CHAPTER I
INTRODUCTION
Brief information of selected drugs is incorporated in this chapter.

1.1 BRIEF INFORMATION OF SELECTED DRUGS:

Various authors defined the term drug in various ways. The most widely accepted definition of the drug is as any chemical agent that affects the living process. Further the term drug includes all chemicals and medicines meant for treatment, mitigation or prevention of diseases in human beings or animals for internal or external use. All substances other than food intended to affect the structure or any function of the human or animal body including contraceptives as well as substances used for destruction of microorganisms and insects, which cause disease in human beings or animals. Pharmaceutical chemistry[^8] 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, the methods of quality control and conditions of their storage. According to their chemical structure or therapeutic action, the drugs may be classified as (i) Anti-infective agents, (ii) Anti-ulcer agents and (iii) Anti-inflammatory agents. The definitions along with examples for different drugs are briefly given below.

1.1.1 Anti-infective agents:

Anti-infective agents treat infection by suppressing or destroying the causative microorganisms namely bacteria, mycobacteria, fungi, protozoa, viruses, etc. Anti-infective agents derived from natural sources are called antibiotics; those produced from synthetic substances are called antibacterials. Antibacterial agents are the agents that are synthetically produced. The first synthetic agents to be produced are sulfonamides. Now the most widely used antibacterial agents are fluoroquinolones
like, norfloxacin, ciprofloxacin, sparfloxacin, gatifloxacin, ofloxacin, levofloxacin, pefloxacin, moxifloxacin, etc.

1.1.2 Antiulcer agents:

The agents that are used in the treatment of ulcers are called antiulcers. There are many categories under this heading out of which proton pump inhibitors like omeprazole, esomeprazole, pantoprazole, lansoprazole, rabeprazole etc are some of them.

1.1.3 Anti-inflammatory agents:

The agents that are used in the treatment of inflammation are called anti-inflammatory agents, which include non steroidal agents like paracetamol, ibuprofen, nimesulide etc, and cox-2 inhibitors like rofecoxib, celecoxib, valdecoxib, etoricoxib etc.

In the present investigation seven drugs, gatifloxacin (GTF), levofloxacin (LVF), moxifloxacin (MXF), esomeprazole (ESZ), pantoprazole (PTZ), rabeprazole (RBZ), etoricoxib (ETR), have been selected and discussed in chapters I to VIII respectively

1.2 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 book called pharmacopoeia (e.g. Indian, IP, United Kingdom, BP, United States, USP, European, EP, Japan, JP and Martindale Extra). The other source information books include Merck index, Remington and 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 a drug is determined after establishing its purity and the quantity of the pure substance in the bulk drug and its formulations.

1.3 Types of pharmaceutical formulations:

The drugs are used in various dosage forms in prophylactic or in therapeutic use. They are formulated as tablets, capsules, dry syrups, liquid orals, creams or ointments, parenterals (injections 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, acacia, gelatin, polyvinyl pyrrolidone, alginic acid, tragacanth, stearic acid, talc, magnesium stearate, waxes, methyl paraben, propyl paraben, sodium benzoate, permitted flavors and colors may be added. In capsules one or more among the diluents, certified dyes, gelatin, plasticizers, preservatives, starch, lactose and talc may be added. In dry syrups and liquid orals, sucrose, sorbitol, 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, water, vegetable oils, mineral oils, simulated oils, propylene glycol, dioxalamines, dimethyl acetamide may be used as vehicles. Any one or more among stabilizers, antioxidants, buffering agents like citrate, acetate, phosphate, co-solvents, wetting, suspending and emulsifying agents like tween-80, sorbitol oleate and preservatives may be added. In lotions, dusting powders and aerosols, talc, silica derivatives, alcohol, preservatives may be added.

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

1.4 Preparation of sample solution for analytical investigation:

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 tablets is to treat them 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 the 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 there by affording favorable conditions for the 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, isooctane and few others have also been used.

