METHOD DEVELOPMENT AND VALIDATION OF ANTIRETROVIRAL DRUGS IN BULK AND PHARMACEUTICAL DOSAGE FORMS

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted
By

Reg No: 261330958

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

OCT 2015

METHOD DEVELOPMENT AND VALIDATION OF ANTIRETROVIRAL DRUGS IN BULK AND PHARMACEUTICAL DOSAGE FORMS

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted
By

C. RAGAVENDRAN

(Reg No: 261330958)

Under the guidance of

Prof. Dr.S.Vadivelan, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACEUTICS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

OCT 2015

Prof.Dr.S.Vadivelan, M.Pharm., Ph.D.,

Associate Professor,

Department of Pharmaceutical Analysis,

Edayathangudy.G.S.Pillay College of Pharmacy,

Nagapattinam – 611 002.

CERTIFICATE

This is to certify that the dissertation entitled **METHOD DEVELOPMENT AND VALIDATION OF ANTIRETROVIRAL DRUGS IN BULK AND PHARMACEUTICAL DOSAGE FORMS** submitted by **C. RAGAVENDRAN** (Reg No: 261330958) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical Analysis, Edayathangudy G.S Pillay College of Pharmacy during the academic year 2014-2015.

Place: Nagapattinam (**Dr.S.Vadivelan, M.Pharm.,Ph.D.,**)

Date:

Prof.Dr.D.Babu Ananth,M.Pharm., Ph.D.,

Principal,

Edayathangudy.G.S.Pillay College of Pharmacy,

Nagapattinam – 611 002.

CERTIFICATE

This is to certify that the dissertation entitled **METHOD DEVELOPMENT AND VALIDATION OF ANTIRETROVIRAL DRUGS IN BULK AND PHARMACEUTICAL DOSAGE FORMS** submitted by **C. RAGAVENDRAN** (Reg No: 261330958) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under the guidance of **Dr.S.Vadivelan, M.Pharm.,Ph.D.,** Associate Professor, Department of Pharmaceutical Analysis, Edayathangudy G.S Pillay College of Pharmacy during the academic year 2014-2015.

Place: Nagapattinam (**Prof.Dr.D.Babu Ananth,M.Pharm., Ph.D.,**)

Date:

ACKNOWLEDGEMENT

I would like to express profound gratitude to **Chevalier Thiru.G.S.Pillay,** Chairman, E.G.S.Pillay College of Pharmacy, and **Thiru. S.Paramesvaran, M.Com., FCCA.,**Secretary, E.G.S.Pillay College of Pharmacy.

I express my sincere and deep sense of gratitude to my guide **Dr.S.Vadivelan, M.Pharm.,Ph.D.,**Department of Pharmaceutical Analysis, E.G.S.Pillay College of Pharmacy, for his invaluable and extreme support, encouragement, and co-operation throughout the course of my work.

It is my privilege to express my heartfelt thanks to **Prof. Dr.D.Babu Ananth, M.Pharm, Ph.D.,** Principal, E.G.S.Pillay College of Pharmacy, for providing me all facilities and encouragement throughout the research work.

I express my sincere gratitude to **Prof. Dr.M.Murugan, M.Pharm., Ph.D.,**Director cum Professor, Head, Department of Pharmaceutics. E.G.S.Pillay College of Pharmacy, for his encouragement throughout the course of my work.

I wish to express my great thanks to **Dr.P.Dheen Kumar, M.Pharm., Ph.D.,** Associate Professor , Department of Pharmaceutical Analysis, E.G.S.Pillay College of Pharmacy, for his support and valuable guidance during my project work.

I would like to extend my thanks to all the **Teaching Staff**and **Non-Teaching Staff**, who are all supported me for the successful completion of my project work.

Last but not least, I express my deep sense of gratitude to my parents, family members and friends for their constant valuable blessings and kindness.

INDEX

S.NO	CONTENTS	PAGE NO
1	INTRODUCTION	1
2	LITERATURE REVIEW	26
3	AIM & OBJECTIVE	24
4	MATERIALS & METHODS	41
5	RESULTS & DISCUSSION	80
6	SUMMARY	85
7	CONCLUSION	90
8	BIBLIOGRAPHY	92

1. INTRODUCTION

1.1 ANALYTICAL CHEMISTRY

Analytical chemistry[1] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation.

The newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis. Most of the instrumental methods fit into one of the three following categories viz., spectroscopy, electrochemistry and chromatography.

Advantages of instrumental methods:

- Small samples can be used
- High sensitivity is obtained
- Measurements obtained are reliable
- Determination is very fast
- Even complex samples can be handled easily

Limitations of instrumental methods:

- An initial or continuous calibration is required
- Sensitivity and accuracy depends on the instrument
- Cost of equipment is high
- Concentration range is limited
- Specialized training is needed

- Sizable space is required

Principle types of instrumentation[2-10]

Spectrometric techniques

- Ultraviolet and visible spectrophotometry
- Fluorescence and phosphorescence spectrophotometry
- Atomic Spectrometry (emission and absorption)
- Infrared Spectrophotometry
- Raman Spectroscopy
- X-Ray Spectroscopy
- Radiochemical Techniques including activation analysis
- Nuclear Magnetic Resonance Spectroscopy
- Electron Spin Resonance Spectroscopy

Electrochemical techniques[11-20]

- Potentiometry
- Voltametry
- Voltametric Techniques
- Stripping Techniques
- Amperometric Techniques
- Colorimetry
- Electrogravimetry
- Conductance Techniques

Chromatographic techniques

- Gas Chromatography

- High performance Liquid Chromatography
- High performance Thin Layer Chromatography

Miscellaneous techniques[21-25]

- Thermal Analysis
- Mass Spectrometry
- Kinetic Techniques

Hyphenated techniques[26-30]

- GC-MS (Gas Chromatography – Mass Spectrometry)
- ICP-MS (Inductivity Coupled Plasma - Mass Spectrometry)
- GC-IR (Gas Chromatography – Infrared Spectroscopy)
- MS-MS (Mass Spectrometry – Mass Spectrometry

1.2 ANALYTICAL METHOD DEVELOPMENT

Method development is done

1) for new products
2) for existing products

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-Pharamcopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure, comparative laboratory data including merit/demerits are made available.

Steps of method development[31]**:**

Documentation starts at the very beginning of the development process, a system for full documentation of the development studies must be established. All data

relating to these studies must be recorded in laboratory notebook or an electronic database.

Analyte standard characterization

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties, toxicity, purity, hygroscopic nature, solubility and stability.

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

c) 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.

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

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.

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, Association of Official Analytical Chemists (AOAC) and American Society for Testing and Materials

(ASTM) publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

Choosing a method

a) Using the information in the literatures and prints, 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.

b) If there is no prior method 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.

Instrumental setup and initial studies

a) The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified.

b) 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.

c) 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.

d) Analysis is done using analytical conditions described in the existing literature.

Optimization[32]

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.

Documentation of analytical figures of merit

The originally determined analytical figures of merit Limit of quantitation (LOQ), Limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

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.

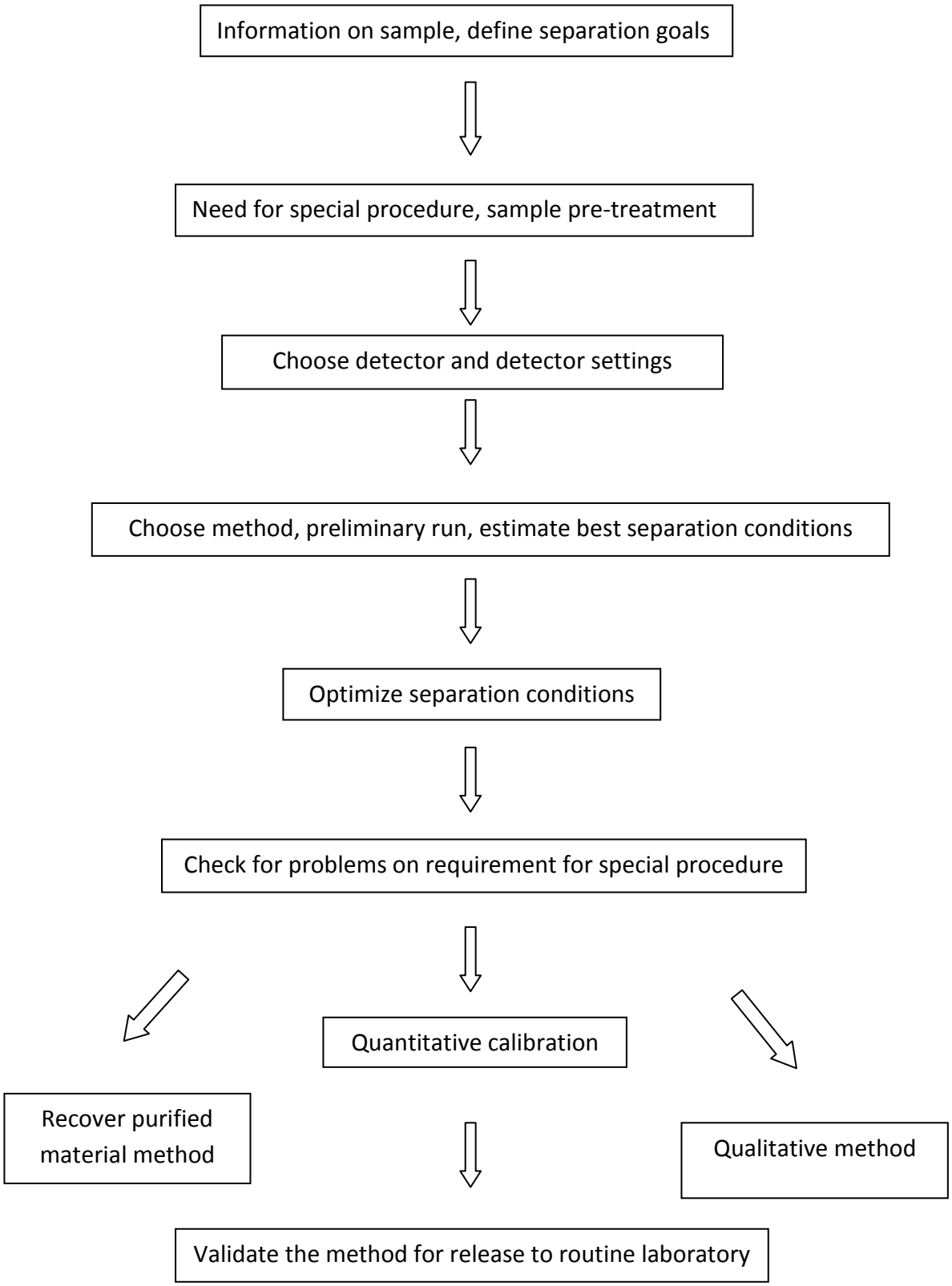
Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

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 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.

Strategy for Method Development:



1.3 Method Validation

Validation is defined as follows by different agencies:

Food and Drug administration (FDA): Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization[33] (WHO): Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

European Committee (EC): Action of providing in accordance with the principles of good manufacturing practice, that any procedure, process, equipment material, activity or system actually lead to the expected results. In brief validation is a key process for effective Quality Assurance.

Analytical method validation

Analytical monitoring of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety efficacy throughout all phases of its shelf life. Such monitoring is in accordance with the specifications elaborated during product development.

Analytical method validation is the corner stone of process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new analytical methods developed are validated.

Steps followed for validation procedures

1. Proposed protocols or parameters for validations are established
2. Experimental studies are conducted

3. Analytical results are evaluated

4. Statistical evaluation is carried out

5. Report is prepared documenting all the results

Table I: Validation Parameters Recommended by International Conference on Harmonization (ICH)

ASSAY TYPE	VALIDATIONS
Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample to that of a reference standard.	Specificity
Different validation characteristics are required for a quantitative test than for a limit test.	Accuracy Precision Specificity Detection limit Quantitation limit Linearity Range
Impurities limits are intended to reflect the purity characteristics of the sample.	Specificity Detection limit
Content / Potency, Dissolution are intended to measure the analyte present in a given sample. A quantitative measurement of the major component (s) in the drug substance.	Accuracy Precision Specificity Linearity Range

In the ICH – 2QA[34] – Text on validation analytical procedures, validation characteristics versus type of analytical procedures are shown in **Table II.**

Table II: Validation Characteristics versus Type of Analytical Procedures Test of Impurities

Type of Procedure	**Identification**	**Quantitation**	**Limit**	**Dissolution Measurement (Content / Potency)**
Accuracy	No	Yes	No	Yes
Precision or Repeatability	No	Yes	No	Yes
Intermediate Precision	No	Yes[a]	No	Yes[a]
Specificity	Yes	Yes	Yes	Yes
Detection Limit	No	No[b]	Yes	No
Quantitation Limit	No	Yes	No	No
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes

a, When reproducibility is performed, intermediate precision is not needed

b, May be needed in some cases

The comparison of different official guidelines in case of parameters required to be validated for different assays is shown in **Table III.**

Table III: Comparative Table Representing FDA, USP and ICH Requirements

Criteria	GMP	FDA	USP	ICH
Accuracy	x	x	x	X
Reproducibility	x			X
Sensitivity	x			
Specificity	x	x	x	X
Linearity		x	x	X
Precision		x	x	X
Detection Limit			x	X
Quantitation Limit			x	X
Range			x	X
Recovery		x		
Ruggedness		x	x	

Analytical methods are required for the identification, batch analysis and storage stability data for active constituents of Pharmaceutical products, and for post-registration compliance purposes. Analytical method development as a first step is carried out to ensure that the API used and the dosage forms that are developed and manufactured for human consumption are meeting the regulated quality norms. Every newly developed method must be validated prior to sample analysis. Validation must also be repeated if a

parameter has been modified or if the validation was strongly performed in another laboratory, to ensure that the methods are transferable. A verification is necessary if the analyst or instrument have been changed, or if the sample type has been modified.

The objective of validation of an analytical method is to demonstrate that the procedure, when correctly applied, produces results that are fit for purpose. Method validation is a practical process designed and experimentally carried out to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range of analysis. Validation provides both assurance and reliability during normal use and documented evidence that the method is 'fit for purpose'. The different validation parameters are as follows.

Accuracy:

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Accuracy of an analytical method is determined by systematic error involved. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. The parameter provides information about the recovery of the drug from sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

Accuracy is calculated the percentage recovery by the assay of the known amount of analyte in the sample or as the difference between the mean and the accepted true value, together with confidence intervals.

For assay method, spiked samples are prepared in triplicate at three intervals over a range of 50-100% of the target concentration. Potential impurities should be added

to the matrix to mimic impure samples. The analyte levels in the spiked samples. The analyte levels in the spiked samples should be determined using the same quantitation procedure as will be used in the final method procedure (i.e. same levels o standards and same number of samples and standard injections).

Precision:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample.

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels: *repeatability, intermediate precision, and reproducibility*.

In the case of *method precision*, six replicates from the same batch are analyzed for the assay and dissolution parameters and observing the amount of scatter in the results. An example of precision criteria of an assay method is that the instrument precision RSD should not be more than 2.0%. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

Repeatability:

Repeatability is the results of the method operating over a short time interval under the same conditions (inter-assay precision). It should be determined from a minimum

of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Intermediate precision:

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

Reproducibility:

Reproducibility refers to the results of collaborative studies between laboratories.

Specificity:

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these extraneous materials.

Limit of Detection:

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually two- or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine LOD: visual non-instrumental methods and a means of calculating the LOD. Visual

non-instrumental methods may include LOD's determined by techniques such as thin layer chromatography (TLC) or titrations. LOD's may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula: LOD = 3.3(SD/S).

Limit of Quantitation:

The Limit of Quantization (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Like LOD, LOQ is expressed as a concentration, with the precision and accuracy of the measurement also reported. Sometimes a signal-to-noise ratio of ten-to-one is used to determine LOQ. This signal-to-noise ratio is a good rule of thumb, but it should be remembered that the determination of LOQ is a compromise between the concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ. This compromise is dictated by the analytical method and its intended use. The calculation method is again based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula: LOQ = 10(SD/S). Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines.

Linearity and Range:

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined

with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results obtained by the method ICH[25] recommended that, for the establishment of linearity, a minimum of five concentrations. It is also recommended that the following minimum specified range should be considered. For assay of a drug substance or a finished product 80-120% of the test concentration should be taken. For an impurity test, the minimum range is from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level.)

Acceptability of the linearity data is often judged by examining the correlation co-efficient and y-intercept of the linear regression line for the response versus concentration plot. The correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at to target level

Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying

method parameters such as percent organic, pH, ionic strength, temperature, etc., and determining the effect (if any) on the results of the method.

The robustness of the methods was determined by performing the assay of the triplicate by deliberately alternating parameters and that the results are not influenced by different changes in the above parameters

Change in column temperature + or - 5^0C

Change in flow rate + or -10%.

Change in organic phase + or -2%.

Change in pH + or -0.2.

The system suitability and the precision of the assay were evaluated for the respective condition. The robustness of an analytical procedure is the measure of its capability to remain unaffected by small, but deliberate, variation in method parameters and providers an indication of its reliability during normal usage.

Chromogenic reagents used in the present investigation.

