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NEW ANALYTICAL METHOD DEVELOPMENT FOR THE ACTIVE PHARMACEUTICAL INGREDIENTS USING VISIBLE SPECTROPHOTOMETRY

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ABSTRACT

Method-A, depends on the oxidation of Teicoplanin with ferric chloride and 1,10-phenanthroline to form a blood red colored chromogen. The Method B is based on reaction of Teicoplanin with ferric chloride 2,2' bipyridyl to form blood colored chromogen. Method C is based on the reduction of Ferric ions of the reagent Ferric chloride to Ferrous ions by the drug. The Method A is based on the formation of golden yellow colored chromogen, due to ion-association of Tenofovir with Metanil Yellow dye in Chlolorform. Method B is based on the formation of golden yellow colored chromgen due to ion-association of Tenofovir with Solochrome Black dye in Chlolorform,. The Method C is based on the formation of blood red colored chromogen with Ferric Chloride and 2,2-Bipyridyl . Methods (A, B and C) and Methods (A, B and C) have been developed for the estimation of Tenofovir Disoproxil Fumerate and Emtricitabine in its pharmaceutical dosage form.

KEYWORDS: Spectrophotometry, Quantitative Analysis, Pharmaceutical Analysis

INTRODUCTION

INTRODUCTION IN ANALYTICAL CHEMISTRY

Analytical chemistry is the science of developing and improving methods for detection and determination of artificial and naturally occurring components in our surroundings and environment as well as within ourselves, in our tissues and body fluids also in Pharmaceutical dosage forms[1]. There are two ways of approaching an analytical problem, either qualitatively or quantitatively.

An analytical method is most often performed in several continuous steps and is sometimes called the analytical chain of events (Figure 1). The chain of events can look very different depending on the application or the intended purpose of analysis, but in general it consists of experimental design and planning, sampling and handling, sample treatment, separation of sample components, detection, evaluation, interpretation of the results, and validation.

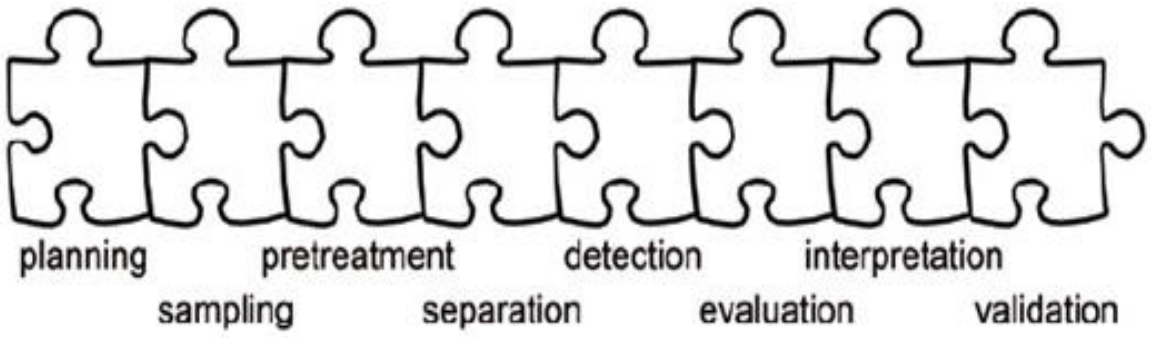

Figure 1: A Schematic Picture Showing the Analytical Chain of Events. Each Step in the Analytical Chain is a Piece of Puzzle

In developing successful analytical methods including the above mentioned events, the analyst should start from the end by asking relevant questions and making them the foundation of analysis. Analysis of matrices like pharmaceutical dosage forms and biological samples will always be challenging, due to their great diversity and complexity[3]. The selectivity gained by separation together with spectrophotometric measurements is necessary to distinguish between similar molecules like drugs and their degradation products as well as the ability to monitor trace levels of the analytes.

