and antiseptic properties and in view of the high yields of the reaction products obtained in its reactions; it has been adopted for use in the determination of many organic
compounds. It acts as a selective oxidizing agent in both acid and alkaline media\textsuperscript{83}. When the reaction is stoichiometric and fast, the solution of CAT is useful in titrimetric determinations in which the end point is detected with either visual indicator\textsuperscript{84-86} or an electrometric technique i.e. potentiometry\textsuperscript{84}. But in many instances direct titration with CAT using a visual or potentiometric end point was not practicable because the oxidation though rapid, was not instantaneous and a back titration procedure was developed\textsuperscript{87}. An excess of standard CAT was added to the analyte solution and was allowed to react for a given time. The excess CAT present in the acidic medium was determined by titrimetry\textsuperscript{87,88}. But titrimetric procedures are not suitable for the determination of compounds at microgram levels. Sastry et al \textsuperscript{89}, reported an indirect spectrophotometric procedure for the determination of some drugs using CAT / Gallocyanine (GC) combination.

In the present investigations the author proposes simple, selective and sensitive indirect spectrophotometric procedure for the determination of SRL using CAT/GC combination. The principle involved in this procedure is quantitative de colourization of GC, an oxidizable dye by excess CAT, which is found to be highly suitable among the several dyes tested (Table 1.03,p. 15-17).

In the second method, CAT has been used as an oxidant in combination with Hae T (M\textsubscript{1a}), to develop visible spectrophotometric methods for the determination of organo sulphur compound (SRL) and the results are incorporated in chapter IV.

\textit{With polyacid complexes: (Method M\textsubscript{3})}

The chemistry of molybdenum is complicated. It forms compounds corresponding to oxidation numbers +2 to +6. The most stable and commonly encountered compounds of molybdenum are derived from its oxide MoO\textsubscript{3}. The molybdenum compounds corresponding to the oxidation states ranging from +2 to +5 are mostly complex species. The tetrahedral anion MoO\textsubscript{4}\textsuperscript{2-} in aqueous
medium which is not strongly oxidized form on acidification exist as isopolyanionic species as a result of polymerization and condensation reactions having an arrangement $\text{Mo}_6$ octahedra as exemplified by $\text{Mo}_7\text{O}_{24}^{6-}$ and $\text{Mo}_8\text{O}_{26}^{4-}$. Molybdate can form heteropolyanionic species such as Phosphomolybdate $\left[\text{PMo}_{12}\text{O}_{40}\right]^{3-}$ phosphomolybdo tungstic acid, $[\text{FC}: 3\text{H}_2\text{O} \cdot \text{P}_2\text{O}_5 \cdot 13 \text{WO}_3 \cdot 5 \text{MoO}_3 \cdot 10\text{H}_2\text{O} \text{ and } 3\text{H}_2\text{O} \cdot \text{P}_2\text{O}_5 \cdot 14 \text{WO}_3 \cdot 4 \text{MoO}_3 \cdot 10\text{H}_2\text{O}]$ and molybdo periodate $\left[\text{I(MoO}_4\right]^{5-}\text{]}$ in the presence of some anions such as $\text{PO}_4^{3-}$, $\text{SiO}_4^{2-}$, $\text{IO}_4^{-}$, $\text{WO}_4^{-}$. The isopolyanionic or heteropolyanionic species undergo reduction to coloured molybdenum species with certain bioactive compounds. The $\lambda_{\text{max}}$ values of reduction products vary from 600nm- 840nm depending upon the reaction conditions (nature and strength of acid or base medium, temperature, time), nature of polyacid (very efficient if the composition of heteroacids are more) and nature of reducing agent (analyte). "Molybdenum blue" is the result of mild reduction of an acidified solution, which contains Mo (VI), either as an iso – or a hetero-polymolybdate anion (or even alkaline conditions) or as a suspension of $\text{MoO}_3$ in water. The "molybdenum blue" contains both oxide and hydroxide, with $\text{Mo O(OH)}_2$, in olive green compound, as one limit and $\text{Mo O}_3$ as the other. The compounds in which the mean oxidation state of Mo is between $5+$ and $6+$ are the blue ones [e.g. $\text{MoO}_2$ (OH) and $\text{MoO}_{25}$ (OH) $_{0.5}$] . The tone of the colour of a reduced solution of Mo (VI) changes between the emerald blue and the deep ultramarine blue, as a function of the concentration of each absorbent particle, which comes from the reduction of Mo (VI).

**FC reagent under alkaline conditions : ($M_3$)**

Among the various heteropolyacids, phosphomolybdo tungstic acid, the well known Folin-Ciocalteu reagent $^{91}$ (F-C reagent) was preferred by a number of workers for the determination of drugs. Allopurinol $^{92}$, caffeine $^{93}$, pentazocine $^{94}$, oxymetazoline, isoxsuprine, orciprenaline, pholedrin, vitamin-K and rutin $^{95}$ are some typical examples of drugs estimated in this manner. Rao et al. $^{96}$ reviewed the applications of this reagent and extended the use of this
reagent to drugs, containing not only phenolic groups but also amino groups and also certain drugs, which contain neither of these groups. The colour formation by FC reagent\(^97,98\) was tentatively explained.

The above reagent (Method M\(_3\)) has been used in the determination of SRL in the present investigations (Table 1.03, p.15-17, chapter IV).

**Blue tetrazolium (Method M\(_{12}\))**

It must be emphasized at first that determinations based on the reduction of colourless tetrazolium salts to highly coloured formanzans in alkaline media are far from specific. However, a limited selectivity can sometimes be attained, provided that the given operative conditions are strictly observed. Factors such as time, temperature and basicity are of the highest importance. It should be also taken into account that the formazan formed can be partly reoxidized by air oxygen\(^99\).

The colourimetric determination of reducing sugars with 2,3,5-triphenyltetrazolium chloride was devised by Mattson and Jensen\(^100\), the method was applied by Fairbridge et al.\(^101\) to the estimation of blood sugar, glucose, lactose, cysteine and ascorbic acid.

Using 2,3,5-triphenyltetrazolium chloride and 2,5-diphenyl 1-3-(4-styrylphenyl) tetrazolium chloride, Avigad et al.\(^102\) showed that, under suitable conditions, these salts are readily reduced by sugars manifesting enediol isomerism, but not by those in which such isomerism is impeded by the presence of a substituent in carbinol group vicinal to the carbonyl group.

Carticosteroids can be determined through their reducing 17-ketol grouping 2,3,5-Triphenyltetrazolium chloride\(^103\) was used at first, but it gives poorly reproducible results. More stable 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium bromide was proposed by Henly\(^104\), but good results are also obtained with blue tetrazolium\(^105\), which is now widely used. The operative
conditions are, however, critical. Under somewhat different conditions and in the heat various 3-ketosteroids with unsaturated ring A react also. These facts were turned to account by Smith et al., who devised a colourimetric method with blue tetrazolium for the analysis of norethindrone, norindrone acetate and norgestrel.

Blue tetrazolium allows a ready determination of thiols. This salt is also reduced by dithiocarbamates which are obtained by reacting a primary or secondary aliphatic amine with carbon disulfide, permitting in this way a colourimetric determination of these classes of amines.

In the present investigation blue tetrazolium has been used for the assay of CTA. The details of investigation are furnished in Table 1.03 p.15-17 and separately in chapter V.