1.5 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, color density or specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physicochemical methods are 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 turbidometry), electrochemical (potentiometry, amperometry, colorimetry and polarography) and chromatography (column, paper, thin layer, gas- liquid, high performance liquid) methods are generally preferable. Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and para magnetic 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. 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, GLC, 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 methods are simple and do not involve high cost. The limitation of many colorimetric procedures is dependant on the reaction upon which these procedures are based rather than on 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 eliminates 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 & ultra violet spectrophotometry and high performance liquid chromatography techniques have been used as tools in the assay of selected drugs.

1.6 GENERAL INFORMATION AND METHODOLOGY FOR THE DEVELOPMENT OF NEW METHODS

1.6.1 VISIBLE SPECTROPHOTOMETRY

Colorimetry and fluorimetric\textsuperscript{10-12} 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. Combined of the steadily resort to physical methods which often necessitates very sophisticated and expensive instrumentation, colorimetric and fluorimetric analysis can be perfected with very simple instrumentation, resulting nevertheless in sensitive, selective and accurate measurements with the addition of speed and simplicity. These are based on chemical reactions, 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

7
of sundry procedures. Analyst, who finds him/herself in need of a colorimetric or fluorimetric method may therefore be obliged to suit tremendous quantity of literature in order to select one procedure which seems to suit his/her needs. The purpose of this thesis is to provide practicing analysts with procedures that were carefully tested for selected drugs. All the data given under results section were actually obtained in our laboratory.

Table 1.01

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>FUNCTIONAL GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Functional groups imparting an acidic nature to substance</td>
<td>Carboxyl - COOH</td>
</tr>
<tr>
<td></td>
<td>Sulphhydryl or - SH</td>
</tr>
<tr>
<td></td>
<td>Thiol</td>
</tr>
<tr>
<td></td>
<td>Imide</td>
</tr>
<tr>
<td></td>
<td>Enol =C= CH (OH)</td>
</tr>
<tr>
<td></td>
<td>Enediol -C (OH) = C (OH)</td>
</tr>
<tr>
<td></td>
<td>Phenolic hydroxyl C₆H₅OH</td>
</tr>
<tr>
<td></td>
<td>Sulphonic acid SO₃H</td>
</tr>
</tbody>
</table>
2. Functional groups imparting basic properties to a substance

<table>
<thead>
<tr>
<th>Primary, secondary and tertiary amino groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNH$_2$</td>
</tr>
<tr>
<td>R$_2$NH</td>
</tr>
<tr>
<td>R$_3$N</td>
</tr>
</tbody>
</table>

3. Functional groups, which exhibit neither acidic nor basic property

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>R CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keto</td>
<td>RCOR</td>
</tr>
<tr>
<td>Alcohol</td>
<td>ROH</td>
</tr>
<tr>
<td>Nitro</td>
<td>RNO$_2$</td>
</tr>
<tr>
<td>Amide</td>
<td>RCONH$_2$</td>
</tr>
<tr>
<td>Ester</td>
<td>RCOOR</td>
</tr>
<tr>
<td>Ether</td>
<td>ROR</td>
</tr>
</tbody>
</table>
1.6.1a Classification of organic reactions: The steps of an organic reaction showing breaking or making of new bonds of carbon atoms in the 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 positive or negative charge. The positively charged reagents attack the regions 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 divided into four types (a) Substitution (b) Addition (c) Elimination and (d) Rearrangement.

1.6.1b Classification of organic reagents: Organic reagents fall into two main groups. An electrophilic reagent (cation, dipolar molecule or molecule that has atoms with incomplete octet) is a species having electron deficient atom or center. The nucleophilic reactions involve the attack of negative or neutral nucleophile on the positively charged products. Various reactions may involve the formation of three main intermediates namely free radicals (from homolytic fission), carbonium ions (C+) and carbanion (C- :) from heterolytic fission which then react with the reagents to form the products.

