CHAPTER – I

INTRODUCTION

1.1 General introduction

Drug is a pharmacological substance which when absorbed into a living organism may modify one or more of its functions and is intended for medicinal usage to assist the diagnosis, cure, treatment, or prevention of disease or ailment. In general the term drug refers to a substance taken for a therapeutic purpose. The Drugs are used in various dosage forms in therapy [1-6]. They are formulated as Tablets, binders, adhesives, capsules, oily, aqueous suspensions, ointments and creams etc.

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 Pharmacopoeia, IP[7]; United kingdom BP[8] and BP[9](Vet); United states pharmacopoeia, USP[10]; European Pharmacopoeia, EP[11]; Martindale extra pharmacopoeia, MEP[12]; Merck Index, MI[13]; Japan Pharmacopeia, JP[14]).

Pharmaceutical analysis [15-17] deals not only with medicaments (drugs and their formulations) but also with their precursors i.e., with the raw materials on whose degree of purity, the quality of medicament depends. The quality of a drug is determined after establishing its purity and the quality of pure substances in the bulk drug and its formulations. There are two possible approaches to reach this goal. They are the determination of the active ingredient content with a highly accurate and precise specific method or the determination of its impurities.

It involves the introduction of more refined and sensitive methods of physiochemical analysis [18-20] such as colorimetry, spectrophotometry covering UV, visible and IR regions, fluorimetry or turbidimetry, NMR and Mass, and chromatography (GLC [21], HPLC [22, 23], TLC [24]) that enables one to assay the quality of drugs more accurately and with the smallest consumption of the analyte, reagent and time.
The modern methods of choice (HPLC, GLC, NMR and Mass) for purity assay involve sophisticated equipment which are costly and pose problems of maintenance. Hence they are not in the reach of most laboratories and small scale industries. The visible spectrophotometric [25-28] or colorometric, or fluorimetric methods are very simple, cheap and are easy to carry out. The limitations in the visible spectrophotometric methods of analysis are dependant up on the chemical reactions rather than the sophistication of the instruments. The visible spectrophotometry is selected as a tool for the assay of selected drugs in the present investigation.
1.1a. Visible Spectrophotometry

The human eye is sensitive only to electromagnetic radiations having a wavelength between 400-800 nm and this region of the spectrum is known as visible spectrum. The visual color is a complementary to the color absorbed (i.e., the color sensation produced by all of the wavelengths minus the wavelength absorbed). The absorption of the radiations is the result of excitation of bonding ($\sigma$, $\pi$) and nonbonding ($n$) electrons. The frequencies of the absorption can be influenced by solvents and by delocalization in conjugated systems. Photometric methods of analysis, based on measuring light absorption of 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. Spectrophotometer (sophisticated than colorimeter) 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 converts radiant energy to electric energy and can eliminate the need for subjective measurements. This limit of detection is lowered by measuring the absorption of a solution at the wavelength of maximum absorption. It is possible to minimise 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. The general methodology for the development of new visible spectrophotometric methods is as follows.

In the past few decades, a number of elegant instrumental techniques such as spectrophotometry, chromatography, electrophoresis etc. were reported which are rapid, selective and having a high degree of accuracy. Among these, spectrophotometry is the most important method, which is widely used for wide variety of materials.
The greatest use of spectrophotometry lies in its application to quantitative measurements. The reasons for this stem from the ease with which most spectrophotometric measurements can be made, their sensitivity and precision and the relatively low cost of instrumental purchase and operation. Direct determinations are made when the analyte molecule contains a chromophore, thus allowing the direct measurement of its absorbance. Standards must be used to determine the absorptivity, so that concentration can be calculated by using the equations or by establishing a calibration plot from which the concentration can be determined by graphical interpretation or by regression analysis. Indirect determinations are commonly used when the analyte molecule does not contain a suitable chromophore. In these instances the analyte is made to quantitatively react with molecules containing a chromophore and correlating the diminution of absorbance with the concentration of analyte or by reacting with a reagent, which produces chromophoric groups.

Spectrophotometric techniques frequently employed in pharmaceutical analysis include UV-Vis, AAS and IR. The theory behind spectrophotometric methods lies on a simple relationship between the color of the substance and its electronic structure. A molecule exhibits absorption in the UV-Vis region when the radiation causes an electronic transition in molecules containing chromophoric groups. In these techniques color is an important criterion for the identification of constituents. The importance of colored solution lies on the fact that the radiation absorbed is the characteristic of the material responsible for absorption and can be determined quantitatively or qualitatively. Nevertheless, a substance that is colorless or faintly colored may be often determined by the addition of chromogenic reagent, imparting intensive color to the species. The quantitative applicability of the absorption method is based on the fact that the number of photons absorbed is directly proportional to the number or concentration of atoms, ions or molecules [29].

In early days spectrophotometric measurements were made using human eye as the detector and undispersed sunlight or artificial light as the light source. The introduction of optical filters, which isolates specific frequencies of light, improved the accuracy and precision of the measurements to some extent. Further improvement of the
measurement came with the use of prism and grating monochromator for wavelength isolation and also photoelectric detectors, phototubes and photomultiplier tubes.

Development of solid-state microelectronics has now made available a wide range of detector type which coupled with the computers, provide highly sophisticated electronic systems.

1.1b Reactions proposed in the present investigation

The nature of organic drugs towards reactivity depends on the presence of functional groups in their molecules [30-31]. Knowing the reactions of functional groups, one can easily analyse any drug with a complicated structure. The structural features (official names, chemical names, structures and analytically useful functional groups) of the selected drugs in the present investigations are given in corresponding chapters. Literature survey on selected drugs showed that there are very few visible spectrophotometric methods of analysis at the time of commencement of these investigations. The analytically useful functional groups of selected drugs have not been fully exploited for developing suitable visible spectrophotometric methods for their assay. The chemical features of selected drug molecules still offer a lot of scope for the development of new visible spectrophotometric methods hopefully with better sensitivity, selectivity, precision and accuracy. The author had made some attempts in this direction and succeeded in developing some new methods (Table.1.1) are present.
### TABLE 1.1 LIST OF PROPOSED VISIBLE SPECTROPHOTOMETRIC METHODS