**Oxidative coupling reactions (Methods M13-M15)**

Among the many reported methods, oxidative coupling procedure involving the use of couplers (Brucine or HaeT) in the presence of an appropriate oxidant (used in the present study IO4- or CAT) under slightly acidic, neutral or slightly alkaline conditions to form highly coloured species were explored for the assay of drugs possessing functional groups such as phenolic hydroxyl, aldehyde, amine or diol in general. In a similar way, DCQC has been suggested for their estimation in the absence of an oxidant. In the present investigation these reagents have been used for the assay of one or more selected drugs (Table 1.03, p.15-17; M13, M14 and M15; chapters II, III and VI).

**Brucine – periodate (Method M13)**

Brucine (2,3-dimethoxystrychnine) under acidic conditions has been reported to be an effective reagent for spectrophotometric determination of nitrates and nitrites, cerium, manganese, cadmium and platinum.
Several modifications have been introduced for the spectrophotometric determination of nitrites and nitrates using this reagent \textsuperscript{114,115}. It was also reported subsequently that in combination with potassium persulphate, brucine can be used for the spectrophotometric determination of halides\textsuperscript{116} and cysteine\textsuperscript{117} and as an indicator in redox titration\textsuperscript{118-120}. Brucine forms a 1:1 coloured complex with p-dimethylamino cinnamaldehyde under acidic conditions\textsuperscript{121}.

Sodium metaperiodate is an effective oxidant for converting methyl substituted p-dihydroxy phenols to o-quinones\textsuperscript{122} and is also colour stabiliser\textsuperscript{123}. Sastry et al. used brucine-periodate reagent for spectrophotometric determination of tryptophan and some sulphur compounds\textsuperscript{124} and for tetracyclines, chloramphenicol and streptomycin\textsuperscript{125}. According to them, periodate converts brucine into bruciquinone, which in turn undergoes nucleophilic attack by the most electron rich portion of the coupler (tryptophan and other mentioned compounds) to yield 1-mono substituted bruciquinone derivatives with an absorption maximum at 500-510 nm as the coloured species.

Since all the selected drugs under the present investigation contain primary or secondary aliphatic amino groups, they were tested with brucine-periodate reagent under acidic conditions. On the basis of this observation the author has developed a method for the assay of GTB, DSP and CTS (Method M\textsubscript{13}) in bulk samples and dosage forms. The details of these spectrophotometric investigations are incorporated in chapters II, III and VI.

**Haematoxylin – CAT (Method M\textsubscript{14})**

Haematoxylin [7,11b-dihydrobenz-(b)-indeno-[1,2-d]-pyran-3,4,6a,9,10-6(H)-pentol] is a catechol derivative isolated form heart wood or log wood (haematoxylin campeachianum) and is an important staining agent\textsuperscript{126} Haematoxylin or its oxidized form hematein (o-hydroxy quinone)\textsuperscript{127} has been
widely used for the detection and determination of several metal ions (aluminium\textsuperscript{128}, arsenic\textsuperscript{129}, tin\textsuperscript{130} and molybdenum\textsuperscript{131}) and organo sulphur compounds (derivatives of thiobarbituric acid, thiols\textsuperscript{132}, thiram, ziram\textsuperscript{133}, thiouracil\textsuperscript{134}, disulfiram\textsuperscript{135}, piroxicam\textsuperscript{136}, phenothiozines\textsuperscript{137}, and thiazides\textsuperscript{138}).

The compounds with phenolic hydroxyl were proved to be remarkable organic analytical reagents characterized by high reactivity tendency towards metal and non-metal ions and oxidizing systems\textsuperscript{139,140}. Some interesting qualitative rules for the light absorption of complexes with phenolic ligands (eg: the catechol units in HaeT, one catechol unit and one enolic form of o-benzoquinone in haematein) were observed partly to the ligand structure and substituents, partly to the resulting composition and structure of complex are evident.

Haematoxylin or its oxidized form has been used as an analytical reagent for the determination of organo-sulphur compounds\textsuperscript{132,133,136}. So preliminary investigations were carried out with haematoxylin in presence of an oxidant (CAT, sodium hypochlorite, H\textsubscript{2}O\textsubscript{2}, Ce(IV), IO\textsubscript{4}\textsuperscript{-}, IO\textsubscript{3}\textsuperscript{-}, Fe(III), Cr(VI) or Fe(CN)\textsubscript{6}\textsuperscript{3-}) or hematein, by varying temperature (25-100°C), pH(1-10) and time (5-60 min.) to develop visible spectrophotometric method for the determination of organo sulphur compound (SRL) in the present investigation. Heating SRL at 70°C for 5 min, with HaeT in presence of CAT and phosphate
buffer (pH 7.0) appeared to be the best experimental condition for getting coloured product with considerable absorbance. In the developed method (Method M₁₄) HaeT – CAT combination has been used for the determination of SRL in its formulations. The results of the investigation are incorporated in chapter-IV.

**DCQC:** (Method M₁₅)

DCQC, 2,6-dichloroquinone N-chlorimide¹⁴¹-¹⁴³ (Gibb's reagent¹⁴⁴) is a general reagent for the determination of phenols, substituted phenols¹⁴⁵, amines¹⁴⁶-¹⁴⁸ and substituted amides¹⁴⁷. It is also used for the determination of thiols¹⁴⁹ (in alkaline medium to give a dichloroquinone sulphinimine), uric acid¹⁵⁰, thiophelline¹⁵¹, thiouracil¹⁴¹ and pyridoxal. The coupling takes place at the para (if it is either free or substituted with electron withdrawing group) or ortho (if para position is blocked) position to the phenolic hydroxyl or amino group in the case of phenols or amines and directly to imido nitrogen in the case of substituted amines. Among the selected drugs for investigation, DSP gave coloured products with DCQC (Table-1.03; p.15-17) and the details of the investigation are presented in chapter III.

**Alkaloid precipitants:** (Methods M₁₆, M₁₇, M₁₈ and M₁₉)

Alkaloids (meaning alkali-like) are generally defined as physiologically active complex molecules of plant origin (with basic nature), in which at least one nitrogen atom form part of a cyclic system. Alkaloids are detected with the aid of group of reactions due to their chemical properties, structure and presence of functional groups. These reactions are based on the ability of the alkaloid to yield simple or complex salts with various acids, heavy metal ions, complex iodates and other substances. The products are insoluble in water and these reactions can be called as precipitation reactions. The reagents, which are used to precipitate alkaloids, are known as alkaloidal precipitants¹⁵². The precipitate is ascribed to the formation of a molecular complex resulting from
the interaction of the unshared pair of electrons on nitrogen in amine with an unoccupied molecular orbital of the alkaloidal precipitant molecules. The complex may be represented as a resonance hybrid of the type \(D^+A^- \leftrightarrow D^+A'\) where \(DA\) is a close association of the electron donor molecule \(D\) and the electron acceptor molecule \(A\), where as in \(D^+A^-\) an electron has been transferred from \(D\) to \(A\).

In the present investigation, it has been observed that one selected drug GTB furnish precipitates with alkaloidal precipitants (Phosphomolybdic acid, (PMA), Method M\textsubscript{16}; Iodine (I\textsubscript{2}) Method M\textsubscript{17}; Ammonium molybdate,( AM) method M\textsubscript{18}; Tannic acid , (TA) method M\textsubscript{19}). Since they posses the nitrogen containing groups( Tertiary amino groups and hetero cyclics).

