DETERMINANTS OF NEVIRAPINE PLASMA LEVELS AND CLINICAL OUTCOMES IN HIV PATIENTS AT KENYATTA NATIONAL HOSPITAL, KENYA

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Reg: U52/74967/2014

A thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in Molecular Pharmacology of the University of Nairobi

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November, 2016

DECLARATION

I, the undersigned, hereby declare that this thesis is my original work and has not been previously presented in its entirety or in part, for the award of any other degree or to any other university.

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DEDICATION

I am grateful to the Almighty God for the blessings in undertaking this work.
I dedicate this thesis to my dear wife and best friend, Jessica for the unwavering support, patience and encouragement, and my two angels, my son Ryan and daughter, Renée for the many questions and smiles.

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LIST OF ABBREVIATIONS AND ACRONYMS

3TC	Lamivudine
ABC	Adenosine triphosphate Binding Cassette
ACN	Acetonitrile
ADRs	Adverse Drug Reactions
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
AZT	Zidovudine
BMI	Body Mass Index
CCC	Comprehensive Care Center
CYP	Cytochrome P450
EFV	Efavirenz
FDA	Food and Drug Administration
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
HQC	High Quality Control standard
KAIS	Kenya AIDS Indicator Survey
KNH	Kenyatta National Hospital
LLOQ	Lower limit of Quantification
LQC	Low Quality Control standard
MeOH	Methanol
MQC	Middle Quality Control standard
MS	Mass Spectrometer
NASCOP	National AIDS and STI Control Program
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NVP	Nevirapine
PB	Phosphate Buffer
PD	Pharmacodynamics
PG	Pharmacogenetics
PK	Pharmacokinetics
SNP	Single Nucleotide Polymorphism
TDF	Tenofovir
ULOQ	Upper Limit of Quantification
UNAIDS	Joint United Nations Program on HIV/AIDS
UV	Ultraviolet light
WHO	World Health Organization

OPERATIONAL DEFINITIONS

Accuracy: Degree of closeness of test results to the true (nominal) value under given conditions

Allele: One member of a pair that makes up a gene at a locus on a chromosome

Analyte: Specific chemical molecule whose measurements are being determined

ATP binding cassette (ABC): Superfamily of efflux drug transporting proteins

Clearance: The removal of a drug from the body through metabolism and excretion

Covariates: Variables likely to explain variance in an outcome of interest

Gene: Unit of heredity passed on from a parent to an offspring

Genotype: Genetic makeup of an organism in terms of specific genes or the entire genome

Genotyping: The process of identifying the genetic makeup of an organism

Heterozygosity: Having different allelic variants of a gene

Upper limit of quantification (ULOQ): Highest amount of analyte that can be determined with acceptable precision and accuracy using an analytical method under specified conditions

Homozygosity: Property of having the same alleles of a gene

Inter individual variability: Differences occurring between individuals

Intra individual variability: Differences occurring within an individual

Linearity: Range of analyte concentration over which a relationship of the parameters can be related using a linear equation and a graphical straight line

Log P: Lipophilicity of un-ionized species in a biphasic environment measured by the oil/water partition coefficient (P) base-10 logarithm

Log S: Aqueous solubility of a compound in moles/litre measured in logarithms to base- 10

Lower limit of quantification (LLOQ): Lowest amount of an analyte that can be determined with acceptable accuracy and precision using an analytical method under specified conditions

Mutation: Spontaneous change in the DNA sequence in an organism's genome

Pharmacokinetics: Drug disposition in an individual encompassing its absorption, distribution, metabolism and excretion

Phenotype: Observable characteristic of an organism determined by the genotype, environment and/or random variation

pKa: Negative base-10 logarithm of the acid dissociation constant (Ka) of a solution; measure of the strength of an acid

Polymorphisms: Genetic variations that occur with a frequency of more than 1% in a population

Population pharmacokinetics: Study of drug concentration variability in plasma between individuals receiving a standard dose of a drug regimen in a population

Precision: Closeness of a series of test results to each other under given conditions

Recovery: Efficiency of extraction of analytical process measured as a percentage of the known amount of an analyte carried in the sample extraction and processing steps of a given quantification method

Selectivity: Ability of a bioanalytical method to differentiate an analyte in a mixture of compounds

Sensitivity: Extent to which an analytical method is able to detect and respond to minimum changes in concentration of the analyte under determination; (also see LLOQ on Page xiv)

Single nucleotide polymorphism (SNP): Variation at one position on the DNA sequence in at least 1% of individuals in a population

Stability: Extent to which the chemical composition of an analyte remains unchanged under specified conditions within a given period of time

Steady-state: Equilibrium state where the rate of input of a drug is equal to the rate of elimination after consistent dosing

ABSTRACT

Background

Nevirapine is a non-nucleoside reverse transcriptase inhibitor used as an antiretroviral agent for the treatment of HIV/AIDS. Current dosing schedules are based on pharmacokinetic studies in Caucasian populations and thus there may be high incidences of toxicity or sub-optimal nevirapine plasma levels that might limit its use and therapeutic success in non-Caucasians.

Objective

This study determined variability of steady-state nevirapine plasma levels and the influence of factors such as genetic polymorphisms, adherence and co-administered antiretroviral drugs in HIV positive patients at the Comprehensive Care Centre (CCC) at Kenyatta National Hospital (KNH). This study also evaluated the influence of nevirapine plasma levels on clinical outcomes such as maximum CD4 cell counts attainable during treatment.

Methods

A high performance liquid chromatography (HPLC) method utilizing ultraviolet (UV) detection was optimized and validated for determination of nevirapine in plasma.

A descriptive one arm cross sectional study was conducted nested in a larger study on 241 HIV patients on nevirapine based antiretroviral therapy (ART) who attended the KNH CCC in 2014. Historical blood samples collected from the patients on ART for at least six months were analyzed using the HPLC-UV method to determine the steady-state nevirapine plasma levels. Previously collected data on baseline sociodemographic characteristics, clinical data for at least six months ART treatment and CYP2B6 genotypes was collated with the nevirapine levels. Descriptive, inferential, and linear regression analysis was performed to determine predictors of nevirapine plasma levels and clinical outcomes.

Results

The HPLC-UV method was selective and sensitive with a limit of quantification of 0.5 µg/mL. The accuracy of the method was 2.3% with inter- and intra-day precision of 7.2% and 4.7% respectively. The analytical range of the method was wide from 0.5 to 25 µg/mL with a recovery of 91.8%.

Steady-state nevirapine plasma levels varied widely among patients (46%) and within patients (28%) with a median of 5.675 µg/mL. Eighteen percent of participants had supra-therapeutic nevirapine plasma levels (above 8.0 µg/mL) while 6.3% had sub-therapeutic nevirapine plasma levels (below 3.0 µg/mL). Seventy eight percent of the patients had nevirapine plasma levels above 4.3 µg/mL reported to offer lasting viral suppression.

This study found that CYP2B6 genotypes may play a significant role in influencing nevirapine plasma levels. Interestingly, homozygous CYP2B6 983CC (10.60 µg/mL) [β =2.619 (95% CI 1.337-3.901; P<0.001] and 516TT (8.450 µg/mL) [β =1.332 (95% CI 0.665-1.999); p<0.000] genotypes had higher nevirapine plasma levels compared to heterozygous (CYP2B6 983TC, 6.858 µg/mL and 516GT, 5.518 µg/mL) and wild type (CYP2B6 983TT, 5.533 µg/mL and 516GG, 5.575 µg/mL) genotypes. Participants who switched to tenofovir based ART regimens had higher nevirapine plasma levels than those who were on stavudine and zidovudine based regimens [β=1.202 (95% confidence interval (CI) 0.402-1.587); p=0.003]. Adherence to nevirapine dosing schedule resulted in an increase in nevirapine plasma levels (p=029).

Nevirapine plasma levels influenced the maximum CD4 cell counts attainable during ART treatment (p=0.0420 but not the occurrence and severity of skin reactions and hepatotoxicity.

Discussion and conclusion:

Nevirapine plasma levels were affected by CYP2B6 genotypes (CYP2B6 983 T>C and 516 G>T) as the SNPs may affect expression and activity of nevirapine metabolizing enzymes. Adherence to dosing schedules may result in higher nevirapine plasma levels whereas those who do not adhere might have erratic nevirapine plasma levels. Nevirapine plasma levels may influence the maximum CD4 cell counts where higher nevirapine plasma levels might lead to greater viral suppression and better immunological recovery.

In conclusion, a simple and improved HPLC-UV method that may be utilized in resource limited settings was used to determine nevirapine in plasma. Nevirapine plasma levels were influenced by CYP2B6 genotypes, adherence and use of tenofovir based ART regimens. Nevirapine plasma levels influenced the maximum immunological response in patients on ART but not the occurrence and severity of skin and hepatotoxic adverse drug reactions. Comprehensive population pharmacokinetic studies may be performed to inform and redesign dosing schedules so as to maximize clinical benefits and minimize occurrence of toxicity in patients using nevirapine based ART in the population.

CHAPTER ONE

INTRODUCTION

1.1 Background

There is still no cure for HIV/AIDS more than three decades after the first case was diagnosed. Therapeutic management of HIV infection aims at reducing the viral load and/or slowing down disease progression to full blown AIDS and/or occurrence of opportunistic infections that may be fatal (WHO, 2016). The use of highly active antiretroviral therapy (HAART) as recommended by the World Health Organization (WHO) has increased the survival period of HIV infected patients. Long term use of HAART may however result in unidentified beneficial or adverse effects of the drugs. In addition, patients having HIV/AIDS for a long period may experience undocumented effects (WHO, 2016).

About 35 million people were living with HIV globally as at 2012 of which almost 71% were in Sub-Saharan Africa (UNAIDS, 2013). The prevalence of HIV in Kenya is estimated at 5.6% among the age group of 15-64 years (NASCOP, 2014). The number of people living with HIV in Kenya who are receiving antiretroviral therapy (ART) as of 2015 were over 900,000 (NASCOP, 2016). The number of patients on ART is expected to increase significantly as WHO guidelines require that all HIV patients should be under HAART regardless of CD4 cell count and stage of the disease (WHO, 2016).

The aim of HAART is to suppress the HIV virus, enhance immune recovery that is reflected in the CD4 cell counts and reduce occurrence of opportunistic infections. The first line ART regimen in resource-limited setting comprise of a non-nucleoside reverse transcriptase inhibitor (NNRTI), which is either efavirenz or nevirapine, combined with two nucleoside reverse transcriptase inhibitors (NRTIs). Nevirapine is also used to prevent-mother-to-child transmission of HIV (WHO, 2016).

Nevirapine is an active drug, the first NNRTI for treatment of HIV-1 virus (Montaner *et al.*, 1998). Nevirapine was licensed after it offered better viral suppression and immune recovery when combined with two NRTIs than a combination of zidovudine and didanosine (Montaner *et al.*, 1998). Nevirapine is well absorbed when taken orally and is mainly excreted as glucuronide conjugates of primary hydroxylated metabolites (Cammett *et al.*, 2009).

Nevirapine plasma levels may be affected by environmental, physiological and genetic factors. Environmental factors such as co-administered drugs, adherence and alcohol intake may affect the pharmacokinetics of nevirapine (Lamorde *et al.*, 2011). Physiological factors such as age, sex, weight, co-morbidities and nutritional status may affect nevirapine plasma levels (Kappelhoff *et al.*, 2005).

Adherence to HAART has been shown to one of the important factors in the success of managing HIV infection (Mannheimer *et al.*, 2006). Assessing the level of adherence to nevirapine based regimens and the its impact on nevirapine plasma levels may lead to better therapeutic outcomes (Hansana *et al.*, 2013).

Nevirapine plasma levels may also be affected by genetic factors that influence its metabolism. Enzymes involved in metabolism of nevirapine are in the Cytochrome P450 (CYP) system. The key isoforms of the CYP system that are involved include CYP2B6 and CYP3A4 (Zanger *et al.*, 2013). The rate of metabolism of nevirapine is affected by genetic polymorphisms in the isoenzymes that may lead to reduced breakdown (Zanger *et al.*, 2013).

The concentration of nevirapine in plasma may indicate the level of viral suppression. High nevirapine plasma levels have been associated with improved virological response and better immunological recovery (Veldkamp *et al.*, 2001). Literature survey reveals that nevirapine plasma levels are determined using chromatographic methods such as liquid and gas chromatography with different modes of detection. Liquid chromatography utilizing mass spectrophotometric detection (LC-MS) is reported to be the most sensitive bioanalytical method (Ren *et al.*, 2010). The high cost of LC-MS instrumentation has led to development of high performance liquid chromatography (HPLC) methods with ultraviolet/visible (UV/Vis) detection for use in resource-limited settings (Hamrapurkar *et al.*, 2010). Furthermore, simultaneous determination of nevirapine together with other antiretroviral (ARV) drugs by HPLC-UV method is preferred as it is efficient (Kumar *et al.*, 2010).

1.2 Problem statement

Determination of the optimal doses of a drug is done using population averaged pharmacokinetics data. The pharmacokinetic data are mainly obtained from Caucasian populations where most clinical studies on drugs are performed and the determined doses may be high or low in non-

Caucasian populations resulting in toxicities or sub-clinical levels. An example is efavirenz where increased toxicity necessitated reduction in the dose from 800 mg to 600 mg (Gatanaga *et al.*, 2007). A number of studies have been performed to determine the pharmacokinetic parameters of nevirapine in African populations (Kappelhoff *et al.*, 2005; Penzak *et al.*, 2007; Svensson *et al.*, 2012). Metabolism of nevirapine is influenced by polymorphisms in CYP2B6 isoenzymes that vary with ethnicity. Evaluating the variability of nevirapine plasma levels and the influence of CYP2B6 genotypes among other covariates such as adherence in the Kenyan population may improve the understanding of the observed phenotypes leading to better clinical use of the drug.

Two studies (Oluka, 2012; Oluka *et al.*, 2015) have been carried out in Kenya to identify the influence of CYP2B6 genotypes on the observed phenotypes of patients using nevirapine containing regimens. Oluka, 2012 evaluated the effect of CYP2B6 516G>T on trough nevirapine plasma levels at six months of treatment in 110 participants. Oluka *et al.,* 2015 examined the influence of CYP2B6 516G>T and 983T>C genotypes on nevirapine plasma levels in 66 women at 12 weeks of treatment in a coastal population.

The current study was undertaken on a larger sample size (241 participants) in a study population with diverse ethnic composition. The present study evaluated the influence of CYP2B6 genotypes and other covariates such as adherence and ART regimens on nevirapine plasma levels in both sexes. Whereas both Oluka, 2012 and Oluka *et al.,* 2015 found a relationship between CYP2B6 genotypes and nevirapine plasma levels, there was no agreement on the influence of nevirapine plasma levels on change in CD4 cell count during treatment. Hence this study evaluated the influence of CYP2B6 genotypes, adherence ART regimens on nevirapine plasma levels.

To analyze nevirapine plasma levels, a simple selective, precise and rapid chromatographic method is required that can be used in resource-limited settings. This study aimed at improving available HPLC-UV methods for quantification of nevirapine in plasma.