Functional groups present in organic drugs determine the way of analyzing them because they are responsible for the properties of substances and determine the identification reaction and the methods of quantitative determination of drugs. Knowing the reactions for detecting functional groups, one can easily analyze any organic drug with a complicated structure. In the present investigation, few visible spectrophotometric methods have been developed for LMV and STV by developing colour in each case with, appropriate reagent. The analytically useful functional groups in the drug have not been exploited completely in developing the new visible

spectrophotometric method and so, the drugs have been selected in the present investigation.

Different type of reagents like Gibbs reagent, MBTH reagent and BPB reagent were used in the present investigation for developing visible spectrophotometric methods.

2, 6 Dichloroquinone chlorimide[35-36]

2, 6 Dichloroquinone chlorimide was also called as Gibbs reagent. Gibbs reagent mainly reacts with phenols, primary amines, secondary amines, aliphatic amines. For the present study the reagent was prepared in methanol.

3-Methyl 2-benzathiozolinone hydrazone[37-39]

MBTH was synthesized by Besthron. MBTH can react with carbonyl compounds and compounds containing amine group. It also forms a strongly electrophilic diazonium salt when acted upon by an oxidizing agent. Ferric chloride has been mostly used as the oxidizing agent for the determination of amines.

Bromophenol Blue[40-41]

As an acid-base indicator its useful range lies between pH 3.0 and 4.6. It changes from yellow at pH 3.0 to purple at pH 4.6; this reaction is reversible. Bromophenol blue is structurally related to phenolphthalein. Bromophenol blue is also used as a dye. At neutral pH, the dye absorbs red light most strongly and transmits blue light. Solutions of the dye therefore are blue. At low pH, the dye absorbs ultraviolet and blue light most strongly and appears yellow in solution. In solution at pH 3.6 (in the middle of the transition range of this pH indicator) obtained by dissolution in water without any pH adjustment, bromophenol blue has a characteristic green red colour. This phenomenon is called dichromatic colour. Bromophenol blue is the substance

with the highest known value of Kreft's dichromaticity index. This means that it has the largest change in colour, when the thickness or concentration of observed sample increases or decreases.

Introduction to Antiretroviral drugs

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. When several such drugs, typically three or four, are taken in combination, the approach is known as **highly active antiretroviral therapy**, or **HAART**. The American National Institutes of Health and other organizations recommend offering antiretroviral treatment to all patients with AIDS. Because of the complexity of selecting and following a regimen, the severity of the side-effects and the importance of compliance to prevent viral resistance, however, such organizations emphasize the importance of involving patients in therapy choices, and recommend analyzing the risks and the potential benefits to patients without symptoms.

Multiple drugs are used in a single patient, sensitive and specific analytical methods are reported for simultaneously determining plasma concentrations[42-45] for as many HIV drugs. One reported method also reveals simultaneous determination of six NRTIs[46-48] and nevirapine. However, only one method has been reported till date for simultaneous determination of lamivudine, zidovudine and nevirapine in human plasma using ion-pair HPLC[49].

The primary objective in the analysis of a antiretroviral drugs is to design and develop methods preferably instrumental ones such as UV spectrometric/colorimetric/ HPLC/

GC[50-52] that are sensitive and reproducible, when applied for analysis of marketed formulations.

Antiretroviral (ARV) drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits.

[1] ***Nucleoside and nucleotide reverse transcriptase inhibitors*** (NRTI) inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation.

[2] ***Non-nucleoside reverse transcriptase inhibitors*** (NNRTI) inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function.

[3] ***Protease inhibitors*** (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virons.

[4] ***Integrase inhibitors*** inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. There are several integrase inhibitors currently under clinical trial, andraltegravir became the first to receive FDA approval in October 2007.

[5] ***Entry inhibitors*** (or fusion inhibitors) interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets. Maraviroc and enfuvirtide are the two currently available agents in this class.

[6] ***Maturation inhibitors*** inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein

into the mature capsid protein. Because these viral particles have a defective core, the virions released consist mainly of non-infectious particles. There are no drugs in this class currently available, though two are under investigation, bevirimat and Vivecon.

[7] ***AV-HALTs*** (Anti Viral Hyper Activation Limiting Therapeutics or 'virostatics') combine immune modulating and antiviral properties to inhibit a specific antiviral target while also limiting the hyper-elevated state of immune system activation driving disease progression.

[8] ***Broad spectrum inhibitors.*** Some natural antiviral, such as extracts from certain species of mushrooms like Shiitake and Oyster mushrooms, may contain multiple pharmacologically active compounds, which inhibit the virus at various different stages in its life cycle. Researchers have also isolated a protease inhibitor from the Shiitake mushroom.

1.4 Formulae for calculations

a) Coefficient of variation (%COV) = S x 100 / X

b) Tailing factor (T_f) = $W_{0.05}$ / 2f

c) Theoretical plates (N) = 5.54(t/w)

d) % area difference = $\frac{Af - Ai}{Ai} \times 100$

e) Detection Limit (DOL) = $\frac{3.3F}{S}$

f) Quantitation Limit (LOQ) = $\frac{10F}{S}$

g) In Accuracy:

i) mg actually added (A) = $\frac{W \times P}{100}$

ii) mg found in spiked sample (F) = $\frac{\mathrm{A_{spl}}}{\mathrm{A_{std}}} \times \frac{W_{spl}}{D_{std}} \times \frac{D_{spl}}{W_{spl}} \times \frac{P}{100} \times TW$

iii) mg recovered (R) = $F - S$

iv) % recovered = $\frac{R \times 100}{A}$

h) Simultaneous equations

$$C_X = \frac{(A_2ay_1 - A_1ay_2)}{(ax_2ay_1 - ax_1ay_2)}$$

$$C_Y = \frac{(A_2ay_1 - A_1ay_2)}{(ax_2ay_1 - ax_1ay_2)}$$

Where,

A_{spl} = Average area response of LMV/ZVD/NVP in sample solution

A_{std} = Average area response of LMV/ZVD/NVP in standard solution

$W_{std/spl}$ = Weight of LMV/ZVD/NVP standard/sample in mg

DS = Dilution of standard

$D_{std/spl}$ = Dilution of sample

P = Potency of LMV/ZVD/NVP Standard (%w/w, on as is basis)

LC = Label claim of E

AW = Average weight

T_f = Peak asymmetry or tailing factor

$W_{0.05}$ = Distance from the leading edge to the tailing edge of the peak measured at a point

5 % of the peak height from the baseline

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

t = Retention time (min)

w = Width at the half height

A_f = Final area

A_i = Initial area

F = Standard deviation of the response

S = Slope of the calibration curve

W = Weight of standard

P = Potency of standard

F-*S* = mg found in spiked sample - mg present in sample as such

TW = Theoretical weight of sample

R= mg recovered

A = mg actually added

X= Mean

S = Standard deviation

Cx = Concentration of lamivudine

Cy = Concentration of zidovudine

ax_1 and ax_2 = Absorptivity of lamivudine at 271.1nm and 264 nm

ay_1 and ay_2 =Absorptivity of zidovudine at 271.1nm and 264 nm

A_1= Absorbance of lamivudine at 271.1nm

A_2= Absorbance of lamivudine at 264 nm

2. OBJECTIVES

- Develop new, simple, sensitive, accurate, and economical analytical method for the determination of Lamivudine, Zidovudine and Nevirapine by HPLC, and validate the proposed method.
- Develop new, simple, sensitive, accurate, and economical analytical method for the determination of Lamivudine by GC, and validate the proposed method.
- Develop few simple, sensitive UV spectrometric/colorimetric methods for the determination of antiretroviral drugs and validate the developed methods.

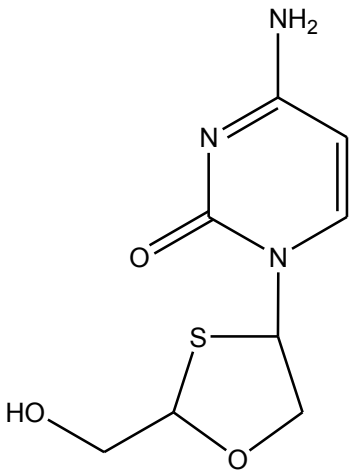
Lamivudine, zidovudine and nevirapine is a relatively new combination. Since multiple drugs are used in a single patient, sensitive and specific analytical methods are needed for simultaneously determining plasma concentrations for as many HIV medications as possible. Till date, numerous analytical methods have been reported for the quantitative determination of lamivudine, zidovudine or nevirapine alone or in combination with other antiviral drugs. One reported method also reveals simultaneous determination of six NRTIs and nevirapine. However, only one method has been reported till date for simultaneous determination of lamivudine, zidovudine and nevirapine in human plasma using ion-pair HPLC.

The primary objective in the analysis of antiretroviral drugs is to design and develop methods preferably instrumental ones such as UV spectrometric/colorimetric/ HPLC/ GC that are sensitive and reproducible, when applied for analysis of marketed formulations.

3. REVIEW OF LITERATURE

The following methods have been reported for the estimation of LMV, ZVD, EFZ, STV and NEV individually and in combination with other drugs.

3.1 Lamivudine (LMV):



Molecular formula: $C_8H_{11}N_3O_3S$

Molecular mass: 229.26 g/mol

Melting point: 175˚C

IUPAC name : 4-amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

Physicochemical properties:

- It is a white crystalline powder
- soluble in methanol and water

Brand Names:

- Lamivir
- Lamidac

Drug Category:

- Anti-HIV Agents
- Nucleoside Reverse Transcriptase Inhibitors
- Reverse Transcriptase Inhibitors
- Reverse transcriptase of hepatitis b Inhibitors

Pharmacology:

Lamivudine is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It needs to be phosphorylated to its triphosphate form before it is active. 3TC-triphosphate also inhibits cellular DNA polymerase.

Basavaiah K[54] ***et al*** developed a titrimetric and spectrophotometric method for the determination of LMV in bulk and tablet dosage form using chloramine-T and two dyes, methyl orange and indigo carmine as reagents. The unreacted oxidant is determined iodometrically. In the spectrophotometric method lamivudine was measured at 610nm. The linearity range was found to be at 3 to 10 µg/ml and 0.35 to 3.0 µg/ml.

Jayaseelan S[55] ***et al*** developed and validated a Bioanalytical method for the estimation of LMV by RP-HPLC with UV detection was developed and validated to separate and detect lamivudine in human plasma using Stavudine as an internal standard. LMV and STV were extracted from human plasma using methanol protein

precipitation and were chromatographed on a Phenomenex C18 (250X4.6mm, 5mμ particle size) column using 20μl injection volume and detection at 270 nm. An isocratic mobile phase consisting of Methanol: Water (85:15%v/v) was used.

Devyani Dube[56] ***et al*** carried out the simultaneous spectrophotometric estimation of LMV and Silymarin in mixture. The method employs formation and solving of simultaneous equation using 270.9 nm and 326.4 nm as two analytical wavelengths. Both the drugs obey Beer's Law in the concentration ranges employed for this method.

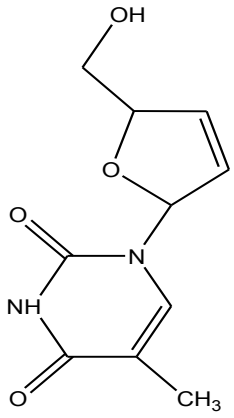
Sockalingam[57] ***et al*** done the simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets. In the UV multi-component spectral method, STV, LMV and NVP was quantified at 266, 271 and 315 nm, respectively. The retention time of STV, LMV and NVP was 2.85, 4.33 and 8.39 min, respectively. In the HPTLC method, the chromatograms were developed using a mobile phase of chloroform: methanol (9:1, v/v).

Bin Fan[58] ***et al*** developed a new high-performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of zidovudine, LMV and NVP in human plasma. Plasma samples were treated using a solid-phase extraction procedure. The compounds were separated using a mobile phase of 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid sodium salt)–acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid on an octylsilane column (150×3.9 mm i.d.) with UV detection at 265 nm.

Abd El-Maaboud[59] ***et al*** developed a simple chemometrics-assisted spectrophotometric method for the simultaneous determination of LMV and STV in

pharmaceutical tablets is described. The UV absorption spectra of the studied drugs, in the range of 200–310 nm, showed a considerable degree of spectral overlapping ($[D_i]^{0.5}$ = 94.9%). Beer's law was obeyed for both drugs in the general concentration ranges of 2–12 and 3–15 μg/ml for LMV and STV, respectively.

3.2 Stavudine (STV):



STV

Molecular formula: $C_{10}H_{12}N_2O_4$

Molecular mass: 224.213 g/mol

Melting point: 160˚C

IUPAC name: 1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione

Physicochemical properties:

- Soluble in water, methanol & chloroform

Brand Names:

- Virostav
- Stag

Drug Category:

- Anti-HIV Agents

Pharmacology:

Stavudine is an analog of thymidine. It is phosphorylated by cellular kinases into active triphosphate. Stavudine triphosphate inhibits the HIV reverse transcriptase by competing with natural substrate, thymidine triphosphate. It also causes termination of DNA synthesis by incorporating into it. Simultaneous use of LMV is not recommended, as it can inhibit the intracellular phosphorylation of stavudine. Other anti-HIV drugs do not possess this property. The oral absorption rate of stavudine is over 80%. Approximately half of stavudine is actively secreted unchanged into the urine and the other half is eliminated through endogenic pathways.

Basavaiah K[60] ***et al*** carried out the rapid titrimetric and spectrophotometric methods for the determination of stavudine in pharmaceuticals using bromate-bromide and three dyes. In titrimetry, aqueous solution of STV was treated with a known excess of bromate-bromide in HCl medium followed by estimation of unreacted bromine by iodometric back titration. Spectrophotometric methods involve the addition of a measured excess of bromate-bromide in HCl medium and subsequent estimation of the residual bromine by reacting with a fixed amount of methyl orange, indigocarmine or thymol blue followed by measurement of absorbance at 520 nm (method A), 610 nm (method B) or 550 nm (method C).

Namita Kapoor[61] ***et al*** described two methods for the simultaneous determination of lamivudine and stavudine in combined pharmaceutical tablets. The first method depends on first derivative UV-spectrophotometry with zero-crossing measurement technique. The first derivative absorbances at 280 and 300 nm were selected for the determination of stavudine and lamivudine, respectively. The second method is based on the separation of both drugs by high performance liquid chromatography using methanol: water (20:80) as the mobile phase at 0.6 ml/min on a reverse phase column with detection at 270 nm.

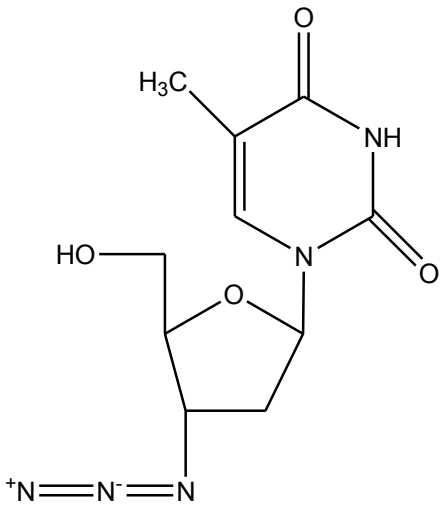
C.P.W.G.M. Verweij-van Wissen[62] ***et al*** developed a reversed phase RP-HPLC method for the simultaneous quantitative determination of the nucleoside reverse transcriptase inhibitors (NRTIs) lamivudine, didanosine, stavudine, zidovudine and abacavir in plasma. The method involved solid-phase extraction with Oasis MAX cartridges from plasma, followed by HPLC with a RP C-18 column and UV detection set at a wavelength of 260 nm. The assay was validated over the concentration range of 0.015–5 mg/l for all five NRTIs.

María Sarasa[63] ***et al*** reported a Sensitive HPLC method for the quantification of STV in human plasma and urine. The methods are linear over the concentration ranges 0.025–25 μg/ml and 2–150 μg/ml in plasma and urine, respectively. An aliquot of 200 μl of plasma was extracted with solid-phase extraction using Oasis cartridges, while urine samples were simply diluted 1/100 with HPLC water. The detection limit is 12 ng/ml in plasma for a sample size of 200 μl.

Ashenafi Dunge[64] ***et al*** developed a validated stability-indicating HPLC assay method The drug was found to hydrolyze in acidic, neutral and alkaline conditions and also under oxidative stress. The major degradation product formed under various conditions was thymine, as evidenced through comparison with the standard and

spectral studies (NMR, IR and MS) on the isolated product. Separation of drug, thymine and another minor degradation product was successfully achieved on a C-18 column utilizing water–methanol in the ratio of 90:10. The detection wavelength was 265 nm.

3.3 Zidovudine (ZVD):



Molecular formula: $C_{10}H_{13}N_5O_4$

Molecular mass: 267.242 g/mol

Melting point: 116 ˚C

IUPAC name: 1-[(2*R*,4*S*,5*S*)-4-azido-5-(hydroxymethyl)oxolan-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione

Physicochemical properties:

- White crystalline powder
- Soluble in water and methanol

Brand Names:

- Zidovir
- Zidomax

Drug Category:

- Anti-HIV Agents
- Nucleoside Reverse Transcriptase Inhibitors

Pharmacology:

Like other reverse transcriptase inhibitors, ZVD works by inhibiting the action of reverse transcriptase, the enzyme that HIV uses to make a DNA copy of its RNA. Reverse transcription is necessary for production of the viral double-stranded DNA, which is subsequently integrated into the genetic material of the infected. The azido group increases the lipophilic nature of ZVD, allowing it to cross cell membranes easily by diffusion and thereby also to cross the blood-brain barrier.

