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HPLC METHOD DEVELOPMENT FOR THE QUANTITATIVE ANALYSIS OF LAMIVUDINE, TENOFOVIR DISOPROXIL FUMARATE AND NEVIRAPINE

BY

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DECLARATION

I declare that this thesis is my own work and idea and that it does not contain materials published by any other investigator except where due references and acknowledgement have been given. I further declare that this work has not been submitted to any institution or body for the award of any degree or certificate.

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DEDICATION

I dedicate this project to the Almighty God, my parents and my family.

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ABSTRACT

An accurate and precise reverse phase HPLC method has been developed for the simultaneous quantification of Lamivudine, Nevirapine and Tenofovir disoproxil fumarate. Phenomenex Synergi C18 (250mm×4.6, 4µm) with methanol: ammonium acetate buffer (adjusted to pH 2.80): acetonitrile in a ratio 50:40:10 v/v was found to achieve suitable separation of the antiretroviral drugs. A flow rate of 1.0ml/min, temperature at 25°C and UV detection at 270nm were used. The run time was 10minutes with retention times of 3.26, 5.42 and 7.55 minutes for lamivudine, nevirapine and tenofovir disoproxil fumarate respectively. The HPLC method developed showed good linearity within the ranges of 10-59µg/ml, 7-42µg/ml and 15-90µg/ml with correlation coefficient of 0.9973, 0.9951 and 0.9968 for tenofovir disoproxil fumarate, nevirapine and lamivudine respectively. The limits of detection were 5.50µg/ml, 3.15µg/ml and 3.93µg/ml for lamivudine, nevirapine and tenofovir disoproxil fumarate respectively. The limits of quantification were 16.68µg/ml, 9.54µg/ml and 10.04µg/ml for lamivudine, nevirapine and tenofovir disoproxil fumarate respectively. The method is accurate in the range 90%- 110% recovery and precise (all %RSD values for inter-day and intraday studies within acceptable criteria). This method detected the antiretroviral drugs in the various formulations within the same retention times as the pure powder samples which informed the specificity of the method. Quantitative analysis of formulations containing the antiretroviral drugs under study were carried out. The percentage content of Tenofovir, lamivudine and Nevirapine in Tenofovir/ Lamivudine fixed-dose combination co-blistered with Nevirapine were 98.55%± 0.17, 105.33%± 0.85 and 99.20%± 1.17 respectively.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral
FDA	Food and Drugs Authority
FDC	Fixed- dose Combination
GC	Gas Chromatography

HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodefiency Virus
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation
ISO	International Organisation for Standardisation
ISO/ IEC	International Organisation for Standardisation/ International Electrotechnical Commission
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MDG	Millennium Development Goals
M.O.H	Ministry of Health
NRTI	Nucleoside/ Nucleotide Reverse Transcriptase Inhibitor
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
PLHIV	People living with Human Immunodeficiency Virus
PMTCT	Prevention of mother to Child Transmission
R^2	coefficient of correlation
RSD	Relative Standard Deviation
U.N	United Nations
USP	United States Pharmacopoeia
UV	Ultra Violet
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Advanced research into the life-cycle and pathogenesis of the HIV virus has led to current advances made in research and development of ARVs that have greatly improved the quality of life and prognosis of people living with HIV following the discovery of the virus in 1981. The introduction of the HAART (use of combination therapy) led to HIV being viewed seemingly as a chronic condition that once well managed is non- fatal (Palmisano and Vella, 2011). Surveys conducted as well as clinical studies in view of the Millennium Development Goal 6 (aimed at combatting HIV/AIDS, malaria and other diseases by stopping the spread of infection by 2015 and to be able to achieve the universal access to treatment for all those in need of it) revealed that the use of the HAART has greatly reduced the prevalence of HIV in terms of new infections and number of deaths. The post-2015 targets set towards HIV/AIDS therefore has at the heart of them the aim to widen access to treatment as the WHO recommends that all people living with the infection should have access to treatment as soon as possible after diagnosis and the organisation also seeks to remove all limitations on the access to treatment for all age groups. This is aimed at ending the AIDS epidemic by 2030 which is captured as the prevention of 21 million deaths related to HIV/AIDS and 28 million people getting newly infected with the virus. Studies have further shown that the use of ARVs by HIV negative partners as prophylaxis reduces significantly the probability of getting infected by the positive partner. These are reasons why the availability of quality

ARVs especially in the sub-Saharan region of Africa which records the highest cases of HIV infections is very important.

HAART came along with issues of adherence with the use of single component formulation in the combination therapy. The aim of improving adherence in conjunction with other advantages of maximizing potency, reducing toxicity and reducing the risk of development of drug resistance made the manufacture of fixed-dose combinations of ARVs gain rapid importance. Manufacturers also considered the production of fixed-dose combination drugs as cheaper compared to producing them as single component drugs (Pujari, 2003).

The quality of a pharmaceutical formulation is the major concern of Quality Assurance having Quality Control at the core. The unavailability of methods for some fixed-dose combination ARVs in the pharmacopoeia has made the development of methods for quality assessment of fixed-dose combination ARVs an area of increased interest in pharmaceutical analysis. Many research works have been done on the quantification of ARVs in FDCs, their related substances, impurities, dissolution methods and many other areas of quality. The need to develop methods for quality assessment is important to ascertain the safety, efficacy and quality of the product. Quantitative assessment especially reduces the risk of toxicity from higher amounts of drugs present, the risk of development of drug resistance and having treatment failure from subtherapeutic amounts of the drugs present in the formulation. The effect of transportation and storage conditions on the amount of drug, the degradability with stress can also be ascertained as well as the compatibility of drugs present in the FDC formulation (that is if one facilitates the breakdown of the other).

Regulatory organisations such as the WHO and the Washington, DC, US FDA have created guidelines for the registration of FDC medicinal products. Some of these guidelines require information on bioavailability and bioequivalence, safety and efficacy of product, the uniformity of content prior to compression, analytical procedures and stability testing.

All these require suitable validated methods to be able to evaluate and provide these information before a marketing authorisation is given. Manufacturers of generic products who therefore do not have access to in-house methods developed by innovator manufacturers will have to research and develop methods that can be used to ascertain the quality of their products (WHO, 2005). The ICH, ISO, USP and other organisations and committees have set guidelines that makes it possible to validate a developed method such that it is credible enough in the absence of pharmacopoeial methods to analyse formulations.

1.2 PROBLEM STATEMENT

Quality control of ARVs is essential to the effective treatment of HIV. The absence of a pharmacopoeial method for the quality assessment of some HAART medication available such as the fixed dose combination of tenofovir disoproxil fumarate and lamivudine, co-blistered with nevirapine may make it difficult to assess its quality (assay) and hence make it difficult to trace formulation related cases of toxicity due to high amounts of active ingredients or drug resistance due to decreased amounts of active ingredients. This has informed the need for the development of a simple and relatively fast HPLC analytical method for the simultaneous quantitative estimation of the above mentioned ARVs in fixed dose combination.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

To develop and validate a method for the quantitative analysis (assay) of tenofovir disoproxil fumarate, lamivudine and nevirapine in the fixed-dose combination formulation.

1.3 2 Specific Objectives

- To obtain adequate information on the ARVs to be analysed as active pharmaceutical ingredients and as a formulation
- To select initial conditions for method development
- To identify the pure samples of ARVs to be used as working standards
- To ascertain the suitable sample preparation method for the ARVs to be analysed as pure forms and in formulation
- To obtain a suitable separation with preliminary runs
- To optimise the separation conditions to improve selectivity, resolution and obtaining a suitable run time
- To validate the method considering the following parameters: Linearity, range, LOQ, LOD, precision (repeatability and intermediate precision), accuracy, stability, robustness and sample carryover To assay the formulation(s) using the developed method.

1.4 JUSTIFICATION

The knowledge of the amount of the individual drugs present in a formulation is very critical to the continuous production of quality medicinal products such as fixed-dose tenofovir disoproxil fumarate and lamivudine co-blistered with nevirapine which is required for treating HIV with improved adherence, maximised potency, reduced toxicity and low risk of development of resistance. It is usually administered to pregnant women in Ghana as treatment for the

women and as a means to preventing transmission of the virus to the child. Hence, it is essential to know how much of the drug is available for its therapeutic effect to be adequate and to prevent the risk of toxicity to both the mother and the unborn child. A validated analytical test method is therefore essential and required for the quantitative analysis of these tablets in routine quality control activity. Local manufacturers with such routine analytical methods made available will also be able to venture into the production of some of these antiretroviral drugs. The availability of the developed analytical test method will help overcome challenges due to unavailability of standard test methods to test formulated medicinal products which may be one of the limitations to their production in the country.

CHAPTER TWO

LITERATURE REVIEW

2.1 HIV/AIDS

The human immunodeficiency virus (HIV) is classified to be a retrovirus which infects the cells of the human immune system and this results in the destruction and impairment in the proper functioning of the cells and hence the immune system. The function of the human immune system is estimated with relation to the number of CD4 cell count, hence when the immune system gradually gets weaker the CD4 count gets lower with the gradual progression of the disease. The progression of the disease which makes the individual susceptible to other infections such as candida infections leads to the most advanced form of the infection known as AIDS characterised by the presence of other diseases such as tuberculosis and certain cancers as well as severe complications and clinical manifestations. Transmission of the virus is mainly by contact of body fluids from an infected person which includes blood, breast milk, semen and vaginal secretions. Predisposing factors that present high risk of acquiring the infection include mainly unprotected sex with an infected person. Others include the use of contaminated sharp objects that may cause a break in the skin, presence of sexually transmitted infections, unsafe blood transfusion amongst others. It has been found that it takes about 2-15 years for an infected person to get the advanced infection and that antiretroviral drugs are able to slow down the process even further.

HIV is treated by combination antiretroviral therapy consisting of three or more antiretroviral drugs. Antiretroviral drugs cause a suppression of the HIV but do

not cure it. It controls the viral replication and load and causes a strengthening of the immune system thereby boosting its functionality. A new guideline published by WHO in 2015 recommends that antiretroviral therapy should be started as soon after diagnosis as possible (WHO, 2013)

2.2 HIV AS A PUBLIC HEALTH ISSUE GLOBALLY

It has been estimated that there are approximately 36.9 million people living with HIV as at the end of 2014 and is estimated to have claimed more than 34 million lives so far with 1.2 million people dying from HIV related conditions in 2014. The number of newly infected people that same year was about 2.0 million. The sub- Saharan region of Africa is reported to be the most infected region having 25.8 million people living with HIV as at 2014. It is reported currently that only 54% of all individuals living with HIV know they are infected. These are some reasons that still present HIV as a public health issue globally (WHO, 2013)

The millennium development goals set in the year 2000 had the sixth goal set to combat HIV/AIDS, malaria and other diseases by stopping the spread of infection by 2015 and to be able to achieve the universal access to treatment for all those in need of it. It has been estimated by the World Health Organisation that the strategies that were put in place to reach these goals have yielded good results seeing about 15.8 million individuals living with HIV been enrolled on the ART by mid-2015. Seventy three per cent (73%) of an estimated 1.5 million pregnant women with HIV worldwide are receiving effective antiretroviral therapy to avoid transmission to their children and new HIV infections have generally dropped by 35%. AIDS related deaths have decreased by 24% and some 7.8 million lives are sustained as reported by the year 2014 and mid-2015

(WHO, 2013). According to a document authored by Ben Schiller, fifteen years later after the setting of the MDGs, the targets of the MDG 6 has been realised. He reported that according to U.N. figures, new infections dropped from about 3.5 million cases in 2000 to 2.1 million cases in the year 2013 accounting for a 40% drop. He also reported that by 2014, 13.6 million people living with HIV/AIDS had access to antiretroviral therapy (Schiller, 2015).

The post-2015 targets set towards HIV/AIDS has at the heart of them to widen access to treatment as the WHO recommends that all people living with the infection should have access to treatment as soon as possible after diagnosis and the organisation also seeks to remove all limitations on the access to treatment all age groups inclusive. This is aimed at ending the AIDS epidemic by 2030 which is captured as the prevention of 21 million deaths related to HIV/AIDS and 28 million people getting newly infected with the virus (WHO, 2013)

2.3 HIV/AIDS IN GHANA

The Guidelines for Antiretroviral Therapy in Ghana records that the first case of AIDS in Ghana was reported in 1986 with subsequent upsurge in cases over the years. It is also reported from research that the predominant mode of transmission of the infection is through sexual intercourse which accounts for about 80% of all transmissions; mother-to-child (vertical) transmission been accounted for 15% of individuals living with the disease and the other 5% attributed to spread through blood and blood products. These findings are from the HIV Sentinel Survey (HSS) that was conducted in 2009 which also captured the female to male ratio of persons living with HIV to be 1.4: 1. An estimated number of 3354 children were also found to be newly infected. Annual AIDS deaths were 20313 and the age bracket between 40- 44 were estimated to be the

highest prevalent group with a prevalent rate of 4.0%. HIV1 and HIV2 were discovered to be present in the Ghanaian population with HIV1 occurring in 91.8% individuals and HIV2 in 5.2% individuals. Both infections occurred in 3.0% individuals.

In response to these alarming figures arising from surveys undertaken over the years, that is, since 2000 to analyse the trend of the infection in the country, interventions which includes primarily promotion of and education on safe sex, proper condom use, improved management of sexually transmitted diseases, safe blood transfusion, infection prevention and control, clinical care and counselling, home supervised care and prevention of mother-to-child transmission (PMTCT) were made and implemented. These interventions were focused towards decreasing new infections and improving on the quality of life of persons living with HIV (PLHIV).

The use of Antiretroviral Therapy (ART) became available in Ghana since June 2003. The treatment sites that helped with the initiation of this therapy were two but has increased to over 138 sites and as many as 33745 number of PLHIV cumulatively were under this therapy as at December 2009 and this most importantly led to a decrease in HIV related morbidity and mortality (M.O.H, 2010)

The current statistics in Ghana according to the Summary of the 2013 HIV Sentinel Survey Report provided by the Ghana AIDS Commission in 2013, the nationwide prevalence of HIV was reported to be 1.3%. Two hundred and twenty two thousand, four hundred and eighty eight (222488) persons which consist of 189931 adults and 34557 children were living with the virus with

7812 new infections recorded the higher number representing adults (5405) and that of children between zero and fourteen years was 2407. The number of annual deaths recorded in this study was lower as compared to the initial study and was found to be 10074 a higher proportion representing adults leaving 184168 children orphaned. The survey reported that 125396 individuals were still in need of antiretroviral therapy and 11682 pregnant women needed PMTCT services. The HIV prevalence for pregnant women who attended antenatal clinic reduced from 2.1% in 2012 to 1.9% in 2013 which was found to be the first recording below 2% in two decades and hence represented an improvement. The age bracket between 45- 49 recorded a prevalence of 3.3% representing the highest prevalence with the age bracket 15- 19 representing the lowest with a percentage of 0.8%. The region recording the highest prevalence of 3.7% was the Eastern Region with the Northern and Upper West regions recording 0.8% representing the lowest. Higher prevalent rates ranging from 11.6% to 0.6% were reported for urban areas (Commission, 2015)

From the statistics, it can be said that HIV/AIDS combatting in Ghana has been quite a success and progressing. The aim to further improve and combat HIV/AIDS prompted the commissioning of the Ghana AIDS Commission to liaise with international organisations, local organisations and any related persons or organisations to support this goal through education and ensuring all persons living with HIV have access to treatment.

2.4 ANTIRETROVIRAL THERAPY IN GHANA

Antiretroviral drugs are the mainstay in antiretroviral therapy. Antiretroviral drugs are grouped into classes mainly based on the different ways they attack

HIV virus lifecycle. The classes of antiretroviral drugs available currently include the Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTI), Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI), Protease inhibitors (PI), Entry inhibitors and the HIV integrase inhibitors.

The antiretroviral drugs according to classes used in Ghana include the Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTI), NonNucleoside Reverse Transcriptase Inhibitors (NNRTI) and Protease inhibitors (PI). Combination therapy (Highly Active Antiretroviral Therapy) is usually advised in this therapy hence where available, fixed dose combinations are preferred to single dose drugs as it improves adherence to treatment. In Ghana, the triple therapy is recommended based on evidence from antiretroviral therapy programmes ran worldwide as well as internal programmes and experience.

The recommended triple therapy regimens are as follows:

- Two Nucleoside or Nucleotide Reverse Transcriptase Inhibitors (NRTI) and one Non-Nucleoside Reverse Transcriptase inhibitor (NNRTI)
- Two Nucleoside or Nucleotide Reverse Transcriptase Inhibitors (NRTI) and one boosted Protease Inhibitor(M.O.H, 2010).

Adherence to ART is essential to improved quality of life and decreased mortality and morbidity. It also decreases the risk of resistance development hence increasing life expectancy and survival of the individual living with HIV.

2.5 ANALYTICAL METHOD DEVELOPMENT

Analytical method development may be referred to as the design of an experimental procedure employing steps and techniques that may be used in the

quantitative or qualitative analysis of compounds which are core aspects in the quality control requirements (Walfish, 2006). The development of analytical methods has over the years been veered towards instrumental analysis with an added advantage of incorporated techniques for separation of components present in a sample under study. Separation may be achieved through chromatography as applied in HPLC, GC and LC. The use of these instruments with selective detectors such as the U.V., photodiode, fluorescent and electrochemical detectors make it easier to detect the separated compounds and assign identities to a particular compound. The mass spectrometer may be employed at other times to facilitate characterisation and identification of samples analysed. Analytical method development has had a lot of applications in pharmaceutical analysis especially in the aspect of quality assurance specifically quality control.