1.6.1c 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, C\textsubscript{6}H\textsubscript{5}, H) and [CH\textsubscript{3}, C\textsubscript{2}H\textsubscript{5}, CH (CH\textsubscript{3})\textsubscript{2}, C (CH\textsubscript{3})\textsubscript{3}] respectively. The mesomeric effect refers to the polarity produced in a molecule as a result of interaction between two $\pi$ bonds or a $\pi$ bond and a lone pair of electrons. This effect is transmitted along a chain. Especially in conjugated systems, the $\pi$ electrons get delocalized 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 (eg. Cl, Br, I, NH, OH, OCH\textsubscript{3}). The -M effect is shown by groups such as COO\textsuperscript{-}, NO\textsubscript{2}, C=\text{N}, SO\textsubscript{3}H 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.

1.6.1d 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 the reaction, the presence of other functional groups 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, 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 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 from that of any of the reactants.

Some examples of more specific reagents are given below.

1. The reagent forms or destroys an acidic, alkaline, oxidizing or reducing property of the functional groups, 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 the 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 colored derivatives. Similarly, a fluorophore may be produced or quenched and the change is measured fluorimetrically.

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.
5. The derivitisation 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 packing and thermal instability. Vast improvement in these respects are easily realized because the polar nature of the compound promotes derivitisation with suitable reagents to replace the polar group with a less polar one, giving sometimes a more sensitive detection response.

6. The reagent forms a derivative suitable for structural investigation or estimation by NMR and MS measurements.

7. Enzymes selectively catalyze specific reactions.

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

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

1.6.1e Chromogenic Reagents And Reactions Used In The Present Investigations:

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 non-destructive 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. The proposed methods of analysis of selected drugs will be discussed in detail in chapters II to VIII.

Different types of chemical reactions like oxidation/reduction (F.C., M7, M14, M22, M28, M35, M37), (CAS, M5) oxidative coupling (Ferric Chloride/MBTH, M41, CAS/MBTH, M6, M15, M23), complex formation (Ferric chloride/ Potassium Ferricyanide, M40, Ferric chloride, M3, M12, M20, Ferric Nitrate, M2, Ferric Alum M1, M11, M19), oxidation followed by complex formation (Ferric chloride/1,10, phenanthroline, M4, M13, M21, M27, M42, Ferric chloride/2,2', Bipyridine, M30, M38), condensation with aromatic aldehydes (Vanillin, M31, M39, PDAB, M29, PDAC, M36), formation of ion pair complexes(BTB, M10, M16, M24, M.O. M9, M17, M25, SBT M8, M18, M 26), formation of an amide from amine (Acetyl Chloride, M34, Acetyl Chloride/DMF, M33, Acetic Acid, M32) have been used. A brief account of the analytical applications of the reagents used in the above methods and the general methodology followed for the establishment of experimental conditions in each case is summarized.

1.6.1f Oxidation/Reduction Reaction: F.C. 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 reactions depend upon pH, composition of the heteropoly acid complex, nature and concentration of the reducing agent, temperature and time. It may be said generally, that the more the number of heteroacids in the complex, the more vulnerable it is to reduction under certain critical conditions. Among the various heteropoly acids, phosphomolybdotungstic acid, the well-known Folin- Ciocalteu
reagent\textsuperscript{17} was preferred by a number of workers for the determination of drugs containing not only phenolic or amino groups but also certain other drugs, which contain neither of these groups. The colour formation by Folin-Ciocalteu 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 utilized for the determination of Gatifloxacin (M\textsubscript{7}, Chapter -II), Levofloxacin (M\textsubscript{14}, Chapter-III), Moxifloxacin (M\textsubscript{22}, Chapter-IV), Esomaprazole (M\textsubscript{28}, Chapter-V) and Pantoprazole (M\textsubscript{35}, Chapter-VI).

1.6.1g Oxidative Coupling : MBTH-Oxidant:

3-Methyl-2-benzothiazolinone hydrazone (MBTH)\textsuperscript{18,19} was synthesized by Besthorn. Although MBTH was first prepared in 1910, its analytical abilities were evidenced only in 1957\textsuperscript{20,21}, and it was introduced as a reagent for colourimetric determination in 1961\textsuperscript{22}. It has since become an analytical tool of considerable versatility.

MBTH can react with carbonyl derivatives through its hydrazone grouping. On the other hand, it forms a strongly electrophilic diazonium salt when acted upon by an oxidising agent. These properties lead the way to colorimetric determination based on the formation of formazans and on diazo coupling.