<table>
<thead>
<tr>
<th>Type of Reaction</th>
<th>Reagent</th>
<th>Method</th>
<th>Drug responded</th>
<th>$\lambda_{\text{max}}$ nm</th>
<th>$\varepsilon_{\text{max}}$ L.mole$^{-1}$ cm$^{-1}$</th>
<th>Beer’s law limits $\mu$g ml$^{-1}$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative</td>
<td>MBTH - NaIO$_4$</td>
<td>M$_1$</td>
<td>LRD</td>
<td>590</td>
<td>8.153 $\times$ 10$^5$</td>
<td>2.0 – 10.0</td>
<td>Chapter II, present work</td>
</tr>
<tr>
<td>Coupling</td>
<td></td>
<td>M$_1$</td>
<td>CYP</td>
<td>550</td>
<td>1.017 $\times$ 10$^4$</td>
<td>5.0 – 25.0</td>
<td>Chapter III, present work</td>
</tr>
<tr>
<td>Oxidative</td>
<td>MBTH - Fe(III)</td>
<td>M$_2$</td>
<td>LRD</td>
<td>630</td>
<td>9.397 $\times$ 10$^5$</td>
<td>5.0 – 25.0</td>
<td>Chapter II, present work</td>
</tr>
<tr>
<td>Coupling</td>
<td>Brucine - IO$_4^-$</td>
<td>M$_3$</td>
<td>LRD</td>
<td>520</td>
<td>3.905 $\times$ 10$^4$</td>
<td>2.5 – 12.5</td>
<td>Chapter II, present work</td>
</tr>
<tr>
<td>Oxidative</td>
<td>IO$_4^-$/[Fe(CN)$_6$]$_3^-$</td>
<td>M$_4$</td>
<td>CYP</td>
<td>520</td>
<td>9.306 $\times$ 10$^5$</td>
<td>4.0 – 16.0</td>
<td>Chapter III, present work</td>
</tr>
<tr>
<td>Coupling</td>
<td>Fe(III)/[Fe(CN)$_6$]$_3^-$</td>
<td>M$_5$</td>
<td>LRD</td>
<td>700</td>
<td>5.425 $\times$ 10$^4$</td>
<td>5.0 – 25.0</td>
<td>Chapter II, present work</td>
</tr>
<tr>
<td>Redox Reaction</td>
<td>AV-H$_2$SO$_4$</td>
<td>M$_6$</td>
<td>LRD</td>
<td>760</td>
<td>3.268 $\times$ 10$^4$</td>
<td>2.5 – 12.5</td>
<td>Chapter II, present work</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP</td>
<td>760</td>
<td>3.199 $\times$ 10$^4$</td>
<td>1.0 – 5.0</td>
<td>Chapter III, present work</td>
</tr>
<tr>
<td>Substitution</td>
<td>CA –MeOH</td>
<td>M$_7$</td>
<td>CYP</td>
<td>535</td>
<td>1.840 $\times$ 10$^4$</td>
<td>2.5 – 12.5</td>
<td>Chapter III, present work</td>
</tr>
</tbody>
</table>
1.2 Objective of the present work

The nature of organic drugs towards reactivity depends on the presence of functional groups in their molecules. Knowing the reactions of functional groups, one can easily analyze any drug with a complicated structure. The structural features (official names, chemical names, structures and analytically useful functional groups) of the selected drugs in the present investigations are given in corresponding chapters. Literature survey on those drugs (Chapter II; Chapter III) showed that there are very few visible spectrophotometric methods of analysis at the time of commencement of these investigations. The analytically useful functional groups of selected drugs have not been fully exploited for developing suitable visible spectrophotometric methods for their assay. The chemical features of selected drug molecules still offer a lot of scope for the development of new visible spectrophotometric methods hopefully with better sensitivity, selectivity, precision and accuracy. The author has made some attempts in this direction and succeeded in developing some new methods (Table 1.1).

1.2a Oxidative coupling reactions (Methods M₁, M₂, M₃& M₄)

Oxidative coupling procedures involving the use of 3-methyl-2-benzothiazolinone hydrazine (MBTH), Brucine and Phenyl hydrazine hydrochloride (PHH) in the presence of an appropriate oxidant under slightly an acidic, neutral or slightly alkaline condition that forms highly colored species. This reaction was explored for the assay of drugs possessing functional groups such as amide, amine in general.

**MBTH – NaIO₄ / Fe(III): (Methods – M₁ & M₂)**

3-Methyl-2-benzothiazolinone hydrazine (MBTH) was synthesized by Besthorn [29]. The first procedure described by Sawicki [30] allowed the determination of aldehydes, with which MBTH condenses to give a blue cation. This technique was later improved, allowing more sensitive determinations [31]. The reaction was applied to the analysis of aliphatic aldehydes [32-34], and the detection of the aldehyde groups in tissue and collagen [35].
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 amine or phenol by electrophilic attack on the most nucleophilic site on the aromatic ring of amine or phenol (i.e., para or ortho position) and the intermediate is spontaneously oxidised in the presence of oxidant to form the colored species.

MBTH also forms a strongly electrophilic diazonium salt when acted upon by an oxidizing agent. These properties led the way to colorimetric determinations based on the formation of formazans. Glyoxal reacts with MBTH in the presence of acetic acid giving yellow diazine, which allows its determination in the presence of unsubstituted monoaldehydes as oxidant(III) in Method M₄ and this method has been applied for the determination of doxorubicin [36]. Phenol was so determined by using the oxidant cerium (IV) ammonium sulphate [37]. This reaction was extended to miscellaneous other phenols, using various oxidants [38-40] and an automated method was proposed by Friested.et al [41]. MBTH can be used for the determination of polyhydroxy compounds [42, 43], aliphatic and alicyclic amines [44]. Azodyes, stilbenes and Schiff bases as well as pyrrole derivatives also react with MBTH under oxidative conditions [45]. This reaction was extended to the determination of bilirubin and its oxidation products such as urobilin and biliverdin [46]. Ferric chloride has been mostly used as an oxidant for the determination of aromatic and heterocyclic amines by Sawicki [46] et al (in neutral conditions) and Pay [47] et al (in acidic conditions). Other oxidants such as periodate (acidic conditions), ammonium persulphate (alkaline conditions) and potassium dichromate (acidic conditions) were employed for the determination of ethylenic compounds and primary alcohols [48](after oxidation with ruthenium tetraoxide) and phenidone [49]. E.I. Kommas [50] suggested ceric ammonium sulphate as an oxidant under acidic conditions for the determination of pharmaceuticals possessing phenol group. Recently Sastry [51] et al reviewed various aspects of MBTH chemistry in pharmaceutical analysis. Oxidative coupling reaction involving MBTH in presence of ferric chloride has been used for the assay of several drugs [52-58].
In the present investigations, the selected two drugs LORTADINE (LRD) and CYPROHEPTADINE HCl (CYP), have responded to the oxidative coupling with MBTH in the presence of NaI04 (Method M1). The probable sequence of reactions and the developed procedure for their assay are presented in corresponding chapters II & III for these drugs.