K Basavaia[65] ***et al*** developed Spectrophotometric Methods for the Determination of Zidovudine in Pharmaceuticals Using Chloramine-T, Methylene Blue and Rhodamine-B as Reagents. The methods use chloramine-T (CAT) and two dyes, methylene blue and rhodamine-B, as reagents and are based on adding of a known excess of CAT to ZVD in hydrochloric acid medium followed by determination of residual oxidant by reacting with a fixed amount of either methylene blue and measuring the absorbance at 665 nm (Method A) or rhodamine B and measuring the absorbance at 555 nm (Method B). In both methods, the amount of CAT reacted corresponds to the amount of ZVD. The absorbance measured is found to increase

linearly with concentration of ZVD. Under the optimum conditions, ZVD could be assayed in the concentration range 1.25-15.0 and 0.25-3.0 mg/ml by method A and method B, respectively.

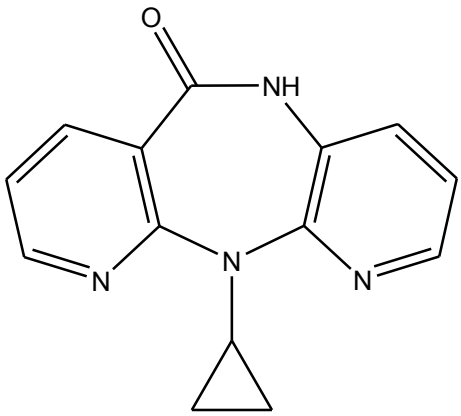
Vaishali P. Nagulwar[66] ***et al*** developed a validated UV spectrophotometric method for the simultaneous estimation of LMV, NVP and ZVD in combined tablet dosage form. The stock solutions were prepared in 0.5M HCl followed by the further required dilutions with distilled water. The lmax for lamivudine, nevirapine and zidovudine were 280.2 nm, 312 nm and 266.8 nm respectively. Linearity in concentration range of 5-25 mg/ml, 5-50 mg/ml and 5-40 mg/mL was shown respectively by the three drugs.

Anantha kumar .D[67] ***et al*** reported a simultaneous determination of LMV, ZVD and Abacavir in Tablet Dosage Forms by RP HPLC Method. Chromatography was carried out on a HiQ Sil C 18 column using a mobile phase consisting of 0.01 M potassium dihydrogen *ortho*-phosphate (pH 3.0) and methanol (55:45 v/v) at a flow rate of 0.8 ml/min. The detection was made at 272 nm and stavudine was used as the internal standard for this study. The retention times for lamivudine, abacavir and zidovudine were found to be 3.8, 6.3, 8.1 min. respectively. The calibration curves were linear over the range 5-250 µg/ml for both zidovudine and abacavir and 5-140 µg/ml for lamivudine.

Vibhuti Kabra[68] ***et al*** developed the simultaneous quantitative determination of zidovudine and nevirapine in human plasma using isocratic, reverse phase high performance liquid chromatography. In the HPLC measurement, sample detection was carried out at 246 nm using an ultraviolet (UV) photo diode array (PDA) detector The compounds were separated using a mobile phase consisting of a pH 3.0 solution

(obtained by adjusting the pH of water with orthophosphoric acid): acetonitrile (73:27 v/v) on a Phenomenex LUNA C18, column (250×4.6 mm i.d., 5µm) at a flow rate of 0.9 ml/min. The total run time for the assay was 10.2 min. The method was validated over the range of 300-9600 ng/ml and 200-6400 ng/ml for ZVD and NVP, respectively.

3.4 Nevirapine (NVP):



Molecular formula: $C_{15}H_{14}N_4O$

Molecular mass: 266.298 g/mol

Melting point: 247 ˚C

IUPAC name: 11-cyclopropyl-4-methyl-5,11-dihydro-6*H*- dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one

Physicochemical properties:

- White crystalline powder
- Soluble in chloroform and methanol

Brand Names:

- Nevivir
- Nevimune

Drug Category:

- Anti-HIV Agents
- Non-Nucleoside Reverse Transcriptase Inhibitors

Pharmacology:

Nevirapine falls in the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretrovirals. Both nucleoside and non-nucleoside RTIs inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzyme's active site, NNRTIs bind allosterically at a distinct site away from the active site termed the NNRTI pocket. Resistance to nevirapine develops rapidly if viral replication is not completely suppressed. As all NNRTIs bind within the same pocket, viral strains which are resistant to nevirapine are usually also resistant to the other NNRTIs, efavirenz and delavirdine.

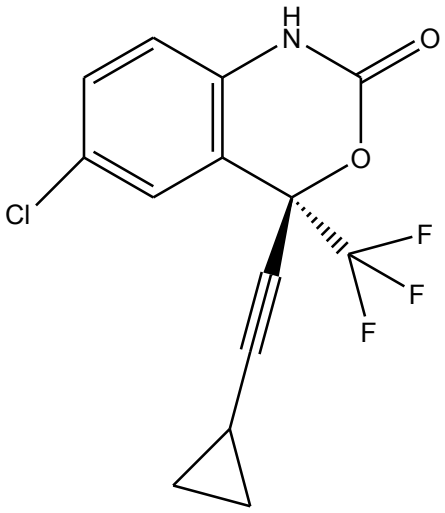
Purnima Hamrapurkar[69] ***et al*** developed a RP-HPLC with ultraviolet detection has been developed and validated for the estimation of nevirapine from human plasma. Chromatographic separation was achieved on Waters RP C18 10 µm column having 250 × 4.6 mm ID with a mobile phase containing 15 mM aqueous phosphate buffer: acetonitrile (65:35 % v/v) in isocratic mode. The flow rate was 1.0 ml / min and effluents were monitored at 283 nm. The retention time of nevirapine and the internal standard was 5.1 min and 6.2 min respectively.

Purnima[70] ***et al*** developed a HPTLC method for the estimation of nevirapine from bulk drug and tablet formulations. The separation was achieved on TLC plates using appropriate solvent system. The spots so developed were densometrically scanned at 283 nm. The linearity of the method was found to be within the concentration range of 2.50µg/ml to 62.50µg/ml.

Wenjing Chen[71] ***et al*** developed a high-performance analytical method based on capillary electrophoresis to investigate interactions between HIV RT (reverse transcriptase enzyme) and NVP was developed Samples containing HIV RT and NVP at various ratios were incubated at 37 °C for 45 min and then separated by CE with Tris–acetate buffer at pH 7.3 containing 0.15% SDS. The binding constants of the interactions between HIV RT and NVP were calculated as $(3.25 \pm 0.16) \times 10^4$ and $(1.25 \pm 0.07) \times 10^2\ M^{-1}$ by Scatchard analysis. HIV RT and NVP have two binding sites.

Peter Langmann[72] ***et al*** reported a sensitive and rapid gas chromatographic method to determine the levels of the HIV-1 non-nucleoside reverse transcriptase inhibitor NVP in human plasma. Quantitative recovery following liquid–liquid-extraction with diethyl ether from 500 µl of human plasma was achieved. Subsequently, the assay was performed with a CP-Sil 5CB capillary column, 15 m×0.32 mm×1.0 µm film thickness with a nitrogen–phosphorous-detector (NPD), Helium 5.0 was used as carrier gas with a constant inlet pressure of 7 psi. Linear standard curves were obtained for concentrations ranging from 10 to 20 000 ng/ml.

3.5 Efavirenz (EFZ):



Molecular formula: $C_{14}H_9ClF_3NO_2$

Molecular mass: 315.675 g/mol

IUPAC name: (4*S*)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1*H*-3,1-benzoxazin-2-one

Physicochemical properties:

- White crystalline powder
- Soluble in methanol and insoluble in water

Brand Names:

- Efavir
- Estiva

Drug Category:

- Anti-HIV Agents
- Non-Nucleoside Reverse Transcriptase Inhibitors

Pharmacology:

Efavirenz falls in the NNRTI class of antiretrovirals. Both nucleoside and non-nucleoside RTIs inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzyme's active site, NNRTIs act allosterically by binding to a distinct site away from the active site known as the NNRTI pocket. Efavirenz is not effective against HIV-2, as the pocket of the HIV-2 reverse transcriptase has a different structure, which confers intrinsic resistance to the NNRTI class. As most NNRTIs bind within the same pocket, viral strains which are resistant to efavirenz are usually also resistant to the other NNRTIs, nevirapine and delavirdine.

Deshpande Anant[73] ***et al*** developed a simple, sensitive and accurate spectrophotometric method was developed in ultraviolet region for the estimation of efavirenz (EFZ) in pure drug, pharmaceutical formulation. Linear response obtained was in the concentration range of 5-40 µg/ml with correlation coefficient of 0.9993, 0.9989 in solvent and plasma respectively. Excellent recovery proved that the method was sufficiently accurate.

Anri Theron[74] ***et al*** reported a novel and robust screening method for the determination of the non-nucleoside reverse transcriptase inhibitor, EFZ, in human saliva and validated based on tandem mass spectrometry (LC–MS/MS). The analytes were separated by high performance liquid chromatography (Phenomenex Kinetex C18, 150 mm × 3 mm internal diameter, 2.6 µm particle size) and detected with tandem mass spectrometry in electrospray positive ionization mode with multiple reaction monitoring. Gradient elution with increasing methanol concentration was used to elute the analytes, at a flow-rate of 0.4 ml/min. The total run time was 8.4 min

and the retention times for the internal standard (reserpine) was 5.4 min and for EFZ was 6.5 min.

Geetha Ramachandran[75] ***et al*** reported a simple and rapid high performance liquid chromatographic method for determination of EFZ in human plasma. The method involved extraction of sample with ethyl acetate and analysis using a reversed-phase C_{18} column (150 mm) with UV detection. The assay was linear from 0.0625 to 10.0 μg/ml. The method was specific for EFZ estimation and the drug was stable in plasma up to one month at −20 °C. The average recovery of EFZ from plasma was 101%. Due to its simplicity, the assay can be used for pharmacokinetic studies and therapeutic drug monitoring of EFZ.

4. MATERIALS AND METHODS

PART A: UV-VISIBLE SPECTROPHOTOMETRIC METHODS FOR LMV AND STV

Method 1: Estimation of lamivudine by MBTH reagent

Method 2: Estimation of lamivudine by Bromophenol blue dye

Method 3: Estimation of stavudine by selective oxidation using Cerium (IV) ammonium sulphate regent

Method 4: Estimation of stavudine by 2, 6-Dichloroquinone Chlorimide (Gibb's regent)

PART B: SIMULTANEOUS ESTIMATION OF LAMIVUDINE, ZIDOVUDINE AND EFAVIRENZ BY UV- SPECTROPHOTOMETRY

Method 5: By Three wavelength spectrophotometry

PART C: RP-HPLC METHOD

Method 6: Simultaneous estimation of lamivudine, Zidovudine and Nevirapine

PART D: GC METHOD

Method 7: Estimation of Lamivudine by Gas Chromatographic method using Ethyl Chloroformate as a Derivatizing reagent

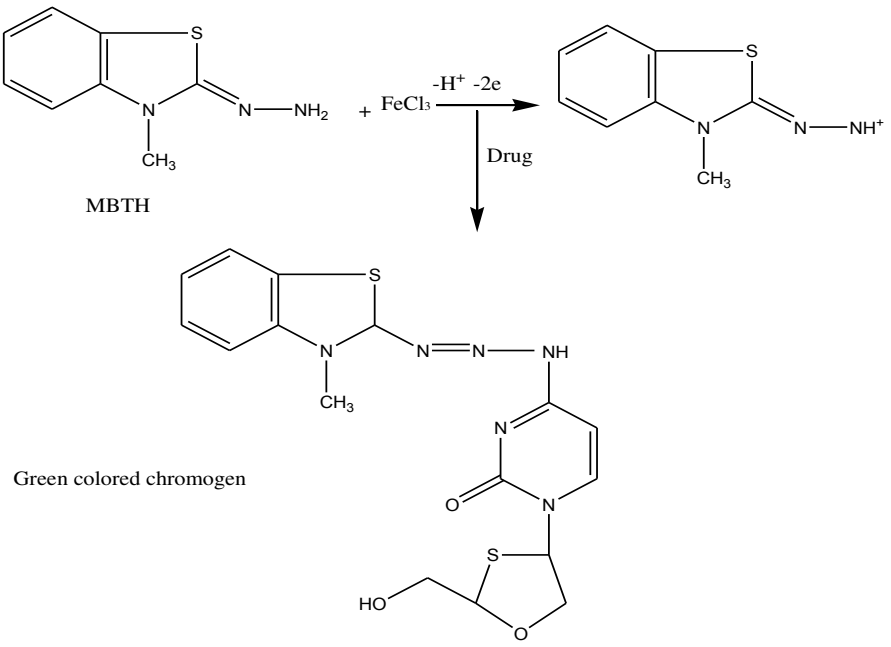
PART A: UV- VISIBLE SPECTROPHOTOMERTIC METHODS

Method 1: ESTIMATION OF LAMIVUDINE BY MBTH REAGENT

1.1 PRINCIPLE INVOLVED

The principle is based on the oxidation followed by coupling of 3-methyl-2-benzothiazolinone hydrazone with LMV in the presence of ferric chloride to form a green colored chromogen. This is an ion catalyzed oxidative coupling reaction of MBTH with the drug. Under reaction conditions, on oxidation, MBTH loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling agent. This intermediate undergoes electrophilic substitution with the drug to form the colored product **(Scheme 1)**.

1.2 REACTION INVOLVED –



Scheme 1: Reaction pathway between Lamuvidine and MBTH reagent.

1.3 PREPARATION OF REAGENTS

1.3.1 MBTH (0.5% w/v)

0.5 g of MBTH was weighed accurately and dissolved in distilled water in 100 ml volumetric flask and volume was made up to mark with water and filtered.

1.3.2 Ferric chloride (1% w/v)

1 g of Ferric chloride was weighed accurately and dissolved in distilled water in 100 ml volumetric flask and volume was made up to mark with water and filtered.

1.4 PREPARATION OF STANDARD CALIBRATION CURVE

1.4.1 Preparation of standard stock solution

Accurately weighed 10.0 mg of lamivudine (bulk drug) was dissolved in 40.0 ml of warm distilled water in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with distilled water to obtain a concentration of 100 µg/ml.

1.4.2 Preparation of calibration curve

Varying aliquots (0.1–0.7 ml) of the standard 100 µg/ml LMV solutions were transferred into a series of 10 ml calibrated flasks by means of a micro burette. To all the calibrated 10 ml flasks, 1 ml of 0.5% MBTH reagent was added. The solutions were swirled and allowed to stand for 5 min. A 1 ml of 1% $FeCl_3$ solution is added to all the flasks, the solutions were swirled and allowed to stand the absorbance was measured at 659 nm against the corresponding reagent blank. The calibration curve was constructed by plotting absorbance against the initial concentration of LMV. The linearity

range or Beer's range follows in the range between 1 to 8 µg/ml **(Fig.1)**. The content of LMV was calculated from the calibration graph.

1.5 ANALYSIS OF TABLET DOSAGE FORM

Ten tablets are taken and finely powdered, each claimed to contain 100 mg (Lamivir). An amount of the powder equivalent to 100 mg of active component was weighed into a 100 ml volumetric flask; about 60 ml of water was added and shaken thoroughly for about 20 min. The volume was made up to the mark with double distilled water, shaken and filtered using filter paper. The filtrate was diluted sequentially to get 0.1 mg/ml of the drug. The resultant solution is also analysed as per the procedure and were statically validated.