The first procedure described by Sawicki et al.\textsuperscript{34} allowed the determination of aldehydes, with which MBTH condenses to give a blue color.

This technique was later improved for more sensitive determinations\textsuperscript{23}. The reaction was applied for the analysis of aliphatic aldehydes\textsuperscript{24-26} and the detection of free aldehyde groups in tissue section and collagen\textsuperscript{27}. Very sensitive results are also obtained with glyoxalic acid\textsuperscript{28}. The method can, of course, be extended to compounds that yield aldehydes upon suitable treatment.
Pyridoxal and its 5'-phosphate can be determined in the presence of each other, in the absence of oxidant. Glyoxal reacting with MBTH in acetic acid medium affords a yellow diazine, which allows its determination in the presence of unsubstituted mono-aldehydes, when no oxidant is present.

It was stated that, upon the action of a suitable oxidant MBTH forms a diazonium salt which can couple with various compounds. Phenol was so determined, the oxidant being cerium (IV) ammonium sulphate\(^{29}\). The reaction was extended to other phenols using various oxidants\(^{30-32}\) and an automated method was proposed by Friestad et al\(^{33}\). MBTH can be used for the determination of polyhydroxy compounds. Aromatic\(^{34,35}\), aliphatic and alicyclic amines\(^{36}\) were determined using MBTH. Azo dyes, stilbenes and schiff’s bases as well as pyrrole derivatives also react under oxidative conditions\(^{37}\). This reaction was extended to the determination of bilirubin and its oxidation products such as urolibin and biliverdin\(^{38}\). MBTH was also used for the indirect determination of compounds such as glycerides\(^{39}\) and glycans\(^{40}\) etc., which yield one of the above mentioned groups on preliminary treatment with certain reagents.E.I. Kommas\(^{41}\) suggested ceric ammonium sulphate as an oxidant under acidic conditions for the determination of 17 pharmaceutical phenols. Ferric chloride has been mostly used for the determination of aromatic and heterocyclic amines by Sawicki et al\(^{42}\) (in neutral conditions) and Pay et al\(^{43}\) (in acidic conditions). Other oxidants such as periodate (acidic conditions), ammonium per sulphate (alkaline conditions) and potassium dichromate (acidic conditions) were used for the determination of ethylenic compounds and primary alcohols (after oxidation with ruthium tetroxide) and phenidone\(^{44}\). Recently Sastry et al\(^{45}\) reviewed various aspects of MBTH chemistry in pharmaceutical analysis.
Under reaction conditions, MBTH loses two electrons and one proton on oxidation forming the electrophilic intermediate, which has been postulated to be the active coupling species. The intermediate reacts with the amine or phenol by electrophilic attack on the aromatic ring of the amine or phenol (i.e. para or ortho position if para position to amino or phenolic hydroxyl group is substituted) and the resulting intermediate is spontaneously oxidized with an oxidant to form the colored species. Oxidative coupling products from MBTH and pholedrine, procainamide hydrochloride or promethazine in the presence of oxidant are indicated.

In the present investigations MBTH was used in the presence of Fe (III) as oxidant (M41) for the determination of Etoricxib (Chapter VIII) and in the presence of Ce (IV) as oxidant (M6, M15, M23,) for the determination of Gatifloxacin (Chapter II), Levofloxacin (Chapter III) and Moxifloxacin (Chapter IV).

1.6.1h Oxidation followed by complex formation reaction: Fe (III) – 2,2'-Bipyridine and Fe (III) – 1,10-Phenanthroline:

Ferric salts (Ferric chloride) play a prominent role in the colourimetric determination of organic compounds. Many phenols, hydroxamic acid esters and more complicate compounds containing the phenolic hydroxyl group in their molecule react with ferric salt in an aqueous, alcoholic or chloroform media to give intense colouration characteristic of each particular phenol. The colour is due to the strongly ionized complex phenolate of trivalent iron, which is formed according to the equation

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

The colour intensity and stability of the complex increases when a – COOH group is present adjacent to phenolic group (ex. Salicylic acid). Addition of acid, glycerol, alcohol and some times excess ferric chloride decreases the degree of
phenolate dissociation (hence the concentration of colour ions decreases), and the colour of the solution vanishes. Alkalizing also destroys the colour by binding the iron ion into hydroxide. Polyhydroxy alcohols or oxyacids react with FeCl₃ to give stabler complexes. Oxyacids react with FeCl₃ to give complex salts of iron, which simultaneously oxidize the oxyacids.

