Chapter I

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

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

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

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

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

In the present study some typical drugs were analyzed for their concentrations, were determined in some aqueous solutions by Spectroscopic and chromatographic techniques to evaluable which one is the better method.
Drugs are chemical entities used in the treatment, cure prevention and diagnosis of diseases or used to otherwise enhance physical and mental well being of human being. Drugs play a vital role in the creation, sustenance and destruction of all living species. Pharmaceutical chemistry is a study of drug-science that makes use of the general laws of chemistry and biology. According to their chemical structure and therapeutic action, the drugs are classified as follows: Anti infective, bacterial, Hypertensive, Diabetic agents. Other groups of drugs such as pharmacodynamic and Lipid lowering compounds are also prokinetics.

In the present investigation of five drugs, Lovastatin (LST), Nicardipine (NCD), Cefipime (CP), Rosuvastatin (RST), Glyburide (GB) have been selected and are presented in 1 to VI chapters, respectively.

Official status of selected drugs

Every country has legislation on bulk drugs and their pharmaceutical formulations that set 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 Kingdom, BP, United States, USP, European, EP, Japanese JP, and Martindale Extra). The other source of information include Merck index, Remington and PDR. Pharmaceutical analysis deals not only with medicament (drugs and in 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 a drug is determined after establishing its purity and the quantity of the pure substances in the bulk drugs and its formulations.

Type of pharmaceutical formulations

The drugs are used in various forms in prophylactic or in therapeutic use. They are formulated as tablets, capsules, dry syrups, liquid orals, creams or Ointments, parenterals (injection in dry or liquid form), lotions, dusting powders, aerosols, etc. In tablets, one or more among the diluents such as starch, lactose, cellulose derivatives, calcium phosphate, mannitol, sorbitol, sucrose, aerosols, acacia, poly vinyl pyrolidine, alginic acid, targacanth, stearic acid, talc, magnesium stearate, waxes, methyl paraben, propyl paraben, sodium benzoate, permitted flavours, and colors may be added. In capsules one or more among the diluents,
certified dyes, gelatin, plasticizers, preservatives, starch, lactose, talc are also added. In dry syrups and liquid orals, sucrose, sorbitols, preservatives, certified colors, and flavors might be added. In creams and ointments, waxes, carbopol, petroleum jelly, surfactants, preservatives, permitted colors, and perfumes might be added. In parenterals, vehicles such as water, vegetable oils, mineral oils, simulated oils, propylene glycol, dioxylamines and dimethyl acetamide are used. Any one or more among stabilizers, anti oxidants, buffering agents like citrate, acetate, phosphate, co-solvents, wetting, suspending and emulsifying agents like tween-80, sorbitol oleate and preservatives may be added. Dusting powders and aerosols, talc, silica derivatives, alcohol, and preservatives are commonly in lotions.

The drugs are applied in some instance in rather small doses and they are often mixed with excipients as combinations. The assay of various dosage forms raises several specials such as skillful sampling and the preparation of sample solutions. Hence standard techniques must be employed to ascertain the homogeneity of the sample before collection for analysis.

**Preparation of sample solution for analytical investigations**

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 the components of the extract or its purification by chromatography are widely used. The most convenient means of extracting drug from tablet is to treat with an appropriate solvent such that the resulting extract can be used directly in the assay method chosen without the interference of associating ingredients. In general, adequate extraction can be achieved when the finely pulverized tablet is agitated or sometimes boiled with solvent for a period from a few minutes to several hours. The solvents are 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 decrease 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 favorable conditions for extraction of the drug by the 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.

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 mostly. 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, color, density or specific gravity (for liquids), moisture content, melting, and boiling points. A physicochemical method is used to study the physical phenomena that occur as a result of chemical reactions. Among the physicochemical methods are optical (refractometry, polarimetry, emission and spectrophotometry, nephelometry or turbidimetry), electrochemical (potentiometry, amperometry, colorimetry and polarography) and chromatography (column, paper, thin layer, gas liquid, high performance liquid) methods are generally preferable. Methods involving nuclear reaction 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 reactions, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have been widely used in pharmaceutical analysis whenever the existing amounts are in mg level and the interferences are negligible. The modern methods (HPLC, GC, NMR, and MS) 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. The colorimetric procedures are simple and do not involve high cost. The limitation of many
colorimetric procedures is dependent on the chemical reaction upon which these procedures are based rather than on the instruments. However, colorimetric methods cannot be extended to all types of drugs either due to non-availability of a suitable reacting reagent or low detection sensitivity. 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.