Moreover, the selected drug Lortadine (LRD), involves in oxidative coupling reaction with MBTH in the presence of oxidant, Fe (III) (Method M2) forming oxidative coupling product and the developed procedure for its assay is presented in corresponding chapter II of the appropriate drug.

**Brucine – Periodate (Method – M3)**

Brucine (2,3–dimethoxystrychnine) under acidic conditions has been reported to be an effective reagent for spectrophotometric determination of nitrates and nitrites [59], cerium [60], manganese [61], cadmium and platinum [62]. Several modifications have been introduced for the spectrophotometric determination of nitrites and nitrates using this reagent [63, 64]. It was also reported subsequently that in combination with potassium persulphate, Brucine can also been used for the spectrophotometric determination of halides [65] and cysteine [66] and as an indicator in redox titration [67-69]. Brucine forms a 1:1 colored complex with p–dimethylaminocinnamaldehyde under acidic conditions [70].

Sodium metaperiodate is an effective oxidant for converting methyl substituted p-dihydroxy phenols to o-quinones [71] and is also color stabilizer. [72] Sastry et al used brucine-periodate reagent for spectrophotometric determination of tryptophan and some sulphur compounds [73], and for tetracyclines, chloropenicol and streptomycin [74]. According to them, periodate converts most electron rich portion of the coupler (tryptophan and other mentioned compounds) to yield 1-mono substituted bruciquinone derivatives with an absorption maximum at 500-510 nm as the colored species. Brucine – periodate reagent gave colored species with the compounds containing either primary or secondary aliphatic amino and aromatic primary amine groups. On the basis of this observation, the author has developed a specific method for the assay of Lortadine (LRD) (Method M3) in bulk samples and dosage forms. The details of the
spectrophotometric investigations of the corresponding drug are incorporated in chapter II.

**NaIO₄/Phenyl hydrazine hydrochloride (PHH)/[Fe(CN)₆]³⁻ (Method - M₄)**

Periodic acid oxidation [75-84] is applicable to compounds having two hydroxyl groups or a hydroxyl and an amino group attached to adjacent carbon atoms and are characterized by the cleavage of the carbon-carbon bond. If the hydroxyl groups or a hydroxyl and an amino group are not vicinal, no oxidation takes place. This selectivity, which is the outstanding characteristic of periodic acid oxidation, adopts the reaction for the presence of vicinal hydroxyl groups [85] and hydroxyl and amino groups. Carbonyl compounds in which the carbonyl group is adjacent to a second carbonyl (α-diketone) or hydroxyl (α-ketol) group are also oxidized. Oxidation of compounds of the types thus far discussed proceeds readily at room temperature. Certain compounds, which show no substantial reaction at room temperature, can be oxidized at elevated temperature [86].

Periodate oxidation can be applied in aqueous solution over a very wide range of pH to small amounts of material in a fairly simple and straightforward fashion. The rapid and generally quantitative nature of the reaction recommends it for a very wide variety of analytical applications. Sodium metaperiodate (IO₄⁻) is considerably soluble in water (12.62g/100mL, 25°C). The solubility of sodium metaperiodate is greatly reduced in alkaline solution because of the formation of disodium metaperodate (Na₂H₃IO₆) [214]. and this effect occurs at pH>5.0. In aqueous solution of sodium metaperiodate at pH 4.0 or below is the most suitable one as the oxidant [87, 88].

The oxidation reaction with periodate are quantitative, certain analytical procedures have been developed based on the determination of aldehydes produced in the reaction [89, 90]. A number of spectrophotometric methods have been developed for the determination of some aldehydes (aldehydes in particular) by sawicki et al. Different types of reagents is involved in developing the spectrophotometric methods for their determination.
Even though there are several procedures based on different principles using several reagents for the determination of aldehydes in particular formaldehyde (existing or formed through some preliminary treatment such as periodate oxidation of compounds possessing vicinal aminol, diol or ketol), the reagent like schryver’s appear to yield highly sensitive and stable chromogen with formaldehyde especially. These methods avoid the distillation or diffusion step and permit the determination of the liberated formaldehyde directly in the reaction medium colorimetrically by oxidative coupling reaction with schryver reaction [91-92] with PHH and hexacyanoferrate.

In the present investigation, of **Cyproheptadine (CYP)**, **(Method M₄)** responded to oxidative coupling reaction with PHH (Phenyl hydrazine hydrochloride) in the presence of hexacyanoferrate (III) giving formazan dye. The details of the investigation of the corresponding drug are incorporated in **chapter III**.

### 1.2b. Redox reactions (Methods M₅& M₆)

**Fe (III)- [Fe(CN)₆]³⁻ (Method - M₅)**

Iron (Fe) exhibits variable valency and exists as ferrous and ferric salts. The former type (Fe (II)) acts as a reductant and involves in complex formation with certain amount of compounds such as o-phenanthroline [93], bipyridyl or triazine [94], hexacyano ferrate (III)[Fe(CN)₆]⁻³[95] giving color species. The latter type Fe (III) functions as an oxidant and also has a tendency to give colored species with potassium thiocyanate, phenolic compounds, oxamic ester [96] and few other bifunctional substrates possessing complexing ability to give four or five membered cyclic compounds.

There are several reports with the use of reagent combination such as Fe (III)/ o-phenanthroline, bipyridyl, triazine or hexacyanoferrate (III) for the estimation of certain bioactive compounds bearing reducing properties [97-102]. In addition, there are reports that cholesterol [103] and few other macrolide antibiotics can be estimated colorimetrically by using Fe (III) salt. In the present investigation, the author has been applied the above method **(Method M₅)** for the assay of **Loratadine (LRD)** are presented in **chapter II**.
**Ammonium Vanadate (AV) - H₂SO₄ (Method - M₆)**

The chemistry of Molybdenum is complicated and it forms compounds corresponding to oxidation numbers +2 to +6. The most stable and commonly encountered compounds of molybdenum are derived from its oxide MoO₃. The molybdenum compounds corresponding to the oxidation states ranging from +2 to +5 are mostly complexs species. The tetrahedral anion MoO₄⁻² in aqueous medium on acidification exists as isopolyanionic species as a result of polymerization and condensation reaction that is exemplified by Mo₇ O₂₄⁻⁶ and Mo₈ O₂₆⁻⁴. Molybdate can form hetero poly anionic species such as Phosphomolybdate [PMo₁₂O₄₀]⁰³⁻, Phosphomolybdotungstate [FC: 3H₂o, P₂O₅.WO₃.5MoO₃.10H₂O and 13H₂O.P₂O₅.5MoO₃.10H₂O] and Molybdoperiodate [I (Mo O₄⁻⁵)₆] in the presence of some anions such as PO₄⁻³, SiO₄²⁻, IO₄⁻, and WO₄⁻.