In addition to precipitation reactions, colour reactions have also been combined to estimate GTB. They are based on the chemical reactions, with either released alkaloidal precipitants from the precipitate with acetone (PMA) or unreacted precipitant in filtrate (I\textsubscript{2} and TA) with chromogenic reagents such as Co(II)-EDTA(for PMA), potassium thiocynate (for AM),PMAP-Cr(VI)(for TA) and PMAP-SAc (for I\textsubscript{2}) (table-1.03, p. 15-17)

**PMA / Co (II)-EDTA: (Method M\textsubscript{16})**

The reduction of PMA by various reducing agents like ascorbic acid, hydrazine hydrate\textsuperscript{153}, to yield molybdenum blue is well known. But the reduction of PMA by Co(II)-EDTA complex has not been exploited only recently. The author has taken this advantage and succeeded in developing chromogenic reagent combination \([\text{Co(II)} - \text{EDTA}]\) as reducing agent for estimating phosphomolybdic acid and in turn GTB. Preliminary investigations were made with this reagent \([\text{Co(II)} - \text{EDTA}]\) in addition to the other two (ascorbic acid and hydrazine hydrate) for the estimation of PMA and in turn GTB. Co(II)-EDTA complex has been found to be sensitive over other two reducing agents.
The present method involves two steps. First step is the quantitative precipitation of GTB with PMA. The second step is the reduction of PMA (released with acetone) from the adduct by Co (II) – EDTA complex to yield molybdenum blue. The information has been given in chapter II.

**I$_2$/PMA – SAc (Method M$_{17}$)**

Iodine is sparingly soluble in water but is quite soluble in solution containing iodide ions. Iodine forms a triiodide complex with iodide.

\[ I_2 + KI \rightarrow KI_3 \]

I$_2$ is a much weaker oxidizing agent, when compared to several other oxidants. In addition I$_2$ functions as precipitant owing to the formation of molecular complexes (brown precipitates) with aliphatic tertiary amines and aromatic nitrogen heterocyclics, probably due to the formation of a periodide (eg: caffeine, C$_{8}$H$_{10}$N$_{4}$O$_{2}$ H,$\text{I}_{4}$).

Sastry et al$^{154}$ have determined vasodilator drug (pentoxifyllin) based on the haloform reaction with a known excess of standard iodine under alkaline conditions and estimated the unreacted iodine at pH 3.0 with PMAP-INH. In the present investigations the author has developed a method (M$_{17}$) by precipitating GTB with iodine followed by estimating the unreacted iodine in the filtrate with PMAP-SAc instead of INH reported earlier. The details of the investigations have been given in chapter II.

**AM/PTC (Method M$_{18}$)**

The study of molybdenum (VI)$^{155}$ has been the subject of many publications. Molybdate ions (MoO$_4^{2-}$) readily form aggregates in solutions and various poly ions are formed as a result of polymerization and condensation reactions. Molybdate forms insoluble complexes (white precipitates) with various organic compounds of basic nature (amines and heterocyclic...
compounds), both in the presence and absence of halide ions. The compounds thus obtained are the molybdenum salts of oxygenated haloacids or the salts of various phosphomolybdic acids.

Sastry et al.\textsuperscript{155} estimated two drugs (Amidoquine and Chloroquine) by precipitating them with ammonium molybdate and estimating the released molybdate (from the ppt with acetone) with potassium thiocyanate. Potassium thiocyanate gives a yellow colour in acidic solutions with ammonium molybdate owing to formation\textsuperscript{155} of Mo(NCS)\textsubscript{6}\textsuperscript{3-}. In present investigations, GTB (Chapter II) has been estimated using this reagent.

**TA/PMAP-Cr(VI) Method (M\textsubscript{19})**

Tannic acid (TA) is known as pentadigalloyl glucose\textsuperscript{156}. Each galloyl unit possesses one adjacent trihydric phenol (pyrogalloyl) and one dihydric phenol.

\[
\begin{align*}
CH_2OR \\
H & \quad OR \\
OR & \quad H \\
OR & \quad H \\
H & \quad OR \\
R = -COC_6H_2(OH)2OCOC_6H_2(OH)_2
\end{align*}
\]

There are reports that tannic acid forms white precipitates\textsuperscript{7} with alkaloids in a neutral or weakly acidic medium. The precipitate may be regarded as an inclusion complex. The precipitate dissolves in an excess of the reagent.

Sastry\textsuperscript{157} et al determined few antimalarials by precipitating them with tannic acid and estimating the released tannic acid from the precipitate (through acetone treatment) with PMAP-Cr(VI).
In the present investigation it has been observed that selected drug GTB gives precipitate with TA/PMAP-Cr(VI) since they possess similar group as in the alkaloids. The author has developed the methods for the assay of GTB. The results are discussed in detailed in the chapter II.

SNP in inner molecular complex formation (Methods M₃₁ & M₃₂)

Sodium nitroprusside (SNP) is known as disodium pentacyanonitrosyl ferrate (II) dihydrate¹⁵⁸, sodium nitro ferric cyanide or sodium nitroprussate.

Sodium nitroprusside in solution is extremely photosensitive and undergoes several reactions, many of which, are undefined. Literature descriptions of the photodecomposition products of nitroprusside are in some cases, contradictory. In direct sunlight, it yields Prussian blue, HCN and NO¹⁵⁹. Wolf and Swinhart have reported the formation of (pentacyanoaquoferrate (III) agreeing with several other papers¹⁶⁰-¹⁶².

\[
[\text{Fe(CN)}_5\text{NO}]^{2^-} + \text{hv} (>300\text{nm}) \rightarrow [\text{Fe}^{III}(\text{CN})_5\text{H}_2\text{O}]^{3^-} + \text{NO}
\]

Photoaquation to yield \([\text{Fe(CN)}_5\text{H}_2\text{O}]^{3^-}\) and NO has been described most frequently as the primary photochemical reaction of nitroprusside ¹⁶³. Photoreduction of \([\text{Fe(CN)}_5\text{NO}]^{2^-}\) to \([\text{Fe(CN)}_5\text{NO}]^{3^-}\) in aqueous solution has been reported ¹⁶⁴. The orange to blue colour change of sodium nitroprusside solution upon standing and exposure to light has been attributed to the change of ferric to ferrous ion¹⁶⁵. When protected from light, aqueous solution of sodium nitroprusside has been reported to be stable for six months ¹⁶⁶.

Sodium nitroprusside is reduced to sodium aquoferrocyanide by the action of alkaline hydroxylamine as in the first step in the preparation of Grote's reagent ¹⁶⁷. The determination of the influence of the exchange of sixth ligand and electron delocalisation. The formation of coloured species of high tinctorial value in the case of present drug experimental conditions may be due to the incorporation of M (GTB or DSP) replacing H₂O present in
Aqueous solution of the nitroprusside reacts with a wide variety of inorganic and organic substances to form usually highly coloured reaction products\textsuperscript{169-178}. The colouration obtained with SNP under different experimental conditions may be due to the formation of ferric ferricyanide (orange yellow) or $\text{[Fe(CN)$_5$H}_2\text{O]}^2^-$, where M is the compound exhibiting liganding properties\textsuperscript{179}.

In the present investigation several procedures involving SNP under varied experimental conditions such as SNP in presence of hydroxylamine, acetaldehyde, acetone or alkali (table 1.03, p.15-17) have been followed. Of them, SNP-acetaldehyde ($M_31$) or SNP-acetone ($M_32$) combination has been preferred for its sensitivity and the details of these investigations for GTB and DSP are incorporated in chapters-II & III.

**Cobalt thiocyanate in coordination complex formation (Method $M_{20}$)**

Cobalt-thiocyanate (CTC) (formed by combination of ammonium thiocyanate and cobalt nitrate) has been proved to be a valuable chromogenic reagent for the detection and determination of amino compounds\textsuperscript{180}. The coloured species formed is the coordination complex of the drug (electron donor) and the central metal atom of cobalt-thiocyanate, which is extractable into nitrobenzene from aqueous solution.