1.3 Research questions

1. Can currently available HPLC-UV methods for quantification of nevirapine plasma levels be improved?
2. What are the steady-state nevirapine plasma levels and how do they vary among HIV patients?
3. Do factors such as CYP2B6 genetic polymorphisms, adherence, age and ART regimens affect nevirapine plasma levels in HIV positive patients on nevirapine based ART?

4. What is the influence of steady-state nevirapine plasma levels on clinical outcomes such as CD4 cell counts and adverse drug reactions in HIV patients?

1.4 Objectives

1.4.1 Main objective

The main objective of this study was to determine nevirapine plasma levels at steady-state, factors associated with its variability and how it influences clinical outcomes in HIV positive patients at Kenyatta National Hospital.

1.4.2 Specific objectives

The specific objectives of this study included:

1. Optimization and validation of a HPLC-UV method for determination of nevirapine in plasma.
2. Determination of the steady-state nevirapine plasma levels in HIV patients.
3. Investigation of the effects of CYP2B6 516G>T and CYP2B6 983T>C genotypes and other covariates such as adherence, age, sex and ART regimens on nevirapine plasma levels.
4. Description of the effect of nevirapine plasma levels on clinical outcomes such as maximum CD4 cell count attained and incidences of adverse drug reactions in HIV patients on nevirapine based ART for not less than six months.

CHAPTER TWO

LITERATURE REVIEW

2.1 Physicochemical properties of nevirapine

Nevirapine (11-cyclopropyl-5, 11-dihyro-4-methyl-6*H*-dipyrido [3, 2-b: 2', 3']-[1, 4] diazepine-6-one) illustrated in **Figure 2.1** is a synthetic organic compound with a dipyridodiazepinone structure (Cheeseman *et al.*, 1993). The chemical formula of nevirapine is $C_{12}H_{14}N_4O$ with a molecular weight of 266.2979. Nevirapine is lipophilic with a partition coefficient of 83 with a $\log_{10}$ P of 1.75. Nevirapine is a weak base (pKa=2.8) and soluble in aqueous solutions at pH less than 3. The water solubility of nevirapine at neutral pH is 0.105 mg/ml with a $\log_{10}$ S of -3.4 mol/L (Drugbank, 2013). The physicochemical properties of nevirapine are coherent with Lipinski's 'Rule of Five' drug like properties (Lipinski, 2000).

Figure 2.1: Chemical structure of nevirapine (Cheeseman *et al.*, 1993)

2.2 Mode of action of nevirapine

Nevirapine is a non-nucleoside reverse transcriptase inhibitor of HIV-1 virus. Reverse transcriptase enzyme is necessary for conversion of single-stranded viral RNA to double-stranded linear DNA which may be incorporated into the genome of the host cell (Cohen *et al.*, 1991). Nevirapine is a non-competitive inhibitor that binds reverse transcriptase and thus blocks the RNA and DNA-dependent polymerase activity by causing a disruption of the polymerase enzyme's catalytic site. Nevirapine binds to the hydrophobic peptide region of HIV-1 reverse transcriptase between the tyrosine amino acid residues at position 181 and 188 on the p66 subunit. Nevirapine binding site is allosteric to the catalytic position and hence does not affect substrate binding (Cohen *et al.*, 1991). Nevirapine acts by slowing the rate of magnesium ion dependent chemical reaction catalyzed by the HIV-1 reverse transcriptase enzyme by altering aspartate carboxyl ligands. The binding of

nevirapine also results in stimulation of HIV-1 ribonuclease (RNase H) and alteration of its cleavage specificity resulting in multiple cleavage that reduces the activity of reverse transcriptase enzyme (Cohen *et al.*, 1991).

2.3 Clinical uses of nevirapine

HAART has revolutionized management of HIV/AIDS resulting in reduction of morbidity and mortality. The recommended first line HAART regimens comprises of three drugs; an NNRTI such as efavirenz (EFV) and nevirapine (NVP) and two NRTIs that include, zidovudine (AZT), abacavir (ABC) and lamivudine (3TC) (WHO, 2016). Tenofovir (TDF) is a nucleotide reverse transcriptase inhibitor (NtRTI) and is used in a similar way as an NRTI. Efavirenz and nevirapine are the commonly used NNRTIs and they have similar clinical effects and hence they are interchangeable (Kappelhoff *et al.*, 2005).

The first line adult ART regimens in Kenya include: TDF (or ABC) + 3TC + EFV (or NVP) or AZT (or ABC) + 3TC + EFV (or NVP). The second line adult ART regimens in Kenya have protease inhibitors (PIs) lopinavir (LPV) and atazanavir (ATZ) boosted with ritonavir (r). The boosted PIs are combined with NRTIs as follows: TDF (or ABC or AZT) +3TC + ATZ/r (or LPV/r) (NASCOP, 2016).

Nevirapine when combined with at least two other antiretroviral drugs (HAART) decreases HIV-1 viral loads, enhances immunological recovery, reduces susceptibility to and development of opportunistic infections in HIV patients. Nevirapine penetrates into sanctuary sites such as the brain and spinal fluids where it may further suppress the HIV virus (WHO, 2016).

Nevirapine is also used to prevent mother-to-child transmission (PMTCT) of HIV where the mother uses HAART and the infant is given nevirapine combined with AZT for the six weeks then nevirapine for another six weeks. Nevirapine may reduce mother-to-child transmission of HIV by over 70% (WHO, 2016). Studies have been carried out on use of nevirapine for post exposure prophylaxis but have not been successful yet due to occurrence of toxic effects in naïve HIV negative patients (Boehringer Ingelheim Pharmaceuticals Inc., 2013).

2.4 Pharmacokinetic properties of nevirapine

2.4.1 Absorption

Absorption of nevirapine into plasma upon oral administration is more than 90% within 4 hours (Riska *et al.*, 1999). The absolute oral bioavailability of a 50 mg tablet of nevirapine is 93± 9% (mean± SD) and 91± 8% for an oral solution (Drugbank, 2013). Peak plasma concentrations of 2± 0.4 µg/mL are attained following a single 200 mg dose. Nevirapine plasma concentrations tend to reduce log linearly leading to a half-life of 45 hours (Drugbank, 2013). Tablets and oral suspension dosage forms of nevirapine have nearly equal absorption. Bioavailability of nevirapine is not affected by food intake.

A two week lead in dosing of 200 mg once daily is adequate in naïve HIV patients during nevirapine initiation. The dose is escalated to 200 mg twice a day after two weeks because nevirapine auto induces cytochrome isoenzymes reducing its half-life from about 45 hours to about 25-30 hours. In multiple dosing of 200 mg twice a day the maximum nevirapine plasma concentration is achieved after 4 hours with a median of 3.1-3.4 mg/L (Drugbank, 2013).

2.4.2 Distribution

Nevirapine is well distributed throughout the body because it is lipophilic and un-ionized at physiological pH. Nevirapine crosses the placenta and is excreted in breast milk. In circulation, about 60% of nevirapine is mainly bound to albumin. Following intravenous administration, the apparent volume of distribution is 21±0.09 L/kg in healthy adults (Drugbank, 2013).

2.4.3 Metabolism and excretion

Nevirapine is metabolized to five key metabolites (**Figure 2.2**) by the Cytochrome P450 monooxygenase system, a superfamily of metabolizing enzymes in the liver. Nevirapine undergoes oxidative metabolism by 2-, 3-, 8- and 12-hydroxylation with subsequent glucuronidation of the hydroxyl groups as shown in Figure 2.2 panels (b), (c), (d), (e) (Riska *et al.*, 1999). The cytochrome P450 isoforms involved in nevirapine metabolism include CYP3A4, CYP2B6, CYP2D6 and CYP2C9 as shown in Figure 2.2 panels (i), (ii), (iii) and (iv). The 12-hydroxynevirapine metabolite undergoes further secondary oxidation to 4-carboxynevirapine as illustrated in Figure 2.2 panels (d), (f) and (iv) (Cammett *et al.*, 2009).

i-iv- Metabolic pathways; **a-f**- Metabolites of nevirapine

Figure 2.2: Nevirapine and its key metabolites (Cammett *et al.,* 2009)

CYP3A4 and CYP2B6 isoenzymes are induced (20-25%) by nevirapine resulting to a 1.5-2 fold increase in nevirapine clearance (Riska *et al.*, 1999). The induction is complete within 2-4 weeks and the plasma concentrations of nevirapine stabilize with a mean steady-state plasma concentration of about 4.5± 1.9 µg/mL (Boehringer Ingelheim Pharmaceuticals Inc., 2013).

About 90% of nevirapine metabolites such as glucuronides are excreted in urine. A small portion of nevirapine metabolites is excreted in the faeces (~10%) and less than 3% of nevirapine is excreted unchanged (Riska *et al.*, 1999).

2.4.4 Toxicity

Adverse drug reactions (ADRs) due to nevirapine have been reported. Mild ADRs experienced during nevirapine initiation include headache, fatigue, nausea, vomiting, rash and insomnia. Serious ADRs include severe skin rash that may progress into Steven Johnson Syndrome (SJS) and toxic epidermal necrosis (TEN); as well as asymptomatic or acute hepatotoxicity (liver necrosis and

hepatitis) (Kesselring *et al.*, 2009). Hepatotoxicity may be evaluated by determining the alanine transaminase (ALT) levels and/or assessing symptomatic presentations such as jaundice.

The concentration of nevirapine in plasma may play a role in the development of ADRs although studies are inconclusive on the association of nevirapine plasma levels and occurrence of adverse events (Kappelhoff *et al.*, 2005). The effect of covariates such as genotype, sex, hepatic impairment, and CD4 levels on development of ADRs has been reported (Kondo *et al.*, 2007; Ratanasuwan *et al.*, 2012). Studies conducted in Kenyan populations reported no association between nevirapine plasma levels and development of toxicity (Oluka *et al.*, 2015).

2.5 Factors affecting nevirapine plasma levels

Factors that affect the pharmacokinetics of nevirapine (**Table 2.1)** include co-administered drugs, alcohol use, sex, age, weight, genotype, adherence, comorbidities and nutrition status (Cooper *et al.*, 2007; Swaminathan *et al.*, 2011).

Females tend to have higher levels of nevirapine compared to males and this may be as a result of differences in body mass and fat distribution between sexes (Zhou *et al.*, 1999). The differences in disposition of nevirapine among sexes may explain why females are more prone to ADRs than males (Cammett *et al.*, 2009). The clearance of nevirapine may be affected by weight (De Maat *et al.*, 2002). Nutritional status may further determine weight and thus affect nevirapine disposition (Ramachandran *et al.*, 2010).

Physiological characteristics tend to vary with age. Hepatic and renal functions are reported to be lower among children and the elderly compared to adults and may affect the pharmacokinetics of nevirapine (Swaminathan *et al.*, 2011). In addition, hepatic function may be affected by preexisting conditions such as liver disease and chronic hepatitis B and/or C that might reduce nevirapine metabolism (Kappelhoff *et al.*, 2005). Furthermore, alcohol intake may affect the liver leading to reduced activity of cytochrome isoenzymes that may affect nevirapine metabolism.

The disposition of nevirapine and thus its levels in plasma may be affected by co-administered drugs (Wang *et al.*, 2008). For example rifampicin, a CYP2B6 enzyme inducer, results in increased nevirapine metabolism leading to reduced concentration of nevirapine at the target site (Stöhr *et al.*, 2008).

Table 2.1: Factors affecting nevirapine plasma levels

Covariates	Effect on nevirapine clearance	Reference
Elderly in age	Decrease	Swaminathan *et al.*, 2011
Female sex	Decrease (13.8-25%)	Zhou *et al.*, 1999
High body mass index	Increase (0.1 L/h/10kg)	De Maat *et al.*, 2002
Hepatic impairment:		Kappelhoff *et al.*, 2005
Hepatitis B	Decrease (19.5%)	
Hepatitis C	Decrease (27.4%)	
Ethnicity:		Stöhr *et al.*, 2008
Caucasians	Increase	
Africans	Decrease	
CYP2B6 genotypes:		Zanger *et al.*, 2013
5l6GG, 983TT	Increase	
516TT, 983CC	Decrease	
Rifampicin co medication	Increase	Lamorde *et al.*, 2011
Malnutrition	Decrease	Ramachandran *et al.*, 2010

2.6 Pharmacogenetics of nevirapine

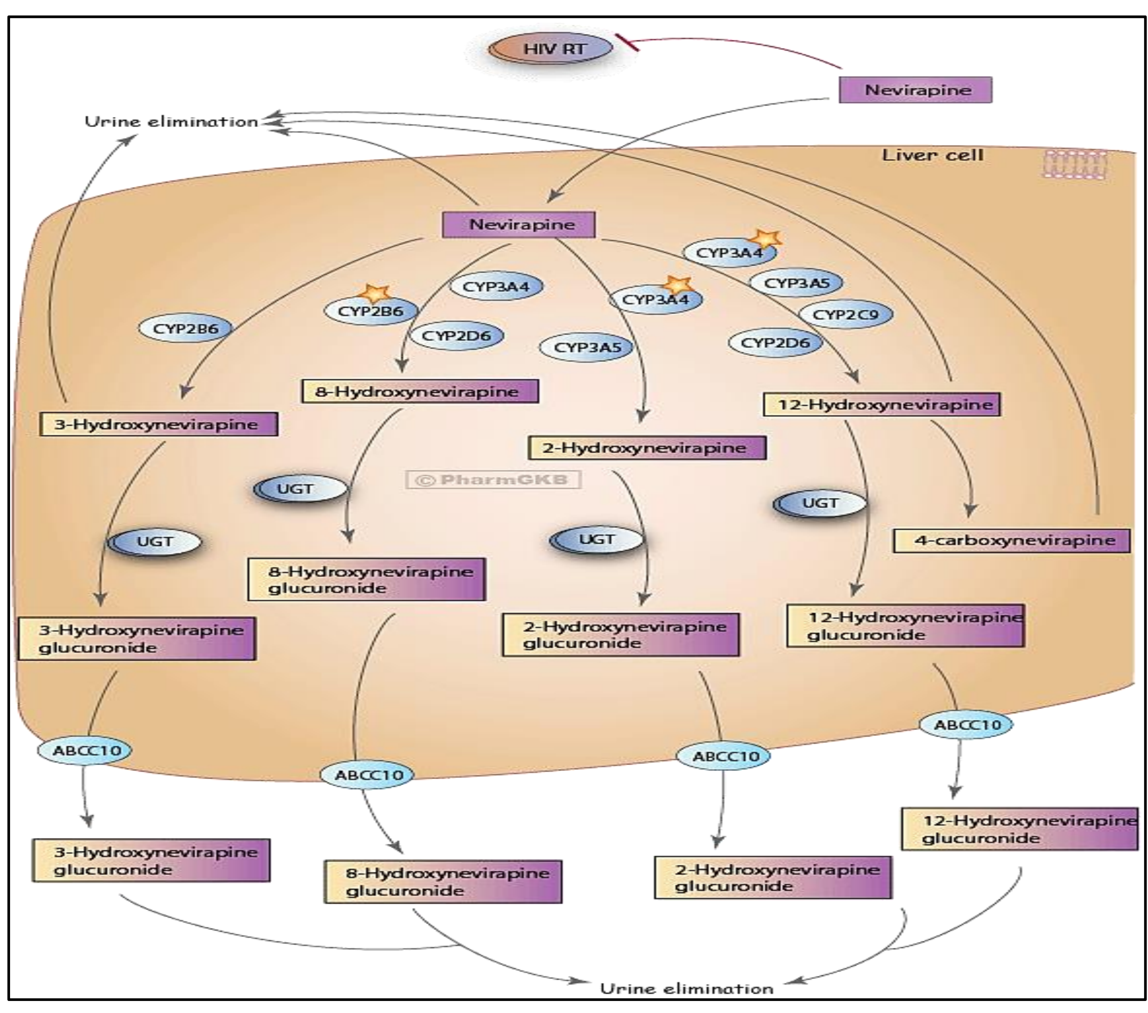
Pharmacogenetics is the study of the influence of genes on pharmacokinetics and pharmacodynamics of a drug. Genes that affect drug pharmacology include those involved in drug transport, metabolism, target receptors and downstream signaling in the target cells. . Mutations such as deletions, insertions and single nucleotide polymorphisms (SNPs) occurring in 1% or more individuals in genes involved in disposition of nevirapine may significantly impact treatment outcomes. Nitrogen bases that form nucleotides in DNA include adenine (A), thymine (T), guanine (G) and cytosine (C). Furthermore, polymorphisms may affect immunogenic responses that in turn increase the likelihood of developing hypersensitivity reactions (Lam *et al.*, 2013).