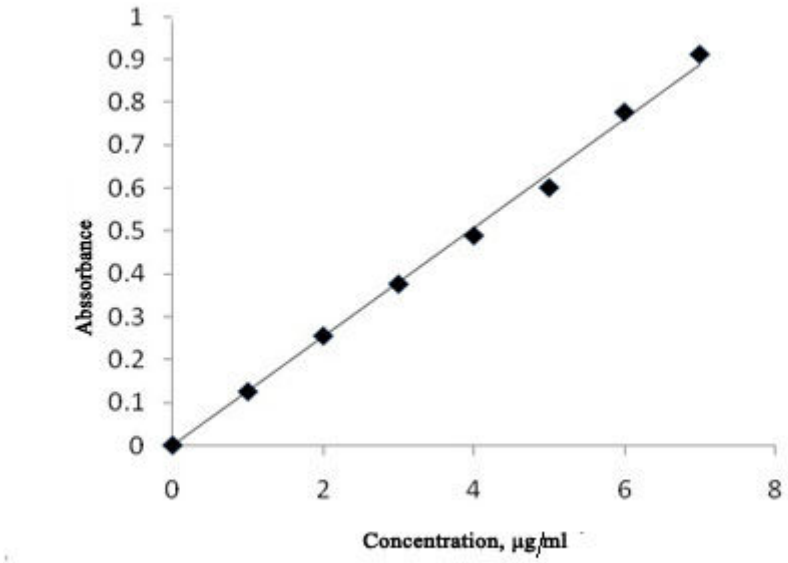


Fig.1: Calibration curve for LMV method 1

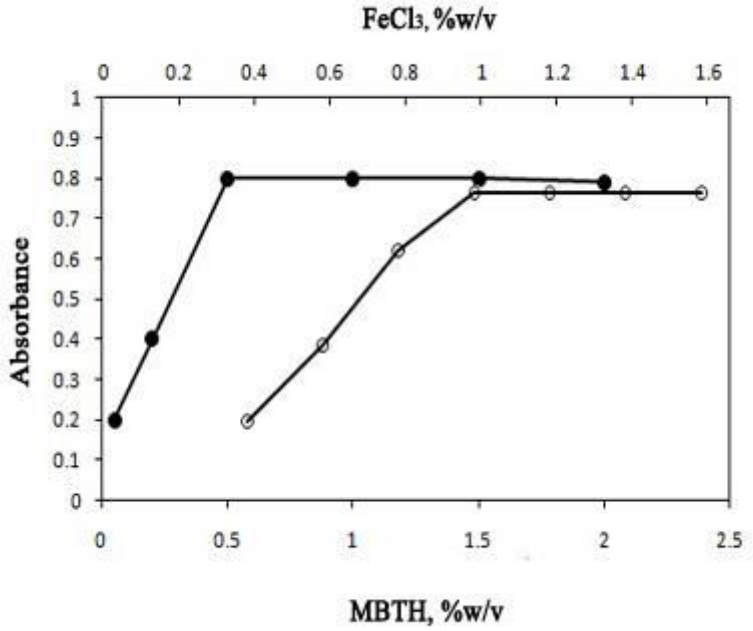


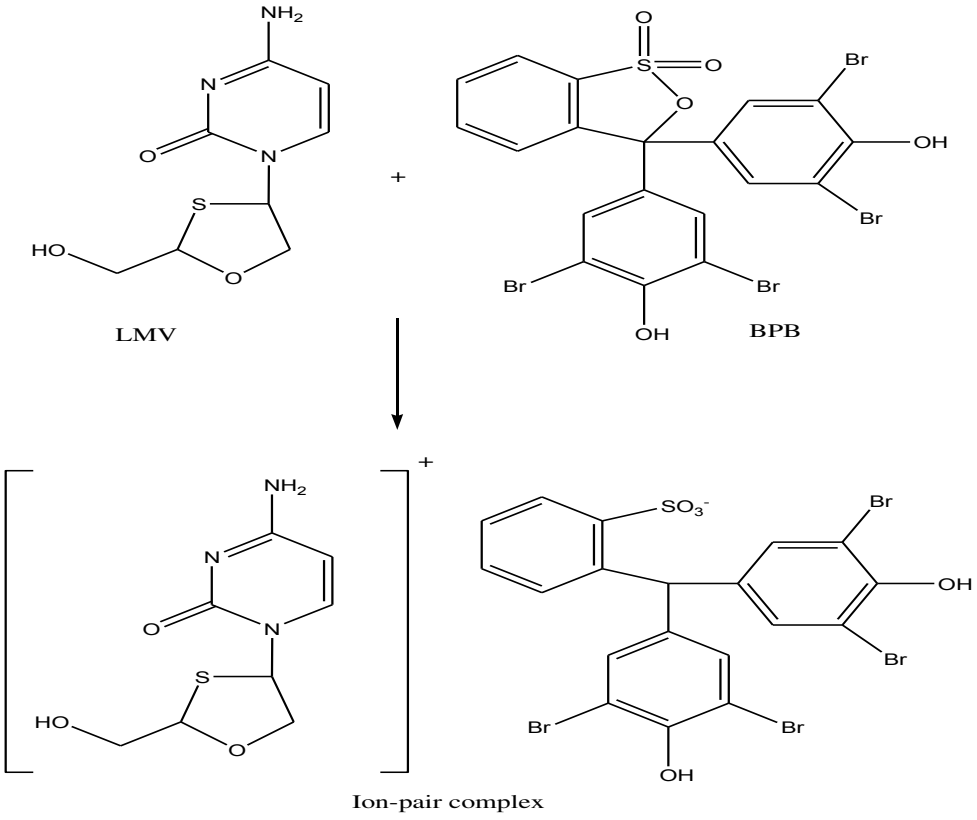
Fig. 2: Effect of MBTH and $FeCl_3$ on the reaction with LMV.

Method 2: ESTIMATION OF LAMIVUDINE BY BROMOPHENOL BLUE DYE

2.1 PRINCIPLE INVOLVED

The principle is based on the application of acidic dyes for the spectrophotometric determination of LMV. The structural formula of LMV feature amine group, suggests the use of acidic dyes (BPB) as chromogenic reagents **(scheme 2)**. The acid dye technique is a general procedure for the quantitative analysis of a variety of pharmaceutical amines. In practice, an aqueous solution containing the amine and a suitable indicator dye is shaken with an organic solvent. The concentration of the resulting ion-pair is then determined spectrophotometrically.

2.2 REACTION INVOLVED



Scheme 2: Reaction pathway between Lamuvidine and BPB dye.

2.3 REAGENTS USED

2.3.1 Bromophenol blue (0.1%) (w/v)

0.1 g of BPB was weighed accurately and dissolved in methanol in 100 ml volumetric flask and volume was made up to mark with methanol and filtering through a Whattman filter paper.

2.4 PREPARATION OF STANDARD CALIBRATION CURVE

2.4.1 Preparation of standard stock solution

Accurately weighed 10.0 mg of lamivudine (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol i.e. 100 µg/ml.

2.4.2 Preparation of calibration curve

Different aliquots (0.1–0.5 ml) of the standard 100 µg/ml LMV solution were transferred into a series of 10 ml calibrated flasks by means of a micro burette. To all the calibrated flasks, 1 ml of 0.1% methanolic solution of BPB was added. The solutions were swirled and allowed to stand for 5 min and the volume was diluted to the mark with methanol and mixed well. The absorbance was measured at 595 nm against the corresponding reagent blank and calibration graph was constructed. The calibration curve was constructed by plotting absorbance against the initial concentration of LMV. The linearity range or Beer's range follows in the range between 1 to 8 µg/ml **(Fig.3)**. The content of LMV was calculated from the calibration graph.

2.5 ANALYSIS OF TABLET DOSAGE FORM

Ten tablets are taken and finely powdered, each claimed to contain 100 mg (Lamivir). An amount of the powder equivalent to 100 mg of active component was weighed into a 100 ml volumetric flask; about 60 ml of methanol was added and shaken thoroughly for about 20 min. The volume was made up to the mark with methanol, shaken and filtered using filter paper. The filtrate was diluted sequentially to get 0.1 mg/ml of the drug. The resultant solution is also analysed as per the procedure and were statically validated.

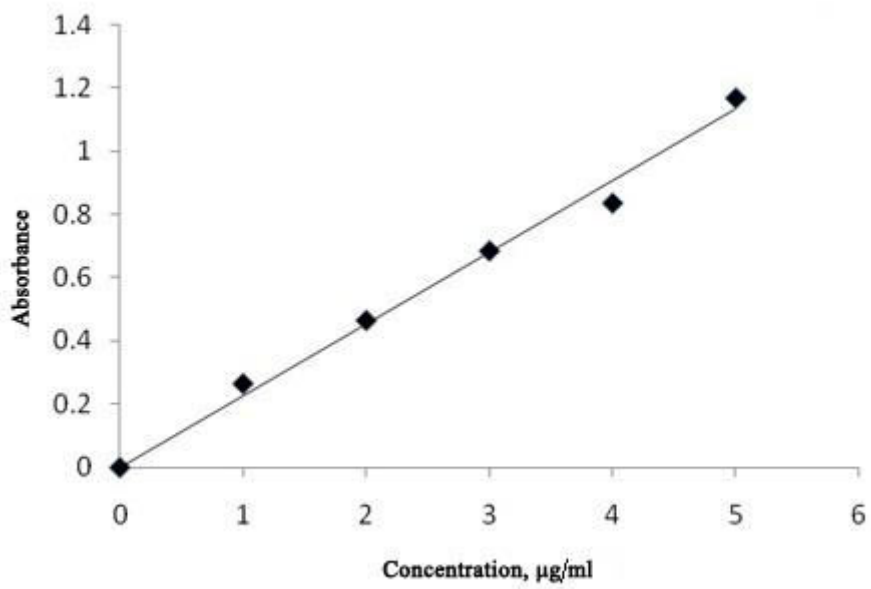


Fig. 3: Calibration curve for LMV method 2

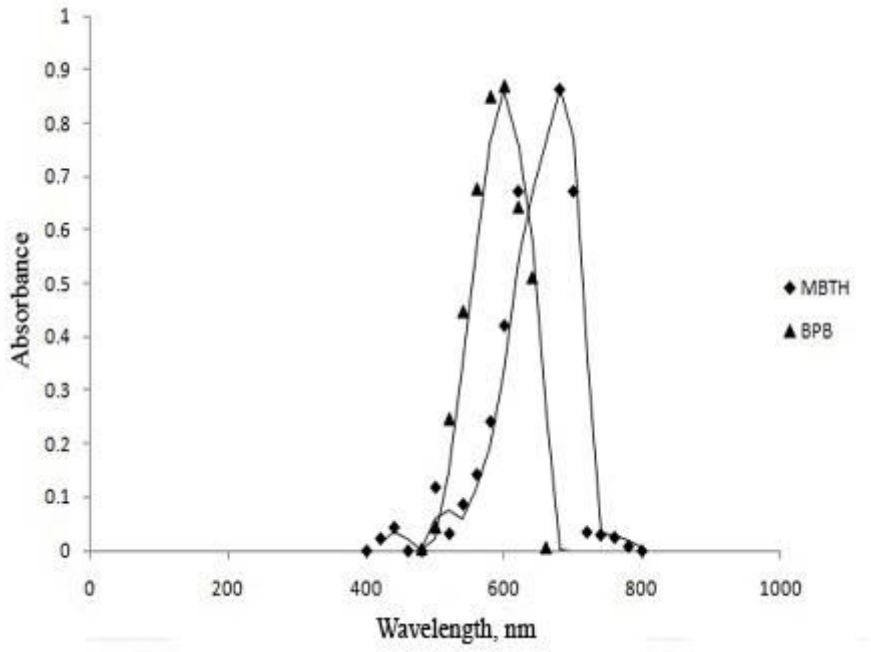


Fig. 4: Absorbance spectra of (LMV-BPB) and (LMV-MBTH) reaction products (initial concentration of LMV was 10 µg/ml).

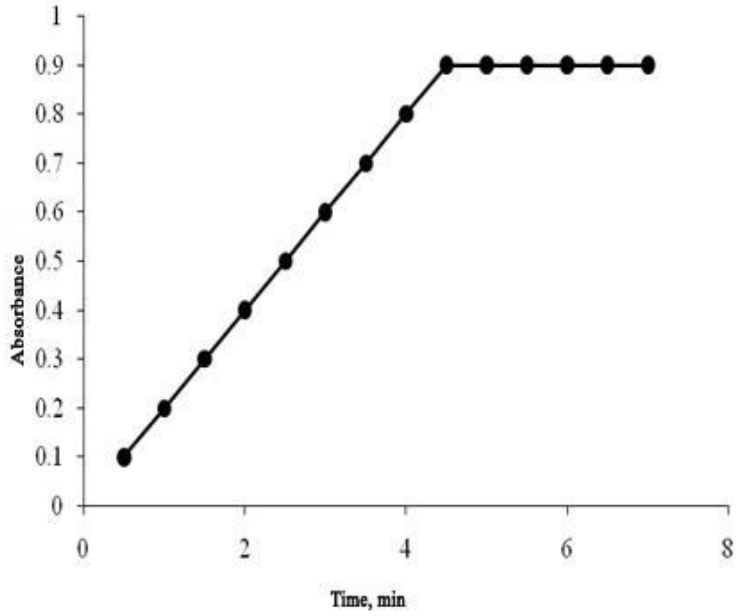


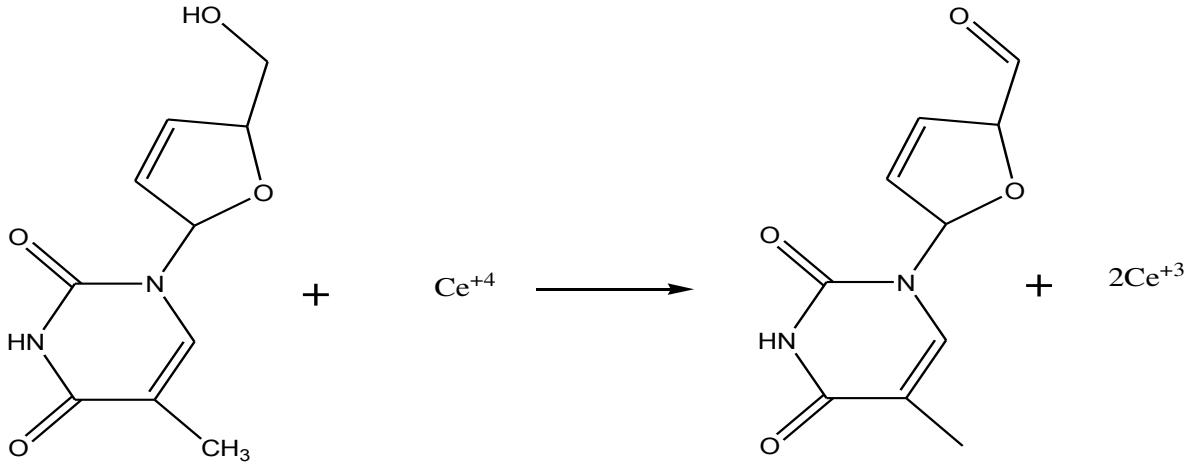
Fig. 5: Effect of time on the reaction of BPB with LMV.

Method 3: ESTIMATION OF STAVUDINE BY SELECTIVE OXIDATION USING CERIUM (IV) AMMONIUM SULPHATE

3.1 PRINCIPLE INVOLVED

Cerium (IV) and iron (III) ammonium sulphates are strong oxidizing agents and are utilized extensively for the determination of organic compounds. When stavudine reacts with Cerium (IV) ammonium sulphate it results in the formation of a oxidized product **(Scheme 3)** which can be quantified spectrophotometrically.

3.2 REACTION INVOLVED



STV Oxidized product

Scheme 3: Reaction pathway between Stavudine and cerium (IV) ammonium sulphate.

3.3 REAGENTS USED

3.3.1 Perchloric acid 4M

33.3 ml of perchloric acid (12M) was accurately taken and diluted in distilled water in 100 ml volumetric flask and volume was made up to the mark with distilled water.

3.3.2 Cerium (IV) ammonium sulphate 0.1% (w/v)

0.1 g of Cerium ammonium sulphate was weighed accurately and dissolved in 4 M perchloric acid in 100 ml volumetric flask and then the volume was made up to mark with 4 M perchloric acid.

3.4 PREPARATION OF STANDARD CALIBRATION CURVE

3.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of STV (bulk drug) was dissolved in 10.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with double distilled water i.e. 1.0 mg/ml (Stock solution A).

From the above stock solution A 10 ml of aliquot was pipette out in 100 ml volumetric flask and the volume was made up to the mark with double distilled water to obtain the final concentration of 100 µg/ml (Stock solution B).

3.4.2 Preparation of calibration curve

Aliquot volumes of standard stock solutions, containing 0.2-1 ml (2-10 µg/ml) drug, were transferred to 10 ml calibrated flasks. 1 ml of 4 M perchloric acid was added each flask followed by 1.0 ml of Ce (IV) solution, mixed well and made up to the mark with distilled water. The absorbance of the resulting solutions was measured after 5 min at 282 nm **(Fig.6)** at 25.5°C against reagent blanks treated similarly. The amount of STV present in the sample solution was computed from its calibration curve. The linearity range or Beer's range follows in the range between 2-10 µg/ml. The content of STV

was calculated either from the calibration graph or corresponding regression equation

3.5 ANALYSIS OF TABLET DOSAGE FORM

Ten tablets are taken and finely powdered claimed to contain 30 mg (Stadine). The accurate quantity of powder equivalent to 100mg of active ingredient was dissolved in 10 ml of methanol later diluted with 50 ml of distilled water and shaken thoroughly for about 5 min. The volume was made up to the mark with distilled water and filtered using Whattman filter paper (Stock solution A). From the above stock solution 10.0ml was pipetted out in 100.0ml volumetric flask and the volume was made up to the mark with warm double distilled water to obtain the final concentration of 100 µg/ml (Stock solution B). From the stock solution B 0.5ml of solution is pipetted in to a 10.0ml volumetric flask and then the resultant solution is also analysed as per the procedure and was validated.