Strategy

‘’How ever beautiful the strategy, you should occasionally look at the results” - Winston Churchill

Development of an analytical method usually, and preferably, starts by setting up experimental conditions and criteria for the method. Knowledge about the sample as well as the analytes to quantified is used to choose appropriate sample treatment, separation, detection, evaluation and validation techniques. Strategy includes the planning of the means to validate the method. When dealing with strategic planning of a new method, the important thing is to understand the intended purpose of the method; what is it supposed to monitor? In what levels? Matrix? Budget and time? Is the purpose of the method to identify or discover compounds in the specific matrix, the criteria for qualitative analysis should be followed. If the purpose instead aims for investigating or monitoring the levels of certain compounds in the intended matrix, a quantitative approach should be performed.

Table 1: Consideration Regarding the Strategy for Analytical Method Development

Event	Examples of consideration
Aim of analysis	Qualitative analysis (identification) Quantitative analysis (relative or absolute quantitation) Off-line analysis or Automated Sampling, Handling, Storage, Direct treatments
Sample treatment[4]	Precipitation(acids, organic modifier, salts) Depletion, Ultrafiltration, Derivatization(masking, ionisation, separation).
Separation	Liquid-Liquid extraction at high alkaline and acid conditions.
Evaluation	External standard calibration, Internal standard calibration, Standard addition method, Derivatization technique.
Validation/Quality Checks	Sensitivity, Precision, Accuracy, Linearity, robustness, Limit of detection, Limit of quantification, etc.

Today’s Challenges

Analysis of pharmaceutical dosage forms will be always be challenging, due to their great diversity and intricacy. Today’s challenges within life science and pharmaceutical analysis lie in the increasing need for tools to discover and monitor upcoming new products and dosage forms. Analyzing complex samples like biological products and biological fluids is a significant challenge even with today’s advanced instrumentation[5]. Rapid, high throughput, sensitive, and selective methods are now a requisite for pharmaceutical analysis. Also the ability to analyze trace mixtures, using an instrumental configuration compatible with sample matrices, emerged as an important feature.

Drug analysis is important in several phases of drug development, such as formulation, stability studies, dissolution studies and quality control. The importance of reliable analytical methods for drug determination in a fast, inexpensive, sensitive and selective way is thus evident. Although there are countless works describing new analytical

methods for determination of drugs that act against diseases & metabolic disorders, a review organizing these works in a systematic and complete way is lacking. In this context, the objective of this thesis is to present the main advances in the development of analytical methods for determination of drugs using, spectrophotometric techniques. Quantitative analysis determines the concentration of a specific analyte in a matrix where other compounds are present. Several analytical methods for determination are based on separating substances from one another by utilizing differences in chemical properties. As pharmaceutical dosage forms contain several substances, apart from the analyte of interest, under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Basic Criteria for New Method Development of Drug Analysis[6]

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

The overall aim of our research is to develop new methods for quantitative determination of drugs in pharmaceutical dosage forms. The emphasis is to find new methods to estimate the active pharmaceutical ingredients by UV-Visible Spectrophotometric methods and to understand the mechanism behind. Hopefully, based on a firm theoretical basis, more selective, efficient, fast and reproducible analytical methods can be developed.

Quality Control and Assurance[7]

The concept of total quality control refers to the process of striving to produce a perfect product by a series of measures requiring an organized effort by the entire company to prevent/eliminate errors in every stage of production. The word quality is derived from the Latin “Qualitas”, which means, incidentally, only the ‘nature’ and ‘inherent characteristics’ of a thing. Quality Control (QC) and Quality Assurance (QA) are integral part of Analytical Research.

"The primary aim of the new analytical method is to produce correct results, not by chance, but at all times and it uses quality control methods to achieve and demonstrate this."Two yardsticks are used in QA viz. Accuracy and Precision. QA is thus by choosing an appropriate specific, sensitive and accurate procedure with precision which involves consideration of several other practical aspects such as the speed, economy and the skill required.

Such procedures assure both accuracy and precision and should be used by the laboratories aiming QA and QC. In research laboratories, no compromise can be made on QA-as today’s is the trendsetter and novel analytical method for tomorrow’s routine analysis. Internal and external quality control measures should be adopted and recorded by each laboratory.