Transport proteins (transporters) play a role in shuttling molecules such as drugs in or out of the cells. Transporters that remove drugs from cells (efflux) contribute to development of resistance as they inhibit accumulation of drugs in target sites. Adenosine triphosphate binding cassette (ABC) family of transporters are the most studied of which ABCB1 (p-glycoprotein or multidrug resistant

protein (MDR1)) has been found to cause multidrug resistance. MDR1 is encoded by the highly polymorphic *ABCB1* gene that has more than 50 SNPs and three insertion/deletion polymorphisms (Lam *et al.*, 2013).

SNPs of *ABCB1* gene include silent polymorphism at 1236C>T and 3435C>T on exons 12 and 26 respectively; and 2677G>A/T on exon 21 that are reported to have no impact on the transport and bioavailability of nevirapine. ABCC10 transports nevirapine and pumps out its metabolites. Polymorphisms in the ABCC10 transporter may affect the levels of nevirapine reaching target molecules and in turn viral suppression (Liptrott *et al.*, 2013).

CYP2B6 and CYP3A4 (**Figure 2.3**) isoenzymes are involved in nevirapine inactivation in the body. Polymorphisms in the genes coding for CYP2B6 and CYP3A4 isoenzymes may lead to reduced activity or loss function of the isoforms.



RT-reverse transcriptase; UGT-uridine glucuronosyl transferase; ABC-ATP Binding Cassette; CYP-Cytochrome P450

Figure 2.3: Metabolic pathway of nevirapine (pharmgkb.org/pathway)

Polymorphisms in CYP3A4 that affect nevirapine metabolism are rare. On the other hand, polymorphisms in CYP2B6 affect nevirapine disposition.

Nevirapine hypersensitivity may be linked to immunogenic factors including the human leukocyte antigen (HLA). Various HLA alleles have been identified in different populations to be risk factors for developing nevirapine hypersensitivity that may lead to Steven Johnson Syndrome (SJS) and toxic epidermal necrosis (TEN). HLA-DRB1*01:01 (Caucasians); HLA-C*08 (Japanese); HLA-C*04 (Chinese); HLA-C*04 and HLA-B*35:05 (Thai); and HLA-C*04 and HLA-C*04:01 (Africans) alleles have been identified (Carr *et al.*, 2013)

2.6.1 Influence of CYP2B6 genotypes on nevirapine disposition

CYP2B6 is a minor cytochrome P450 isoenzyme accounting for 2-10% of the hepatic CYP content and metabolizes about 4% of drugs used clinically. CYP2B6 exhibits intra- and inter- individual variability due to genetic polymorphism, as well as induction and inhibition by many compounds. Other non-genetic factors also affect CYP2B6 functionality. CYP2B6 is one of the most polymorphic enzymes with great ethnic variability. Genetic polymorphisms affect *CYP2B6* gene regulation, transcription, splicing, expression and activity. Some of the variants are combined in haplotypes as they affect several functional levels simultaneously. Currently, 63 variants and 70 haplotypes of CYP2B6 have been identified (PharmGKB.org, 2015).

CYP2B6 516G>T and CYP2B6 983T>C variants have been implicated in the variance of nevirapine plasma levels. Common SNPs of CYP2B6 affecting nevirapine metabolism include change from guanine nucleotide to thymine at position 516 in one or both alleles (516G>T). Persons with CYP2B6 516GG genotype are said to have the wild type (normal) variant while those with 516GT and 516TT are termed heterozygous and homozygous mutated variants respectively. Another CYP2B6 SNPs that affect metabolism of nevirapine is 938T>C where 983TT is termed the wild type variant while 983TC and 983CC are heterozygous and homozygous variants respectively (Zanger *et al.*, 2013). Homozygosity of 516TT and /or 983CC results in increased nevirapine plasma levels due to reduced activity of CYP2B6 isoform. Distribution of these CYP2B6 SNPs varies with ethnicity and are more prevalent in the African than Caucasian and Asian populations (Turpeinen *et al.*, 2012). Studies have established that CYP2B6 516G>T SNP has a high prevalence in African populations (>30%) whereas CYP2B6 983C>T SNP has a low to moderate prevalence (4-11%) in blacks and has not been reported in Caucasians (Zanger *et al.*, 2013).

Studies have reported a high prevalence of CYP2B6 516G>T across ethnic groups in Kenya of more than 30%. This prevalence infers the need to assess the impact CYP2B6 516G>T and 983T>C polymorphisms on nevirapine plasma levels and clinical outcomes in the population and ascertain whether there is need for monitoring of individuals with such variants (Oluka *et al.*, 2015).

Therefore, evaluation of genetic factors prior to initiation of nevirapine based therapy may be a guide to achieve optimum therapeutic effect and minimize ADRs either by dose adjustment or avoiding use of nevirapine. Evaluation of factors affecting the disposition of nevirapine may be used to personalize the dosing schedule.

2.7 Quantification of nevirapine by HPLC methods

Determining levels of a drug in plasma may be important in predicting the expected effect of the medication in terms of the therapeutic effect or occurrence of ADRs. Studies correlating nevirapine plasma levels with viral suppression and occurrence of ADRs have shown that high nevirapine plasma levels may result in greater viral suppression and better therapeutic activity (Veldkamp *et al.*, 2001). Studies are not conclusive on the relationship between nevirapine plasma levels and development of ADRs (Kappelhoff *et al.*, 2005).

Methods used to determine nevirapine in plasma (**Table 2.2**) differ in approach and cost. The methods for quantification are mainly used to analyze nevirapine in tablet dosage forms with differences in sensitivity, selectivity, analytical range and run times.

Table 2.2: Validation parameters of some methods for quantification of nevirapine

Method	**LLOQ** (ng/mL)	**Precision** (%)	**Accuracy** (%)	**Reference**
LC-MS	1	<14	95-114	Ren *et al.*, 2010
GC-MS	10	2-8	96-109	Vogel *et al.*, 2010
HPLC-Diode array	50	1-9	90-99	Kabra *et al.*, 2009
HPLC-UV	50	3	97-99	Hamrapurkar *et al.*, 2010

LLOQ – Lowest limit of quantification; **LC**-Liquid chromatography; **MS**-mass spectrometer detection; **GC**-Gas chromatography; **HPLC**-High performance liquid chromatography; **UV**-ultraviolet light

Liquid chromatography with mass spectrophotometric detection (LC-MS) is the gold standard for nevirapine bioanalysis. However, LC-MS instrumentation may be too costly for resource-limited settings. HPLC-UV gives comparable results to LC-MS method and because of its low cost; it is the most widely used technique. HPLC-UV methods may have high lowest limit of quantification (LLOQ) resulting in low sensitivity (Ren *et al.*, 2010).

Simultaneous determination of plasma levels of different antiretroviral drugs using HPLC-UV methods has been reported. Concurrent quantification is efficient and advantageous as one can analyze several drugs at once (Fan *et al.*, 2002). The challenge is obtaining a suitable mobile phase to achieve good separation and with no interferences by other drugs in the matrix in a short period of time.

Varying chromatographic conditions are used in HPLC-UV/VIS analysis. The conditions depends on the cost, samples to be analyzed, available materials and reagents. Reported methods in literature and pharmacopoeias for quantifying nevirapine may be adopted or optimized for analysis of nevirapine in tablet form and/or plasma. A summary of some methods for quantification of nevirapine using HPLC-UV with flow rates between 0.8 to 1.3 mL/min and injection volumes of 20 to 90 µL is presented in **Table 2.3**.

Table 2.3: Examples of HPLC-UV methods for quantification of nevirapine

Column length (C_{18}, 4.6 mm ID)	**Mobile Phase (% v/v)**	**Wavelength (nm)**	**Reference**
150 mm	MeOH: PB (70:30)	227	Ranaware *et al.*, 2012
	A=NH_4 Ace (pH 4.5), B=MeOH (gradient elution)	270	International Pharmacopoeia, 2015
	NH_4 Ace: MeOH (Gradient and isocratic)	265	Anjali *et al.*, 2012
250 mm	PB: ACN (76: 24)	282	Minzi *et al.*, 2010
	PB: ACN (65:35)	283	Hamrapurkar *et al.*, 2010
	MeOH: ACN: H_2O (50:30:20)	225	Ravisankar *et al.*, 2013
	PB: ACN (45:55)	270	Kumar *et al.*, 2010

ID-internal diameter, **ACN**-Acetonitrile, **H_2O**-Water, **NH_4** Ace-Ammonium acetate, **PB**-phosphate buffer, **MeOH**-methanol.

The chromatographic conditions during method optimization are guided by several factors that include: lowest concentration of analyte to be analyzed; expected range of concentrations to be analyzed; number of samples to be assayed hence expected run time; quantity of the sample hence the injection volume; time taken for sample work up; cost of reagents; and available resources.

2.8 Studies done in Kenya on nevirapine plasma levels

The influence of CYP2B6 516G>T and 983T>C genotypes on nevirapine plasma levels in the Kenyan population has been evaluated in two studies (Oluka, 2012; Oluka *et al.*, 2015). These two studies found a relationship between CYP2B6 genotypes and nevirapine plasma levels.

Oluka, (2012) carried out a study in 110 participants, composed mainly of central highlands Bantus, and evaluated the effect of CYP2B6 516G>T genotype on variability of nevirapine levels at six months of treatment. The study reported a strong correlation between CYP2B6 genotypes and nevirapine plasma levels. Use of social drugs was reported to affect nevirapine plasma levels while age and weight had no influence. The study also found no association between nevirapine plasma levels with toxicity and change in CD4 levels (Oluka, 2012).

Oluka *et al.,* (2015) assessed the influence of CYP2B6 516G>T and 983T>C genotypes on nevirapine plasma levels in 66 women, composed mainly of coastal Bantus, at 12 weeks of treatment. The study assessed the effect of CYP2B6 genotypes on nevirapine plasma levels and subsequently on CD4 cell count and viral load. Oluka *et al.,* (2015) found a strong correlation between CYP2B6 genotypes and nevirapine plasma levels. Nevirapine plasma levels predicted change in CD4 cell count and immunological response (Oluka *et al.*, 2015).

2.9 Study justification

Patients undergoing HAART may fail to achieve adequate immune response or experience adverse drug reactions depending on nevirapine plasma levels. Determining factors that influence nevirapine plasma levels such as CYP2B6 genotypes and adherence may enhance the therapeutic benefits of this drug. Establishing an association between predictors and nevirapine plasma levels may be useful in therapeutic drug monitoring. Determining predictors such CYP2B6 genotypes is costly and thus this study considers alternatives of genotyping where a correlation between genotype and nevirapine plasma levels is established. Use of steady-state nevirapine plasma levels may be an alternative to CYP2B6 genotyping in resource-limited settings in therapeutic drug monitoring.

Pharmacokinetic studies place great emphasis on timed plasma samples. However, it is not always possible to obtain with accuracy, the duration after drug administration a sample was taken. This study evaluated the utility of randomly obtained samples when nevirapine plasma levels had attained steady-state. Samples in this study were taken regardless of knowledge of time the medicines were taken.

Oluka, (2012) and Oluka *et al.*, (2015) gave conflicting association between nevirapine plasma levels and CD4 response thus this study seeks to investigate and clarify this association. This study also evaluates the effect of covariates such as adherence on nevirapine plasma levels. Levels of nevirapine at steady-state were also determined in a large sample of HIV patients.

Patients with HIV usually use nevirapine combined with at least two other antiretroviral drugs mainly nucleoside reverse transcriptase inhibitors such as lamivudine, zidovudine, tenofovir and abacavir. In addition, other medications for treating and/or preventing opportunistic infections such as cotrimoxazole or dapsone are used by HIV patients. The presence of co-medications, metabolites and other compounds in plasma calls for highly selective methods for the quantification of nevirapine in plasma. Therefore, existing HPLC-UV methods can be improved and used to analyze nevirapine plasma levels in resource-limited settings. Improved HPLC-UV methods are likely to be invaluable in pharmacokinetics and bioequivalence studies.

CHAPTER THREE

OPTIMIZATION AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF NEVIRAPINE IN PLASMA

3.1 Introduction

Nevirapine plasma levels are determined using chromatographic methods such as gas and liquid chromatography utilizing different modes of detection. HPLC-UV/VIS systems are available in most analytical laboratories and are preferred in resource-limited settings (Hamrapurkar *et al.*, 2010). Available HPLC-UV methods for determination of Nevirapine in plasma may be improved to be utilized in resource limited settings. This study aimed at optimizing and validating a simple and selective HPLC-UV method for quantifying Nevirapine in plasma.

The initial chromatographic conditions for this study were an isocratic mobile phase consisting of 15 mM potassium dihydrogen orthophosphate (pH=5.0) and acetonitrile (45:55% v/v) at a flow rate of 1 mL/min. The stationary phase was reversed phase octadecylsilane (C_{18}) packed in a column measuring 250 mm in length, 4.6 mm internal diameter, 5 µm particle size and 110 Å pore size set at a temperature of 23ºC. The ultraviolet (UV) detection wavelength was set at 270 nm while the injection volume was 20 µL with carbamazepine as the internal standard in the analysis (Kumar *et al.*, 2010).

Poor separation and co-elution of nevirapine with compounds in plasma occurred when the initial chromatographic conditions were used. To improve efficiency in separation of nevirapine, the chromatographic conditions were optimized. To obtain a reliable method for determining nevirapine plasma levels, the optimized HPLC-UV method was validated using the FDA, 2001 guideline. This study optimized a HPLC-UV method and validated it for subsequently determination of nevirapine plasma levels in HIV patients.