In the present investigation, GTB forms coloured complex with CTC. The details of its estimation are presented in chapter II.
Quinones as chromogenic reagents (Methods M_{21}, M_{22}, M_{23} & M_{30})

There are many reactions involving a π-acceptor and a lone pair of π-donor in which the donor is dehydrogenated and the π-acceptor is reduced. From the standpoint of reactions, in which the transition state resembles the molecular complex, three elementary reactions are of interest: electron transfer, hydride transfer and hydrogen atom transfer. Single electron transfer has been directly observed in the absence of subsequent reactions of the radical ions produced.

\[ D + A \xrightarrow{\text{equilibrium}} DA \xrightarrow{\text{equilibrium}} D^+ + A^- \]

The position of equilibrium depends on the donor, the acceptor and the solvent. The formation of ions being favoured by donors of low ionization potential, acceptors of high electron affinity and solvents with strong solvating powers.

Quinones are good electron acceptors and amines are good electron donors. The formation of outer complexes of electron donor – acceptor (EDA) complexes between quinones and amines is well known. Since both the donor and acceptor are often very reactive, chemical reactions can occur between them. The products of such reactions may then mask or replace the anticipated EDA complex. There is good evidence for the formation of weak outer (EDA) complexes particularly in poorly ionizing solvents such as cyclohexane and CCl₄ (CHCl₃ has been used occasionally because of the low solubility of many quinones in the more inert solvent). The complexes are usually characterized by an intermolecular charge – transfer absorption band that often appears in the visible region. The energy of the band (hvCT) for a given complex agrees well with the electron donating and accepting properties of the two components. In some cases more than one intermolecular charge-transfer transition is observed. These could correspond to transitions from the highest filled and penultimate filled levels in the donor to the lowest empty level in the acceptor. Many of the
quinone-aromatic amine systems form weak outer complexes, usually though not always, with a 1:1 stoichiometry\textsuperscript{181}. The basic structures in these are stacks of alternate quinone and amine molecules with their planes parallel or nearly so, though the molecules are not normally stacked vertically above one another\textsuperscript{181}. If the quinone has a sufficiently high electron affinity, and the donor, a sufficiently low ionization potential, then the electron may be transferred from the donor to the acceptor moiety in the ground state. This will produce in principle a pair of ion radicals (radical anion and radical cation), although there may be some interaction between the two\textsuperscript{182}. The complexes with tertiary amines persist for a long time without further chemical reaction, although such subsequent reactions are dependent to a great extent on the nature of the solvent\textsuperscript{183,184}. In solvents of intermediate ionizing power, for example, ethylene glycol, dimethyl ether, acetonitrile, tetrahydrofuran or benzotrifluoride, the ions, ion pairs and acceptor complex are observed simultaneously in equilibrium. With most aromatic amines, the EDA complex fades with time because of other reactions, which lead eventually to substitution (usually substitution in the 2 and 5 positions). Semipolar and polar solvent media facilitate the formation of radical ions and substitution.

The general conclusion is that the electron affinity of the conjugated acceptor species will increase\textsuperscript{185,186} with

1. The electron withdrawing ability of the substituent.

2. The number of substituents present, depending on their respective positions in the molecule and

3. The extent of conjugation in the molecule, ethylenic compounds, for example, being better acceptors than aromatic compounds, p-benzoquinones\textsuperscript{187} with various electron withdrawing substituents such as DDQ (2,3-dichloro, 5,6-dicyano-1,4-benzoquinone), TQ(2,3,5,6-tetrachloro-1,4-benzoquinone), chloranil), DHQ (2,5-dihydroxy 3,6-
dichloro-1, 4-benzoquinone, chloranilic acid) are well known as acceptors and extensive work was performed in the study of nature of coloured..

\[
\text{DDQ} : R^1 : R^2 : \text{Cl}; R^3 : R^4 : \text{CN} \\
\text{DHQ} : R^2 : R^3 : \text{OH}; R^1 : R^4 : \text{Cl} \\
\text{TQ} : R^1 : R^2 : R^3 : R^4 : \text{Cl}
\]

Species formed with \( n \) (eg. aliphatic primary, secondary and tertiary amines) and \( \pi \) (eg. aromatic primary, secondary and tertiary amines) donors and some of these reactions have been utilized in the colourimetric assays of donors\(^{188-190}\). The products which are observed in the interaction of aliphatic amines with \( p \)-benzoquinones are normally the corresponding amino-\( p \)-benzoquinones (usually 2,5 disubstituted in primary amines, monosubstituted in secondary amines\(^{191,192}\)). The reactions of several amino acid esters\(^{193}\) with various \( p \)-benzoquinones in ethanol solution have been studied. In all cases the disubstitution product was observed. During the reaction, the formation of the semiquinone ion was observed. The semiquinone ion has also been detected in the studies of Lautenberger and Miller\(^{191}\) and by Yamaoka Nagakura\(^{194}\).

The intermediate state of the \( \sigma \)-complex found between chloranil – methyl amine (I) and chloranil-methoxide (II) can be proposed as
The reaction of p-benzoquinones with tertiary aliphatic amines is of particular interest. Henbest and his co-workers\textsuperscript{195} showed that the blue product formed was 2-diethyl amino vinyl-3,5,6-trichloro-p-benzoquinone from the interaction of triethylamine and chloranil.

\[
\begin{array}{c}
\text{Cl} & \text{Cl} & \text{Cl} \\
\text{Cl} & \text{Cl} & \text{O} \\
\text{Cl} & \text{Cl} & \text{C} = \text{C} \\
\text{O} & \text{H} & \text{NET}_2
\end{array}
\]

Similar blue colour formation has been observed in the case of aliphatic secondary amines with chloranil as well, only in the presence of acetaldehyde. The N-alkyl vinyl amine obtained by condensing the amine with acetaldehyde reacts with chloranil to give vinyl amino substituted quinone to give blue colour. Chloranil has a fairly high electron affinity (1.4ev) and it is known to be a strong electron acceptor forming complexes with various Lewis bases (vinyl amino substituted base). In the present investigation, GTB which possesses a secondary amine group has been involved in the formation of vinyl amino substituted with acetaldehyde and yields coloured substituted product with chloranil (Method M\textsubscript{30}) (Table 1.03, p.15-17).

53
In the present investigation three quinones ((DDQ (M21), DHQ (M22) and TQ (M23)) have been tried in the assay of drugs (Table 1.03, p.15-17) selected and details concerning the superiority of quinone for selected drug GTB is presented in Chapter II.

Condensation reactions (Methods M24, M25 and M26)

It is well known that aromatic aldehydes form coloured condensation product (Schiff base) with aromatic primary amines in particular. It has been observed by suitable alteration of experimental conditions, others such as hydrazine\(^{196}\) and it's mono substituted derivatives, primary alkyl amines\(^{197}\), amino acids\(^{198-200}\) converted to pyrrole derivatives\(^{201,202}\), nitro aromatic derivatives reduced before hand to amines\(^{203-205}\), indole derivatives\(^{206}\), primary heterocyclic amines and m-diphenol\(^{207-213}\) also develop colour with aromatic aldehydes. These observations have led to numerous applications of aromatic aldehydes such as p-dimethyl amino benzaldehyde (PDAB), p-dimethyl amino cinnamaldehyde (PDAC) and Vanillin (p-hydroxy-m-methoxybenzaldehyde) as analytical reagents. In the present investigations Vanillin (M24), PDAB (M25) and PDAC (M26) have been used as reagents for the estimation of DSP, CTA and CTS. The details of these investigations are presented in chapter III, V and VI.