Internal quality control measures by using controls, replications and random sample check determine the drifts occurring in the daily tests. External quality control methods measures periodic analysis of unknown samples from reputed QC programmes can be done. The variance index scores are the measure of the performance.

QA & QC develop and follow standard internal operating procedures directed toward assuring the quality, safety, purity and effectiveness of the drug supply.

Quality of Analytical Methodologies

The nature of the analytical methods may be physical, chemical, microbiological, biological or combination of these types. The analytical methods used for the assessment of potency and purity of drugs may be divided broadly into:

- Biological and microbiological methods
- Physico-chemical methods including instrumental methods.

The biological and microbiological methods are tedious, cumbersome and also expensive; are replaced by physicochemical methods. The classical physicochemical methods like gravimetry and titrimetry are either non-specific or not sensitive enough for monitoring the very low concentrations of drugs in biological fluids. An important development in pharmaceutical chemistry is the introduction of more refined and sensitive instrumental methods of analysis[8] such as Spectroscopy (UV-Visible Spectrophotometry, IR, NMR, MS, fluorimetry, nephelometry & turbidimetry), Chromatography (TLC, GLC, and HPLC) that enable one to perform the assay of drugs and formulations more accurately and with the smallest possible consumption of the analyte, reagents and time. The selection of analytical method may be based on one or more of the following design criteria: accuracy, precision, sensitivity, selectivity, robustness, ruggedness, scale of operation, analysis time, availability of equipment and cost.

Many organic compounds absorb in the ultra-violet region of the spectrum and pre-treatment involves only separation of interferences. Some elements in the periodic table absorb strongly in the visible or UV, at least in certain oxidation states and preliminary steps may involve redox reactions as well as separations[9]. Development of absorption by means of inorganic reagents is occasionally possible. The colored complexes formed by metal ions with organic reagents, many of them were metal-chelates, offer most impressive variety of Spectrophotometric methods, and they are especially useful in the field of trace analysis in biological fluids and in pharmaceutical dosage forms. In some regards, the low aqueous solubility of many of the metal chelate compounds is disadvantageous, but on the other hand, extraction of metals into nonaqueous solvents by means of chelating agents may lead to very powerful analytical methods. Reasonable absorbance values are generally obtained with chloroform solutions whose metal concentrations are on the order of a few µg/mL. The solvent used in Spectrophotometric methods poses a problem in some regions of spectrum. There is no solvent which is transparent throughout the infrared region. However, in UV/Visible region most of the organic solvents have UV-cutoff points are below 210 nm and water is an excellent solvent in that region, due to transparency throughout the spectrum.

Direct Spectrophotometric determinations such as colorimetric analysis or ultra-violet determination is widely used in pharmaceutical analysis. The estimation of an analyte's concentration based on its absorption of ultraviolet or visible radiation is one of the most frequently encountered quantitative analytical methods. One reason for its popularity is that many organic and inorganic compounds have strong absorption bands in the UV/Visible radiation. An additional advantage to UV/Visible absorption is that in most cases it is relatively easy to adjust experimental and instrumental conditions so that Beer's law is obeyed. The applications of Beer's law for the quantitative analysis of samples in environmental chemistry, clinical chemistry, industrial, forensic and in pharmaceutical chemistry are numerous. The scale of operation for molecular UV/Visible absorption is routinely used for the analysis is generally better than that of IR absorption. It is routinely used for the analysis of trace analytes in macro and meso samples. The analysis of a sample by molecular absorption spectroscopy is relatively rapid, although additional time may be required when it is necessary to use

a chemical reaction to transform a nonabsorbing analyte into an absorbing form. The cost of UV/Vis instrumentation is relatively less. The selectivity and sensitivity of analyte towards the absorption of light can be increased by converting it into a chromogenic derivative, by adopting a suitable chemical reaction, which also prevents the interferences.