An analytical method is usually developed when methods to analyse the drugs are not available in the Pharmacopoeias. These may occur when a new drugs or existing drugs which has been modified in a way are required to be introduced onto the market but have not had their specifications included yet in the pharmacopoeia as further research may have to be made in terms of its effectiveness, toxicities (newer ones for structurally modified drugs) and development of resistance which may inform its continuous stay on the market and hence its inclusion in the pharmacopoeia. This creates a need for an analytical method to be developed within the time between introduction of the drug onto the market and its inclusion in the pharmacopoeia to be able to access quality of drug before standards are officially set for it (P. Ravisankar, 2014). Also, with the need to fight against drug resistance and the need for more

effective yet less harmful treatment there has been an upsurge in the use of combination drug therapy where multiple medication are used to treat the same condition as various clinical studies have indicated its advantages in disease treatment without relapses (Oncosec, 2013).

In order to improve adherence fixed-dose combinations are becoming rapidly important and are increasingly manufactured currently for various conditions which includes the ARVs. Manufacturers also consider the production of fixeddose combination drugs as cheap compared to producing them as single component drugs (WHO, 2005). Some of these formulations however do not have standard procedures for analysis available in the pharmacopoeias making it beneficial to develop analytical methods for their quality to be assessed.

A developed analytical method should be:

- simple
- solid and well understood,
- scientifically sound
- described in details in a step-by-step way to ensure reproducibility (good documentation)
- robust and uncomplicated
- precise and accurate
- relatively fast and cost effective (Services, 2009)

Most of the qualities stated above require experimental investigations once the method is developed to assess and ensure it meets the above stated criteria. These investigations are summarised as method validation.

2.6 METHOD DEVELOPMENT USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Developing a method using HPLC requires the use of a suitable column and solvent system to most of the time cause separation of components of sample under study, identify them and then quantify the desired component present using information obtained from the chromatogram obtained. Separation is usually achieved either through isocratic or gradient elution of desired components through the column with relative differences in their retention onto the column.

2.7 IDENTIFICATION OR QUALITATIVE ANALYSIS IN HPLC METHOD DEVELOPMENT

The retention time is used to identify the components separated in comparison with known reference samples using tests performed under the same conditions (same mobile phase composition, column, flow rate, injection volume and wavelength of detection). Identification can also be achieved with the use of specific selective detectors for the components in the sample under study. For instance, a component that is detected at a particular wavelength using an ultraviolet detector produces a particular spectrum which may be used for identification. Also, a component that absorbs light and emits it at a particular wavelength may be analysed using a fluorescence detector. The use of mass spectrometer as a detector also encourages identification using fragmentation patterns.

2.8 QUANTITATIVE ANALYSIS IN HPLC METHOD DEVELOPMENT

The peak area is a parameter that is most often used to quantify the sample under study. In other cases, the peak height may be used especially where trace amounts of the sample are being analysed. However, the peak area gives most accurate results so often preferred. The amount of analyte is usually quantified in relation to its known reference sample analysed under the same conditions. A series of concentrations of reference sample are analysed and responses obtained. A plot of the various concentrations against response obtained as peak area provides a means of transforming the relationship concentration and response into a linear fit. An equation of the line obtained generally represented as y = mx+c may be used to determine the concentration of analyte in the sample under study where y represents the detector response and x represents the concentration. Another way of quantifying the analyte present is by using the equation below and this is usually preferred when the concentrations of the standard and the sample are the same.

$$\frac{\text{Concentration of sample}}{\text{Concentration of standard}} = \frac{\text{Area of sample}}{\text{Area of standard}}$$

2.9 PROCESS OF METHOD DEVELOPMENT USING HPLC

Developing an analytical method with the HPLC instrument has become useful and is used often these days. The approach to each developed method may be different but there is often a general pattern that is usually followed. These include:

- Obtaining adequate information on the sample(s) to be studied
- Clearly outlining the separation targets or what the separation is eventually to be used for, example for qualitative or quantitative analysis, isolation and characterisation amongst others

- Sample preparation
- Selection of appropriate detector and knowing the range within which the sample will be detected
- Preliminary work on separation
- Optimizing the most suitable separation conditions obtained
- Validation of the method
- Assessment of its ability to achieve the aim for which the method is developed (Snyder et al., 2012)

2.9.1 Information on Sample

There is always a need to review any information available on the analyte(s) under study before proceeding to develop the method. Information may be obtained if it is a drug from its respective monograph in the pharmacopoeias. Others may be obtained from previous research works done on the analyte of interest. Information on the structure of the compound, the molecular weight, the pKa, the wavelength of maximum absorption if it can be detected by UV, the solubility profile amongst others are important to note. Adequate information gathered usually makes it easier to infer the necessary initial conditions (whether reverse phase or normal phase, solvents to be used for elution) to be used for preliminary separation runs.

In other instances, it may be necessary to use knowledge acquired from previous experiments performed by the researcher. This approach is essentially used when the identity of analyte(s) present in the sample is/are unknown. In this case it may be essential to perform trials to arrive at initial conditions to be used for separation. The use of the two approaches however and their successful blend aid in arriving at a separation method faster (Snyder et al., 2012).

2.9.2 Separation Targets

Separation targets give an indication of the level of separation required. A separation that is aimed at quantitative study will require that all sample components are well resolved (specificity) clearly resolving any accompanying impurity or degradant from the actual analyte under study. A quantitative study should ensure that the separation leads to the development of a method that is accurate and precise. A preparative HPLC required for isolation and further characterisation will also require a certain level of separation. Two or more analytes present in a sample will require taking into consideration a suitable run time to appropriately separate the different components. The separation targets will also aid in determining the appropriate HPLC equipment to use, for example, the equipment should have a pump system that will facilitate the use of the gradient mode if gradient elution will be required for the separation of the analytes. The sample matrix within which the analyte is present will affect the conditions necessary for the level of separation required. A careful analysis of separation targets is important before the start of the method development and requires that the researcher asks certain questions which when answered satisfactorily will ensure a smooth initiation of the method development process (Snyder et al., 2012).

2.9.3 Sample Preparation and Selection of Suitable Detector

Samples used for HPLC run generally should be injected as a solution. These samples when dissolved may require further dilutions before injection. It is important to note that the solvent used for dissolution of the sample may have an effect on the responses obtained (shape of the peak). It is therefore considered best to use a dissolution solvent that is close to the composition of the mobile phase. This reduces baseline disturbance and other problems such as peak

splitting and band broadening. Samples that come as liquids may have analytes present in matrices such as urine, blood as well as environmental samples such as samples from water bodies. These will require some pre- treatment such as with the use of solid phase extraction to remove unwanted substances and to concentrate the desired analytes before their use in analysis. Samples that come as tablet formulation will usually require crushing the tablets and extracting the desired analyte using the appropriate solvent. Certain samples may require buffering to keep them stable and maintain their integrity in solution example proteins; others may also require injection of sample at cold temperatures. All these factors are to be investigated before initiating method development.

The appropriate detector that will specifically and selectively detect the analyte under study is often desired. This information may be obtained from literature or obtained practically by running the analyte using various detectors. Varying wavelengths may also be used (use of variable- wavelength UV detector) to establish which detector and at which point set on the detector will ensure satisfactory detection of the analyte. It will also be desirable to investigate if the analyte will require derivatives to be formed to enhance absorption. The structure of a compound if known makes detector selection relatively easier

(Snyder et al., 2012)

2.9.4 Preliminary Work on Method Development

Initial conditions such as choice of solvent(s) to be used for elution (mobile phase) and the column are the two essential tools based on information acquired on analyte or empirical approach. Preliminary work involves running samples containing analyte(s) to obtain separation of components of the sample. Here,

the composition of mobile phase is changed until the composition that suitably separates the components is found. It is usually preferable to start with two solvent compositions (binary system) before moving in to a three solvent composition mobile phase (tertiary system). A 100% solvent (one solvent) composition is not usually recommended as it may just carry certain components with it through the column with no relative retention thereby not achieving separation. An isocratic mode is used initially; unsatisfactory separation will lead to the use of gradient elution. It is important that the final conditions settled on gives the most suitable separation and is able to generate reproducible chromatograms especially with respect to retention time (Snyder et al., 2012).

2.9.5 Optimizing the Separation Method Developed

A good separation should have a good resolution and a satisfactory run time. Optimising the method developed should therefore ensure a good balance between the two parameters. System elements such as the column dimension, particle size and packing in the column will have an effect on the resolution of the components of the sample. It may therefore be essential in this step to use other columns if available to ascertain the one that gives the best resolution. The resolution may also have slight influence from the pH of the mobile phase; adjusting the pH may also be essential to ensure good resolution. The run time is usually affected by the length of the column, the flow rate as well as temperature. It is desirable that run times are not so long and therefore the flow rate, length of column and temperature may be optimised in such a way that a reasonable run time is used which does not affect the resolution of the peaks (Walfish, 2006).

2.10 METHOD VALIDATION

Validation of an analytical method refers to setting up or finding documented proof that provides a high degree of confidence that a specific developed method and the supporting instruments used in the development of the method will constantly and reproducibly yield results that reflect exactly the quality characteristics of the substance tested (Shabir, 2004). In other words, validation demonstrates the scientific soundness and confirms by examination that an analytical test method used to perform a specific test is suitable for its intended use. Method validation is essential to be done:

- Before a newly developed analytical test method is established or introduced for regular use
- Where an existing established method is to be carried out under different conditions for which the method was initially validated
- Whenever it is observed overtime that the existing method is changing with time beyond the initial scope of the method (Kalra, 2011).

Many international and local regulatory bodies, organisations as well as committees such as the FDA, WHO and ICH have guidelines which usually suggest that any analytical method developed and used for the analysis of any pharmaceutical substance during pharmaceutical development should be stability indicating and validated. The ISO/IEC 17025 contains a chapter on method validation with a list of elements (parameters) that should be examined during validation. The ICH has also designed and established a document based on the validation of analytical procedures which provides definitions and detailed methodology of eight validation parameters (Kalra, 2011).

Parameters for Method Validation

Linearity

Linearity refers to the validation measurement that is tested in order to investigate and hence demonstrate that there is a direct relationship (proportional) between the concentration of the sample under study and the response obtained within a given working range. The ICH guidelines require that for the establishment of linearity, a minimum of five concentrations obtained from a stock or standard solution should be used. Each concentration is required to have three replicate readings. Results obtained are analysed via least square regression calculations where it is necessary to transform results obtained to get a linear fit. The co-efficient of correlation is calculated and submitted in order to prove the direct relationship between concentration and response obtained. The y-intercept of the linear regression line and the slope are also important to be computed and submitted for use in subsequent calculation of the limit of detection and the limit of quantification which are also validation parameters. The y-intercept is required to be less than a few per cent of the response obtained for the analyte at a particular target level and not significantly different from zero (ICH, 2005).

Range

The ICH document on validation of analytical methods refers to the range of an analytical procedure to be the points or values (which are actually different concentrations of the analyte in a sample) that are contained within two extremes, (the upper and lower limit concentrations) these concentrations included. Within this interval, it is to be proven that the analytical procedure has a suitable level of precision, accuracy and linearity (ICH, 2005). The range is

dependent on the intended use of the developed test method. It is then said to conform to accepted standards when the test method provides a satisfactory level of linearity, accuracy and precision when used to analyse samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

Accuracy

Accuracy of a test method shows the degree of scatter (closeness of agreement or level of difference) between the value which is accepted as the conventional true value (an accepted reference value) and the average of a set of measured values. The accuracy is usually analysed and confirmed over the specified range of the test method. ICH recommends that accuracy data should be evaluated using a minimum of nine determinations over a minimum of three concentration levels across the specified range. Accuracy is required to be reported as per cent recovery after the assay of a known added quantity of analyte to that present in the sample. Another way of reporting it may also be as the difference between the mean and the accepted true value together with confidence intervals. For an active drug substance accuracy may be determined by using the developed test method to analyse the active drug substance of known purity example the reference sample. Another way is the compare the results obtained from the developed test procedure with that of an established (well-characterised procedure) which has its accuracy known, stated and defined. Accuracy may also be determined once the precision, linearity and specificity have been and analysed (ICH, 2005)

Precision

Precision is the validation element that seeks to indicate the closeness of agreement between repeated measurements obtained through multiple sampling of a homogenous sample under the prescribed conditions. The measure of precision can be examined in three different areas which include repeatability, intermediate precision and reproducibility. The statistical tool used to analyse precision data is the variance, the standard deviation or the relative standard deviation (the coefficient of variation) of repeated measurements.

The ICH guidelines on validation of analytical methods require that a minimum of nine determinations be used to determine the precision usually covering the range for the analytical test method developed or having a target (100%) concentration, a minimum of six findings are obtained from analysis and used (ICH, 2005).

Repeatability

Repeatability indicates the precision of a developed analytical method when repeated measurements from analysis are recorded under unchanged operating conditions over a relatively short period of time. This means analysis carried out on the same instrument or equipment and by the same analyst. This may also be termed intra-day precision (ICH, 2005).

Intermediate Precision

This indicates the precision of a developed analytical method when repeated measurements from analysis are recorded under different conditions within the same laboratory. This usually is observed over a period of days to weeks. The different conditions that may be investigated includes measurements recorded on different days (inter-day precision), operation of procedure by different

analysts and analysis carried out on a different instrument or instrument parts or equipment (ICH, 2005).

Reproducibility

Reproducibility is a validation measurement that indicates precision of an analytical method that has been developed when analysis is carried out in different laboratories. This parameter is usually important when the method developed is been standardised. It expresses the precision of the method under different environmental condition (temperature and humidity), with analysts with different experience and thoroughness, different instruments and equipment amongst others (ICH, 2005)

Limit of Detection (LOD)

This refers to the lowest quantity of analyte in a sample which can be detected (seen or picked as a response) from analysis on an instrument or equipment but not essentially quantified. When using chromatography as a method of analysis, the limit of detection is observed as the amount of analyte injected that gives a response with a peak height which is two or three times higher than the base line noise level (Huber, 2007). The ICH also documented other methods that may be used for the analysis of this parameter. It includes:

- Visual Inspection: This method involves the analysis of samples of known concentrations and by observation detecting and determining the lowest concentration at which the analyte can be detected with certainty.
- Standard Deviation of the response and the slope obtained from the calibration curve. With this method, the LOD is calculated for using the formula below:

$$\frac{3 \times \text{Standard deviation of the response}}{\text{Slope of the calibration curve}} \quad 3.$$

The standard deviation can be obtained by computing the residual standard deviation of the regression line or computing the standard deviation of yintercepts of the regression line from a carefully studied calibration curve generated from the analysis of samples having analyte quantities within the range of the LOD and their responses (ICH, 2005)

Limit of Quantitation (LOQ)

Limit of quantitation as a validation parameter refers to the minimum quantity or amount of analyte in a sample that is capable of been quantitatively measured with acceptable precision and accuracy. The ICH guidelines describe several procedures that may be used to determine this parameter and some of them are described below.

Using the standard deviation of the response and the slope obtained from the calibration curve. The mathematical formula that may be used to determine the LOQ here is:

$$\frac{10 \times \text{Standard deviation of the response}}{\text{Slope of the calibration curve}}$$

The standard deviation estimated either by analysing a suitable number of blank samples and standard deviation calculated from the responses obtained or by computing the residual standard deviation of the regression line or computing the standard deviation of y- intercepts of the regression line from a carefully studied calibration curve generated from the analysis of samples having analyte quantities within the range of the LOQ and their responses.

Visual Evaluation: This is used mainly for non-instrumental methods but may be applied with instrumental methods. This method involves the analysis of samples of known concentrations and by observation detecting and determining the lowest concentration at which the analyte can be detected with certainty.

Another approach is signal-to-noise approach: This is done by analysing samples with known low concentrations and blank samples. The responses obtained for the low concentrations analysed and that of the blank samples are compared and the lowest concentration at which the analyte can be quantified with certainty is established. The conforming signal-to-noise ratio required 10:1 (Huber, 2007)

Robustness

The robustness of a developed method is the validation parameter that investigates the influence or effect of small but intentional changes in method parameters or conditions on responses obtained. It is preferable that the method remains relatively unaffected by these changes and should be within the previously specified tolerance range. This gives an indication of its reliability during routine use (ICH, 2005). Some parameters that may be varied and investigated include pH, wavelength of detection, mobile phase composition, flow rate, temperature and column with the use of chromatography (Huber, 2007).

The Relative Standard Deviation (RSD) of a series of measurement obtained from the analysis of a known concentration of the sample is the statistical tool used to assess the robustness of a method as well as other system suitability

parameters such as the resolution factor, the tailing factor and the number of plates in the case of chromatography.

Specificity

Specificity is the validation parameter that requires that a developed method when used to analyse a particular analyte in a sample produces a response that represents that analyte only even in the presence of other components which may include excipients, impurities, degradants or matrix (example urine and blood).

