DEVELOPMENT AND VALIDATION OF A METHOD FOR THE ASSAY AND DISSOLUTION OF A FIXED DOSE COMBINATION OF LAMIVUDINE, TENOFOVIR AND EFAVIRENZ TABLET

By

Bwiro Joseph

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Pharmacy in Quality Control and Quality Assurance of Muhimbili University of Health and Allied Sciences

Muhimbili University of Health and Allied Sciences

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled **Development and validation of a method for the assay and dissolution of a fixed dose combination of lamivudine, tenofovir and efavirenz tablet** in (Partial) fulfillment of the requirement for the degree of Master of Pharmacy in Quality Control and Quality Assurance of Muhimbili University of Health and Allied Sciences.

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…….

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DECLARATION AND COPYRIGHT

I, **Bwiro Joseph**, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other university for a similar or any other degree award.

Signature…… Date ……

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DEDICATION

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ABSTRACT

A reverse phase-high performance liquid chromatographic method was developed and validated for the simultaneous determination of lamivudine, tenofovir disoproxil fumarate and efavirenz in lamivudine, tenofovir and efavirenz finished formulation product. The method was developed by altering various organic solvents such as acetonitrile and methanol, column, detection, flow rate and temperature. An isocratic elution mode with a mixture of acetonitrile and water in the ratio of (55:45 % v/v) was selected for the mobile phase with a nucleosil (Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5µm) column as stationary phase for simultaneous separation of lamivudine, tenofovir disoproxil fumarate and efavirenz. The separation was achieved at a flow rate of 1 mL/min and detection wavelength of 252 nm at room temperature. Further, different dissolution media were investigated for optimal release of lamivudine, tenofovir disoproxil fumarate and efavirenz from lamivudine, tenofovir and efavirenz tablets. The optimization of dissolution medium was preceded by establishment of the sink concentration for efavirenz which was found at 0.5% sodium dodecyl sulphate in water with a release of more than 75% of each of the three active pharmaceutical ingredients at 37°C with a paddle method, 75 rpm at 45 min. The analytical method was validated and the linear range was found in the concentration range of 0.05 to 0.12 mg/mL of lamivudine, tenofovir disoproxil fumarate and efavirenz with regression coefficient (r^2) of 0.9984 which met the acceptance criteria of r^2 equal or greater than 0.98. The % rsd for the intra-day precision were 1.08%, 1.23% and 1.46% for lamivudine, tenofovir disoproxil fumarate and efavirenz respectively. The % rsd for the inter-day precision were 1.95%, 1.99% and 1.67% for lamivudine, tenofovir disoproxil fumarate and efavirenz respectively. The test method had an acceptable level of accuracy for the assay of lamivudine, tenofovir disoproxil fumarate and efavirenz in lamivudine, tenofovir and efavirenz tablets from 50 % to 120 % of test concentration with % rsd less than 2% for all three active pharmaceutical ingredients. The test solution remained stable when stored at 4°C for 72 hours. The method was robust as it remained largely unaffected by small variations in temperature and mobile phase. All of these assessed parameters complied with the acceptance criteria hence indicated the usefulness of the reverse phase-high performance liquid chromatographic method for determination of assay and dissolution release testing for finished formulation product which contain lamivudine, tenofovir disoproxil fumarate and efavirenz active substances.

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List of Acronyms and Abbreviations

ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
APIs	Active pharmaceutical ingredients
APV	Amprenavir,
ART	Antiretroviral therapy
ARV	Antiretroviral
ATV/r	Ritonavir boosted atazanavir
AZT, ZID	Zidovudine
CMC	Critical micelle concentration
d4T	Stavudine
ddI	Didanosine
DLV	Delavirdine
DNA	Deoxyribonucleic acid
EFV	Efavirenz
ESI--MS	Electrospray-mass spectrometry
FDA	U.S. Food and Drug Administration
FDCs	Fixed-dose combinations
FTC	Emtricitabine
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography

ICH	International conference on harmonization
IDV	Indinavir
LC	Liquid chromatography
LMV, 3TC, LAM	Lamivudine
LPV	Lopinavir
LPV/r	Ritonavir boosted lopinavir
LTE	Lamivudine, Tenofovir, Efavirenz
M8	Active nelfinavir metabolite
n	Number of replicate experiments
NIR-CI	Near infrared chemical imaging
NNRTI	Non nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
NVF, NFV	Nelfinavir
NVP	Nevirapine
PEP	Post exposure prophylaxis
Pharm R & D Lab	Pharmaceutical Research and Development Laboratory
PIs	Protease inhibitors
Rpm	Revolution per minute
RTV	Ritonavir
SDS	Sodium dodecyl sulfate
SQV	Saquinavir
TDF, TNF	Tenofovir disoproxil fumarate
TFA	Trifluoroacetic acid

UV	Ultra violet
W.H.O	World Health Organization

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1.0 BACKGROUND INFORMATION

1.1 INTRODUCTION

It is three decades since the human immunodeficiency virus type 1 (HIV-1) imposed a threat especially to the third world countries including Tanzania. Since then the global cumulative death toll from HIV and/or acquired immunodeficiency syndrome (AIDS) has been greater than 25 million globally, where approximately 33 million people worldwide are living with HIV/AIDS, of which about 68% (22.5 million) live in sub-Saharan Africa (1).

Highly active antiretroviral therapy (HAART) has brought new hope for those people who live with HIV/AIDS by decreasing the morbidity and mortality among people infected with HIV. Highly active antiretroviral therapy also has improved the quality of life among the people who live with HIV/AIDS. Toxicity, potency, adherence, and resistance are still matters of intense research, which need to improve in order to overcome the current limitations of available drugs (2). Combination therapy is preferred to be the gold standard for the treatment of AIDS so as to maximize potency, minimize toxicity, diminish the risk for resistance development and reduction of pill burden to once-daily dosing so as to optimize the patient's compliance and reduce the treatment costs (3). The increasing number of antiretroviral drugs leads to increasing possibilities of combinations for the antiretroviral therapy (ART) of HIV-1 infected patients. Thus, it is of interest to determine the most potent combination of antiretroviral drugs for the first ART to delay the development of drug resistance (4). There is a number of antiretroviral (ARV) drugs that have been approved for the treatment of HIV/AIDS whereas fixed-dose combinations (FDCs) have been formulated to reduce toxicity and at the same time to increase tolerability, convenience and compliance to the patients (1). In resource-poor settings such as Tanzania the use of monotherapy in the treatment of HIV infection is not recommended. Therefore first-line FDCs used are largely combinations of two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI). The effectiveness of these combinations decreases over time, requiring a switch to combinations that retain potency in the presence of viral resistance (1). Increasing access to second-line FDCs and new developments in first-line ARV therapy are cost challenges especially in resource poor countries like Tanzania (1).

Antiretroviral drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits. Currently there are about six classes of antiretroviral drugs namely; nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) examples include lamivudine, emtricitabine, tenofovir, abacavir, zidovudine and didanosine; non-nucleoside reverse transcriptase inhibitors (NNRTIs) including delavirdine, efavirenz, etravirine and nevirapine; protease inhibitors (PIs) including drugs such as darunavir, indinavir, nelfinavir, atazanavir, saquinavir and fosamprenavir; entry or fusion inhibitors example enfurvirtide; CCR5 receptor antagonists example maraviroc; integrase inhibitors example raltegravir and maturation inhibitors which include drugs such as alpha interferon, bevirmat and vivecon (5).

Recommended drugs for first line treatment for adults and adolescent ART naïve patients include combination of zidovudine(AZT), stavudine(d4T), lamivudine(3TC), emtricitabine(FTC), tenofovir(TDF), nevirapine(NVP) and efavirenz (EFV).

The recommended first line regimen by WHO for ART-naive individuals eligible for treatment include; AZT + 3TC + EFV; AZT + 3TC + NVP; TDF + 3TC (or FTC) + EFV; TDF + 3TC (or FTC) + NVP (6).

For the second line regimen, WHO recommends a boosted protease inhibitor (bPI) plus two nucleoside analogues (NRTIs) where as ATV/r and LPV/r are the preferred bPIs for the second-line ART.

Also WHO recommends simplification of second NRTI options; if d4T or AZT has been used in first-line therapy, use TDF + (3TC or FTC) as the NRTI backbone in second- line therapy (6).

If TDF has been used in first-line therapy, use AZT + 3TC as the NRTI backbone in second- line therapy (6).

The default first line regimen in Tanzania is zidovudine (AZT) 300 mg + lamivudine (3TC) 150 mg twice daily and efavirenz (EFV) 600 mg once daily at night (7). For women of child bearing age, nevirapine (NVP) 200 mg twice a day is given instead of efavirenz because efavirenz has been reported to be associated with teratogenicity in early pregnancy (7). Antiretroviral drugs for those patients who have been exposed to antiretroviral therapy should be discussed with an antiretroviral expert before they are enrolled in the Care and Treatment Centre (CTC) and (re)started on treatment.

The preferred first line treatment options for children include; AZT + 3TC + NVP for children under 3 years old; AZT + 3TC + EFV or NVP for children 3 years old or more; abacavir (ABC) + 3TC) + EFV for children 3 years old or more or NVP for children under 3 years; d4T + 3TC + NVP available as fixed dose combination (FDC) for children (7).

The recommended second line regimen for infants and children who have failed their first line regimen include; didanosine (ddI) + abacavir + ritonavir boosted lopinavir (LPV/r) or nelfinavir (NVF).

The second line antiretroviral therapy in adults and adolescents in Tanzania includes NRTIs and PIs; ABC, ddI, TDF with 3TC or FTC, LPV/r and ritonavir boosted atazanavir (ATV/r) (7).

The second line NRTI choice for adults and adolescents depends on the first line regimen. For patients on AZT or d4T in first line, the default second line option is to use TDF + 3TC or FTC with ritonavir boosted PI either LPV/r or ATV/r.

For patients who were initiated on TDF in the first line because of intolerance to AZT and d4T, the default second line option is to use ABC + ddI combined with a ritonavir boosted PI either LPV/r or ATV/r (7).

1.2 Generic fixed dose triple combination

The Pharmaceutical Research and Development Laboratory (R & D) is working on a new fixed dose ARV combination which contains lamivudine which is a NRTI, tenofovir disoproxil fumarate which is a NtRTI and efavirenz which is a NNRTI (LTE) recommended by WHO as the first line ARV combination regimen (6). The fixed dose combination of LTE is recommended due to its high value on the simplicity of its use such as potential for one pill once daily and the treatment of HIV/HBV co-infection (6). Currently there is no such combination commercially available in the Tanzanian market, but the combination is in phase 3 clinical trial and the safety and efficacy of TDF in combination with lamivudine and efavirenz has already been reported.

It is anticipated to be a clinically potent combination that is free of short-term irritating toxicity and also of low production cost. The limited published data have indicated that emtricitabine and lamivudine have equivalent potency, and randomized controlled trials have produced evidence of

the efficacy of lamivudine combined with tenofovir disoproxil fumarate in a regimen containing either the non-nucleoside reverse transcriptase inhibitor efavirenz or a protease inhibitor lopinavir/ritonavir (8).

The efficacy and potency of efavirenz has been established in many clinical trials (9-11) and cohort studies; its pharmacokinetics allows for a convenient once-daily administration. Efavirenz is one of the first agents to be included in once-daily regimens in HIV patients due to its efficacy, potency and pharmacokinetics which allows once daily dosing which is convenient to the patients (11).

1.2.1 Lamivudine

Lamivudine (3TC, LMV) is a white to off-white crystalline solid with a solubility of approximately 70 mg/mL in water at 20°C (12). It has a molecular formula of $C_8H_{11}N_3O_3S$ and a molecular weight of 229.3 g/mole.

Lamivudine (Figure 1) is a synthetic nucleoside analogue that is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate which is a structural analogue of deoxycytidine triphosphate, the natural substrate for viral reverse transcriptase. Lamivudine triphosphate competes with naturally occurring deoxycytidine triphosphate for incorporation into viral DNA by reverse transcriptase and once incorporated, causes premature termination of viral DNA synthesis (13).

Lamivudine is the (-) enantiomer of a dideoxy analogue of cytidine. Lamivudine has also been referred to as (-) 2′, 3′-dideoxy, 3′-thiacytidine (13). The chemical name of lamivudine is (-) 4-amino-1-[(2*R*, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] pyrimidin-2(1*H*)-one (14).

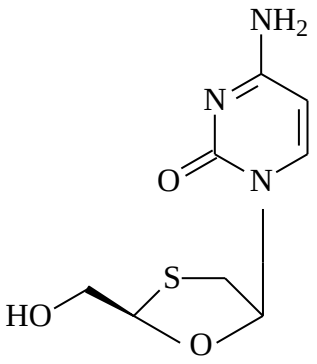

Figure 1: Chemical structure of lamivudine

1.2.2 Tenofovir disoproxil fumarate:

Tenofovir disoproxil fumarate (Figure 2) is a fumaric acid salt of the bis-isopropoxycarbonyloxymethyl ester derivative of tenofovir. The chemical name of tenofovir disoproxil fumarate is 9-[(R)-2 [[bis [[(isopropoxycarbonyl) oxy]-methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1) (15, 16). Tenofovir disoproxil fumarate is a salt of an oral prodrug of tenofovir. Tenofovir disoproxil was developed to increase bioavailability because tenofovir was not well absorbed from the intestine (17).

Tenofovir disoproxil fumarate is a white to off-white crystalline powder with a solubility of 13.4 mg/mL in water at 25 °C (15, 16).

The partition coefficient (log p) for tenofovir disoproxil is 1.25 and the pKa is 3.75. All dosages are expressed in terms of tenofovir disoproxil fumarate except where otherwise noted (16).

Tenofovir disoproxil fumarate has a molecular formula of $C_{19}H_{30}N_5O_{10}P \bullet C_4H_4O_4$, a molecular weight of 635.52 g/mole (16, 17).

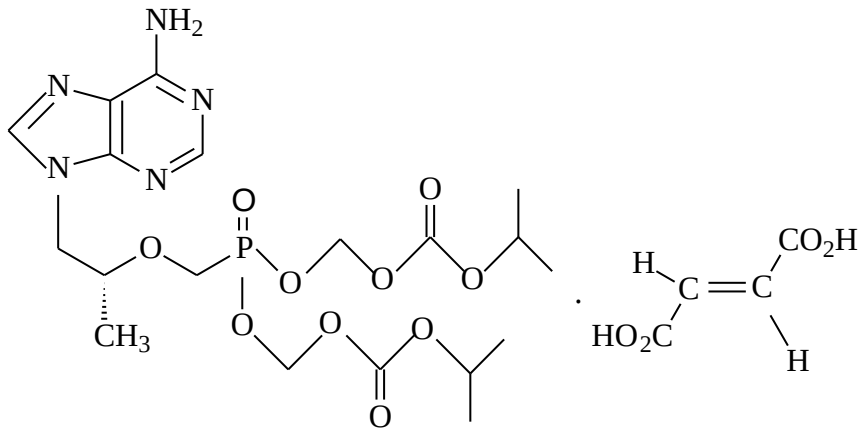

Figure 2: Chemical structure of tenofovir disoproxil fumarate

1.2.3 Efavirenz

Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used as part of highly active antiretroviral therapy (HAART) for the treatment of a human immunodeficiency virus (HIV) type 1 (7). Human immunodeficiency virus infection that has not previously been treated, the United States Department of Health and Human Services Panel on Antiretroviral Guidelines currently recommends the use of efavirenz in combination with lamivudine/zidovudine (combivir) or tenofovir/emtricitabine (truvada) as the preferred NNRTI-based regimens in adults and adolescents. Efavirenz is also used in combination with other antiretroviral agents as part of an expanded post exposure prophylaxis (PEP) regimen to prevent HIV transmission for those exposed to materials associated with a high risk for HIV transmission (7). The adult dose of efavirenz is 600 mg once a day. It has neurological and psychiatric adverse effects thus it is recommended to be taken at bedtime in attempt to reduce the above adverse effects (18). Efavirenz was combined with the popular HIV medication Truvada. The combination of these three medications was approved by the U.S. Food and Drug Administration (FDA) in July 2006 under the brand name Atripla® which provides HAART in a single tablet taken once a day offering a simplified drug regimen for many patients.

Efavirenz chemically (Figure 3) is named as (4*S*)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2, 4-dihydro-1H-3, 1-benzoxazin-2-one. Its molecular formula is $C_{14}H_9ClF_3NO_2$ (19).

