CHAPTER – I

INTRODUCTION ON ANTI-HIV DRUGS, PHYICO-CHEMICAL METHODS (SPECTROPHOTOPMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY) UESED IN THE ASSAY OF DRUGS

ANTIRETROVIRAL DRUGS, SPECTROPHOTOMERTIC AND RP-HPLC METHODS - GENERAL INTRODUCTION

1.01 INTRODUCTION ON HIV DRUGS

Antiretroviral drugs are the medications used for treatment of infections by retroviruses, primarily human immunodeficiency viruses (HIV) that can lead to acquire immunodeficiency syndrome (AIDS). It could hardly be foreseen that within 28 years of the virus being discovered we would now, at hand have 25 anti-HIV compounds that are categorized [**Table.1.01,P.4-10**] into six groups. These drugs are licensed (thus formally approved) in the market for the treatment of AIDS. The reverse transcriptase associated with HIV is actually the target for three classes of inhibitors: Nucleoside reverse transcriptase inhibitors (NRTIs), Nucleotide reverse transcriptase inhibitors (NtRTIs), and Non-nucleoside reverse transcriptase inhibitors (NNRTIs). The NRTIs and NtRTIs interact with the catalytic site of the enzyme, whereas the NNRTIs interact with an allosteric site located at a short distance from catalytic site [1].

There are at present seven NRTIs that have been formally approved for the treatment of HIV infections: Xidovudine, Didanosine, Xalcitabine, Lamivudine, Abacavir, Stavudine and Emtricitabine **[Table.1.01, P.4-10]**. All the NRTIs could be considered as 2',3'-dideoxynucleoside analogues and act in a similar fashion. NtRTIs should be clearly distinguished from the NRTIs as they are nucleotide analogues, which means that they only need two phosphorylation steps to be converted to their active form. Also, they contain a phosphonate group that cannot be cleaved by esterases, which would make it more difficult to cleave off these compounds, once incorporated at the 3'-terminal end, compared with their regular nucleotide counterpart. At present there are only one NtRTIs

that have been formally approved for the treatment of HIV infections: **Tenofovir [Table1.01, P.4-10]**.

NNRTIs attack the same target enzyme as NRTIs. However, rather than integrating themselves into the transcribed DNA, NNRTIs attach themselves to reverse transcriptase and prevent the enzyme from converting RNA to DNA. Unlike NRTIs, which must be phosphorylated to prevent HIV from infecting the cell, NNRTIs are active in the form administered. The four NNRTIs presently available for the treatment of HIV infections are Eefavirenz, Nevirapine, Delavirdine and Etravirine [1, 2] **[Table1.01, P.4-10]**. During the later stages of the HIV growth cycle, the Gag and Gag-Pol gene products are translated into polyproteins, and these become immature budding particles. Protease is responsible for cleaving these precursor molecules to produce the final structural proteins of the mature virion care. Protease inhibitors (PIs) are active against both HIV-1 and HIV-2; unlike the NRTIs, however, they do not need intracellular activation. The ten PIs currently available for the treatment of HIV infections are Ritonavir, Indinavir, Saquinavir, Nelfinavir, Amprenavir, Lopinavir, Fosamprenavir, Atazanavir, Tipranavir and Darunavir [1- 6].

The process of HIV-1 entry into host cell is complex; each step forms a potential target for inhibition. Viral attachment to the host cell entails binding of the viral envelope glycoprotein complex gp160 to its cellular receptor CD4. At present there are only two entry inhibitors (fusion inhibitors and co-receptor inhibitors) that have been formally approved for the treatment of HIV infections: Enfuvirtide and Maraviroc [1-6]. Althought integrase has been pursued for many years as a potential target for the development of new anti-HIV compounds, the first integrase inhibitor **(**INIs) licensed for clinical use,

raltegravir, has only recently been approved. Raltegravir is a pyrimidinone analog that binds integrase, a viral enzyme essential to the replication of both HIV-1 and HIV-2. It is licensed for use in treatment-experienced adult patients infected with strains of HIV-1 resistant to multiple other agents. The growing demand for these agents stimulate a search for new even more effective drugs, but also calls for higher level of quality control of these therapeutic substances and preparations, so that they are in the highest possible degree free from any impurities that may come from the production process, as well as from decompositions products of active or auxiliary substances. Therefore, it seems appropriate to develop new analytical methods regarding their qualitative and quantitative analysis. For this aim, different analytical methods were used for determining anti-HIV drugs.

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

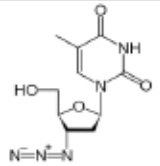
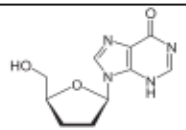
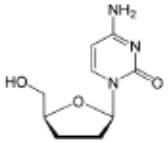
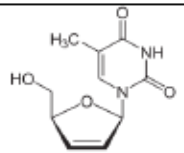
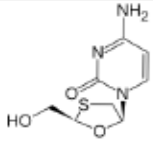
Categories	Generic name	Structure formulae	Brand name	Manufacturer
Nucleoside reverse transcriptase inhibitors (NRTIs)	Zidovudine		Retrovir	GlaxoSmithKline
	Didanosine		Videx (tablet) Videx EC (capsule)	Bristol-Myers Squibb Bristol-Myers Squibb
	Zalcitabine		Hivid	Hoffmann-La Roche
	Stavudine		Zerit	Bristol-Myers Squibb
	Lamivudine		Epivir	GlaxoSmithKline

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

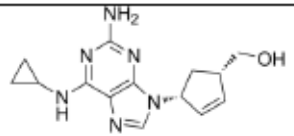
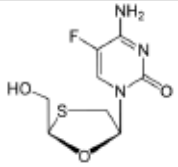
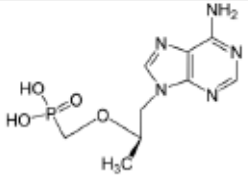
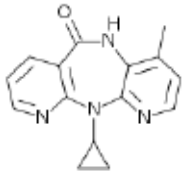
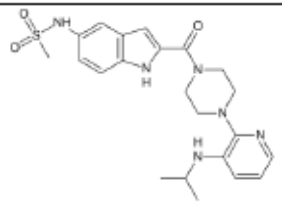
	Abacavir		Ziagen	GlaxoSmithKline
	Emtricitabine		Emtriva	Gilead Sciences
Nucleotide reverse transcriptase inhibitors (NtRTIs)	Tenofovir		Viread	Gilead Sciences
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Nevirapine		Viramune	Boehringer Ingelheim
	Delavirdine		Rescriptor	Pfizer

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

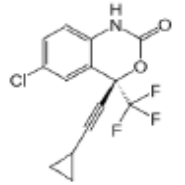
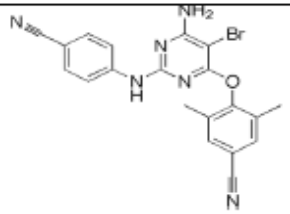
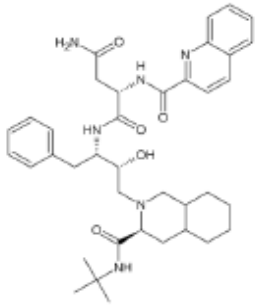
	Efavirenz		Sustiva Stocrin	Bristol-Myers Squibb Merck
	Etravirine		Intelence	Tibotec Therapeutics
	Saquinavir		Invirase (hard gel capsule) Fortovase (soft gel capsule)	Hoffmam-La Roche Hoffmann-La Roche

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

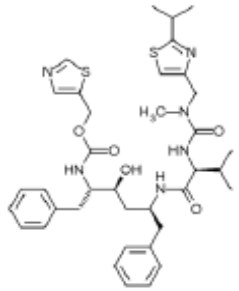
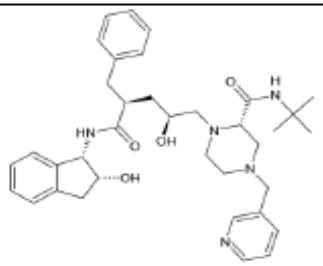
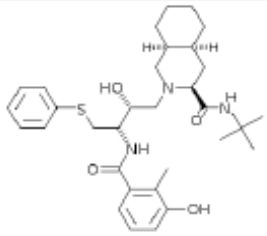
Protease Inhibitors (PIs)	Ritonavir		Norvir	Abbott Laboratories
	Indinavir		Crixivan	Merck
	Nelfinavir		Viracept	Agouron Pharmaceuticals