Identification tests that are capable of differentiating between compounds with similar structural characteristics may be used to perform specificity tests. A comparison of samples with known analyte(s) with their respective known reference standards may be carried out ensuring that tests are performed under same conditions. It will be of importance to compare results obtained for a positive test to negative tests where the presence of the identified analyte (from comparative studies with reference standard) is investigated for in a sample that does not contain the analyte (ICH, 2005)

A method developed using chromatography requires that chromatograms are used to establish specificity. Individual components should be identified and labelled appropriately. The resolution of two compounds which elute closest to each other (critical separation) may be used to investigate and establish specificity in chromatography (Huber, 2007). Peak purity test which usually employs the use of selective detectors such as photodiode, mass spectrometer and fluorescence detectors may also be useful to illustrate a response obtained

due to a particular chromatographed analyte and to demonstrate that a peak obtained does not belong to more than one component (ICH, 2005)

Stability

Stability is a validation parameter used to ascertain the consistency in responses or results obtained over a particular period of time using the same sample solution. This study is important as certain compounds are very unstable and may decompose in solution especially during sample preparation or within a period of time prior to the analysis of the compound or even on storage under specific conditions. In such cases, it is important to determine the optimum sample pre- treatment and preparation method that keeps the integrity of the sample prior to analyse. It is also essential to determine the length of time that an analyte will remain intact in solution for accurate results to be obtained during analysis. The effect of storage at room temperature or cold temperatures on the analyte may also be investigated here over a period of hours, days and sometimes weeks.

The Relative Standard Deviation (RSD) of a series of measurement obtained from the analysis of a known concentration of the sample is the statistical tool used to establish the stability (Huber, 2007).

2.11 SAMPLE CARRYOVER

Sample carryover is a major problem that affects the accuracy and precision of an analytical test method. It is an important test that should be performed when using the chromatography instruments such as the high performance liquid chromatography (HPLC) and the liquid chromatography- mass spectrometry (LC/MS). HPLC carryover may be categorised as auto sampler carryover and column carryover. The auto sampler carryover is often as a result of absorption

and or trapping of earlier run sample on the auto sampler needle, the injection port, the transfer tubes, the sample loop or the injector valve. Auto sampler carryover will usually have the same retention time as the analyte in a previously analysed sample. This mostly affects the accuracy in responses and usually shifts it towards the positive bias and this impacts significantly on samples of lower analyte concentrations when many samples beyond the calibration ranges are analysed (high concentration samples). Column carryover however is caused by residual amounts of previously ran samples present on the column. This when left on column may sometimes get decomposed causing the original form of the analyte to be changed and hence it may not always be seen here that the carryover sample has the same retention time as that of the sample under study. This therefore produces random errors and mainly affects the precision of the method (Hughes et al., 2007)

Carryover should therefore be investigated and eliminated or reduced during method development and validation and routinely during analysis while using these instruments to prevent the influence of these extraneous peaks on the accuracy and precision of the method. The study of carryover may be done by injecting a sample of high analyte concentration, followed by the injection of the blank. Careful examination of the blank injection response generated is done to ascertain the presence of any peaks that are identical to that of the components of the sample under study. Ideally there should not be any peaks present, since peaks present from previous analysis will add on to current peaks generated during analysis therefore producing errors in results generated. However, if peaks of analyte(s) are seen in the blank, the carryover of the peaks in the blank

is calculated and expressed as a percentage of the response of the concentrated sample. The acceptance criteria for sample carryover is ≤ 1% (Kassaye and Genete, 2013)

2.12 RELATED WORKS DONE ON METHOD DEVELOPMENT FOR ANTIRETROVIRAL DRUGS

Literature reveals much work that has been done concerning method development and validation for separating and quantifying antiretroviral drugs. The use of varied instruments and techniques have been exploited for analysis of the antiretroviral drugs as pure compounds, as bulk and formulated products, as single component products as fixed-dose combination and in other sample matrices especially human plasma. Below is a review of some works that have been done.

A reverse HPLC method for the simultaneous analysis of lamivudine and zidovudine in an FDC formulation has been developed by Santosh Kumar M. et.al. The mobile phase, consisting of 50mM potassium dihydrogen phosphate and methanol in a ratio 65:35, was used to achieve separation and hence quantitative analysis of the individual drugs (Santosh Kumar et al., 2011).

Rajkumar B. et.al reported on a reverse HPLC method developed and validated for the simultaneous quantitative analysis of lamivudine, zidovudine and efavirenz tablets using acetonitrile and potassium dihydrogen orthophosphate buffer (0.02M) adjusted to pH 3.2 using orthophosphoric acid in a proportion 30:70 v/v pumped through column at a flow rate of 1.0 ml/min and a detection wavelength of 275nm using a UV detector (Rajkumar et al., 2014).

Work has been done on developing a method and validating it for lamivudine and tenofovir in combination dosage formulation. It was reported to be a novel reverse HPLC method using a tertiary mobile phase system consisting of trimethylamine buffer (pH 5): acetonitrile: methanol in a ratio 30:40:30%v/v in an isocratic mode. Elution was carried out on a Symmetry C8 column with a flow rate of 0.8ml/min and detection at 260nm. (Kumar K. A. et.al, 2012)

A UV- VIS spectrophotometry method as well as a titrimetric method has been developed for the quantification of lamivudine in bulk and formulation. The titrimetric method involved the use of starch as an indicator, sodium thiosulphate as the titrant and lamivudine as the analyte in the presence of 1M sulphuric acid, chloramines- T solution and 10% potassium iodide. A colorimetric estimation in which coloured complexes formed between lamivudine and p- Dimethyl Amino Benzaldehyde (formation of rose red coloured complex) as well as 3-Methylbenthiazolinone-2-(3H)-hydrazone (formation of blue complex) were carried out. Absorbances of coloured complexes were taken at 570nm and 630nm respectively. Using methanol as dissolution solvent and blank, lamivudine content was estimated using UV at a wavelength of 270nm (Ravi Kumar et al., 2014)

The only isocratic liquid chromatographic method that has been developed for tenofovir, lamivudine and nevirapine was made available in November, 2015. A work done by Anumolu PD.et.al which is a reverse phase method using acetonitrile and phosphate buffer at a pH of 3.0 in the ratio (40:60) with a flow rate of 1.0 ml/ min at a UV detection wavelength of 252nm (PD et al., 2015)

A gradient elution method for the estimation of tenofovir, lamivudine and nevirapine consists of a reverse phase liquid chromatography method using two mobile phase systems; mobile phase A consisting of mixed ammonium phosphates (1.15g of ammonium dihydrogen phosphate and 1.32g of di ammonium phosphate in one litre) pH adjusted to 2.8 with dilute trifluoroacetic acid and mobile phase B consisting of a mixture of methanol and acetonitrile in a ratio 10: 90%v/v. The flowrate used was 0.8 ml/min and detection was done at 260nm. This was further used for stability studies of the antiretroviral drugs by force degradation method (Rama Prasad et al., 2012)

An assay method developed using reverse HPLC covers the simultaneous quantitative determination of Protease inhibitors which include indinavir, amprenavir, nelfinavir, saquinavir, ritonavir, lopinavir and non- nucleoside reverse transcriptase inhibitors which are nevirapine, delavirdine and efavirenz was reported. The method is a linear gradient method using 25% phosphate buffer pH 4.5, 60% acetonitrile, 15% methanol and 0.75ml trifluoroacetic acid with a flow rate of 0.9- 1.1ml run for 30 minutes (Bwiro, 2011).

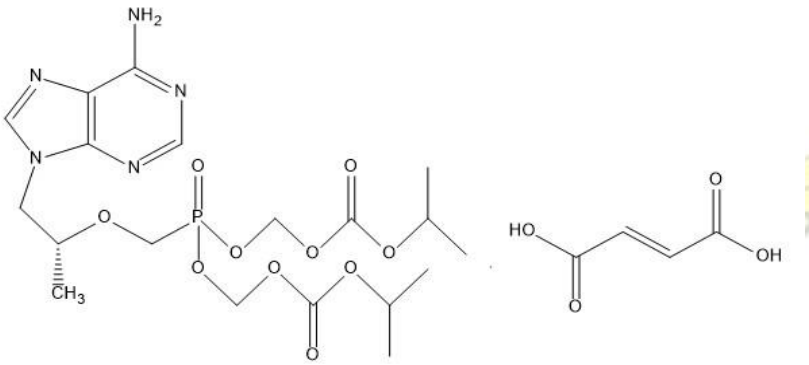
An LC/MS method that is capable of simultaneously analysing saquinavir, nelfinavir, indinavir, ritonavir and amprenavir (protease inhibitors) as well as nevirapine, delavirdine and efavirenz (NNRTIs) was reported. The electrospray ionisation method was employed and a linear gradient method using water and acetonitrile was used. The run time was ten minutes and the sample matrix was in human plasma (Villani et al., 2001).

An LC/MS analytical method developed to estimate the amount of tenofovir and emtricitabine in the plasma was reported. A gradient elution method using

acetonitrile and water with formic acid 0.05% on a reverse phase column was employed. The electrospray ionisation method was used to ionise samples in the mass spectrometer for fragmentation and detection (Gomes et al., 2008)

Literature review of method development for antiretroviral drugs reveals that more work was done outside Africa. This gives the indication that more work should be done in the area of developing quick and economical methods for analysing antiretroviral drugs to improve their production in this part of the world.

2.13 TENOFOVIR DISOPROXIL FUMARATE



(*R*)-(((((1-(6-amino-9*H*-purin-9-yl)propan-2-yl)oxy)methyl)phosphoryl)bis(oxy))bis(methylene) diisopropyl dicarbonate fumarate
Chemical Formula: $C_{23}H_{34}N_5O_{14}P$
Exact Mass: 635.18
Molecular Weight: 635.51

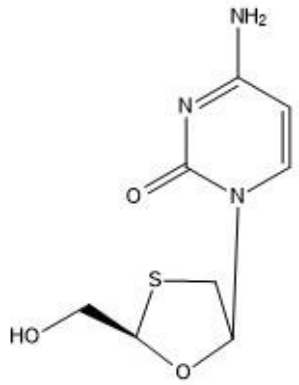
Figure 2.1 Structure of tenofovir disoproxil fumarate showing its IUPAC name, chemical formula and molecular mass (Source: ChemDraw Ultra 12.0)
Chemically known as [[(1R)-2(6- Amino- 9H-purin-9-yl)-methylethoxy] methyl] phosphonate, bis (isopropyloxycarbonyloxymethyl ester), fumarate (1:1), tenofovir disoproxil fumarate is the only nucleotide analogue that is available for use in antiretroviral therapy. It is a white to almost white crystalline powder which is slightly soluble in water, soluble in methanol and very slightly soluble in dichloromethane. Tenofovir has a pKa value of 3.75. It is a derivative of adenosine S'-monophosphate and its structure is seen to lack a complete ribose ring. It is administered as a disoproxil fumarate prodrug

because tenofovir has poor bioavailability as tenofovir which is not well absorbed from the intestines; the fumaric acid salt of tenofovir therefore increases its bioavailability. In- vivo, the prodrug is converted to tenofovir which is then acted upon by cellular kinases causing it to be phosphorylated to tenofovir diphosphonate which is the active metabolite responsible for the activity of tenofovir. Tenofovir disallows the infection of susceptible healthy host cells but do not have any effect on cells that are already infected with the HIV virus. It usually enters cells and prevents the replication of viral genome in two ways;

- Competitive inhibition which involves preventing the incorporation of the host's nucleotide and
- Termination of the process of elongation of nascent proviral DNA

It inhibits both HIV1 and HIV2 and has favourable safety and tolerability profiles that make it a significant component of any combination regimen used in antiretroviral therapy. Tenofovir is approved and recommended as a component combination antiretroviral therapy for adults and has been seen to contribute significantly in especially three- drug regimen with other antiretrovirals which usually include other nucleoside analogues, protease inhibitors and/or non-nucleoside reverse transcriptase inhibitors (Brunton et al., 2008)

2.14 LAMIVUDINE



4-amino-1-((2*R*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl)pyrimidin-2(1*H*)-one
Chemical Formula: $C_8H_{11}N_3O_3S$
Exact Mass: 229.05
Molecular Weight: 229.26

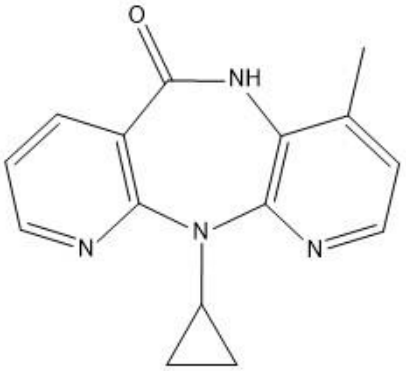
Figure 2.2 Structure of lamivudine showing its IUPAC name, chemical formula and molecular mass (Source: ChemDraw Ultra 12.0)

Lamivudine is a drug belonging to the nucleoside reverse transcriptase inhibitor class of antiretrovirals. It is chemically known as 2(1H)-Pyrimidinone,4-amino1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] -(2R- cis)- (-)- 1- [(2R, 5S)-2- (Hydroxymethyl)- 1,3- oxathiolan-5-yl] cytosine. It is a white to almost white powder which is soluble in water, sparingly soluble in methanol and slightly soluble in ethanol. It has a pKa of 4.24- 4.3. It is a cytosine analogue that is available for the treatment of HIV1 and HIV2 infections. It is also active against hepatitis B virus. It is manufactured as the pure cis (-) enantiomer which is the more potent molecular form of the drug and significantly safer (less toxic). Its active metabolite exists as intracellular triphosphate derivative and has low affinity for human DNA polymerase. Lamivudine also disallows infection of susceptible healthy host cells but does not have any effect on cells that are already infected with the HIV virus.

Lamivudine inhibits both HIV1 and HIV2 and is known to have activity (broad spectrum) against other retroviruses. Some are also used in the treatment of

hepatitis B infection as they are active against the HEP B virus or the herpes viruses. Lamivudine has been shown to contribute to sustained virologic effects when used in combination therapy especially with zidovudine as it restores sensitivity to zidovudine. This drug is one of the safest and has few significant side effects. Trials that were carried out confirm the benefits of lamivudine in especially three-drug regimen with other nucleoside analogues, protease inhibitors and/ or non-nucleoside reverse transcriptase inhibitors based on its safety, convenience and efficacy(Brunton et al., 2008).

2.15 NEVIRAPINE



11-cyclopropyl-4-methyl-5*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6(11*H*)-one
Chemical Formula: $C_{15}H_{14}N_4O$
Exact Mass: 266.12
Molecular Weight: 266.30

Figure 2.3 Structure of nevirapine showing its IUPAC name, chemical formula and molecular mass (Source: ChemDraw Ultra 12.0) Nevirapine is an antiretroviral belonging to the non-nucleoside reverse transcriptase inhibitor class of antiretrovirals. Its chemical name is 11cyclopropyl-4-methyl-5, 11- dihydro [3, 2-b: 2', 3'-e] [1, 4] diazepin-6-one hemihydrate. It is anhydrous in nature and is a white to almost white substance with a pKa value of 2.8. It is highly soluble at pH < 3; amphoteric in nature and classified as a weakly basic drug. It is practically insoluble in water but slightly soluble in methylene chloride and methanol. This drug is available and approved for the treatment of HIV1 infection in adults and children in combination with other

antiretroviral agents. Monotherapy with this drug is not recommended as resistance can rapidly develop with its use as a single agent. It is also not to be used as the sole addition to a failing regimen. Nevirapine is an essential antiretroviral used in the prevention of mother- to- child transmission of HIV as it readily crosses the placenta. Nevirapine also disallows infection of susceptible host cells with the virus but unlike the Nucleoside/ Nucleotide

Reverse Transcriptase Inhibitors does so by non-competitive inhibition. Nevirapine and its related NNRTIs are strain specific and therefore act mainly against HIV1 infection and do not have to undergo the process of intracellular phosphorylation to become active. It does not have any effect on the host cell's DNA polymerase. Resistance to Nevirapine can develop rapidly even as early as a few days or weeks. Side effects such as elevated hepatic transaminases, severe and fatal hepatitis have been associated with the use of nevirapine more especially in women and in pregnancy. Nevirapine is usually not to be initiated in women with CD4 counts less than 250 per mm3 or men with CD4 count less than 400 per mm3 unless it is very important to start provided its benefits outweighs its risks (Brunton et al., 2008).

CHAPTER THREE

METHODOLOGY

3.1 MATERIALS

3.1.1 Pure Samples and Formulations

Lamivudine, Nevirapine and Tenofovir disoproxil fumarate pure powders with purities 100.2%, 100.1% and 100.2% respectively were obtained from Danadams Pharmaceutical Industry Limited, Spintex, Ghana. Tenofovir disoproxil fumarate/ Lamivudine tablets 300mg/ 300mg + Nevirapine tablets USP 200mg and Tenofovir disoproxil fumarate/ Lamivudine tablets 300mg/ 300mg manufactured by Mylan Laboratories Limited, India were used.

3.1.2 Reagents

Acetonitrile (HPLC grade) from Fisher Scientific, Methanol (HPLC grade) from Fisher Scientific, Ammonium Acetate from E. Merck and Acetic Acid Glacial (100%) from VWR International Limited, Merck House were used.

3.1.3 Equipment and Instrumentation

The HPLC system comprises of a SHIMADZU prominence UFLC series system consisting of LC–20A quaternary pump (part G1311A), DGU-20A_5 inline vacuum degasser (part no. G1322A) and SPD-20A ultra violet detector. Data acquisition was by the LC solutions software Version A.10.02 Build 1757. The chromatographic separation (method development) and validation was carried out using a reverse phase C18 column, Phenomenex Synergi 4µ Hydro-PP 80A 723105-34 5375-068, 250mm×4.6. An electronic analytical balance (Mettler Toledo AB204-S/FACT), a digital pH meter (Mettler Toledo seven compact pH/Ion S220) and a sonicator were used.

3.2 METHODS

3.2.1 Identification of Pure Samples

Lamivudine pure powder (2mg) was triturated with 200mg of finely powdered and dried potassium bromide R using an agate mortar and pestle. The resulting mixture was spread uniformly in a die and compressed using a hydraulic press. The disc obtained was then placed in the sample holder and run to obtain a spectrum which was compared to that of the chemical reference standard obtained from Danadams Pharmaceuticals Industry Limited. The same procedure was followed for the identification of nevirapine and tenofovir disoproxil fumarate pure powders.