In the present investigation, visible spectrophotometry and high performance liquid chromatography techniques have been used as tools in the assay of selected drugs.

**General information and methodology for the development of**

**New method**

**Part—A**

**Visible spectrophotometry**

Colorimetric\(^{33,36}\) and fluorimetric analysis of compounds and drugs have been used extensively in various fields and it may be stated that they will remain great value in the future. Combination of the steady resort to physical methods that often necessitates very sophisticated and expensive instrumentation, Colorimetric and fluorimetric analysis can be perfected with very simple instrumentation, resulting nevertheless in sensitive and selective, an accurate measurements with the addition of speed and simplicity. These are based on chemical reaction, characteristics of the various functional groups. The molecule bearing such a group reacts with suitable reagent to give a colored or fluorescent species. Numerous reactions of this kind have been described in the literature and a particular reaction has given rise to a whole lot sundry procedures. Analyst, who finds him/herself in need of a colorimetric or fluorimetric method, may therefore be obliged to suit his/her needs. The purpose of this thesis is to provide practising analysts with procedures that were carefully tested for selected drugs. All the data given under results section were actually obtained in this laboratory.
Classification of functional groups in drugs

A general feature of organic drugs is the presence of functional groups in their molecules. Knowing the reactions of functional groups\textsuperscript{17-45}, one can easily analyze any drug with a complicated structure. A survey of literature on selected drugs (LST, NCD, CP, RST, and GB) revealed that there are very few visible spectrophotometric methods for their estimation at the time of commencement of this investigation.

The analytically important functional groups of selected drugs do not seem to be fully exploited for designing visible spectrophotometric methods for their determinations. The chemical features of selected drug molecules still offer a lot of scope for the development of new UV and visible spectrophotometric and HPLC methods hopefully with better sensitivity, selectivity, precision and accuracy. The author has made some attempts in this direction and succeeded in developing some new methods having advantages of one or more of the above desirable features. Among the several HPLC methods reported for the selected drugs estimation of many of them, are in biological fluids and very few in pharmaceutical formulations.

Selection of reagents for organic analysis

Several papers are being published every year on the reaction and possible applications of new and old organic reagents in organic analysis (inclusive of drugs). The selection of an appropriate reagent for a particular analytical situation is still a challenging problem. The choice of a particular reagent depends on careful consideration of such factors as the scale of economies of the reaction, the presence of other functional groups besides the chosen one that might be adversely affected by the reagents, the deactivation of the reaction center by steric and electronic effects, the instability or high reactivity of the desired product, the rate of the reaction, position of equilibrium as in the case of reversible reaction and other related factors. The objective is to get the best yields possible. The selection of a reagent for the determination of a particular compound is made after a literature survey for methods that have been under consideration. If much information is not found in this way, the reagent that acts most rapidly and stoichiometrically or at least giving reproducible results can be chosen after investigation of the performance of plausibly selected ones on a pure sample of the compound sought.
Reagent selectivity for a particular functional group (in selected drug) is normally the minimum requirement specificity for a single compound containing the functional group is often desirable, not only to isolate it from other compounds containing the same functional group but also to eliminate the effects of interfering compounds.

The general objective of reagent is the formation of one or more derivatives having measurable chemical or physical properties as completely different as possible from that of any of the reagents. Some examples of more specific reagents go as follows.

1. The reagent forms or destroys an acidic, alkaline, oxidizing or reducing propriety of the functional group, the amount of change being determined titrimetrically or spectrophotometrically.