Classification of Organic Reactions[10 -16]

An organic reaction may be presented by the general schemes

Substrate + attacking reagent ⟶ intermediate ⟶ Products

The steps of an organic reaction show the breaking of an existing bond or making of new bonds of carbon atom in the reactant (called substrate) leading to the formation of the final products through intermediates which in some cases have only a transitory existence. More often than not, the attacking reagents carry positive or negative charge. The positively charged reagents attack the regions of high electron density in the substrate molecules while the negatively charged reagents attack the regions of low electron density.

Organic reactions are generally classified into four types; substitution (at saturated or unsaturated carbon atom) or displacement reactions which may be initiated by attack of electrophiles, nuclephiles or free radicals. Elimination reactions involve loss of atoms or groups of atoms from a molecule and generate unsaturated centres in the product. Addition reactions (characteristic of unsaturated compound i.e. existence of a π bond) are distinguished on the basis of the attacking reagent (nucleophile, electrophile or a free radical).

Rearrangement reactions involve the migration of an atom or groups of atoms (carbanions, carbonium ions or free radicals) from one site to another within the same molecule.

Classification of Organic Reagents

Organic reagents fall into two main groups: An electrophilic reagent (a cation, a dipolar molecule or a molecule which has atoms with incomplete octet) is a species having an electron deficient atom or centre and may be of two types – positive electrophiles (H^+, Br^+, Cl^+, NO_2^+, NH_4^+, H_3O^+, R_3O^+,

Ar-N^+ ≡ N etc.) and neutral electrophiles (BF_3, $AlCl_3$, $FeCl_3$, SO_3

R—C—Cl etc)

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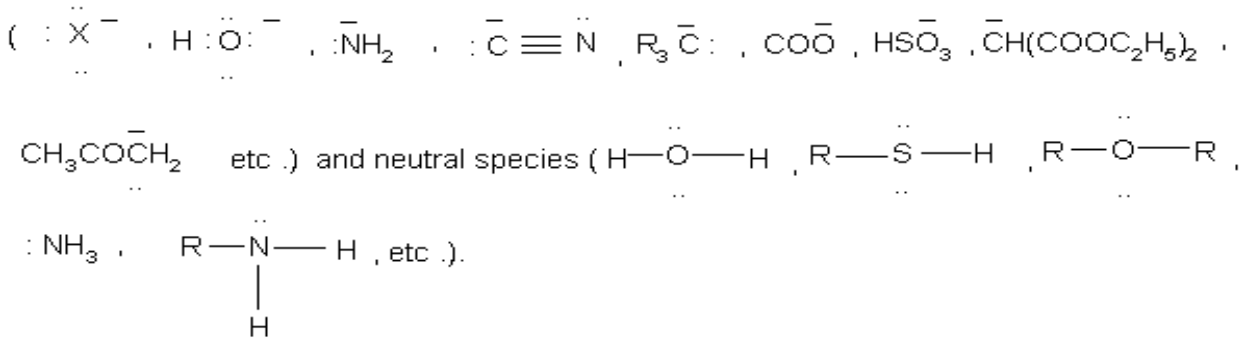
An electrophilic reagent is most likely to attack a molecule at the point of high electron density. Nucleophilic reagents are electron rich and can be classified into negatively charged species.



The attack of negatively charged or neutral nucleophiles on the positively charged substrates results in the formation of neutral or positively charged products. A nucleophile reagent is most likely to attack a molecule at the point of low electron density.

The often transitory intermediates formed during the course of the various organic reactions may be free radicals (from homolytic fission) or carbocations (C-C+) and carbanions (-C :) (from heterolytic fission) which then undergo further change to form the products.

OBJECTIVES

Having established the methodology, and the range over which the calibration is applicable, the final task in any good analytical process must be validation of the procedure. Essentially three processes are available to the user, which may be selected as required.

Check Using Standard Reference Materials (SRM)

Standard reference materials are used in complex matrices e.g. biological fluids, foods, sea water, etc, in which all the analytes of interest have been determined. The quoted concentrations are validated by various statistical processes, and are usually expressed as a definitive figure with an attached ± tolerance. This is usually the preferred procedure, but it is often limited by the availability of a suitable material.