3.2.2 HPLC Method Development

HPLC Conditions

The chromatographic separation of the drugs was done using a reverse phase C18 column, Phenomenex Synergi (250×4.6 mm, 4µ). A ternary system of mobile phase consisting of acetonitrile, methanol and ammonium acetate buffer (0.02M, pH adjusted to 2.8 using acetic acid) in a ratio 10: 50: 40 v/v was used. The mobile phase was pumped through the column at a flow rate of 1ml/min and the volume of injection was 10ul for every injection. The wavelength of detection was set at 270nm for identification and quantification of analytes. The column was kept at constant temperature of 25° C in a column oven. The run time for the separation was 10 minutes. Sample preparation was done using a mixture of 10: 40: 50 v/v acetonitrile: ammonium acetate buffer (0.02M adjusted to pH 2.8): methanol as dissolution solvent. Extraction of drugs from formulation was done using methanol.

Ammonium Acetate Buffer Preparation

The solution was prepared by weighing 1.5416g of ammonium acetate into a beaker using the analytical balance. It was dissolved in about 100ml of distilled water and transferred into a 1000ml volumetric flask. Five hundred millilitres (500ml) of filtered distilled water was added to it and then the solution adjusted to pH 2.8 using acetic acid with continuous stirring and monitoring using the pH meter. The solution was then made up to volume to obtain 0.02M buffer strength. This was then filtered through a 0.45 µm membrane filter.

Mobile Phase Preparation

The solvents were separately poured into solvent reservoirs A, C and D representing methanol, acetonitrile and buffer reservoirs and programmed to pump them in a ratio of 50:10:40 v/v respectively.

3.2.3 Validation of the HPLC Method

The method developed for separation, detection and quantification was validated by the ICH guidelines (ICH, 2005). Parameters validated include linearity, the range, the limit of detection and quantitation, specificity, stability, robustness, accuracy and precision.

Preparation of Stock Solution of Pure Powders of Lamivudine, Nevirapine and Tenofovir Disoproxil Fumarate

Seventy-five milligrams (75mg) of Lamivudine, 35mg of nevirapine and 50mg of tenofovir disoproxil fumarate were weighed accurately and transferred into a 100ml volumetric flask that contained 50ml of the dissolution solvent. It was then sonicated for 10 minutes at 37°C and then allowed to cool. It was made up to volume after sufficient cooling and filtered through a 0.45um membrane filter. The stock solution was stored in a reagent bottle at room temperature.

From this solution 3ml was taken and transferred into a 50ml volumetric flask and made up to volume with dissolution solvent. This produced a solution having 45µg/ml, 21µg/ml and 30µg/ml concentration of lamivudine, nevirapine and tenofovir disoproxil fumarate concentration serving as working concentration during validation.

Linearity

The working standard solution was diluted using the dissolution solvent to obtain six solutions within the range of 10-59µg/ml, 7- 42µg/ml and 15-90µg/ml for tenofovir disoproxil fumarate, nevirapine and lamivudine respectively. Triplicate injections were done for each solution and peak areas recorded. A graph of peak area was plotted against concentration of analytes. The equation of the line was determined and correlation of regression deduced by Microsoft Excel 2013.

Limit of Detection and Quantification

The limits of detection and quantification were deduced from the intercept on the y- axis slope of the calibration curves of the linearity curve (ICH guidelines, 2005). The formula below was used for calculating the LOD and LOQ.

$$\text{LOD} = \frac{3.3 \times \text{Standard deviation of the response}}{\text{Slope of the calibration curve}}$$

$$\text{LOQ} = \frac{10 \times \text{Standard deviation of the response}}{\text{Slope of the calibration curve}}$$

Accuracy

The stock solution of tenofovir disoproxil fumarate, nevirapine, and lamivudine was diluted to obtain 24µg/ml, 30µg/ml, 36µg/ml of tenofovir disoproxil fumarate; 16.8µg/ml, 21µg/ml, 25.2µg/ml nevirapine and 36µg/ml, 45µg/ml,

54µg/ml lamivudine using the dissolution solvent. These concentrations represent 80%-120% concentration levels of the working standard for validation for each analyte. Triplicate injections were made for each solution, and the response determined. The accuracy was determined by calculating the recovery.

Robustness

Diluting the mixed stock solution to 45µg/ml lamivudine, 21µg/ml nevirapine and 30µg/ml tenofovir disoproxil fumarate, injections was done at a wavelength of 268nm and 272nm while leaving all other parameters constant.

The robustness of the method was also determined by changing the flow rate to 0.9ml/min and 1.1ml/min whilst leaving all other parameters constant.

Precision

Repeatability (Intra- day Precision)

The stock solution of tenofovir disoproxil fumarate, nevirapine, and lamivudine were diluted to obtain 10µg/ml, 30µg/ml, 59µg/ml of tenofovir disoproxil fumarate; 7µg/ml, 21µg/ml, 42µg/ml nevirapine and 15µg/ml, 45µg/ml, 90µg/ml lamivudine using the dissolution solvent. These concentrations represent concentration levels covering the specified range for each analyte. Triplicate injections were made for each solution, and the response determined. The injections were repeated three different times within 24 hours. The RSDs were calculated.

Intermediate precision (Inter-day Precision)

The working standard solution was injected six consecutive times on three different days. The RSDs were calculated.

Stability

The stability of the solutions was determined by comparing the responses obtained with freshly prepared working standard solution, which represents time 0 and then responses obtained at times 1,2,3,4,5 and 6 hours.

Specificity

Specificity was done by injecting blank dissolution solvent and then injecting the working standard solution. Identification of analytes using their relative retention times was demonstrated.

3.2.4 Sample Carryover

Sample carryover was investigated by the initial injection of a blank sample followed by the injection of a solution with tenofovir disoproxil fumarate, nevirapine and lamivudine (2mg/ml, 2mg/ml and 1mg/ml respectively). This was injected three times and then the blank sample was run to determine the presence of any peak due to the analytes under study.

3.2.5 Assay of Antiretroviral Drugs in Formulations

Uniformity of Weight

This was carried out according to the British Pharmacopoeia (2013) specifications for the uniformity of weight of tablets.

Fixed Dose Combination Tenofovir Disoproxil Fumarate and Lamivudine Tablets

Twenty tablets were weighed individually and together. The deviations in mass of individual tablets were calculated for and expressed as a percentage deviation with reference to the average weight of the tablet. The tablets were crushed uniformly using a porcelain mortar and pestle for weighing and sample preparation.

Co-Blistered Nevirapine Tablets

The uniformity of weight procedure described above was performed using ten tablets.

Preparation of Mixed Sample Solution of Tenofovir Disoproxil Fumarate and Lamivudine

An accurately weighed powdered tablet sample containing an equivalent of 75mg of lamivudine was transferred into a 100ml volumetric flask. About 50 ml of methanol was added to it and sonicated for 10 minutes at 37°C to enhance dissolution. It was allowed to cool and made up to volume. The resulting solution was then filtered through a Whatman No. 1 filter paper, the first 5ml of the filtrate discarded. 3ml of the resulting filtrate was measured and transferred into a 50ml volumetric flask and made up to volume using the dissolution solvent.

Preparation of Co- Blistered Nevirapine Sample Solution

An accurately weighed powdered tablet sample containing 35mg of nevirapine was transferred into a 100ml volumetric flask. About 50 ml of methanol was added to it and sonicated for 10 minutes at 37°C to enhance dissolution. It was allowed to cool and made up to volume. The resulting solution was then filtered through a Whatman No.1 filter paper, the first 5ml of the filtrate discarded. 3ml of the resulting filtrate was measured and transferred into a 50ml volumetric flask and made up to volume using the dissolution solvent.

Assay Method

Triplicate injection was made for all sample solutions prepared. Responses were recorded and using the equation of line obtained from calibration curves, the

actual concentration of antiretroviral drugs in the various formulations determined.

CHAPTER FOUR

RESULTS

4.1 IDENTIFICATION OF PURE SAMPLES

4.1.1 Tenofovir Disoproxil Fumarate

The presence of carbonyl groups present in tenofovir shows the carbonyl (C=O) stretch occurring around 1700cm^{-1} and 1750cm^{-1}. The one at the longer wavenumber representing the C=O associated with the ester groups is very intense in the spectrum. Another distinct feature is the strong C-O absorption in the fingerprint region occurring around 1250cm^{-1}. A C-H stretch which may be owing to the methyl (CH_3) present was also seen at 3000cm^{-1}. A hydroxyl (-OH) stretch is also seen at 3300cm^{-1} and is quite a broad peak. (Refer to APPENDIX: Figures B1 and B2; pages 77-78)

4.1.2 Lamivudine

A broad peak spanning from about 3500cm^{-1} to about 2000cm^{-1} was noticed in both the reference spectrum and the pure sample spectrum. An intense but broad peak around 1650cm^{-1} was seen in both spectra and this demonstrates the presence of a carbonyl in the structure of the compound. The C=C bond is observed around 1500cm^{-1} and the fingerprint region which is characteristic for a particular compound is similar for both spectra. (Refer to APPENDIX: Figures B3 and B4; pages 79-80)

4.1.3 Nevirapine

A peak observed around 3300cm^{-1} represents the secondary amine in both the reference and sample spectrum. The intense peak observed around 1700cm^{-1} indicates the presence of a carbonyl. The presence of the sp^2 hybridised =C-H

in aromatics occurs around 3000cm^{-1} to 3100cm^{-1} in both spectra. The nature of spectrum considering the functional group region and fingerprint region of the spectrum obtained for nevirapine pure powder was very similar to that of the reference standard. (Refer to APPENDIX: Figures B5 and B6; pages 81-82)

4.2 SELECTION OF INITIAL CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions were evaluated using a reverse phase C18 column, Phenomenex Synergi (250×4.6 mm, 4µm). The appropriate mobile phase was discovered after several compositions of solvents which include methanol, acetonitrile, buffer 1(phosphate) and buffer 2 (ammonium acetate) were tried in isocratic mode. A run time of 10 minutes was deduced from the preliminary trials. Column temperature 25°C was maintained throughout the study.

Table 4.1 Preliminary trials in method development

Trial	Mobile phase composition	Flow rate (ml/min)	Buffer pH	Comment
1a.	Methanol: Buffer (1) (80:20)	1.0	3	Good peaks, tailing.
1b.	Methanol: Buffer (1) (75:25,70:30,65:35, 55:45)			Tailing, peak splitting of lamivudine and minimal resolution between peaks, decreasing retention time, clogging of column over time.
2	Methanol: Buffer (2) (90:10,80:20,70:30, 65:35, 60: 40 55: 45)	1.0	3	Decreasing retention time with decreasing methanol, unsatisfactory resolution, broad peaks

3	Methanol: Buffer (2): Acetonitrile (30:60:10, 30:50:20)	1.0	2.60	Broad peaks, tailing, unsatisfactory resolution, increasing run time
4	Methanol : Buffer (2) : Acetonitrile (50:40:10)	1.2	3.3	Decreased retention time, inadequate resolution, relatively symmetrical peaks
5	Methanol : Buffer (2) : Acetonitrile (50: 40: 10)	1.0	2.8	Symmetrical peaks, good resolution between analytes, convenient run time.

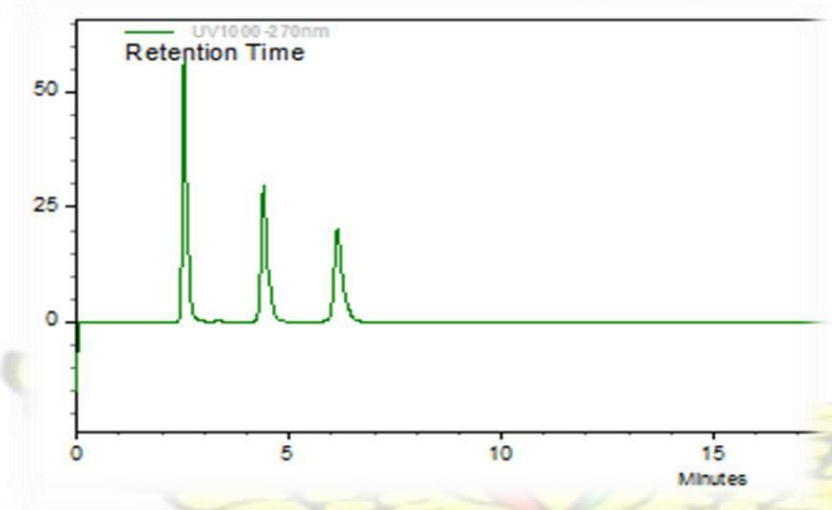


Figure 4.1 Chromatogram showing optimised separation of lamivudine, nevirapine and tenofovir (from left to right) using developed method

Table 4.2 Mean retention time of pure samples

PURE SAMPLE	MEAN RETENTION TIME (MINUTES)
Lamivudine	3.2648± 0.008
Nevirapine	5.4232± 0.003
Tenofovir disoproxil fumarate	7.5552± 0.003

Table 4.3 Calibration equations and correlation coefficients for pure samples

PURE SAMPLE	CALIBRATION EQUATION	CORRELATION COEFFICIENT	RANGE (µg/ml)
Lamivudine	y= 14487x+ 38521	0.9968	15-90
Nevirapine	y= 9363.1x+ 11417	0.9951	7- 42

Tenofovir	y= 5283.1x+8161.5	0.9973	10-59

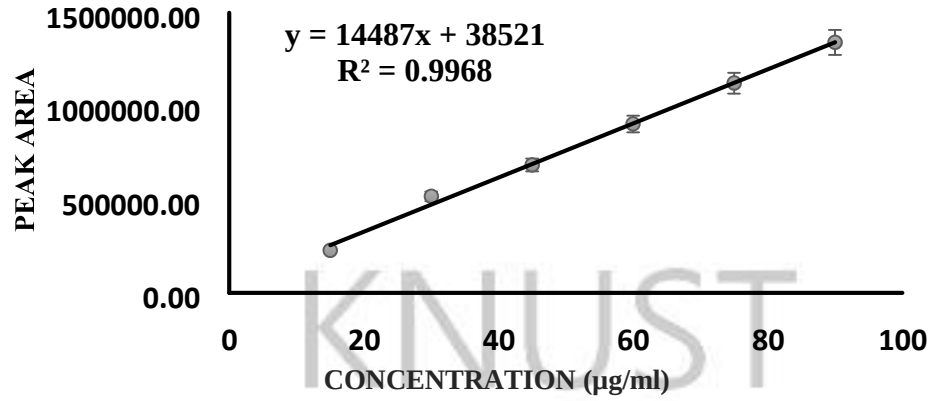


Figure 4.2 Calibration graph for Lamivudine

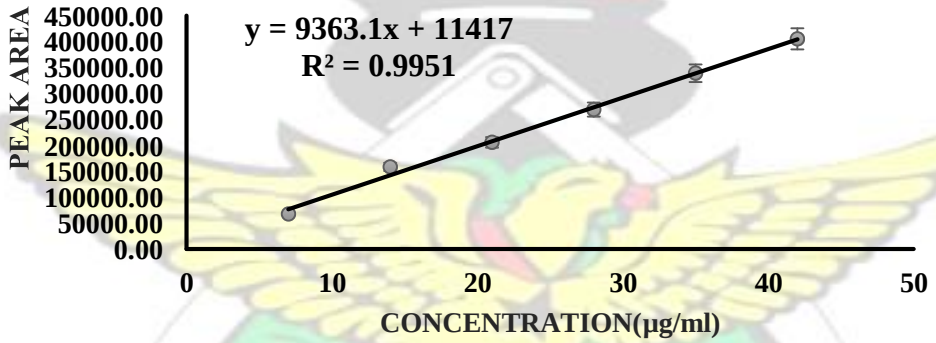


Figure 4.3 Calibration graph for Nevirapine

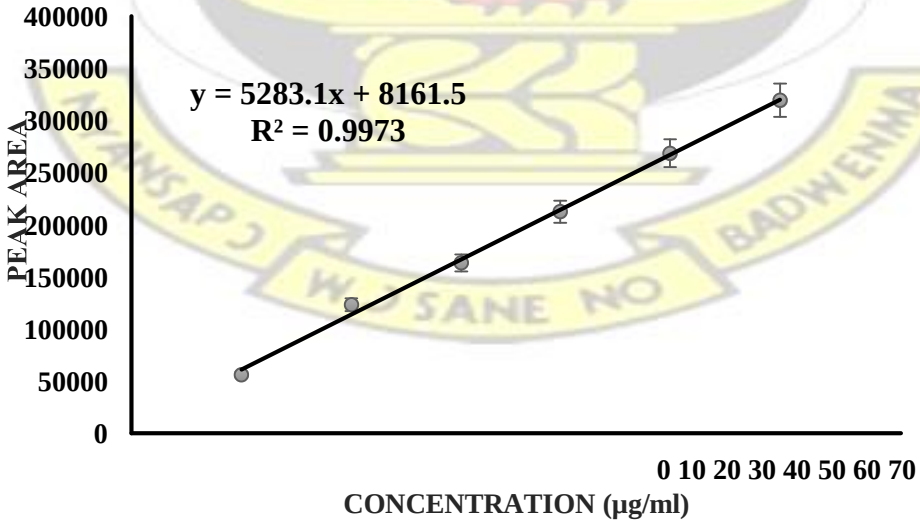


Figure 4.4 Calibration graph for Tenofovir disoproxil fumarate

Table 4.4 Limit of detection (LOD) and quantification (LOQ) of the pure samples

PURE SAMPLE	LOD (µg/ml)	LOQ (µg/ml)
Lamivudine	5.5032	16.6762
Nevirapine	3.1496	9.5443
Tenofovir disoproxil fumarate	3.9267	10.0433