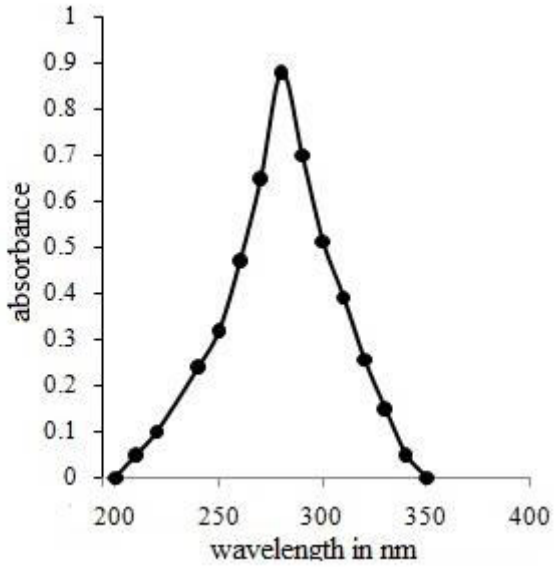


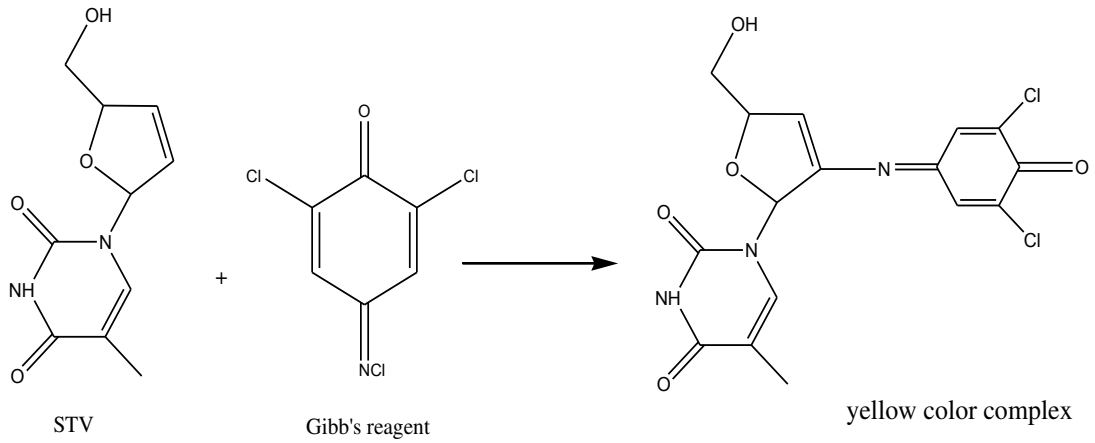
Fig. 6: The graph showing the maximum absorbance for method 3

Method 4: ESTIMATION OF STAVUDINE BY 2, 6-DICHLOROQUINONE CHLORIMIDE OR GIBB'S REGENT

4.1 PRINCIPLE INVOLVED

The principal involved is Gibb's reagent mainly reacts with phenols, primary amines, secondary amines, and aliphatic amines to form a colored complex. The first step of reaction is formation of the corresponding quinine imines. Quinone imines are condensation products of quinone chlorimines with phenols in aqueous alkaline media. Imide portion of Gibb's reagent reacts with phenolic compounds gives corresponding products. STV possesses different functional groups such as hydroxyl group and amino group. An attempt has been made to determine STV by reacting at the Gibb's reagent.

4.2 REACTION INVOLVED



STV Gibb's reagent yellow color complex

Scheme 4: Reaction pathway between stavudine and Gibb's reagent.

4.3 REAGENTS USED

4.3.1 2, 6-Dichloroquinone Chlorimide or Gibb's regent 0.5% (w/v)

0.5 g of Gibb's reagent was accurately weighed transferred into a 100 ml calibrated volumetric flask and diluted to the mark with Isopropanol

4.4 PREPARATION OF STANDARD CALIBRATION CURVE

4.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of STV (bulk drug) was dissolved in 10.0ml of methanol in 100ml volumetric flask and sonicated for about 15min to enhance the solubility and volume was made up to the mark with double distilled water i.e. 1.0 mg/ml (Stock solution A).

From the above stock solution-A 10 ml of aliquot was pipette out in 100 ml volumetric flask and the volume was made up to the mark with distilled water to obtain the final concentration of 100 µg/ml (Stock solution B).

4.4.2 Preparation of calibration curve

Aliquots of STV ranging from 0.1- 1.2 ml (2.0- 12 µg/ml) were pipette into as series of 10 ml volumetric flask. To each flask, 1ml of Gibb's reagent was added to each volumetric flask and made up to the volume with distilled water. The absorbance was measured at 528 nm (Fig.7) against a reagent blank. The amount of STV present in the sample solution was computed from its calibration curve. The calibration curve was constructed by plotting absorbance against the initial concentration of STV. The linearity range or Beer's range follows in the range between 2.0-12 µg/ml. The content of STV

was calculated either from the calibration graph or corresponding regression equation.

4.5 ANALYSIS OF TABLET DOSAGE FORM

Ten tablets are taken and finely powdered claimed to contain 30 mg (Stadine). The accurate quantity of powder equivalent to 100 mg of active ingredient was dissolved in 10 ml of methanol later diluted with 50 ml of distilled water and shaken thoroughly for about 5 min. The volume was made up to the mark with distilled water and filtered using whattman filter paper (Stock solution A). From the above stock solution 10.0 ml was pipetted out in 100.0 ml volumetric flask and the volume was made up to the mark with warm double distilled water to obtain the final concentration of 100 µg/ml (Stock solution B). From the stock solution B 0.5 ml of solution is pipetted in to a 10.0ml volumetric flask and then the resultant solution is also analysed as per the procedure and was statically validated using the regression equation

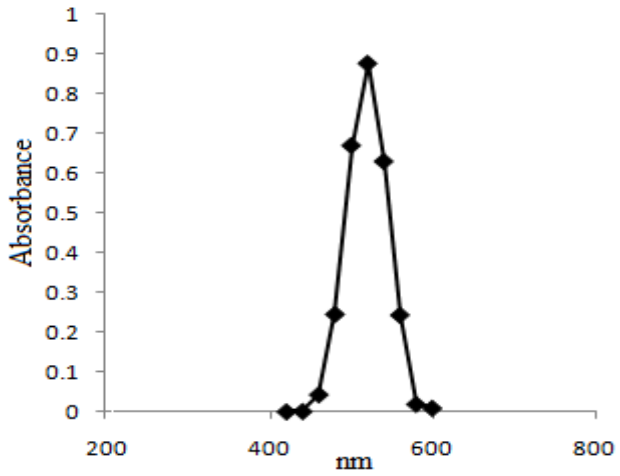


Fig. 7: The graph showing the maximum absorbance method 4

PART B: SIMULTANEOUS ESTIMATION OF LAMIVUDINE, ZIDOVUDINE AND EFAVIRENZ BY UV- SPECTROPHOTOMETRY

Method 5: ESTIMATION LAMIVUDINE, ZIDOVUDINE AND EFAVIRENZ BY THREE WAVELENGTH SPECTROPHOTOMETRY

5.1 PRINCIPLE INVOLVED

The principle involved is the Overlain spectra suggest that lamivudine, zidovudine and efavirenz show absorbance maximum at 271.7 nm, 264 and 246 nm (Fig.8) and efavirenz does not show absorbance at 265 nm (λ_{max} of zidovudine) and 271.7 nm (λ_{max} of lamivudine) lamivudine and zidovudine was estimated by simultaneous equations. Efavirenz is estimated at 246 nm. For this purpose the standard calibration curve of efavirenz was prepared at 246 nm in the concentration range of 2-20 µg/ml. A reference solutions containing exactly the same concentration of lamivudine and zidovudine as determined from the absorbance measurement at 271.7 nm and 264 nm was prepared. The absorbance of the sample solution containing lamivudine, zidovudine and efavirenz was measured at 246 nm against the reference solutions containing lamivudine and zidovudine.

5.2 EQUATIONS

$$C_X = \frac{(A_2ay_1 - A_1ay_2)}{(ax_2ay_1 - ax_1ay_2)} \text{ ------- equation 1}$$

$$C_Y = \frac{(A_2ay_1 - A_1ay_2)}{(ax_2ay_1 - ax_1ay_2)} \text{ ------- equation 2}$$

Where Cx = concentration of lamivudine

Cy = concentration of zidovudine

ax_1 and ax_2 are the absorptivity of lamivudine at 271.1nm and 264 nm

ay_1 and ay_2 are the absorptivity of zidovudine at 271.1nm and 264 nm

A_1= absorbance of lamivudine at 271.1nm

A_2= absorbance of lamivudine at 264 nm

5.3 REAGENTS USED

5.3.1 Selection of solvent (95% methanol)

After assessing the solubility of drug in different solvents 95% methanol was used as common solvent for developing spectral characteristics.

5.4 PREPARATION OF STANDARD CALIBRATION CURVE

5.4.1 Preparation of standard stock solution

The standard stock solution of lamivudine, zidovudine and efivarenz was prepared by dissolving 25 mg of each drug in 25 ml volumetric flask and 10 ml of methanol was added to it and sonicated for 5 minutes and the volume was made up to 25 ml with methanol (Stock solution A). 0.1 ml of the stock solution A was taken into a 10 ml volumetric flask and further diluted to get standard solutions of 10 µg/ml.

5.4.2 Preparation of calibration curve

Aliquots of LAM, ZVD and EFZ ranging from 0.2-2 ml (2.0- 20 µg/ml) were pipette into as series of 10ml volumetric flask. The volume was made up to the mark at with methanol the absorbances were measured at 271.7 nm, 264 nm and 246 nm respectively for LAM, ZVD and EFZ. The amount of LAM, ZVD and EFZ present in the sample solution was computed from its calibration curve the calibration curve was constructed by plotting absorbance against the

initial concentration of LAM, ZVD and EFZ. The linearity range or Beer's range follows in the range between 2.0- 20 µg/ml. The content of LAM, ZVD and EFZ was calculated either from the calibration graph or corresponding regression equation. Overlain spectra suggest that lamivudine and efavirenz and zidovudine show absorbance maximum at 271.7 nm, 264 and 246 nm and efavirenz does not show absorbance at 265 nm (λ_{max} of zidovudine) and 271.7 nm (λ_{max} of lamivudine) lamivudine and zidovudine was estimated by following simultaneous equations.

Efavirenz is estimated at 246 nm. For this purpose the standard calibration curve of efavirenz was prepared at 246 nm in the concentration range of 2-20 µg/ml. A reference solutions containing exactly the same concentration of lamivudine and zidovudine as determined from the absorbance measurement at 271.7 nm and 264 nm was prepared. The absorbance of the sample solution containing lamivudine, zidovudine and efavirenz was measured at 246 nm against the reference solutions containing lamivudine and zidovudine. The concentration of efavirenz is obtained from the calibration curve plotted at 246 nm.

5.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets (LAZID-E) were taken, their average weight was determined, and were crushed to fine powder. Then powder equivalent to 50 mg of lamivudine and 100 mg of zidovudine was put into a 100 ml volumetric flask and dissolved in 30 ml methanol with vigorous shaking for 5-10 minutes. Finally volume was adjusted with same solvent up to 100 ml. This solution was transferred to a 100 ml of volumetric flask through a whatman #41 filter paper. The residue was washed twice with methanol, and combined filtrate was made

up to the 100 ml mark with methanol. The solutions were further diluted with methanol to get a solution containing 10 µg/ml of lamivudine. This was analyzed at 271.7 nm and 264 nm wave lengths and the values of absorptions were substituted in equations 1 and 2 to obtain the content of lamivudine and zidovudine. Having determined the concentration of lamivudine and zidovudine reference solutions containing exactly the same concentrations of lamivudine and zidovudine (µg/ml) as contained in the stock solution was prepared. The absorbance of the stock solution was measured at 246 nm against reference solution in the spectrum mode of the instrument and the concentration of efavirenz was obtained from the calibration curve of efavirenz.

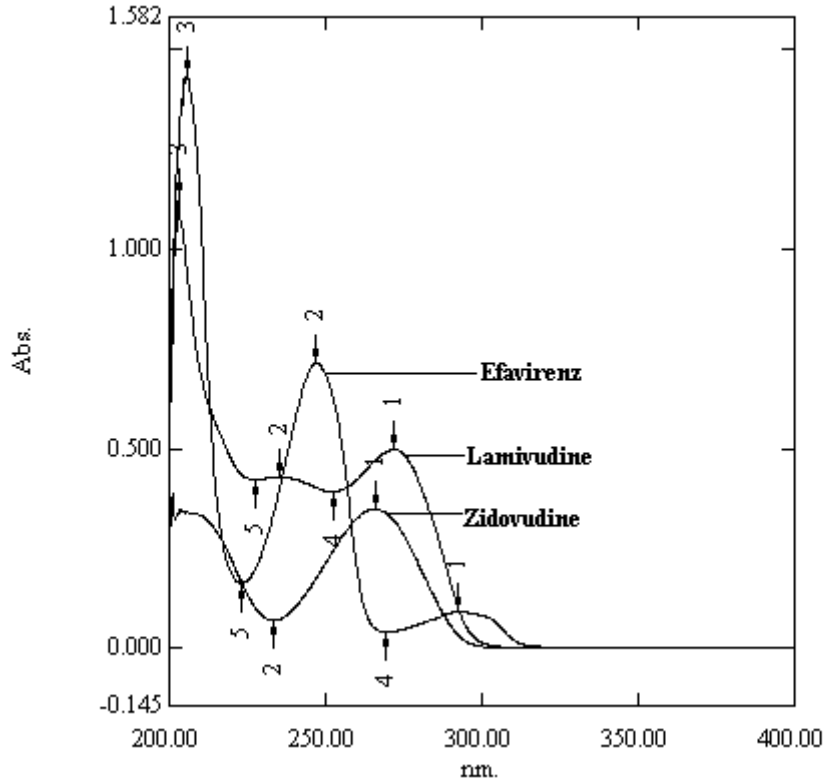


Fig. 8: Overlain spectra of efavirenz, lamivudine and zidovudine.

PART C: RP-HPLC METHOD

Method 6: SIMULTANEOUS ESTIMATION OF LAMIVUDINE, ZIDOVUDINE AND NEVIRAPINE

6.1 PRINCIPLE INVOLVED

The principal involved in HPLC is, when a mixture of two components is applied on the stationary phase, the components with strong affinity for stationary phase binds to it and the one with less affinity for stationary phase separated out. The degree of separation depends on the surface area of the adsorbent so the particle size of the column should be small.

6.2 EQUATIONS

Amount in mg found (F) = $\frac{A_{spl}}{A_{std}} \times \frac{W_{spl}}{D_{std}} \times \frac{D_{spl}}{W_{spl}} \times \frac{P}{100} \times TW$

Theoretical plates (N) = $5.54(t/w)$

Tailing factor (T_f) = $W_{0.05} / 2f$

% area difference = $\frac{Af - Ai}{Ai} \times 100$

6.3 REAGENTS USED

6.3.1 Phosphate buffer pH 3

Potassium dihydrogen phosphate was used to prepare phosphate buffer. 340 mg of potassium dihydrogen ortho phosphate was accurately weighed and transferred into a 250 ml volumetric flask to obtain 0.01M solution and finally

the PH was adjusted to 3 by adding drop by drop orthophosphoric acid (Maximum up to 2 drops).

6.4 PREPARATION OF STANDARD CALIBRATION CURVE

6.4.1 Preparation of standard stock solution

Standard stock solution (1000 µg/ml) of Lamivudine, Zidovudine and Nevirapine were prepared separately in methanol. The working standard solutions were prepared and further diluted so as to contain a mixture of Lamivudine, Zidovudine and Nevirapine, within the linearity range from 5.0-100.47 µg/ml for lamivudine, 10.0-170.0 µg/ml for zidovudine and 5.0-60.0 µg/ml for nevirapine respectively**.**

6.4.2 Preparation of calibration curve

Aliquots of LAM, ZVD and NVP ranging from 0.1- 2 ml (2.0- 100 µg/ml) were pipette into as series of 10ml volumetric flask. The volume was made up to the mark at with methanol the absorbances were measured at 280.0 nm. The amount of LAM, ZVD and NVP present in the sample solution was computed from its calibration curve the calibration curve was constructed by plotting absorbance against the initial concentration of LAM, ZVD and NVP. Linearity was 5.0-100.47 µg/ml for lamivudine, 10.0-170.0 µg/ml for zidovudine and 5.0-60.0 µg/ml for nevirapine respectively**.**

6.4.3 Chromatographic conditions maintained

The quantification was carried out using **Shimadzu** HPLC 2010, C_{18} (250 × 4.6mm, 5µ) column and mobile phase comprising of methanol and phosphate buffer (pH 3) in proportion of 45:55 (v/v). Flow rate was maintained at rate of 0.8 ml/min and the effluent was monitored at 280 nm. The retention time of

Lamivudine Zidovudine and Nevirapine were 3.12, 5.01 and 7.08 min respectively. The software used was **LC solution.**

6.5 ANALYSIS OF TABLET DOSAGE FORM

Ten tablets, CYTOM-N (ALKEM), each containing 150 mg of Lamivudine, 300 mg of Zidovudine and 200 mg of Nevirapine were weighed and finely powdered. Powder equivalent to 15 mg of Lamivudine, 30 mg of Zidovudine and 20 mg of Nevirapine was weighed respectively and transferred to a standard volumetric flask. The contents were mixed thoroughly and filtered through a 0.45 µm membrane filter. 10 µl of the sample was injected in to HPLC system for the analysis. The results are reported in Table 15. The chromatogram of tablet is as shown in figure 10.