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

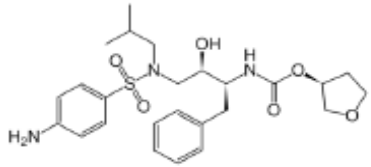
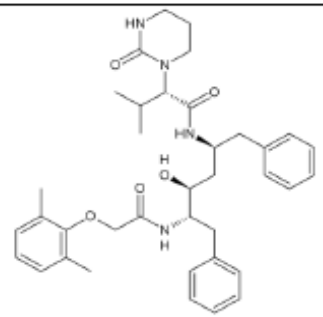
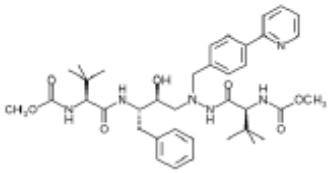
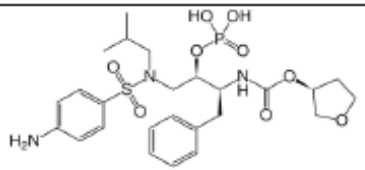
	Amprenavir		Agenerase	GlaxoSmithKline
	Lopinavir (Tritonavir)		Kaletra	Abbott Laboratories
	Atazanavir		Reyetaz	Bristol-Myers Squibb
	Fosamprenavir		Lexiva Telzir	GlaxoSmithKline GlaxoSmithKline

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

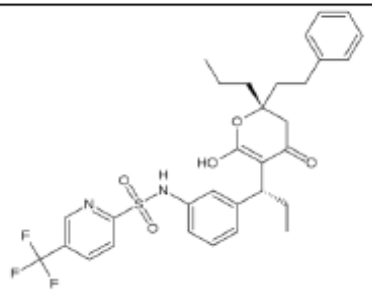
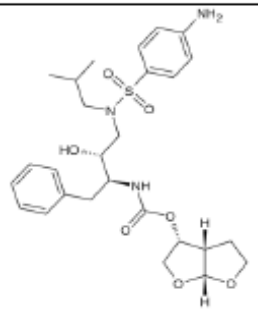
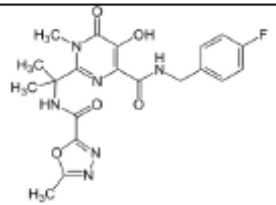
	Tipranavir		Aptivus	Boehringer Ingelheim
	Darunavir		Prezista	Tibotec, Inc.
Integrase inhibitors (INIs)	Raltegravir		Isentress	Merck&Co., Inc.

Table.1.01.
Approved anti- HIV Drugs and their structural formulaes

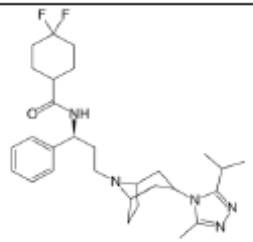
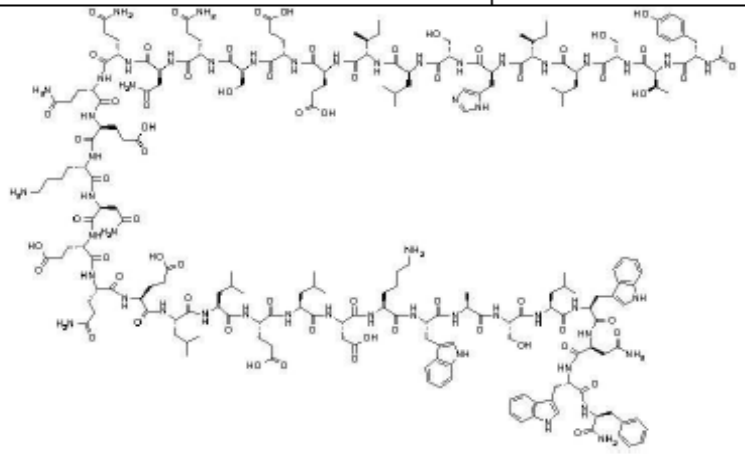
Cell entry inhibitors and co-reseptor inhibitors	Maraviroc		Celsentri Selzentry	Pfizer Pfizer	
	Enfuvirtide			Fuzeon	Hoffmann-La Roche&Trineris

TABLE. 1.02

LIST OF PROPOSED VISIBLE SPECTROPHOTOMETRIC METHODS

Type of Reaction	Reagent	Method	Drug responded	References
Oxidative Coupling	MBTH-IBDA	M_{1a}	Stavudine	Chapter II
			Lamivudine	Chapter III
			Efavirenz	Chapter V
	MBTH-Ce(IV)	M_{1b}	Stavudine	Chapter II
			Efavirenz	Chapter V
	MBTH-$NaIO_4$	M_{1c}	Stavudine	Chapter II
			Zidovudine	Chapter IV
			Efavirenz	Chapter V
Oxidative Coupling	IO_4^- /PHH/$[Fe(CN)_6]^{-3}$	M_2	Stavudine	Chapter II
Oxidative Coupling	Brucine-IO_4^-	M_3	Stavudine	Chapter II
			Lamivudine	Chapter III
			Zidovudine	Chapter IV
Redox Reaction	Fe(III) – o-Phen	M_4	Stavudine	Chapter II
Redox Reaction	AV-H_2SO_4	M_5	Efavirenz	Chapter V
Redox Reaction	Fe(III)/$[Fe(CN)_6]^{-3}$	M_6	Efavirenz	Chapter V

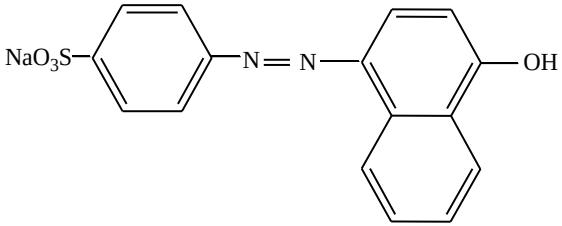
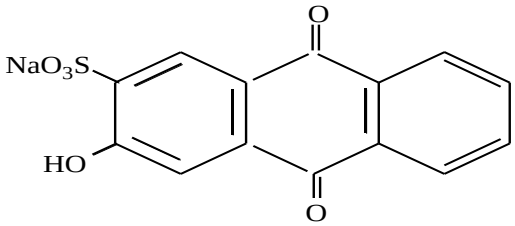
TABLE. 1.02

LIST OF PROPOSED VISIBLE SPECTROPHOTOMETRIC METHODS

Type of Reaction	Reagent	Method	Drug responded	References
Condensation reaction	Isatin-H_2SO_4	M_7	Zidovudine	Chapter IV
Condensation reaction	Vanillin-H_2SO_4	M_8	Zidovudine	Chapter IV
Nucleophilic Substitution Reaction	NQS	M_9	Stavudine	Chapter II
			Efavirenz	Chapter V
Charge transfer reaction	PCA	M_{10}	Stavudine	Chapter II
			Lamivudine	Chapter III
			Zidovudine	Chapter II
Ion Association Complex formation	TPooo	M_{11a}	Stavudine	Chapter IV
			Lamivudine	Chapter III
			Zidovudine	Chapter IV
			Efavirenz	Chapter V
Ion Association Complex formation	ARS	M_{11b}	Stavudine	Chapter II
			Lamivudine	Chapter III
			Zidovudine	Chapter IV
			Efavirenz	Chapter V
Diazo Coupling	HNO_2 - PGNL	M_{12a}	Lamivudine	Chapter III

TABLE.1.03

CHEMICAL FEATURES OF DYES USED IN ION ASSOCIATION COMPLEX FORMATION

S.No	Dye name / CI No.	Chemical category	Structure	Chemical name
1	Tropaeoline ooo (Tpooo) / 14600	Azodye		*Benzenesulphonic acid,4[(4-hydroxy-1-naphthalenyl) azo]-, mono sodium salt*
2	Alizarine Red S (ARS) / 58005	Anthraquinone dye		*2-Anthrcene sulphonic acid - 9,10-dihydro-3,4- dihydroxy -9,10-dioxo, mono sodium salt*

1.02: PART –A: SPECTROPHOTOMETRY

The methods for the estimation of anti-HIV drugs are classified into physical, chemical, physico-chemical and biological ones. Physical methods involve the study of the physical properties such as solubility, transparency or degree of turbidity, color density, specific gravity etc. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation, redox reactions etc. Titration in non-aqueous media and complexometry are also being used in pharmaceutical analysis. Physico-chemical methods involve the study of the physical phenomena that occurs as a result of chemical reactions [7-9]. These include spectrophotometric and chromatographic methods.