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

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

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

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

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

7. Enzymes selectively catalyze specific reaction.

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

In few instances the less reactive functional group may be converted to more reactive functional group through preliminary reaction (e.g., reduction of $-\text{NO}_2$ to $-\text{NH}_2$ hydrolysis of acyl substituted functional group of amine or phenol to free amino or phenolic hydroxyl group respectively).

Features of chemical reactions and reagents of interest

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

Dyes as analytical reagents

In the present investigations dyes have been used either freely or in combination with an oxidant in the assay of selected drugs. Dye may be defined as a colored substance which when applied to the fiber gives it a permanent color resistant to the action of light, water and soap. Because of their commercial importance a very large number of dyes have been placed in the market. The color index sponsored jointly by the "society of dyers and colorists" lists about 4500 different dyes and pigments. They have assigned names according to the method of application and given a color index number according to their structures. Each manufacturer however usually labels his products with registered trademark. The dyes are categorized according to common parent structure$^{46}$. The chemical categories of dyes are given in Table 1.1.
Table 1.1
Chemical categories of dyes

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Category of the dye</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nitro dyes</td>
<td>Naphthol yellow S</td>
</tr>
<tr>
<td>2</td>
<td>Nitroso dyes</td>
<td>Fast green O</td>
</tr>
<tr>
<td>3</td>
<td>Tropaeoline</td>
<td>Tropaeoline OOO*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naphthol blue black</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naphthalene blue 12 BR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Congo red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erichrome Black T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tartrazine</td>
</tr>
<tr>
<td>4.</td>
<td>Thiazoles</td>
<td>Primuline</td>
</tr>
<tr>
<td>5.</td>
<td>Diphenyl methanes</td>
<td>Auramine</td>
</tr>
<tr>
<td>6.</td>
<td>Triphenyl methane and analogous Dyes</td>
<td>Fast green FCF*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromo phenol blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromo cresol green</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bromothymol violet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erioglaucine A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rosaaanilne</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrmine G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>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</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lissammine blue BF</td>
</tr>
<tr>
<td>10.</td>
<td>Phenoxazines</td>
<td>Celestine blue*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gallocyanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>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*</td>
</tr>
<tr>
<td>14.</td>
<td>Indigoids</td>
<td>Indigotin</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>Phythalocyanines</td>
<td>Monastrial fast blue BS</td>
</tr>
<tr>
<td>19.</td>
<td>Cyanines</td>
<td>Kryptocyanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Astraphloxine FF</td>
</tr>
<tr>
<td>20.</td>
<td>Miscellaneous dyes</td>
<td>Quinoline yellow</td>
</tr>
</tbody>
</table>

Recently, chemists extended their dyes study to correlate visual color with structural features of molecule in 1976, Witt pointed out that two types of groups are usually present in highly colored compounds (Chromophores) and color intensifying groups (auxochromes).
Table: 1.2
List of important chromophores and auxochromes

<table>
<thead>
<tr>
<th>Chromophores</th>
<th>Auxochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acidic</td>
</tr>
<tr>
<td>-N=N-</td>
<td></td>
</tr>
<tr>
<td>-N=O</td>
<td>Phenolic -OH</td>
</tr>
<tr>
<td>C=O</td>
<td></td>
</tr>
<tr>
<td>-COO⁻</td>
<td>-COOH</td>
</tr>
<tr>
<td>C=C, C=N, C=S</td>
<td>-SO₃H</td>
</tr>
</tbody>
</table>