3.2 Materials and methods

HPLC analysis was performed at the National Quality Control Laboratory (NQCL), Nairobi, Kenya. Preparation of the samples and extraction by protein precipitation was done at the University

of Nairobi - African Institute of Biomedical Science Technology (UoN-AiBST) Laboratory in the Department of Pharmacology and Pharmacognosy, School of Pharmacy, University of Nairobi.

3.2.1 Instrumentation

A Merck Hitachi LaChrom HPLC system (Hitachi Ltd, Tokyo, Japan) was used for analysis and comprised of the following components: an L-7100 quaternary low pressure gradient pump; an L-7400 variable wavelength UV/Vis detector; an L-7200 variable injection volume autosampler; and an L-7350 thermostatic column oven. The above components are supported by a D-7000 software interface module. Instrument control and data processing were performed on an IBM compatible Windows based desktop personal computer running a HPLC System Manager (HSM) software version 4.1 (Merck KGaA, Darmstadt, Germany and Hitachi Instruments Inc., San Jose, USA) coupled to the HPLC system interface module.

The mobile phase solutions were degassed for 20 minutes by sonication using a DC 200h MRC ultrasonic water bath (MRC Ltd, UK) sonicator. The sonicator was also used to aid dissolution of the drugs and mixing of solutions. A 3540 Jenway pH meter (Bibby Scientific Ltd, Stone, Staffordshire, UK) was used to adjust pH of buffer solutions.

Spiked plasma samples were mixed by vortexing for 20 seconds using an SN 70918146 Denley Vibromix (Thermo Electron Corporation, UK) vortex mixer. Precipitated plasma samples were centrifuged using a D-37520 Osterode Biofuge *pico* (Heraeus Instruments, Germany) centrifuge.

An AUW220D Shimadzu analytical balance (Shimadzu Corporation, Japan) was used to weigh working chemical reference standards and salts for preparation of mobile phase buffers.

3.2.2 Reagents and solvents

Analytical grade potassium dihydrogen orthophosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and ammonium acetate (Merck Chemicals Pty Ltd., Gauteng, South Africa), were used to prepare buffers during method optimization. HPLC grade methanol and acetonitrile (Rankem, Avantor Performance Materials Ltd, India) were used for preparation of the mobile phase and solubilization of organic compounds. Aqueous solutions were prepared using ultra-purified water prepared in house by consecutive reverse osmosis and ultra-filtration on an Arium 61316 Water System (Sartorius AG Göttingen, Germany). Frozen pooled plasma was obtained from the National Blood Transfusion Services, Nairobi.

Working chemical reference standards of nevirapine, zidovudine, lamivudine, stavudine, tenofovir, efavirenz, trimethoprim, sulphamethoxazole and carbamazepine were donated by the NQCL, Kenya and Universal Corporation Limited. The reference standards were stored under refrigeration (2-8°C) and protected from light.

3.2.3 Preparation of standard and buffer solutions

3.2.3.1 Calibration standard solutions

A stock solution of 1 mg/mL of nevirapine was prepared by dissolving 20 mg of nevirapine in 20 mL mixture of methanol and water (50:50% v/v). This stock solution was stored at -20°C and subsequently used to prepare the working calibration standards at different concentrations. The working calibration standards were used to prepare spiked plasma samples summarized in **Table 3.1.** Spiked plasma samples were prepared by adding 40 µL of the working standars to 360 µL of plasma (10 fold dilution).

Table 3.1: Working calibration standards and spiked nevirapine plasma samples

Working calibration standards (µg/mL)	Spiked plasma samples (µg/mL)	Working calibration standards (µg/mL)	Spiked plasma samples (µg/mL)
5	0.5	75	7.5
7.5	0.75	100	10.0
10	1.0	150	15.0
25	2.5	200	20.0
50	5.0	250	25.0

3.2.3.2 Internal standard solutions

Carbamazepine (**Figure 3.1**) was used as the internal standard (IS) owing to its chemical similarity to nevirapine in terms of structure, physicochemical properties and chemical stability. A fixed concentration of 5 µg/mL carbamazepine was added to all samples that were analyzed. The IS peak area was used to diagnose problems during sample preparation and analysis (Kappelhoff *et al.*, 2003).

A stock solution of 1 mg/mL carbamazepine was prepared in a methanol: water mixture (50:50% v/v) and stored at -20°C. This stock solution was subsequently used to prepare a working solution of 5 µg/mL by diluting 1 ml to 200 mL using acetonitrile.

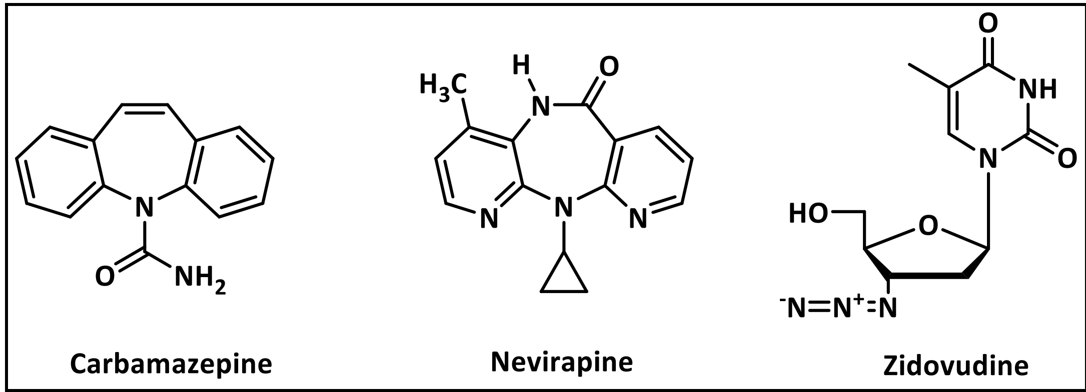


Figure 3.1: Chemical structures of nevirapine, zidovudine and carbamazepine

3.2.3.3 Quality control standard solutions

The quality control (QC) standards of nevirapine were used for validation of the method (FDA, 2001). Three different concentrations were prepared. The first concentration was 3 times the lowest limit of quantification (LLOQ) and designated the low QC (LQC) standard. The second concentration was at the mid-range of the calibration curve and was termed the middle QC (MQC) standard. The third concentration was at the high end of the calibration curve and it was designated high QC (HQC) standard.

A 1 mg/mL nevirapine stock solution was prepared in methanol: water (50:50% v/v) mixture and subsequently used to prepare working QC standards. The working solutions were used to prepare spiked plasma samples. The concentrations of the QC working standard solutions and spiked plasma samples are summarized in **Table 3.2**.

Table 3.2: Quality control working standards and spiked nevirapine plasma samples

QC label	QC working standards (µg/mL)	Spiked plasma QC samples (µg/mL)
LLOQ	5	0.5
LQC	15	1.5
MQC	125	12.5
HQC	225	22.5

3.2.3.4 Buffer solutions

The salts used to prepare buffers solutions investigated during optimization of the HPLC-UV method were; potassium dihydrogen orthophosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and ammonium acetate. Concentrations of the salts in the buffers were: 15 mM and 20 mM of KH_2PO_4; 20 mM K_2HPO_4 and 20 mM ammonium acetate. The buffers were prepared by accurately weighing the respective salts and dissolving in HPLC grade water.

3.2.4 Sample work-up by protein precipitation

Blood samples were centrifuged at 5000xg for 5 minutes and the plasma extracted and refrigerated at -20ºC. The plasma was thawed thawed immediately before use. Acetonitrile containing 5 µg/mL carbamazepine was added to all plasma samples in the ratio of 1:2 (plasma: acetonitrile) and vortexed for about 20 seconds to precipitate the proteins. The mixture was then stored for 10 minutes at -20ºC to enhance precipitation and then centrifuged at 10000xg for 6 minutes. The supernatant was then withdrawn for analysis. **Figure 3.2** summarizes the protein precipitation process.

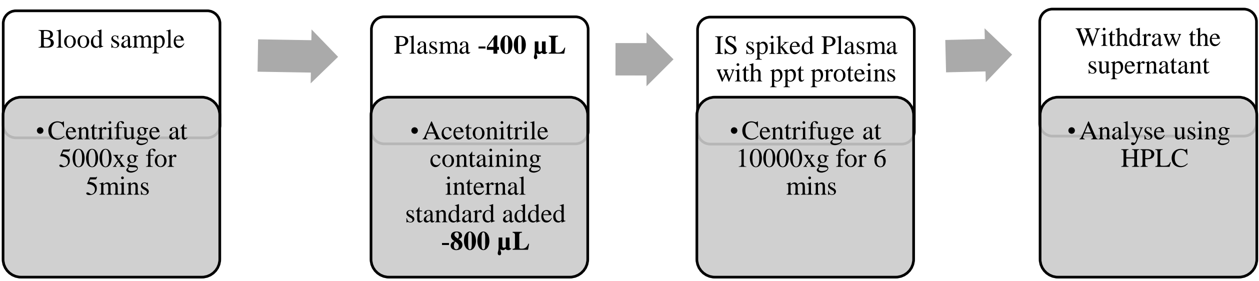


Figure 3.2: Plasma sample preparation and extraction process

3.2.5 Chromatographic conditions

Optimization of the HPLC-UV method aimed at obtaining a suitable analytical approach to determine nevirapine in plasma. The method reported by Kumar *et al.,* (2010) HPLC-UV method for simultaneous determination of zidovudine, lamivudine and nevirapine with a short run time of six minutes was used as the starting point. A sample containing 10 µg/mL of nevirapine, zidovudine and lamivudine was used in evaluation of mobile phases.

The maximum flow rate during optimization of the HPLC-UV method was 1.5 mL/minute to maintain column back pressure at less than the set upper limit of 250 bar. Long analyses with back

pressures above 250 bar would have damaged the column and compromised its integrity. At the beginning of HPLC-UV method optimization, the flow rate of the mobile phase was fixed at 1.0 mL/min and the column temperature was set at 40ºC. The flow rate was assessed at 0.8, 1.0 and 1.2 mL/min. The following injection volumes were evaluated at 0.5, 5 and 10 µg/ml nevirapine and carbamazepine with the other parameters being fixed: 10, 20, 30, 40, 50, 70 and 100 µL.

3.2.5.1 Selection of column and detection wavelength

A HyperClone® BDS C_{18} column (150 mm x 4.6 mm ID, 5 µ) (Phenomenex, Torrance, California, USA) was selected as the preferred stationary phase for the method owing to its stability over a wide pH range (pH 1-9). The column was silica based and expected to be more efficient in separation in addition to giving better peak shapes compared to synthetic polymer based columns.

Wavelengths used in literature were assessed with other chromatographic conditions being fixed and included: 246, 254, 265, 270, 275 and 282 nm. The peak areas were used to select the optimum detection wavelength for zidovudine, lamivudine, nevirapine and carbamazepine.

3.2.5.2 Mobile phase composition

The International Pharmacopoeia method for determination of nevirapine, lamivudine and zidovudine in fixed dose combination tablets and several other methods in literature for simultaneous determination of nevirapine, zidovudine and lamivudine in dosage forms and plasma were evaluated in coming up with an optimized method for the analysis.

Mobile phases consisting of different solvents and buffers were assessed to obtain one with the best separation and resolution. Solvents and their mixtures used included water, acetonitrile, methanol and phosphate buffer. Proportions used in the separation consisted of a maximum of three solvents. Selected mobile phases evaluated are presented in **Table 3.3**. Gradient elution was done by varying composition of the mobile phase at different run times.

Table 3.3: Mobile phases for optimization of the HPLC-UV method

Mobile Phase Code	Percentage (%) composition		
	PB	ACN	MeOH
A	45	55	-
B	55	30	15
C	65	20	15
D	67	33	-
E	70	30	-
F	75	25	-
G	80	20	-
H	85	15	-
I	90	10	-

PB-phosphate buffer, **ACN-**acetonitrile, **MeOH**-methanol

3.2.5.3 Effect of the inorganic aqueous buffer and organic modifiers

The effect of the inorganic aqueous buffer on separation of nevirapine was tested and compared with unbuffered mobile phase. Potassium dihydrogen orthophosphate (KH_2PO_4) buffer which is used extensively on reverse phase liquid chromatography with wide-ranging buffering capacity (pH 3-13) was chosen. Low concentrations of the buffer were used to minimize its precipitation in mixtures with organic solvents.

The effect of low pH (4.5) was assessed using 20 mM potassium dihydrogen phosphate (KH_2PO_4) while 20 mM dipotassium hydrogen phosphate (K_2HPO_4) was used to evaluate the influence of high pH (8.4). Varying proportions of 20 mM KH_2PO_4 and 20 mM K_2HPO_4 were used to evaluate intermediate pH ranges (5.0, 6.8). Ammonium acetate (20 mM), an alternative buffer was investigated to improve separation at pH 5.0.

The effect of organic modifier concentration was evaluated by checking the influence of methanol and acetonitrile on the retention times at a fixed pH and buffer concentration. The effect of different proportions of methanol and acetonitrile on separation was assessed.

3.2.6 Validation of the HPLC-UV method

The optimized HPLC-UV bioanalytical method was validated according to the FDA guidelines (FDA, 2001). The following parameters were assessed during validation of the HPLC-UV method: linearity of the calibration curve; sensitivity; accuracy; precision; selectivity; recovery; carryover; and sample stability.

3.2.6.1 Linearity and criteria for acceptance of the calibration curve

The calibration curve was obtained by plotting the nominal concentrations of nevirapine against the ratio of the analyte and internal standard peak areas. The calibration standards (CS) with known concentrations of nevirapine were added to blank plasma. The concentrations of the spiked plasma samples were: 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10, 15, 20 and 25 µg/mL. Six replicates were used to determine all calibration standards. The relative deviation from the nominal concentration and coefficient of variation at each concentration was determined.

The concentration of the calibration standards were back calculated based on simple linear regression. For the standard curve to be acceptable, 75% of the back calculated concentrations of the calibration curve should fall within ± 15% of the nominal value except at LLOQ where an upper limit of 20% was considered acceptable.

Residuals were generated and subjected to model diagnostic tests after linear regression. Diagnostic tests examined whether assumptions that underlie linear regression are violated. The core assumption asserts that the residuals should be homoscedastic and normally distributed with a mean of zero. Homoscedasticity implies that the variance of the residuals is constant and should not be dependent on the independent variable nor show any pattern. Homoscedasticity was checked by plotting the residuals against the nominal levels of nevirapine in spiked plasma samples used to generate the calibration curves.

3.2.6.2 Sensitivity

Two fold dilutions were made at the lowest concentration of the calibration curve to determine lowest limit of quantification (LLOQ) which is a measure of sensitivity. The concentrations of nevirapine used to determine LLOQ were 0.005, 0.01, 0.025 and 0.05 µg/mL. The ratio of the test and internal standard peak areas was obtained from chromatograms of these concentrations. The accuracy and coefficient of variation for each concentration was determined.