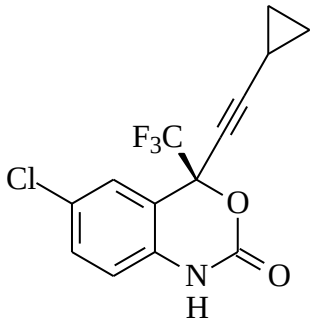

Figure 3: Chemical structure of efavirenz

1.3 Regulatory requirement for new formulation

1.3.1 Dissolution test

A drug must be in solution before it can be absorbed from the gastro-intestinal tract. It therefore follows that a tablet may meet disintegration standards yet be therapeutically inactive (20). Hence where there may be problems in dissolving the active ingredient, the tablet is subjected to a dissolution test.

Drug dissolution testing is an integral part of pharmaceutical development and routine quality control monitoring of drug release characteristics (21, 22). The profiles, obtained from dissolution rate studies, have also been used in an attempt to characterize the *in-vitro* behaviour of drug with success. They have to be performed under precisely specified conditions such as temperature, volume, and stirring rate that mimic process in the gastrointestinal tract (22). The British Pharmacopeia 2005 permits three types of apparatus, the rotating basket, the paddle and the flow through cell methods. In general acidic media are used with basic drugs (such as 0.1M hydrochloric acid for quinine sulphate), more alkaline media with acidic drugs (such as pH 6.8 buffer for phenoxymethylpenicillin) and water for non-ionizing molecules such as digoxin (23).

1.3.2 Assay

Assay is the measure of the amount of a substance or active pharmaceutical substance in a sample. The British Pharmacopeia 2005 gives room for the analyst to employ alternative methods including methods of micro analysis in any assay or test if it is known that the method used will give a result of equivalent accuracy. For preparations other than those of fixed strength, the quantity to be taken for an assay is usually expressed in terms of the active ingredient (23).

1.3.3 Related substances

In U.S Pharmacopoeia 2010, tests for the presence of impurities and foreign substances are provided to limit such substances to amounts that are objectionable under conditions in which the article is customarily employed.

1.4 Problem statement

With the continuing spread of HIV infection, particularly in resource-poor settings, cost-effective treatment for its management is a high priority. In developing countries such as Tanzania cost is a huge factor that limits ARVs drug access resulting in high rates of new infection and subsequent mortality. The development of a triple fixed dose combination of lamivudine, tenofovir and efavirenz tablet has created a need to have an analytical method for simultaneous analysis for assay and dissolution testing.

1.5 Rationale

Knowledge of assay and dissolution for newly formulated pharmaceutical products such as LTE tablets is of critical importance for routine quality control and kinetics of the drug. For a drug to be absorbed from the gastro-intestinal tract it should be in solution state, it therefore follows that a tablet may meet assay and disintegration standards yet be therapeutically inactive. Validated method for assay determination and dissolution of LTE tablets is required for routine analysis of the tablets. Fixed dose combination of lamivudine, tenofovir and efavirenz will be of much relief to the patients due to the fact that the reduced number of tablets and a once daily dosing will increase patient adherence thus reducing resistance and side effects resulting from long term use of the ARVs.

2.0 Literature review on analytical methods available for lamivudine, tenofovir disoproxil fumarate and efavirenz

A reverse-phase high-performance liquid chromatography (HPLC) assay for the simultaneous quantitative determination of HIV-protease inhibitors (PIs) (indinavir,IDV; amprenavir, APV; saquinavir, SQV; nelfinavir, ritonavir, RTV; and lopinavir) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (nevirapine, delavirdine, DLV; and efavirenz) was made possible through a linear gradient (36-86%) of 25% phosphate buffer (pH 4.5), 60% acetonitrile, 15% methanol, and 0.75 mL trifluoroacetic acid (TFA), with a gradient mobile phase flow rate (0.9-1.1 mL) over 30 min run time (24).

A simple, high-performance liquid chromatographic method has been developed and validated for the quantitative determination of efavirenz in human plasma. The analysis was *via* UV detection at 250 nm using a reversed-phase C8 analytical column and an isocratic mobile phase consisting of phosphate buffer (pH 5.7)-acetonitrile that resolved the drug and internal standard (L-737, 345) from endogenous matrix components and potential co-administered drugs. This validated assay is being used in pharmacokinetic studies of efavirenz (25).

An assay was developed for quantification of all currently approved non-nucleoside reverse transcriptase inhibitors and protease inhibitors, including the new protease inhibitor darunavir and the active nelfinavir metabolite M8 which used liquid chromatographic method coupled with tandem mass spectrometry (26). The sample pretreatment consisted of a protein precipitation with a mixture of methanol and acetonitrile using only 100 μL plasma. Chromatographic separation was performed on a reversed-phase C18 column (150mm x 2.0 mm, particle size 5 μm) with a quick stepwise gradient using an acetate buffer (pH 5) and methanol, at a flow rate of 0.25 mL/min. The analytical run time was only 10 min. The triple quadrupole mass spectrometer was operated in the positive ion mode and multiple reaction monitoring was used for drug quantification. The method was validated over a range of 0.1 μg/mL to 20 μg/mL for efavirenz, amprenavir, atazanavir, indinavir, lopinavir and nelfinavir. The method is now successfully applied for routine therapeutic drug monitoring and pharmacokinetic studies in HIV-infected patients (26).

An analytical technique using liquid chromatography (LC) coupled with electrospray-mass spectrometry (ESI-MS) has been developed for the simultaneous determination of five protease inhibitors (PIs): saquinavir, indinavir, ritonavir, nelfinavir, and amprenavir; and the non nucleoside reverse transcriptase inhibitors (NNRTIs): nevirapine, delavirdine, efavirenz, in human plasma. This assay allows the elution and identification of these drugs in a single run (10 mins) using a linear gradient with water and acetonitrile. High-performance liquid chromatography separation was achieved on a C18 reversed-phase column, with a linear gradient elution followed by mass spectrometry detection (27).

Lamivudine and zidovudine in human serum and in pharmaceutical formulations could be described by three new simple, precise, rapid and selective determination methods where as the first method was based on the compensation technique presented for the derivative spectrophotometric determination of binary mixtures with overlapping spectra. Using ratios of the derivative maxima or the derivative minimum, the exact compensation of either component in the binary mixture and human serum could be achieved, followed by its determination (28). The second method was differential derivative spectrophotometry which comprised of measurement of the difference absorptivities derivatized in the first order (deltaD1) of a tablet extract in 0.1 N NaOH relative to that of an equimolar solution in methanol at wavelengths of 246 nm and 263 nm, respectively. The third method was based on HPLC on a reversed-phase column using a mobile phase of 0.01M sodium dihydrogen phosphate: methanol: acetonitrile (4: 2: 3 v/v/v), with detection at 285 nm. Repeatability and reproducibility studies for each compound showed no significant differences at 95% confidence level. The proposed methods were used for the simultaneous determination of drugs in human serum samples and binary mixtures with good recoveries (28).

Quantitative analysis of lamivudine tablets was carried out by using high performance liquid chromatography with detection at 270 nm, using a stationary phase LiChrospher 100 (RP-18, 125 x 4mm, 5 μm Merck Darmstadt, Germany). The mobile phase was composed of methanol and acetate buffer (60:40 % v/v), pH 6.5 at a flow rate of 1.0 mL/min (29).

Research shows that spectrophotometric method is as accurate as the HPLC method for estimation of lamivudine in tablet/capsule. Hence laboratories that do not have HPLC equipment can also undertake lamivudine and other drug estimations using spectrophotometer (30). Non-destructive measurement technique such as near infrared chemical imaging (NIR-CI) can also be used to determine the composition of lamivudine in lamivudine tablets (31).

A comparison of different methods for dissolution test used by five different manufacturer laboratories of lamivudine tablets was made, evaluated, and discussed. Dissolution medium (water and hydrochloric acid pH 1.2), apparatus (paddles and baskets) and time (30 and 60 min) were analyzed. The determination was accomplished by spectrophotometry at 270 nm. After the comparative analysis of the results, optimal dissolution conditions were determined as follows: water as dissolution medium, paddles at the stirring speed of 50 rpm and time of 30 min. The method was applied to the dissolution test of samples from eleven batches of tablets, produced by five different laboratories (32).

Literature survey has revealed a number of analytical methods which included liquid chromatography with tandem mass spectrometry (33, 34), HPLC with solid phase extraction (35) and first order UV spectrophotometry derivative methods (36).

Liquid chromatographic method was developed and validated for the analysis of tenofovir disoproxil fumarate and its related substances (37). The gradient method used a base deactivated C18 column (Hypersil BDS column; 250mm x 4.6 mm I.D.) maintained at a temperature of 30°C. The mobile phases consisted of acetonitrile, tetrabutylammonium/phosphate buffer pH 6.0 and water: (A; 2:20:78 v/v/v) and (B; 65:20:15 v/v/v). The flow rate was 1.0 mL/min and UV detection was performed at 260 nm. Good separation of TDF and 21 impurities was achieved (37).

A sensitive and specific method for the quantitation of tenofovir in human plasma by liquid chromatography/electrospray ionization mass spectrometry was developed and validated where as tenofovir was shown to be stable under normal storage and assay conditions; no degradation was seen when stored at -20 ^{0}C or -80 ^{0}C for up to 6 months, and after 16 h at room temperature in the injection matrix (38).

A validated liquid chromatography coupled with a mass spectrometer method was used for the determination of plasma concentrations of tenofovir and emtricitabine in HIV infected patients.

Chromatographic separation was achieved with a gradient (acetonitrile and water with formic acid 0.05%) on an Atlantis 4.6 mm x 150 mm, reversed phase analytical column. Detection of tenofovir and emtricitabine was achieved by electrospray ionization mass spectrometry in the positive ion mode (39).

From literature, most of the methods described are largely from the West. Few methods are from countries such as Africa and Asia where fixed dose combinations (FDCs) are in use. Therefore, there is a need to develop methods that are affordable within developing countries (which are mostly using FDCs) and where analytical equipments are limited.

3.0 OBJECTIVES

3.1 General objective:

- To develop and validate a method for the assay and dissolution of triple combination of lamivudine, tenofovir and efavirenz tablets.

3.2 Specific objectives:

- To develop and validate an analytical method for simultaneous assay of lamivudine, tenofovir disoproxil fumarate and efavirenz in LTE fixed dose combination tablets.
- To optimize the in-vitro dissolution method and media suitable for optimal release of all three active pharmaceutical substances.

4.0 METHODS

4.1 Instruments

During method development different equipments were used such as high performance liquid chromatographic systems Varian ProStar with auto sampler and UV/VIS detection, made in Holland and Hewlett Packard (HP) 1090 series II with auto sampler and diode array detection (DAD), made in Germany. Dissolution was performed by using a dissolution tester, ERWEKA® DT 600 GmbH, D-63150 Heusenstamm made in Germany. Weight measurements were performed by analytical balance, Adventure® Ohaus Corp. Pine Brook, NJ, USA. Distillation system used RO- Purification System Millipore® made in France for water purification.

4.2 Reagents and reference standards

4.2.1 Reagents

Reagents and chemicals used during method development were mainly HPLC and few were analytical grade. Reagents such as acetonitrile, methanol and triethylamine were HPLC grade manufactured by Fisher Scientific, UK Limited. Sodium dihydrogen phosphate was from Medlab distributors Dar es Salaam, Tanzania, and glacial acetic acid manufactured by Scharlau Chemie, Spain, ammonium acetate manufactured by Loba Chemie Pvt. Ltd, Mumbai, India. Distilled water was in house prepared by reverse osmosis and/or double distillation by using RO-Purification System Millipore® made in France. Sodium dodecylsulfate (SDS) pharmaceutical grade was from Fisher Scientific UK Limited. Microcrystalline cellulose manufactured by FMC BioPolymer, Philadelphia. Sodium carboxymethyl cellulose and polyvinylpyrrolidone cross-linked from China Associate Co. Ltd, Shenzhen, China. Magnesium stearate manufactured by Shandong Liaocheng Ehua Medicine Co. Ltd, China.

4.2.2 Reference standards

Lamivudine batch number DBH010-4-B14-100112 (manufactured 02.01. 2010 expiry date 01.01. 2012), efavirenz batch number DBH012-5-A16A-091101 (manufactured 21.11. 2009 expiry date 20.11. 2011) and tenofovir disoproxil fumarate batch number DH101-4-091101b

(manufactured 15.11. 2009 expiry date 14. 11. 2011) all from Desano Chemical Pharmaceutical Co. LTD, Shanghai-China were used as secondary reference standards.

4.2.3 Test samples

Lamivudine, tenofovir and efavirenz tablets batch 1b manufactured on 29.04.2009 by Pharmaceutical Research and Development Laboratory of MUHAS-Tanzania. Desano Chemical Pharmaceutical Co. LTD, Shanghai-China kindly provided the active pharmaceutical ingredients which were used to make tablets for the test samples.

4.3 Procedures

4.3.1 Development of assay method and dissolution medium

A high-performance liquid chromatographic (HPLC) qualitative and quantitative method for simultaneous analysis of lamivudine, tenofovir disoproxil fumarate and efavirenz was developed and validated in accordance with USP and ICH Q2 R1(40,41). During method development tenofovir disoproxil fumarate and efavirenz assay methods were tried with the purpose of achieving simultaneous separation of all three active components in LTE tablet. High performance liquid chromatographic systems were used, Varian with auto sampler and UV/VIS detection, made in Holland and Hewlett Packard (HP) 1090 series II with auto sampler and diode array detection (DAD), made in Germany were used for analysis of the active pharmaceutical ingredients under this study during method development.

Tenofovir disoproxil fumarate assay method from Desano (42) was first considered and tried to simultaneously separate all three active pharmaceutical ingredients in the LTE tablet by using the mobile phase consisting of sodium dihydrogen phosphate and triethylamine buffer with the pH of 2.3 adjusted by using orthophosphoric acid and acetonitrile (buffer-acetonitrile, 60:40 % v/v). The analysis was performed by using a nucleosil (Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5μm) column, detection at 260 nm and a flow rate of 1 mL/min.

Different ratios of ammonium acetate solution buffer at pH of 3.0 and acetonitrile such as (30:70 % v/v), (40:60 % v/v), (45:55 % v/v), (50:50 % v/v), (55:45 % v/v), (60:40 % v/v) and (70:30 % v/v) were tried to find out which system could give better separation by using a nucleosil

(Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5μm) column, at a wavelength of 252 nm and a flow rate of 1 mL/min.

Efavirenz assay method from Desano was tried with the mobile phase containing acetonitrile-water (55:45 % v/v) (42). The analysis was done by using a nucleosil (Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5μm) column, at a wavelength of 252 nm and a flow rate of 1 mL/min. Different ratios of acetonitrile and water such as (30:70 % v/v), (40:60 % v/v), (45:55% v/v) , (50:50 % v/v), (55:45 % v/v), (65:35% v/v) and (70:30 % v/v) were also tried to find out which ratio could result to better separation.

After the development of the analytical method for simultaneous analysis of LTE active pharmaceutical ingredients, then dissolution medium for each single APIs in LTE tablet was used to test whether specific dissolution media for lamivudine, tenofovir disoproxil fumarate and efavirenz were able to simultaneously release all the three APIs in the LTE tablets. Lamivudine, tenofovir and efavirenz tablets were dissolved in 0.1 M hydrochloric acid (HCL) medium, in water medium and lastly dissolved in sodium dodecyl (SDS) media which contained 1g, 3g, 5g, 10g, 15g and 20g equivalent to 0.1%, 0.3%, 0.5%, 1.0%, 1.5% and 2.0% of sodium dodecyl sulphate in water. Sodium dodecyl sulphate media at 0.1%, 0.3%, 0.5%, 1.0%, 1.5% and 2.0% were used to test minimum concentration of SDS enough to simultaneously release all APIs in the LTE tablet. Dissolution tests were performed at 37°C by using a paddle method at 75 rpm for 120 min.

4.3.2 Validation of the assay method

The developed method for simultaneous analysis of lamivudine, tenofovir disoproxil fumarate and efavirenz from LTE tablets was validated in accordance with USP and ICH Q2 R1 (40, 41). The method was validated for specificity and/or selectivity, linearity, accuracy and precision, stability of solution and robustness. A validation protocol was prepared with reference to the Pharm R & D validation master plan having the ICH guidelines and USP for registration of human medicines as human reference documents (40, 41).