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

**Electronic transition in absorbing species**

<table>
<thead>
<tr>
<th>Transition in energy level involving sigma, pi and n electrons</th>
<th>Involving 'd' &amp; 'f' electrons</th>
<th>Charge – transfer spectral absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma - \sigma^<em>$, n- $\sigma^</em>$, n-$\pi^<em>$, $\pi$-$\pi^</em>$ (imp). $\sigma^<em>$ and $\pi^</em>$ are antibonding orbital while 'n' involves non-bonding orbital having an energy in between bonding and anti bonding orbitals. The polarized force between solvent and species lower the energy levels of excited and unexcited states. As (n-sigm$^<em>$ or sigma-sigma$^</em>$ require much higher energies, they are seen in a vacuum – UV and are harder to observe)</td>
<td>Transition metals involve electronic transition among different energy levels of d-orbitals $t_2g (d_{xy}, d_{xz}, d_{yz})$ and $e_g (d_{x^2-y^2}, d_{z^2})$ are split by delta in presence of ligands. $I^- &lt; Br^- &lt; Cl^- &lt; F^- &lt; OH^- &lt; Oxalate^2- &lt; H_2O &lt; SCN^- &lt; NH_3 &lt; en &lt; NO_2 &lt; CN^-$ (crystal field theory)</td>
<td>In charge transfer complexes, components should be both electron donor and electron acceptor which in turn involves transfer of electron acceptor which in turn involves transfer of electrons to give absorption radiation (longer wavelength)</td>
</tr>
</tbody>
</table>

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

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

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

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

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

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

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

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

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

\[
\text{R COO C}_2\text{H}_5 + \text{NH}_2\text{OH} \rightarrow \text{R CO NHOH + C}_2\text{H}_5\text{OH}
\]

\[
\text{R CO NH}_2 + \text{NH}_2\text{OH} \rightarrow \text{NH}_3 + \text{R CO NHOH}
\]

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

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

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

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

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

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

\[ 3 \text{C}_10\text{H}_7\text{OH} + 2\text{FeCl}_3 \rightarrow \text{HOC}_{10}\text{H}_6\text{C}_10\text{H}_6\text{OH} + 2 \text{FeCl}_2 + 2 \text{HCL} \]

Other phenols also form phenolates of iron with partial oxidation.

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

\[ \text{Fe}^{2+} + [\text{Fe(CN)}_6]^{3-} \rightarrow \text{Fe}_3[\text{Fe(CN)}_6]_2 \]

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

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

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

**Oxidation/reduction reaction**

**FC Reagent: Folin-Ciocaltaeu Reagent**

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

The color formation by Folin ciocaltaeu (FC) reagent with organic compounds may be explained in the manner based on the analogy with the reports of earlier workers.

In the present investigation FC Reagent was used for the determination of CP, Glyburide.

**Diazoo coupling reactions**

The diazonium salts derived from p-amino benzoic acid, p-nitroaniline, p-sulphanilic acid (Pauli reagent), 2-aminobenzothiazole, 3-phenyll-5-nitrossamine, 2, 4 thiadiazole, 4-amino6chloro1, 3benzenedisulphonamide, benzocaine, dapsone and sulphaanilamide are commonly used reagents for direct coupling procedures. The aryl amine coupling reagents are converted to their diazonium salts with HCl and sodium nitrite. The reaction is usually carried out in an ice bath, the excess nitrate is removed by reaction with sulphamic acid or ammonium sulphamate, and the pH is adjusted for the coupling reaction. The reagent is used immediately since most diazonium salts are not stable.
The most common source of interference in analysis using the direct coupling procedure is impurities in the sample, which also couple with the diazonium salt and exhibit some absorbance at the wavelength chosen for the analysis. Interference of this type can only be avoided by employing a separation step prior to color development.

Several compounds of pharmaceutical importance are analyzed by direct coupling with a diazonium salt. They include, levarterenol, 8-hydroxy quinoline, isonazide, 3-amino-1H-1, 2, 4-triazole, estradioldipropionate, tyrosine, thiamine, salbutamol, nydri

Diazotization of the analyte and coupling

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

\[ H^+ + HONO \rightarrow H_2O + NO^+ \rightarrow NOX + NO_2 \]
\[ NOX + ArNH_2 \rightarrow X^- + Ar N^+H_2-N=O \rightarrow H^+ + Ar-N-N=O \rightarrow H^+ + Ar-N=N-OH \rightarrow Ar-N^+ \equiv N + H_2O \]

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

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

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

A number of drugs have also been determined by first converting them to a diazotizable species (either by hydrolysis or reduction) which is then coupled in the usual manner. Chloramphenicol contains a nitro group, which can be reduced to an amine with a suitable reducing agent, (metallic zinc/acid, stannous chloride or sodium dithionate). Reduction to the amine followed by the Bratton-Marshall reaction has been used for the analysis of this drug.