Table 4.5 Measure of accuracy using recovery

SAMPLE	CONCENTRATION (µg/ml)	MEAN %RECOVERY	RSD%
Lamivudine	36.0	99.30± 0.34	0.3378
	45.0	100.80± 0.32	0.3203
	54.0	97.90± 0.46	0.4699
Nevirapine	16.8	102.99± 0.45	0.4368
	21.0	99.71± 0.72	0.7210
	25.2	98.22± 0.51	0.5205
Tenofovir disoproxil fumarate	24.0 30.0	101.75± 0.28	0.2700
	36.0	98.58± 0.71	0.7182
		98.18± 0.26	0.1805

4.3 PRECISION

Table 4.6 Intra- day precision (Repeatability)

SAMPLE	CONCENTRATION (µg/ml)	MEAN PEAK AREA	RSD%
Lamivudine	15	258033.00	0.2184
	45	696530.11	0.2043
	90	1363172.11	0.1569
Nevirapine	7	67984.67	0.9336
	21	203734.00	1.7226
	42	405342.67	0.4120
Tenofovir disoproxil fumarate	10	55857.56	0.7498
	30	163930.67	0.3851
	59	317435.00	0.2685

Table 4.7 Inter-day Precision (Intermediate Precision)

SAMPLE	DAY	MEAN PEAK AREA	RSD%
Lamivudine	1	695818.50	0.5331
	2	697241.67	0.9456
	3	695828.17	0.5177
Nevirapine	1	204734.00	1.2091
	2	207297.67	1.0511
	3	208786.83	0.5325
Tenofovir disoproxil fumarate	1	162629.20	0.2906
	2	161947.70	0.9317
	3	161415.70	0.3039

4.4 ROBUSTNESS

Table 4.8 Effect of changes in wavelength and flow rate on developed method

CONDITIONS	MEAN PEAK AREAS			%RSD		
(Wavelength & Flow rate)	LAM	TEN	NEV	LAM	TEN	NEV
268 nm	706701.67	163237.00	204186.00	0.1976	0.1465	0.6848
270 nm	696530.00	163297.67	207478.67	0.2043	0.6962	0.9746
272 nm	690664.33	163219.67	199845.33	0.1365	0.1448	0.2923
0.9ml/min	699463.00	164203.00	210140.33	0.1758	0.0268	0.3874
1.0ml/min	696530.00	163297.67	209682.00	0.2042	0.6962	0.9643
1.1ml/min	693209.33	162488.33	197181.00	0.4126	0.2448	0.6604

Table 4.9 Change in retention time with changing flow rate

SAMPLE	FLOW RATE (ml/min)	MEAN RETENTION TIME	RSD%
Lamivudine	0.8	4.42	0.43
	1.0	3.26	0.05
	1.2	2.20	0.82
Nevirapine	0.8	6.38	0.52
	1.0	5.42	0.03
	1.2	3.42	0.64
Tenofovir disoproxil fumarate	0.8	8.26	0.04
	1.0	7.56	0.03
	1.2	4.78	0.76

4.5 SPECIFICITY

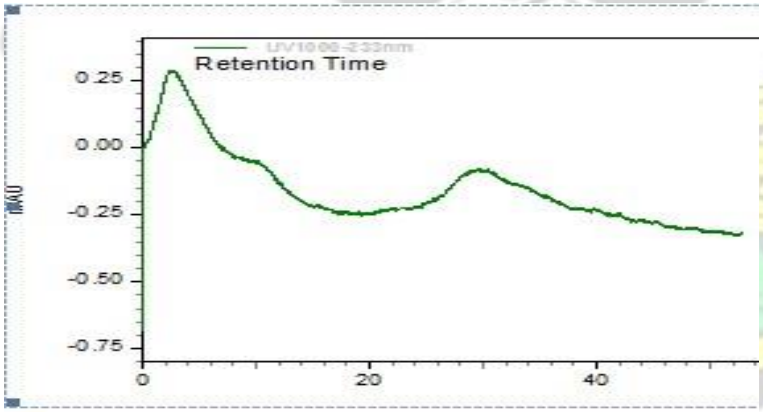


Figure 4.5 Chromatogram of blank sample injection

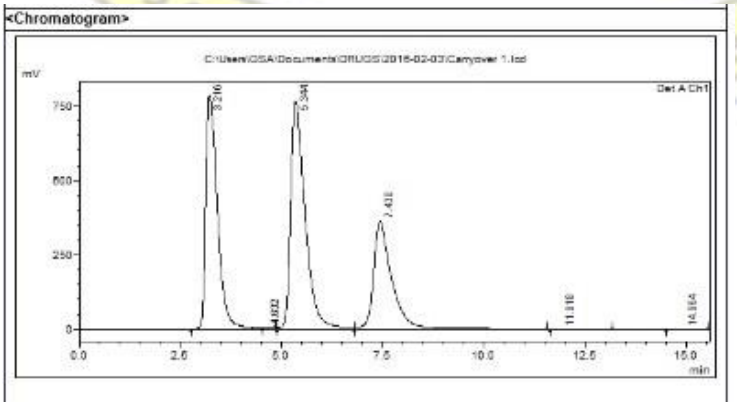


Figure 4.6 Chromatogram showing peaks obtained from the injection of pure sample solutions of lamivudine, nevirapine and tenofovir (from left to right) with retention times 3.26, 5.42 and 7.55 minutes respectively.

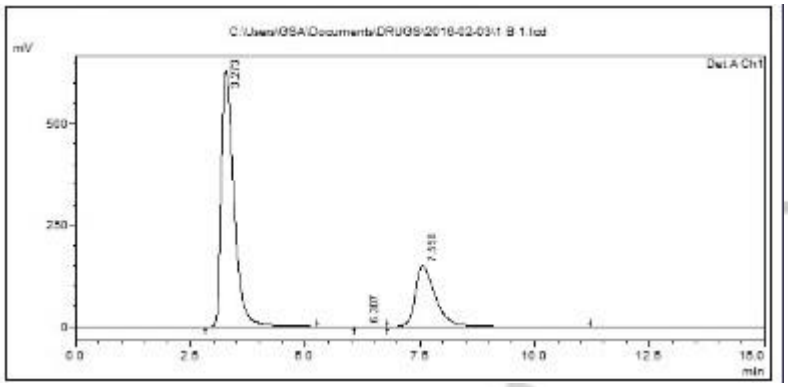


Figure 4.7 Chromatogram showing the peaks of lamivudine and tenofovir in a sample solution prepared from FDC tablet of lamivudine and tenofovir.
Retention times are 3.27 and 7.55 minutes respectively.

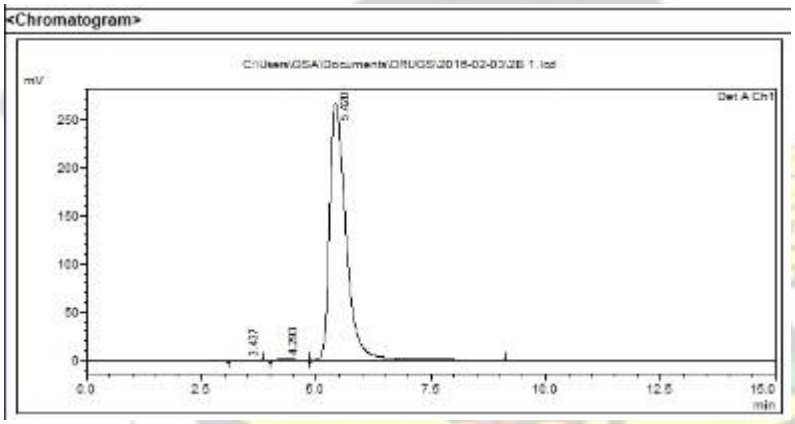


Figure 4.8 Chromatogram showing the peak of Nevirapine in a sample solution prepared from co- blistered Nevirapine tablet with a retention time, 5.42 minutes.

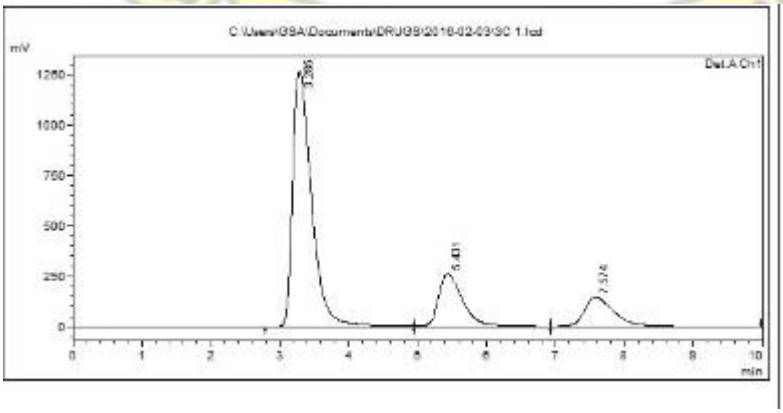


Figure 4.9 Chromatogram showing the peaks of Lamivudine, Nevirapine and Tenofovir (from left to right) in a sample solution obtained from single component tablets of the above mentioned antiretroviral deliberately prepared together. Retention times are 3.28, 5.43 and 7.56 respectively.

4.6 STABILITY

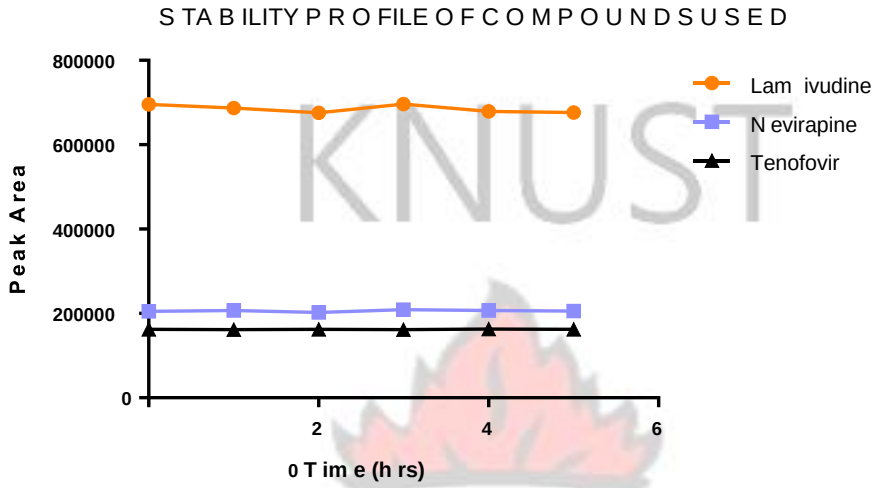


Figure 4.10 A graph demonstrating the stability of Lamivudine, Nevirapine and Tenofovir disoproxil fumarate over a six-hour period.

4.7 SAMPLE CARRYOVER

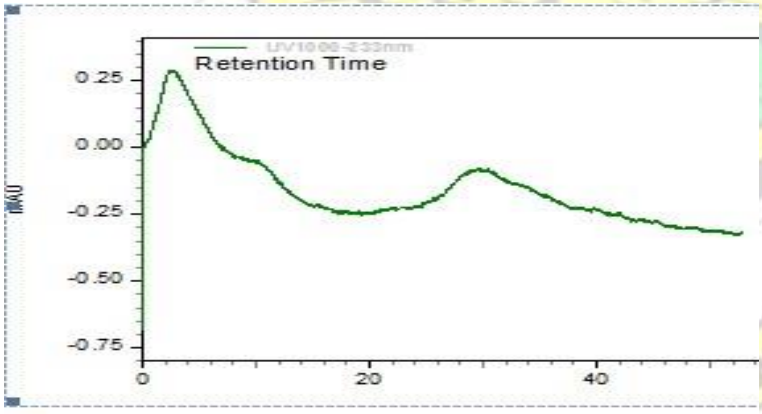


Figure 4.11 Chromatogram of blank sample injection

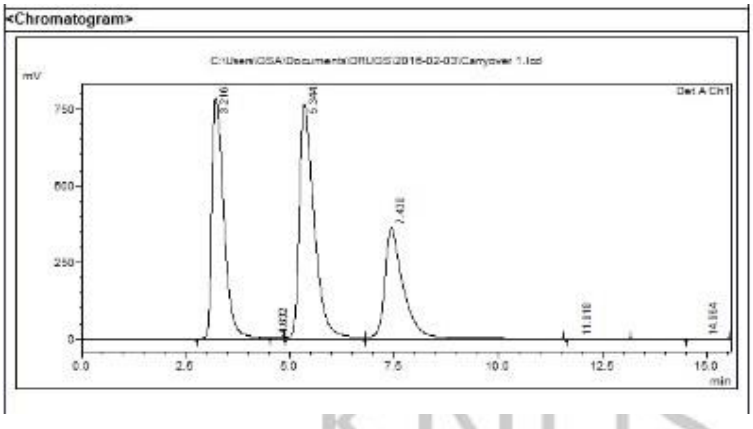


Figure 4.12 Chromatogram showing the peaks of lamivudine, nevirapine and tenofovir (from left to right) at high concentrations.

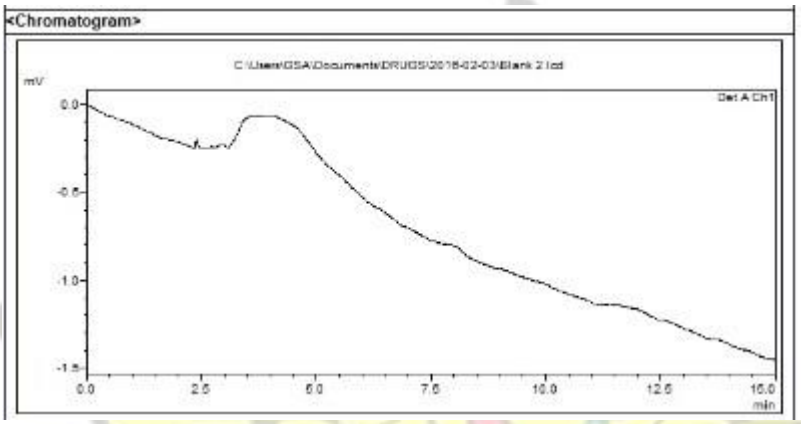


Figure 4.13 Chromatogram of blank sample after injection of high concentration of analytes showed the absence of any peak due to the analyte(s).

4.8 ASSAY OF ANTIRETROVIRAL DRUGS IN FORMULATIONS

Table 4.10 Percentage content obtained for Tenofovir, Lamivudine and Nevirapine compared with standard content according to USP (2008)

TENOFOVIR/ LAMIVUDINE + NEVIRAPINE	CONTENT	STANDARD CONTENT	INFERENCE
TENOFOVIR	98.55± 0.17	90- 110	Passed
LAMIVUDINE	105.33± 0.85	90- 110	Passed
NEVIRAPINE	99.20± 1.17	90- 110	Passed

Table 4.11 Percentage content obtained for Tenofovir and Lamivudine

compared with standard content according to USP (2008)

TENOFOVIR/ LAMIVUDINE	CONTENT	STANDARD CONTENT	INFERENCE
TENOFOVIR	97.50± 0.75	90- 110	Passed
LAMIVUDINE	97.72± 0.09	90 -110	Passed

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Identification of Pure Powder of Lamivudine, Nevirapine and Tenofovir Disoproxil Fumarate

Infrared spectroscopy was used in the identification of the pure compounds. The spectra obtained were compared to information of spectra of the chemical reference standards obtained from Danadams Pharmaceuticals Industry Limited. The nature of the spectrum of individual pure powders as well as the wavenumbers at which specific functional groups occur compared to that of the chemical reference standards were used in the identification of the pure powder samples. The results showed that peaks occurring at specific wavenumbers in the functional group region in the spectra for pure powders were similar to that in the spectra of the chemical reference standards. The fingerprint region which is characteristic of a compound and aids significantly in identifying the compound were also similar in both spectra. (Refer to APPENDIX: Figures B1 to B6; pages 77- 82)

5.1.2 Method Development

Literature search was carried out on the physico-chemical properties of antiretrovirals under study. Properties such as solubility and pKa were taken into consideration in selecting initial conditions. In addition, previous works done on these antiretrovirals in other combination medications and as single components were reviewed. The following reagents and solvents were then selected to be used: methanol, phosphate buffer, ammonium acetate buffer and acetonitrile. Methanol was solely chosen initially without acetonitrile because

research shows that methanol which is more polar than acetonitrile reduces the risk of solid buffer precipitation, is more affordable and less toxic. The buffers were to modify the mobile phase system in terms of ionic strength and polarity. Trials were carried out using a combination of phosphate buffer (adjusted to pH 3 with orthophosphoric acid) and methanol. For the 20:80v/v ratio, relatively good peaks especially for tenofovir and nevirapine with a split peak for lamivudine was observed. Elution of the first compound was always less than one minute for combinations involving methanol and phosphate buffer unless flow rate was reduced. Decreasing the methanol composition as seen in Table 4.1 above resulted in worsening tailing, peak splitting for lamivudine, minimal resolution between peaks and decreasing retention time. It was observed that phosphate buffered mobile phases consistently caused clogging of the column. This led to the need to regenerate the column many times. The ammonium acetate buffer which was usually used from literature for lamivudine assay and other combinations such as zidovudine, nevirapine and lamivudine in other works was explored.