4.3.2.1 Specificity and/or selectivity

Placebo tablets with same composition as that of the LTE tablets without actives were prepared and examined for possible excipient interferences. The test sample was injected twice.

4.3.2.2 Linearity

Linearity was evaluated for the assay of lamivudine, tenofovir and efavirenz (LTE) by preparing five standard concentrations of LTE ranging from 50% to 120% (0.05 to 0.12 mg/mL) of lamivudine, tenofovir disoproxil fumarate and efavirenz using serial dilutions from a stock solution. Three injections were made at each concentration, adequately bracketed by the standard.

4.3.2.3 Accuracy and precision

Accuracy and precision were evaluated for the assay method for drug substance and drug product. For the drug substance validation, six solutions of the controls were made at each level of the controls 50%, 100% and 120% which were prepared at 0.025 mg/mL lamivudine, 0.025 mg/mL tenofovir and 0.05 mg/mL efavirenz corresponding to the 100 % level of the calibration curve. Accuracy was determined by evaluating the recovery of the analyte at these levels from a spiked placebo solution.

For the assay method for the drug product, six replicate samples solutions equivalent to 0.025 mg/mL Lamivudine, 0.025 mg/mL Tenofovir and 0.05 mg/mL Efavirenz were prepared, duplicate injections were made for each sample. The assay results (% recovery) for each sample, the mean and relative standard deviations (% rsd) of the six samples were calculated.

4.3.2.4 Stability of solutions

Solution stability was evaluated for LTE standards for 72 hours by observing change in peak areas with time.

4.3.2.5 Robustness of the method

The capacity of the analytical method to remain unaffected by small variations in method parameters was determined by varying temperature, flow rate and mobile phase composition. These factors were varied over a narrow range at three levels such that analysis was done at a temperature of 25°C, 30°C and 35°C , flow rate of 0.8 mL/min, 1.0 mL/min and 1.2 mL/min,

mobile phase composition of 54:46, 55:45 and 56:44 acetonitrile: water. In each case as one factor was varied, the other factors were held constant.

4.3.2.6 Forced degradation

Forced degradation tests were done to challenge the separation ability of the method to separate potential degradation products of lamivudine, tenofovir disoproxil fumarate and efavirenz under stressful conditions such as oxidation, base and acid. The degradation conditions would form degradation products resembling those seen by the drug substance or drug product under accelerated temperature storage.

4.3.2.7 Peroxide stress

Lamivudine, tenofovir and efavirenz finished formulation products were dissolved in 3% hydrogen peroxide then refluxed for 2 hours. The refluxed solution was then diluted to nominal assay concentration with the mobile phase and then analysed for the degradation products using Hewlett-Packard 1090 series II diode array detector.

4.3.2.8 Base stress

Lamivudine, tenofovir and efavirenz finished formulation products were dissolved in 0.1M sodium hydroxide then refluxed for 1 h. The refluxed solution was then diluted to nominal assay concentration with the mobile phase and then analysed for the degradation products using Hewlett-Packard 1090 series II diode array detector.

4.3.2.9 Acidic stress

Lamivudine, tenofovir and efavirenz finished formulation products were dissolved in 0.1M hydrochloric acid then refluxed for 1 h. The refluxed solution was then diluted to nominal assay concentration with the mobile phase and then analysed for the degradation products using Hewlett-Packard 1090 series II diode array detector.

5.0 RESULTS AND DISCUSSION

5.1 Method development

5.1.1 Assay method development

Preliminary experiments explored the existing methods for individual drug to find out if they would be selective for the triple fixed dose combination. Tenofovir disoproxil fumarate assay method from Desano (42) was first considered and tried to simultaneously separate all three active pharmaceutical ingredients in the LTE tablet by using the mobile phase consisting of sodium dihydrogen phosphate and triethylamine buffer with the pH of 2.3 adjusted by using orthophosphoric acid and acetonitrile (buffer-acetonitrile, 60:40 % v/v). The analysis was performed at a wavelength of 260 nm, flow rate of 1 mL/min and a nucleosil (Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5µm) column. This method did not achieve good separation, only lamivudine and tenofovir disoproxil fumarate were eluted but not efavirenz (Figure 4).

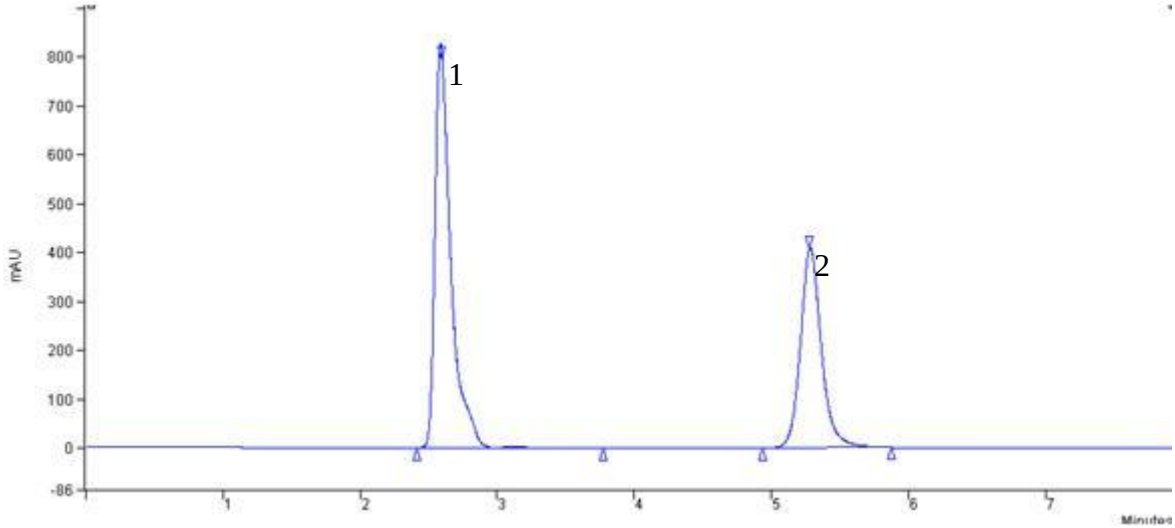

Figure 4: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: sodium dihydrogen phosphate and triethylamine buffer-acetonitrile (60:40 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 260 nm. Flow rate 1 mL/min. Peak 1: Lamivudine. Peak 2: Tenofovir disoproxil fumarate.

Different ratios of ammonium acetate solution buffer at pH of 3.0 and acetonitrile such as (30:70 % v/v), (40:60 % v/v), (45:55 % v/v), (50:50 % v/v), (55:45 % v/v), (60:40 % v/v) and (70:30 % v/v) were used to find out which system could give better separation. At acetonitrile: buffer (30:70 % v/v), lamivudine and tenofovir disoproxil fumarate co-eluted (Figure 5), at acetonitrile: buffer (50:50 % v/v), lamivudine and tenofovir co-eluted (Figure 6). At acetonitrile: buffer (60:40 % v/v), lamivudine, tenofovir disoproxil fumarate and efavirenz were separated with retention time of 3.8, 8.0 and 24.3 minutes respectively but the separation was poor between tenofovir and lamivudine (Figure 7).

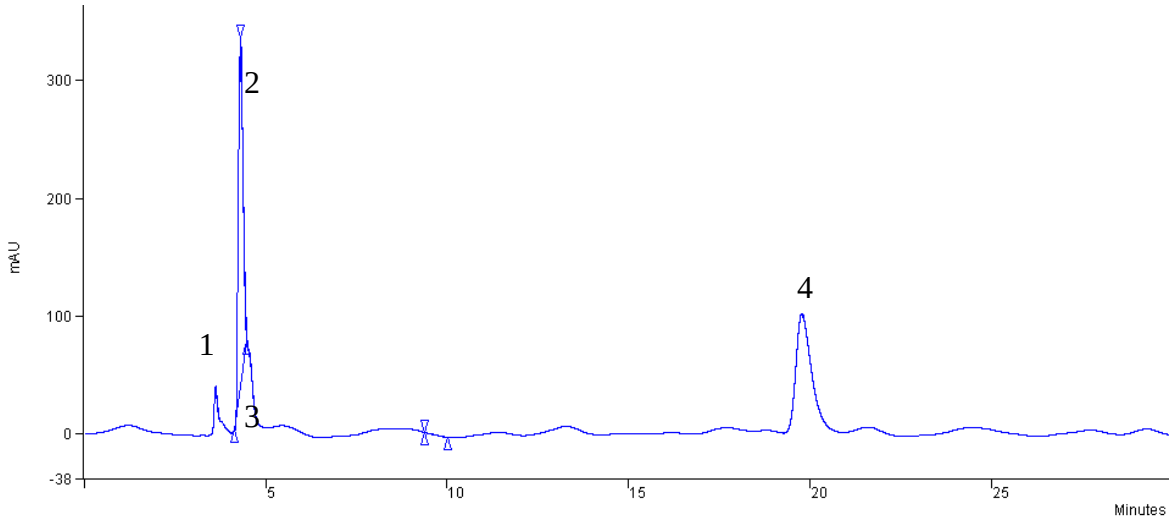

Figure 5: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-ammonium acetate solution (30:70 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

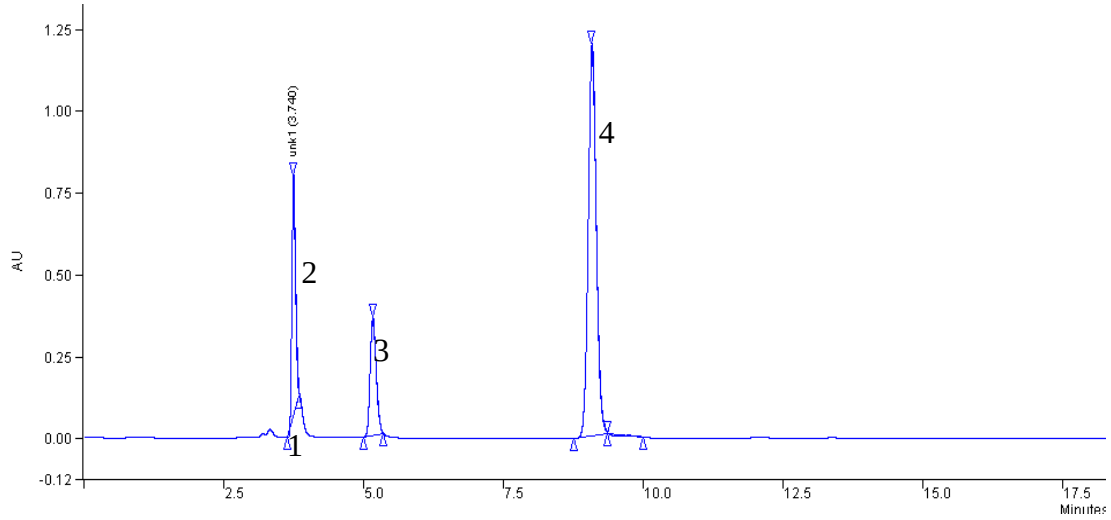

Figure 6: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-ammonium acetate solution (50:50 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

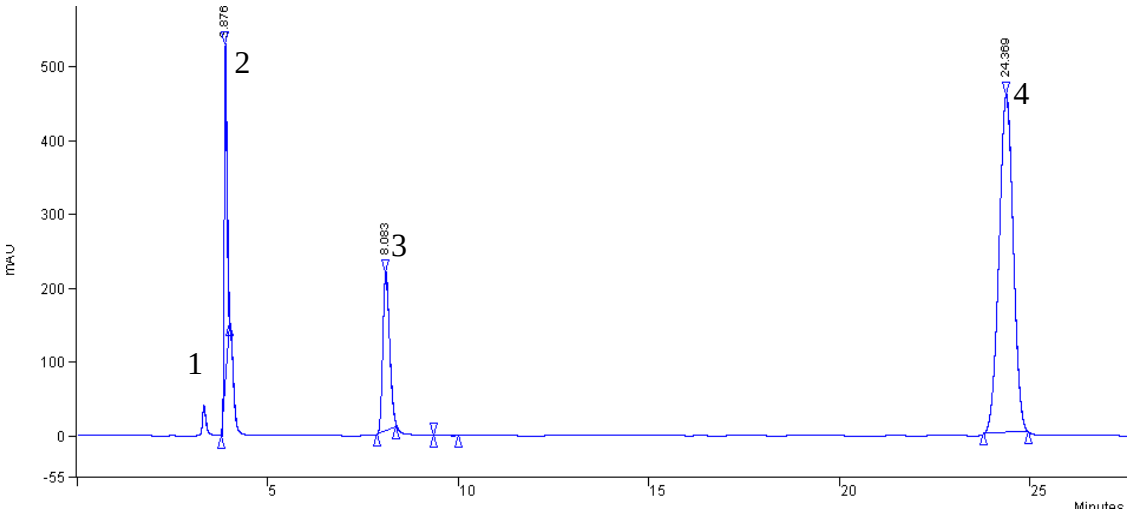

Figure 7: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile–ammonium acetate solution (60:40 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

The method was switched to efavirenz assay method from Desano which used acetonitrile and water (55:45 % v/v) for the mobile phase (40). The analysis was done by using a nucleosil (Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5µm) column at a wavelength of 252 nm and a flow rate of 1 mL/min. Different ratios of acetonitrile and water such as (30:70 % v/v), (40:60 % v/v), (45:55% v/v) , (50:50 % v/v), (55:45 % v/v), (65:35% v/v) and (70:30 % v/v) were used to find out which ratio could result to better separation and the results are shown in Figure 8, 9, 10, 11 and 12. At a ratio of acetonitrile: water (30:70 % v/v), lamivudine was not eluted but efavirenz was eluted with a retention time of 25 min (Figure 8). At a ratio of acetonitrile: water (40:60 % v/v), only tenofovir and efavirenz were eluted, efavirenz had a retention time of 20 min (Figure 9). As the concentration of acetonitrile increased in the mobile phase, retention time of efavirenz decreased (Figure 10). The decrease in retention time of efavirenz with the increase in acetonitrile concentration was associated with the increase in solubility of efavirenz in the organic solvent.

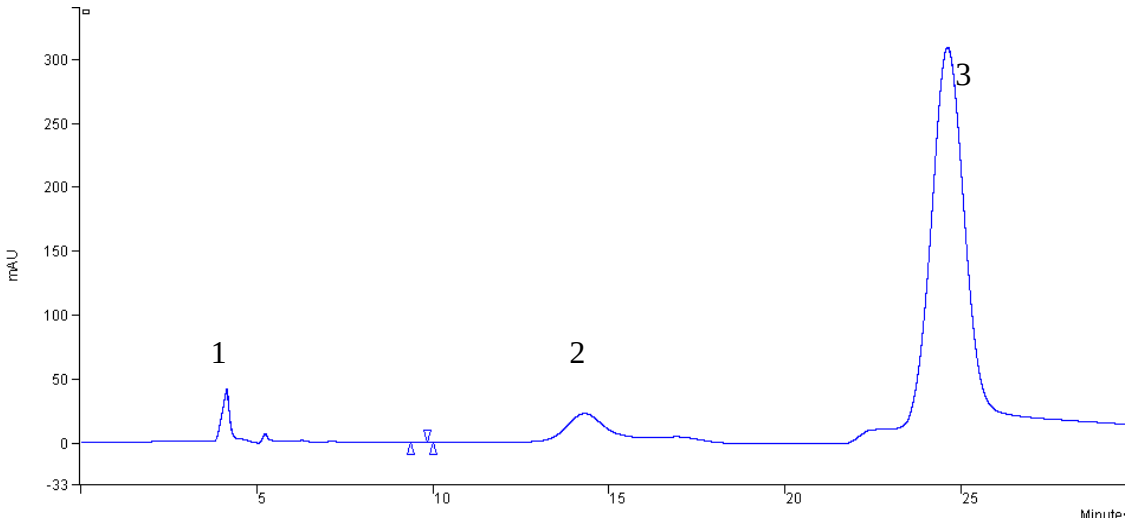

Figure 8: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (30:70 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Tenofovir disoproxil fumarate. Peak 3: Efavirenz.