In the past few decades, a number of elegant instrumental techniques such as spectrophtometry, 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 [10].

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.

I) Criteria for Satisfactory Spectrophotometric Analysis

In order to have successful and satisfactory result, the process of analysis needs careful operations. Since the color development in spectrophotometry involves diverse type of reactions, a number of points need to be ensured before applying the method for a particular application. Some of the points have to be considered are presented here

Choice of solvent

The first point is the selection of the solvent which is to be used in colorimetric or spectrophotometric determinations. It must be a good solvent for the substance under determination. Before using a particular solvent, it must be ensured that it does not interact with the solute. The solvent must not show significant absorption at the wavelength to be employed in the determination. For inorganic compounds, water normally meets these requirements, but for majority of organic compounds, it is necessary to use an organic solvent. All solvents show absorption at some point in the ultraviolet region and care must be taken to choose a solvent for a particular determination which does not absorb in the requisite wavelength region. Any impurities present in the solvents may affect the absorption at certain wavelength and it is therefore, essential to employ materials of the highest purity.

Choice of wavelength

The second point is the selection of analytical wavelength at which measurements are to be carried out. In order to enhance the sensitivity of the method and signal to noise ratio, the wavelength of maximum absorbance is chosen as analytical wavelength. After setting the analytical wavelength, the color developing reagent and the absorbing product must be stable for a considerable period of time.

Calibration curve

The third point is the construction of a calibration curve for the constituents being determined. Calibration is one of the most important steps in drug analysis. For this purpose, suitable quantities of the constituents are taken and treated in exactly the same way as the sample solution for the development of color, followed by the measurement of the absorption at the optimum wavelength. The absorbance is then plotted against concentration of the constituents. A

straight line is obtained if Beer's law is followed. This calibration curve may then be used to determine the constituents under the same conditions. The calibration curves need checking at intervals.

II) Reactions proposed in the present investigation

The nature of organic drugs towards reactivity depends on the presence of functional groups in their molecules [11-13]. 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.1.02, P.11, 12**). having advantages of one or more of the above desirable features.

i) Oxidative coupling reactions (Methods M_{1a}, M_{1b}, M_{1c}, M_2 and M_3)

Oxidative coupling procedures involving the use of either **3-methyl-2-benzothiazolinone hydrazone [MBTH]** with various oxidants **(M_{1a}, M_{1b}** and **M_{1c}), Phenyl hydrazine hydrochloride (PHH) (M_2)** and **Brucine (M_3)** in the presence of an appropriate oxidant under slightly acidic, neutral or slightly alkaline conditions to form highly colored species were explored

for the assay of drugs possessing functional groups such as phenolic hydroxyl, aldehyde, amine or diol in general.

a) MBTH - Oxidant (Method – M_{1a}, M_{1b} and M_{1c}):

3-Methyl-2-benzothiazolinone hydrazone Hydrochloride (MBTH) was synthesized by Besthorn[14]. The first procedure described by Sawicki[15] allowed the determination of aldehydes, with which MBTH condenses to give a blue cation. This technique was later improved, allowing more sensitive determinations[16]. The reaction was applied to the analysis of aliphatic aldehydes and the detection of the aldehyde groups in tissue and collagen. 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 oxidized 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, when oxidant is present. Phenol was so determined using the oxidant cerium (IV) ammonium sulphate. This reaction was extended to miscellaneous other phenols, using various oxidants. MBTH can be used for the determination of polyhydroxy compounds, aromatic amines [18], aliphatic and alicyclic amines. Azodyes, stilbenes and Schiff bases as well as pyrrole derivatives also react with MBTH under oxidative conditions. This reaction was extended to the determination of bilirubin and its oxidation products such as urobilin and biliverdin. Ferric chloride has been mostly used as an oxidant for the determination of aromatic and heterocyclic amines 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 [19](after oxidation with ruthenium tetraoxide) and phenidone. E.I. Kommas [20] suggested ceric ammonium sulphate as an oxidant under acidic conditions for the determination of pharmaceuticals possessing phenol group. Sastry [21] 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 [22-28].

The author had attempted to develop new visible spectrophotometic methods for the selected drugs [**Stavudine (STV), Lamivudine(LMV), Zidovudine(ZDV) and Efavirenz(EFZ)],** which possesses secondary amino group, involve the oxidative coupling reaction with **MBTH** in the presence of various oxidants which include **IBDA[Method M_{1a}], Ce(IV) [Method M_{1b}]** and **$NaIO_4$[Method M_{1c}]** forming colored products. The probable sequence of reactions and the developed procedure for their assay are presented in corresponding **chapters II,III,IV** & **V** of the corresponding drugs.

b) $NaIO_4$ / (PHH)/$[Fe(CN)_6]^{-3}$(Method M_2)

Periodic acid oxidation [29] 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 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. Certain compounds, which show no substantial reaction at room temperature, can be oxidized at elevated temperature.

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 (IO_4^-) is considerably soluble in water (12.62g/100mL, 25^oC). The solubility of sodium metaperiodate is greatly reduced in alkaline solution because of the formation of disodium metaperiodate ($Na_2H_3IO_6$). This effect occurs at pH>5.0.Aqueous solution of sodium metaperiodate at pH 4.0 or below is the most suitable one as the oxidant. The oxidation reaction with periodate are quantitative. Certain analytical procedures have been developed for the determination of aldehydes utilizing periodate oxidation. Different types of reagents are 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), appear to yield highly sensitive and stable chromogen with formaldehyde especially. This method permits the determination of the liberated formaldehyde directly in the reaction medium colorimetrically by oxidative coupling reaction with schryver reaction [30] with PHH and hexacyanoferrate (III) and this method has been applied for the determination of doxorubicin [31].

In the present investigation, **Stavudine (STV)** responded to oxidative coupling reaction with PHH in the presence of hexacyanoferrate (III) giving formazan dye. The details of the investigation of the corresponding drug (**STV**) are incorporated in **chapter II.**

c) Brucine – Periodate (Method – M_3):

Brucine (2,3 – dimethoxystrychnine)[32] under acidic conditions has been reported to be an effective reagent for spectrophotometric determination of nitrates ,nitrites, cerium, manganese, cadmium and platinum. It was also reported subsequently that in combination with potassium persulphate, it is used for the spectrophotometric determination of halides and cysteine and as an indicator in redox titration. Brucine forms a 1:1 colored complex with p–dimethylamino cinnamaldehyde under acidic conditions.

Sodium metaperiodate is an effective oxidant for converting methyl substituted p-dihydroxy phenols to o-quinones and is also color stabilizer. Sastry et al used brucine-periodate reagent for spectrophotometric determination of tryptophan and some sulphur compounds, tetracyclines, chlorophenicol and streptomycin [33]. 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, the author has developed a specific method for the assay of the selected drugs **{Stavudine(STV),Lamivudine(LMV)** and **Zidovudine(ZDV)(Method M_3)** in bulk samples and dosage forms. The details of the spectrophotometric investigations of the corresponding drugs are incorporated in **chapter II, III** and **IV** respectively.

ii) Redox reactions (Methods M_4, M_5 & M_6)

a)Fe (III)/o-Phenanthroline (Method- M_4)

Ferric salt converts into a ferrous salt upon oxidation of a compound and can be easily detected by the usual reagent for divalent iron, potassium ferricyanide[34], o-phenanthroline ,

bipyridyl or triazine [35]. o-Phen forms a complex with Fe(II) which in turn functions as a better oxidant than Fe(III) itself. The reduction product is tris complex of Fe (II), well known as ferroin. Based on its complexing tendency and oxidizing properties, ferric salt was suggested in the estimation of several drugs.