Using it to diazotize an aromatic amine, coupling the diazotized amine with a substrate and measuring the product spectrophotometrically can determine nitrous acid. The Griess method for nitrite employs sulphanilic acid as the source of coupling agent and α-naphthyl amine as the substrate. This reaction has been used for the analysis of pharmaceutical preparations including gelatin capsules and can also be used for nitrate after a reduction step. A typical procedure will consist of an alkaline hydrolysis, followed by acidification, diazotization of coupling agent and finally coupling with the substrate. Isosorbide dinitrate has been determined using sulphanilic acid and α-naphthyl amine (Griess method), and also using
p-nitroaniline as the coupling agent and azulene as the substrate. The later combination has also been used to analyze pentaerythritol tetranitrate.

In the present investigation diazotization methods M1, M2 have been used for the determination of NCD, and CP.

**Dyes in ion association complex formation: (method: m₄, m₅, m₆)**

The term molecular complex is used to describe a variety of association products of two or more molecules. In recent years, extensive attention has been given to a large number of complexes formed by weak interaction of certain classes of organic compounds functioning as electron donors (bases), while others act as electron acceptors, (acids). The forces, which lead to the formation of molecular complex, include forces such as dipole and induced dipole interactions, London dispersion forces, hydrogen bonding and dative bonding interactions. The donor-acceptor complexes (where composition can be represented by integral ratios of the components) are in many instances so unstable that they cannot be isolated in the pure state at ordinary temperatures but exist only in solutions in equilibrium with their components. They can however, usually be detected readily because of differences in the physical properties (e.g. absorption spectra, solubility in organic solvents) from those of the pure components. The ion association complex or adduct is a special form of molecular complex resulting from two components extractable into organic solvents from aqueous phase at suitable pH. One component is a chromate (dye or metal complex) possessing charge (cationic or anionic nature) and so insoluble in organic solvents. The other is colorless, possessing opposite charge (anionic or cationic) to that of chromate.

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

Therefore, compounds may be determined in binary mixtures: the less polar amine is estimated with bromophenol blue, that the sum of the two amines is given by bromocresol purple. Mixtures of diphenyl hydri ne and ephedrine were also analyzed.

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

The same principle has been applied for the determination of secondary and tertiary aliphatic amines with tropacolin and of tertiary aliphatic amine with erythrosine. Several dyes belonging to different chemical classes have been used for the determination of basic drugs. Very few dyes (for Diltiazem, Timolol and prazosin) or one (for Mebevarine and Amlodipine) have been reported for the selected drugs. Few dyes have been utilized for the development of new extractive spectrophotometric methods for the determination of selected drugs in the present work. The acidic dyes have been summarized in Table 1.1. In the present investigation BTB (M₄) was used for the determination of LST, NCD, and RST. Methyl orange (M₅) was used for the determination of LST, NCD, RST and solochrome black-T was utilized for the determination of LST, NCD, RST.

Methodology in UV-visible spectrophotometry

In ultra violet (200-380 nm) and visible (380-800 nm) spectroscopy absorption of radiation is the result of excitation of bonding (sigma and pi) and non bonding (n) electrons. The frequencies of the absorption can be influenced by solvents and by delocalization in conjugated systems. Spectrophotometer is an instrument for measuring the intensity of light of various wavelength transmitted by a solution. The intensity of light is determined by electric detectors, which convert...
radiant energy to electric energy and can eliminate the need for subjective measurements. The limit of detection is lowered by measuring the absorption of a solution at the wavelength maximal absorption. It is possible to minimize the effect of foreign colored substances by working at a suitable wavelength and greater precision can be obtained in spectrophotometry than other methods. The fundamental principle of visible spectrophotometry lies in that light of a definite interval of wavelength passes through a cell with a colored solution or solvent and falls on the photoelectric cell that converts the radiant energy into electric energy measured by a galvanometer. Photometric methods of analysis, based on measuring light absorption molecules in a solution is proportional to the intensity of incident light and to the concentration or number of the absorbing species in the path of the beam.