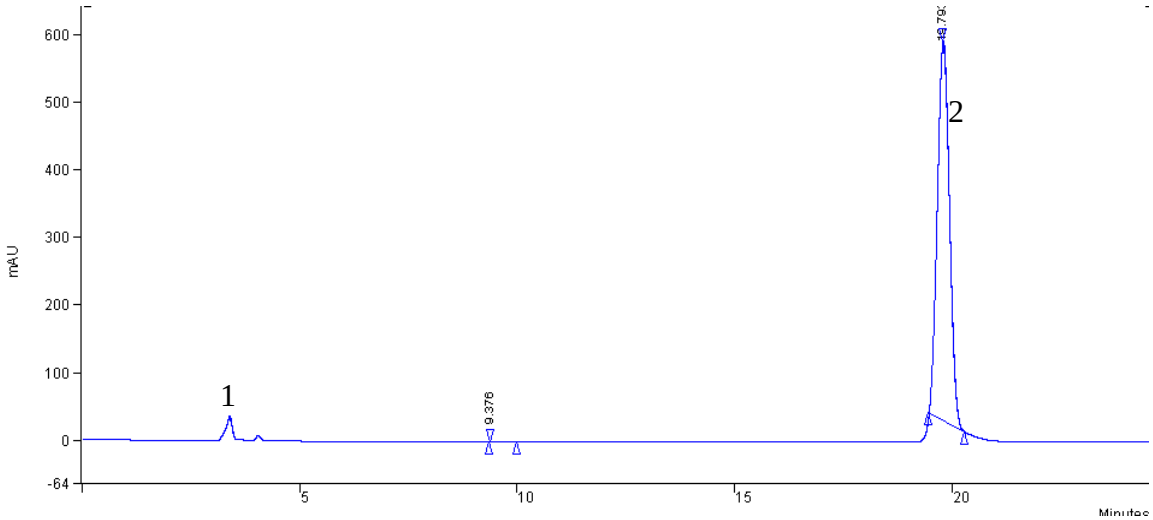

Figure 9: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (40:60 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Efavirenz.

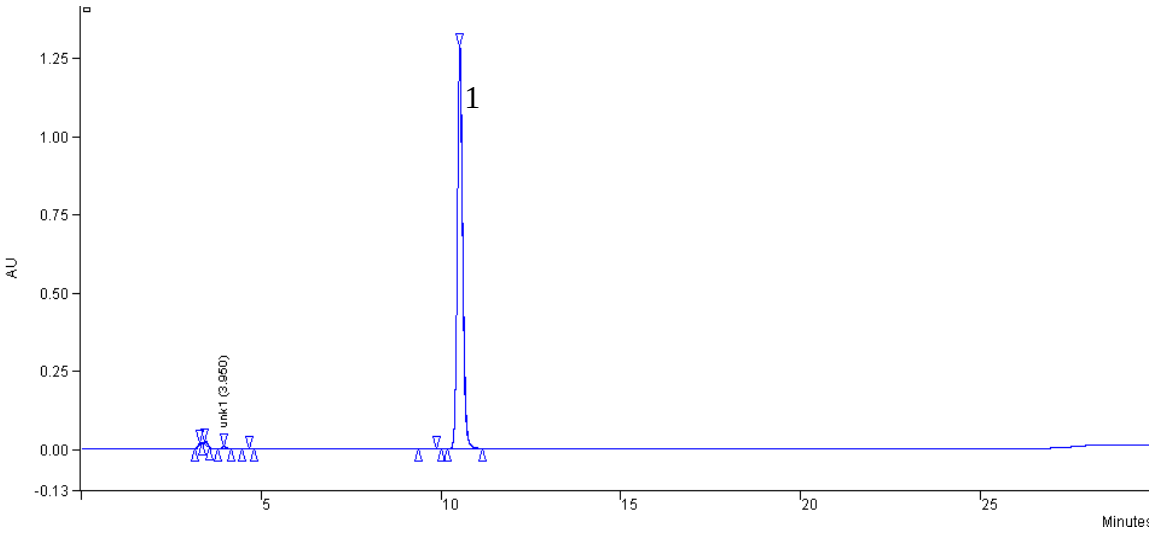

Figure 10: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (50:50 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1= Efavirenz.

At a ratio of acetonitrile: water (70:30 % v/v), lamivudine, tenofovir disoproxil fumarate and efavirenz co-eluted (Figure 11). Efavirenz assay method was able to simultaneously analyze lamivudine, tenofovir disoproxil fumarate and efavirenz with better separation at a ratio of acetonitrile: water (55:45 % v/v) (Figure 12).

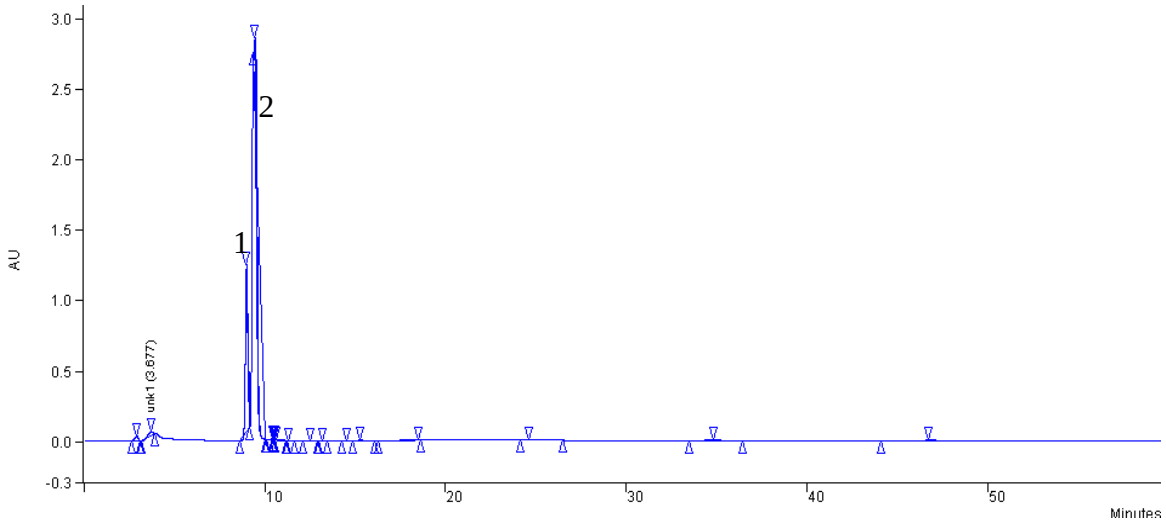

Figure 11: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (70:30 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1 and 2: Lamivudine and tenofovir disoproxil fumarate co- eluted with efavirenz.

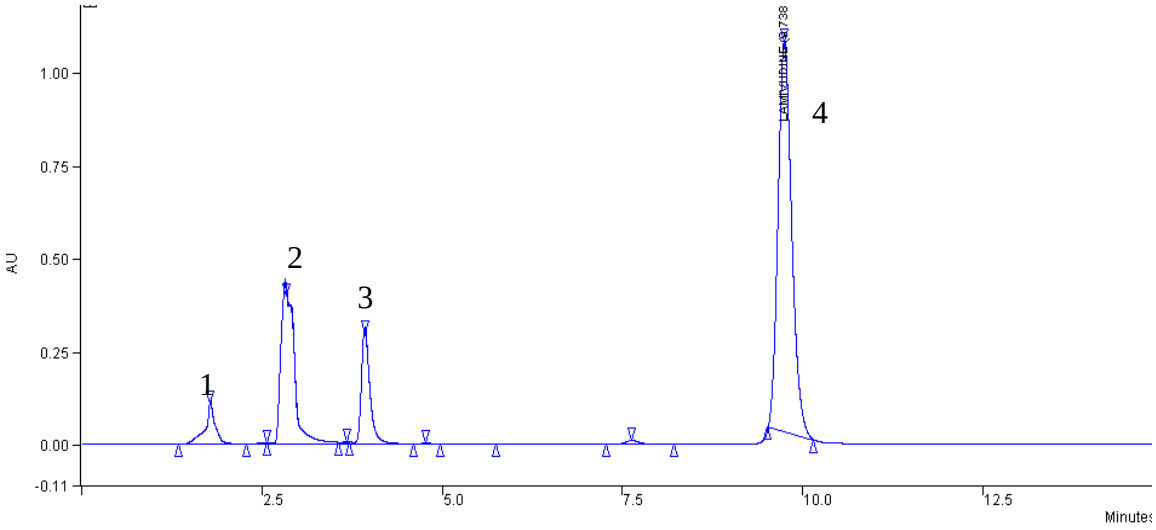

Figure 12: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

Variation of acetonitrile concentration significantly affected retention time of efavirenz such that at a ratio of acetonitrile: water (30:70 % v/v and 40:60 % v/v) the retention time of efavirenz was 25 and 20 min, respectively. At acetonitrile: water (55:45 % v/v) the mobile phase offered the shortest analysis time where as the last peak which is efavirenz eluted at around 10 min at 40°C. When the temperature was reduced to 30°C efavirenz retention time increased to about 13 min. At room temperature the retention time of the last peak was 13.6 min (Figure 13). The appropriate analysis time for the efavirenz method was agreed to be 20 min.

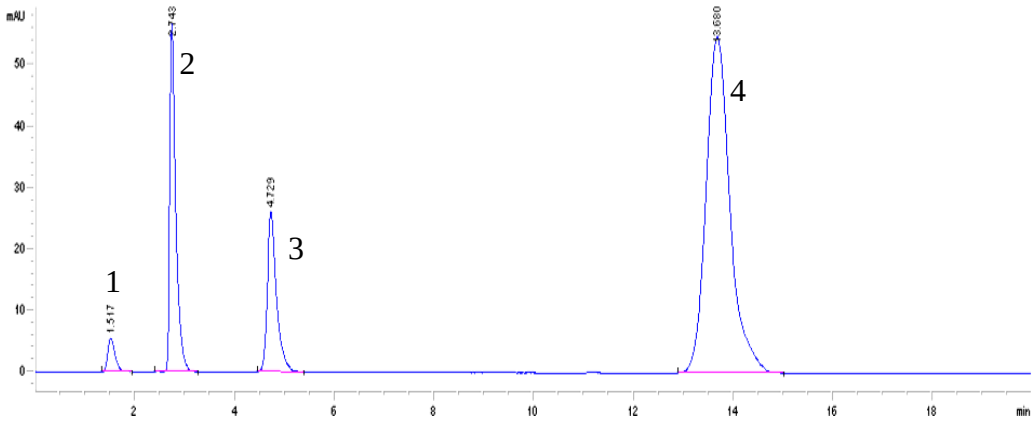

Figure 13: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

Therefore, a rapid HPLC assay method for simultaneous analysis of lamivudine, tenofovir disoproxil fumarate and efavirenz was successful developed through different trials with efavirenz assay method. The method used acetonitrile-water (55:45 % v/v) for the mobile phase, column: C18 4.6 mm x 250 mm x 5 µm, detection: 252 nm and flow rate 1 mL/min. The typical chromatograms for lamivudine, tenofovir disoproxil fumarate and efavirenz from LTE reference standard and LTE tablet are shown in Figures 14 and 15 respectively.

Table 1 : Chromatographic conditions for the assay of lamivudine, tenofovir and efavirenz tablets

Parameter	Condition
Mobile phase:	acetonitrile -water (55:45 % v/v)
Column:	C18, 4.6 mm x 250 mm x 5µm
Temperature:	ambient temperature*
Injection volume:	20 µL
Analysis time:	20 minutes
Detector	252 nm DAD (diode array detector)
Flow rate	1.0 mL/min

* In our laboratory the temperature ranged from 25ºC-28ºC

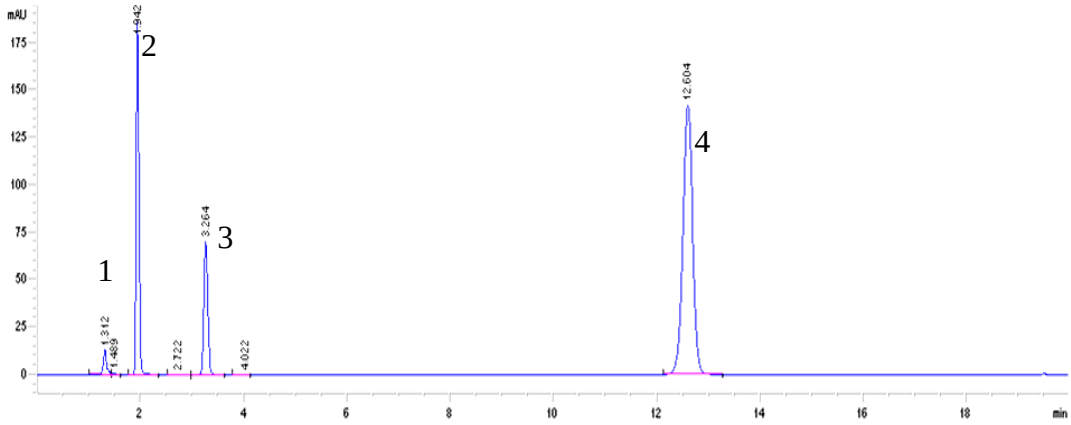

Figure 14: The typical chromatogram of a reference standard solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

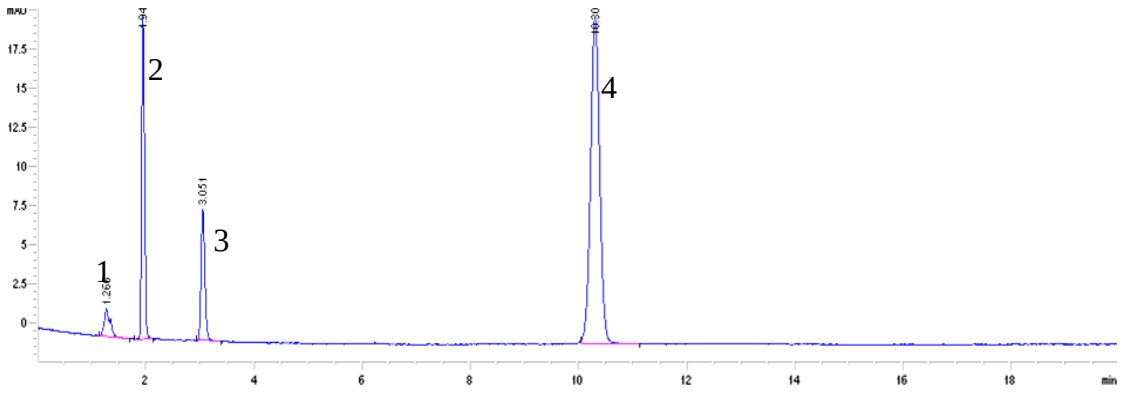

Figure 15: The chromatogram of LTE tablet solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

5.1.2 Forced degradation

Lamivudine, tenofovir and efavirenz tablets were prone to degradation when subjected to 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and in 3% hydrogen peroxide.

5.1.2.1 Peroxide stress

When 0.1 mg/mL of LTE tablet solution was stressed in hydrogen peroxide (3%) for 2 h, unknown peaks at around 2.7 and 16.3 min were observed (Figure 17) when compared to the LTE reference standard (Figure 16). Therefore the tablets were prone to degradation when stressed in peroxide hence not stable in peroxides.

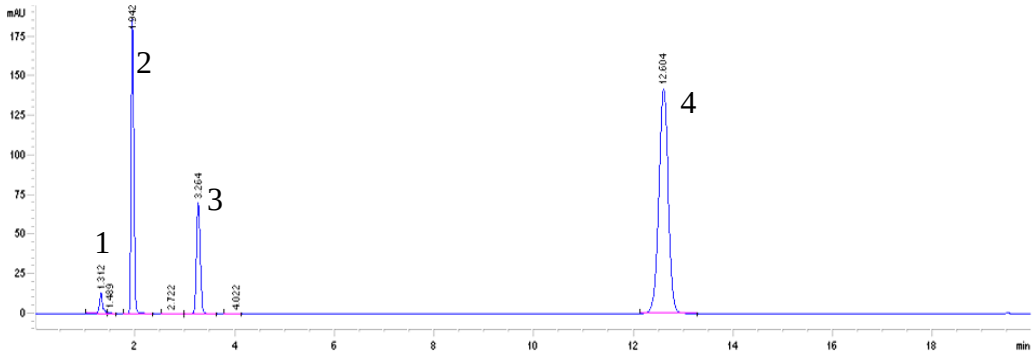

Figure 16: A chromatogram of a reference standard solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

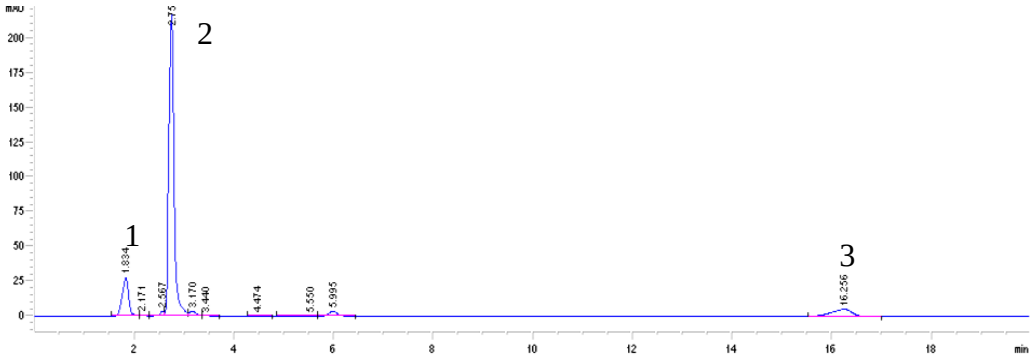

Figure 17: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz stressed with 3% hydrogen peroxide. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peaks 2 and 3: Unknowns.