Ferric chloride can also oxidize phenols. It oxidizes the hydroquinone to quinone, which then gives quinhydrone. Naphthols are oxidized by FeCl₃ to give sparingly soluble dinaphthols, 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, ferric salt converts into ferrous salt. It can easily be detected by the usual reagent for divalent iron such as potassium ferricyanide, orthophenanthroline, 2,2'-bipyridine or triazine.

2,2'-Bipyridine forms a complex of low tinctorial 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). Based on its complexing tendency and oxidizing properties, ferric salt was suggested in the estimation of several drugs.

In the present investigation the reduced drug was treated with excess ferric salt under specified experimental conditions. The ferrous salt was estimated with 2,2'-bipyridine (Esomeprazole, M₃₀, Chapter V, Rabeprazole, M₃₈, Chapter VIII). It was also estimated with 1, 10-Phenanthroline (Gatifloxacin, M₄ Chapter II, Levofloxacin M₁₃, Chapter III, Moxifloxacin M₂₁, Chapter IV, Esomeprazole M₂₇, Chapter V).
1.6.1i Condensation with aromatic aldehydes:

P-dimethylaminobenzaldehyde (PDAB), P-dimethylaminocinnamaldehyde (PDAC), Vanillin:

Certain amines condense with various aldehydes in strongly acidic media to give products that are oxidisable to give color. Among the many aldehydes that have been shown to react are p-dimethyl amino benzaldehyde, p- dimethyl amino cinnamaldehyde, benzaldehyde, piperonal, vanillin, formaldehyde, salicylaldehyde, paraldehyde, p- acetyl amino benzaldehyde, m- and p- nitro benzaldehyde, m-amino benzaldehyde and metaldehyde. The most common oxidant used is atmospheric oxygen, but the process has been hastened by the addition of hydrogen peroxide, nitrites, nitrates, ferric ion and several other metal ion catalysts. Of all the aldehydes, best results have been obtained with p- dimethylaminobenzaldehyde, p-dimethyl aminocinnamaldehyde and vanillin.

The reaction of p-dimethylaminobenzaldehyde, p-dimethylamino cinnamaldehyde and vanillin with indoles and pyrrole has been used qualitatively and quantitatively for many years. The reaction with aromatic amines to give schiff bases is equally well documented. Not so well known, the Wasicky reaction, utilizing 94% aqueous sulphuric acid solution, gives a colour reaction with nitrogen compounds and found that in a more dilute aqueous system aromatic compounds react in the presence of mineral acid, provided the-NH₂ group is directly attached to the benzene nucleus. No reaction occurs with (i) aliphatic amines and aminoacids (ii) N- substituted aromatic amines (iii) heterocyclic amino compounds or (iv) amino derivatives of cycloparaffins (ex. cyclohexyl amine).

In the present investigation PDAB (M₂₉) reagent system was utilized for the determination of Esomeprazole (Chapter V), PDAC (M₃₆) reagent system was utilized
for the determination of Pantoprazole (Chapter VI) and Vanillin (M₃₁, M₃₉) reagent system was utilized for the determination of Esomeprazole (Chapter V) and Rabeprazole (Chapter VII).

1.6.1j Complex formation with ferric ion

1.6.1j.(a) Ferric nitrate, Ferric Alum and Ferric chloride:

Ferric salts (ferric nitrate, ferric alum or ferric chloride) play a prominent role in the colorimetric determination of organic compounds. Many phenols, hydroxamic acid esters and more complicated compounds containing the phenolic groups in their molecule react with ferric salt in an aqueous, alcoholic or chloroform media to give intense colouration, characteristic of each particular phenol (ranging from green through blue and violet to red). The colour is due to the formation of strongly ionized complex phenolate of trivalent iron.