The methodology for the development of new visible spectrophotometric methods is outlined.

**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 scanning with a 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 chromogenie reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknown should be on a definite time schedule.
Optimization of analytical method

In each type of basic reaction, the colored species is formed or the final color of the reaction mixture whose absorbance is measured and thus the sensitivity of the method, rate of color formation and stability is affected by the concentration of the reagent in the solution, nature of 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 investigations are furnished in subsequent chapters.

Calibration

Calibration is one of the most important steps in bioactive compound analysis. A good precision and accuracy can only be obtained when a good calibration procedure is used. 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 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.

\[ Y = a + bx \]

In calibration univariate regression is applied, which means that all observations are dependent upon a single variable \( X \).
The method of least squares

Least square regression analysis can be used to describe the relationship between response (y) and concentration (x). The relationship 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.

Adapting the convention that the x values relate to the controlled 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 the 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 \( a, b_1, \ldots, b_m \) must be estimated in such a way that the model fits the experimental data points \((x_i, y_i)\) 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 \((x_i, y_i)\) can be represented as

\[ Y_i = \alpha + \beta X_i + e_i \]

The signal \( Y_i \) is composed of deterministic component predicted by linear model and a random component \( e_i \). One must now find the estimates of \( \alpha \) and \( \beta \) of the two values \( \alpha \) and \( \beta \). This can be done by calculating values \( a \) and \( b \) for which \( e_i^2 \) is minimal. The component \( e_i \) represent the differences between the observed \( Y_i \) values and the predicted \( Y_i \) values by the model. The \( e_i \) are called the residuals, \( \alpha \) and \( b \) are the intercept and slope respectively.

\[
\begin{align*}
b &= \frac{n \sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n \sum_{i=1}^{n} x_i^2 - \left[ \sum_{i=1}^{n} x_i \right]^2} \\
a &= \frac{n \sum_{i=1}^{n} y_i \sum_{i=1}^{n} x_i^2 - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i y_i}{n \sum_{i=1}^{n} x_i^2 - \left[ \sum_{i=1}^{n} x_i \right]^2}
\end{align*}
\]
Standard error on estimation, \( (S_e) \)

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

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

\( y_i \) and \( \bar{y}_i \) are the observed and predicted values, respectively. Standard deviations on slope \( (S_b) \) and intercept \( (S_a) \) 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 concentrations etc. It is important to understand how uncertainties in the slope are influenced by the 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 on slope, \( (S_b) \)**

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

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

Where \( \bar{X}_i \) is the arithmetic mean of \( X_i \) values

**Standard deviation on slope, \( (S_a) \)**

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

\[
S_a = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2}{(n-2)}} \frac{1}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x}_i)^2}} \frac{\sqrt{\sum_{i=1}^{n} x_i^2}}{n}
\]

Where \( \bar{X}_i \) denote the arithmetic 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 \(y\).

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

Selectivity of the method

Matrix and interference effects may disturb the determination of an analyte. Some of the excipients, incipient and additives present in pharmaceutical formulations may sometimes interfere in the assay of drug and in such instance appropriate separation procedure is to be adopted initially; the selectivity of the method is separation procedure. 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 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

Knowledge of the sensitivity of the color is important and the following terms are commonly employed for expressing sensitivity,

According to Bougher – Lambert – Beer’s Law

\[
A = \log \frac{\text{Intensity of incident radiations}}{\text{Intensity of transmitted light}} = \varepsilon c t
\]

The absorbance \((A)\) is proportional to the concentration \((c)\) of the absorbing species, if absorptivity \((\varepsilon)\) and thickness of the medium \((t)\) are constant. Where \(c\)
is in moles per liter, the constant is called molar absorptivity. Beer's law limits and $\lambda_{\text{max}}$ values are expressed as $\mu g.ml^{-1}$ and 1 mole$^{-1}.cm^{-1}$, respectively.