The lowest concentration on the calibration curve in which the precision was less than 20% with a relative deviation from the nominal concentration of 20% was considered as the LLOQ. The concentration where the peak of the analyte was distinguishable from that of the blank matrix and had a measurable coefficient of variation was the lowest limit of detection (LOD).

3.2.6.3 Accuracy and precision

Accuracy was determined by the percentage relative deviation of the determined concentrations from nominal levels. Six replicate samples containing known amounts of nevirapine QC concentrations, the LLOQ, LQC, MQC and HQC were assessed and the mean value of each was calculated. The deviation from the nominal values was acceptable if it was less than 15%, and less than 20% for the LLOQ.

Precision was determined by the intra- and inter- day coefficient of variation that was used to assess reproducibility and repeatability of the analytical method. Univariate ANOVA was used to determine variance for both intra- and inter- day precision. The coefficient of variation of less than 15% was considered acceptable except for LLOQ where less than 20% was acceptable.

3.2.6.4 Selectivity

Selectivity of the method to the test analyte and internal standard was determined in the presence of interfering endogenous and exogenous substances found in plasma. Exogenous substances found in plasma include drugs that are commonly used by HIV/AIDS patients. The effect of endogenous substances found in plasma was evaluated by analyzing six pooled blank plasma matrix samples and plasma spiked with known concentration of nevirapine at LLOQ. The selectivity of the method in the presence of interferences was calculated as follows.

$$\text{Selectivity} = \frac{\text{Peak area of the interference at the retention time of NVP/internal standard}}{\text{Mean peak area of NVP at LLOQ/internal standard}}$$

Acceptance criteria was set at a maximum of 20% interference of the area at the retention time compared to the area at LLOQ.

Interference by drugs used by HIV/AIDS patients was evaluated by spiking plasma with the various drugs and determining the peak areas at the retention time of nevirapine or carbamazepine. The peak areas at the retention time were compared with the area of the analyte at LLOQ. The drugs used to assess selectivity included 10 µg/mL nevirapine, zidovudine, lamivudine, stavudine,

tenofovir, sulphamethoxazole, trimethoprim and carbamazepine (IS). The acceptance criterion was set at ≤ 20% interference of the area at the retention time compared to the area at LLOQ.

3.2.6.5 Recovery and carryover effect

Recovery was evaluated to determine the efficiency of extraction of the analyte from the plasma matrix, that is, on the QC samples at LLOQ, LQC, MQC and HQC concentrations. The peak area ratio of the analyte and internal standard of extracted spiked plasma samples and of unextracted QC samples (diluent used instead of plasma) were compared. The unextracted QC samples were prepared by adding the working QC standards to a mixture of water: methanol (50:50%) rather than plasma. Recovery was calculated as follows:

$$\text{Percentage recovery} = \frac{\text{Peak area ratio of the extracted spiked plasma samples x 100}}{\text{Peak area ratio of the unextracted QC standards.}}$$

Six replicate determinations were done and a recovery of ≥ 70% was considered acceptable

Carryover was assessed to check the amount of analyte retained in different components of the chromatographic system after each run that would be transferred and detected in subsequent injections. This was determined by extracting three plasma QC samples at HQC. Immediately after running each HQC sample, a blank extracted plasma sample was run. The carryover was then calculated as the percentage of the peak area at the retention time of the analyte compared to the mean peak area at LLOQ as follows.

$$\text{Carry over (\%)} = \frac{\text{Peak at retention time of NVP in blank plasma}}{\text{Mean peak area of nevirapine at LLOQ}}$$

Six replicate determinations were done. The criteria for acceptance was a response ≤20% at LLOQ and ≤2% of area at the retention time of internal standard. If the response due to sample left in the needle was ≥20% of the drug response at LLOQ, then the carryover was not to be greater than 1% of HQC.

3.2.6.6 Stability

Short term (bench top, room temperature), freeze-thaw and long term stability were determined. QC standards were aliquoted and analyzed before and after stability testing to determine the change in concentration (conc). The percentage change was calculated as follows:

$$\text{Percentage change} = \frac{\text{Conc (comparison sample) - Conc (stability sample) x 100}}{\text{Conc (comparison sample)}}$$

Bench top/short term stability was determined by preparing 3 aliquots of the QC standards. These were frozen at -20°C for 24 hours then thawed and left at room temperature for 4-24 hours followed by analysis.

Freeze- thaw stability was determined using aliquots of the QC standards that were frozen and thawed for 3 cycles. Each cycle consisted of freezing for 24 hours then storage at room temperature until thawing had taken place, then refreezing for 12-24 hours. The concentrations were then determined after the three cycles.

Long term stability was determined by preparing 3 aliquots of LQC and HQC standard samples. The first aliquots were analyzed two days before starting method validation. The second aliquots were analyzed after two months during quantification of nevirapine in patient samples. The last aliquot was analyzed at five months after concluding the quantification of nevirapine. The percentage change in the concentration of the QC standards during the stability test was evaluated.

3.2.7 Quality assurance

All data obtained during method validation were entered in an Excel spreadsheet and double checked by the investigator during data entry. The validation was done in accordance with set protocols (FDA, 2001). Any deviation from the standards and protocols was recorded, reviewed and those that affected validity of the study were documented.

3.2.8 Statistical analysis

The data obtained during method validation were analyzed using Microsoft® Office Excel 2013 for Windows. The peak area ratios of the analyte and internal standard were used in the analysis unless absolute unmodified peak areas were specified for analysis.

The calibration curves were obtained by plotting the ratio of peak areas of nevirapine and carbamazepine (IS) against the nominal concentration. The data was fitted using simple linear regression. The linear model with a coefficient of determination (R^2) greater than 0.99 was considered acceptable. Unknown concentrations were interpolated from the calibration curve. Residuals generated during fitting of the calibration curves were plotted against nominal nevirapine concentrations and their distribution evaluated for homoscedasticity using Microsoft® Office Excel 2013.

Precision was determined using the coefficient of variation. The e intra-and inter-day variance in in the determined nevirapine concentrations of QC samples was assessed using univariate ANOVA performed in STATA version 10. Accuracy was measuring by the calculated relative deviation of the analyte from the nominal concentration. Selectivity, recovery, carryover and stability were also determined.

3.3 Results

The results of this study are reported in two parts: HPLC-UV method optimization of chromatographic conditions; and HPLC-UV method validation.

3.3.1 HPLC-UV method optimization of chromatographic conditions

3.3.1.1 Mobile phase

Gradient elution of nevirapine was found to be optimum. The mobile phase consisted of two solutions (A and B) that were a mixture of 20 mM KH_2PO_4 buffer (PB) pH 4.5±0.05 and acetonitrile (ACN) in different proportions. Solution A had PB: ACN (85: 15 % v/v) and solution B had PB: ACN (67:33 % v/v). Gradient elution with a run time of 11.0 minutes was performed as presented in **Table 3.4**.

Table 3.4: Mobile phase composition for gradient elution by the HPLC-UV method

Time (minutes)	**Solution (%)**	
	A	**B**
0.0	100	0
3.5	100	0
4.4	0	100
9.0	0	100
9.2	100	0
11.0	100	0

Solution A- KH_2PO_4 buffer: Acetonitrile (85: 15 % v/v);
Solution B- KH_2PO_4 buffer: Acetonitrile (67:33 % v/v)

The optimum buffer was 20 mM KH_2PO_4 with unadjusted pH of 4.5±0.5. The elution sequence was not significantly affected by pH and there was no need of pH adjustment during buffer preparation. Assessment of 20 mM ammonium acetate alternative buffer resulted in a negligible difference in elution sequence and separation.

3.3.1.2 Wavelength and injection volume

The optimum detection wavelength was 270 nm. This wavelength gave high absorbance with large peak areas for zidovudine and nevirapine with fair intensity for carbamazepine (Kumar *et al.*, 2010).

The optimum injection volume was 30 µL. Peak broadening was observed especially for carbamazepine at injection volume of more than 40 µL at concentrations above 10 µg/mL. Low injection volumes resulted in less drug reaching the detector hence difficulty in analyzing low concentrations of nevirapine at concentrations below 0.5 µg/mL.

3.3.1.3 Flow rate and temperature

The optimum flow rate was 1.2 mL/min. High flow rates resulted in higher back pressures that would have greatly reduced column lifespan and were avoided while low flow rates led to longer run times. The optimum column temperature was 40^oC. Temperatures below 30^oC resulted in increased drift and noise affecting the sensitivity of the method. Temperatures greater than 40^oC did not improve separation. A summary of optimized chromatographic conditions is presented in **Table 3.5.**

Table 3.5: Chromatographic conditions for the quantification of nevirapine

Parameter	Optimized chromatographic conditions		
Column	HyperClone® BDS C_{18} (150 mm x 4.6 mm ID, 5 µ)		
UV Detection Wavelength	270 nm		
Temperature	40ºC		
Flow Rate	1.2 mL/min		
Injection Volume	30 µL		
Inter-run wash	Once after every run		
Run Time	11 minutes		
Mobile Phase	Gradient elution (PB: ACN)		
	Time (minutes)	PB: ACN (85: 15% v/v) %	PB: ACN (67: 33% v/v) %
	0.0	100	0
	3.5	100	0
	4.4	0	100
	9.0	0	100
	9.2	100	0
	11.0	100	0

PB-20 mM potassium dihydrogen orthophosphate buffer pH 4.5±0.5; **ACN**-Acetonitrile; **ID**-Internal Diameter

The separation and resolution of nevirapine and carbamazepine in spiked plasma samples under optimized chromatographic conditions is illustrated in **Figure 3.3.**

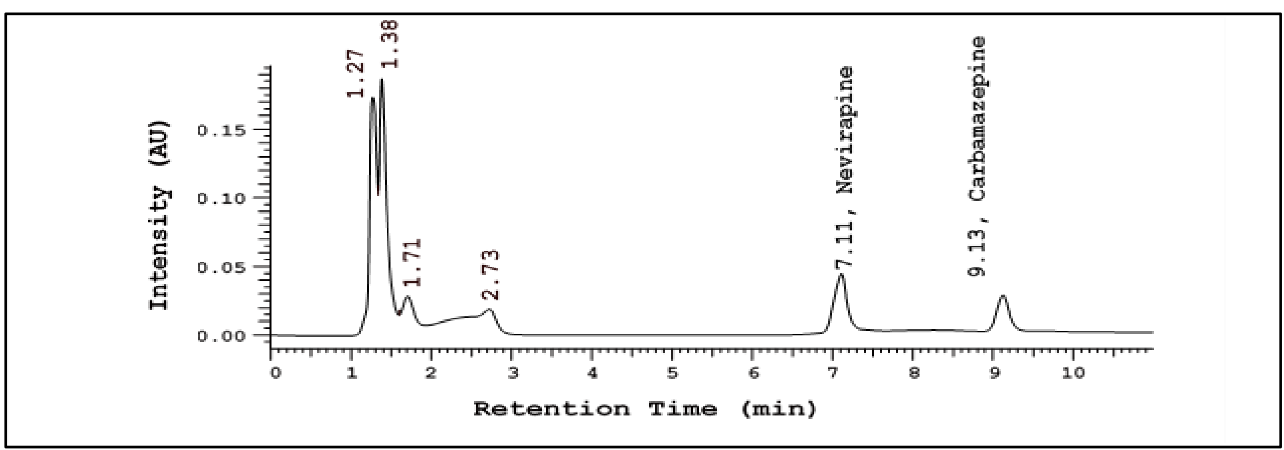


Figure 3.3: Typical chromatogram of nevirapine and carbamazepine in plasma (10.0 and 5.0 µg/mL respectively)

The main challenge encountered in optimizing the analytical HPLC-UV method was the poor resolution between sulphamethoxazole and nevirapine. The gradient elution was the best approach in resolving sulphamethoxazole, nevirapine and carbamazepine. Some of the plasma samples contained high levels of sulphamethoxazole which eluted as a broad peak as illustrated in the chromatogram in **Figure 3.4**.

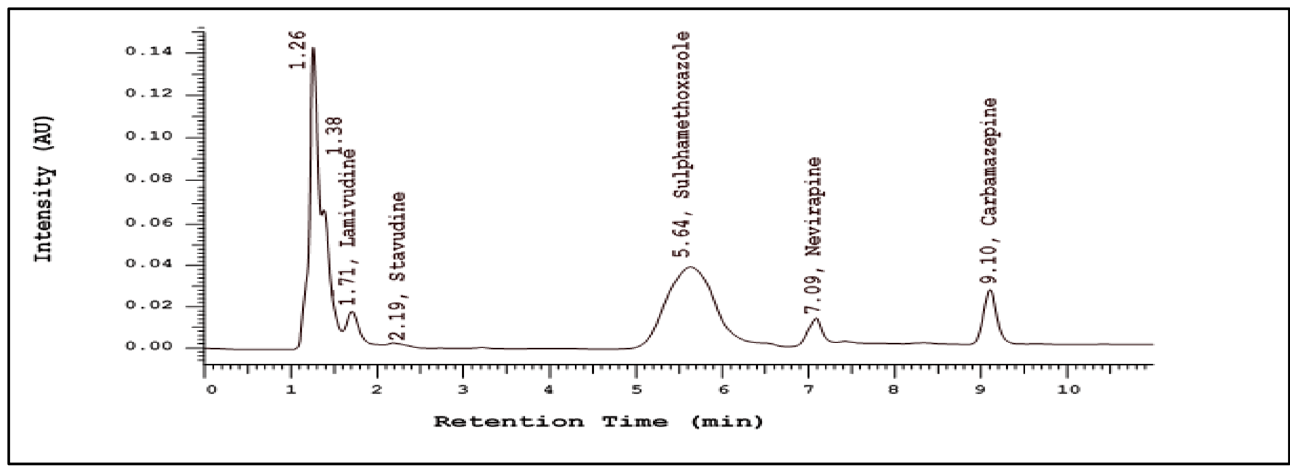
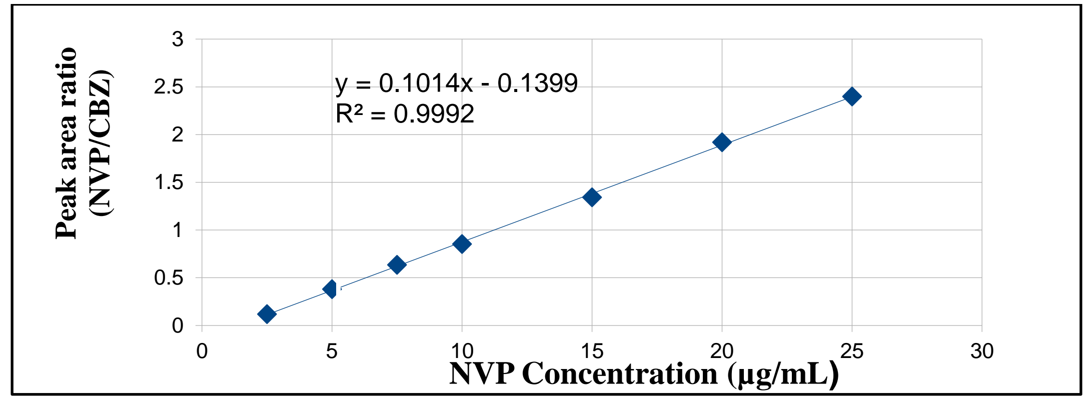


Figure 3.4: Typical chromatogram of plasma samples of patients on sulphamethoxazole

3.3.2 HPLC-UV method validation

3.3.2.1 Calibration curve and linearity

The simple regression equation was y= 0.1014x – 0.1399 with a coefficient of determination of 0.9992 (R^2>0.99). This model (**Figure 3.5**) gave a low accuracy at the low concentrations and was used for high concentrations (**Table 3.6**).