In the present investigations, (**Method M_4**), the selected drug (**Stavudine**) is treated with excess of Fe(III) salt under specified experimental conditions. Acting as oxidant Fe(III) undergoes reduction to Fe(II) in oxidizing (**Stavudine**) which corresponds to the drug concentration. Fe(II) was estimated by the usual reagent for divalent iron, o-Phenanthroline. The details of the investigations, scheme of reactions are compiled in **chapter II**.

b)Ammonium Vanadate (AV) - H_2SO_4 (Method – M_5)

The orthovanadate ion $[VO_4]^{-3}$ occurs only at very high pH. It is such a strong base that the first step in its protonation, forming $[HVO_4]^{-2}$, is already complete at pH 12. When the pH is gradually lowered to one successive protonation takes place that ultimately leads to the formation of the pale yellow cationic species, usually formulated as VO_2^{-1}. Due to the great tendency of vanadate to oligomerize, the protonated monomers $[HVO_4]^{-2}$, $[H_2VO_4]^{-1}$, and VO^{+}_2 (except at very low pH) are predominant only in highly diluted solution. The chemistry of vanadium is complicated. It forms compounds corresponding to oxidation numbers +2 to +5. The most stable and commonly encountered compounds of Molybdenum are derived from its oxide $[VO_4]^{-3}$ .The vanadium compounds corresponding to the oxidation states ranging from +2 to +5 are mostly complexes species. The isopolyanionic or hetero polyanionic species of vanadium undergo reduction to coloured vanadium species with certain bioactive compounds. The λ_{max} values of reduction products vary from 600nm – 850 nm 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). “Vanadium greenish

Blue" is the result of mild reduction of an acidified solution, which contains V(VI), either as an iso-or a hetero polymolybdate anion (or even alkaline conditions). In the present investigation, the author has developed colored product of maximum intensity with the selected drug **Efavirenz(EFZ)** under specified experimental conditions, when treated with **Ammonium Vanadate (AV) (Method M_5)**. The details of the investigation have been compiled in corresponding chapter of the responded drug **chapter V**.

c)Fe (III)- $[Fe(CN)_6]^{-3}$ (Method - M_6)

Iron (Fe) 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 [34], bipyridyl or triazine [35] hexacyano ferrate (III)$[Fe(CN)_6]^{-3}$ 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 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-phenantheroline, bipyridyl, triazine or hexacyanoferrate (III) for the estimation of certain bioactive compounds bearing reducing properties [36-41]. In the present investigation, the author has been applied the above method (**Method M_6**) for the assay of **Efavirenz(EFZ)** and the results are presented in **chapter V.**

iii) Condensation reactions

a) Isatin - Sulphuric acid (Method – M_7)

Isatin (1H – indole – 2,3 – dione) was first obtained by Erdman and Laurent in 1841 as a product from the oxidation of indigo by nitric and chromic acids. The synthetic versatility of isatin has led to the extensive use of this compound in organic synthesis. Three reviews have been published regarding the chemistry of this compound: the first by Sumpter, in 1954 and second by

Popp in 1975, and the third on the utility of isatin as a precursor for the synthesis of other heterocyclic compounds[42]. The synthetic versatility of isatin has stemmed from the interest in the biological and pharmacological properties of its derivatives.

Isatin is known to be a color reagent for the aminoacid proline, forming a blue derivative[43]. This property has been exploited for the determination of the level of this aminoacid in pollens[44-46] and other vegetal materials[47] using paper chromatography, or for the detection of polymer bound compounds possessing proline residues[48]. It has also been used in a colorimetric screening test for human serum hyperprolinemia, in a colorimetric assay of HIV-1 proteinase and for the estimation of the age of bones in crime investigations. As isatin produces a fluorogenic derivative when reacted with tryptophan, it has been used for the detection of this amino acid by thin layer chromatography[49]. It is also useful for the detection of 3,4-dehydroproline, which is oxidized by isatin and further reacted with *p*-dimethylaminobenzaldehyde to give a colored derivative[50]. In a similar manner, isatin-3-hydrazone has been studied for the colorimetric determination of steroids[51], including deoxycorticosterone. A further application of isatin in steroid analysis is its use as a colored marker in the Sephadex LH-20 chromatographic separation of steroidal blood components[52]. 1-Chloromethylisatin has been used as a derivatizing agent for alcohols , small chain and fatty carboxylic acids including indole and compounds containing acidic C-H bonds for their analysis by RP-HPLC or TLC. Isatin has been used in the determination of the enzymatic activity of ketopantoyl-lactone reductase and other fungal carbonyl reductases[53,54], as it is a substrate of these enzymes that is reduced to a dioxindole in a reaction that can be monitored by colorimetry. Ketopantoyl-lactone reductase, also named as isatin hydrolase, can be used to remove unwanted isatin from the broth of the microbial production of indigo[55,56].

In the present investigation, a visible spectrophotometric method has been developed for **Zidovudine(ZDV)**, which possesses primary amine moiety using isatin and sulphuric acid in acetic acid medium. The details of these investigations are presented in **chapter IV**.

b) Vanillin (Method – M_8)

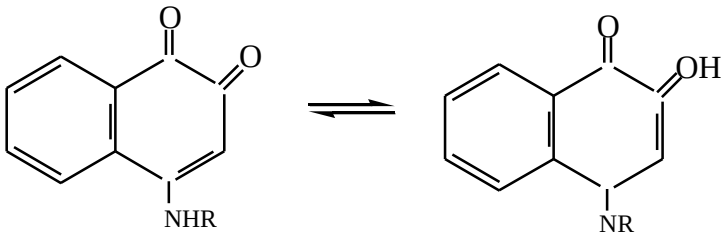
It is well known that aromatic aldehydes form colored condensation product (schiff base) with aromatic primary amines in particular. It has been observed by suitable alteration of experimental conditions, others such as hydrazine and its mono substituted derivatives, primary alkyl amines converted to pyrrole derivatives[57-59], nitro aromatic derivatives reduced before hand to amines[60,61], primary heterocyclic amines and m-diphenol[62] also develop color with aromatic aldehydes. These observations have led to application of aromatic aldehyde, Vanillin (p-hydroxy-m-methoxy benzaldehyde) as analytical reagent in the analysis of pharmaceutical dosage forms. In the present investigation the selected drug **Zidovudine(ZDV)**, it was observed that **Vanillin (BH)** under certain established experimental conditions produce color of maximum intensity in methanol and the results of these investigations are presented in **chapter IV** of the appropriate drug.

iv) Nucleophilic substitution reaction

a)Reaction with NQS (Method M_9)

The reaction of 1, 2-Napthaquinone-4-Sulphonic acid (NQS) with primary aromatic amine was reported by Boniger. Replacement of the sulphonate group of the naphthoquinone sulphonic acid by an amino group gives N-alkylamino naphthoquinone[63]. This reaction has been applied to the characterization of primary aromatic amines and later formed the basis for colorimetric determinations of amino acids , sulphonamides , primary or secondary aliphatic and aromatic amines. In the case of primary amine,the adduct can be represented by either the amino quinone

structure(I), the quinone imine structure(II) or an equilibrium mixture of the two obviously, only the quinone structure can be taken into account with a secondary amine.

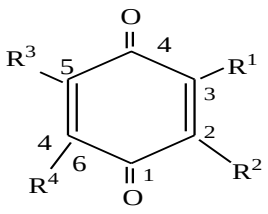


Aminoquinone Quinoneimine

It may be concluded that, under the conventional analytical conditions, equilibrium between two forms (I and II) may intervene for the derivatives obtained from primary amines, but the quinone structure is mostly favored. The selected drugs **Stavudine(STV**) and **Efavirenz(EFZ)**were estimated with this reagent and the details are presented in **chapters II** & **V**.

v) Charge transfer reactions with quinones (Method – M_{10})

The charge transfer complex forming reactions are based on that **"π acceptors react with the basic nitrogenous compounds as n-donors to form charge transfer complexes or radical anions according to the polarity of the solvent used"** and these reaction has been widely studied recently. The basic mechanism involves the molecular interactions between electron donors and electron acceptors which are generally associated with the formation of intensely colored charge-transfer complexes, which absorb radiation in the visible region.