The present investigation deals with the estimation of Gatifloxacin (M₁, M₂, M₉, Chapter II), Levofloxacin (M₁₁, M₁₂, Chapter III) and Moxifloxacin (Chapter IV M₁₉, M₂₀).

1.6.1j.(b) Fe (III) – K₃Fe (CN)₆:

Ferric salts (ferric nitrate, ferric alum or ferric chloride) play a prominent role in the colorimetric determination of organic compounds. Many phenols, hydroxamic acid esters and more complicated compounds containing the phenolic groups in their molecule react with ferric salt in an aqueous, alcoholic or chloroform media to give intense colouration, characteristic of each particular phenol (ranging from green through blue and violet to red). The colour is due to the formation of strongly ionized complex phenolate of trivalent iron.

The other phenols also form phenolates of iron with partial oxidation. Acting as an oxidant ferric salt converts into ferrous salt. The usual reagents for divalent iron
are potassium ferricyanide, 1,10-phenanthroline, 2,2'-bipyridine and triazine etc. The reaction of ferric ion with potassium ferricyanide is given below.

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

The present investigation deals with the estimation of Etoricoxib (M48, Chapter VIII).

1.6.1k Complex formation with acetonium ion:

Acetonium ion react with secondary amines to form an amide, which acquires a yellowish, colored complex. Acetyl chloride and acetic acid are used in the present investigation.

\[
\begin{align*}
\text{H} & \quad \text{COCH}_3 \\
\text{N} + \text{CH}_3\text{COCl} & \quad \longrightarrow & \quad \text{N} + \text{HCl}
\end{align*}
\]

The present investigation deals with the estimation of Esomeprazole (M32, Chapter V), Pantoprazole (M33, M34, Chapter VI).

1.6.1l Ion Association Complex Formation With Dyes:

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 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). The forces, which lead to the formation of molecular complex, include forces such as dipole and induced dipole interaction, London dispersion forces, hydrogen bonding and dative bonding interactions. The donor-acceptor complexes (where composition can be represented by integral ratios of the components) can however, usually be detected readily because of differences in physical properties (e.g. absorption spectra, solubility in organic solvents) from those of the pure components. The ion – association complex or adduct 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 colorless, 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 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 colour can be altered or intensified upon acidification or re-extracted into a buffer. The presence of hydrophilic substituents such as \(-\text{OH}\) or \(-\text{COOH}\) often prevent 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. Relatively polar 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 diphenylmethane or phenothiazine.

According to the same principle, acid compounds can be determined colorimetrically with basic dyes. P-Toluensulfonic acid, camphorsulfonic 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 tropacoline OO and of tertiary aliphatic amines with erythrosine. Several dyes belonging to different chemical classes have been used for
the determination of basic drugs. Based on the above principles in the present investigation Solochrome Black- T (SBT), Methyl Orange (M.O) and Bromothymol Blue (BTB) were used for the estimation of Gatifloxacin (M₈, M₉, M₁₀, Chapter II), Levofloxacin (M₁₆, M₁₇, M₁₈, Chapter III) and Moxifloxacin (M₂₄, M₂₅, M₂₆, Chapter IV).

1.6.2. ULTRA VIOLET SPECTROPHOTOMETRY:

In ultra violet (200-380 nm) and visible (300-800 nm) spectroscopy, absorption of radiation is the result of excitation of bonding (σ, π) and non bonding (n) electrons. The frequencies of the absorption can be influenced by solvents and by delocalisation in conjugated systems. Spectrophotometer is an instrument for measuring the intensity of light of various wavelengths 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 of 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 a solvent 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, utilizes the principle that the amount of light absorbed by a substance in 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.
Based on the above principle in the present investigation Etoricoxib (M43, Chapter VIII) was estimated by U.V. method.

1.6.2.1 METHODOLOGY:

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 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 wavelengths.

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.

1.6.2.1a 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 used in different procedures of present investigation are furnished in subsequent chapters.
1.6.2 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 sample cannot be measured directly, but is determined using another physical 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 $y = b(x)$ 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 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 analysis is applied, which means that all observations are upon a single variable $X$.