The isopolyanionic or hetero polyanionic species undergo reduction to colored molybdenum species with certain bioactive compounds. The λ_max values of reduction products vary from 600nm – 840nm depending upon the reaction conditions (nature and strength of acid or base medium, temperature, time) nature of poly acid (very efficient if the composition of hetero acids are more) and nature of reducing agent (analyte). “Molybdenum blue” is the result of mild reduction of an acidified solution, which contains Mo (VI), either as an iso-or a hetero polymolybdate anion (or even alkaline conditions) or as a suspension of MoO₃ in water. The “Molybdenum blue” contains both oxide and hydroxides with MoO (OH)₂ in olive green compound, as one limit and MoO₃ as the other.

In the present investigations, the author has developed colored product of maximum intensity with the selected two drugs **Lortadine (LRD)** and **Cyproheptadine (CYP)**, under specified experimental conditions, when treated with **Ammonium Vanadate (AV) (Method M₆)**. The details of the investigation have been compiled in corresponding chapters of the responded drugs **chapters II and III**.
1.2c. Charge transfer reactions with quinones (Method – M\textsubscript{7})

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

\[
\text{D}^+ \text{A} \quad \text{AD} \quad \text{D}^+\text{A}^- \\
\]

The position of equilibrium depends on the donor, the acceptor and the solvent, the formation of ions being favored by donors of low ionisation potential, acceptor of high electron affinity and solvents with strong solvating powers. Amines are good electron donors and Quinone’s are good electron acceptors. The formation of outer complexes or electron donor-acceptor (EDA) complex between Quinone and amine is well known. Since both the donor and acceptor are often very reactive, chemical reaction can occur between them. The products of such reactions may replace the anticipated EDA complex. There is good evidence for the formation of weak outer (EDA) complexes particularly in poorly ionizing solvents such as cyclohexane and CCl\textsubscript{4} (CHCl\textsubscript{3}) has been used occasionally because of the low solubility of many Quinone’s in the more inert solvent. The complexes are usually characterized by an intermolecular charge-transfer absorption band which often appears in the visible region.

The energy of the band (h\nu\text{CT}) for a given complex agrees well with the electron donating and accepting properties of the two components [104]. In some cases more than one intermolecular charge-transfer transition [105] is observed. These could correspond to transition from the highest filled and penultimate filled levels in the donor to the lowest empty level in the acceptor. Many of the Quinone-aromatic amine systems forms weak outer complexes, usually though not always with a 1:1 stoichiometry [106]. The basic structures in these complexes are stacks of alternate Quinone and amine molecules with their planes parallel or nearly so though the molecules are not normally stacked.
above one another [107]. If the quinone has a sufficiently high electron affinity, and the
donor, a sufficiently low ionization potential, then the electron may be transferred form
the donor to the acceptor moiety in the ground state. This will produce in principle a pair
of ion radicals (amino radical and semi Quinone ion), although there may be some
interaction between the two [108]. The complexes with tertiary amines persist for a long
time without further chemical reaction, although such subsequent reaction are dependent
to a great extent, on the nature of the solvent [109,110] In solvents of intermediate
ionizing power, for example, ethylene glycol, dimethyl ether, acetonitrile, tetrahydrofuran
or benzotri fluoride, the ions/ ion pairs and acceptor complex are observed simultaneously
in equilibrium. With most aromatic amines, the EDA complex fades with time because of
other reactions which lead eventually to substitution (usually substitution in the 2 and 5
positions). Semi polar and polar solvent media facilitate the formation of radical ions and
substitution.

The general conclusion is that the electron affinity of the conjugated acceptor species
will increase [111,112] with

1. The electron withdrawing ability of the substitute.

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

3. An extent of the conjugation of ethylenic compound is better acceptors than aromatic
   compounds.

Some substituted quinones such as p-benzoquinone [113], fluoranil [114], tetracyanobenzoquinone [115], 1, 1, 2, 2-tetracyano-1, 4 naphthaquinone; dimethane [116], 7, 7,8,8-tetracyanoquinonedimethane[117,118], N-arylsulfonyl-1,4benzoquinonemonoamine
[119], p-benzoquinone chlorimide [120], 2,5-dichlorobenzoquinone[121] and p-N-
methylbenzoquinone monoamine [122], DDQ [123,124], chloranilic acid [125] and
chloranil [126] have been used in the studies of amines. The EDA complexes formation
with aromatic amines and quiriones are usually characterised by an intermolecular charge
- transfer absorption band which often appears in the visible region.
The chemical reaction between quinones and aromatic amines is usually
distributed in the 2-and 5-positions (chloranil - aniline). Nagakura and Coworkers [127-
129]propose that these are sigma of the type. In this case the monosubstituted Quinone is
probably the final product.

\[
\begin{align*}
\text{O} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{Cl} \\
\text{H} & \quad \text{H} & \quad \text{H} & \quad \text{Cl} \\
\text{N} & \quad + \\
\text{O} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{Cl}
\end{align*}
\]

Generally, the C-T transitions (DDQ < TQ < DHQ) reflects the high electron affinity of
quinones (DDQ > TQ > DHQ). Yamaoka and Nagakura [130] studied for formation of
ions from chloranil and tributylamine in ethanol. Nagakura and Coworkers [131] have
investigated the reaction of aniline and 1, 3, 5-triamino benzene with TQ to yield the
substitution products (2, 5-di). Dwivedi and Rao [132] have found that reactivity in the
reaction of p-substituted anilines with chloranil. The rate of reaction of aniline and
chloranil increases with increasing solvent polarity. Similar vinylic substitution reactions
of TQ compounds have been reported by Causquis and Thiband [133,134]. The products
which are observed in the interaction of aliphatic amines with p-benzoquinones are
normally the corresponding amino-p-benzoquinones (usually 2, 5-disubstituted imines in
primary amines; monosubstituted in secondary amines). During the reaction the
formation of the semiquinone ion was observed. The absorption which develops a broad
band at 520nm is primarily due to dianion of chloranilic acid (2, 5 - dihydroxy -3, 6 -
dichloro - p - benzoquinone) which is a precursor of chloranilic acid. In this region there
is also a weak absorption band due to the 2, 5 di (amino ester) substituted 3, 6 -
dichloroquinone which is mainly distinguished by the more intense band in the near
ultraviolet The semiquinone ion has also been detected in the studies of Lautenberger and
Mille[135] and by Yamouka and Nagakura[131]. The reaction of p-benzoquinones with
tertiary aliphatic amines is of particular interest. Henbest and his Coworkers [136] who
showed that the blue product formation Joined was 2-diethylamino vinyl – 3, 5, 6 -
trichlorobenzoquinone from the interaction of triethyl amine and chloranil. Henbest [136]
suggested that the hydrogen transfer step in the initial enamine formation might take place with EDA complex involving amine and chloranil. Similar blue color formation was observed in the case of aliphatic secondary amine with chloranil as well only in the presence of acetaldehyde.