(DHQ) Chloranilic acid: $R^1 = R^4 = Cl$, $R^2 = R^3 = OH$

Many drugs are easy to determine by spectrophotometry based on colored charge transfer (CT) complexes formed with electron acceptors. A variety of electron donating compounds[π-acceptors] have been reported as analytical reagents to yield charge-transfer complexes leading to numerous applications in the development of simple and convenient spectrophotometric methods for the determination of many drugs in pharmaceutical formulations [64-78].

In the present investigations **p-CA** used as selective reagent in the proposed methods for the determination of the cited drugs. The details of the investigation have been incorporated in **chapter II, III** and **IV** respectively.

vi) Ion association complex formation (Method M_{11a} and **M_{11b})**

The Ion association complex extraction has been applied to the estimation of numerous compounds; possessing basic moieties (secondary or tertiary amino groups) by using an acid dye as a reagent and a chlorinated solvent as an extractant. The structure of the species formed may depend upon the experimental conditions (Concentration of the components, pH of the aqueous phase). The selectivity of the reaction may increase by using appropriate organic solvent as an extractant, which then depends upon parameters such as the polarities of the amine and of the dye.

Several acidic dyes belonging to different chemical classes have been used for the assay of basic drugs [79-81]. According to the same principle basic dyes [82] can be used for the assay of acidic drugs. Many pharmaceutical compounds have been determined by the formation of an ion-pair complex [83-85]. These methods involve the formation of colored ion – associate complex between drug and reagent and the colored product was extracted with pure chloroform.

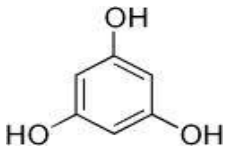
TPooo and ARS have been used as active reagents for the determination of different drugs [86-89]. Preliminary investigations were carried out by the author using **two acidic dyes [TPooo** and **ARS]** by extraction spectrophotometric technique for the assay of selected drugs. The chemical features of the dyes used in the ion association complex formation are given in (**Table.1.03, P.10,11**).

The selected drugs [**Stavudine(STV), Lamivudine(LMV), Zidovudine(ZDV)** and **Efavirenz(EFZ)**] has responded with two acidic dyes (**TPooo** and **ARS**), forming ion association complexes which are extractable into chloroform from the aqueous phase. The λ_{max} and $\in_{max}$ values obtained with the above two acidic dyes with the responded drugs are compiled in (**Table.1.02, P.6-9**).

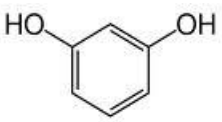
In the present investigations, the author has successfully developed the procedures for the four selected drugs [**Stavudine (STD), Lamivudine (LMV), Zidovudine (ZDV)** and **Efavirenz(EFZ)**] with [**acidic dyes TPooo** and **ARS**] (and the details of these investigations are incorporated in **chapters II, III, IV & V** of the individual drugs.

vii) Diazo coupling reaction (Method – M_{12a} & M_{12b})

The diazo coupling reaction is defined "**as a proton eliminating condensation of diazonium salt with another compound possessing an active hydrogen atom"**. The coupling of a diazonium salt formed from aromatic amine takes place in mild acid, weak alkali and strong alkali conditions respectively. Diazocoupling and formation of diazonium salts [**Nitrosation reaction in which nitrous acid (formed insitu from sodium nitrite and hydrochloric acid) reacts with primary amines (aromatic) to diazonium salt]** have opened the way to a great number of colorimetric determinations.



Phloroglucinol



Resorcinol

The formation of the diazo coupling reaction product from diazonium salt from aromatic amine and compound having active hydrogen atom [Phloroglucinol or Resorcinol] is the basis for the determination of several drugs [90-91] in bulk and pharmaceutical formulations.

In the present investigation the same diazocoupling reactions has been used by the author for the determination of the selected drug **Lamivudine[LMV][Method M_{12a}** for Phloroglucinol & **M_{12b}** for Resorcinol] and the details of these investigations have been incorporated in **chapter III** of the corresponding drug.

III) Validation of the developed methods

Validation methodology aims to demonstrate that a method of analysis corresponds to the use for which it has been elaborated and therefore, its characteristic features, established by laboratory tests, meet the demands so that the method can be applied. The main parameters for validation are **specificity/selectivity, linearity, precision**, **accuracy, sensibility and robustness.**

i)Selectivity

Selectivity is defined as, "the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample The definition of selectivity is quite similar to the definition of specificity: "the ability to assess unequivocally the analyte in the presence of components which might be expected to be present .

ii)Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A calibration curve is the relationship between instrument response and known concentrations of analyte.

iii)Precision

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term 'set' is defined as 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.

Percent of relative standard deviation (%R.S.D) = (SD/Mean) x 100

iv)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.

a) Absolute method: Taking amounts of the constituents and proceeding according to specified instructions carries out the test for accuracy of the method. The difference between the means of an adequate number of results and amount of constituent actually present, usually expressed as parts per hundred (%) is termed as % error. The constituent in question will be determined in the presence of other substances, and it will therefore be necessary to know the effect of these upon the determination. This will require testing the influence of a large number of probable compounds in the chosen samples, each varying amounts. In a few instances, the accuracy of the method controlled by separations (usually solvent extraction or chromatography technique) involved.

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

To evaluate the accuracy of the method, one often compares the method being investigated of 'test method' with an existing method called the 'reference method. **Student t-test** is used to

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

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

has a student t-distribution with (n -1) degrees of freedom, where di = X_R (Reference method) – x_T. (Test method) and s_d is the standard deviation.

F-test is useful to test the significance of the proposed method by evaluating the difference between 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

$$\mathbf{F = S_T^2 / S_R^2}$$

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 literature table, one can conclude that the procedures are not significantly different in precision at given confidence level.

v) Sensitivity

Sensitivity is often described in terms of the molar absorptivity (ε, L $mol^{-1}cm^{-1}$). The objective numerical expression of the sensitivity of spectrophotometric methods is the molar absorptivity (ε) at the wavelength (λ max)of maximum absorbance of the colored species, which is given by the equation **(ε) = A / c l.** Sandell's sensitivity [92] is the concentration of the analyte (µg mL^{-1}) which will give an absorbance of 0.001 in a cell of path length 1.0cm and is expressed as µg cm^{-2}.

vi) Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection is the smallest concentration of a solution of an element that can be detected with 95 % certainty. This is the quantity of the element that gives a reading equal to twice the standard deviation of a series of ten determinations taken with solutions of concentrations which are close to the level of the blank. Several approaches for determining the detection limit are possible, depending on whether the procedure is an instrumental or non-instrumental. The limit of quantitation (LOQ) is determined by the analysis of sample of known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. The LOQ was calculated as follows: 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

$$\mathbf{LOD = 3S_a / b}$$

$$\mathbf{LOQ=10S_a / b}$$

vii) Ringbom plot

Ringbom plot [93] gives relative error coefficient (i.e. plot of log C α Transmittance) the main limitations of Ringbom plot is that it provides no information concerning the concentration range of good precision unless it is combined with ΔT versus T relation. The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increases at the extremes of the transmittance scale.

vii) Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". The variable parameters in spectrophtometric technique may involve volume of reagents, order of addition of reagents, temperature, pH composition and stability.

1.02: PART-B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

I) General introduction

Chromatography (Chroma means 'color' and graphein means to 'write') is a technique used to separate and analyze complex mixtures. The components to be separated are distributed between two phases: a stationary phase and a mobile phase, which percolates through the stationary bed. The phases are chosen such that components of the sample have differing solubilities in each phase. A component, which is quite soluble in the stationary phase, will take longer time to travel through it than a component, which is not highly soluble in the stationary phase but soluble in the mobile phase. As a result of these differences in mobilities, sample components separate from each other as they travel through the stationary phase. The separated

molecules leave the column and get detected by one or more on-line electrical devices with signals proportional to the concentration of the analytes [94-98].

Liquid Chromatography] is an analytical Chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase to differing degrees due to differences in adsorption, ion exchange, partitioning or size. These differences will allow the mixture components to be separated from each other by using these differences to determining the transit time of the solutes through a column.