Ninhydrin/ Ascorbic acid (Method M27)

Various mild reducing agents, including photochemical reduction converts ninhydrin to hydrindantin, the bimolecular hemiacetal. Ascorbic acid appears to the reagent of choice. The hydrindantin acts as an antioxidant and prevents interference by dissolved oxygen.
The ninhydrin reaction is one of the most useful reactions of \(\alpha\) - amino acids. An intensely coloured product (Ruhemann’s purple) is produced when a \(\alpha\) - amino acid is heated with ninhydrin\(^{214}\) (indane - 1,2,3 – trione hydrate, NH). The reaction proceeds in two steps.

The purple colour development is based on Ruhemann’s purple strecker degradation\(^{215}\) in weakly acidic solution to the anion formed by the reaction of liberated ammonia with ninhydrin and its reduction product hydrindantin. The blue coloured product can be used to quantify small amounts of amino acid in solution or on paper strips. Proline and hydroxyl proline (imino acids) do not give Ruhemann’s purple, but yield coloured product in a different mechanism.

It was later observed that the stability of the reduction product hydrindantin, formed in the first step could be enhanced by making use of reducing agents such as stannous chloride\(^{216}\) or ascorbic acid\(^{216}\) (AA) etc. So in subsequent methods\(^{217}\), ninhydrin along with a reducing agent has been used as a chromogenic reagent.

Ammonium salts, dilute ammonia solutions, and some amines give a blue colour under certain conditions, apparently because of an inter molecular oxidation of the ninhydrin in the presence of AA at pH 5.0.
In the present investigation, ninhydrin and ascorbic acid combination at pH 5.0 has been utilized for the determination of DSP which possess primary amino group and the results are presented in Table 1.03, p.15-17 and Chapter III.

**Nucleophilic Substitution reaction with NQS (Method M2a)**

The reaction of 1,2-Napthaquinone-4-Sulphonic Acid (NQS) with primary aromatic amine was reported by Boniger\textsuperscript{218}. Replacement of the Sulphonate group of the napthaquinone sulphonic acid by an amino group gives N-alkylamino napthaquinone\textsuperscript{219}.

This reaction has been applied to the characterization of primary aromatic amines and later formed the basis for colourimetric determinations of amino acids\textsuperscript{220,221} sulphonamides\textsuperscript{222-224}, primary or secondary aliphatic and aromatic amines\textsuperscript{219,225}.

In the case of primary amine, the adduct can be represented by either the amino quinone structure (I), the quinone imine structure (II) or an equilibrium mixture of the two. Obviously, only the quinone structure can be taken into account with a secondary amine.

![Diagram of Aminoquinone and Quinoneimine](image-url)
It may be concluded that, under the conventional analytical conditions, an equilibrium between two forms (I and II) may intervene for the derivatives obtained from primary amines, but the quinone structure is mostly favoured.

The selected drugs GTB, DSP and CTS were estimated with this reagent and the details are presented in Table 1.03, p. 15-17 and Chapters II, III and VI respectively.

*Dehydration and internal salt formation (Citric acid / Ac₂O); (Method M₂₉)*

Tertiary aliphatic amines develop a colour with a mixture of citric acid and acetic anhydride²²⁶ and the reaction may be applied to determinations²²⁷. By the dehydration of citric acid with sulphuric acid (or acetic anhydride) aconitic acid is normally prepared. It has the trans configuration and on heating, it is readily decarboxylated to aconitic acid. On dehydration with acetic anhydride, it give cis-aconitic anhydride and α, γ-anhydro aconitic acid²²⁸. The former one involves in the formation of red-violet colour internal salt²²⁹ with aliphatic tertiary amine. In the present investigations, GTB and CTS (method M₂₉) have been estimated with this reagent and the results of their investigations are incorporated in chapters. II & VI.

1.08 General methodology for the development of new visible Spectrophotometric methods:

Development of a method:

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

After selection of the analytical wavelength, the chromogenic reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknowns should be on a definite time schedule.

**Optimization of analytical method**

The basic reactions of the spectrophotometric methods in the present investigation include (a) ion-association complex formation (b) oxidation followed by charge-transfer complex formation (c) reduction (d) oxidative coupling (e) complex formation. In each type of basic reaction, the coloured species is formed or the final colour of the reaction mixture whose absorbance is measured and thus the sensitivity of the method, rate of colour formation and stability is affected by the concentration of the reagent in the solution, the nature of the solvent, temperature, pH of the medium, order of addition of reactants and intervals between additions. For simple systems, having no interaction between variables, the one variable at a time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum conditions. The OVAT approach requires all variables but one to be held constant while a univariate search is carried out on the variable of interest. The details of fixing optimum conditions used in different procedures of present investigation are furnished in subsequent chapters.
Calibration

Calibration is one of the most important step in bioactive compound analysis. A good precision and accuracy can only be obtained when a good calibration procedure is adopted. In the spectrophotometric methods, the concentration of a sample cannot be measured directly, but is determined using another physical measuring quantity ‘Y’ (absorbance of a solution). An unambiguous empirical or theoretical relationship can be shown between this quantity and the concentration of an analyte. The calibration between $Y = g(x)$ is directly useful and yields by inversion of the analytical calculation function.

The calibration function can be obtained by fitting an adequate mathematical model through the experimental data. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. In practice, however, many deviations from this ideal calibration line may occur. For the majority of analytical techniques the analyst uses the calibration equation

$$Y = a + bx$$

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

The method of least-squares

Least-squares regression analysis can be used to describe the relationship between response ($Y$) and concentration ($X$). The relatationship can be represented by the general function.

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

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

We adopt the convention that the ‘x’ values relate to the controlled on
independent variable (e.g. the concentration of a standard) and the Y values to
the dependent variable (the response measurements). This means that the ‘x’
values have no error. On the condition that errors made in preparing the
standards are significantly smaller than the measuring error (which is usually
the case in analytical problems). The values of the unknown parameters a, b,
...bₙ must be estimated in such a way that the model fits the experimental data
points (xᵢ, yᵢ) as well as possible.

The true relationship between x and y is considered to be given by a
straight line. The relation between each observation pair (xᵢ, yᵢ) can be
represented as

\[ yᵢ = α + β xᵢ + eᵢ \]

The signal yᵢ is composed of a deterministic component predicted by
linear model and a random component eᵢ. One must now find the estimates ‘a’
and ‘b’ of the two values α and β. This is done by calculating values of ‘a’ and
‘b’ for which \( eᵢ^2 \) is minimal. The component eᵢ represent the differences
between the observed yᵢ values and the predicted yᵢ values by the model. The
eᵢ are called the residuals, a and b are the intercept and slope respectively

\[
b = \frac{n \sum_{i=1}^{n} xᵢ yᵢ - \sum_{i=1}^{n} xᵢ \sum_{i=1}^{n} yᵢ}{n \sum_{i=1}^{n} xᵢ^2 - \left( \sum_{i=1}^{n} xᵢ \right)^2}
\]

\[
a = \frac{\sum_{i=1}^{n} yᵢ \sum_{i=1}^{n} xᵢ^2 - \sum_{i=1}^{n} xᵢ \sum_{i=1}^{n} xᵢ yᵢ}{n \sum_{i=1}^{n} xᵢ^2 - \left( \sum_{i=1}^{n} xᵢ \right)^2}
\]
Standard error of estimate, $S_e$:

The standard error of estimate is a measure of the difference between experimental and computed values of the dependent variable. It can be represented by the following equation.

\[ S_e = \sqrt{\frac{1}{n-2} \sum_{i=1}^{n} (y_i - \hat{y}_i)^2} \]

$y_i$ and $\hat{y}_i$ are the observed and predicted values respectively.