\[
\text{(TQ) Chloranil: } R^1 = R^2 = R^4 = \text{Cl} \\
\text{DHQ) Chloranilic acid: } R^1 = R^4 = \text{Cl}, R^2 = R^3 = \text{OH} \\
\text{DDQ: } R^1 = R^2 = \text{Cl}, R^3 = R^4 = \text{CN}
\]

In the present investigation, the colored species formation in the method (Method – M₇) for the assay of Cyproheptadine (CYP) (Table 1.03, P. 3) appear to be due to the formation of either radical ion or monosubstituted derivative which possess aliphatic secondary amino group respectively. The details of the investigation have been incorporated in chapter III.

1.3. General methodology for the development of new visiblespectrophotometric methods:

i. 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 wavelength in the visible region are laid off along the axis of ordinates. A characteristic of an absorption spectrum is a position of the peaks of light absorption by the substance and also the intensity of absorption, which is determined by the absorptivity at definite wavelength. After
selection of the analytical wavelength, the chromogenic reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknowns should be on a definite time schedule.

ii. **Optimization of analytical method** [137]

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.

iii. **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 \( x \).

iv. The method of least squares [138,139]

Least-squares regression analysis can be used to describe the relationship between response (\( y \)) and concentration (\( x \)) 138,139. 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.

We adopt the convention that the \( x \) values relate to the controlled on independent variable (e.g. the concentration of a standard) and the \( y \) values to the dependent variable (the response measurements). This means that the \( x \) values have no error. On the condition that 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 \( a^1 \) and \( b^1 \) of the two values \( \alpha \) and \( \beta \). This can be observed by calculating the 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, \( a \) and \( b \) are the intercept and slope respectively.
\[
\begin{align*}
    a &= \frac{\sum_{i=1}^{n} y_i \sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i y_i\right)}{n \sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2} \\
    b &= \frac{n \sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n \sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2}
\end{align*}
\]

v. 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 \(y_i\) are the observed and predicted values, respectively. Standard deviations on 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.

vi. 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.
\[ S_b = \sqrt{\frac{n \sum (y_i - \bar{y})^2}{(n-2)}} \quad \text{and} \quad \frac{1}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2}} \]

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

v. Standard deviation on intercept, \( S_a \)

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

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

Where \( x_i \) denote the arithmetic mean of \( x_i \) values

vi. 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 \( y \).

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

vii. 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 instances appropriate separation procedure is to be adopted initially. The selectivity of the method is ascertained by
studying the effect of a wide range of excipients and other additives usually present in the pharmaceutical formulations to be determined under optimum conditions.

Initially, interference studies are carried out by the determination of fixed concentration of the drug several times by the optimum procedure in the presence of a suitable (1-100 fold) molar excess of the foreign compound under investigation and its effect on the absorbance of the solution is noticed. The foreign compound is considered to be interfering at these concentrations if it constantly produces an error of less than 3.0% in the absorbance produced in pure solution.

viii. 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

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

**Intensity of transmitted light**

The absorbance (A) is proportional to the concentration (c) of the absorbing species, if absorptivity (\(\varepsilon\)) 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 \(\varepsilon_{\text{max}}\) values are expressed as \(\mu g.ml^{-1}\) and \(1 \text{ mole}^{-1}.\text{cm}^{-1}\), respectively.

Sandell's sensitivity [140] 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 \(\mu g.cm^{-2}\)).

ix. Limit of detection [141]

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

$$LOD = 3S_a / b$$

x. **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.

xi. **Precision**

Precision refers to the reproducibility of measurement within a set, i.e., 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 terms employed is the standard deviation of a population of observation. Standard deviation is the square root of, the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set the standard deviation $S$, is given by

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

Standard deviation has the same units as the property being measured. The square of standard deviation is called 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.

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

xiii. **Absolute method**

The test for accuracy of the method is carried out by taking varying amounts of the constituents and proceeding according to specified instructions. The difference between the means 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.

xiv. **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.

**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.'
xv. Student t-test

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

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

If this is the case the quantity has a student t-distribution with (n-1) degrees of freedom, where \( d_i = X_R \) (Reference method) – \( X_T \) (Test method) and \( S_d \) is the standard deviation.

xvi. 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 \( S_T^2 \) (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 - 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.
1.4 INTRODUCTION ON APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE VALIDATION OF DRUGS

1.4a. PART-B: INTRUMENTATION OF HPLC

High performance liquid chromatography [142,143,144] is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows using a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

HPLC employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch. The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process, if diffusion is minimized, a faster and effective separation can be achieved. The technique of high performance liquid chromatography is so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

- Speed (many analysis can be accomplished in 20 min or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
Ideal for the substances of low viscosity

Easy sample recovery, handling and maintenance.

Instrumentation leads itself to automation and quantification (less time and less labour)

Precise and reproducible

Integrator itself does calculations.

1.4b. Types of HPLC Techniques:

Based on modes of chromatography

i. Reverse phase chromatography

ii. Normal phase chromatography

Based on principle of separation

i. Adsorption chromatography

ii. Ion exchange chromatography

iii. Size exclusion chromatography

iv. Affinity chromatography

v. Chiral phase chromatography

Based on modes of chromatography

i. Reversed Phase Chromatography: [145]

In 1960s, chromatographers started modifying the polar nature of the silanol group by chemically reacting silicon with organic silanes. The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica in now reversed
and it is non-polar or the nature of the phase is reverted, the chromatographic separation carried out with such silica is referred to as reversed-phase chromatography[146].

Reversed phase liquid chromatography (RPLC) is considered as the method of choice for the analysis of pharmaceutical compounds for several reasons, such as its compatibility with aqueous and organic solutions as well as with different detection systems and its high consistency and repeatability. Sensitive and accurate RPLC analysis, whether in the pharmaceutical or bioanalytical field, necessitates the use of stationary phases which give symmetrical and efficient peaks. Therefore, manufacturers of stationary phases are continuously improving and introducing new RPLC products, and the selection of various types of reversed phase stationary phases is high. The needs for consistency as well as the globalization of the pharmaceutical companies require that the methods will be transferred from site to site, using either the same column brands or their equivalents. Therefore, an extensive categorization or characterization of the rich selection of stationary phases has been done in recent years.