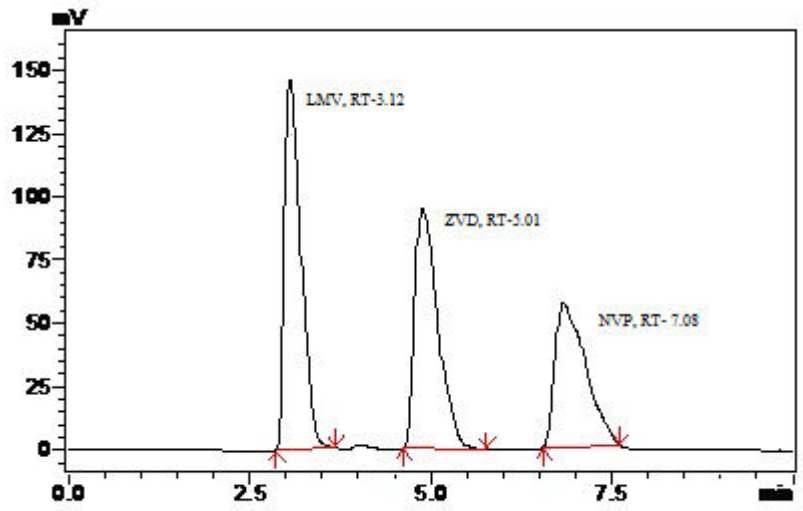


Fig. 9: Typical Chromatogram of Lamivudine, Zidovudine and Nevirapine in mixed standard solution

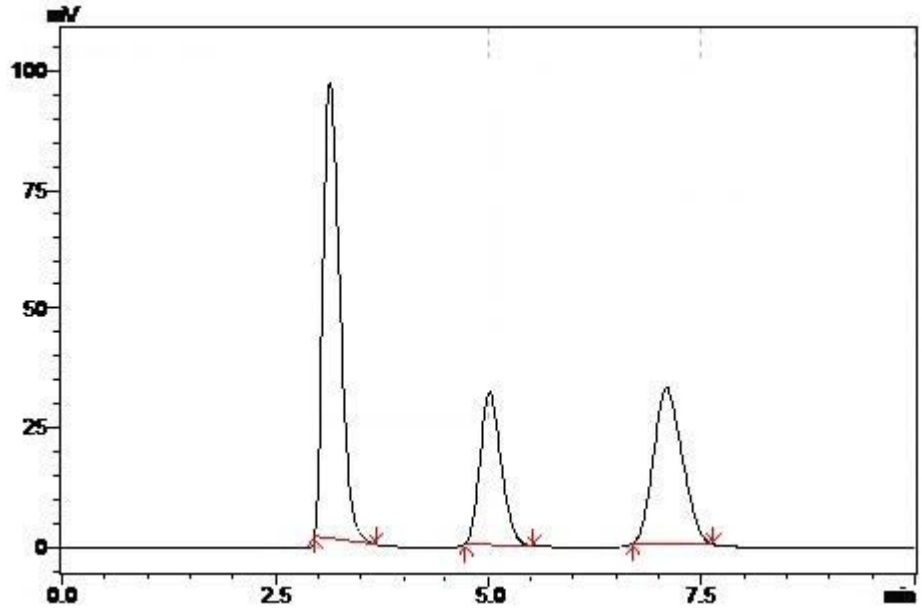


Fig. 10: Typical Chromatogram of Lamivudine, Zidovudine and Nevirapine in Tablet dosage form

PART D: GC METHOD

Method 7: ESTIMATION OF LAMIVUDINE BY GAS CHROMATOGRAPHIC METHOD USING ETHYL CHLOROFORMATE AS A DERIVATIZING REAGENT

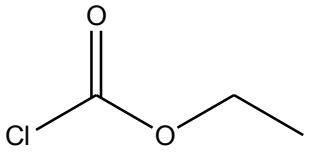
7.1 PRINCIPLE INVOLVED

LMV analysis was performed after derivatization with ECF (precolumn derivatization). The method development for the assay of LMV was based on its chemical properties. LMV is a polar molecule and, therefore, a polar solvent methanol was used as the diluent. PHZ was used as an internal standard. The capillary column coated with 5% diphenyl/ 95% dimethyl polysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations.

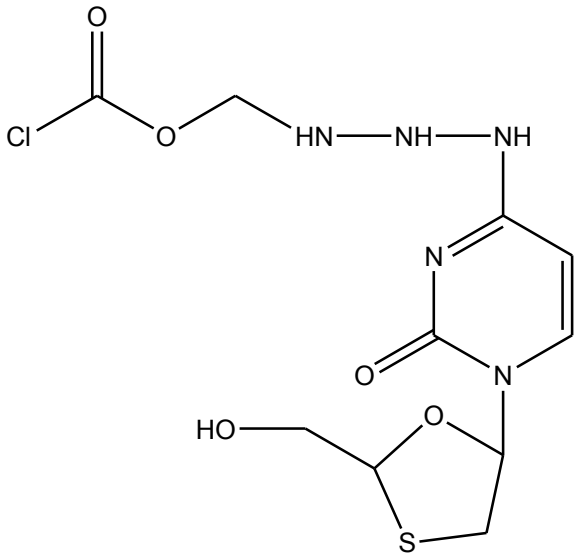
The compounds LMV and PHZ reacted with reacted with ECF to form a volatile product **(scheme 5)** and eluted from a capillary GC column, each has single peak. The reaction was carried out in methanol. A better GC response (average peak height/peak area) was observed using an aqueous solution containing pyridine as the reaction medium. The effect of pH on the derivatization was examined between 1-10 at unit interval.

7.2 REACTION INVOLVED

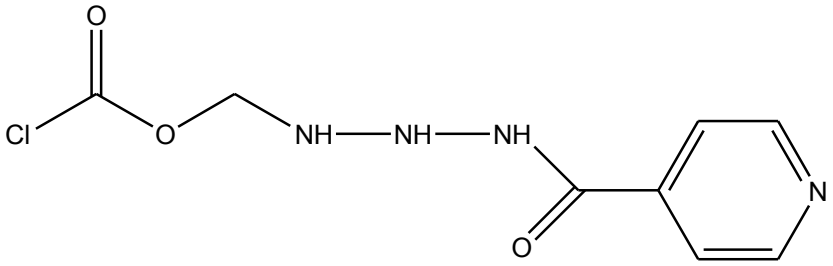
(a)



(b)



(c)



Scheme 5: Structure diagram of the derivative of ECF (a) ECF (b) LMV (c) PHZ

7.3 REAGENTS USED

7.3.1 Phenyl Hydrazone (Internal standard):

1mg of phenyl hydrazone was accurately weighed and transferred into a 10 ml volumetric flask and further 0.5 ml was withdrawn and transferred into another 10ml volumetric flask to get a final concentration of 0.05 mg/ml.

7.3.2 Chloroform: Normal chloroform of analytical quality from Mark was used for extraction.

7.3.3 Ethyl Chloroformate

Ethyl chloroformate (ECF) was used as a precolumn derivitizing reagent.

7.4 PREPARATION OF STANDARD CALIBRATION CURVE

7.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of LMV (bulk drug) was dissolved in 10.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15min to enhance the solubility and volume was made up to the mark with methanol i.e. 1.0 mg/ml (Stock solution A).

7.4.2 Preparation of calibration curve

From the stock solution A serial dilutions were done to obtain 10 to 50 ng/ml. Internal standard phenyl hydrazone 0.05 mg/ml was prepared. The different concentrations of the internal standards resulted from adjustment of the height of its peaks to those of analytes. The solutions were kept at low temperatures and were protected from light. To each volumetric flask ethyl chloroformate 1ml was added and further heated to 70˚C for 5 minutes. The solutions were further extracted for with chloroform and redissolved in methanol

7.4.3 Chromatographic conditions

GC studies were carried out on SHIMADZU model 2014 (shimadzu Technologies, Japan) coupled with a split/split less injector, operated in a split-mode and FID. The computer with GC solutions software controlled the gas chromatograph, and a Cannon laser printer was used. Rtx-5 capillary column (cross bond 5% diphenyl/ 95% dimethyl polysiloxane) with a length of 30 meters and an internal diameter of 0.25 mm was used throughout the study.

The GC-FID parameters used in the method development were based on the boiling point. The injection port and detector temperature were set to 100˚C and 250˚C, respectively. Different temperature programs were investigated for GC oven. The end of this investigation, the best temperature program was selected for a good resolution.

Manual split injection (split ratio 4:1) of approximately 3 µl sample was performed at an inlet temperature of 150˚C. The detector temperature was set to 250˚C. After injection the oven temperature was increased quickly from 50˚C to 100˚C, which was held for 1 min, then programmed within 5 min to 250˚C at a rate of 20˚C per min which was held for 4min.

Nitrogen at a flow rate of 2 ml/min was used as a carrier gas. Synthetic air (flow rate of 100 ml/min), hydrogen (25 ml/min) were fed to the FID. All the gases used in these studies were of Pharamcopoeial purity.

7.5 ANALYSIS OF TABLET DOSAGE FORM

The studies were conducted on the drug LMV, Hepitec (100mg, GSK) and Viramid (100mg, Lupin). The tablets were powdered, and an amount of

powder equivalent to the average weight of a tablet was mixed with methanol and shaken for 15 min at a frequency of approximately 3 cycle's s^{-1}. The solution was then filtered through 0.2 µm whatman filter paper. The prepared solutions were of concentration 10 , 30 and 50 ng/ml, sample solution were obtained by following the same procedure as in the construction of calibration graph. The solutions were heated to 70˚C for 5 minutes. Further the solutions were extracted with chloroform and redissolved in methanol. The internal standard of concentration 50 µg was added to each of the sample solution. The chromatogram obtained is shown in the **fig.12.**

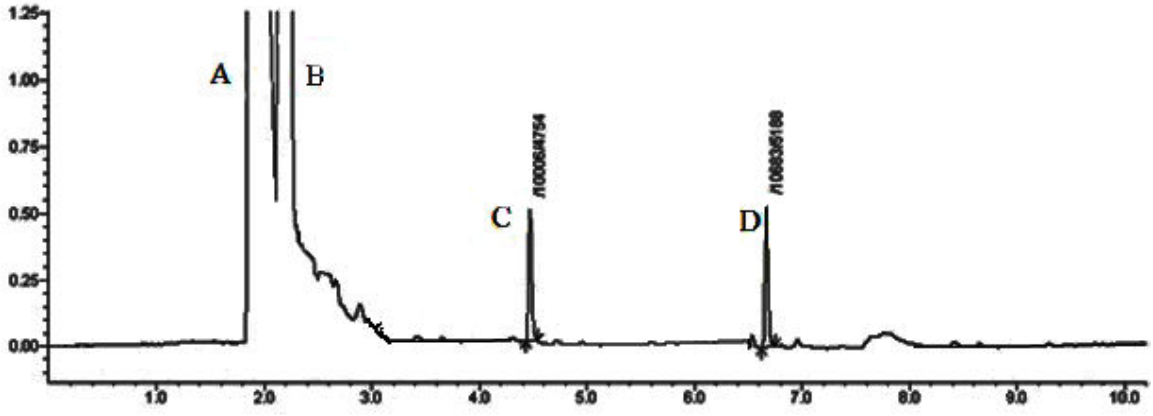


Fig. 11: Chromatogram obtained from Lamivudine (LMV) and Phenyl hydrazine (PHZ) solution, Methanol (A), ethyl chloroformate (B), lamivudine (C) and phenyl hydrazine (D).

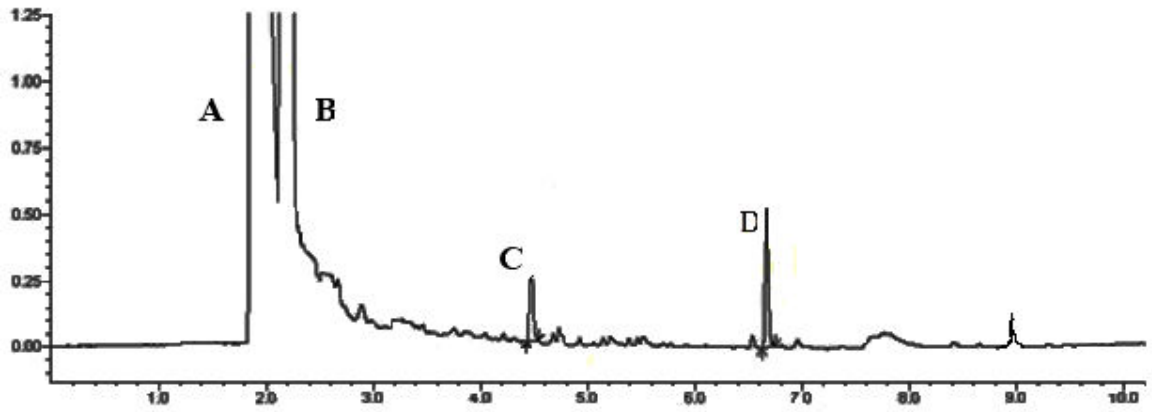


Fig.12: Chromatogram obtained from Lamivudine (LMV) tablet solution. Methanol (A), ethyl chloroformate (B), lamivudine tablet (C) and phenyl hydrazine (D).

5. RESULTS

PART A: UV-VISIBLE SPECTROPHOTOMETRIC METHODS FOR LMV AND STV

Method 1: Estimation of lamivudine by MBTH reagent

Method 2: Estimation of lamivudine by Bromophenol blue dye

Table 1: Analytical and validation parameters for the assay of LMV

Parameter	**Method 1**	**Method 2**
Colour	Red	Green
λ_{max}(nm)	595 nm	659 nm
Beer's law range (µg/ml)	1-5	1-7

Molar absorptivity(L mol^{-1} cm^{-1})	$0.6075x10^3$	0.286×10^3
Limit of detection (µg/ml)	0.1	0.3
Limit of quantification (µg/ml)	0.3	0.9
Intercept	0.050	0.045
Slope b ± S_b	0.0023	0.0013
Correlation coefficient, R	0.9913	0.9967

Table 2: Intra-day precision and intra-day error of the methods 1 and 2

Method	**LMV(µg/ml) taken**	**LMV(µg/ml) found**	**Relative error (%)**	**RSD (%)**
	1	0.9	0.6	0.4
BPB	2	1.92	0.13	0.2
	3	2.89	0.1	0.2
	4	3.91	0.15	0.1
MBTH	5	4.96	0.11	0.1
	6	5.88	0.25	0.1
	7	7.97	0.25	0.1

Table 3: Assay of drug in pharmaceutical formulations (tablets)

Preparation	**Label claim (mg per tablet)**	**Percentage found (%)+ SD**	
		MBTH (Method 1)	BPB (Method 2)
VIROLAM	100	99.9±0.5	99.8±0.5
LAMIDAC	100	99.9±0.4	99.6±0.4
LAMIVIR	150	99.9±0.2	99.4±0.2

Method 3: Estimation of stavudine by selective oxidation using Cerium (IV) ammonium sulphate

Method 4: Estimation of stavudine by 2, 6-Dichloroquinone Chlorimide (Gibbs regent)

Table 4: Optical characteristics of spectrophotometric methods 3 and 4

Parameter	Method 3	Method 4
Color	Colourless	Yellow colour
λ_{max}(nm)	282	528
Beer's law range (µg/ml)	2-10	2-12
Molar absorptivity (l $mol^{-1}cm^{-1}$)	3.17 x 10^4	3.32 x 10^4
Sandell's sensitivity (µg cm^{-2})	0.170212	0.0775
Limit of detection (µg/ml)	0.8712	0.984
Limit of quantification (µg/ml)	2.64	2.98
Correlation coefficient, R	0.991	0.9967
Slope b	0.010	0.055
Intercept a	0.184	0.158

Table 5: Results of determination of STV in formulations and statistical comparison for method 3 and method 4

Pharmaceutical dosage form	Labelled Amount	Amount found by proposed methods (mg)
Stadine (Emcure, ARV),		
Method-3	100 mg	99.6
Method-4	100 mg	99.8
Stag (Hetero, Genx)		
Method-3	100 mg	98.1
Method-4	100 mg	99.7
Virostav (Ranbaxy, India)		
Method-3	100 mg	98.91
Method-4	100 mg	99.4

Table 6: Accuracy and method precision data for the developed method 3 and 4

Drug	S.no	Label Claim (mg)	Amount found*	% Purity*	Avera-ge (%)	S.D	R.S.D[a]	RSD[b]	S.E.M
Method-3 **Stadine (stavudine)**	1	100	98.642	99.321	99.47	0.4344	0.4379	0.8456	0.1773
	2		101.604	100.82					
	3		97.057	100.05					
	4		98.542	99.271					
	5		94.164	97.082					
	6		100.604	100.30					
Method –4 **Stag (stavudine)**	1	100	97.17	99.8	98.34	0.376	0.362	0.723	0.124
	2		100.2	100.43					
	3		94.23	96.22					
	4		98.22	99,16					
	5		99.21	99.43					
	6		99.53	99.23					

SD: Standard deviation; SEM. Standard error of mean; RSD. Relative standard Deviation; a. intraday precision, b. inter day precision.