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

**Standard deviation of slope, $S_b$:**

The standard deviation of slope is proportional to standard error of estimate and inversely proportional to the range and square root of the number of data points.

\[ S_b = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n-2}} \]

Where $\bar{x}_i$ denote the arithmetic mean of $x_i$ values.
Standard deviation of intercept, $S_a$:

Intercept values of least squares fits of data are often used to evaluate additive errors between or among different methods.

$$
S_a = \sqrt{\frac{\sum_{i=1}^{n} (\hat{y}_i - \bar{y})^2}{(n-2)}} \left( \sqrt{\frac{1}{\sum_{i=1}^{n} (x_i - \bar{x})^2}} \right) \left( \sqrt{\frac{\sum_{i=1}^{n} x_i^2}{n}} \right)
$$

Where $\bar{x}_i$ denote the arithematic mean of $x_i$ values.

**Correlation Coefficient, (r)**

The correlation coefficient $r(x, y)$ is more useful to express the relationship of the chosen scales. To obtain a correlation coefficient the covariance is divided by the product of the standard deviation of $x$ and $y$.

$$
r = \left( \frac{\sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})}{(n-1)} \right) / \left( \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2(y_i - \bar{y})^2}{(n-1)^2} \right)
$$

**Selectivity of the Method:**

The determination of an analyte may be disturbed by matrix and interference effects. Some of the excipients, incipients and additives present in pharmaceutical formulations may sometimes interfere in the assay of drug and in such instances appropriate separation procedure is to be adopted initially. The selectivity of the method is ascertained by studying the effect of a wide range of excipients and other additives usually present in the Pharmaceutical formulations to be determined under optimum conditions.
Initially, interference studies are carried out by the determination of fixed concentration of the drug several times by the optimum procedure in the presence of a suitable (1-100 fold) molar excess of the foreign compound under investigation and its effect on the absorbance of the solution is noticed. The foreign compound is considered to be non-interfering at these concentrations, if it constantly produces an error of less than 3.0% in the absorbance produced in pure solution.

**Linearity and sensitivity of the method:**

A knowledge of the sensitivity of the colour is important and the following terms are commonly employed for expressing sensitivity.

According to Bouger – Lambert – Beer’s Law, log intensity of incident raditations

\[ A = \log \frac{\text{intensity of incident light}}{\text{intensity of transmitted light}} = \varepsilon \text{ct} \]

The absorbance (A) is proportional to the concentration (C) of the absorbing species if absorptivity (ε) and thickness of the medium (t) are constant. When C is in moles per litre, the constant is called molar absorptivity. Beer’s Law limits and C\text{max} values are expressed as μg/ml and 1 mole⁻¹ cm⁻¹ respectively.

Sandell’s sensitivity \(^{61}\) refers to the number of μg of the drug to be determined, converted to the coloured product, which in a column solution of cross section 1 cm² shows an absorbance of 0.001 (expressed as μg/cm²)

**Limit of detection\(^{233}\):**

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

\[
\text{LOD} = 3 \frac{S_a}{b}
\]

**Ringbom's Plot**

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increases at the extremes of the transmittance scale. The scope of plot 'C' versus T, i.e. Ringbom plot gives relative error coefficient (i.e., plot of log \( C \alpha T \))

The main limitation of Ringbom's plot is that it provides no information concerning the concentration range of good precision unless it is combined with \( \Delta T \) versus T relation. The above expression valid whether the Beer's law is followed or not.

**Precision and Accuracy**

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

**Precision**

Precision refers to the reproducibility of measurement within a set, that is, to the scatter or dispersion of a set about its central value. The term 'set' is defined as referring to a number (n) of independent replicate measurements of some property. One of the most common statistical term employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the
mean, divided by one less than the number of results in the set.

The standard deviation $S$, is given by,

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

Standard deviation has the same units as the property being measured.

The square of standard deviation is called the variance ($S^2$). The Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e. $S/x$. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

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

**Accuracy**

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

**Absolute method**

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

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

**Comparative method**

In the analysis of pharmaceutical formulation or solid laboratory prepared samples of desired composition, the content of the constituent sought has been determined by two or more (proposed and official or reference) supposedly ‘accurate’ methods of essentially different character can usually be accepted an indicating the absence of an appreciable determinate error. The general procedure for the assay of commercial samples either in the proposed or reference methods comprises of various operations which include sampling, preparation of solutions, separation of interfering ingredients if any and the method for quantitative assay.

**Recovery experiments (Standard addition method)**

A known amount of the constituent being determined is added to the sample, which is analysed for the total amount of constituent present. The difference between the analytical results for samples with and without the added constituent gives the recovery of the amount of added constituent. If the recovery is satisfactory our confidence in the accuracy of the procedure is enhanced.

**Evaluation of Precision and accuracy by comparison of two procedures**

To evaluate the accuracy of the method, one often compares the method being investigated or ‘test method’ with an existing method called the ‘reference method’.

**Student t – test**

Student t – test is used to compare the means of two related (paired) samples analysed by reference and test methods. It gives answer to the correctness of the null hypothesis with a certain confidence such as 95% or
99%. If the number of pairs (n) are smaller than 30, the condition of normality of x is required or at least the normality of the difference (d). If this is the case the quantity.

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

has a student t-distribution with (n-1) degrees of freedom, where \( d = x_R - x_T \) (reference method) - (test method) and \( S_d \) is the standard deviation.

**F - Test**

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

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

It is conventional to calculate the F-ratio by dividing the large variance by the smaller variance in order to obtain a value equal or larger than unity. If the calculated F-value is smaller than the F-value from the table, one can conclude that the procedures are not significantly different in precision at given confidence level.

**Study of composition of the coloured species**

Slope analysis method \(^{238,239}\) and mole ratio methods\(^69\) were followed for finding out the composition of the components in the ion-association complexes and charge transfer complexes respectively.
PART-B

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography (HPLC)\textsuperscript{240-248} is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result, the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially. The main components of the High performance liquid chromatography are shown in the schematic diagram.

Solvent container-pump-damping unit- precolumn-injection port
\[...............................\text{Thermostat}..................\text{Column}\]
\[...............................\text{Recorder .... Detector}\]
\[...............................\text{Effluent}\]

Columns:

The heart of the system is the column. The choice of the common packing material and mobile phases depend on the physical properties of the drug. The column selection a flow chart in table 1.08 can assist one in determining which columns to examine. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C8 or C18 column (eg. Zorbax RX C8) and determine if it provides good separations. If this column does not provide good separation or the mobile phase is unsatisfactory, alternate methods or columns should be explored. Reverse phase columns differ by the carbon chain lengths, degree of end capping and percent carbon loading. Diol, cyano and amino groups can also be used for reverse phase chromatography.
<table>
<thead>
<tr>
<th>Sample</th>
<th>LC mode</th>
<th>Column choice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basic or Acidic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse phase-ion pair</td>
<td>C18,C8,C6,C4,C2,TMS,CN,amino(not for carbonyl compounds),phenyl, HamiltonPRP-1 (PH1-13)</td>
</tr>
<tr>
<td></td>
<td>Acidic Ion suppression</td>
<td>C18,C8,C6,C4,C2,TMS,CN,amino(not for carbonyl compounds),phenyl, HamiltonPRP-1 (PH1-13)</td>
</tr>
<tr>
<td>Ionizable</td>
<td>Ion Exchange</td>
<td>Strong Anion exchange</td>
</tr>
<tr>
<td></td>
<td>Anionic</td>
<td>Strong Cation exchange</td>
</tr>
<tr>
<td></td>
<td>Cationic</td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>Normal phase</td>
<td>Increasing polarity of bonded phases diol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH$_2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica</td>
</tr>
<tr>
<td></td>
<td></td>
<td>alumina</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increasing polarity of bonding phase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-4</td>
</tr>
<tr>
<td></td>
<td>Reverse phase</td>
<td>phenyl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hamilton PRP-1</td>
</tr>
</tbody>
</table>
Mobile phase:

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. Table 1.09 lists the physical properties of organic solvents commonly used for HPLC. Isocratic methods are preferable to gradient methods. Gradient methods will some times be required when the molecules being separated have vastly different partitioning properties. When a gradient elution method is used, care must be taken to ensure that all solvents are miscible.