During 1970's, most chemical separations were carried out using a variety of techniques including open-Column Chromatography, Paper Chromatography and Thin Layer Chromatography. However, these Chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time pressure Liquid Chromatography began to be used to decreased flow through time, thus reducing separation time of compounds being isolated by Column Chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure debated. High-pressure Liquid Chromatography quickly improved with the development of column packing materials. Additional convenience of on-line detectors became rapidly a powerful separation technique and is today called as High Performance Liquid Chromatography (HPLC). The HPLC is the method of choice in the field of analytical chemistry and it has both advantages and disadvantages.

Advantages

- HPLC separations can be accomplished in a matter of minutes, in some cases even in seconds.
- High resolution of complex sample mixture into individual components.

- Rapid growth of HPLC is also because of its ability to analyse substances that are unsuitable for Gas Liquid Chromatographic (GLC) analysis due to non-volatility or thermal-instability.
- Depending on sample type and detector used, it is frequently possible to measure 10^{-9} g or 1ng of sample. With special detectors, analysis down to 10^{-12} pg has been reported.

Disadvantages

- HPLC instrumentation is expensive and represents a major investment for many laboratories.
- It requires a proficient operator to handle the instrument.
- HPLC cannot handle gas samples.
- HPLC is poor identifier. It provides superior resolution but it does not provide the information that identifies each peak.

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, ion exchange and size exclusion chromatography.

In the normal phase mode, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

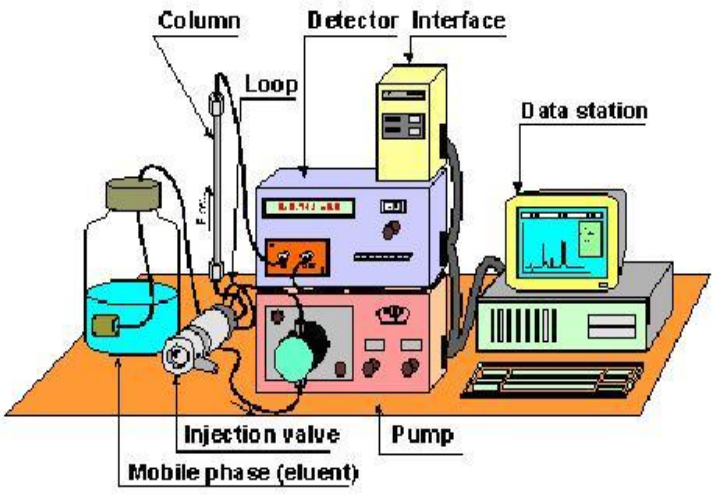
Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile

phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C_{18}, $C_{8,}$ C_4, etc., (in the order of increasing polarity of the stationary phase).

In ion exchange chromatography, the stationary phase contains ionic groups like NR_3^+ or SO_3^- , which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Size exclusion chromatography separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

II) Instrumentation of HPLC



Schematic diagram of HPLC equipment

The Basic HPLC Instrumentation of HPLC involves the following **Components [**97,98]

a) Solvent delivery system: A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation eluting power increases with increasing polarity of the solvent but for reversed phase separation, eluting power decreases with increasing polarity. A degasser is needed to remove dissolved air and other gases from the solvent. Special grades of solvents are available for HPLC and these have been purified carefully in order to remove absorbing impurities and particulate matter to prevent these particles from damaging the pumping or injection system or clogging the column.

b) Pumps

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system.

1. Displacement pump: It produces a flow that tends to independent of viscosity and back pressure and also output is pulse free. But it possesses limited capacity (250 mL).

2. Reciprocating pump: It has small internal volume (35 to 400 µl), their high output pressure (upto 10,000 psi) and their constant flow rates. But it produces a pulsed flow.

3. Pneumatic or constant pressure pump: They are pulse free; suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

(c) Sample injection system: Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

- Loop injection: In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.
- Valve injection: In which, a variable volume is introduced by making use of an injection valve.
- On column injection: In which, a variable volume is introduced by means of a syringe through a septum.

d) Chromatographic column: The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10mm inside diameter containing stationary phase at particle diameter of 25 µm or less. Columns with an internal diameter of 5mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing: The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

1. Porous, polymeric beds: Porous, polymeric beds based on styrene divinyl benzene co-polymers used doe ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

2. Porous layer beds: Consisting of a thin shell (1-3 µm) of silica or modified silica on a spherical inert core (e.g. Glass). After the development of totally porous micro particulate packings, these have not been used in HPLC.

3. Totally Porous silica particles (dia. <10 µm): These packing have widely been used for analytical HPLC in recent years. Particles of diameter >20 µm are usually dry packed. While particles of diameter <20 µm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

(e) Detectors: The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. Detectors are usually of two types:

1. Bulk property detectors: It compares overall changes in a physical property of the mobile phase with and without an eluting solute. e.g. refractive index, dielectric constant or density.

2. Solute property detectors: It responds to a physical property of the solute which is not exhibited by the pure mobile phase. e.g. UV absorbance, fluorescence or diffusion current. Such detectors are about 1000 times more sensitive giving a detectable signal for a few nanograms of sample. The following types of solute property detectors can be used in HPLC analysis

(a)Refractive index detectors: They measure the ability of sample molecules to bend or refract light. This property is called refractive index. Detection occurs when light is bent due to samples eluting from the column and this is read as a disparity between the two channels.

(b) Ultraviolet (UV) detectors: They measure the ability of samples to absorb light. This can be established at one or several wavelengths.

i) **Fixed wavelength**: Measures one wavelength, usually 254nm.

ii) **Variable wavelength**: Measures one wavelength at a time, but can detect over a wide range of wavelengths.

iii) **Diode array**: Measures a spectrum of wavelengths simultaneously.

(c)Fluorescent detectors: They measure the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence.

(d)Radiochemical detectors: Involves use of radio labeled material usually tritium (^{3}H) or carbon 14 (C^{14}). It operates by detection of fluorescence along with beta - particle ionization.

(e)Electrochemical detectors: Used in analysis of compounds that undergoes oxidation or reduction reactions. They measure the difference in electrical potential when the sample passes between the electrodes.

III) HPLC method development [99]

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-Pharmacopoeial) products are also developed to reduce the cost and time for better precision and accuracy. Trial runs are conducted, method is optimized and validated.

Steps involved in method development:

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

1. Analyte standard characterization: All known information about the analyte and its structure is collected i.e., physical and chemical properties.

a) The standard analyte (100% purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).

b) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.

c) Only those methods (Spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements: The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology: The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

4. Choosing a method: Using the information in the literatures and prints, a new methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples. If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies: The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified. Always new consumables (e.g. solvents, filters and gases) are used. For example, method development is never started on a HPLC column that has been used

earlier. The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

6. Optimization: During optimization one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit: The originally determined analytical figures of merit are Limit of Quantitation (LOQ), Limit of Detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples: The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis: Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average ± standard deviation) from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100 % recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

IV) HPLC method validation [100-106]

Method validation study include system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

a. System Suitability: Prior to the analysis of samples of each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and Precision. The requirements for system suitability are usually developed after method development and validation have been completed.

b. Linearity: The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

c. Precision: Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample". A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types (i) repeatability (ii) intermediate precision (iii) reproducibility Repeatability is the precision of a method under the same operating conditions over a short period of time. Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

d. Accuracy: The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy (i) Comparison to a reference standard, recovery of the analyte spiked into blank matrix and standard addition of the analyte.

e. Specificity / selectivity: 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 selective refers to a method which 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. The analyte should have no interference from other extraneous components and be well resolved from them. A representative Chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

f. 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 normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method. For the determination of ruggedness, the degree of reproducibility of test result is determined as function of the assay variable. This reproducibility

may be compared to the precision of the assay under normal condition to obtain a measure of the ruggedness of the analytical method.

g. Robustness: The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters in HPLC technique may involves flow rate, column temperature, sample temperature, pH and mobile phase composition.

h. Limit of Detection and limit of quantification: Limit of Detection (LOD) 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 LOD and LOQ 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 LOD from the area counts of the analyte. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

Limit of Quantification: Limit of Quantitation (LOQ) 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 LOQ are possible depending on whether the procedure is a non-instrumental or instrumental. LOD and LOQ are determined based on the slope the standard deviation of the intercept (S_a), and the slope of calibration curve (b) by the following equations

LOD = $3S_a$ / b **LOQ=$10S_a$ / b**

j. Stability: To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g., one day, one week, one month, depending on need). Therefore, a few hours of standard and sample solution stability can be required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability.

k. Performance calculations

Calculating the following values are used to access overall system performance.