Table 7: Comparison of the results between methods proposed 3 and 4

Parameter	Method-3	Method-4
Percentage recovery (%)	99.20	99.41
Accuracy	99.47	98.34
Standard deviation	0.4344	0.376
Standard error of mean	0.1773	0.124
intraday precision	0.4379	0.362
inter day precision	0.8456	0.723

PART B: SIMULTANEOUS ESTIMATION OF LAMIVUDINE, ZIDOVUDINE AND EFAVIRENZ BY UV- SPECTROPHOTOMETRY

Method 5: By Three wavelength spectrophotometry

Table 8: Optical characteristics data and validation parameters of method 5

Parameters	Values		
	Lamivudine	Zidovudine	Efavirenz
Working λmax	271.7 nm	265 nm	246nm
Beers law limit (µg/ml)	2-20	2-20	2-20
Absorptivity*	0.0726	0.0298	0.0179
Correlation coefficient*	0.979	0.990	0.974
Intercept*	0.0312	0.0673	-0.038
Slope*	0.351	0.117	0.108
LOD*(µg/ml)	0.106	0.051	0.148
LOQ*(µg/ml)	0.324	0.28	0.432
Intra-day* (precision) (%COV)	0.275	0.231	0.341
Inter-day (precision) (%COV)	0.983	0.986	0.670

Table 9: Result of recovery studies of method 5

Method	Recovery level (Added amount)	Percent recovery ± SD #		
		Lamivudine	**Zidovudine**	**Efavirenz**
Three wavelength spectrophotometry	80%	98.99 ± 0.0456	100.20 ± 0.1045	101.20 ± 0.355
	100%	99.85 ± 0.0345	101.20 ± 0.7567	98.97 ± 0.432
	120%	99.50 ± 0.2321	100.10 ± 0.6578	98.90 ± 0.870

Average of three estimations at each level of recovery, SD: standard deviation

Table 10: Analysis data of tablet Formulation of method 5

Method	Drug	Label Claim mg/tab	Amount found* mg/tab	Label claim (%)	S.D*	% COV
Three wavelength spectrophotometry	**LMV**	150	148.23	99.03	0.6574	0.634
	ZDV	300	299.99	99.95	0.4522	0.462
	EFZ	600	600.23	100.38	0.162	0.151

* Average of six estimations of tablet formulation, COV coefficient of variation

PART C: RP-HPLC METHOD

Method 6: Simultaneous estimation of lamivudine, Zidovudine and Nevirapine

Table 11: Determination of Lamivudine, Zidovudine and Nevirapine in Tablet dosage form of method 6

	Label Amount(mg)	Amount(µg) Taken	Found	%RSD	%W/W
Lamivudine	150	15	149.67	0.211	99.7
Zidovudine	300	30	298.82	0.118	99.6
Nevirapine	200	20	19.86	0.266	99.3

Table 12: Recovery Studies of Lamivudine, Zidovudine and Nevirapine of method 6

Name of the Drug	Sample ID	Amount added (µg/ml)	Amount recovered (µg/ml) n=3	Percentage recovery	Average percentage recovery
Lamivudine	80%	10	9.84	98.4	98.67
	100%	15	14.8	98.6	
	120%	20	19.8	99.0	
Zidovudine	80%	20	19.7	98.5	98.23
	100%	30	28.9	96.5	
	120%	40	39.89	99.72	
Nevirapine	80%	10	9.98	99.8	98.53
	100%	20	19.3	96.5	
	120%	30	29.8	99.3	

Table 13: Intraday Precision of Lamivudine, Zidovudine and Nevirapine of method 6

Parameters	Lamivudine	Zidovudine	Nevirapine
Mean	236.335	2.567	1.0
S.D	0.6819	0.0228	0.0089
%RSD	0.288	0.88	0.89

Table 14: Inter day Precision of Lamivudine, Zidovudine and Nevirapine of method 6

Parameters	Lamivudine	Zidovudine	Nevirapine
Mean	236.99	2.642	1.015
S.D	0.058137	0.02682	0.00268
%RSD	0.02453	1.015	0.264

Table 15: LOD and LOQ of Lamivudine, Zidovudine and Nevirapine of method 6

Parameters	Lamivudine (µg)	Zidovudine (µg)	Nevirapine (µg)
Limit of detection	0.116	0.129	0.018
Limit of quantitation	0.348	0.388	0.055

Table 16: System Suitability Parameters of method 6

Parameters	Lamivudine	Zidovudine	Nevirapine
Theoretical plates/meter	793.641	1218.767	1353.339
HETP	189.002	123.007	110.873
Tailing factor	1.638	1.801	2.105
Rt	3.12	5.01	7.08
Resolution	0.0	3.670	2.985

PART D: GC METHOD

Method 7: Estimation of Lamivudine by Gas Chromatographic method using Ethyl Chloroformate as a Derivatizing reagent

Table 17: Retention times of LMV and PHZ of method 7

Compound name	Retention time (min)
Lamivudine	4.5
Phenyl hydrazine	6.8

Table 18: Results from analysis of LMV in pure drug of method 7

Parameter	Value
Linearity (ng)	10-50
LOD (ng)	0.54
LOQ (ng)	1.64
Precision	1.53
Recovery %	99.2

Table 19: Precision and accuracy of LMV by GC-FID method

Added amount (ng/ml)	Within-day			Between-day		
	Found ±SD (ng/ml)	Accuracy	Precision RSD%[a]	Found ±SD (ng/ml)	Accuracy	Precision RSD%[a]
10	10.12 ± 0.039	3.20	3.02	9.87±0.05	4.80	3.82
20	20.42± 0.136	1.47	1.79	20.12±0.172	2.93	2.23
30	31.21± 0.391	4.56	1.52	30.31±0.752	5.12	2.86

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation,
a average of six replicate determinations, Accuracy: (%relative error) (found-added)/added x100

Table 20: Recovery values of LMV by GC-FID method in pharmaceutical preparations

Commercial preparation		LMV tablet		
Method	Added (ng/ml)	Found ±SD (ng/ml)	Recovery (%)	RSD%[a]
GC-FID	10	9.52 ± 0.192	96.5.0	3.78
	15	15.13 ± 0.263	100.86	2.59
	20	20.11 ± 0.631	101.2	2.47

SD: Standard deviation of six replicate determinations, RSD: Relative standard derivation
a average of six replicate determinations

6. DISCUSSION

Method 1: Estimation of lamivudine by MBTH reagent

Method 1 is based on the oxidation followed by coupling of 3-methyl-2-benzothiazolinone hydrazone with LMV in the presence of ferric chloride to form a green colored chromogen. Actually, this is an iron catalyzed oxidative coupling reaction of MBTH with the drug. Under reaction conditions, on oxidation, MBTH loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling agent. This intermediate undergoes electrophilic substitution with the drug to form the colored product. The reaction path way is represented in **scheme 1**. The linearity range or Beer's range follows in the range between 1 to 8 µg/ml **(Fig.1)**

Effect of MBTH Concentration

The studying of MBTH concentrations revealed that the reaction was dependent on MBTH reagent **(Fig.2)**. The absorbance of the reaction solution increased as the MBTH concentration increased, and the highest absorption intensity was attained at MBTH concentration of 0.5 % (w/v). Higher MBTH concentrations up to 1.25 % had no effect on the absorption values. Further experiments were carried out using 0.5 % MBTH.

Effect of $FeCl_3$ Concentration

The studying of $FeCl_3$ concentrations revealed that the reaction was dependent on $FeCl_3$ reagent **(Fig.2).** The absorbance of the reaction solution increased as the $FeCl_3$ concentration increased, and the highest absorption intensity was attained at $FeCl_3$ concentration of 1 % (w/v). Higher $FeCl_3$ concentrations up

to 1.6 % had no effect on the absorption values. Further experiments were carried out using 1 % $FeCl_3$.

Method 2: Estimation of lamivudine by Bromophenol blue dye (BPB).

The method is based on the application of acidic dyes for the spectrophotometric determination of LMV. The structural formula of LMV feature amine group, suggests the use of acidic dyes (BPB) as chromogenic reagents. The acid dye technique is a general procedure for the quantitative analysis of a variety of pharmaceutical amines. In practice, an aqueous solution containing the amine and a suitable indicator dye is shaken with an organic solvent. The concentration of the resulting ion-pair is then determined spectrophotometrically. Few studies have reported the analysis of pharmaceutical compounds through formation of ion-pair, without extraction, followed by spectrophotometric estimation. The reaction path way is represented in **scheme 2**. When added in increasing concentrations of LMV to a fixed concentration of BPB there is a proportional increase in absorbance at the respective λ_{max}. Preliminary experiments were performed to fix the upper concentrations of the dye that could be determined spectrophotometrically.

Effect of BPB Concentration

Various concentrations of BPB were tried and the absorbance maximum was found with 0.1% of BPB. Higher BPB concentrations up to 1 % had no effect on the absorption values. Further experiments were carried out using 0.1 % BPB. The colour intensity increased with time. The maximum absorbance was seen only after 5 minutes and there was stable after 5 minutes as shown in the

(**Fig. 5**.) All the experimental procedures were carried out in room temperature.

Method 3: Estimation of stavudine by selective oxidation using Cerium (IV) ammonium sulphate

Cerium (IV) and iron (III) ammonium sulphates are strong oxidizing agents and are utilized extensively for the determination of organic compounds. The reagent blank has negligible absorbance in the range used for detection of the STV. Beer's law is obeyed in the range of 2–10 µg/ml. The apparent molar absorptivity of the drug was found to be 3.17 x 10^4 l $mol^{-1}cm^{-1}$. STV on oxidation with Cerium (IV) or iron (III) in acidic medium had shown a maximum absorption at 282 nm with the reagents **(Scheme 3).**

Method 4: Estimation of stavudine by 2, 6-Dichloroquinone Chlorimide (Gibbs regent)

Oxidation of STV was attempted in the present study for the development of spectrophotometric method for its determination. The method 4 is based on the reaction between the Gibb's reagent and STV. The Gibb's reagent reacts with STV and results in the formation of yellow colored complex (Scheme 4). The reagent blank has negligible absorbance in the range used for detection of the STV. Beer's law is obeyed in the range of 2–12 µg/ml. The apparent molar absorptivity of the drug was found to be 3.32 x 10^4 l $mol^{-1}cm^{-1}$. Optimization of the spectrophotometric conditions was intended to take into account the various goals of method development. Analytical conditions were optimized

via a number of preliminary experiments. The effect of the Gibb's reagent concentration was studied and found that 0.5% gave good absorbance values so further experiments were carried out using 0.5 % Gibb's reagent.

Method 5: By Three wavelength spectrophotometry

During the time of preparation of samples the maintenance of room temperature and the samples should be freshly prepared. The samples prepared before 3 days cannot be used as the drug had shown a reduce in absorbance at its maximum absorption point. The standard solution are prepare only between 2-20 µg/ml above this range the absorbances were out of Beer's range.

The validity and reliability of proposed methods are assessed by recovery studies. Sample recoveries for both the methods are in good agreement with their respective label claims, which suggest non-interference of formulation additives in estimation.

Method 6: Simultaneous estimation of lamivudine, Zidovudine and Nevirapine by HPLC

Present study indicates the suitability of reversed-phase column procedure for the simultaneous determination of Lamivudine, Zidovudine and Nevirapine in combined dosage form. The chromatographic conditions were optimized by making a change in mobile phase composition, change in pH, and changes in the buffers used in the mobile phase. Different ratios phosphate buffer and methanol were experimented in order to optimize the mobile phase. Finally a mixture of methanol and phosphate buffer (adjusted to pH-3) in the ratio of 55:45 was taken into consideration. All system suitability parameters

(Theoretical plates, tailing factor and resolution) were accurate. The run time was set at 10 min as the three drugs appeared on the chromatogram below 10 min. The developed LC method was found to be specific for simultaneous estimation of Lamivudine, Zidovudine and Nevirapine.

Method 7: Estimation of Lamivudine by Gas Chromatographic method using Ethyl Chloroformate as a Derivatizing reagent

LMV analysis was performed after derivatization and the internal standard technique was used for computation. The method development for the assay of LMV was based on its chemical properties. LMV is a polar molecule and, therefore, a polar solvent methanol was used as the diluent. The capillary column coated with 5% diphenyl/ 95% dimethyl polysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations.

The compounds LMV and PHZ reacted with reacted with ECF to form a volatile product **(Scheme 5)** and eluted from a capillary GC column, each has single peak. The reaction was carried out in methanol. A better GC response (average peak height/peak area) was observed using an aqueous solution containing pyridine as the reaction medium. The effect of pH on the derivatization was examined between 1-10 at unit interval. It was observed that derivatization occurred at pH value above 6. The reaction mixture was sonicated at room temperature (30˚C) for 5-20 min at an interval of 5 min and the optimum response was observed within 15 min. Chloroform was used for extraction of derivatives, as reported for related compounds. The recovery studies were done and the percentage recovery of LMV was found to be 99.2%

7. SUMMARY

Several drugs are available in the form of pharmaceutical formulations to control diseases. Methods of assay for controlling the concentration of these chemicals in the medicine and in the living body are necessary. Pharmaceutical analysis occupies a pivotal role in statutory certification of drugs and their formulations either by the industry or by the regulatory authorities. The complexity of the problem encountered in pharmaceutical analysis coupled with importance of achieving the selectivity, speed, cost, simplicity, precision and accuracy results in new methods of analysis being quickly adopted by pharmaceutical industry.

Formulations containing combinations of drugs for potentiating or complementing another in therapy are on the increase. In some cases, no precise analytical methods are reported and quite often the reported procedures need improvements or changes keeping in the view of the advances.

Among several instrumental techniques (HPLC, GC, Fluorimetry, NMR, mass spectroscopy covering IR, UV and visible regions) available for assay of drugs, visible spectrophotometric methods depend only on the nature of chemical reaction utilized for colour development and not on sophistication of the equipment. GC method is highly selective and sensitive compared to spectroscopic or other chromatographic methods. GC method is also cost effective as expensive solvents are not required and it is a versatile tool for qualitative and quantitative analysis of drugs and pharmaceuticals.

Due to the importance of analysis, present analytical method has been developed for some of the widely used antiretroviral drugs such as lamivudine,

zidovudine, stavudine, nevirapine and efavirenz. Hence we planned o develop HPLC, GC and spectrophotometric methods.

There is a wide scope for the development of new analytical methods for the assay of the antiretroviral drugs. Spectrometric (part A&B), HPLC (part C) and GC (part D) techniques have been used as a tool in the present thesis work. The above tools have been used for the development of new analytical methods for the assay of drugs mentioned by exploiting their characteristics, physical and chemical properties (dependent on basic moieties and functional groups present in each drug).

The contents of the thesis have been divided into eight chapters and appropriate reference has been placed at the end of the ninth chapter.

Chapter-1 opens with the introduction giving a brief account of various aspects like chemotherapy, instrumental methods, concepts of spectroscopy, various reagents, types of chromatographic methods and method validation. The introduction includes brief account on selected drugs.

Chapter- 2 explains the objective of present investigation adopted for selected drug.

Chapter-3 for review of literature gives details on reported methods, clinical studies under different experimental conditions, drug introduction and therapeutic importance of selected drug for present work. Also it gives information about the necessity to investigate new analytical methods for Lamivudine, Zidovudine, Stavudine, Nevirapine and Efavirenz for the quantitative estimation.

Chapter-4 contains four parts. Part A gives information regarding UV spectroscopic method (1 - 4). It briefly explains about principle involved in the

method, proposed reaction, reagents and materials used, preparation of standard solutions, preparation of calibration curve, analysis of tablet dosage form. Primary amine group in Lamivudine was exploited in the present investigations for the development of the methods.

In method 1 method is based on oxidation followed by coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with LMV in the presence of ferric chloride to form green colored chromogen exhibiting absorption maximum at 659 nm.

In method 2 method is based on the interaction of LMV with 0.1% methanolic solution of bromophenol blue (BPB) to form a stable, red-colored, ion-pair complex peaking at 595 nm

In method 3 method is based on the selective oxidation of this drug with either Ce^{IV} in acid medium and shown absorption maxima at 282 nm.

In method 4 method is based on the formation of a colored oxidative coupling product between 2, 6-dichloroquinone chlorimide and the drug; it had shown absorption maxima at 528 nm. The method obeyed Beer's range in a concentration range of 2-12 μg/ml.

Part B describes the simultaneous estimation of lamivudine, zidovudine and efavirenz in pure and tablet dosage form by using methanol as a solvent was developed and validated. LMV, ZVD and EFZ shown absorbance maximum at 271.7 nm, 264 nm and 246 nm. Validation study reveals that the method is specific, accurate, precise and reproducible. All the three drugs obey Beer's law in the concentration range 2 μg to 20 μg.

Part C describes the HPLC method for assay of LMV, ZVD and NVP in bulk and tablet formulation is carried out. The assay method is found to be specific for the

LMV, ZVD and NVP tablets. The method is found to be linear in the specified range for LMV, ZVD and NVP tablets.