5.1.2.2 Base stress

When 0.1 mg/mL of LTE tablet solution was stressed in 0.1M sodium hydroxide for 1 h, three extra unknown peaks at 2.8, 3.2 and 16.3 min were observed (Figure 18) when compared to the LTE reference standard (Figure 16). All three active pharmaceutical ingredients were largely affected when subjected to basic conditions therefore the tablets were not stable in basic conditions.

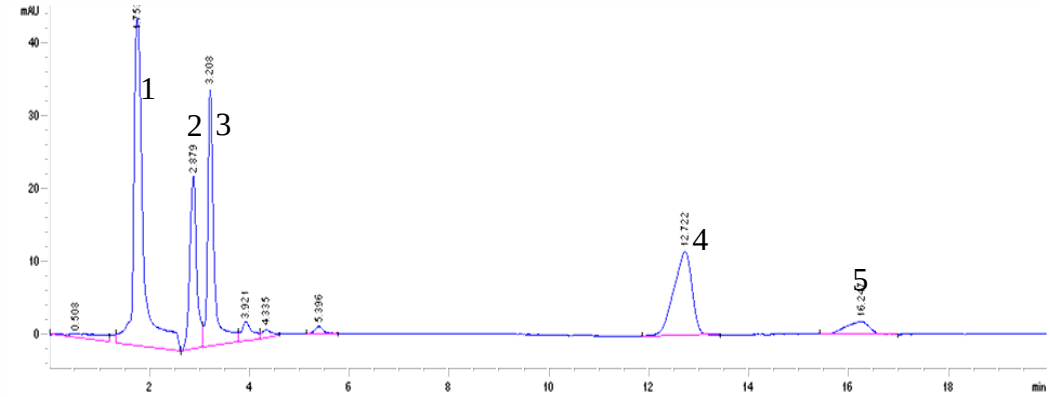

Figure 18: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz stressed with 0.1 M sodium hydroxide. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peaks 2 and 3: Unknowns. 4: Efavirenz. Peak 5: Unknown.

5.1.2.3 Acid stress

When 0.1 mg/mL of LTE tablet solution was stressed in 0.1M hydrochloric acid for 1 h; unknown peaks at 4.3, 7.6 and 15.2 min were observed (Figure 19) when compared to the LTE reference standard (Figure 16). LTE tablets were not stable in acidic conditions (Figure 19).

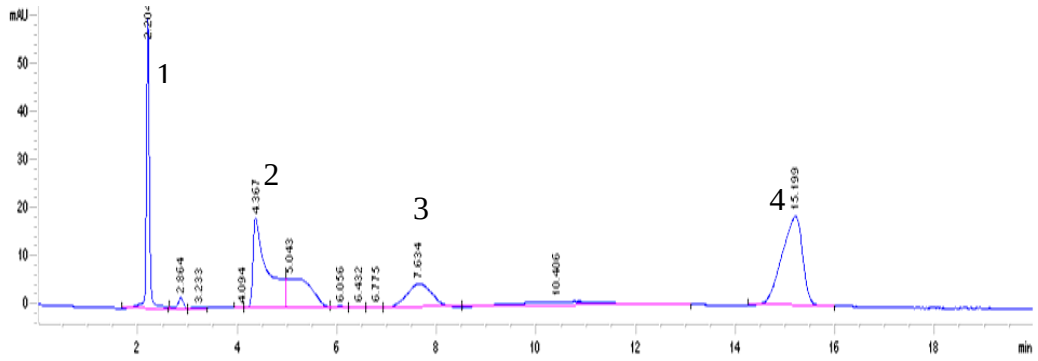

Figure 19: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz stressed with 0.1 M hydrochloric acid. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peaks 2, 3 and 4: Unknowns.

5.1.3 Dissolution medium

The dissolution profile of LTE tablets was performed to determine the optimal release of the APIs in LTE tablets. Lamivudine, tenofovir and efavirenz tablets were dissolved in different dissolution media; 0.1M hydrochloric acid, water and 2% sodium dodecyl sulphate (SDS). Efavirenz is poorly soluble in acid and water, but its solubility was improved with SDS. Different amounts of SDS ranging from 0.1% to 2% SDS were used to determine the optimal release of LTE with a paddle method at 75 rpm and a temperature of 37°C. Optimization of LTE release in different concentrations of SDS was preceded by determining the sink conditions of efavirenz in different concentrations of SDS (Table 2 and Figure 20). From the table it was observed that at a concentration range of 0.5 to 2.0% SDS media, more than 75% efavirenz could be released from the drug matrix without saturating the dissolution system. Lamivudine and tenofovir disoproxil fumarate sink conditions were not determined because from the three active pharmaceutical ingredients under this study only efavirenz was considered critical because of its poor solubility. Therefore from the sink condition results, a concentration of 0.5% SDS was enough to dissolve LTE tablets containing lamivudine 150 mg, tenofovir disoproxil fumarate 150 mg and efavirenz 300 mg (Table 2 and Figure 20).

Table 2: Sink conditions for efavirenz in different concentrations of sodium dodecyl sulphate

Concentration of sodium dodecyl sulphate (%)	Concentration of efavirenz (mg/L)	Sink concentration of efavirenz (mg/L)
0.3	508.64	169.55
0.5	1122.32	374.11
0.8	1889.36	627.79
1.0	2446.48	815.49
1.2	2947.08	982.36
1.5	4101.72	1367.24
2.0	4747.64	1582.55

Dissolution conditions: Temperature 37°C, 100 rpm, paddles method, 1 hour.

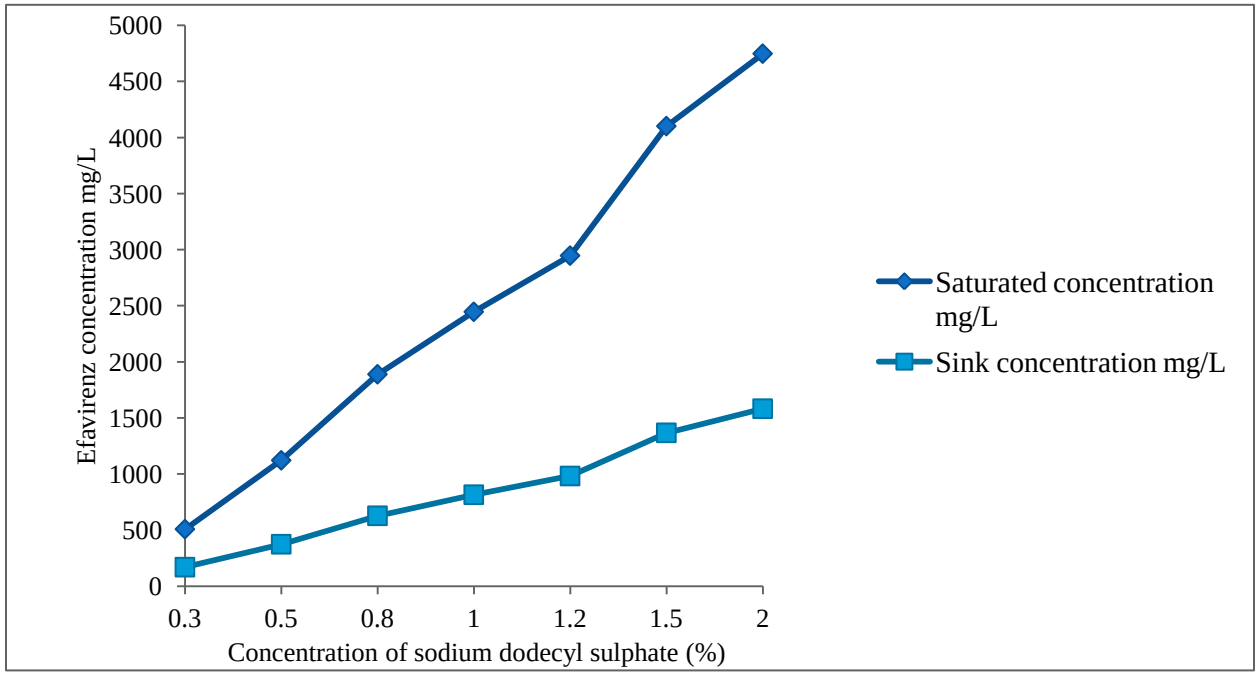

Figure 20: Sink conditions for efavirenz in different concentrations of sodium dodecyl sulphate.

The release of all the three APIs in LTE tablets was more than 75% at 45 min in 0.3%, 0.5%, 1.0%, 1.5% and 2.0% concentrations of SDS (Figures 21, 22 and 23). In 0.1% SDS dissolution medium, lamivudine and tenofovir disoproxil fumarate release was more than 75%, but for efavirenz the release was only 18.30% at 45 min and 20% at 120 min. Efavirenz release was poor at 0.1% SDS dissolution medium because of poor solubility in water and also the concentration of SDS was below CMC. As the concentration of SDS increased, efavirenz solubility improved therefore its release increased up to more than 75% at 45 min.

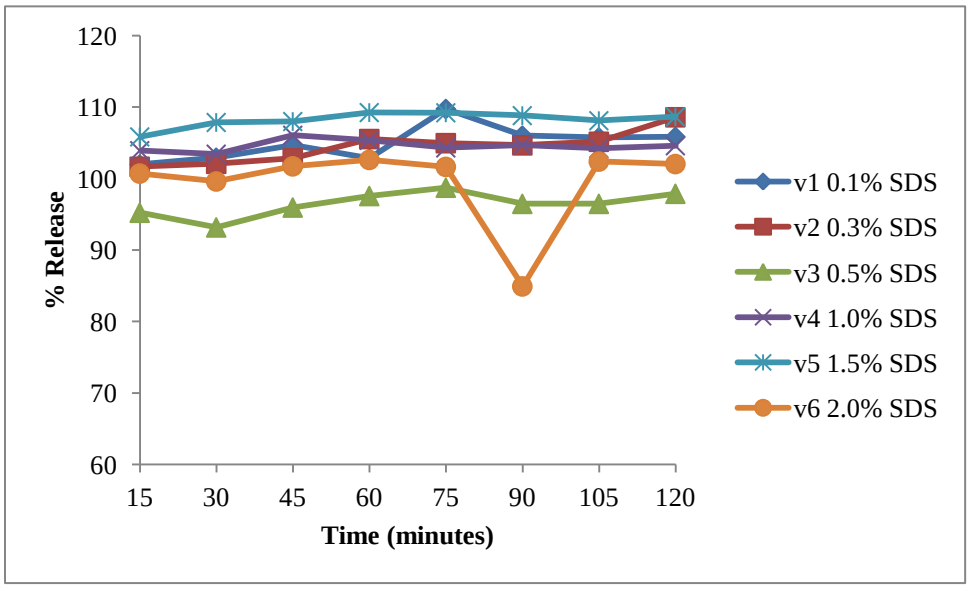

Figure 21: Dissolution profile of lamivudine from lamivudine, tenofovir and efavirenz tablets in dissolution media containing different concentrations of sodium dodecyl sulphate. v1: vessel 1, v2: vessel 2, v3: vessel 3, v4: vessel 4, v5: vessel 5, v6: vessel 6.

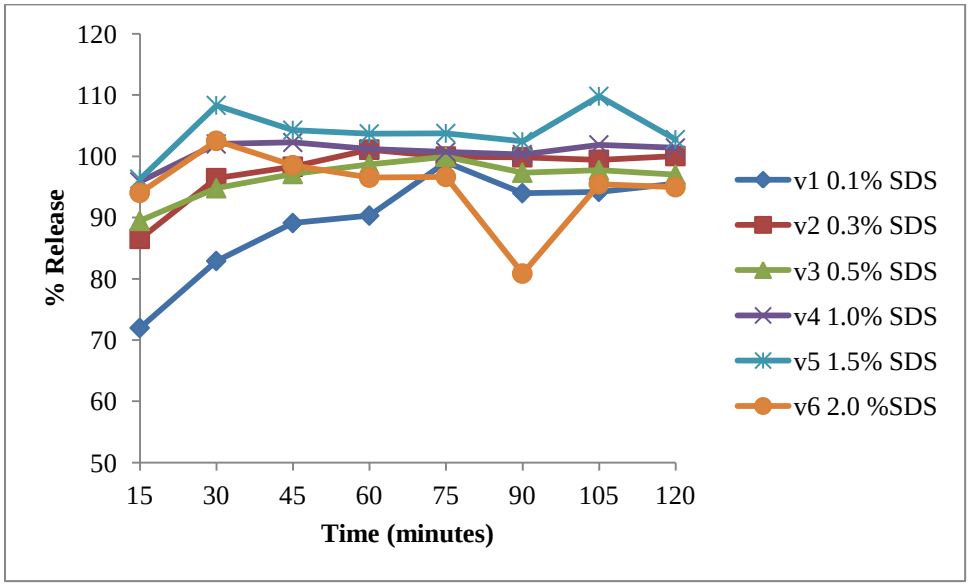

Figure 22: Dissolution profile of tenofovir disoproxil fumarate from lamivudine, tenofovir and efavirenz tablets in dissolution media containing different concentrations of sodium dodecyl sulphate. v1: vessel 1, v2: vessel 2, v3: vessel 3, v4: vessel 4, v5: vessel 5, v6: vessel 6.

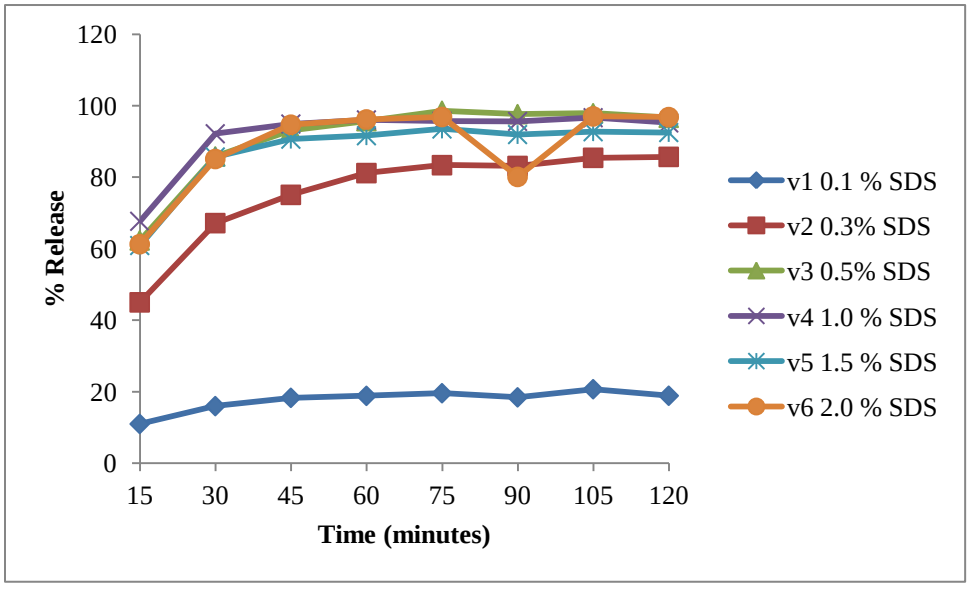

Figure 23: Dissolution profile of efavirenz from lamivudine, tenofovir and efavirenz tablets in the dissolution media containing different concentrations of sodium dodecyl sulphate. v1: vessel 1, v2: vessel 2, v3: vessel 3, v4: vessel 4, v5: vessel 5, v6: vessel 6.