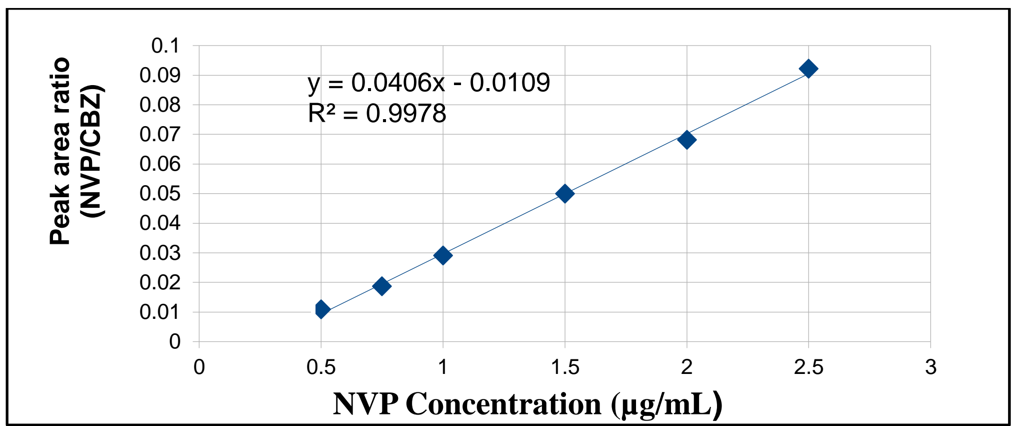


Standard Error (SE) of gradient = 0.0012 (1.23%), SE of the Y intercept = 0.0179 (-12.79%)

NVP-nevirapine; **CBZ**-carbamazepine

Figure 3.5: Calibration curve for high concentrations of nevirapine (2.5-25 µg/mL)

A second model (**Figure 3.6**) was generated by dividing the data set into two: low concentrations of below 2.5 µg/mL and high concentrations above 2.5 µg/mL. Two separate simple linear regression models were generated each with its own gradient and coefficient of determination. The second model gave better accuracy for the low concentrations (**Table 3.6**). Hence the second model with a simple regression equation y= 0.0406x – 0.0109 and a coefficient of determination of 0.9978 (≥0.99) was used for determining low concentrations.



Standard Error (SE) of gradient = 0.0009 (2.33%), SE of the Y intercept = 0.0015 (-13.36%); **NVP**-nevirapine; **CBZ**-carbamazepine

Figure 3.6: Calibration curve for low concentrations of nevirapine (0.5-2.5 µg/mL)

The relative deviation using the two simple linear regression modes at low and high nevirapine plasma levels is shown in **Table 3.6**.

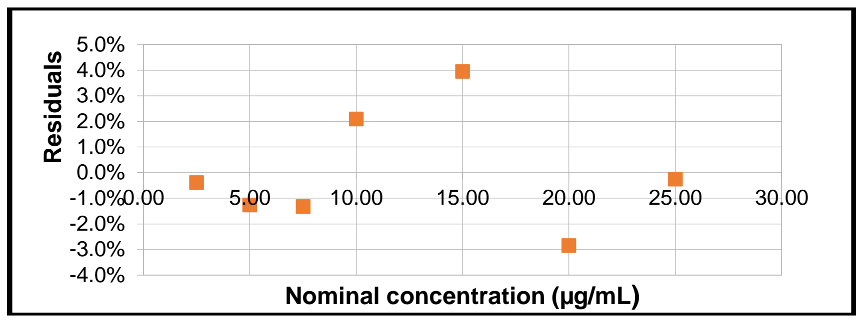
Table 3.6: Comparison of accuracy at low and high concentrations

Nominal Conc	**Interpolated Conc (µg/mL) and Relative deviation (% RD)**			
	A		**B**	
	Conc	**RD**	**Conc**	**RD**
0.5068	0.5387	6.30	1.4867	193.39
0.7601	0.7306	-3.88	1.5635	105.69
1.0135	0.9869	-2.62	1.6660	64.38
1.5203	1.5015	-1.23	1.8718	23.13
2.0270	1.9495	-3.82	2.0510	1.18
2.5338	2.5428	0.36	2.2883	-9.69
5.0675	9.6325	90.08	5.1237	1.11

Conc: Concentration; **A**-Interpolated levels using low concentration curve (RD) (0.5-2.5 µg/mL); **B**- Determined levels using high concentration curve (RD) (2.5-25 µg/mL)

When the high concentrations curve was used to interpolate the levels of nevirapine in spiked plasma samples, the relative deviation from the nominal concentration was within acceptable ≤15% limit above 2.0 µg/mL. On the other hand, when the low concentrations curve was used to determine concentrations of nevirapine in the spiked plasma samples, the accuracy was within the acceptable ≤15% limit between 0.5-2.5 µg/mL. Therefore, during interpolation of nevirapine plasma levels in the HIV patients, the low concentration curve was used for peak area ratios that gave concentration below 2.25 µg/mL while high concentrations curve was used in those that gave a concentration above 2.25 µg/mL. The optimal regression model was the one with a break point or a point of inflection at 2.5 µg/mL. Unknown concentrations were interpolated from the calibration curves in **Figures 3.5 and 3.6**.

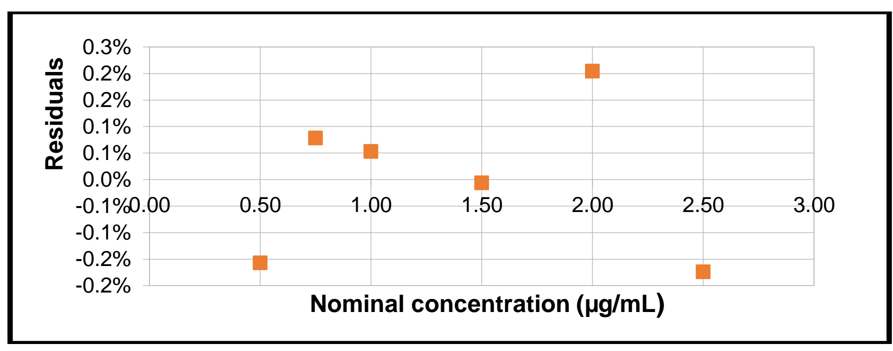
The standard deviation of the residuals was 2.5% and 7.7% for the high and low concentration calibration curves respectively (**Figures 3.7 and 3.8**).



Standard deviation of the residuals = 2.5%

Figure 3.7: Scatter plot of residuals and high nominal concentrations (2.5-25 µg/mL)

The standard deviations of the residuals were within the limit of ± 15%. The residuals were normally distributed. There was no clear pattern between residuals and the nominal concentration and hence there was homoscedasticity.



Standard deviation of the residuals = 7.7%

Figure 3.8: Scatter plot of residuals and low nominal concentrations (0.5-2.5 µg/mL)

3.3.2.2 Accuracy and precision

The accuracy (% relative deviation, RD) of the HPLC-UV method was (mean ± SD) 2.27% ± 3.84 and ranged between -2.14 and 4.89%. Intra-day precision was 4.68% ± 1.18 with a range of 3.78 to 6.02% while inter-day precision was 7.15% ± 4.37 with a range of 4.55 to 12.20%. Accuracy and precision of the HPLC-UV method are summarized in **Table 3.7**. The accuracy and precision were within accepted limits of less than or equal to 15% and less than or equal to 20% for LLOQ.

Table 3.7: Accuracy and precision of the method

Label (n=6)	Nominal Concentration (µg/ml)	Mean observed (µg/ml)	Accuracy (% RD)	Intra-day precision (% CV)	Inter-day precision (% CV)
LLOQ	0.5068	0.5898	16.39	5.31	6.62
LQC	1.5203	1.5945	4.89	4.25	4.55
MQC	12.6688	13.184	4.07	3.78	4.70
HQC	22.8038	22.3151	-2.14	6.02	12.20

3.3.2.3 Sensitivity

The limit of detection (LOD) was 0.25 µg/mL while the lowest limit of quantification (LLOQ) was 0.5 µg/mL as presented in **Table 3.8**. Above the LLOQ, the precision and accuracy were within accepted limits.

Table 3.8: Lowest limit of quantification and limit of detection of nevirapine

Concentration (µg/ml)	Accuracy (% RD), n=6	RSD (%), n=6	Label
0.25	68.15	17.69	**LOD**
0.5	16.39	5.31	**LLOQ**
0.75	6.1	0.79	
1.0	-11.62	5.91	
1.5	4.89	4.25	

RD; relative deviation; **RSD-**relative standard deviation;
LOD- limit of detection; **LLOQ**-lowest limit of quantification

3.3.2.4 Selectivity

There was no interference by endogenous substances found in plasma at the retention time of nevirapine and carbamazepine. **Figure 3.9** shows the chromatogram of blank pooled plasma.

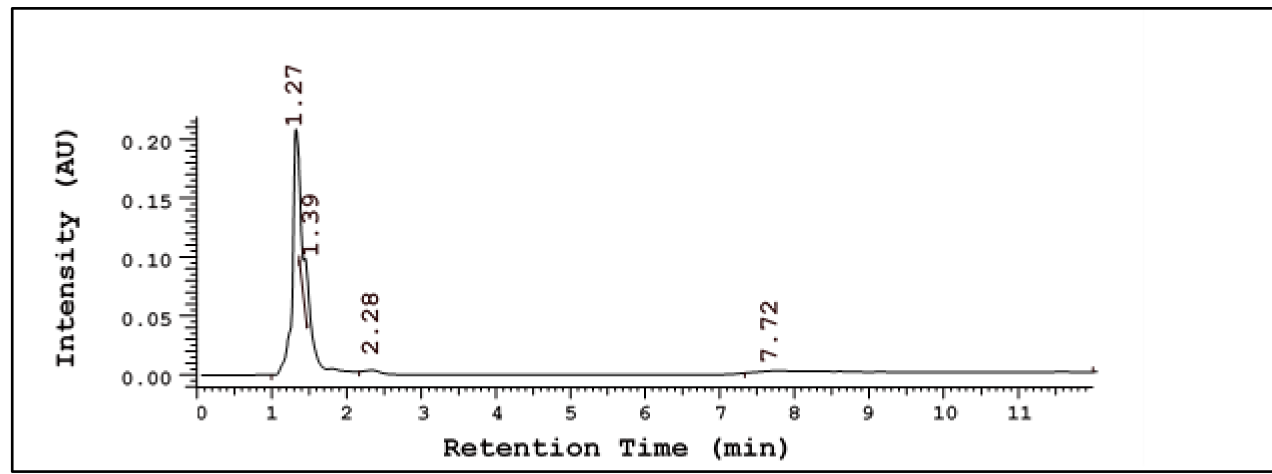


Figure 3.9: Typical chromatogram of pooled plasma

Separation and resolution at LLOQ is illustrated in **Figure 3.10** and shows no interferences by compounds in plasma.

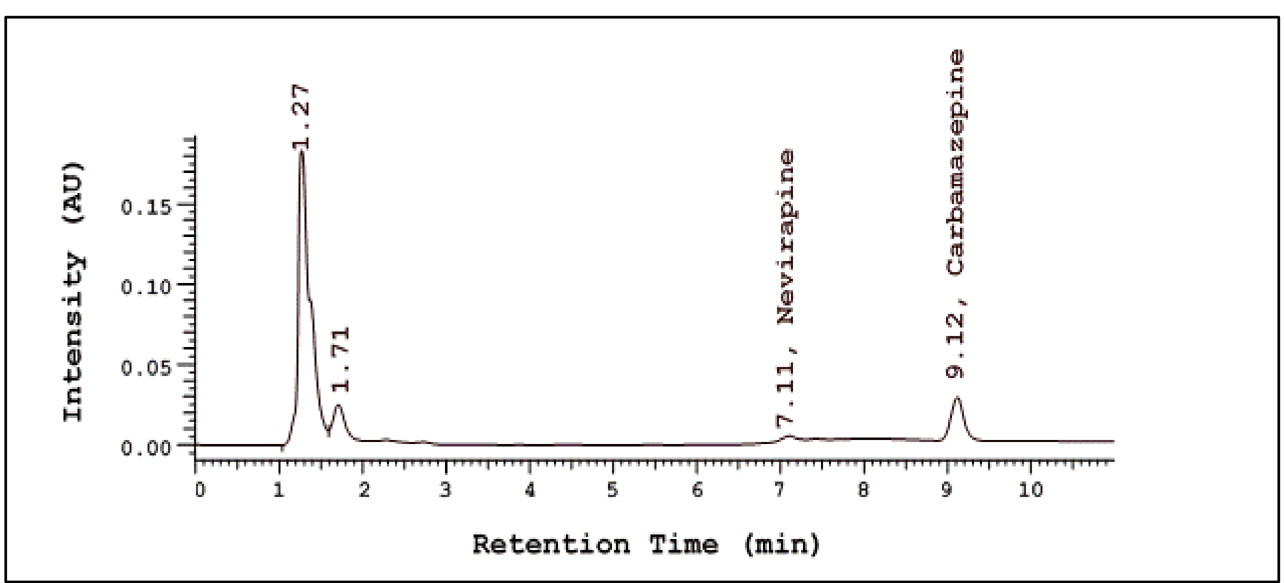


Figure 3.10: Typical chromatogram at LLOQ concentration of nevirapine in plasma

In addition, there was no interference by other drugs at the retention times of nevirapine and carbamazepine. **Figure 3.11** shows the retention times of some drugs used by HIV patients that were analyzed.