The stationary phase in the Reversed Phase chromatographic columns is a hydrophobic support that is consisted mainly of porous particles of silica gel in various shapes (spherical or irregular) at various diameters (1.8, 3, 5, 7, 10 µm etc.) at various pore sizes (such as 60, 100, 120, 300). The surface of these particles is covered with various chemical entities, such as various hydrocarbons (C1, C6, C4, C8, C18, etc). In most methods used currently to separate medicinal materials, C18 columns are used, which sometimes are called ODS (octedecylsilane) or RP-18. A polar solvent is used as mobile phase.

The parameters that govern the retention in Reversed Phase systems are the following:

A. The chemical nature of the stationary phase surface.
B. The type of solvents that compose the mobile phase
C. pH and ionic strength of the mobile phase
A. The chemical nature of the stationary phase: [147]

The chemical nature is determined by the size and chemistry of hydrocarbon bonded on the silica gel surface, its bonding density (units of µmole/m²), and the purity and quality of the silica gel support. As a rule, the more carbons in a bonded hydrocarbon the more it retains organic solutes (as long as similar % coverage is compared). The higher the bonding density the longer the organic solutes are retained. A column is considered relatively hydrophobic if its bonding density exceeds 3 µmole/m².

Very important modifiers of the stationary phase's surface are surface-active substances used as mobile phase's additives, acting as ion-pair reagents. These are substances such as tri-ethylamine or tetrabutylamine or hexyl, heptyl, octyl sulfonate. They are distributed between the mobile phase and the hydrophobic surface and cover it with either positive (alkyl amines) or negative (alkylsulfonates) charges. This change of the surface into charged surface affects the retention significantly, especially on charged species in the sample.

B. Composition of the mobile phase

As a rule, the weakest solvent in Reversed Phase is the most polar one is water and the other polar organic solvents are strong solvents, where the order of solvent strength follows more or less their dielectric properties, or polarity. The less polar the solvent added to the mobile phase, the stronger it gets, shortening the retention times.

C. PH and ionic strength of the mobile phase: [148, 149]

When the samples contain solutes of ionizable functional groups, such as amines, carboxyls, phosphates, phosphonates, sulfates and sulfonates, it is possible to control their ionization degree with the help of buffers in the mobile phase. As a rule, the change of an ionizable molecule to an ion makes it more polar and less available to the stationary phase.
ii. **Normal Phase Chromatography**

In normal phase chromatography, the stationary phase is polar adsorbent. The mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol group at the end in normal phase separations. These OH groups are statistically distributed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase.

This forms a weak bond with many molecules in the vicinity when any of the following interactions are present. Dipole-induced dipole, dipole-dipole, hydrogen bonding, π-complex bonding. These situations arise when the molecule has one or several atoms with lone pair electrons or a double bond. The adsorption strengths and hence ‘K’ value (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatic < organic < halogen compounds < sulphides < ethers < esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample molecule but also on stearic factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as aminopropyl, cyanopropyl and diol phases are the stationary phases alternative to silica gel in normal phase chromatography.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phase and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in the increased homogeneity of the stationary phase surface.

Polar modifiers such as acetic acid or triethylamine (TEA) are added to the mobile phase, to deactivate the more polar adsorption sites on the surface of stationary phase, which in turn will improve peak shape as well as the reproducibility of the retention times.
Based on principle of separation:

i. Adsorption Chromatography

The stationary phase is an adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps.

ii. Ion-Exchange Chromatography

Separation is based on the charge-bearing functional groups, anion exchange for sample negative ion, or cation exchange - for sample positive ion. Gradient elution by pH is common.

iii. Affinity Chromatography

Separation is based on a chemical interaction specific to the target species. The more popular reversed phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvent(s). Affinity chromatography, common for macromolecules, employs a ligand (biologically active molecule bonded covalently to the solid matrix) which interacts with its homologous antigen (analyte) as a reversible complex that can be eluted by changing buffer conditions.

iv. Chiral Chromatography

Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on a chiral stationary phase. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug.

v. Ion-Pair Chromatography: [150,151,152]

Ion Pair Chromatography (IPC) is used to separate ionic analytes on a Column. An Ion Pair reagent is added to modulate retention of the ionic analytes. Ion-pair chromatography is commonly used in combination with UV detection, in which case it is
referred as reverse phase ion-pair chromatography (RPIPC). Ion Pair Chromatography (IPC) is used to separate ionic analytes on a reversed phase column. An Ion Pair reagent is added to modulate retention of the ionic analytes.

1.4c. Components of HPLC:

The individual components HPLC and their working functions are described below.

a. Mobile phase and reservoir

b. Solvent degassing system

c. Pump

d. Injector

e. Column

f. Detector

g. Data system

Fig: 1.01 Block diagram of HPLC
a. **Mobile Phase and Reservoir**

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air.

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations, eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. 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:

It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.

Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.

The mobile phase should have a pH 2.5 and pH 7.0 to maximize the lifetime of the column.
Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible minimizes the absorbance of buffer. Since trifluoroacetic acid, acetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products without chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, which does not absorb above 200 nm.

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.

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

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filters, vacuum degassing with an air-soluble membrane, helium purging ultra signification or purging or combination of these methods. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

c. Pump

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The most important advantages are: higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle pickings that require less pressure. Flow rate stability is another important pump feature that distinguishes pumps. Very stable flow rates are usually not essential for analytical chromatography.

However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable flow rate. An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps
include such state-of-the-art technology as electronic feedback and multiheaded configurations. It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

d. Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, and loss in efficiency or all of these. It is always best to remove particles from the sample by filtering over a 5 μm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. Sample introduction techniques can be used with a syringe or an injection valve.

e. Column

The heart of the system is the column. The choice of common packing material and mobile phases depends on the physical properties of the drug. 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 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. Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 μm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile
phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed. Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment. In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new, and to retain the chromatogram. If questionable results are obtained later, the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

f. Detector

Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low Volume (~10μl) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data. The most commonly used detector in LC is the ultraviolet absorption detector. A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority samples. Other detectors in common use include: Photo Diode Array UV detector (PDA), refractive index (RI), fluorescence (FLU), electrochemical (EC). The RI detector is universal but also the less sensitive one. FLU and EC detectors are quite sensitive (up to 10-15 pmole) but also quite selective.
g. Data System

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimization and deconvolution (i.e. resolution) of overlapping peaks. Finally, software safeguards can be designed to reduce accidental misuse of the system.