In acidic medium, the release of lamivudine and tenofovir disoproxil fumarate was more than 70% at 45 minutes but there was no release for efavirenz which made acidic medium not a suitable medium for simultaneous release of all three active pharmaceutical ingredients (Figures 24-26).

When water was used as a dissolution medium for LTE tablets, lamivudine and tenofovir disoproxil fumarate release were more than 70% at 45 min, for efavirenz there was no release throughout the dissolution profile time of 90 min. Poor release of efavirenz was associated with its poor solubility in water (Figure 24-26).

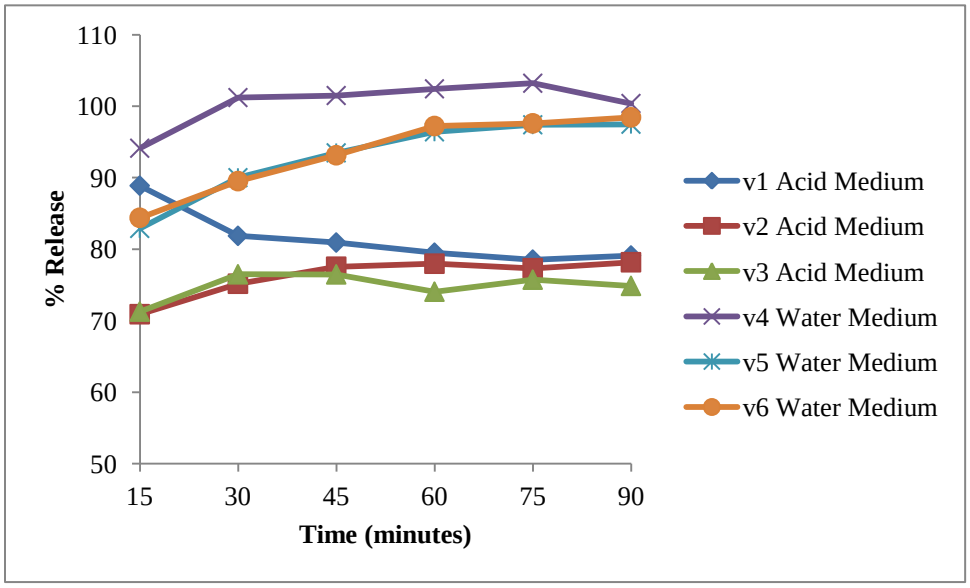

Figure 24: Dissolution profile of lamivudine from lamivudine, tenofovir and efavirenz tablets in 0.1M hydrochloric acid (vessel 1, 2 and 3) and in water (vessel 4, 5 and 6) dissolution media. v1: vessel 1, v2: vessel 2, v3: vessel 3, v4: vessel 4, v5: vessel 5, v6: vessel 6.

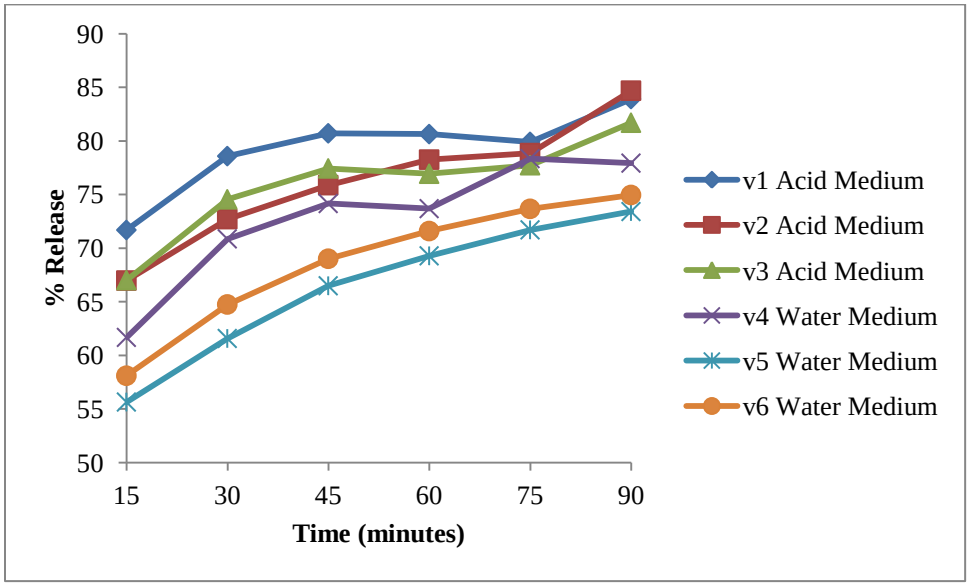

Figure 25: Dissolution profile of tenofovir disoproxil fumarate from lamivudine, tenofovir and efavirenz tablets in 0.1 M hydrochloric acid (vessel 1, 2 and 3) and in water (vessel 4, 5 and 6) dissolution media. v1: vessel 1, v2: vessel 2, v3: vessel 3, v4: vessel 4, v5: vessel 5, v6: vessel 6.

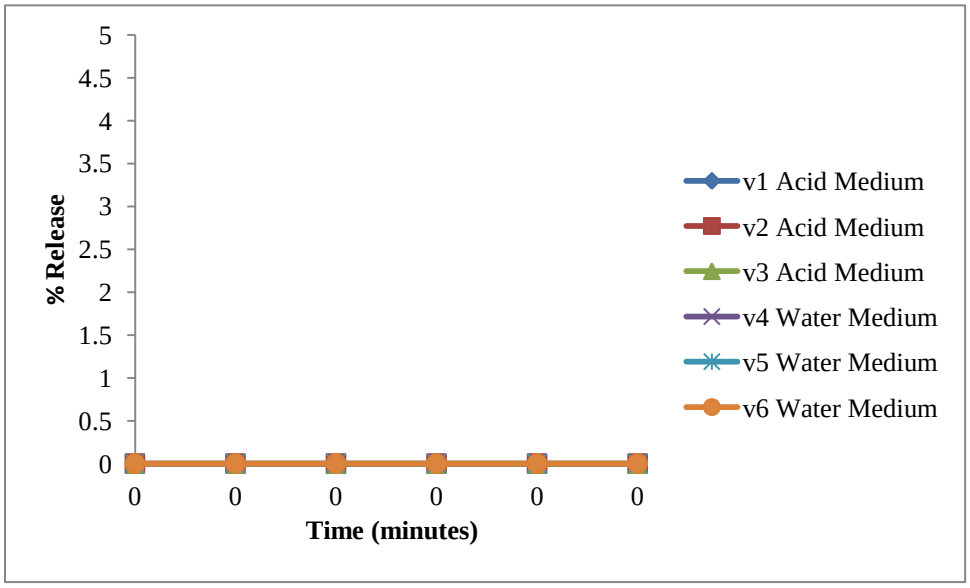

Figure 26: Dissolution profile of efavirenz from lamivudine, tenofovir and efavirenz tablets in 0.1 M hydrochloric acid (vessel 1, 2 and 3) and in water (vessel 4, 5 and 6) dissolution media. v1: vessel 1, v2: vessel 2, v3: vessel 3, v4: vessel 4, v5: vessel 5, v6: vessel 6.

5.2 Method validation

The assay and dissolution method was validated for specificity and/or selectivity, linearity, accuracy and precision, stability of solution, and method robustness as per the validation protocol. Peroxide, base and acid stress were done to determine the capacity of the method to resolve lamivudine, tenofovir disoproxil fumarate and efavirenz peaks from their degradation products.

5.2.1 Specificity and/or selectivity

From the chromatograms obtained, the method was specific for the assay and dissolution testing and was selective for the active pharmaceutical ingredients for which there were no blank interferences. The chromatograms (Figure 27, 28) show that the method was acceptable for routine assay and dissolution of the LTE tablets.

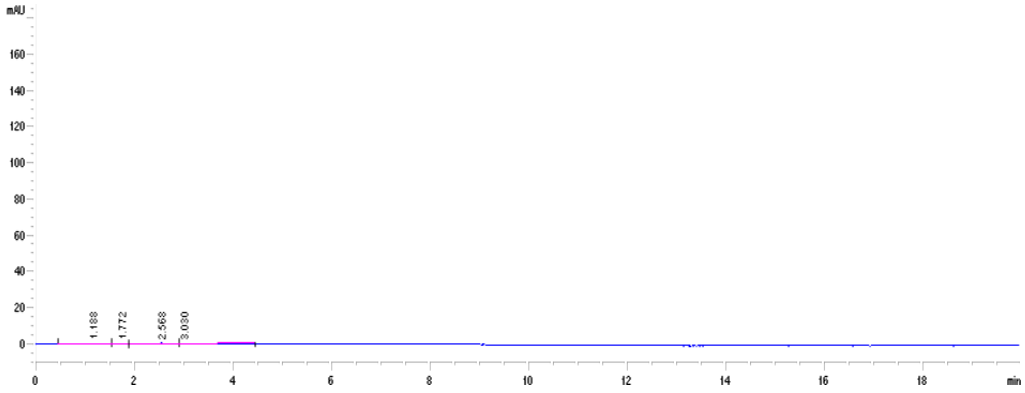

Figure 27: A chromatogram of a placebo solution containing all excipients in the lamivudine, tenofovir and efavirenz tablet formulation. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C18, 4.6 mm x 250 mm x 5 μm. Detection: 252 nm. Flow rate 1 mL/min.

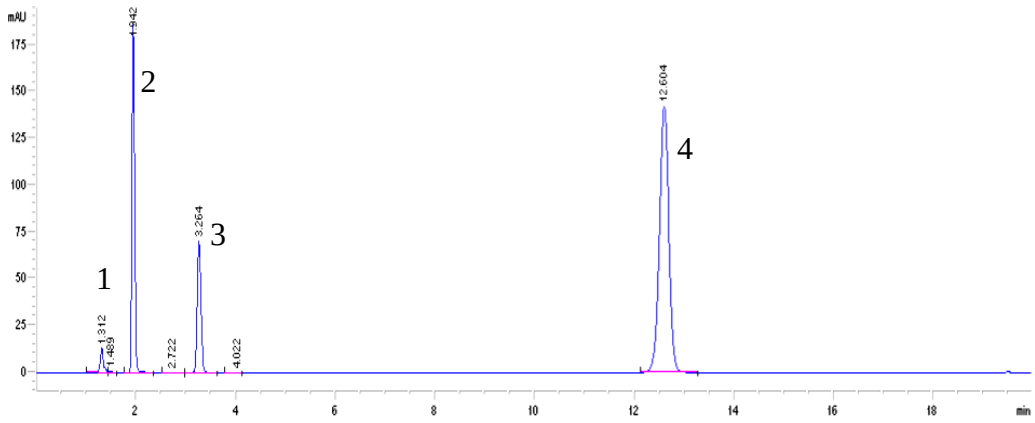

Figure 28: A chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C18, 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

5.2.2 Linearity

The linearity was evaluated by injecting standard solutions of lamivudine, tenofovir disoproxil fumarate and efavirenz ranging from 50% to 120% of the nominal concentrations. Regression coefficients (r^2) were greater than 0.98 and there were no curvature in the residuals plot. The *y* intercept did not significantly depart from zero where as the area response of *y* intercept was less than 5% of the response of the midrange concentration value (C/y*100). Linearity tests were within the acceptance criteria, therefore the tests passed (Table 3).

Table 3: Intraday precision for lamivudine, tenofovir disoproxil fumarate and efavirenz obtained from LTE tablets

Parameter	Lamivudine	Tenofovir disoproxil fumarate	Efavirenz	Acceptance criteria
x-coefficient	4875.600	3153.100	15692.000	
y-intercept	-7.165	-7.206	-49.918	
r^2	0.998	0.998	0.998	≥ 0.98
% C/y	1.760	2.760	3.890	≤ 5%

5.2.3 Precision

Intra-day (repeatability) and inter-day method precision were determined according to the test procedures predetermined in the validation protocol which included sample preparation. The results were summarized in tables 4 and 5.

Table 4: Intraday precision for lamivudine, tenofovir disoproxil fumarate and efavirenz obtained from LTE tablets

	Peak areas at 100% concentration level		
Test sample	Lamivudine	Tenofovir disoproxil fumarate	Efavirenz
Mean	499.36	243.77	1545.60
sd	5.41	3.00	22.51
% rsd	1.08	1.23	1.46

n=6

The % rsd for the intra-day precision shown in table 4 were within the acceptance criteria which required the % rsd of the assay to be equal or less than 2.0%.

Table 5: Inter-day precision for lamivudine, tenofovir disoproxil fumarate and efavirenz obtained from LTE tablets

	Peak areas at 100% concentration level		
Test sample	Lamivudine	Tenofovir disoproxil fumarate	Efavirenz
Mean	493.89	243.00	1534.49
sd	9.65	4.83	25.65
% rsd	1.95	1.99	1.67

n=18

The % rsd for the inter-day precision shown in table 5 were within the acceptance criteria which required the % rsd of the assay to be equal to or less than 2.0%.

5.2.4 Accuracy

The recovery results indicated that the test method has an acceptable level of accuracy for the assay of lamivudine, tenofovir disoproxil fumarate and efavirenz in LTE tablets from 50 % to 120 % of test concentration. The values are shown in Table 6.

Table 6: Recovery values for lamivudine, tenofovir disoproxil fumarate and efavirenz from a placebo mixture spiked with LTE reference standard

	Concentration levels	50%	100%	120%
Lamivudine	Mean recovery	94.32	98.75	100.53
	sd	0.25	0.08	0.09
	% rsd	0.26	0.08	0.09
Tenofovir disoproxil fumarate	Mean recovery	96.23	100.20	101.51
	sd	0.55	0.10	0.14
	% rsd	0.58	0.10	0.13
Efavirenz	Mean recovery	97.84	98.46	100.87
	sd	0.31	0.08	0.29
	% rsd	0.32	0.08	0.28

n=6

The mean recoveries on the 50% concentration level for all three APIs were less than 98%, therefore the test did not meet the acceptance criteria which required the mean recovery to be equal or greater than 98% and less or equal to 102%. The mean recoveries on the 100% and 120% concentration levels for all three APIs were greater than 98%; therefore the tests met the acceptance criteria. The percentage rsd at 50%, 100% and 120% concentration levels were less than 2% for all three APIs, therefore the % rsd were within the acceptance criteria.

5.2.5 Stability of solutions

Solution stability was evaluated for LTE tablets for 72 hours at room temperature and refrigerator (4 °C) by observing change in peak areas with time. The stored test solutions at room temperature and refrigerator were each injected twice at 24 and 72 hours and their peak areas recorded. The assay was expressed as a percentage related to peak area quotient of freshly prepared solution (Table 7).

Table 7: Assay of stability of test solution at room temperature and refrigerator

	Fresh solution	25°C/24 h	25°C/72 h	4°C/24 h	4°C/72 h
Lamivudine	100.00%	94.70%	77.20%	94.60%	94.90%
Tenofovir disoproxil fumarate	100.00%	90.20%	90.80%	92.30%	91.80%
Efavirenz	100.00%	97.80%	97.10%	101.60%	100.00%

After 72 hours the test solution stored in the refrigerator were more stable as compared to the sample solution stored at room temperature without light protection. The decrease in percentage assay with respect to increase in time might be associated with the decrease of the active pharmaceutical ingredients as a result of formation of degradation products of the respective active pharmaceutical ingredients due to effect of light and temperature. The chromatograms of samples stored at room temperature and in refrigerator (Figures 30 and 31) showed no new emerging peaks when compared to the freshly prepared sample solution (Figure 29). The fact that there were no new emerging peaks implies that the degradation products do not absorb UV light at the wavelength of detection. Therefore it was recommended to keep the test solutions protected from light and under refrigerated conditions when stored for more than 24 hours to avoid further loss of the APIs.