The use of ammonium acetate buffer adjusted to pH 3 and methanol in various ratios showed the separation of peaks without splitting and relatively good retention with the first compound eluting after two minutes. However, resolution of peaks was not good enough, the peaks were quite broad and it was observed here that with decreasing methanol composition the retention time decreases. This may have been due to the fact that the compounds got ionised in acidic environment and as such became more polar. The buffer was significantly aqueous based and as such with the decrease in methanol and increase in buffer composition, the analytes become more soluble in the solvent

system making them elute faster and hence reducing their retention times. Further research was done which indicated that employing acetonitrile reduced back pressure and often resulted in slightly better peak shape. Also, acetonitrile is reported not to form polar-polar or ionic interactions with solutes as may occur with the use of methanol which is protic and therefore improves selectivity for polar analytes. It was also shown to produce better early peak retention time reproducibility at low compositions in the mobile phase system. With all these advantages discovered, acetonitrile was introduced and added to the mobile phase system in low composition. Method development at low pH < 3 but not below 2 is usually recommended. This pH ensures silanol groups on a reverse phase column are protonated and thus minimises peak tailing by eliminating silanol/ base interaction (Agilent, 2015). It is based on this that the conditions obtained above were optimised. Lowering the pH of the buffer to 2.8 produced symmetrical peaks with good resolution between analytes. The mean retention times for lamivudine, nevirapine and tenofovir disoproxil fumarate were 3.26, 5.42 and 7.56 minutes respectively. A run time of 10 minutes was therefore used for the analysis. In accounting for the order of elution of lamivudine, nevirapine and nevirapine, the molecular weight, functional groups on the compound which makes it acidic or basic (relating to pKa) and the pH of mobile phase system may be factors to take note of. The reported pKa value from literature search indicates 4.24-4.3 and 13.8 for lamivudine, 2.8 for nevirapine and 3.8 and 6.7 for tenofovir. The pH of the aqueous buffer in the mobile phase system was 2.8. Tenofovir is found to exist in more of its unionised form (about 90%) at a lower pH and this may be due to the presence of the many acidic functional groups (from the fumaric acid groups and the

many carboxylic acid groups in the disoproxil portion of the molecule) which makes the drug more acidic than basic and hence less ionised in a low pH medium. The largely unionised form renders it more lipophilic and hence it will interact more with the reverse stationary phase as compared to the aqueous mobile phase. Tenofovir also has highest molecular weight compared to the other two compounds, with a molecular weight of about 635.51g/mol and therefore the proportion that is ionised will move at a relatively slower rate as compared lamivudine and nevirapine. All these may contribute to reasons why tenofovir eluted last compared to the others. The pKa of Nevirapine is reported to be 2.8 and with the aqueous buffer adjusted to a pH 2.8, a 50% ionisation may be occurring here which gives it intermediate polarity and hence interact equally with the mobile phase and the stationary phase. Its molecular weight is higher than that for lamivudine, with a molecular weight of 266.30g/mol and therefore its ionised species will move at a relatively slower rate but faster than tenofovir. Lamivudine with a pKa reported to be 4.24-4.3 and 13.8 is weakly basic and hence when present in an acidic medium will be more ionised and hence more polar, thereby interacting more with the mobile phase than the stationary phase. It is the least bulky compared to the others with a molecular weight of 229.26 g/mol, hence its ionised form will move at a relatively faster rate compared to the others and therefore accounting for its earliest elution compared to nevirapine and tenofovir disoproxil fumarate. The flow rate used was 1.0ml/ min at 25°C.

Sample preparation was investigated concurrently with the preliminary runs. It started initially with the use of methanol only with sonication, filtration and injection. This however constantly produced split peaks or shouldering peaks

as well as broad bands. An amount of buffer was then added to the dissolution solvent to investigate the effect on peak shape. The results showed improved peak shape with minimal splitting and shouldering. The mobile phase composition was then used to prepare the samples for the study and very good peaks (symmetrical) were obtained. Initial peak splitting, shouldering and broadening may have been as a result of using 100% methanol for the preparation (Charde et al., 2014). This indicated that the higher proportion of methanol in the injection solvent than in the mobile phase disrupted the analyte peak. Therefore using the mobile phase as the dissolution solvent eliminated the problems faced earlier.

5.1.3 Method Validation

The method developed was validated for linearity, LOD, LOQ, accuracy, specificity, accuracy, precision (inter-day and intra-day), robustness, stability and sample carryover.

Linearity, Range, LOD and LOQ

Linearity of the developed method was investigated by injecting six concentration solutions of the pure standard of lamivudine, nevirapine and tenofovir disoproxil fumarate. The regression coefficient of correlation (r^2) value obtained for the above mentioned antiretroviral drugs were 0.9968, 0.995 and 0.9973 respectively. Since all the values were ≥ 0.995, the test for linearity has passed as the values are within the acceptance criteria and hence it can be said that there is a strong relationship (proportional and linear) between the responses and the concentrations injected. The range within which there is an acceptable degree of linearity, accuracy and precision for lamivudine,

nevirapine and tenofovir dixoproxil fumarate are 15µg/ml- 90µg/ml, 7µg/ml-42µg/ml and 10µg/ml – 59µg/ml respectively.

The lowest quantity of analyte in Lamivudine, Nevirapine and Tenofovir disoproxil fumarate solution which can be detected (seen or picked as a response) but not essentially quantified from the HPLC instrument using this method is 5.5032µg/ml, 3.1496µg/ml and 3.9267µg/ml respectively. The minimum quantity or amount of Lamivudine, Nevirapine and Tenofovir disoproxil fumarate in a sample that is capable of being quantitatively measured with acceptable precision and accuracy using this method is 16.6762 µg/ml, 9.5443µg/ml and 10.0433µg/ml. They all fall within the specified range for the analytical method developed.

Precision Repeatability

This was analysed as an intra- day precision where replicate sampling was done on three concentrations spanning the specified range over a period of time in the same day. Three determinations in the day was carried out and the results expressed as mean peak areas of the replicate injections in all three determinations in the day were in close agreement with each other and did not differ much. The %RSD calculated for the intra-day precision as shown in Table 4.5 reveals that all the values obtained are less than 2% which indicates that the test for repeatability passed. The acceptance criterion for precision is %RSD ≤ 2%.

Intermediate Precision

The element of investigation here was the closeness of agreement of response and in this case peak area on different days, that is for three consecutive days.

The mean peak areas did not differ significantly from each other for all three days and the %RSD been less than 2% for all days indicates that the method passed the intermediate precision test.

Accuracy

The results obtained for accuracy expressed as recovery results shows that the developed method has a satisfactory level of accuracy for the assay of the pure sample within an 80% to 120% range of the working concentration of lamivudine, nevirapine and tenofovir. The recovery results as shown in Table 4.5 obtained for accuracy at each level are consistent and precise and show the closeness of the mean concentration obtained after test to the true concentration value of the analytes that was prepared. The criterion for recovery is usually 100%± 10 for a pure sample. All the recoveries at all levels fell within the range of acceptance. It is also recommended that there is consistency in the values obtained at each concentration level. The results obtained depict consistency in recoveries obtained with the use of the developed analytical test method. The %RSD at all the concentration levels fell within the acceptance criteria of ≤ 2%. The standard deviations also fall within the range required for satisfactory quality control work which is ≥ ±2. The test method develop can be said to be accurate.

Robustness

The flow rate and the wavelength of detection of analytes were deliberately changed to observe their effects on the analytical method. The retention time increased with decreased flow rate (0.8 ml/min). The retention time was slightly prolonged as analytes had to take a relatively longer time to elute from the column. The converse of this happened with increased flow rate to 1.2 ml/min.

This was evident by the shift in retention times of lamivudine from 3.26 to 2.20, nevirapine 5.43 to 3.42 and tenofovir 7.56 to 4.78 minutes when flow rate was increased to 1.2ml/min. Reducing flow rate to 0.8 ml/min increased lamivudine, nevirapine and tenofovir retention times to 4.42, 6.38 and 8.26 minutes respectively. A slight increase in peak areas was also observed for flow rate 0.8ml/min. There was still good resolution between the peaks and selectivity maintained in all cases. All RSDs were also within the acceptance criteria of ≤ ±2%.

The changes made in wavelength showed no significant changes in the peak areas of the analytes and all RSD values were within acceptance limits. This indicates that the developed method is robust.

Specificity

The chromatograms generated for the blank sample (dissolution solvent) showed the absence of any interference. The method was observed to be specific for the assay testing as it selectively identified the analytes under study in the various tablets assayed without any blank interference. The retention times were used to identify the presence of the analytes under study in the various chromatograms generated and all of them indicate the specificity and the selectivity of the method for the analytes under study.

Stability

The solution stability was assessed for the pure sample solution for 6 hours at room temperature by observing for any changes in peak area with time. The peak areas recorded for the solution with time was compared to the freshly prepared solution at time 0. The chromatograms obtained over the six-hour

period for the various analytes showed no other additional peaks which means that analytes did not break down on standing at room temperature for six hours. In addition to this, the peak areas obtained for the solution over the period of time was relatively consistent.

5.1.4 Sample Carryover

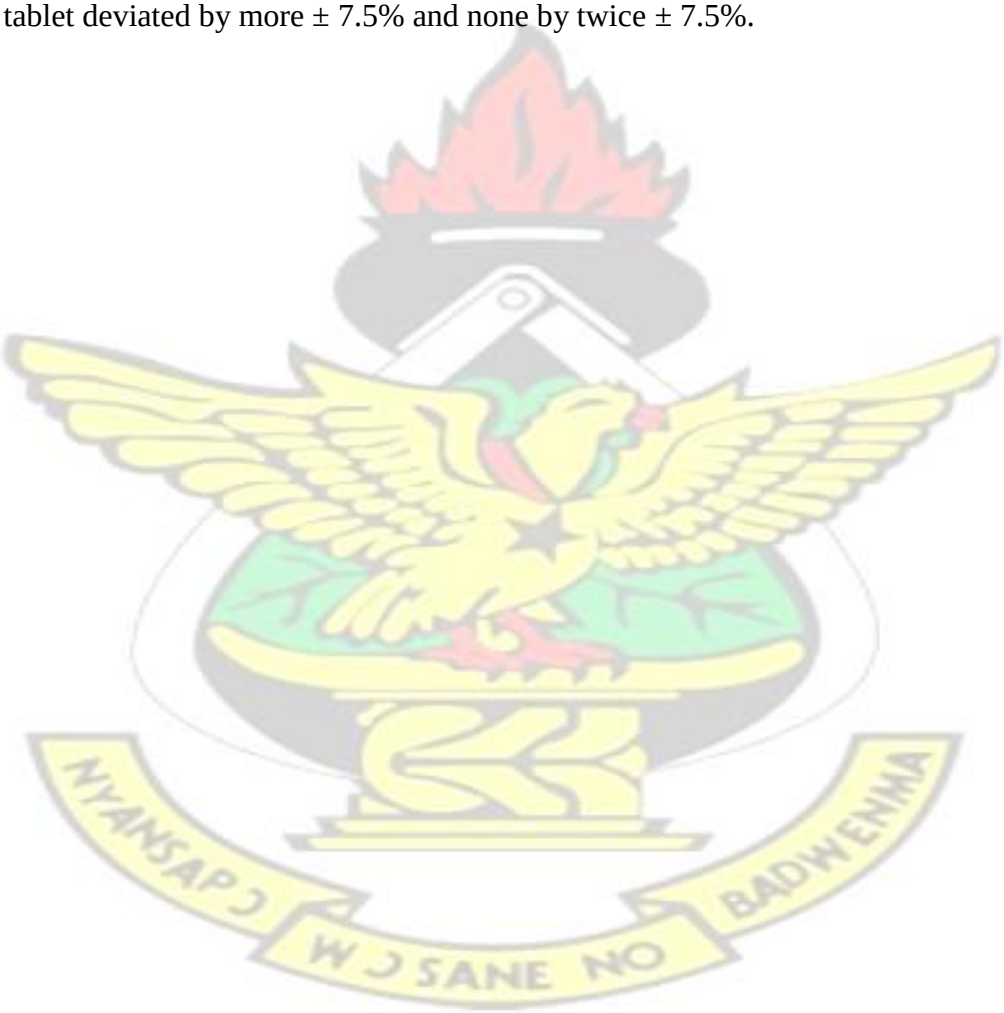
The chromatogram generated for the blank sample after the column had been stressed with high concentrations of the analyte by three replicate injections indicates the absence of any peak for any analyte with no peak area integration and value recorded. It can therefore be said that the needle wash adequately cleanses the needle such that there is no transfer of residual analyte due to adsorption or residual analyte present on/in the needle during injection. Also, any analyte residual contamination of column is eliminated in between run. The results obtained from the study can be said to be a true representation of what is injected and hence results obtained are reliable.

5.1.5 Assay of Antiretroviral drugs in Formulations

Uniformity of Weight

The uniformity of weight test is a pharmacopoeial test done to determine the uniform distribution of active ingredient and excipients in a dosage form in a given batch of manufactured drugs. This ensures consistency and accuracy in drug formulation manufactured and hence administered to patients. According to the British Pharmacopoeia for a tablet (coated or uncoated) with strengths 250mg and above, not more than two tablets should deviate from the average weight by ±5% and not one should deviate by more than ±10% (Pharmainfo.net, 2011). Results obtained from the uniformity of weight test shows that the batch of fixed dose Tenofovir and Lamivudine tablets (300mg/300mg) passed the

uniformity of weight test (Refer to APPENDIX C pages 83-84). This implies that the tablets manufactured in the given batch had active ingredients and excipients consistently distributed uniformly in the various formulations. For tablets with active ingredients of mass more than 80mg but less than 250mg, not more than two tablets should deviate from the average weight by more than ±7.5% and none should deviate by twice ±7.5% (Pharmainfo.net, 2011). The batch of Nevirapine (200mg) also passed the uniformity of weight test as no tablet deviated by more ± 7.5% and none by twice ± 7.5%.



Assay

Tenofovir/ Lamivudine + Nevirapine

The percentage content of a drug in a formulation refers to the actual amount of the active ingredient present in a formulation expressed as a percentage usually measured against the label claim of the amount of drug present. The percentage content of a drug is analysed to ensure drugs produce the required therapeutic effect as well as avoiding toxicities due to overdose or treatment failure due to under dose. It is therefore important that drugs meet the specification limits set in monographs.

According to the United States Pharmacopoeia, the percentage content of Tenofovir and Lamivudine and Nevirapine in tablets should fall within the limits 90%- 110% (USP, 2008). From the analysis conducted the content obtained for Tenofovir and Lamivudine was 98.55%± 0.17, 105.33%± 0.85 and 99.2%± 1.17 respectively. These fell within the acceptable range and hence the tablets comply with the monograph's specifications for content. Tablets passing the percentage content and uniformity of weight test mean that each tablet contains the required amount of active ingredient as well as excipients. This signifies correct weighing was done, good manufacturing procedures were followed in production and therefore there is an eventual reduction in the risk of toxicities or treatment failure and development of resistance due to overdose or under-dose of drug respectively.

Tenofovir/ Lamivudine

The percentage content obtained for tenofovir and lamivudine here were 97.50%± 0.75 and 97.72%± 0.09 which all fell within the acceptable specification range for content. These tablets therefore when administered will

provide the adequate dose for treatment and hence improve the quality of life of people living with HIV and preventing the transmission of new infections to children in pregnant women and to partners who are HIV negative.

5.2 CONCLUSION

An accurate, precise and selective reverse HPLC method for the simultaneous estimation of lamivudine, nevirapine and tenofovir disoproxil fumarate has been developed and validated. The method was developed based on adequate information obtained on the drugs to be studied which included their solubility profiles, their pKa(s), and structure as well as previous research works that had been done on them individually or in other FDC formulations. The optimised conditions used to develop the method include the use of a C-18 Column, a ternary phase mobile phase system consisting of methanol: ammonium acetate buffer (pH adjusted to 2.8): acetonitrile in a ratio 50:40:10%v/v, flow rate of 1.0 ml/min, wavelength of detection 270nm, temperature set at 25° and a run time of ten minutes. The average retention time obtained for lamivudine, nevirapine and tenofovir disoproxil fumarate was 3.26, 5.42 and 7.55 minutes respectively which represents a suitable separation for further analytical work. The sample preparation was done using the mobile phase.

Infrared spectroscopy was used to identify the pure powders of tenofovir disoproxil fumarate, lamivudine and nevirapine used in the study. The peaks obtained in the functional group region and fingerprint regions (characteristic for a particular compound) of the infrared spectra of the pure powders were similar to those present in the spectra of the reference standards. The comparison of spectra of the pure powders to that of the reference standards made it possible to identify the pure powders.

The method was validated per the ICH guidelines and passed all tests for the various validation parameters.

a) The range within which there was satisfactory linearity, accuracy and precision of the method is 10-54µg/ml for tenofovir disoproxil fumarate, 7-42µg/ml for nevirapine and 15-90µg/ml for lamivudine.
b) The limits of detection were 5.50µg/ml, 3.15µg/ml and 3.93µg/ml for lamivudine, nevirapine and tenofovir disoproxil fumarate respectively. The limits of quantification were 16.68µg/ml, 9.54µg/ml and 10.04µg/ml for lamivudine, nevirapine and tenofovir disoproxil fumarate respectively.
c) The developed method is specific and selective based on the similarity of retention times of the antiretrovirals in formulation and pure powders and the absence of blank interferences.
d) The antiretrovirals in this study were found to be stable for at least six hours after their solutions were prepared.
e) There was no evidence of sample carryover and this confirms the accuracy and precision of responses obtained with the use of the HPLC instrument.