1.4d. ANALYTICAL METHOD VALIDATION

Method validation can be defined as (ICH) “Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics”.

Method validation is an integral part of, accuracy and preciseness of its intended purpose and demonstrating that analytical procedures are the method development; it is the process by which a method is tested by the developer or user for reliability suitable for their intended use that they support the identity, quality, purity, and potency of the drug substances and drug products Data thus generated become part of the methods validation package submitted to Center for Drug Evaluation and Research (CDER). Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose[153,154].
Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations, and throughout the life of the drug product. Data that are generated for acceptance, release, stability, or pharmacokinetic will only be trustworthy if the methods used to generate the data are reliable. The process of validation and method design also should be clearly in the development cycle before important data are generated. Validation should be on going in the form of re-validation with method changes.

Though many types of HPLC techniques are available, the most commonly used method, the reversed-phase HPLC with UV detection, is selected to illustrate the parameters for validation. The criteria for the validation of this technique can be extrapolated to other detection methods and chromatographic techniques. For acceptance, release or stability testing, accuracy should be optimized since the need to show deviation from the actual or true value is of the greatest concern.

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 includes namely

a. System suitability
b. Specificity
c. Accuracy
d. Precision
e. Linearity
f. Limit of Detection
g. Limit of Quantitation
h. Ruggedness
i. Robustness
a. System Suitability

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns, analysts) is suitable for the intended application.

Similar to the analytical method development, the system suitability test Strategy should be revised as the analysts develop more experience with the assay. In general, consistency of system performance. (E.g.: Replicate injections of the standard) and chromatographic suitability. (Eg: Tailing factor, column efficiency and resolution of the critical pair) are the main components of system suitability.

During the early stage of the method development process some of the more sophisticated system suitability tests may not be practical due to the lack of experience with the method. In this stage, usually a more "generic" approach is used. For example, evaluation of the tailing factor to check chromatographic suitability, and replicate injections of the system suitability solution to check injection precision may be sufficient for an HPLC impurities assay. As the method matures more experience is acquired for this method, a more sophisticated system suitability test may be necessary.

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors; resolution and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of system suitability "sample" that is a mixture of main components and expected by-products.
Table: 1.2 System suitability parameters and recommendations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity Factor (k’)</td>
<td>The peak should be well-resolved from other peaks and the void volume, generally k’ 1 to 20</td>
</tr>
<tr>
<td>Repeatability</td>
<td>RSD ≤ 1% for N ≥ 5 is desirable.</td>
</tr>
<tr>
<td>Relative retention</td>
<td>Not essential as long as the resolution is stated.</td>
</tr>
<tr>
<td>Resolution (Rₛ)</td>
<td>Rₛ of &gt; 2 between the peak of interest and the closest eluting potential interfering (impurity, excipient, degradation product, internal standard, etc.</td>
</tr>
<tr>
<td>Tailing Factor (T)</td>
<td>T of &gt;0.5 and ≤ 2</td>
</tr>
<tr>
<td>Theoretical Plates (N)</td>
<td>N &gt; 3000</td>
</tr>
</tbody>
</table>

b. Specificity/Selectivity: [155]

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.
Specificity is the ability of a method to discriminate between the analyte(s) of interest and other components that are present in the sample. Studies are designed to evaluate the degree of interference, if any, which can be attributed to other analytes, impurities, degradation products, reagent "blanks" and excipients. This provides the analyst with a degree of certainty that the response observed is due to the single analyte of interest. The degree of specificity testing varies depending on the method type and the stage of validation. Specificity should be evaluated continually through the drug development process. Specificity is sometimes used interchangeably with the term "selectivity". The argument over which term is more correct is one of semantics. Although there is some dissention, the term "specificity" has been adopted by the regulatory guidance documents and should be used to prevent further confusion.

Non-Interference of Placebo

This portion of specificity evaluation applies to the finished drug product only. Excipients present in the formulation should be evaluated and must not interfere with the detection of the analyte. Individual solutions of each excipient prepared at several times the normal concentration of the component in the drug product ensure that any detector response from the excipient will be readily visible. Injecting individual solutions of each excipient into the HPLC system in comparison with a standard solution of the analyte is one means of performing this experiment. The absence of a peak eluting at the retention time of the active ingredient is sufficient to demonstrate specificity for excipients.

Challenge Study

Injecting solutions of known process impurities, degradation products, intermediates, homologues, dimers, etc. further challenges the specificity of a method. Identification of these compounds may require an extensive search in order to identify all possible species that may be present in the sample. For new chemical entities (NCE), this information may not be readily available. Probable suspects should be identified by careful review of the synthetic route and manufacturing process to identify any likely species that may be present in the sample.
Degradation Studies

Degradation studies involve exposing the sample to a variety of stressed conditions to further evaluate the specificity of degradation products. In this study, the drug substance, drug product, and the combined excipients (or placebos) are each exposed to the stressed conditions. These may include, but are not limited to, heat, light, acidic media, alkaline media, and oxidative environments. Other conditions may be used depending on the nature and chemistry of the test subject. Forced degradation is usually evaluated with not more than 20% degradation of the drug substance, although more may be acceptable depending on the particular properties of the drug. A reasonable effort should be made to degrade samples in order to identify possible degradation products. If the planned experiments do not show any appreciable degradation, the strength and/or exposure time of the stress condition may be increased, but degradation is not required for every condition studied. There is a point beyond which the stress condition becomes extreme and unrealistic. Sound scientific judgment should be used to determine the extent and degree of degradation studies.

c. Accuracy

Accuracy is the measure of how close the experimental value is to the true value. Accuracy should be established across the specified range of the analytical procedure.

Assay

Drug Substance

Several methods of determining accuracy are available:

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material);

b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined.

c) Accuracy may be inferred once precision, linearity and specificity have been established.
**Drug Product**

Several methods for determining accuracy are available:

a) Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analyzed have been added.

b) In cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined.

c) Accuracy may be inferred once precision, linearity and specificity have been established.

**Impurities (Quantitation)**

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities. In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

**Recommendations**

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations / 3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.
d. Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. Ruggedness as defined in USP XXII <1225>, 1990 incorporates the concepts described under the terms "intermediate precision", "reproducibility" and "robustness" of this guide.

Repeatability

(i) Injection Repeatability

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be partially controlled by monitoring the specification for injection reproducibility (system suitability testing).