1.6.2.1c The method of least squares$^{52,53}$:

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

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

We adopt 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 size). The values of the unknown parameters $a_1,$
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 a 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 is done by calculating values of \(\alpha\) and \(\beta\) for which \(\sum e_i^2\) 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 \(\beta\) are the intercept and slope respectively. The equations given for intercept and slope of the line are as follows.

Slope (\(\beta\)) = 
\[
\frac{\sum_{i=1}^{n} X_i Y_i - \sum_{i=1}^{n} X_i \sum_{i=1}^{n} Y_i}{\sum_{i=1}^{n} X_i^2 - (\sum_{i=1}^{n} X_i)^2}
\]

Intercept (\(\alpha\)) = 
\[
\frac{\sum_{i=1}^{n} Y_i - \sum_{i=1}^{n} X_i \sum_{i=1}^{n} Y_i}{\sum_{i=1}^{n} X_i^2 - (\sum_{i=1}^{n} X_i)}
\]

1.6.2.1d 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.

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

\(y_i\) 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 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.

### 1.6.2.1e 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.

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

Where \( x_i \) is the arithmetic mean of \( x_i \) values.

### 1.6.2.1f Standard deviation of intercept, \( S_a \):

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

\[
S_a = \sqrt{\frac{\sum_{i=1}^{n} (Y_i - \hat{Y}_i)^2}{(n - 2)}} \sqrt{\frac{1}{\sum_{i=1}^{n} (x_i - \bar{x}_i)^2}} \sqrt{\frac{\sum_{i=1}^{n} X_i^2}{n}}
\]

Where \( x_i \) is the arithmetic mean of \( x_i \) values.

### 1.6.2.1g 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 \( y \).
1.6.2.1h Selectivity of the method:

Matrix and interference effects may disturb the determination of an analyte. 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 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.

1.6.2.1i 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 Bouger-Lambert-Beer's law, log intensity of incident radiations

\[ A = \log \left( \frac{\text{Intensity of incident light}}{\text{Intensity of transmitted light}} \right) \]

\[ = c \cdot t \]

The absorbance (A) is proportional to concentration (C) of the absorbing species, if absorptivity (C and thickness of the medium (t) are constant. When c is in moles per liter, the constant is called molar absorptivity. Beer's law limits and C\text{max} values are expressed as \( \mu g \cdot ml^{-1} \) and mole \(^{-1}\) respectively.
Sandell’s sensitivity refers to number of µg of the drug to be determined, converted to the colored product, which in a column solution of cross section 1cm² shows an absorbance of 0.001 (expressed as µgcm⁻²).

1.6.2.1j Limit of detection⁵⁴:

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 (Sₐ), which must be related to LOD, and the slope of the calibration curve, b by

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

1.6.2.1k Ringbom’s plot⁵⁵⁵⁶:

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increases at the extremes of the transmittance scale. The slope of plot ‘C’ versus T, i.e. Ringbom’s plot gives relative error coefficient (i.e. plot of log C versus T).

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

1.6.2.1l Precision and accuracy⁵⁷:

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 criterion for judging analytical procedures by their results.

1.6.2.1m 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 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 deviation 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

$$S = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} \left( X_i - \overline{X} \right)^2}$$

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

The square of standard deviation is called Variance ($S^2$). The relative standard deviation is the standard deviation expressed as fraction as percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = $S \times 100 / x$

1.6.2.1n 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. These methods include absolute method and the comparative method.
1.6.2.1o Absolute method:

Taking different amounts of the constituents and proceeding according to specified instruction carries out the test for accuracy of the method. "The difference between the means of an adequate number of results and the amount of constituent actually present, usually expressed as parts per hundred (%) and 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.

1.6.2.1p Comparative method:

In the analysis of pharmaceutical formulation or solid laboratory prepared samples of desired composition, the content of 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 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.

1.6.2.1q 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 deference 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.

1.6.2.1r 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.