Part D describes the a novel gas chromatographic (GC) method has been developed for the quantitative estimation of lamivudine (LMV) both in bulk drug and pharmaceutical dosage forms. Ethyl chloroformate (ECF) was used as a precolumn derivitizing reagent. Phenylhydrazine (PHZ) was used as an internal standard. GC separation was carried out on a Rtx-5 capillary column (cross bond 5% diphenyl/ 95% dimethyl polysiloxane) with a length of 30 meters and a internal diameter of 0.25 mm with flame ionization detector. The recovery studies were done and the percentage recovery of LMV was found to be 99.2%

Chapter 5 explains the results that are divided into four parts.. In part A, B, C and D result obtained in each of the UV-Visible spectrophotometric method, HPLC and GC method for estimation of Lamivudine, Stavudine, Zidovudine, Nevirapine and Efavirenz are summarized in **Tables 1-20**.

Chapter 6 consists of discussion which is divided into four parts. Part A and B discusses about developed visible spectrophotometric methods. Part C discusses about developed HPLC method. Part D discusses about developed GC method.

Chapter 7 explains the conclusion of the proposed methods for the quantitative estimation of Lamivudine, Stavudine, Zidovudine, Nevirapine and Efavirenz. A set of seven methods has been developed for the purpose and these methods are validated in terms of sensitivity, accuracy and precision.

The methods can also be used for the routine determination of Lamivudine, Stavudine, Zidovudine, Nevirapine and Efavirenz in bulk drug and pharmaceutical formulations.

Chapter 9 contains the references from which the information of Lamivudine, Stavudine, Zidovudine, Nevirapine and Efavirenz and introduction of general methodology was collected.

Chapter 10 contains the list of paper communicated so far to various journals.

8. CONCLUSION

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. When several such drugs, typically three or four, are taken in combination, the approach is known as **highly active antiretroviral therapy**, or **HAART**. The American National Institutes of Health and other organizations recommend offering antiretroviral treatment to all patients with AIDS.

Although various UV-visible methods have been reported for the estimation of LMV, ZDV and STV it was found that the reagents used in the present study were not used and the methods developed are much sensitive and less time consuming compared to the methods previously develop. The simultaneous estimation of LMV, ZDV and EFZ with UV spectrophotometer using triple point method was not investigated.

The work deals with four UV-Visible spectrophotometric methods i.e. oxidation reaction with Cerium (IV) ammonium sulphate, visible spectrophotometric method with BPB, GIBBS reagent and coupling reaction with MBTH reagent.

The methods are validated in terms of sensitivity, accuracy and precision.

A. Comparative Sensitivity

   1> 2 > 3 > 4

B. Comparative accuracy:

   3> 4 >2 > 1

C. Comparative precision:

   3 > 4 > 1 > 2

Simultaneous estimation of LMV, ZVD and NVP by RP-HPLC was also developed. The retention times of the drugs were less when compared to other methods developed. In terms of sensitivity, accuracy and precision the present developed method has shown good results when compared to the existing method.

Lamivudine was quantified by Gas Chromatographic method using Ethyl Chloroformate as a Derivatizing reagent. There were no GC methods reported for lamivudine. LMV analysis was performed after derivatization and the internal standard technique was used for computation. The method development for the assay of LMV was based on its chemical properties. The method developed is very sensitive as the limit of detection is very less **(Table 18).**

Results of analysis of the pharmaceutical formulations revealed that the proposed methods are suitable for their analysis with no interference from the usual additives.

All the methods were found to be linear, precise, accurate, specific and all proved to be sensitive, convenient and effective for the determination of LMV, ZVD, STV, NVP and EFZ in bulk and pharmaceutical dosage forms.

9. BIBLIOGRAPHY

1. Sharma B. Instrumental methods of chemical analysis. 19 ed: Goel Publishing House; 2003.
2. Willard H. Instrumental method of analysis. 7th Edition ed. New Delhi: CBS Publishers and Distributors; 1986.
3. Remington. The Science and practice of pharmacy. 21st edn. (1). 2006.
4. Satoshkar R, Bhandarkar S, Ainapure S. Pharmacology and pharmacotherapeutics. 15th edn. Popular Prakashan. Mumbai. 1996.
5. Wolff M.E. Berger's a Medicinal Chemistry. 4th Edn. Wiley Interscience. New York. 1981.
6. Doserge R.F. Wilson and Gisvold's Text book of organic medicinal and pharmaceutical chemistry. 8th Edn. Lippincott Company. 1982.
7. Korolkovas A. Essentials of medicinal chemistry. 2nd Edn. Wiley Interscience. New York. 1988.
8. Topliss J.G. Quantitative structure Activity relationships of Drugs. vol. 19. Academic Press. London. 1983.
9. Goodman A. and Gilman, Rall. T.W, Nies A. The Pharmacological Basis of Therapeutics, 8th Edn. Pergamon Press. New York. 1988.
10. The drugs and cosmetics act and rules. Government of India publications.1984.
11. Indian pharmacopoeia. Indian pharmacopoeia commission. Ghsziabad.2007.
12. British Pharmacopoeia. Medicines and health care product regulatory agency. London 2010.
13. Reynolds J. E. F. Martindale- The Extra Pharmacopoeia. 28th Edn. The Pharmaceutical Press. London.1982.

14. Ashutosh Kar. Pharmaceutical analysis.1st Edn. Vol I. CBS Publishers and Distributers. New Delhi. 2007.

15. Beckett A. H. Stenlake J. B. Practical pharmaceutical chemistry. 4th Edn. CBS Publishers and Distributers. New Delhi. 2007.

16. Ravi Shankar S. Pharmaceutical analysis. 3rd Edn. Rx Publications. Tirunelvi. 2006.

17. Chatwal G. R, Anand S.K. Instrumental methods of chemical analysis. 5th Edn, Himalaya Publishing House. Mumbai, 1979.

18. Sethi P. D. Quantitative analysis of drugs in Pharmaceutical Formulations.3rd Edn. CBS Publishers. New Delhi. 1986.

19. Sharma B. K. Instrumental method of chemical analysis. 26th Edn. GOEL Publishing house. New Delhi.2007.

20. Gary D.Christian. Analytical chemistry. 4th Edn. John Wiley And Sons Publications. New York. 1986.

21. Foye W. O. Principles of medicinal chemistry. 6th Edn. Wolter Kluwer Pvt. Ltd. New Delhi. 2008.

22. Douglas A. Skoog, Donal M. West, F. James Holler, Stanley R. Crouch. Fundamentals of analytical chemistry. 8th Edn. Cengage Learning India Pvt. Ltd. New Delhi. 2010.

23. David C. Lee, Michael Webb. Pharmaceutical analysis. Black Well publishing. 1994.

24. Finar I. L. Organic chemistry. 6th Edn. Pearson Education. New Delhi. 2004.

25. Willard H, Merritt L, John A. Dean, Frank A. Settle. Instrumental methods of analysis. 7th Edn. CBS Publishers and Distributers. New Delhi. 1986.

26. James E. Gearien, Bernard F. Grabowski. Methods of drug analysis. Lea and Febiger. USA. 1969.

27. Basett J, Denney R. C, Jerry G. H, Mendham J. Vogel's text book of Quantitative inorganic analysis. 4th Edn. England. London Group.1986.

28. John A. Adamoules. Chromatographic analysis of pharmaceuticals. Marcel Dekker. Inc. New York. 1990.

29. Fulton G. Kitson, Barbara S. Larsen, Charles N. McEwen. Gas chromatography and mass spectroscopy-A practical Guide. Academic Press. London. 1996.

30. Raymond P. W. Scott. Gas Chromatography. Library 4 Science. UK. 2003.

31. Marvin C. McMaster. HPLC-A Practical User's Guide. 2nd Edn. John Wiley & Sons. Inc. Hoboken. New Jersey. 2007.

32. Thomas H. Stout, John G. Dorsey. High-Performance Liquid Chromatography. Eurand America. Inc. Vandalia. Ohio. 2002.

33. Michael E. Analytical method Development and validation.

34. Quality Assurance of Pharmaceuticals. Geneva; 1999.

35. WHO Essential drug list: LSN dispersible tablets. geneva; 2009.

36. Method validation guidelines international conference on harmonization. Geneva; 1996.

37. Svobodova D, Krenek P, Fraenkl M and Gasparic J. Colour reaction of phenols with the gibbs reagent. The reaction mechanism and decomposition and stabilisation of the reagent. Microchimica Acta 1977; 67 (3-4): 251-264.

38. Gadkariem EA, Ibrahim KEE, Kamil NAA, Haga MEM and El-Obei HA. A new spectrophotometric method for the determination of methyldopa. Soudi pharmaceutical journal 2009; 17(4): 289-29.

39. Gasparic J, Svobodova D and Pospisilova M. Investigation of the color reaction of phenols with the MBTH Reagent-Identification of organic compounds. Microchimica Acta 1977; 67(3-4): 241-250.

40. Aziz unnisa, venu gopala raju K. New spectrophotometric methods for estimation of Ethacridine lactate in pharmaceutical formulations. International Journal of Chemical Tech 2010; 2(2): 1271-1274.

41. Rekha Rajeevkumar, Rajeev kumar , Nagavalli D. Spectrophotometric Method for Quantitative Estimation of Moprolol in Bulk and Pharmaceutical Preparation. International Journal of Chemical Tech Research 2009; 1(4): 1068-1071.

42. Kreft S, Kreft M. Physicochemical and physiological basis of dichromatic colour". Naturwissenschaften 2007; 94(11): 935.

43. Kreft S, Kreft M. Quantification of dichromatism a characteristic of color in transparent materials. Journal of the Optical Society of America 2009; 26: 1576–1581.

44. Gurtman A, Borrego F, Klotman ME, Simultaneous determination of lamivudine/zidovudine/nevirapine in human plasma using RP-HPLC. Seminars in Nephrology 1998; 18(4): 459-480.

45. Schrive JC, Plasse. Bioanalytical Method development and validation of lamivudine by RP-HPLC method. Journal of Chromatography B 1994; 657: 233.

46. Marchei E, Valvo L, Pellegrini M, Pacifici R, Tossini G, Zyccaro P. Bioanalytical method development and Validation of lamivudine by rp-hplc method. Journal of Pharmaceutical and Biomedical Analysis 2002; 29: 1081.

47. Esrela CR, Salvadori MC, Suarez-Kurtz G G, Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis 2004; 18(1-2): 1147.

48. Marier JF, Dimarco M, Guilbaud R, Dodard C, Morelli G, Tippabhotla SK, Singla AK, Thudi NR, Monif T. Pharmacokinetics of lamivudine, zidovudine, and nevirapine administered as a fixeddose combination formulation versus coadministration of the individual products, Journal of Clinical Pharmacology 2007; 47(11): 1381.

49. Li Z, Ding C, Ge Q, Zhou Z, Zhi X, Liu X. Simultaneous determination of lamivudine, stavudine and nevirapine in human plasma by LC-MS/MS and its application to pharmacokinetic study in clinic. Biomed Chromatography. 2010; 23(2): 1232-1238.

50. Notari S, Bocedi A, Ippolito G, Narciso P, Pucillo LP, Tossini G, Donnorso RP, Gasparrini F, Ascenzi P, Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography. Journal of Chromatography B. 2006; 831(1-2): 258-66.

51. Rezk NL, Tidwell RR, Kashuba AD, Simultaneous determination of six HIV nucleoside analogue reverse transcriptase inhibitors and nevirapine by liquid chromatography with ultraviolet absorbance detection, Journal of Chromatography B 2003; 791(1-2): 137-47.

52. Aymard G, Legrand M, Trichereau N, Diquet B.J, Determination of twelve antiretroviral agents in human plasma sample using reversed-phase high-performance liquid chromatography. Chromatography B Biomed Science Application 2000; 744(2): 227-40.

53. Fan B, Stewart JT, Determination of zidovudine/lamivudine/nevirapine in human plasma using ionpair HPLC, Journal of Pharm Biomed Analalysis 2002; 28(5): 903-908.

54. Basavaiah K. and Somashekar BC. Titrimetric and spectrophotometric determination of lamivudine in pharmaceuticals. Indian journal of chemical technology 2006; 13: 7-12.

55. Jayaseelan S, Suresh S, Sathishkumar G, Sekar V and Perumal P. Bioanalytical method development and validation of lamivudine by RP-HPLC method. International Journal of ChemTech Research 2010; 2(1): 163-167.

56. Devyani dube and Vyas SP. Simultaneous spectrophotometric estimation of lamivudine and silymarin in mixture. International journal of pharmacy and pharmaceutical sciences 2009; 1(2): 107-111.

57. Sockalingam .A and Narayana reddy I. Simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets. Journal of Pharmaceutical and Biomedical Analysis 2007; 22: 232-123.

58. Bin Fan and James Stewart T. Determination of zidovudine/lamivudine/nevirapine in human plasma using ion-pair HPLC. Journal of Pharmaceutical and Biomedical Analysis 2001; 28(5): 903-908.

59. Abd El-Maaboud Mohamed and Workalemahu M. Determination of lamivudine and stavudine in pharmaceutical preparations using chemometrics-assisted spectrophotometry. Saudi Pharmaceutical Journal 2009; 17(4): 275-281.

60. Basavaiah K, Ramakrishna V, Somashekar C, Urdigere R. Sensitive and rapid titrimetric and spectrophotometric methods for the Determination of stavudine

in pharmaceuticals using bromate-bromide and three dyes. Annals of the Brazilian Academy of Sciences 2008; 80(2): 253–262.

61. Namita K, Sateesh K, Ramesh P. Simultaneous determination of lamivudine and stavudine in antiretroviral fixed dose combinations by first derivative spectrophotometry and high performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis 2006; 41(3): 761-765.

62. Verweij-van Wissen C.P.W.G.M, Aarnoutse R.E, Burger D.M. Simultaneous determination of the HIV nucleoside analogue reverse transcriptase inhibitors lamivudine, didanosine, stavudine, zidovudine and abacavir in human plasma by reversed phase high performance liquid chromatography. Journal of Chromatography B 2005; 816(1-2): 121-129.

63. María S, Neus R, Xavier C. Determination of stavudine in human plasma and urine by high-performance liquid chromatography using a reduced sample volume. Journal of Chromatography B: Biomedical Sciences and Applications 2000; 746(2): 183-189.

64. Ashenafi Dunge, Nishi Sharda, Baljinder Singh, Saranjit Singh. Establishment of inherent stability of stavudine and development of a validated stability-indicating HPLC assay method. Journal of Pharmaceutical and Biomedical Analysis 2005; 37(5): 1115-1119.

65. Basavaiah K, Anil kumar. Simple Spectrophotometric Methods for the Determination of Zidovudine in Pharmaceuticals Using Chloramine-T, Methylene Blue and Rhodamine-B as reagents. E-Journal of Chemistry 2006; 3(12): 173-181.

66. Vaishali P, Kishor Bhusari P. A validated UV spectrophotometric method for the simultaneous estimation of Lamivudine, Nevirapine and zidovudine in combined tablet dosage form. Journal of Pharmacy Research 2009; 2(4): 666.

67. Anantha Kumar D, Srinivasa Rao G, Seshagiri Rao J.V.L.N. Simultaneous Determination of Lamivudine, Zidovudine and Abacavir in Tablet Dosage Forms by RP HPLC Method. E-Journal of Chemistry 2010; 7(1): 180-184.

68. Vibhuti K, Vivek A, Chandrabose K, Piyush T. Simultaneous quantitative determination of zidovudine and nevirapine in human plasma using isocratic, reverse phase high performance liquid chromatography. Tropical Journal of Pharmaceutical Research 2009; 8(1): 79-86.

69. Purnima H, Mitesh P, Priti Patil, Nitul Shah. Determination of Nevirapine in Human Plasma by High Performance Liquid Chromatography with Ultraviolet Detection. International Journal of Pharma Tech Research 2010; 2(2): 1316-1324.

70. Purnima D, Hamrapurkar, Mitesh D, Nitul Shah. Quantitative Estimation of Nevirapine By High Performance Thin Layer Chromatography. Journal of Pharmacy Research and Health Care 2009; 1(2): 197-216.

71. Wenjing C, Wujie L, Ling X. Study on the interaction between HIV reverse transcriptase and its non-nucleoside inhibitor nevirapine by capillary electrophoresis. Journal of Chromatography B 2010; 878(20): 1714-1717.

72. Langmann P, Diana S, Thomas V, Steffen De, Michael Z, Hartwig K. Rapid determination of nevirapine in human plasma by gas chromatography. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 2002; 767(1): 69-74.

73. Deshpande A, Dhawale S, Gurav S, Sandeep W, Ajay G, Kamlesh N. Spectrophotometric Estimation of Efavirenz in Formulation and Biological Fluid. International Journal of Research in Pharmaceutical Sciences 2010; 1 (4): 402-406.

74. Anri Theron, Duncan C, Malie Rheeders, Michelle V. Determination of salivary efavirenz by liquid chromatography coupled with tandem mass spectrometry. Journal of Chromatography B 2010; 878(28): 2886-2890.

75. Geetha Ramachandran, Hemanth Kumar A.K. Soumya S, Venkatesan P, Kumaraswami V, David J. Simple and rapid liquid chromatography method for determination of efavirenz in plasma. Journal of Chromatography B 2006; 835(1-2): 131-135.