The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested.

The information is provided as part of the validation data and as a system suitability test. The specification, as the coefficient of variation in % or relative standard deviation (RSD), set here will determine the variation limit of the analysis. The tighter the value, the more precise or sensitive to variation one can expect the results. This assumes that the chromatograph does not malfunction after the system suitability testing has been performed. Keep in mind, however, that it does not consider variations due to the drug product manufacturing and laboratory sample preparation procedures. The set of four duplicate samples were injected sequentially. Variations in peak area and drift of retention times are noted.

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 terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set.

The standard deviation $S$, is given by

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

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

The square of standard deviation is called 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.

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

**Recommendations**

As part of methods validation, a minimum of 10 injections with an RSD of 2% is recommended. With the methods for release and stability studies, an RSD of 2% for precision of the system suitability tests for at least five injections ($n=5$) for the active drug either in drug substance or drug product is desirable. For low-level impurities, higher variations may be acceptable.

(ii) **Analysis Repeatability**

Determination, expressed as the RSD, consists of multiple measurements of a sample by the same analyst under the same analytical conditions.

For practical purpose, it is often combined with accuracy and carried out as a single study.

(iii) **Intermediate Precision**

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used
during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

Intermediate precision in the test method can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications.

e. Linearity

The linearity of a method is its ability to obtain test results that are directly proportional to the analyte concentration over a given range. For HPLC methods, the relationship between analyte concentration and detector response (peak area or height) is used to make this determination.

Concentration Ranges

The concentration range used for linearity should be large enough to encompass the desired range of the method. A minimum of five concentration ranges should be investigated and a plot of the detector response vs. the sample concentration should be generated. It is important that the concentration ranges selected for the linearity study are relatively equally spaced throughout the range of the method (e.g., 50%, 75%, 100%, 125% and 150%), and not clustered, as this will provide a skewed estimation of linearity.

Acceptance Criteria

Acceptance criteria should be evaluated to ensure that they are meaningful when compared with the performance of the method. Table: 1.3 gives a list of suggested acceptance criteria for use in evaluating method linearity. The ranges in Table: 1.3 is suggestions only and should be adjusted to ensure that all specification limits are within the validated linear range for any given method. Under most circumstances, regression coefficient (r) is 0.999. Intercept and slope should be indicated.
Statistical Analysis

Linearity data should be evaluated using appropriate statistical methods. A simple regression line of the detector response vs the analyte concentration is the most common means of evaluation. Regulatory agencies require the submission of the correlation coefficient, y-intercept, slope of the regression line, and the residual sum of squares for linearity evaluation. A graphical representation of the linearity data should also be generated. Additional analysis of the deviation of the actual values from the regression line is suggested, especially when the method uses a single-point calibration standard. The percent y-intercept is calculated by dividing the y-intercept by the detector response at the nominal concentration expressed as a percentage. For single-point calibration, this value should be less than 1-2% to ensure accurate results.

<table>
<thead>
<tr>
<th>Test</th>
<th>Level</th>
<th>Range</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td>5</td>
<td>50% to 150%</td>
<td>R &gt; 0.999,</td>
</tr>
<tr>
<td>Dissolution</td>
<td>5-8</td>
<td>10% to 150%</td>
<td>R &gt; 0.99,</td>
</tr>
<tr>
<td>Impurity</td>
<td>5</td>
<td>LOQ to 2%</td>
<td>R &gt; 0.98</td>
</tr>
</tbody>
</table>

f. Limit of Detection

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product.

Limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation.
by detector manufacturer. At low levels, assurance is needed that the detection and quantitation limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detectability, extraneous peak(s) could "disappear/appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

**Based on Visual Evaluation**

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

**Based on Signal-to-Noise**

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

**Based on the Standard Deviation of the Response and the Slope**

The detection limit (DL) may be expressed as:

$$ DL = \frac{3.3 \sigma}{S} $$

Where,  
$\sigma$ = the standard deviation of the response  
$S$ = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways, for example

**Based on the Standard Deviation of the Blank**

Analyzing an appropriate number of blank samples and calculating the standard deviation of these responses perform measurement of the magnitude of analytical background response.

**Based on the Calibration Curve**

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

**Recommendations**

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

**g. Limit of Quantification**

Limit of Quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the Quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.
Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The Quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

\[ QL = \frac{10 \sigma}{S} \]

Where,

\[ \sigma = \text{the standard deviation of the response} \]

\[ S = \text{the slope of the calibration curve} \]

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways.

Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responseses
Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of intercepts of regression lines may be used as the standard deviation.

Recommendations

The quantitation limit and the method used for determining the quantitation limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit. Otherwise the information that is expressed as % area or height of the drug substance peak from the same HPLC chromatogram will be biased. It should also be noted that the extraneous peak using area count does not consider the detection response that depends on the UV extinction coefficient or absorptivity of the compound.

h. Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments, reagents, elapsed assay times, assay temperatures, or days. It is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Recommendations

The ruggedness of an analytical method is determined by analysis of aliquots from homogeneous lots in different laboratories, by different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The degree of reproducibility of test results is then determined as a function of the assay variables. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the method.
i. Robustness

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.

In the case of gas chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.

Recommendations

Data obtained from studies for robustness, though not usually submitted, are recommended to be included as part of method validation.
General Recommendation

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the test method. For dissolution or release profile test methods using an external standard method, k', T and RSD are minimum recommended system suitability tests. For acceptance, release, stability, or impurities/degradation methods using external or internal standards, k', T, R_S and RSD are recommended as minimum system suitability testing parameters. In practice, each method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system. Additional tests may be selected at the discretion of the applicant or the reviewer.

HPLC methods for drug substance and drug product, methods should not be validated as a one-time situation, but methods should be validated and designed by the developer or user to ensure ruggedness or robustness throughout the life of the method. The variations due to the drug product manufacturing process, the laboratory sample preparation procedure and the instrument performance contribute to the accuracy of the data obtained from the analysis. With proper validation and tight chromatographic performance (system suitability) criteria, an improvement in the reliability of the data can be obtained. Variations except from the drug product-manufacturing process will be minimized only with good reliable validated methods can data that are generated for release, stability, and pharmacokinetic is trust-worthy.
References


153. Practical Ion Chromatography an Introduction, Dipl.-Ing. Claudia Eith, Prof. Dr. Maximilian Kolb, Prof. Dr. Andreas Seubert, page.18

154. High Performance Liquid Chromatography (HPLC) in the pharmaceutical analysis by Shulamit Levin, Medtechnica Feb 2010