Check Using Values Achieved by an Unrelated Technique

The analyte in question is determined by another technique, the fundamental principle of which is totally different to UV-Visible spectrophotometry. Chromatography (HPLC or TLC) is often the chosen technique. This procedure relies upon the essential validation of the alternative technique.

Addition of Known Amounts of the Analyte of Interest at the Start of the Procedure and then Determination of the Actual Amounts at the End

These 'recoveries' are usually expressed as a percentage of the original concentration added, and will, of course, reflect interference on the determination by the matrix. Ideally recoveries of about 100% should be achieved.

Establishment of Optimum Conditions of the Method[104]

The basis of most Spectrophotometric methods, is usually

- Complex formation reaction.
- An oxidation-reduction process
- A catalytic effect.

In each type of reaction, the yield of colored species whose absorbance is measured and thus the sensitivity of the method, rate of color formation and stability is effected by the concentration of the reagent in the solution, the nature of the solvent, the temperature, the pH of the medium, order of addition of reactants and waiting periods.

It is necessary to establish the optimum conditions in the procedure to be developed through control experiments by varying one among the above parameters and keeping others constant at a time and measuring the absorbances at $\lambda_{max.}$

The range of different parameters within which attainment of high absorption at λ_{max} coupled with maximum intensity and stability of the colored species is achieved, are known as the optimum conditions.

RESEARCH METHODOLOGY

The Ph.D Synopsis comprises of **11 visible spectrophotometric methods using 8 different chromogenic reagents.** The respective methods are indicated below in tabular form.

S.No	Reagent[s] Used	Methods
1.	1,10-Phenanthroline/Ferric Chloride	Method-A of Teicoplanin.
2.	2,2"-Bipyridyl/Ferric Chloride.	Method-B of Teicoplanin.
3.	$FeCl_3$/Potassium ferricyanide.	Method-C of Teicoplanin.
4.	PDAC in Methanol/H_2SO_4	Method- A of Emtricitabine
5.	MBTH/Cerric Ammonium Sulfate.	Method-B of Emtricitabine
6.	Mordant Black/Phthalate Buffer	Method-C of Emtricitabine
7.	Mordant Black/Phthalate Buffer	Method –A of Atazanavir
8.	Solochrome Black T/Phthalate Buffer	Method-B of Atazanavir
9	Metanill Yellow Dye/Phthalate Buffer.	Method-A of Tenofovir.
10	Solochrome Black Dye/chloroform	Method-B of Tenofovir.
11.	2,2"-Bipyridyl/Ferric Chloride	Method-C of Tenofovir.

Chapter-1 opens with a brief account of Analytical chemistry, Quality control and Quality assurance of pharmaceutical products, different analytical techniques, and importance of instrumental techniques. An examination of various factors that have to be taken into account during the course of development of new visible Spectrophotometric methods of assay is presented. The factors include the classification of analytically useful functional groups in the drugs, classification of organic reagents and reaction types, selection of reagents for organic analysis, and systematic investigation to be performed in the development of new visible spectrophotometric methods. The systematic investigations include parameters fixation (spectral characteristics of the colored species, effect of pH, reagent concentration and order of addition, keeping time and temperature during each addition, effect of solvent, rate of color formation and its stability, calibration curve and optimum concentration range), sensitivity (Sandell's sensitivity, molar extinction coefficient and Ringbom plots), interference studies, precision (standard deviation, percent range of error, significance testing by F-tests), accuracy (comparison of proposed and reference methods, percentage recovery studies, testing of significance by t-tests), role of the reagents used in the investigation and Method validation parameters, their definitions and equations to calculate the results.

Each one of the three remaining chapters (Chapter 2 –5) describes the investigations performed by the author for the development of visible spectrophotometric methods for one of the three mentioned drugs. A brief account of the physicochemical properties, therapeutic importance and available formulations of the concerned drug is given in each chapter, followed by a survey of literature on the reported physicochemical methods of analysis. The details of systematic investigations (parameters fixation, sensitivity, precision, accuracy, interference studies and nature of the colored species) performed by the development of the proposed methods for the concerned drug are described in each chapter.