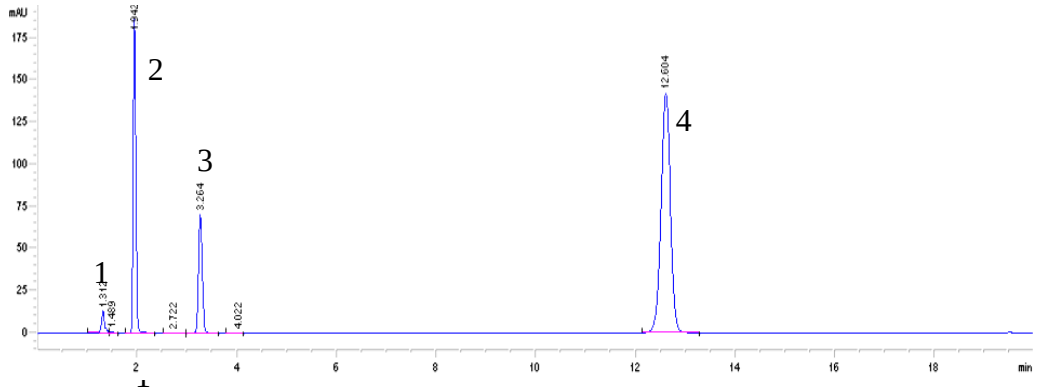

Figure 29: Chromatogram of a freshly prepared test solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

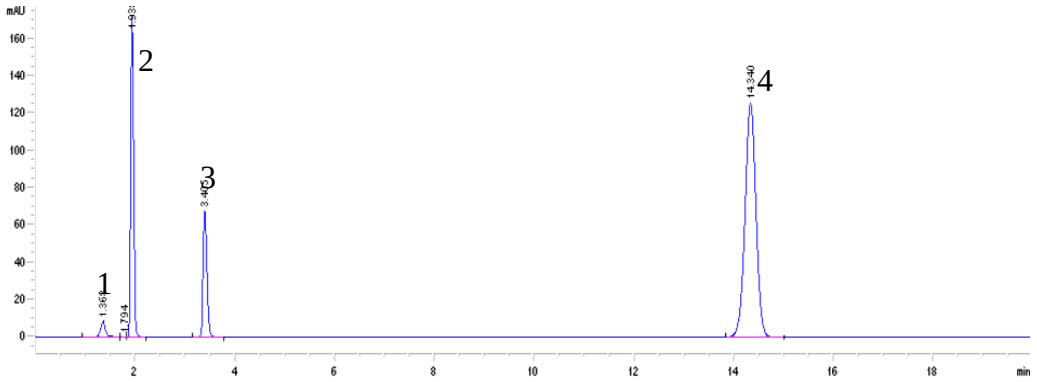

Figure 30: Chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz at room temperature. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18, 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

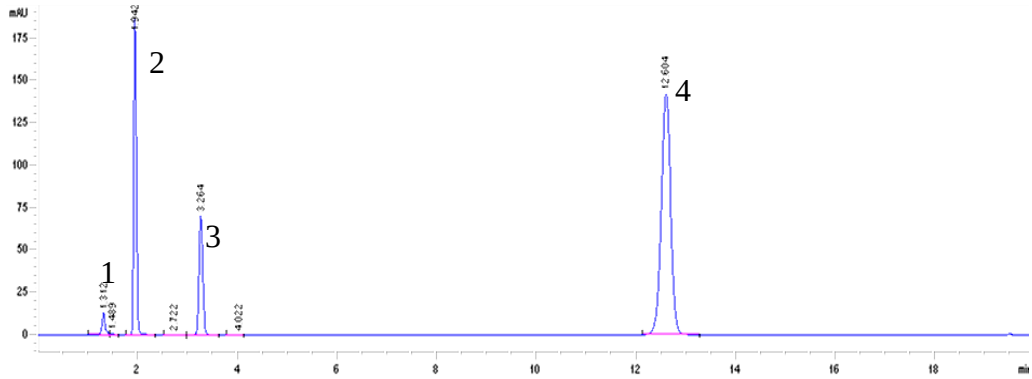

Figure 31: Chromatogram of a solution containing 0.025mg/mL of lamivudine, 0.025 mg/mL tenofovir disoproxil fumarate and 0.05 mg/mL efavirenz stored in refrigerator. Mobile phase: acetonitrile-water (55:45 % v/v). Column: C 18 4.6 mm x 250 mm x 5 µm. Detection: 252 nm. Flow rate 1 mL/min. Peak 1: Tenofovir. Peak 2: Lamivudine. Peak 3: Tenofovir disoproxil fumarate. Peak 4: Efavirenz.

5.2.6 Robustness of the method

Resolution and capacity factors of lamivudine, tenofovir disoproxil fumarate and efavirenz were determined to examine the effect of small changes in the chromatographic conditions/system on these chromatographic parameters. Retention times of lamivudine and tenofovir disoproxil fumarate were not very much affected by the change in the mobile phase composition. Only retention time of efavirenz was affected by small variation of the mobile phase composition, increase in acetonitrile concentration from 54 to 55% reduced the retention time from 14.3 to 11.4 min (Table 8). Retention time of efavirenz was affected by variation of acetonitrile in the mobile phase because acetonitrile (organic solvent) increase solubility of efavirenz which is poorly soluble in water. The capacity factors for lamivudine and tenofovir disoproxil fumarate were not affected much by change in mobile phase concentration. Efavirenz capacity factor was affected by slight change of the mobile phase composition; it decreased as the concentration of acetonitrile increased (Figure 32). Resolution of lamivudine, tenofovir disoproxil fumarate and efavirenz were slightly affected by small variation in the mobile phase composition (Figure 33).

Table 8: The effect of mobile phase composition (acetonitrile:water) on the chromatographic parameters of lamivudine, tenofovir disoproxil fumarate and efavirenz

Active pharmaceutical ingredient	% Acetonitrile	Retention time (min)	Resolution (R)	Capacity factor (k´)
Lamivudine	54	1.938	0.734	0.417
	55	1.941	0.882	0.479
	56	1.929	0.607	0.488
Tenofovir disoproxil fumarate	54	3.405	3.124	1.489
	55	3.264	3.120	1.488
	56	3.143	2.765	1.425
Efavirenz	54	14.340	3.124	9.482
	55	12.623	3.120	8.611
	56	11.438	2.765	7.826

Column: C 18, 250 mm x 4.6 mm x 5 µm

Column temperature: 30°C

Flow rate: 1 mL/min

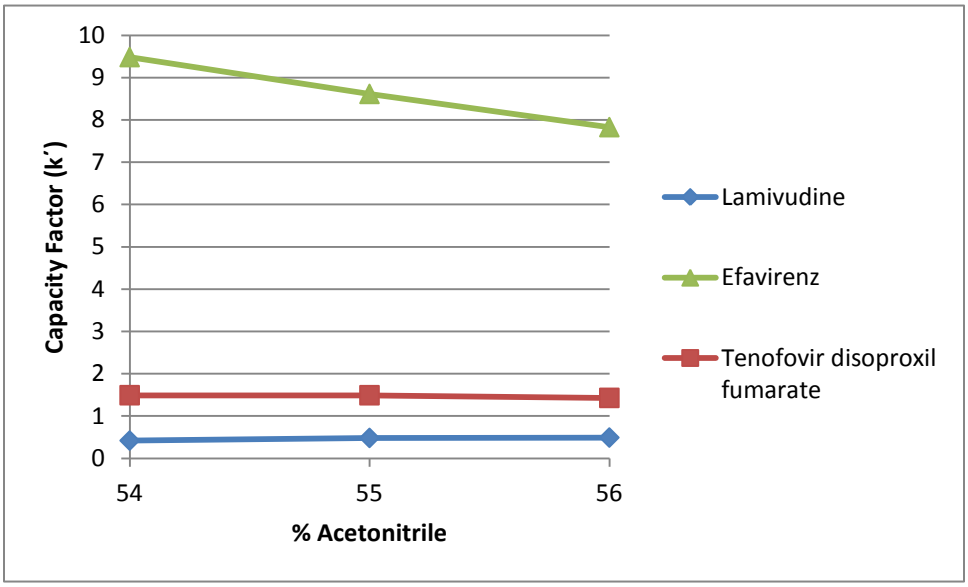

Figure 32: Effect of mobile phase composition on the capacity factors for lamivudine, tenofovir disoproxil fumarate and efavirenz. Mobile phase: acetonitrile: water (55:45, 56:44 and 54:46 % v/v). Column: C18, 250mm x 4.6 mm x 5nm. Detection: 252 nm. Column temperature: 30°C. Flow rate 1.0 mL/min.

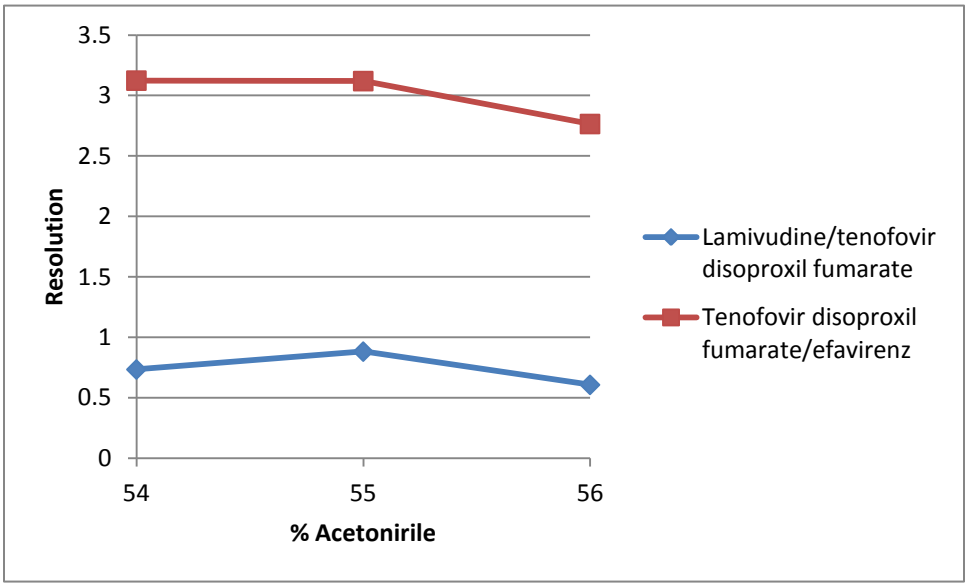

Figure 33: Effect of mobile phase composition on the resolution factors for lamivudine, tenofovir disoproxil fumarate and efavirenz. Mobile phase: acetonitrile: water (55:45, 56:44 and 54:46 % v/v). Column: C18, 250mm x4.6 mm x 5nm. Detection: 252 nm. Flow rate 1.0 mL/min.

The column temperature was varied at intervals of ±5°C of the nominal temperature which is 30°C (Table 9). The retention time of efavirenz decreased from 13.0 to 11.9 min as temperature was increased from 25 to 35°C. Retention times of lamivudine and tenofovir disoproxil fumarate were not much affected by change in temperature (Figure 34). Capacity factors for tenofovir disoproxil fumarate and efavirenz were affected by temperature change; as the temperature increases the capacity factors for tenofovir disoproxil fumarate and efavirenz decreased (Figure 34). Resolution of all the three APIs under this study decreased as the temperature decreased (Figure 35).

Table 9: The effect of column temperature on the chromatographic parameters of lamivudine, tenofovir disoproxil fumarate and efavirenz

Active pharmaceutical ingredient	Column temperature	Retention time (min)	Resolution (R)	Capacity factor (k´)
Lamivudine	25°C	1.935	0.672	0.487
	30°C	1.941	0.882	0.479
	35°C	1.943	0.871	0.519
Tenofovir disoproxil fumarate	25°C	3.278	2.786	2.520
	30°C	3.264	3.120	1.488
	35°C	3.250	3.970	1.519
Efavirenz	25°C	13.030	2.786	9.015
	30°C	12.623	3.120	8.611
	35°C	11.867	3.970	8.278

Column: C 18, 250 mm x 4.6 mm x 5 µm

Mobile phase: acetonitrile-water (55:45 % v/v)

Flow rate: 1 mL/min

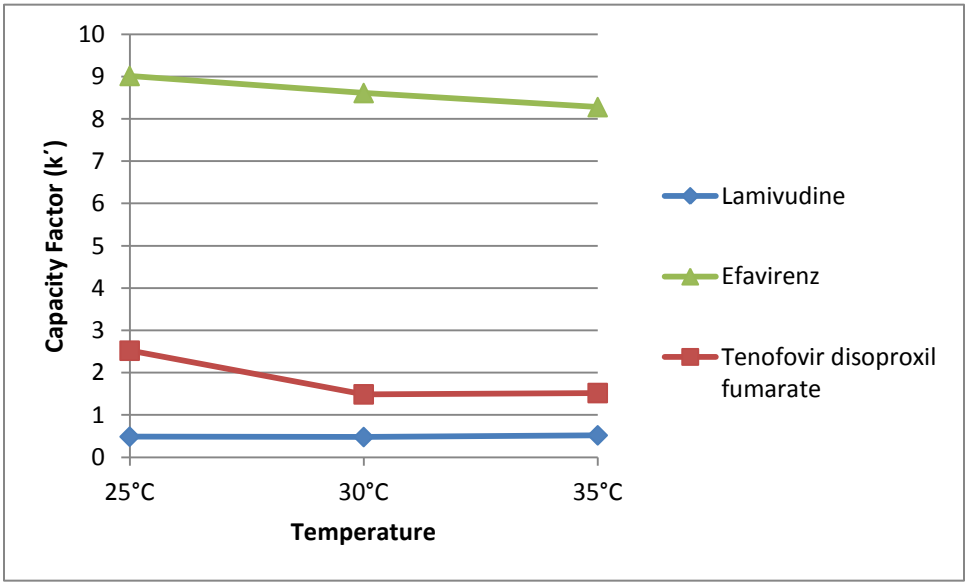

Figure 34: Effect of column temperature on the capacity factors for lamivudine, tenofovir disoproxil fumarate and efavirenz. Mobile phase: acetonitrile: water (55:45 % v/v). Column: C18, 250mm x4.6 mm x 5nm. Detection: 252 nm. Flow rate 1.0 mL/min.

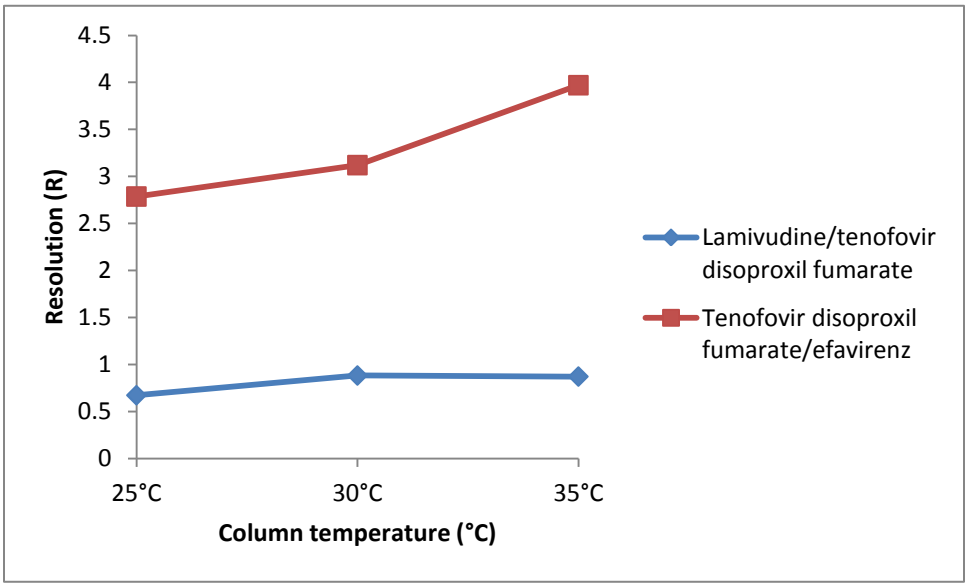

Figure 35: Effect of column temperature on the resolution factors for lamivudine, tenofovir disoproxil fumarate and efavirenz. Mobile phase: acetonitrile: water (55:45 % v/v). Column: C18, 250mm x4.6 mm x 5nm. Detection: 252 nm. Flow rate 1.0 mL/min.

Small variation in the flow rate has proven to affect the retention times for lamivudine, tenofovir disoproxil fumarate and efavirenz (Table 10). The retention times for the APIs under this study decreased with the increase in flow rate and increased with the decrease in the flow rate. The capacity factors for lamivudine, tenofovir disoproxil fumarate and efavirenz remained unaffected by small variation of the flow rate (Figure 36). Resolution of lamivudine, tenofovir disoproxil fumarate and efavirenz was affected with the change in the flow rate (Figure 37).