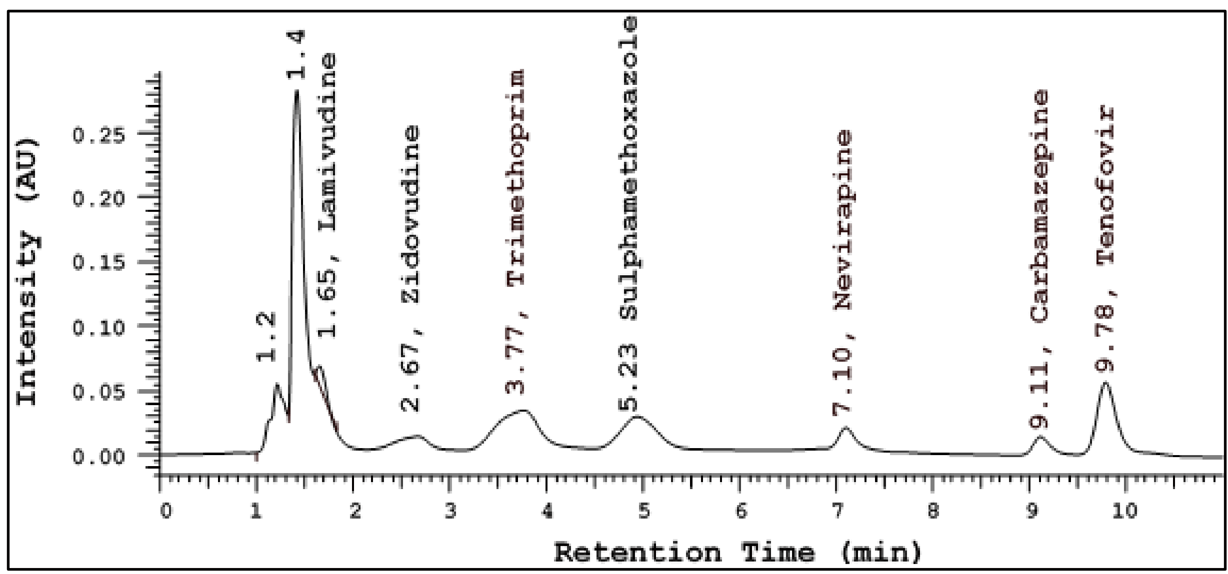


Figure 3.11: Typical chromatogram of some drugs used by HIV patients

The retention times of the drugs were compared to carbamazepine internal standard and relative retention time obtained are in **Table 3.9.**

Table 3.9: Selectivity of some drugs used by HIV patients

Drug	Retention time (minutes)	Relative retention time	Concentration (µg/mL)
Lamivudine	1.63	0.18	10
Stavudine	2.06	0.23	10
Zidovudine	2.67	0.29	10
Trimethoprim	3.77	0.41	10
Sulphamethoxazole	5.23	0.57	10
Nevirapine	7.10	0.78	5
Carbamazepine	9.11	1.00	5
Tenofovir	9.78	1.07	15

3.3.2.5 Recovery and Carryover

The recovery of nevirapine from the plasma matrix was 91.84%±4.12 with a range of 89.19 to 96.57%. The recovery was within the set limit of more than 70% as presented in **Table 3.10**.

Table 3.10: Recovery of nevirapine in plasma samples

Label	Concentration (µg/ml)	Mean % Recovery (n=6)
LLOQ	**0.5**	72.56
LQC	**1.5**	89.75
MQC	**12.5**	89.19
HQC	**22.5**	96.57

The carryover was 0% when a blank plasma sample was run after analyzing samples containing high concentrations of nevirapine.

Stability of nevirapine samples

The results for benchtop, freeze-thaw and long term stability tests are summarized in **Table 3.11**. Nevirapine was stable with minimum loss under different environmental conditions. The mean loss (± SD) during bench top and freeze - thaw stability testing was 5.8%±5.0 and 8.8%±4.0 respectively. Nevirapine (in plasma) was stable when stored at -20ºC for five months with a mean loss (± SD) of 5.9%±0.8.

Table 3.11: Change in concentration during stability testing of nevirapine

Stability (n=6)	Label	Spiked plasma (µg/ml)	Comparison sample (µg/ml)	Stability sample (µg/ml)	Percent change
Bench top	HQC	22.804	24.184	23.936	1.03
	MQC	12.669	14.709	13.673	5.42
	LQC	1.520	1.709	1.521	11.02
Freeze and thaw	HQC	22.804	21.628	19.629	9.24
	MQC	12.669	13.167	11.503	12.64
	LQC	1.520	1.433	1.367	4.62
Long term	HQC	22.804	24.184	22.901	5.29
	LQC	1.520	1.71	1.599	6.46

LQC-Low quality control standard; **MQC**-Middle quality control standard; **HQC**-High quality control standard.

3.4 Discussion

The optimized HPLC-UV method utilizing UV detection had a short run time of 11 minutes. Therefore it was possible to analyze many patient samples. This HPLC-UV method was selective with no observable interference by endogenous substances found in plasma nor by other drugs commonly taken by HIV/AIDS patients. The calibration curve had a point of inflection (break point) at 2.5 µg/mL hence two simple regression models were used to fit the curve. The linearity of the calibration curves was between 2.5 and 25 µg/mL (R^2=0.9992) for high concentrations and 0.5 to 2.5 µg/mL (R^2=0.9978) for low concentrations. The range of concentrations that can be analyzed by this method is 0.5 to 25 µg/mL. This range is wide compared to other methods where higher

concentrations had to be diluted before analysis (Kabra *et al.*, 2009; Minzi *et al.*, 2010). The residuals of both curves were homoscedastic hence a good fitting of the curve.

The sensitivity of the method was good with the lowest limit of quantification of 0.5 µg/mL and limit of detection of 0.25 µg/mL. This sensitivity was comparable with other reported methods for quantifying nevirapine in plasma (Minzi *et al.*, 2010; Oluka *et al.*, 2015; Ranaware *et al.*, 2012).

The recovery of the analyte from plasma was 89.2-96.6% which was above the 70% limit. These recoveries were comparable to other reported methods (Kabra *et al.*, 2009; Oluka *et al.*, 2015). The method was accurate, repeatable and reproducible having an accuracy of 97.9-104.9% with intra-day precision of 3.8-6.0% and inter-day precision of 4.6-12.2%. This precision and accuracy is within set limits and comparable to other methods (Hamrapurkar *et al.*, 2010; Kabra *et al.*, 2009; Oluka *et al.*, 2015).

Nevirapine was stable with benchtop stability of 89.0-99.0%, freeze and thaw stability of 87.4-95.4% and long term stability of 93.5-94.7%. This stability was comparable to other methods (Kappelhoff *et al.*, 2003). This minimal loss due to environmental changes meant that when the plasma was stored for long periods under refrigeration, factors such as power outages or freeze-thaw during transport, were likely to have a minimal effect on nevirapine concentration. The undetectable analyte between subsequent injections meant that there was negligible remnants of the drug after injection therefore minimum variance due to leftovers when samples with high and low nevirapine levels were run consecutively.

3.5 Conclusion

In conclusion, the optimized chromatographic conditions culminated into a HPLC-UV method that was simple and can be used in routine analytical laboratories. The parameters of the optimized HPLC-UV method were within acceptable criteria of the FDA validation guidelines (FDA, 2001).

CHAPTER FOUR

DETERMINATION OF NEVIRAPINE PLASMA LEVELS IN HIV PATIENTS

4.1 Introduction

Nevirapine plasma levels in HIV patients are indicative of the amount of drug reaching the target receptors. The concentration of nevirapine in plasma is associated with the extent of viral suppression. High nevirapine plasma levels have been reported to offer greater viral suppression (Veldkamp *et al.*, 2001). Determining factors that predict nevirapine plasma levels, which may vary in different populations, such as CYP2B6 genotypes could be invaluable in maximizing its therapeutic use. Subsequently, better use of nevirapine may result in improved virological, immunological and clinical outcomes of patients receiving antiretroviral therapy (ART). The study aimed at determining the variability and predictors of nevirapine plasma levels in the study population. Subsequently, the influence of nevirapine plasma levels on clinical outcomes such as maximum CD4 cell counts is described.

4.2 Methods

Nevirapine plasma levels in the study population were determined using the HPLC-UV method described in chapter three. Factors that may influence nevirapine plasma levels were evaluated using bivariable and linear regression analysis. The impact of nevirapine plasma levels on treatment outcomes was assessed.

4.2.1 Study design and site

The study design was a nested single arm cross sectional study. This study was nested in a previous study carried out on patients receiving nevirapine based ART regimens who attended the Comprehensive Care Centre (CCC) at the Kenyatta National Hospital (KNH).

KNH is the largest public teaching and referral hospital in Kenya located at the Upper Hill area. The CCC offers specialized treatment and care for HIV/AIDS patients in addition to being a center for research. The CCC clinic has more than 10000 HIV patients on care and treatment. Nevirapine plasma levels in historical samples were determined using HPLC-UV method at the NQCL. Sample

preparation by protein precipitation was done in the Uon-AiBST laboratory in the Department of Pharmacology and Pharmacognosy.

4.2.2 Study population

The study population consisted of adult HIV positive patients who had been on nevirapine based ART for not less than six months and attended the CCC at KNH in the year 2014.

Inclusion and exclusion criteria

The patients that were included in the study were: HIV infected males and females on ART; aged between 18 and 55 years; on a nevirapine based regimen for 6 months or more; and who gave voluntary informed consent. Patients who were not on nevirapine based regimens; who did not give informed consent; and had not been on HAART for at least six months were excluded from the study.

4.2.3 Sample size, sampling, participant recruitment and data collection

Sample size, sampling, participant recruitment, collection of baseline characteristics, assessment of skin reactions and hepatotoxicity of the study population has been reported by Makori *et al.,* (2015). An estimated minimal sample size of the study was 139 was used. Participants were sampled by convenient sampling method and those who gave informed consent included in the study. A total of 241 participants met the eligibility criteria and their data abstracted. Random blood samples (5 ml) were collected from the participants. The blood samples were collected irrespective of the duration after drug administration as there may be minimum variability in nevirapine plasma levels at steady-state with time after taking the dose (Nellen *et al.*, 2008). The blood samples were subsequently centrifuged at the CCC laboratory to obtain 2 ml blood cells and 3 ml plasma that were stored under refrigeration (-20ºC). The stored historical plasma samples were used in this study for determination of nevirapine plasma levels in the historical cohort.

Data collected in the larger study included marital status, level of education, alcohol use and smoking status. In addition, data on sociodemographic characteristics such as age, sex, height, weight and ethnicity was collected. Baseline liver function tests, renal function tests, history of hepatotoxicity, skin reactions and presence of comorbidities illnesses of the historical cohort had been evaluated. Data on the type of ART regimens the participants were initiated on, switched to and were currently using when the blood samples had been collected. Baseline CD4 cell counts

before patients were initiated on ART and changes in CD4 cell counts during ART treatment until the blood sample was collected for analysis had been evaluated.

The level of adherence had been assessed in two ways: using the CASE adherence tool; and self-reported adherence. The CASE adherence tool had three questions concerning: the number of days per week the participant missed at least one dose; frequency of delaying more than two hours to take a dose; and the duration of failing to take the drug (Mannheimer *et al.*, 2006). The adherence measured by the CASE tool was also summed into an index score (1 to 14).

The severity of the skin reactions was graded from mild to severe depending on clinical presentation and effect on normal functional activities. Hepatotoxicity was measured by elevation of ALT levels. Baseline ALT levels were determined and changes during ART treatment recorded.

4.2.3.1 Genotyping

Genotyping was performed by Angima, (2015). Blood from the historical samples was genotyped for genetic polymorphism of CYP2B6. DNA was extracted using the PureLink® Genomic DNA Mini Kit at the Uon-AiBST Laboratory at the Department of Pharmacology and Pharmacognosy at the School of Pharmacy, University of Nairobi. The extracted DNA was amplified using real-time polymerase chain reaction (PCR) and genotyping performed using Taqman® Genotyping Assay mix at the Kenya Medical Research Institute Virology Laboratory. The SNPs that were assayed using allelic discrimination were CYP2B6 516G>T and 983T>C.

4.2.4 Nevirapine plasma levels in study population

Nevirapine plasma levels were determined by the validated HPLC- UV analytical method described previously at the NQCL. The HPLC-UV method was validated using the FDA bioanalytical guidelines. Patient samples were thawed extracted using protein precipitation and analyzed. Plasma samples spiked with LQC and HQC quality control standards of nevirapine were prepared freshly daily and run together with patient samples. The calibration curves obtained during method validation were used to determine nevirapine plasma levels in µg/mL using the formula:

$$X = \left(\frac{Area1}{Area2} - a\right)/b$$

Where;

X = Unknown concentration, Area1= Peak area of nevirapine, Area2=Peak area of CBZ, a = intercept and b=slope

4.2.5 Definitions of variables

Patients were termed to have adhered to taking medication if they reportedly took medication on time or if they had an adherence index score of 13 and above on the CASE tool or if they took or rarely delayed taking medication within two hours of the stipulated time. Patients were non adherent if they reportedly delayed taking medication on time or had an adherence index score of less than 13 on the CASE tool or if they delayed most of the time in taking medication within two hours of the stipulated time.

CYP2B6 983T>C and CYP2B6 516G>T genotypes were described as: wild type (983TT or 516GG), heterozygous (983TC or 516GT), or homozygous mutated (983CC or 516TT). CYP2B6 genotypes were combined and metabolic phenotypes assigned (Haas *et al.*, 2009). Participants were described as 'extensive metabolizers (EM)' if there was no allele variant at either positions (983TT or 516GG). Participants who had a single variant allele at any position (516 or 983) but not in the both loci were termed as 'intermediate metabolizers (IM).' On the other hand, participants who had two homozygous variant alleles at the positions (983CC, 516TT, or 983TC with 516GT) were described as 'slow metabolizers' (Haas *et al.*, 2009).

The highest CD4 cell counts value attained during ART treatment was considered the maximum CD4 cell counts attainable. Adverse skin reactions were considered mild if symptoms caused minimum interference with normal functional activities; moderate if symptoms caused more than minimum interference in normal functional activities; severe if symptoms caused inability to perform normal functional activities; and life threatening if symptoms caused inability to undertake normal functional activities and medical intervention was required to prevent permanent disability or death.

The severity of liver toxicity was measured using the fold elevation of ALT levels above the upper limit of normality (ULN) of 40 IU/L based on the AIDS Clinical Trial group (ACTG) grading system. The grades for ALT were as follows: normal (less than 50 IU/L); mild (50 – 100 IU/L); moderate (101 – 200 IU/L); severe (201 – 400 IU/L) and very severe (greater than 400 IU/L).

The therapeutic range of nevirapine plasma levels was considered to range from 3.0 to 8.0 µg/mL (Duong *et al.*, 2004). Patients with levels below 3.0 µg/mL were considered to have sub-therapeutic nevirapine plasma levels while those above 8.0 µg/mL were considered to have supra-therapeutic levels. Patients with nevirapine plasma levels above 4.3 µg/mL that is reported to offer lasting viral were termed to have durable viral suppression. Nevirapine plasma levels between 3.0 and 4.3

µg/mL are reported to result in selection of resistant strains and patients with these levels were termed to be in the mutant selection window (Duong *et al.*, 2004).

4.2.6 Variables and outcomes

The outcome variable was variability in steady-state nevirapine levels. The predictor variables were: age, gender, body mass index (BMI), switching regimens, ART regimen, adherence, comorbidities, CYP2B6 genotypes and ethnicity among others. The effect of nevirapine plasma levels on occurrence of skin reactions, elevation of ALT and the maximum CD4 cell count attained by the patients was a secondary outcome of interest.

4.2.7 Data management

Unique patient identifiers rather than patient names or outpatient numbers were used to ensure confidentiality. Any document linking the collected data to the patient files including the raw data were kept under lock and key and only accessible by the investigator or on request by regulatory teams such as the Ethics and Research Committee (ERC) and the quality control team for audit purposes.