Sandell's sensitivity$^{182}$ refers to the number of $\mu g$ of the drug to be determined, converted to the colored product, which in a column solution of cross section 1 cm$^2$ shows an absorbance of 0.001 (expressed as $\mu g$ cm$^{-2}$).

**Limit of detection**$^{142}$

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 the intercept ($S_d$), which may be related to LOD and the slope of calibration curve, $b$, by

$$\text{LOD} = 3S_d/b$$

**Ringbom's plot**$^{143, 144}$

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increase at the extremes of the transmittance scale. The slope of plot $C$ versus $T$, i.e. Ringbom plot gives relative error coefficient (i.e. plot of $\log C$ vs $T$) the main limitations Ringbom plot is that it provides no concerning the concentration range of good precision unless it is combined with delta $T$ versus $T$ relation. The above expression is valid whether 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 time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important critical for judging analytical procedures by their results.
**Precision**

Precision refers to the reproducibility of measurement within a set that is to the scatter of dispersion of a set about its central value. The term 'set' is defined referring to a number (n) of independent replicate & measurements of some property. One of the most common statistical terms employed is the standard deviation population of observation. Standard deviation is the square root of the sum of squares 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 variance ($S^2$). Relative standard deviation is the standard deviation expressed as a fraction of the, mean, i.e. $s/X$. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

\[\%	ext{ Relative standard deviation} = S \times \frac{100}{X}\]

**Accuracy**

Accuracy normally refers to the difference between the mean xof the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (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**

Taking amounts of the constituents and proceeding according to specified instructions carries out the test for accuracy of the method. The difference between the mean of an adequate number of results and amount of constituent actually, present, usually expressed as parts 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 varying amounts. In a few instances, the
accuracy of the method controlled by separations (usually solvent extraction or chromatography technique) involved.

**Comparative method**

In the analysis of pharmaceutical formulations (or solid laboratory prepared samples of desired composition), the content of the constituent sought (expressed as percent recovery) has been determined by two or more (proposed and official or reference) supposedly "accurate" methods of essentially different character can usually be accepted as 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 that 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 analyzed for the total amount of constituent present. The difference between the analytical results for sample with and without the added constituent gives the recovery of the amount of the added constituent. If the recovery is satisfactory, 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 of '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 analyzed 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 small than 30, the condition 'normality of x is required or at least the normality of the difference (d_i). If this is the case the quantity has a student

\[ t = \frac{\bar{d}_i}{s_d / \sqrt{n}} \]
t-distribution with \((n-1)\) degrees of freedom, where \(d_i = X_R(\text{Reference method}) - X_T(\text{Test method})\) and \(s_d\) is the standard deviation.

**F-test**

By the f-test the significance of the difference in variances of reference and test methods can be tested. Let us suppose that one carried out \(n_1\) replicate measurements by test methods and \(n_2\) replicate measurements by using reference method. If the null hypothesis is true then the estimates \(S_T^2\) (variance of the 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 variances

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

It is conventional to calculate the F-ratio by dividing the larger variance by the smallest variance in order to obtain a value equal or larger than unity. If the calculated F-values are smaller than 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 colored species**

Slope analysis method\(^{148, 149}\) and mole ratio methods 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

High Performance Liquid Chromatography (HPLC) 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.

Operculum ......Injection Port ......Column ..........Detector ..........Effluent ..........Thermostat ......Recorder