1. **Relative retention :** $\alpha = (t_2 - t_a) / (t_1 - t_a)$

2. **Theoretical plates:** $n = 16 (t / W)^2$

3. **Plates per meter:** $N = n / L$

4. **Height equivalent to theoretical plate (HEPT):** L/n

5. **Resolution:** $R = 2 (t_2 - t_1) / (W_2 + W_1)$

6. **Peak asymmetry:** $T = W_{0.05} / 2f$

Where, **α = Relative retention**

t_2 = Retention time of the second peak measured from point of injection

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

t_a = Retention time of the inert peak not retained by the column, measured from point of injection

n = Theoretical plates

t = Retention time of component

W = Width of the base of the component peak using tangent method

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

$\mathbf{W_2}$ = Width of the base of component (peak - 2)

$\mathbf{W_1}$ = Width of the base of component (peak -1)

T = Peak asymmetry (Tailing factor)

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

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

N = Plates / meter

L = Column length, in meters

1.04. The aim of the present investigation

The objectives of this study were to develop a simple, economic, rapid, precise, and accurate visible spectrophotometric and high performance liquid chromatographic methods with good sensitivity for quantitative analysis of the selected drugs {Stavudine [STV], Lamivudine [LMV], Zidovudine[ZDV] and Efaverinz [EFZ]} in pure and in pharmaceutical dosage forms.

BIBLIOGRAPHY

[1] E. D. Clercq, "Anti-HIV drugs: 25 compounds approved within 25 years after discovery of HIV," International Journal of Antimicrobial Agents, **33**, 307-320, 2009.

[2] J. E. Gallant, "Initial therapy of HIV infection," Journal of Clinical Virology, **25**, 317-333, 2002.

[3] B. G. Kalzung, S. B. Masters, A. J. Trevor (Eds), Basic & Clinical Pharmacology, 11th Ed., McGraw Hill Medical, New York, 2010.

[4] M. R. Sadaie, R. Mayner, J. Doniger, "A novel approach to develop anti-HIV drugs: adapting non-nucleoside anticancer chemotherapeutics," Antiviral Research, **61**, 1-18, 2004.

[5] D. J. Back, S. H. Khoo, S. E. Gibbons, H. Reynolds, J. F. Tjia, C. Merry, "Therapeutic drug monitoring of anti-HIV drugs," International Congress Series, **1220**, 145-160, 2001.

[6] X. Tan, C. K. Chu, F. D. Boudinot, "Development and optimization of anti-HIV nucleoside and prodrugs: A review of their cellular pharmacology, structure-activity relationships and pharmacokinetics," Advanced Drug Delivery Reviews, **39**,117-151, 1999.

[7]H. H. Willard, L. L. Merritt, Jr., J. A. Dean & F. A. Settle, Jr., Instrumental Methods of Analysis, 6th Edn., CBS Publishers, New Delhi, India, 1986.

[8] R. A. Day & A. L. Underwood, Quantitative Analysis, 6th Edn., Prentice Hall,New Delhi 1998.

[9] J. Besset, R. C. Denney, G. H. Jeffery & J. Mendham, Vogel's Text Book of Quantitative Inorganic Analysis, 6th Edn., Longman Group, England, 2000.

[10] W. J. Blaedel & V. M. Meloche, "Elementary Quantitative Analysis – Theory and Practice", 2nd Edn., Harper and Row, New York ,1984.

[11]Cheronics, N.D. and Ma, T.S., "Organic functional group analysis by micro and semimicro methods", Inter science, New York, 1985.

[12]Fiegl, F., "Spot tests in organic analysis", 6th Edn., elsevier, London, 1986.

[13]Siggia, S. and Hanna, J.G., "Quantitative organic analysis via functional groups", 4th Edn., Wiley Inter science, Nottingholm, 1979.

[14]Besthorn. S.L. and Kasture.A.U., "Talanta"., **40**, 1525, 1993.

[15]Sawicki. E, Hauser. T.R, Satnly. T.W, Elbert. W. and Fox. F.T., "Anal. Chem"., **33**, 1574, 1961

[16]Hauser. T.R. and Commizs. R.L., "Anal. Chem"., **36,** 679, 1984.

[17].Pays. M. and Beligean. M., "Ann. Pharm. Fr".,**28**, 193, 1970.

[18].Sastry. C.S.P, Thirupathi Rao. T. and Sailaja. A., "Talanta".,**38**, 1057, 1991.

[19].Pessey. M. and Baltos. J., "Ann. Pharm. France"., **28**, 153, 1970.

[20].Kommos. E. and Michael. E., "Arch. Pharm. Chem. Sci. Ed"., **10**, 146, 1982.

[21].Sastry. C.S.P. and Sastry. B.S.,"The Eastern Pharmacist"., **29(345),** 31, 1986.

[22].Sastry.C.S.P, Srinivas.Y, Subba Rao.P.V., "MikrochimActa". **126**, 63-67, 1997.

[23].Murthy.T.K, Reddy.M.N, Rao.Y.S, and Sankar.D.G., "Ind. Jour. of Pharmaceutical sciences"., **64(5),** 491-493, 2002.

[24].Sastry.C.S.P, Naidu.P.Y and Murty.S.S.N., "Talanta"., **44(7),**1211-1217, 1997.

[25].Sultan.M., "IL Farmaco"., **57(11),**865-870, 2003.

[26].Sankar.D.G, V.V.N.Reddy.M, Kumar J.M. Rajendra, and Murthy.T.K., "Ind. Jour.of Pharmaceutical sciences"., **64(5),** 504-506, 2002.

[27].Lakshmi Chilukuri.S.R, and N.Reddy.Manda.,"Mikrochim Acta"., **132**, 1-6, 1999.

[28].Sastry.C.S.P, and Ramamohanarao.A., "Mikrochim Acta [Wein]"., **1**,237-244, 1989.

[29].Dryhurst.G., "Periodate oxidation of diol and other functional groups"., Ed. Belcher.R and Anderson.D.M.W., Pergamom Press, London, 1970.

[30].Perez and Bartol.J., "Colorimetric And Fluorimetric Analysis Of Organic Compounds And Drugs", Marcel Dekker, New York, 504, 1974.

[31].C.S.P.Sastry, Jana.S.V.M.Lingeswara rao., "Talanta"., **43(11),** 1827-1835,1996.

[32].Pessey. M. and Baltos. J., "Ann. Pharm. Forance"., **28**, 153, 1970.

[33].Nabi Syed. A, Siddiqui. R, and Nizam. A.A., "Chemia. Analityezna (Warsa)", **25**, 643, 1980.

[34]. A., "Anal .Lett"., **20**, 427, 1987.

[35].Sastry. C.S.P, Sankar. D.G, Reddy. M.N, and Aruna. M., "Indian Jour. Pharm. Sci."., **50**, 178, 1988.

[36].Raggi. M.A, Cavrini. A.M, and Dipietra Lacehe. D., "Pharm. Acta Helv"., **63**, 1988.

[37].Reddy.M.N, Sankar.D.G, Rao.G.D, and Sreedhar.K.,"The Eastern Pharmacist"., **34**, 127, 1991.

[38].Sastry. C.S.P, Krishna. D.M, and Sailaja. A., "The Eastern Pharmacist"., **35**, 179, 1992.

[39].Issopulos. P.B., "Analyst"., **114**, 627, 1989.

[40].Murthy.T.K, Reddy.M.N, Rao.Y.S, and Sankar.D.G.,"Ind.Jour.of Pharmaceutical sciences"., **64(5),** 491-493, 2002.

[41].Mohamed Abd El-Ghaffar, Dina El-Sherbiny, Dalia El-Wass eefand Saadia El- Ashry., "Jour.of Food and Drug analysis"., **16(2),** 26-35, 2008.

[42].Sastry, C.S.P., Sankar, D.G., Reddy, M.N. and Aruna, M., Indian J. Pharm. Sci., **50**, 178, 1988.

[43].Shulka, V.K.S., Pande, V.C. and Sharma, J.P., Z. Anal. Chem., 5, 539, 1972.