Table 10: The effect of flow rate on the chromatographic parameters of lamivudine, tenofovir disoproxil fumarate and efavirenz

Active pharmaceutical ingredient	Flow rate (mL/min)	Retention time (min)	Resolution (R)	Capacity factor (k´)
Lamivudine	0.8	2.418	0.822	0.494
	1.0	1.941	0.882	0.479
	1.2	1.623	1.105	0.490
Tenofovir disoproxil fumarate	0.8	4.062	3.312	1.510
	1.0	3.264	3.120	1.488
	1.2	2.728	3.891	1.505
Efavirenz	0.8	15.655	3.312	8.676
	1.0	12.623	3.120	8.611
	1.2	10.510	3.891	8.651

Column: C 18, 250 mm x 4.6 mm x 5 µm

Mobile phase: acetonitrile-water (55:45 % v/v)

Column temperature: 30°C

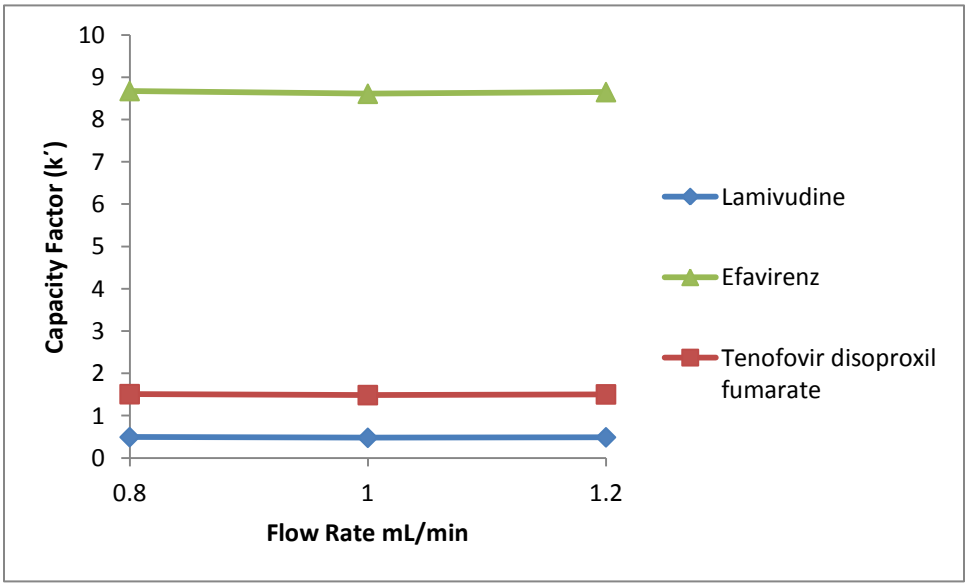

Figure 36: Effect of flow rate on the capacity factors for lamivudine, tenofovir disoproxil fumarate and efavirenz. Mobile phase: acetonitrile: water (55:45 % v/v). Column: C18, 250mm x4.6 mm x 5nm. Detection: 252 nm. Flow rate: (0.8, 1.0 and 1.2mL/min).

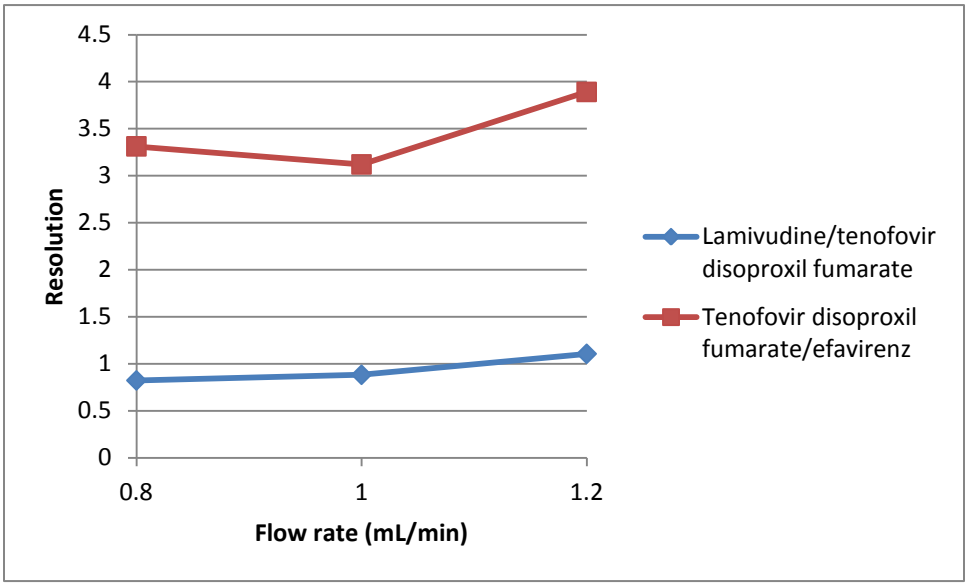

Figure 37: Effect of flow rate on the resolution of lamivudine, tenofovir disoproxil fumarate and efavirenz. Mobile phase: acetonitrile: water (55:45 % v/v). Column: C18, 250mm x4.6 mm x 5nm. Detection: 252 nm. Flow rate: (0.8, 1.0 and 1.2mL/min).

6.0 CONCLUSION

A simple high performance liquid chromatographic method for simultaneous analysis of lamivudine, tenofovir disoproxil fumarate and efavirenz was developed and underwent an extensively single laboratory validation (SLV) for specificity and/or selectivity, linearity, accuracy, precision, robustness and stability of test solution. An isocratic elution mode with a mixture of acetonitrile-water (55:45 % v/v) was selected as the mobile phase. The analysis was performed with a nucleosil (Macherey Nagel-Germany) C18 (4.6 mm x 250 mm x 5μm) column as stationary phase for simultaneous separation of lamivudine, tenofovir disoproxil fumarate and efavirenz. Other chromatographic conditions included a flow rate of 1 mL/min with the detection wavelength of 252 nm at room temperature. The dissolution medium for LTE tablets was such that 0.5% of sodium dodecyl sulphate in water was enough to achieve a release of more than 75% of each of the three active pharmaceutical ingredients in the LTE tablets with a paddle method at 37°C, 75 rpm at 45 min. The linear range was found in the concentration range of 0.05 to 0.12 mg/mL with regression coefficient (r^2) of 0.9984 for lamivudine, tenofovir disoproxil fumarate and efavirenz which met the acceptance criteria of r^2 equal or greater than 0.98. The % rsd for the intra-day precision were 1.08%, 1.23% and 1.46% for lamivudine, tenofovir disoproxil fumarate and efavirenz respectively. The % rsd for the inter-day precision were 1.95%, 1.99% and 1.67% for lamivudine, tenofovir disoproxil fumarate and efavirenz respectively. The test method had an acceptable level of accuracy for the assay of lamivudine, tenofovir disoproxil fumarate and efavirenz in lamivudine, tenofovir and efavirenz tablets from 50 % to 120 % of test concentration with % rsd less than 2% for all three active pharmaceutical ingredients. The test solution remained stable when stored at 4°C for 72 hours. The method remained largely unaffected by small variations in temperature and flow rate. The mobile phase should be accurately measured for best results because the mobile phase composition had more effect on the retention time of efavirenz due to its poor solubility in water. Cost implication for the method application is relatively low as it does not involve any costly sample extraction procedure. The method uses distilled water as one of the solvents which is relatively of low cost as compared to other solvents. The analysis time is short, approximately 20 min. From the findings, the method is suitable for routine quality control tests for assay and dissolution in

pharmaceutical products which contain lamivudine, tenofovir disoproxil fumarate and efavirenz active substances in resource poor settings laboratories.

7.0 RECOMMENDATIONS

Further developments should be done on the analytical method for impurity separation due to the fact that the analytical method developed under this study did not effectively resolve the active pharmaceutical ingredients of lamivudine, tenofovir and efavirenz finished product from their potential degradation products when challenged by forced degradation.

REFERENCES

1. Pinheiro S., Antunes O., Fortunak J., A survey of the syntheses of active pharmaceutical ingredients for antiretroviral drug combinations critical to access in emerging nations, Antiviral Res. 2008; 79:143-65
2. Cahn P., Emtricitabine: A new nucleoside analogue for once-daily antiretroviral therapy, Expert Opin Invest Drugs. 2004; 13:55-68
3. De Clercq E., Emerging anti-HIV drugs, Expert Opin Invest Drugs. 2005; 10:241-73
4. Drogan D., Rauch P., Hoffmann D., Walter H., Metzner K., The antiretroviral potency of emtricitabine is approximately 3-fold higher compared to lamivudine in dual human immunodeficiency virus type 1 infection/competition experiments in vitro, Antiviral Res. 2010; 86:312-5
5. Antiretroviral drugs; http://en.wikipedia.org/wiki/Antiretroviral_drug (Last accessed: October 16, 2011)
6. Antiretroviral Therapy for HIV infection for adults & adolescents; http://www.who.int/hiv/pub/arv/adult2010/en/index.html (Last accessed: August 19, 2011)
7. The United Republic of Tanzania. National guidelines for the management of HIV and AIDS, 2009; 3rd Edition: 131-76.
8. Gazzard G., Use of tenofovir disoproxil fumarate and emtricitabine combination in HIV-infected patients, Expert Opin Pharmacother. 2006; 7:793-802
9. Graeme J., Efavirenz: shifting the HAART paradigm in adult HIV-1 infection, Expert Opin Invest Drugs. 1999; 8:473-486
10. Brookie M., Miguel G.,Efavirenz – Still First-line King?, Expert Opin Drug Metab Toxicol. 2008; 4: 965-972
11. Palacios R., Aguilar I., Hidalgo A., Santos J., Didanosine, lamivudine-emtricitabine and efavirenz as initial therapy in naive patients, Expert Rev Anti Infect Ther. 2006; 4:965-71
12. María L., Cuestas M., Oubiña J., Sosnik A., Drug delivery systems and liver targeting for the improved pharmacotherapy of the hepatitis B virus (HBV) infection, Pharm. Res. 2009; 27:189
13. Lamivudine information, Health mash; http://www.healthmash.com/health/lamivudine.html (Last accessed: August 19, 2011)

14. WHO Drug Information, 2005; 19:2; http://apps.who.int/medicinedocs/en/d/Js7917e/7.html (Last accessed: August 19, 2011)
15. Branch E., Floyd M., Honeywell M., Tenofovir: The first nucleotide analog for HIV-1, 2002; 27:359-61
16. Gilead Sciences. Full prescribing information: TRUVADA, 2009; 14
17. Pogány J., Pharmaceutical development for multisource (generic) pharmaceutical products 42nd meeting of the WHO Expert Committee on Specifications for Pharmaceutical Preparations; 2007 Oct 15-19; Geneva Switzerland; 2008. 11-2
18. Poupard M., Ngom Gueye F., Thiam D., Ndiaye B., Girard M., Delaporte E., Quality of life and depression among HIV-infected patients receiving efavirenz- or protease inhibitor-based therapy in Senegal, HIV Med. 2007; 8:92-5
19. Efavirenz Wikipedia; http://en.wikipedia.org/wiki/Efavirenz (Last accessed: August 19, 2011)
20. Amin A., Hamdard J., Nagar H., Pharmaceutical technology: Biopharmaceutics -New Delhi - http://nsdl.niscair.res.in/bitstream/123456789/749/1/Revised++Biopharmaceutics.pdf (Last accessed: August 20, 2011)
21. Quereshi A., McGilveray J., A critical assessment of the USP dissolution apparatus suitability test criteria, Bureau of Drug Research. 1995, 21:905-924
22. Mandloi D., Tyagi P., Rai V., Dey S., Ashada R., Mohanraj P., Method development and validation of RP- HPLC in the application of *invitro* dissolution study of lamivudine in bulk drug and tablet formulation, J. Chem. Pharm. Res**.** 2009; 1: 286-287
23. British Pharmacopoeia. London: The Stationery Office; 2005. 2:1111
24. Rezk L, Tidwell R, Kashuba D. High-performance liquid chromatography assay for the quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma, J. Chromatogr., B: Anal Technol Biomed Life Sci. 2004; 805:241-7
25. Saras-Nacenta M., Lopez-Pua Y., Lipez-Cortes F., Mallolas J., Gatell M., Carne X., Determination of efavirenz in human plasma by high-performance liquid chromatography with ultraviolet detection, J Chromatogr B Biomed Sci Appl. 2001; 763:53-9

26. Ter Heine R., Alderden-Los G., Rosing H., Hillebrand J., Van Gorp C., Huitema D., Fast and simultaneous determination of darunavir and eleven other antiretroviral drugs for therapeutic drug monitoring: method development and validation for the determination of all currently approved HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry, Rapid Commun Mass Spectrom. 2007; 21:2505-14
27. Villani P., Feroggio M., Gianelli L., Bartoli A., Montagna M., Maserati R., Antiretrovirals: simultaneous determination of five protease inhibitors and three nonnucleoside transcriptase inhibitors in human plasma by a rapid high-performance liquid chromatography--mass spectrometry assay, Ther Drug Monit. 2001; 23:380-8
28. Erk N., Derivative-differential UV spectrophotometry and compensation technique for the simultaneous determination of zidovudine and lamivudine in human serum, Pharmazie. 2004; 59:106-11
29. Beck C., Athayde L., Cardoso G., HIV/AIDS treatment and physicochemical quality control of medicines: evaluation of non-generic lamivudine + zidovudine tablets manufactured in Brazil, 2007; Braz J Infect Dis.11:540-3
30. Hemanth K., Sudha V., Swaminathan S., Ramachandran G., Comparison of HPLC and spectrophotometric methods for estimation of antiretroviral drug content in pharmaceutical products, Indian J Med Res. 2010; 132:390-4
31. Lopes B., Wolff C., Bioucas-Dias M., Figueiredo A., Determination of the composition of counterfeit heptodin tablets by near infrared chemical imaging and classical least squares estimation, Anal Chim Acta. 2009; 641:46-51
32. Fernandes C., Junqueira G., Campos M., Pianetti A., Dissolution test for lamivudine tablets: optimization and statistical analysis, J Pharm Biomed Anal. 2006; 42:601-6
33. Tracy K., Lane B., Jennifer K., Peter L., Ray M., Delahunty T.,Courtney F., Liquid chromatography-tandem mass spectrometric determination of tenofovir-diphosphate in human peripheral blood mononuclear cells, J. Chromatogr., B: Anal Technol Biomed Life Sci. 2006; 843: 147-56

34. Delahunty T., Bushman L., Fletcher V., Sensitive assay for determining plasma tenofovir concentration by LC/MS/MS, J. Chromatogr., B: Anal Technol Biomed Life Sci. 2006; 830: 6-12
35. Rezk N., Crutchley R., Kashuba A., Simultaneous quantification of emtricitabine and tenofovir in human plasma using high-performance liquid chromatography after solid phase extraction, J. Chromatogr., B: Anal Technol Biomed Life Sci. 2005; 822: 201-208
36. Atul S., Charushila B., Sunjay S., Application of UV-spectrophotometric methods for estimation of tenofovir disoproxil fumarate in tablets, Pak J Pharm Sci. 2009; 22:27-9
37. Ashenafi D., Chintam V., Van Veghel D., Dragovic S., Hoogmartens J., Adams E., Development of a validated liquid chromatographic method for the determination of related substances and assay of tenofovir disoproxil fumarate, J Sep Sci. 2010; 33:1708-16
38. Bennetto-Hood C., Long C., Acosta P., Development of a sensitive and specific liquid chromatography/mass spectrometry method for the determination of tenofovir in human plasma, Rapid Commun Mass Spectrom. 2007; 21:2087-94
39. D'Avolio A, Sciandra M, Siccardi M, Baietto L, Gonzalez de Requena D., Bonora S., A new assay based on solid-phase extraction procedure with LC-MS to measure plasmatic concentrations of tenofovir and emtricitabine in HIV infected patients, J Chromatogr Sci. 2008; 46:524-8
40. The United States Pharmacopoeia, United States: Board of Trustees; 2010. 33: R-659-63
41. International Conference on Harmonization. ICH Q2 (R1): Validation of analytical procedures: Text and Methodology, 2005:1-13
42. Shanghai Desano Chemical Pharmaceutical Co. LTD. Technical information package for efavirenz and tenofovir disoproxil fumarate assay methods: 5-6