Chapter-2 begins with the introduction giving brief account of Teicoplanin. Existing analytical methods reveal that relatively little attention was paid in developing visible spectrophotometric methods by exploiting thoroughly useful functional groups in Teicoplanin. The chemical features of analytically useful groups of Teicoplanin offers a lot of scope for the development of new methods, hopefully with better sensitivity, precision and accuracy, which prompted the author to carry out investigations in this accord. Three methods based on different chemical reactions have been developed. Method-A, depends on the oxidation of Teicoplanin with ferric chloride and 1,10-phenanthroline to form a blood red colored chromogen. The Method B is based on reaction of Teicoplanin with ferric chloride 2,2' bipyridyl to form blood

colored chromogen. The Method C is based on the reduction of Ferric ions of the reagent Ferric chloride to Ferrous ions by the drug, which further in the presence of potassium ferricyanide as oxidizing agent produces blue colored chromogen measured at 700 nm, against reagent blank. These Methods exhibit maximum absorption at 510 nm, 520 nm and 720 nm respectively and obey the Beer's law in the concentration range of 5-30 mcg/ml, 5-50 mcg/ml and. 1-10 mcg/ml respectively. The Methods have been statistically evaluated and were found to be precise and accurate. The proposed methods are economical and sensitive for the estimation of Teicoplanin in bulk drug and in its formulations.

Chapter-3 records the details of the development of - Three simple, accurate, rapid and sensitive methods (A, B and C) have been developed for the estimation of Tenofovir Disoproxil Fumerate in its pharmaceutical dosage form. The Method A is based on the formation of golden yellow colored chromogen, due to ion-association of Tenofovir with Metanil Yellow dye in Chlolorform, which exhibits λ_{max} at 425 nm. Method B is based on the formation of golden yellow colored chromgen due to ion-association of Tenofovir with Solochrome Black dye in Chlolorform, which exhibits λ_{max} at 438 nm. The Method C is based on the formation of blood red colored chromogen with Ferric Chloride and 2,2-Bipyridyl which shows absorption maximum at 523 nm. The absorbance-concentration plot is linear over the range of 10-60 mcg/ml for Method A, 10-60 mcg/ml for Method B and 2-9 mcg/ml for Method C. Results of analysis for all the methods were validated statistically and by recovery studies. The proposed methods are economical and sensitive for the estimation of Tenofovir Disoproxil fumerate in bulk drug and in its formulations.

Chapter-4 starts with the introduction giving brief account of Atazanavir. There was few methods reported utilizing visible Spectrophotometric method, during the time of commencement of this work. Two simple accurate, rapid and sensitive methods have been developed for the estimation of Atazanavir in the pharmaceutical dosage forms. The Method-A is based on reaction of Atazanavir with Mordant Black II to form an ion-association colored complex at pH-2.4. The method B is based on the reaction of Atazanavir with Solochrome black-T to form an ion-association colored complex in the presence of Potassium Phthalate buffer system. These Methods exhibits maximum absorption at 537nm and 491nm respectively and obeys the Beer's law in the concentration range of 5-90mcg/ml, 10-120mcg/ml respectively. The Methods have been statistically evaluated and were found to be precise and accurate. The proposed Methods are economical and sensitive for the estimation of Atazanavir in the bulk drug and in its formulations.