[44].Barakat, M.Z., E1-Wahab, M.F.A and E1-Sadar, M.M., Anal. Chem., **27**, 236, 1955.

[45].Mazzuchin, A., Thibert, R.J., and Pedley, E.G., Mikro Chim. Acta, **285**, 1971.

[46].Sastry, C.S.P., Srinivas, K.R. and Krishna Prasad, K.M.M., Mikro Chim. Acta, **122 (1)**, 77, 1996.

[47].Sastry, C.S.P. and Lingeswara Rao, J.S.V.M., The Eastern Pharmacist, **34 (459),** 117, 1996.

[48].Dryhurst, G., "Periodata Oxidation of diol and other functional groups" edt., Belcher, R. and Anderson, D.W.W., Pergamon Press London, 1970.

[49].Sastry, C.S.P., Sastry, B.S., The Eastern Pharmacist, **29**, 67, 1986.

[50].Sastry, C.S.P., Naidu, P.Y., Murthy, S.S.N., Indian Drugs, **33(12),** 607, 1996.

[51].Wolfe, S., Ingold, C.F., and Lemieuk, R.U., J. Am. Chem. Soc., **103**, 938 & 940, 1981.

[52].Patai., “The Chemistry of diazonium and diazo groups”., Wiley, New York, 1978.

[53].Appaiah.K.M, Kapur.P.O, and Nagaraja.K.V., “Jour.Assoc. Off. Agric. Chem”., **66**, 105, 1983.

[54]. Sastry. C.S.P, Ramakrishna.R., “Ind.Chem.Jour”., 13, 27, 1978.

[55]. Bapal.S, Ei-Nenacy. E.A, Soliman.S.A., “Talanta”., 25,290,1975

[56]. Goswami.A.K., “Analyst”., **99**, 657, 1974.

[57].Sastry, C.S.P., Sastry, B.S., The Eastern Pharmacist, **29**, 67, 1986.

[58].Sastry, C.S.P., Naidu, P.Y., Murthy, S.S.N., Indian Drugs, **33** (12), 607, 1996.

[59].Martin, T. and Patel, J.A., Amer. J. Hosp. Pharm., **26**, 51, 1969.

[60].Wolfe, S., Ingold, C.F., and Lemieuk, R.U., J. Am. Chem. Soc., **103**, 938 & 940, 1981.

[61].Nanji, D.R. and Norman, A.G., J. Soc. Chem. Ind., (London), **45**, 337T, 1976.

[62].Sastry, C.S.P., Sankar, D.G., Reddy, M.N. and Aruna, M., Indian J. Pharm. Sci., **50**, 178, 1988.

[63].Sastry, C.S.P., Prasad, T.N.V. and Rao, E.V., Indian J. Pharm. Sci., **49**, 95, 1987.

[64]. Mohamed G G, Nour El-Dien F A F and Mohamed N A, Spectrochim Acta (A), **68(5),** 1244-1249, 2007.

[65]. Hasani M and Akbari S, Spectrochim Acta (A), **68(3),** 409-413, 2007.

[66]. Wu H and Du L M, Spectrochim Acta (A), **67(3-4)**, 976-979, 2007.

[67]. Khaked E, Talanta, **75(5),** 1167-1174, 2008.

[68]. El-Zaria M E, Spectrochim Acta (A), **69(1)**, 216-221, 2008.

[69]. A. A. Gouda., Talanta, **80**, 51-157,2009.

[70]. K. Elmorsy., Talanta, **75**, 1167-1174 ,2008.

[71]. I.A. Darwish., Anal. Chim. Acta, **549**, 212-220 ,2005.

[72]. R.Nafisur, and K.Mohammad., J. Anal. Chem. **60**, 636-643, 2005.

[73]. M. Walash, M. Sharaf-EI Din, M. E. S. Metwalli, and M.RedaShabana., Arch. Pharm. Res. **27**, 720-726, 2004.

[74]. P. Y. Khashaba, S. R. El-Shabouri, K. M. Emara, and A. M.Mohamed., J. Pharmaceut. Biomed. Anal. **22**, 363-376, 2000.

[75]. H. E. Abdellatef. J. Pharmaceut. Biomed. Anal. **17**, 1267-1271, 1998.

[76]. G. A. Saleh., Talanta, **46**, 111-121, 1998.

[77]. N. A. El Ragehy, S. S. Abbas, and S. Z. El-Khateeb. Anal. Lett. **30**, 2045-2058, 1997.

[78].K. Basavaiah and S. Abdulrahman Thai J. Pharm. Sci., **34** 134-145, 2010.

[79].Sastry, C.S.P. and Naidu, P.Y., Talanta, **46**, 1357, 1998.

[80].Sastry, C.S.P. and Sastry, B.S., The Eastern Pharmacist, **29**, 67, 1986.

[81].Ramakrishna, R: and Sastry, C.S.P., Talanta, **26**, 867, 1979.

[82].Siraj, P., Ramakrishna, R., Murthy, S.S.N. and Sastry, C.S.P., Nat. Acad. Sci. Lett., **2(11)**, 413, 1979.

[83].Pindur, U., Arch . Pharm., **314**, 337, 1981.

[84].Tormos, G.V., Belmore, K.A., CaVA. M.P., J.Am. Chem. Soc., **115**, 11512, 1993.

[85].Trigoso. C. I., Ibenez. N., Stockert, J.C.J. Histochem. Cytochem., **41**, 1157, 1993.

[86].Datta. S., Datta. S.C., J.Chronatogr., **170**, 228, 1979.

[87].Sastry, C.S.P., Sastry, B.S., The Eastern Pharmacist, **29**, 67, 1986.

[88].Sastry, C.S.P., Naidu, P.Y., Murthy, S.S.N., Indian Drugs, **33 (12),** 607, 1996.

[89]. Ingold, C.F., and Lemieuk, R.U., J. Am. Chem. Soc., **103**, 938 & 940, 1981.

[90].Chandra Singh, U. and Subbaratnam, N.R., Spectrochimica Acta,Part A; Molecular spectroscopy, 37 A (1) 1, 1981.

[91].Dryhurst.G., "Periodate oxidation of diol and other functional groups"., Ed. Belcher.R and Anderson.D.M.W., Pergamom Press, London, 1970.

[92].Sandell, E.B., "Colorimetric determination of traces of metals", Inter Science, New York, 1950.

[93].Ringbom, A., Z. "Anal. Chem"., 115, 332, 1938.

[94].Sethi P.D, HPLC Quantitative Analysis of Pharmaceutical formulations, CBS Publisher and Distributor, New Delhi, 5, 1996.

[95].K. Albert. On-line LC–NMR and related techniques. Eds. Wiley, Chichester, UK, 2002.

[96].Kazakevich Y, Lobrutto R. HPLC for Pharmaceutical Scientist. 4th ed. New York: Wiley & Sons Inc., 10-14, 2007.

[97].Lindsay S. High Performance Liquid Chromatography. 1st ed. London: John Wiley & Sons, 45-75, 1991.

[98].Lough WJ, Wainer IW. High Performance Liquid Chromatography: fundamental principles & practice. Glasgow (UK): Blackie Academic & Professional, 2-28, 1995.

[99].Snyder LR, Kirkland JJ, Joseph LG. Practical HPLC Method Development. 2nd ed. New York: Wiley & sons, 46-51, 1997.

[100].Loyd L,Snyder R.,Joseph.JGlajch, Practical HPLC Method Development, **2,** 27,29, 2004.

[101].Michael E, Schartz IS, Krull. Analytical method development and Validation. 3rd ed. London: John Wiley & sons, 25-46, 2004.

[102].International Conference on Harmonization, Validation of Analytical Procedures: Methodology. Federal Register,1-8, 1996.

[103].International Conference on Harmonization, Draft Guidelines on Validation of Analytical Procedures, Definitions and Terminology. Federal Register 1260, 1995.

[104].ICH, Specifications:. International Conference on Harmonization, IFPMA, Geneva, 1999.

[105].International Conference on Harmonization (ICH), Guidance for Industry, Q1A (R2): Stability Testing of New Drug Substances and Products, IFPMA, Geneva, 2003.

[106].M. Mulholland, Trends Anal. Chem., **7**, 383,1988.