The following points should also be considered when choosing a mobile phase:

1. It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis
2. Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.
3. The mobile phase should have a pH 2.5 and pH 7.0 to maximize the lifetime of the column
4. Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible.
5. Minimize the absorbance of buffer. Since trifluoroacetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products with out chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, which does not absorb above 200nm.
6. Use volatile mobile phase when possible to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, formic acid, acetic acid and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220 nm.
Table 1.10

PHYSICAL PROPERTIES OF COMMON HPLC SOLVENTS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>MW</th>
<th>BP</th>
<th>RI (25°C)</th>
<th>UV\textsuperscript{a} cut off (nm)</th>
<th>Density g/ml (25°C)</th>
<th>Viscosity cP (25°C)</th>
<th>Dielectric constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>41.0</td>
<td>82</td>
<td>1.342</td>
<td>190</td>
<td>0.787</td>
<td>0.358</td>
<td>38.8</td>
</tr>
<tr>
<td>Dioxane</td>
<td>88.1</td>
<td>101</td>
<td>1.420</td>
<td>215</td>
<td>1.034</td>
<td>1.26</td>
<td>2.21</td>
</tr>
<tr>
<td>Ethanol</td>
<td>46.1</td>
<td>78</td>
<td>1.359</td>
<td>205</td>
<td>0.789</td>
<td>1.19</td>
<td>24.5</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>88.1</td>
<td>77</td>
<td>1.372</td>
<td>256</td>
<td>0.901</td>
<td>0.450</td>
<td>6.02</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.0</td>
<td>65</td>
<td>1.326</td>
<td>205</td>
<td>0.792</td>
<td>0.584</td>
<td>32.7</td>
</tr>
<tr>
<td>CH\textsubscript{2}Cl\textsubscript{2}</td>
<td>84.9</td>
<td>40</td>
<td>1.424</td>
<td>233</td>
<td>1.326</td>
<td>0.44</td>
<td>8.93</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>60.1</td>
<td>82</td>
<td>1.375</td>
<td>205</td>
<td>0.785</td>
<td>2.39</td>
<td>19.9</td>
</tr>
<tr>
<td>n-propanol</td>
<td>60.1</td>
<td>97</td>
<td>1.383</td>
<td>205</td>
<td>0.804</td>
<td>2.20</td>
<td>20.3</td>
</tr>
<tr>
<td>THF</td>
<td>72.1</td>
<td>66</td>
<td>1.404</td>
<td>210</td>
<td>0.889</td>
<td>0.51</td>
<td>7.58</td>
</tr>
<tr>
<td>Water</td>
<td>18.0</td>
<td>100</td>
<td>1.333</td>
<td>170</td>
<td>0.998</td>
<td>1.00</td>
<td>78.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: The wavelength at which the absorbance of 1 cm cell is 1.0.

Ionizable compounds in some cases can present some problems when analyzed by reverse phase chromatography. Two modifications of the mobile phase can be useful in reverse phase HPLC for ionizable compounds. One is called ion suppression and other ion pairing chromatography. In both techniques, a buffer is used to ensure that the pH of the solution is constant and usually at least 1.5 pH units from a pKa of the drug to ensure that one form predominates. If pH is approximately equal to pKa, peak broadening can occur. In ion suppression chromatography, the pH of the aqueous portion of the mobile phase is adjusted to allow the neutral form of the drug to predominate. This ensures that the drug is persistent in only one form and results in improvement of the peak shape and consistency of retention times. In ion pairing chromatography, the pH of the mobile phase is adjusted so that the drug is completely ionized. If necessary to improve peak shape or lengthen retention time, an alkyl sulfonic acid salt or bulky anion such as trifluoroacetic acid is
added to the ion pair to cationic drugs or a quaternary alkyl ammonium salt is added to ion-pair to anionic drugs. Ion pairing chromatography also allows the simultaneous analysis of both neutral and charged compounds.

**Temperature:**

Room temperature is the first choice. Elevated temperatures are sometimes used to reduce column pressure or enhance selectivity. Typically, temperatures in excess of 60°C are not used.

**Retention time:**

Due to a number of samples assayed in the course of preformulation study, it is advisable to have as short a retention time as possible. However, the retention time should be long enough to ensure selectivity. When choosing the optimum mobile phase, considerations should be given to the retention time of degradation products. So that these compounds do not elute in the solvent front and remain in the column.

**Detectors:**

The detection of UV light absorbance offers both convenience and sensitivity for molecules. When a chromophore the wavelength of detection for a drug should be based on its UV spectrum in the mobile phase and not in pure solvents, the most selective wavelength for detecting a drug is frequently the longest wavelength maximum to avoid interference from solvents, buffers and excipients. Other method of detection can be useful or required in some instances.
1. Solute specific detectors (UV, Visible, fluorescence, electrochemical, infrared, radioactivity),

2. Bulk property detectors (refractive index, viscometric, conductivity),

3. Desolvation detectors (flame ionization etc.),

4. LC-MS detectors, and

5. Reaction detectors

Performance calculations:

Calculating the following values (which can be included in a custom report) used to access overall system performance.

1. Relative retention
2. Theoretical plates
3. Capacity factor
4. Resolution
5. Peak asymmetry
6. Plates per meter

The following information furnishes the parameters used to calculate these system performance values for the separation of two chromatographic components. (Note: Where the terms W and t both appear in the same equation they must be expressed in the same units).

Relative retention (Selectivity):

\[
\alpha = (t_2-t_a)/(t_1-t_a)
\]
Theoretical plates:

\[ n = 16 \left( \frac{t}{W} \right)^2 \]

Capacity factor

\[ K' = \frac{t_2}{t_a} - 1 \]

Resolution:

\[ R = 2 \frac{(t_2 - t_1)}{(W_2 + W_1)} \]

Peak asymmetry:

\[ T = \frac{W_{0.05}}{2f} \]

Plates per meter:

\[ N = \frac{n}{L} \]

HETP: \[ L/n \]

Where \[ \alpha = \text{Relative retention.} \]

\[ t_2 = \text{Retention time of the second peak measured from point of injection.} \]

\[ t_1 = \text{Retention time of the first peak measured from point of injection.} \]

\[ t_a = \text{Retention time of an inert peak not retained by the column, measured from point of injection.} \]

\[ n = \text{Theoretical plates.} \]

\[ t = \text{Retention time of the component.} \]
$W =$ Width of the base of the component peak using tangent method.

$K' =$ Capacity factor.

$R =$ Resolution between a peak of interest (peak 2) and the peak preceding it (peak 1).

$W_2 =$ Width of the base of component peak 2.

$W_1 =$ Width of the base of component peak 1.

$T =$ Peak asymmetry, or tailing factor.

$W_{0.05} =$ Distance from the leading edge to the tailing edge of the peak, measured at a point 5 % of the peak height from the baseline.

$f =$ Distance from the peak maximum to the leading edge of the peak.

$N =$ Plates per meter.

$L =$ Column length, in meters.