The reverse HPLC method was applied in an assay for the quantitative estimation of the drugs in tenofovir/ lamivudine fixed dose combination co-blistered with nevirapine and tenofovir/ lamivudine fixed dose combination only. The percentage content obtained for tenofovir and lamivudine in Tenofovir/Lamivudine fixed-dose combination were 97.50%± 0.75 and 97.72± 0.09 respectively. The percentage content of tenofovir, lamivudine and nevirapine in Tenofovir/Lamivudine fixed-dose combination co-blistered with nevirapine were 98.55%± 0.17, 105.33%±

0.85 and 99.20%± 1.17 respectively. The method is therefore useful for routine quality control for the assay of antiretroviral drugs containing tenofovir disoproxil fumarate, lamivudine and nevirapine.

5.3 RECOMMENDATIONS

1. Collaborative studies may be carried out for the standardisation of the method.
2. Other isocratic methods may be developed using relatively cheaper reagents.

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APPENDICES

Appendix A

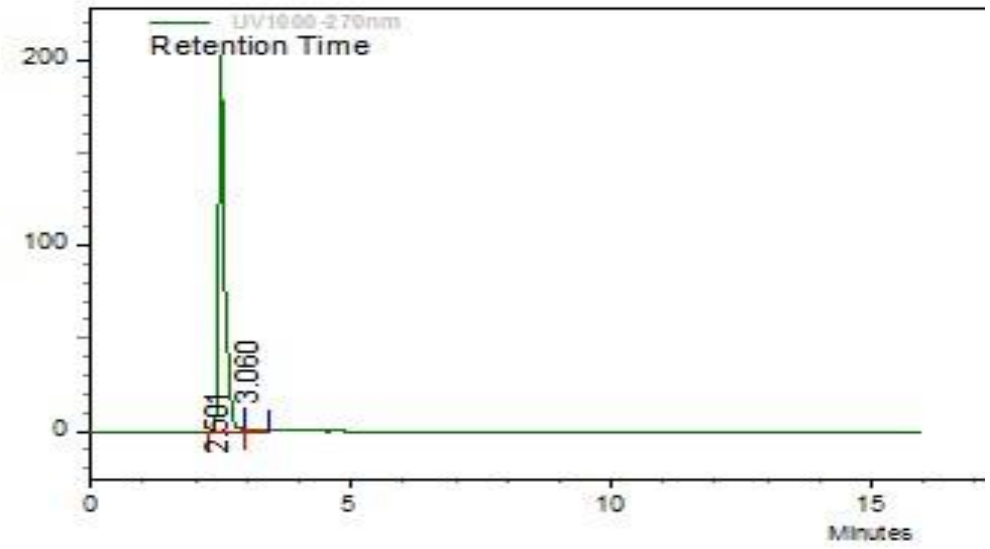

Figure A.1 Chromatogram of lamivudine using developed method

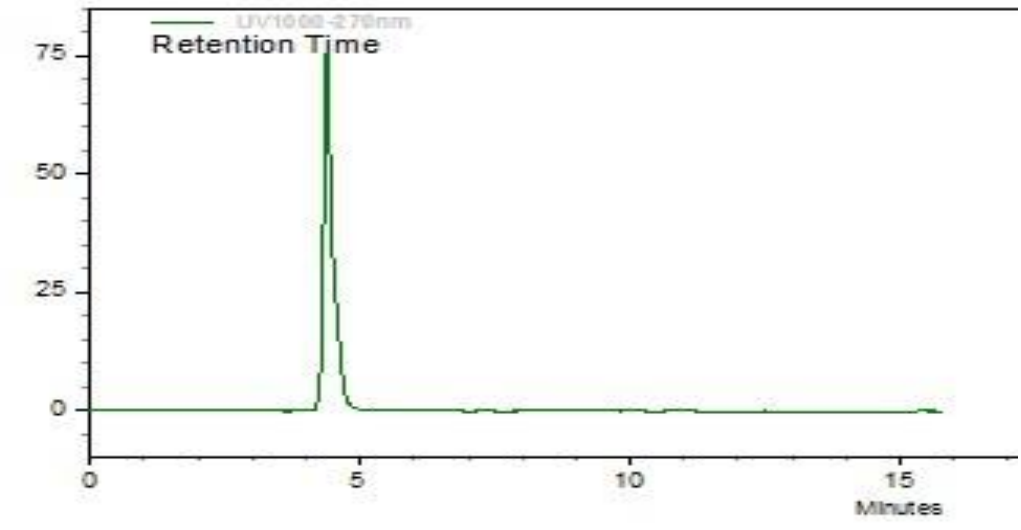

Figure A.2 Chromatogram of nevirapine using developed method

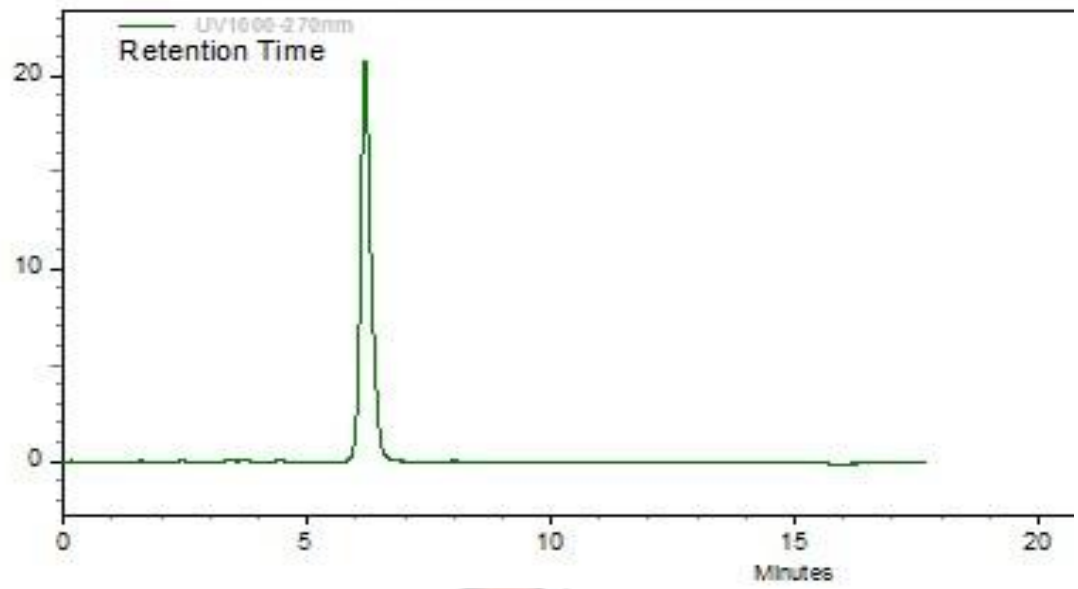

Figure A.3 Chromatogram of tenofovir disoproxil fumarate using developed method

P.S.: Chromatograms for individual pure samples were obtained using a Thermo Finnigan Spectra System HPLC Instrument. Initial method development was done using the above mentioned instrument. It however broke down and therefore the SHIMADZU prominence UFLC series system was used for subsequent work on validation and analysis of formulation. This is responsible for slight differences in retention times seen in chromatograms in the thesis presented.

Appendix B

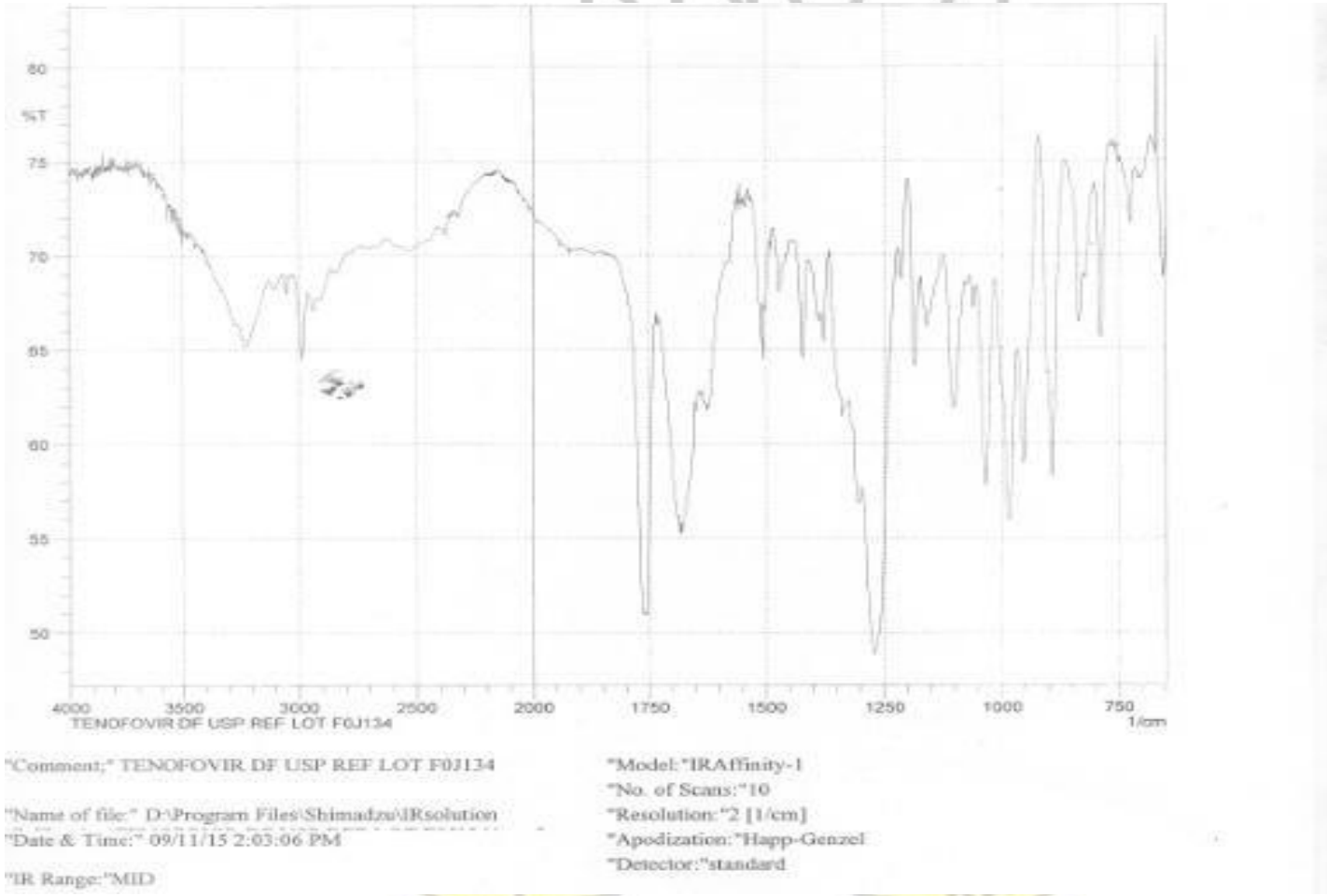

Figure B.1 Infra- red spectrum of tenofovir disoproxil fumarate chemical reference standard.