Chapter-5 deals with the Spectrophotometric method development of an antiviral drug Emtricitabine. Emtricitabine is a nucleoside reverse transcriptase inhibitor related to cytosine with antiretroviral activity against HIV^2. It also active against hepatitis B virus. It is used with other anti-retrovirals for combination therapy of HIV infection. Three simple, accurate, rapid and sensitive methods (A, B and C) have been developed for the estimation of Emtricitabine in its pharmaceutical dosage form. The Method A is based on the formation of orange red colored chromogen, due to reaction of Emtricitabine with p-Dimethyl amino benzaldehyde (PDAC) reagent in the presence of methanol, which exhibits λ_{max} at 538 nm. Method B is based on the reaction of Emtricitabine with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in the presence of Cerric ammonium sulphate to form a green colored chromogen, which shows maximum absorbance at 635 nm. The Method C is based on the formation of reddish brown colored chromogen with Mordant Black Dye in the presence of Phthalate buffer, which shows absorption maximum at 520 nm. The absorbance-concentration plot is linear over the range of 1-10 mcg/ml for Method A, 1-18 mcg/ml for Method B and 5-90 mcg/ml for Method C. Results of analysis for all the methods were validated statistically and by recovery studies. Literature survey reveals only HPLC methods deals with biological fluids and in fixed pharmaceutical dosage forms. The proposed methods are economical and sensitive for the estimation of Emtricitabine in bulk drug and in its formulations.

The Spectrophotometric analytical data given in Chapters (2-5) reveal that the proposed methods were simple,

selective and sensitive. In case of Teicoplanin and Atazanavir, the presently proposed methods constitute valuable contributions. In the case of other three drugs namely- Tenofovir and Emtricitabine the presently proposed methods have advantages over the already reported methods with respect to simplicity, selectivity and sensitivity. All the methods proposed have reasonable precision and accuracy. In conclusion, the proposed methods are simple, sensitive, accurate and economical for the routine estimation of respective active pharmaceutical ingredients in their bulk and in its formulations. Much of the thesis work is published, in reputed national and international-journals.

The overall aim of our research is to develop new methods for quantitative and qualitative determination of drug and related compounds in pharmaceutical formulations. The emphasis is to find new principles for the chromogen formation by using UV-Visible Spectrophotometric methods and to understand the mechanism behind. Hopefully, based on a firm theoretical basis, more selective, efficient, fast and reproducible analytical methods can be developed.

Among several instrumental techniques [HPLC,GLC, fluorimetry, NMR, mass spectroscopy, Spectrophotometry covering IR, UV and visible (colorimetry) regions] available for the assay of drugs, visible Spectrophotometry combines the advantages of low cost and simplicity with the possibility of achieving high sensitivity and selectivity with good precision, accuracy and reliability.

Keeping the simplicity in view, the candidate has examined the present state of development of instrumental methods of analysis for some of the widely used antiviral and antibacterial drugs (Teicoplanin, Atazanavir, Emtricitabine and Tenofovir Disoproxil Fumerate). Although one or two visible Spectrophotometric methods have been developed for above drugs, but they suffer from one disadvantage or the other, such as low sensitivity, lack of selectivity and/ or simplicity etc. So the candidate has chosen this task for his Ph.D work and succeeded in developing useful visible Spectrophotometric for four drugs such as Teicoplanin, Atazanavir, Emtricitabine and Tenofovir Disoproxil Fumerate.

A detailed account of all analytical methods existing for the drug is made to avoid duplication of the method developed. Details about the structure of the drugs and their physicochemical properties are also collected to find out the stability and homogeneity of the sample solutions. UV-Visible spectrophotometry is widely used for the analysis of Chromophores [group of atoms characterized by strongly absorbing electronic transitions]. The Choice of these analytes results from the presence of analytically potential functional groups, aromatic ring resonance of the selected drugs along with simplicity of the spectra and their direct relationship to molecular functional groups results in easy & robust calibrations. The author has made successful attempt in exploiting these features in development of new analytical method. Aromatic nature of the selected compounds is one of the most strongly absorbing chromophores, which result from the effect of double bond conjugation/aromatic ring resonance also one of the striking features in choosing the analytes.

CONCLUSIONS

The overall aim of our research is to develop new methods for quantitative and qualitative determination of drug and related compounds in pharmaceutical formulations. The emphasis is to find new principles for the chromogen formation by using UV-Visible Spectrophotometric methods and to understand the mechanism behind. Hopefully, based on a firm theoretical basis, more selective, efficient, fast and reproducible analytical methods can be developed

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