1.6.2.1s Students’ 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 smaller than 30, the condition of $x$ is required at least the normality of the difference ($d$). If this is the case the quantity

$$ t = \frac{d_1}{S_d / \sqrt{n}} $$

has a student $t$-distribution with $(n - 1)$ degrees of freedom, where $d_1 = x_r$ (Reference method) – $x_t$ (Test method) and $S_d$ is the standard deviation.

1.6.2.1t 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 test methods and $n_2$ replicate measurements by using reference method. If the null hypothesis is true, then the estimates $S^2_T$ (variance of the test method) and $S^2_R$ (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.

$$ F = \frac{S^2_T}{S^2_R} $$
It is conventional to calculate 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 - value is smaller than F - Value from the table one can conclude that the procedures are not significantly different in precision at given confidence level.

1.6.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)⁵⁹:

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.

Solvent container – pump – damping unit – precolumn – injection port

................................................Thermostat............................................Column

| Recorder-----------------------Detector

| Effluent

1.6.3.1 Columns:

The heart of the system is the column. The choice of common packing material and mobile phases depend on the physical properties of the drug. The column selection can assist on 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 (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.

1.6.3.2 Mobile phase:

Mobile phases used for HPLC typically are mixtures of organic solvents and
water or aqueous buffers. Table 1.02 lists the physical properties of organic solvents
commonly used for HPLC. Isocratic methods are preferable to gradient methods.
Gradient methods will sometimes 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 perception, 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, acetic acid or
formic acid absorb at shorter wavelengths, they may prevent detention of
products with out chromospheres above 220 nm. Carboxylic acid modifiers
can be frequently replaced by phosphoric acid, which does not absorb above
200 nm.

6. Use volatile mobile phases 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 1.2**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>MW</th>
<th>BP</th>
<th>RI</th>
<th>UV&lt;sub&gt;a&lt;/sub&gt; cut-off</th>
<th>Density g/ml (25°C)</th>
<th>Viscosity cP (25°C)</th>
<th>Dielectric constant</th>
<th>UV&lt;sub&gt;a&lt;/sub&gt; cut-off (nm)</th>
<th>Density g/ml (25°C)</th>
<th>Viscosity cP (25°C)</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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;</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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></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>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ionizable compounds in some cases can present some problems when analysed by Reverse phase chromatography. Two modifications of the mobile phase can be useful in reverse phase HPLC for ionisable 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 boarding 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 paring 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.

1.6.3.3 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.

1.6.3.4 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.

1.6.3.5 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 methods 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
5. Reaction detectors

1.6.3.6 Performance calculations:

The following values (which can be included in a custom report) are 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).

\[ \alpha = \frac{(t_2 - t_a)}{(t_1 - t_a)} \]

Theoretical plates:

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

Capacity factor:

\[ K^1 = \frac{(t_2/t_a)}{} - 1 \]

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: \( \frac{L}{n} \)

Where 
- \( a \) = Relative retention
- \( t_2 \) = Retention time of the second peak measured from point of injection.
- \( t_1 \) = Retention time of the first peak measured from point of injection.
- \( t_a \) = Retention time of an inert peak not retained by the column, measured from point of injection.
- \( n \) = Theoretical plates.
- \( t \) = Retention time of the component.
- \( W \) = Width of the base of the component peak using tangent method.
- \( k^l \) = Capacity factor
- \( R \) = Resolution between a peak of interest (peak 2) and the preceding it (peak 1)
- \( W_2 \) = Width of the base component peak 2.
- \( W_1 \) = Width of the base 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 base line.
- \( f \) = Distance from the peak maximum to the leading edge of the peak.
- \( N \) = Plates per meter.
- \( L \) = Column length, in meters.

1.6.3.7 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:**

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

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

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

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

**1.6.3.8 Internal standard method:**

\[ Y = \frac{\text{Component area or height}}{\text{Internal standard area or height}} \]

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

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

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

**1.6.3.9 Information relating to the developed methods:**

In the present investigation, the author has developed five (M44 – M48) HPLC methods by using with or without the use internal standard for single or combined formulations of drugs. The technique has been utilized for the development of one method for the assay of five selected drugs. The three internal standards that were used in the present study are ciprofloxacin, omeprazole and celecoxib.