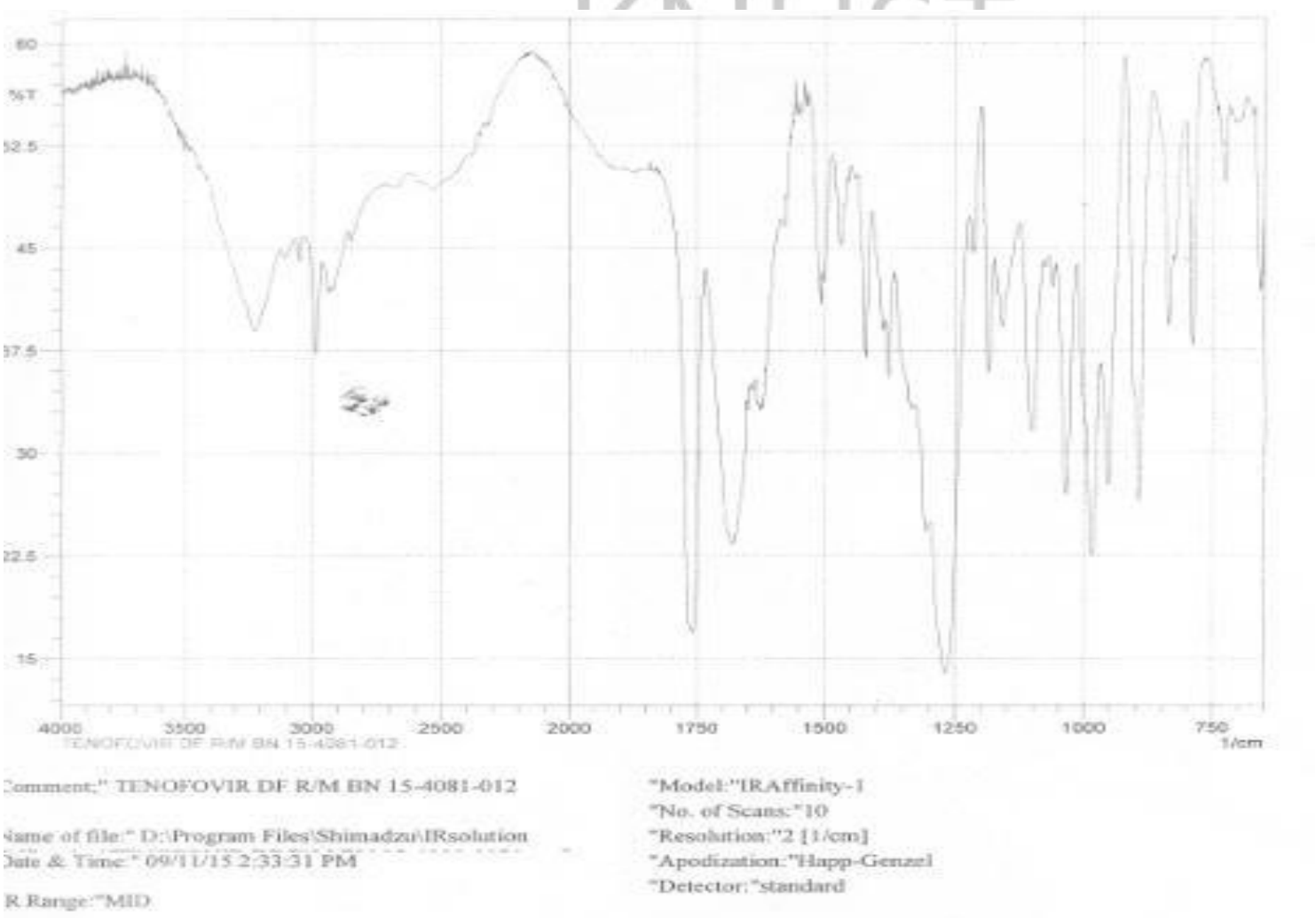

Figure B.2 Infra-red spectrum of tenofovir disoproxil fumarate pure sample

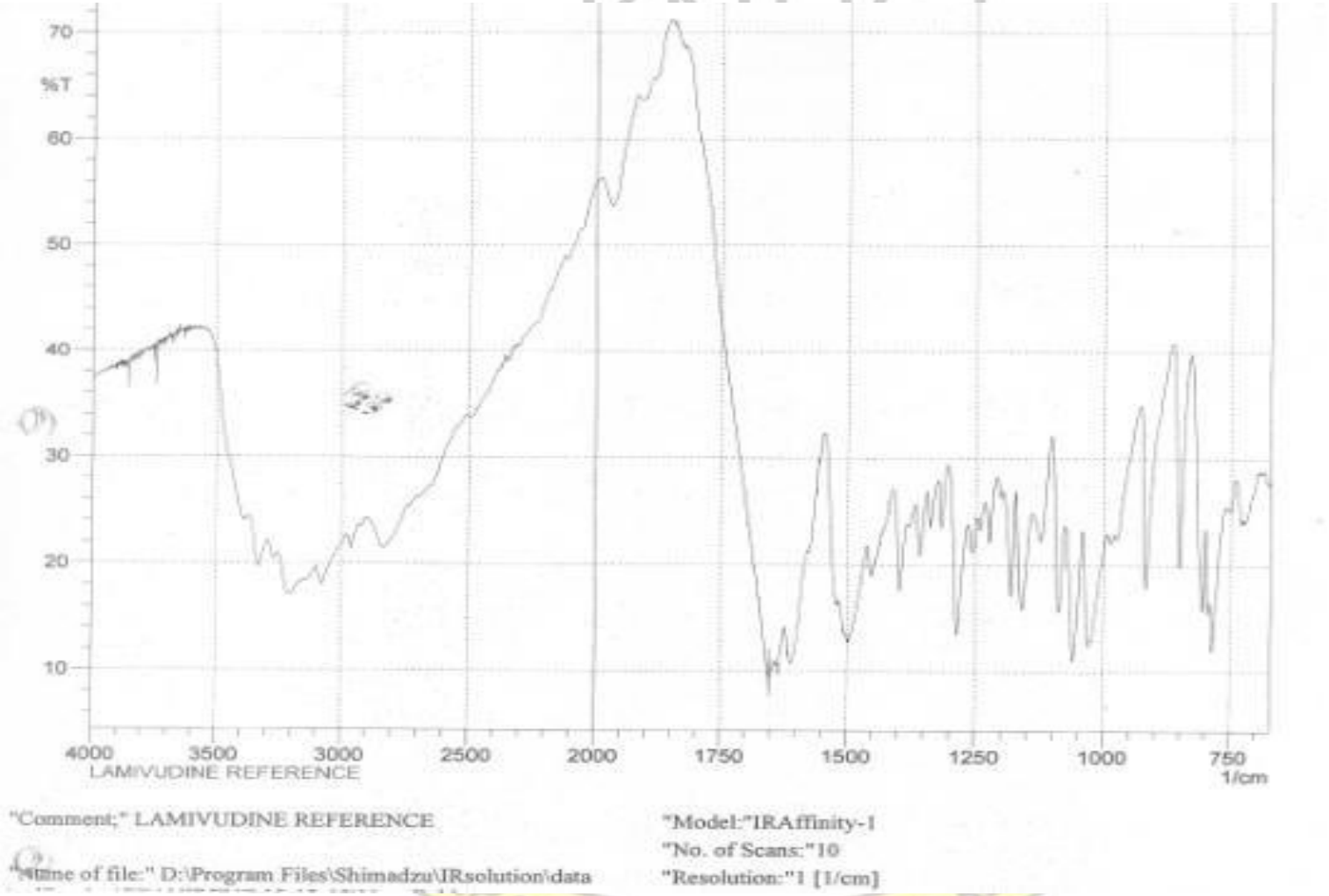

Figure B.3 Infra- red spectrum of lamivudine chemical reference standard

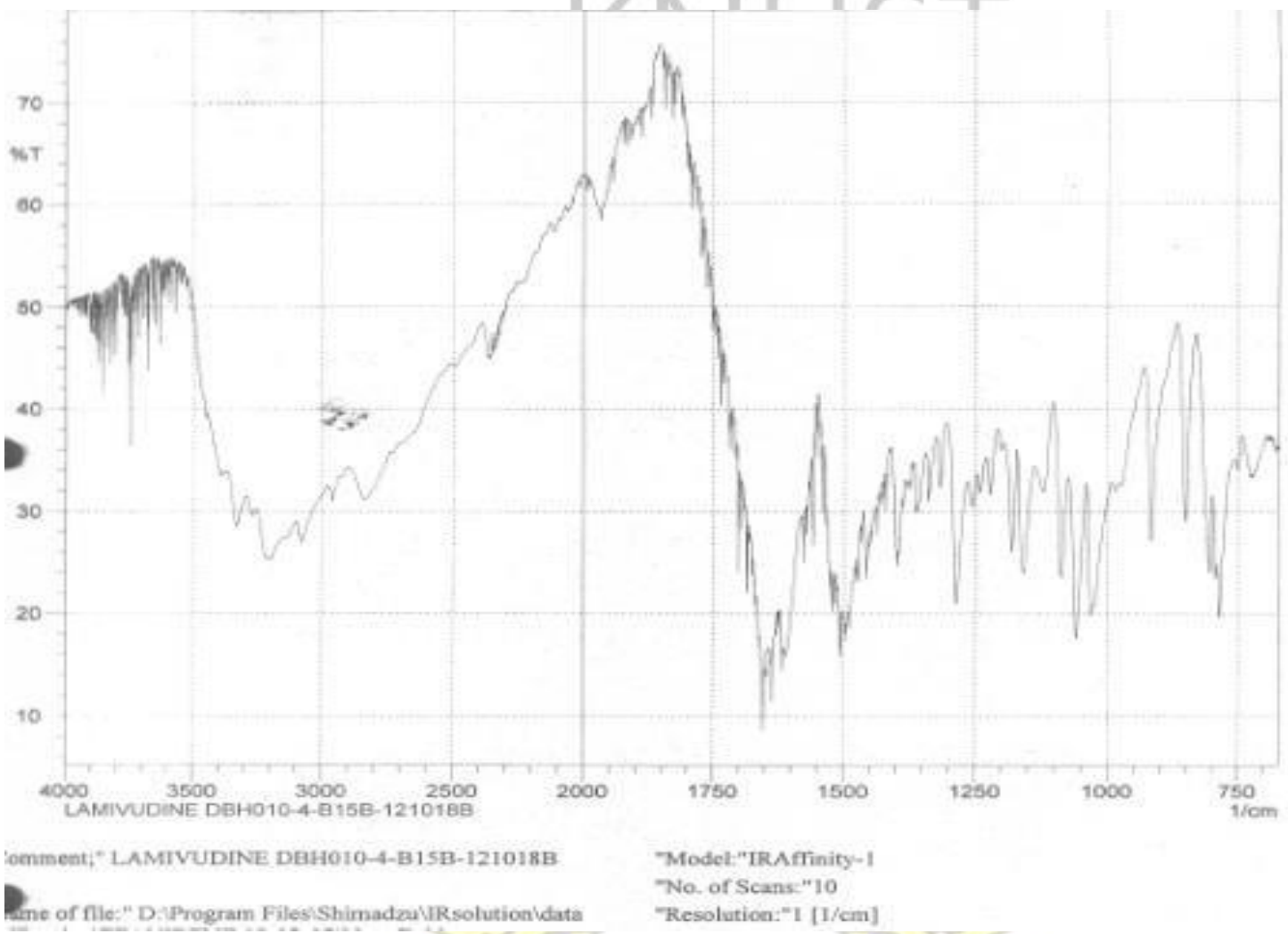

Figure B.4 Infra- red spectrum of lamivudine pure powder

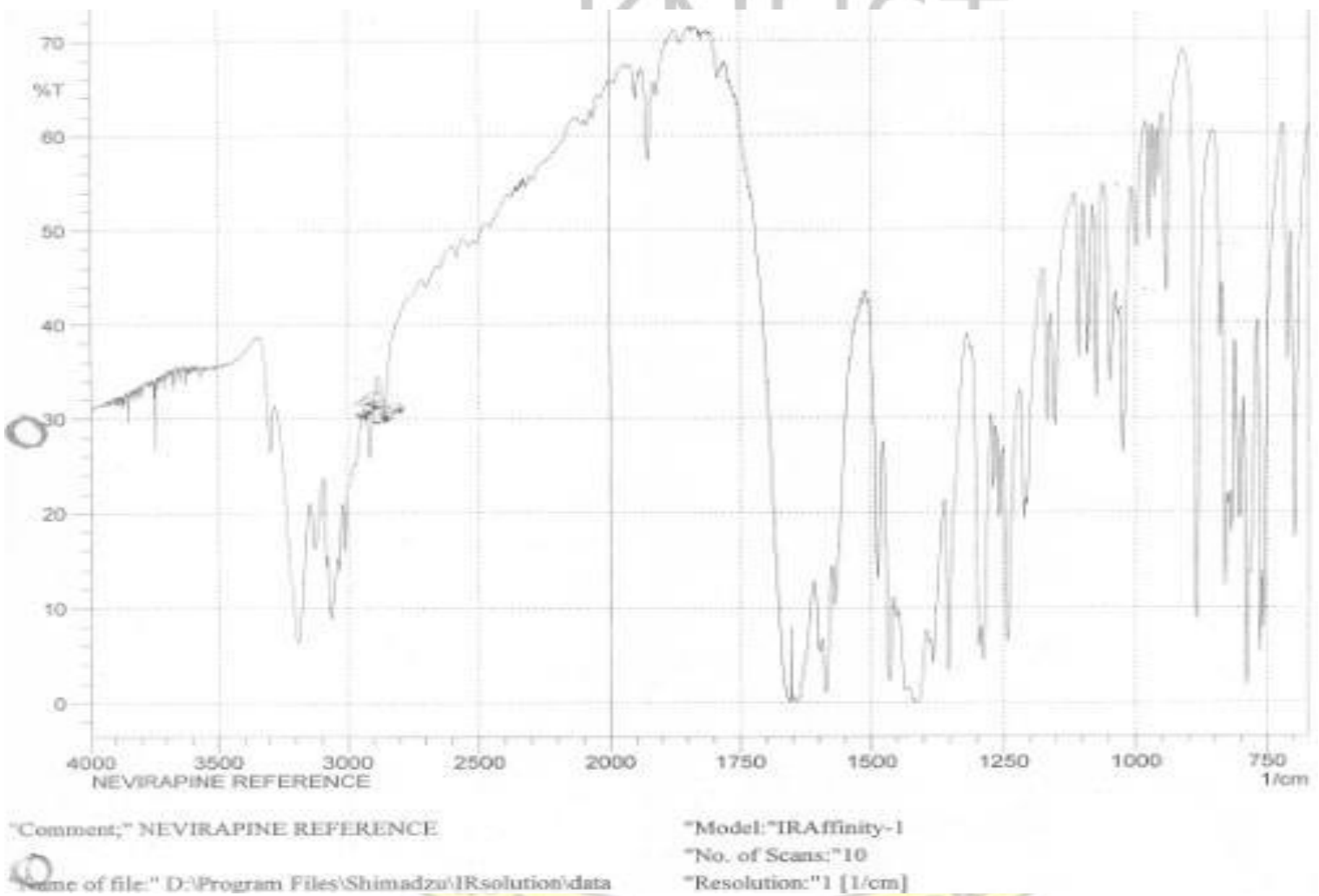

Figure B.5 Infra- red spectrum of Nevirapine chemical reference standard

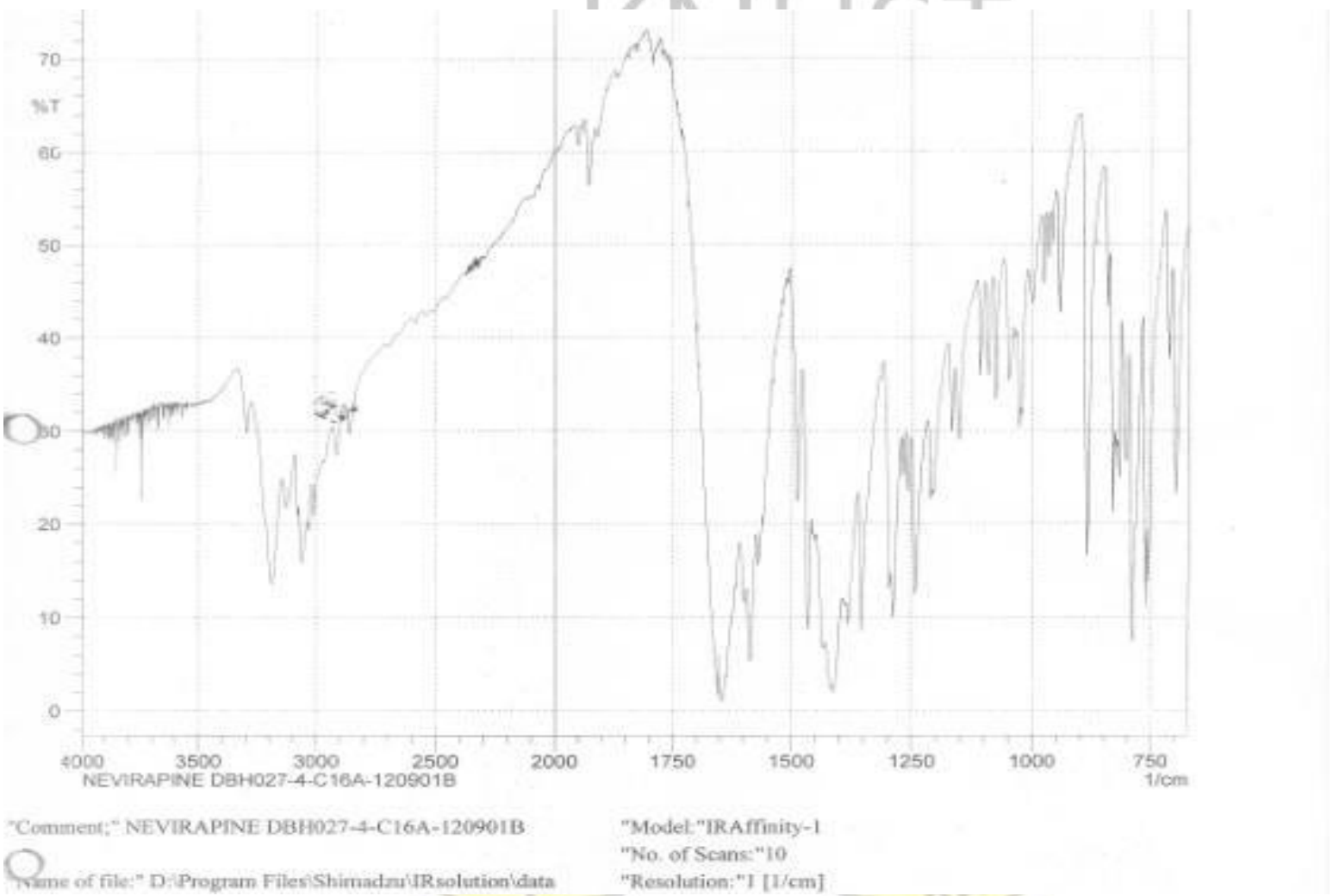

Figure B.6 Infrared spectrum of nevirapine pure powder

Appendix C

UNIFORMITY OF WEIGHT

Table C.1 Tenofovir/ Lamivudine (300mg/ 300mg)

Tablet number	Individual tablet weight/g (x)	Deviation (x-a)	% Deviation (x-a)/a × 100
1	1.0888	-0.0544	-4.75
2	1.1148	-0.0284	-2.48
3	1.0897	-0.0535	-4.68
4	1.1026	-0.0406	-3.55
5	1.1118	-0.0314	-2.74
6	1.0769	-0.0096	-0.84
7	1.1069	-0.0363	-3.17
8	1.1092	-0.0340	-2.97
9	1.1129	-0.0303	-2.65
10	1.0951	-0.0481	-4.20
11	1.0922	-0.0510	-4.46
12	1.0940	-0.0492	-4.30
13	1.0924	-0.0508	-4.44
14	1.1218	-0.0214	-1.87
15	1.1254	-0.0178	-1.55
16	1.0972	-0.0460	-4.02
17	1.1022	-0.0410	-3.58
18	1.0716	-0.0197	-1.72
19	1.1060	-0.0372	-3.25
20	1.1116	-0.0316	-2.76

Weight of 20 tablets = 22.8631g

Average weight = $\frac{22.8631}{20}$ g = 1.1432g

Weight of powder taken

300mg ≡ 1.1432g

75mg ≡ 0.2858g

Table C. 2 Uniformity of weight for co-blistered Nevirapine tablets (200mg)

Tablet number	Individual tablet weight/g (x)	Deviation (x-a)	% Deviation (x-a)/a ×100%
1	0.8144	0.0081	1.00
2	0.8066	0.0003	0.03
3	0.8021	-0.0042	-0.52
4	0.8033	-0.0030	-0.38
5	0.8028	-0.0035	-0.44
6	0.8069	0.0006	0.07
7	0.8089	0.0026	0.32
8	0.8058	-0.0005	-0.07
9	0.8005	-0.0058	0.72
10	0.8120	0.0057	0.70

Weight of 10 tablets = $\frac{8.0633}{10}$ g = 0.80633g

200mg ≡ 0.80633g

35mg ≡ 0.1411g

Appendix D

Table D.1 ACCURACY

Sample calculation using Lamivudine

Conc. (µg/ml)	Peak area			Actual conc. (µg/ml)			% recovery			Mean
				1	2	3	1	2	3	
36	557522	557580	554140	35.84	35.83	35.59	99.51	99.53	98.87	99.30
45	688382	699492	698986	44.86	45.63	45.59	99.68	101.39	101.31	100.80
54	802497	801745	808861	52.74	52.68	53.17	97.66	97.56	98.47	97.90

Calculating actual concentration

Using equation of the line, x = (y – c)/ slope

X= (557522 – 38521)/ 14487 = 35.84µg/ml

% recovery = (35.84µg/ml/ 36µg/ml) × 100 = 99.51%

Table D.2 Intraday Precision

Sample using lamivudine

NO. OF RUNS	PEAK AREA								
	1ST DETERMINATION			2ND DETERMINATION			3RD DETERMINATION		
	15	45	90	15	45	90	15	45	90
1	257301	698382	1347496	258001	697982	1367601	257651	698182	1357548
2	259971	687492	1357501	257409	697424	1368511	258690	692458	1363006
3	258518	707986	1378103	256998	689915	1359821	257758	698950	1368962
MEAN	258596.67	697953.33	1361033.33	257469.33	695107	1365311	258033	696530	1363172

CONC.	MEAN PEAK AREAS DETERMINATIONS			MEAN	%RSD (µg/ml)
	1	2	3		
15	258596.67	257469.33	258033	258033	0.2184

45	697953.33	695107	696530	696530.11	0.2043
90	1361033.33	1365311	1363172	1363172.11	0.1569

Table D.3 Inter- day Precision

Sample using lamivudine (45µg/ml)

No. of runs	Peak area on various days		
	1	2	3
1	697982	698382	697988
2	697424	687492	689984
5	689915	707986	699121
4	698182	697953	698943
5	692458	695107	693512
6	698950	696530	695421
Mean	695818.50	697241.67	695828.17
%RSD	0.5331	0.9456	0.5177

Table D.4 Assay

Sample using lamivudine (45µg/ml) for tenofovir/ lamivudine (300mg/300mg)

Serial code.	Peak area 1	2	3	**Mean**	**Actual conc. (µg/ml)**	**Content (%)**
Assay 1A	715704	718547	725561	701286.67	47.04	104.53
Assay 1B	727834	719705	726561	719937.33	47.37	105.26
Assay 1C	730207	731329	731329	724700	47.80	106.22

Assay 1A, 1B and 1C represent three different weights of the same crushed powder taken equivalent to 450mg of lamivudine.

Actual concentration= (701286.67 - 38521)/ 14487 = 47.04µg/ml

Mean content = $\frac{(104.53 + 105.26 + 106.22)}{3}$ = 105.33%w/w