**Linear Fit:**

A linear calibration fit determines the best line (linear regression) for a series of calibration points. A minimum of two calibration points is required to determine a linear fit. The equation for calculating the uncorrected amount is:

$$Y = aX + b$$

**Area / Amount factor definition:**

External Standard Method:

$$Y = \text{Component area or height}$$

$$a = \text{Slope of the calibration line}$$
\[ X = \text{Uncorrected amount} \]

\[ b = \text{Y-axis intercept of the calibration line} \]

**Internal Standard Method:**

\[ Y = \text{Component area or height / internal standard area or height} \]

\[ a = \text{Slope of the calibration line} \]

\[ X = \text{Uncorrected amount ratio} \]

\[ b = \text{Y-axis intercept of the calibration line} \]

**Information relating to the developed methods:**

In the present investigation, the author has developed three \((M_{33} - M_{35})\) HPLC methods by using with or without the use of internal standard for single or combined formulations of drugs. The technique has been utilized for the development of one or more among three methods (three columns and three mobile phases) for the assay of three selected drugs. (GTB, DSP and SRL)

**Validation of analytical methods:**

The objective of the method of validation process is to provide evidence that the method does what it is intended to.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including (a) recovery (b) response function (c) sensitivity (d) precision (e) accuracy.
(a) Recovery:

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

Absolute recovery = \frac{\text{response of analyte spiked in to matrix (processed)}}{\text{response of analyte of pure standard (unprocessed)}} \times 100.

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

(b) Response function:

In chromatographic methods of analysis peak area or peak height may be used as the response function to define the linear relationship with concentration known as the calibration model. It is essential to verify the calibration model selected to ensure that it adequately describes the relationship between response function (y) and concentration (x). The difference between the observed y-values and the fitted y-value or residual, should be examined for a minimum of six unique concentrations. A plot of studentised residual (raw residual/ standard error) Vs log concentration will then show how well the model describes the date.

(c) Sensitivity:

The method is said to be sensitive if small changes in concentration cause larger changes in response function. The sensitivity of an analytical
method is determined from the slope of the calibration line. The limits of quantitation (LOQ) or working dynamic range of bioanalytical method are defined as the highest and lowest concentrations, which can be determined with acceptable accuracy. It is suggested that, this be set at ± 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

(d) Precision:

The precision of an analytical method is a measure of the random error and is defined as the agreement between replicate measurements of the sample. It is expressed as the percentage coefficient of variation (% CV) or relative standard deviation (RSD) of the replicate measurements.

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

Precision can be considered as having a within assay batch component or repeatability which defines the ability to repeat the same methodology with the same analyst, using the same equipment and the same reagents in a short interval of time, e.g. within a day. This is also known as intra-assay precision.

The ability to repeat the same methodology under different conditions, e.g. change of analyst, reagent or equipment, or on subsequent occasions, e.g. across several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay precision. The reproducibility of a method is of most interest to the analyst, since this will give better representation of the precision during routine use as it includes the variability from many sources.
<table>
<thead>
<tr>
<th>Mode of Chromatography/Column</th>
<th>Mobile phase composition</th>
<th>Method</th>
<th>Drugs Responded</th>
<th>Internal standard/Combination</th>
<th>Method characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acquity UPLC BEH C-18</td>
<td>Acetonitrile, water, trifluoroacetic acid and triethyl amine 55:45:0.1:0.1</td>
<td>M₃₃</td>
<td>GTB</td>
<td>–</td>
<td>220</td>
<td>1-4</td>
</tr>
<tr>
<td>Hypersil BDS Phenyl</td>
<td>Methanol and buffer (272 mg of potassium dihydrogen phosphate and 2.554gm of disodium hydrogen phosphate) 50:50</td>
<td>M₃₄</td>
<td>DSP</td>
<td>–</td>
<td>254</td>
<td>1-3</td>
</tr>
<tr>
<td>Inertsil ODS 3V, C18</td>
<td>0.01M sodium dihydrogen phosphate(1.379grams/1000ml), acetonitrile and methanol 5:5:4</td>
<td>M₃₅</td>
<td>SRL</td>
<td>–</td>
<td>225</td>
<td>1-3</td>
</tr>
<tr>
<td>Hypersil BDS RP C18</td>
<td>0.02 M Dipotassium Hydrogen ortho phosphate and MeoH 10:90</td>
<td>-</td>
<td>GTB</td>
<td>-</td>
<td>246</td>
<td>25-300</td>
</tr>
<tr>
<td>C 18</td>
<td>pH 6.5 buffer and acetonitrile 55:45</td>
<td>-</td>
<td>GTB</td>
<td>-</td>
<td>220</td>
<td>10-60</td>
</tr>
<tr>
<td>Capillary ODS Hypersil</td>
<td>Aqueous ammonium acetate</td>
<td>-</td>
<td>GTB</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------</td>
<td>---</td>
<td>-----</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Waters X- Terra C 18</td>
<td>Acetonitrile - water containing 0.1% Formic acid 70:30</td>
<td>-</td>
<td>GTB</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenomenex Synergi 4μ MAX- RP80A C12</td>
<td>Acetonitrile -1% Formic acid 30:70</td>
<td>-</td>
<td>GTB</td>
<td>Deuterated Gefitinib (D8-ZD1839)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inertsil ODS3</td>
<td>Acetonitrile / ammonium acetate</td>
<td>-</td>
<td>GTB</td>
<td>Deuterated Gefitinib (D8-ZD1839)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metrosep A supp 5 ion exchange</td>
<td>Water : Acetonitrile 80:20</td>
<td>-</td>
<td>DSP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IC - pak Anion HR</td>
<td>2 m mol/L - 1 nitric acid (adjust pH = 2.70 with 5 mol/L -1 nitric acid)</td>
<td>-</td>
<td>DSP</td>
<td>-</td>
<td>230</td>
<td>3.82-45.82</td>
</tr>
<tr>
<td>C 18</td>
<td>Acetonitrile and the NaOH solution of 0.4% EDTA (21:79)</td>
<td>-</td>
<td>DSP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxy ethyl methacrylate polymer</td>
<td>-</td>
<td>-</td>
<td>DSP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C 18</td>
<td>Water- Acetonitrile 88:12 (adjusted pH 2.0 with phosphoric acid)</td>
<td>-</td>
<td>SRL</td>
<td>-</td>
<td>238</td>
<td>40-400</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------------------------------------</td>
<td>----</td>
<td>-----</td>
<td>----</td>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td>Ion exchanger</td>
<td>Mix of org. solvent and buffer solution (such as MeCN, MeOH or EtOH) and buffer 0.03 - 0.1 mol/L Na dihydrogen phosphate solution with pH of 2.5-6 at volume ratio (55:45)-(5:95)</td>
<td>-</td>
<td>SRL</td>
<td>-</td>
<td>230-245</td>
<td>265</td>
</tr>
</tbody>
</table>
For validation of new analytical method for routine use, it is suggested that precision be assessed at four unique concentrations in replicates of six, on four separate conditions i.e. 4 x 6 x 4. This approach will allow the data for individual analytes to be analyzed by a one-way analysis of variance, which gives estimates of both the intra-assay and the inter-assay precision of the method at each concentration. To be acceptable, both measures should be within ± 15% at all concentrations.

(e) Accuracy:

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

\[
\% \text{ Bias} = \frac{[ (\text{Measured value minus True value}) \times 100]}{\text{True value}}
\]

Since the true value is not known for real samples, an approximation is obtained, based on spiking drug-free matrix to a nominal concentration. The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug-free matrix samples. For the validation of a new analytical method for the use, the measured concentrations will be those obtained during the estimation of precision i.e. from the 4 x 6 x 4 experiment. All results other than those rejected for analytical reasons, i.e. poor chromatography, should be used in the calculation and accuracy of the method should be within ± 15% at all concentrations.