Columns

The heart of the system is the column. The choice of the common packing material and mobile phase depend upon the physical properties of the drug. The column selection, a flow chart in Table 1.4 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 separation with a standard C-8 or C-18 column. (E.g. Zorbax, Rx C-8) 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 length; 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>Basic or Acidic</td>
<td>Reverse phase-ion pair</td>
<td>C18, C8, C6, C4, C2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)</td>
</tr>
<tr>
<td></td>
<td>(Allows neutral and charged compounds to be simultaneously analyzed)</td>
<td></td>
</tr>
<tr>
<td>Ionizable</td>
<td>Ion Separation</td>
<td>C18, C8, C6, C4, C2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anionic</td>
<td></td>
<td>Strong anion exchange</td>
</tr>
<tr>
<td>Cationic</td>
<td></td>
<td>Strong cation exchange</td>
</tr>
<tr>
<td>Normal phase</td>
<td></td>
<td>Increasing polarity of Bonded phases</td>
</tr>
<tr>
<td>Neutral</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td></td>
</tr>
<tr>
<td>Reverse phase</td>
<td>Silica</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alumina</td>
<td>Increasing Polarity of bonding phase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenyl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TMS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamilton PRP-1</td>
<td></td>
</tr>
</tbody>
</table>
Mobile phases used for HPLC typically are mixtures of organic solvent and water or aqueous buffers. Table 1.5 lists the physical properties of organic solvents commonly used for HPLC. Isocratic methods are preferable to gradient methods. Gradient methods will sometime be required when the molecules being separated have vastly different portioning properties. When a gradient elution method is used care must be taken to ensure that all solvents are miscible.

The following points should 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 preparation, which can damage HPLC equipment.
3. The mobile phase should have a pH 2.5 and pH 7.0 to maximize the life time 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.
6. Since trifluoroacetic acid, acetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products without chromophores above 220nm. Carboxylic acids modifier can be frequently replaced by phosphoric acid, which does not absorb above 200nm.
7. Use volatile mobile phases when possible to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, and formic acid, acetic acid and trifluoroacetic acid.
8. Some caution is needed as these buffers absorb below 220nm.

Based on the nature of the stationary and mobile phases and use of different columns, in the present investigation five HPLC methods (M10 to M15) have been reported.
Table: 1.5

Physical properties of common HPLC solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>M.W (25°C)</th>
<th>B.P</th>
<th>R.I</th>
<th>UV^a Cut</th>
<th>Density g/ml(25°C)</th>
<th>Viscosity C^p (25°C)</th>
<th>Dielectric constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>41.02</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>CH2C12</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>7.58</td>
</tr>
</tbody>
</table>

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 the 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 1.5 PH units from a pK_a of the drug to ensure that one form predominates. If PH is approximately equal to pK_a, 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 sulphonic 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 neither reduce column pressure nor 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 pre formulation 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, consideration 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 detections of UV light absorbance offer both convenience and sensitivity for molecules. In 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 the 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 detector (Reractive 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 systems performance values for the separation of two chromatographic components. (Note: Where the terms W and t appear in the same equation they must be expressed in the same units).

Relative Retention (Selectivity): \[ \alpha = \frac{(t_2 - t_a)}{(t_1 - t_a)} \]

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

Capacity factor: \[ K' = \frac{(t_2 - t_a) - 1}{t} \]

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

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

Plates per meter: \[ N = n/L \]

HETP: \[ \frac{L}{n} \]

A : Relative retention

T_2 : Retention time of the second peak measured from the point of injection

t_1 : Retention time of the first peak measured from the point of injection

t_a : Retained by the Column measured from point of injection

N : Theoretical plates

T : Retention time of the component
\[ Y = a X + b \]

**External standard method**

\[ Y = \text{component area or height} \]

\[ A = \text{slope of calibration line} \]

\[ X = \text{uncorrected amount} \]

\[ b = \text{y-axis intercept of the calibration line} \]
**Internal standard method**

\[ Y = \text{component area or height/} \text{Internal standard area or height} \]

\[ a = \text{slope of calibration line} \]

\[ X = \text{uncorrected amount} \]

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

**Validation of analytical data**

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, and 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.

**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 or 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 + response of analyte spiked in a matrix (processed)/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.

**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 calibration \((x)\). The differences between the observed \(y\) values and the fitted \(y\) values 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 data.

**Sensitivity**

The method is said to be sensitive, small changes in concentration causes 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 \(\pm 15\%\) 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.

**Precision**

The precision of analytical method is 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.

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

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

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. 4x6x4. 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 inter-assay precision of the method at each concentration. To be acceptable, both measures should be within ± 15% at all concentrations.

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.

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

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*6*4 experiment. All results other than those rejected for analytical reasons, i.e., poor chromatography, should be used in the calculations and accuracy of the method should be within ± 15% at all concentrations.
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