The data were backed up weekly at a separate secure site. The results of nevirapine plasma concentrations were entered into an Excel® spreadsheet and the data transferred to the existing STATA version 10 database. The database was password protected to control access.

4.2.8 Quality assurance

All data obtained during quantification of nevirapine were double checked by the investigator during data entry. The precollected data in the existing database was checked for errors before utilization in the study. Any deviation from the standards and protocols was recorded, reviewed and those which might have affected the validity of the study were documented.

4.2.9 Statistical analysis

Data analysis was divided into the following two sections; a descriptive, exploratory and linear regression analysis of levels of nevirapine plasma concentration in relation to potential predictor variables; and Bivariable and linear regression analysis of nevirapine plasma levels in relation to clinical outcomes.

4.2.9.1 Descriptive, exploratory and linear regression analysis of nevirapine plasma levels

Nevirapine plasma concentrations of the study population were tested for normality using the Shapiro-Wilk test. Nevirapine concentration in plasma was expressed as the mean and standard deviation (if normally distributed) or as the median and interquartile range (if not normally distributed). Inter- and intra-patient variance in nevirapine plasma levels was assessed using repeated measures ANOVA.

Differences in the mean or median nevirapine concentration plasma were compared across categorical potential predictors like genotype, age, BMI, comorbidities, sex, ethnicity and co medications using the unpaired student's t-test or the Mann-Whitney inferential tests. Where there were more than two categories in a variable, either one way ANOVA or Kruskal-Wallis test as appropriate was used for inferential data analysis.

The correlation between nevirapine plasma levels and continuous predictor variables was determined. The findings of exploratory data analysis were used to identify key predictors of nevirapine plasma levels for subsequent use in linear regression analysis.

Linear regression analysis was performed using nevirapine plasma levels as the outcome variable. Manual stepwise forward model building was done to identify the most important predictors of nevirapine plasma levels. This analysis was performed using STATA version 10 software. The level of significance was set at 0.05.

4.2.9.2 Analysis of nevirapine plasma levels in relation to clinical outcomes

Bivariable analysis was performed where differences in the mean or median levels of nevirapine were compared across categorical treatment outcomes that included the occurrence and severity of skin reactions and hepatotoxicity using unpaired student's t-test or the Mann- Whitney inferential tests. Where there were more than two categories in a variable, either one way ANOVA or Kruskal-Wallis test as appropriate was used for inferential data analysis. The correlation between the maximum CD4 cell count attained by the participants during treatment and nevirapine plasma levels was determined.

Linear regression was performed using nevirapine plasma levels as a predictor of treatment outcomes. Logistic regression was performed on ALT levels greater that normal to determine the effect of nevirapine plasma levels.

4.2.10 Ethical considerations

Approval to analyze the historical blood samples was sought from the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (KNH-UoN ERC) and was granted as per reference letter P655/10/2015 (Appendix A). This study was nested in a previous study in which ethical approval was granted by the KNH-UoN ERC as per reference letter P10/01/2014 (Appendix B).

4.3 Results

Results of this study are presented in four parts: baseline characteristics of the study population; steady-state nevirapine plasma levels; factors affecting nevirapine plasma concentrations; and impact of steady-state nevirapine plasma levels on clinical outcomes. The baseline characteristics, adherence, ART regimens, and CYP2B6 genotypes of the historical cohort have been reported and a summary is presented in this study (Angima, 2015; Makori *et al.*, 2015).

4.3.1 Baseline characteristics of the study population

The baseline characteristics of the historical cohort study population are shown in **Table 4.1**. A total of 241 study participants took part in the study of with a median age of 39 years [interquartile range (IQR) 35, 44]. Females in the study cohort were 185 (76.8%) while 56 (23.2%) were males. The median weight was 62 kg [IQR 56, 70] with median body mass index (BMI) of 23.38 kg/m^2 [IQR 21.34, 25.95]. Participants with college education (degree and diploma) were 76 (31.6%) while 117 (48.6%) and 48 (19.9%) had secondary and primary education respectively. About 184 (76.8%) were Bantus while 43 (17.9%) and 1 (0.4%) were Nilotes and Cushites respectively. Participants with comorbidities were 59 (24.5%) while 182 (75.5%) had no other illnesses.

Table 4.1: Sociodemographic and clinical characteristics of the study population (Makori *et al.*, 2015)

Variable	Characteristic	Median [IQR] or n (%)
Age at diagnosis (years)		39 [35, 44]
Sex	Female	185 (76.8)
	Male	56 (23.2)
Weight at diagnosis (kg)		62 [56, 70]
Height (cm)		162[158, 168]
BMI (kg/m^2)		23.4 [21.3, 25.9]
Marital status	Married	155 (64.3)
	Single	57 (23.7)
	Divorced	4 (1.7)
	Widowed	24 (10.0)
	Separated	1 (0.4)
Education	Degree	19 (7.9)
	Diploma	57 (23.7)
	Secondary	117 (48.6)
	Primary	48 (19.9)
Employment status	Unemployed	18 (7.5)
	Employed	108 (44.8)
	Self-employed	115 (47.7)
Smoking	No	236 (97.9)
	Yes	5 (2.1)
Alcohol use	No	167 (69.3)
	Yes	74 (30.7)
Ethnicity	Kikuyu	98 (40.8)
	Luo	41 (17.1)
	Kamba	40 (16.8)
	Luhya	25 (10.4)
	Meru/Embu	5 (2.1)
	Kisii	16 (6.7)
	Kalenjin	2 (0.8)
	Cushite	1 (0.4)
Comorbidities	None	182 (75.5)
	Hypertension	36 (14.9)
	Diabetes	3 (1.2)
	PUD	4 (1.7)
	Chronic pain	5 (2.1)
	Other conditions	8 (3.6)
Duration of therapy (years)		4.8 [3.3, 6.6]
ALT at initiation of HAART		22 [17, 32]
	Normal (≤40 IU/L)	209 (86.7)
	Elevated (≥40 IU/L)	26 (10.8)
	Missing	6 (2.5)
CD4 cell counts x 10^9/L		206 [127, 270]
	≤250	158 (65.6)
	≥250	68 (28.2)
	Missing entries	15 (6.2)

4.3.1.1 Adherence of study population

The adherence of the study population is shown in **Table 4.2**. About 194 (80.5%) adhered to ART dosing schedule while 47 (19.5%) participants did not adhere. The self – reported adherence was similar to that measured by the CASE tool. About 91 (37.8%) participants reportedly always took the medication on time. The number of patients who took medication two hours before or after the stipulated time in most cases were 3 (1.2%), while 147 (61.0%) participants rarely delayed taking the dose.

Table 4.2: Adherence to medication in the study population

Variable	Characteristic	n (%)
CASE tool adherence	Adhered	194 (80.5)
	No-adherence	47 (19.5)
Self-reported adherence	adhered	210 (87.1)
	Non-adherence	31 (12.9)
Delays taking the dose	Most of the time	3 (1.2)
	Rarely	147 (61.0)
	Never	91 (37.8)

4.3.1.2 ART regimens of the study population

The median duration on which the patients had been on antiretroviral therapy (ART) was 4.8 [IQR 3.3, 6.6] years. Most of the participants (n=85, 35.3%) were initiated on a stavudine based ART regimen. Due to toxicity of stavudine, 80 participants were switched to tenofovir based regimens (n=80, 87%). A large number of the study participants (n=148, 61.4%) were on a combination therapy of tenofovir, lamivudine and nevirapine. **Table 4.3** summarizes the type of regimens the participants were initiated on, switched to and were taking at the time of recruitment.

Table 4.3: Antiretroviral regimens of the study population

Regimen	Initiated n (%)	Current n (%)	Switched n (%)
TDF/3TC/NVP	71 (29.5)	148 (61.4)	80 (87.0)
AZT/3TC/NVP	78 (32.4)	82 (34.0)	11 (11.9)
D4T/3TC/NVP	85 (35.3)	10 (4.2)	-
ABC/3TC/NVP	-	1 (0.4)	1 (1.1)
TDF/3TC/EFV	3 (1.2)	-	-
AZT/3TC/EFV	3 (1.2)	-	-
ABC/3TC/EFV	1 (0.4)	-	-
Total	**241 (100)**	**241 (100)**	**92 (100)**

TDF-tenofovir; **3TC**-lamivudine; **AZT**-zidovudine; **NVP**-nevirapine; **D4T**-stavudine; **EFV-**efavirenz; **ABC**-abacavir; **Initiated**-initial ART regimen; **Current**- ART regimen at the time of study; **Switched-** ART regimen changed to.

4.3.1.3 CYP2B6 genotypes and allele frequencies of the study population

CYP2B6 genotypes and allelic frequencies (**Table 4.4**) of the study participants have been previously reported (Angima, 2015). The prevalence of CYP2B6 516GG wild type, GT heterozygous and TT homozygous genotypes were 89 (45.9%), 73 (37.6%) and 32 (16.5%) respectively. On the other hand, the prevalence of CYP2B6 983 TT wild type, TC heterozygous and CC variants was 183 (89.7%), 19 (9.3%) and 2 (1%) respectively.

Table 4.4: CYP2B6 983T>C and 516G>T genotypes and allele frequencies of the study population (Angima, 2015)

Characteristic	n (%)	Characteristics	n (%)
CYP2B6 983T>C		**CYP2B6 516G>T**	
Genotypes		**Genotypes**	
TT	183 (89.7)	GG	89 (45.9)
TC	19 (9.3)	GT	73 (37.6)
CC	2 (1)	CC	32 (16.5)
TOTAL	204 (100)	TOTAL	194 (100)
Alleles		**Alleles**	
T	385 (94.4)	G	251 (64.7)
C	23 (5.6)	T	137 (35.3)
TOTAL	408 (100)	TOTAL	388 (100)

4.3.2 Steady-state nevirapine plasma levels of the study population

4.3.2.1 Distribution of nevirapine plasma concentrations in the study population

A total of 251 blood samples were obtained from 240 participants. The additional 11 second samples were obtained on a second visit and were used for the determination of intra patient variability.

Nevirapine concentration in plasma was quantified in 240 participants. The determined nevirapine plasma concentrations were not normally distributed (P<0.001) (appendix D). There were two sub populations as presented in **Figure 4.1**. One sub-population had nevirapine plasma concentration ranging from 0.5 to 12.5 µg/mL (n=226, 94.2%), with median levels of 5.621 [IQR 4.404, 6.817] µg/mL. The second sub group, had nevirapine concentrations of 12.5 to 19 µg/mL (n=14, 5.8%), with median levels of 13.66 [IQR 13.047, 15.267] µg/mL.

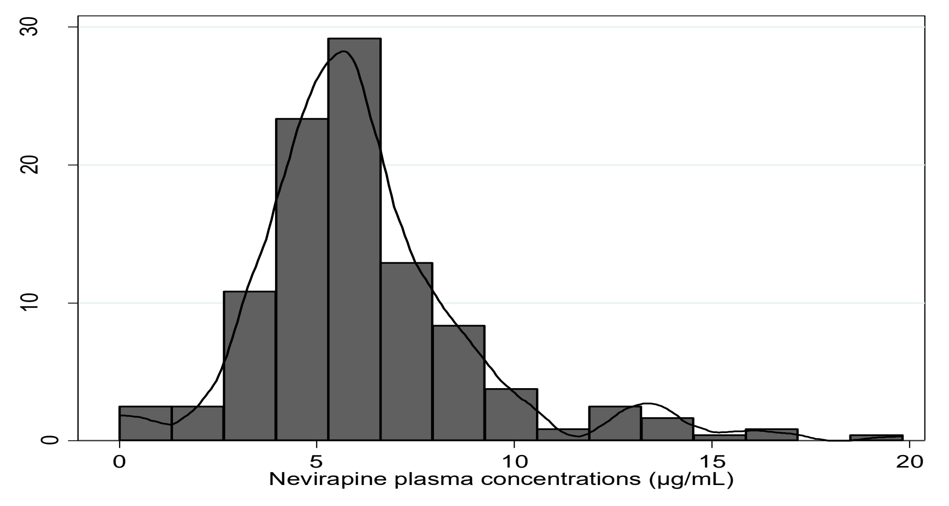


Figure 4.1: Distribution of nevirapine plasma levels in the study population

The median nevirapine plasma concentration was 5.675 [IQR 4.537, 7.203] µg/mL. There were 5 participants (2.1%) who had nevirapine plasma concentration below the detection limit. Three participants exhibited high concentrations at 16.288, 16.673 and 19.821 µg/mL. **Table 4.5** shows nevirapine plasma levels in the study population.

Table 4.5: Nevirapine plasma concentrations in the study population

Statistic	Concentration (µg/mL), n=240
First quarter (25%)	4.537
Median (50%)	5.675
Third quarter (75%)	7.203
Range	0.454-19.821

4.3.2.2 Variability of nevirapine plasma levels in the study population

The coefficient of variation in levels of nevirapine between patients was 46.0%. When levels of nevirapine were analyzed in samples obtained on two different occasions, the inter-occasion variability was 27.4%. There was a strong correlation between levels of nevirapine levels measured

on two different days of 0.761. When variability within a participant was assessed, the intra- patient coefficient of variation was 26.7%.

4.3.2.2.1 Distribution of nevirapine plasma levels according to therapeutic categories

The therapeutic range of nevirapine plasma levels is 3.0 to 8.0 μg/mL. About 162 (74.7%) participants had nevirapine plasma levels within the therapeutic range. The number of participants with sub-therapeutic nevirapine plasma levels below 3.0 μg/mL was 14 (6.5%). Those having supra-therapeutic nevirapine plasma levels above 8.0 μg/mL were 41 (18.9%). **Table 4.6** summarizes the number of participants at different nevirapine plasma levels therapeutic categories.

Table 4.6: Categories of therapeutic nevirapine plasma levels in the study population

Nevirapine plasma levels (μg/mL)	Median [IQR] μg/mL	Females (%)	Males (%)	Total (%)
Therapeutic range (3.0 – 8.0)	5.518 [4.568, 6.290]	127 (78.4)	35 (21.6)	162 (74.7)
Sub-therapeutic (<3.0)	2.111 [0, 2.512]	9 (64.3)	5 (35.7)	14 (6.5)
Supra-therapeutic (>8.0)	9.695 [8.504, 13.047]	31 (75.6)	10 (24.4)	41 (18.9)
Durable viral suppression (>4.3)	6.196 [5.449, 7.808]	131 (80.0)	37 (20.0)	168 (77.4)
Mutant selection (3.0 - 4.3)	3.868 [3.633, 4.069]	27 (77.1)	8 (22.9)	35 (16.1)

The number of participants with nevirapine plasma levels between 3.0 and 4.3 μg/mL that results in mutant selection were 35 (16.1%). The number of participants with nevirapine plasma levels above 4.3 μg/mL reported to offer lasting viral suppression were 168 (77.4%).

4.3.2.2.2 Nevirapine plasma levels in relation to sex, body mass index and ART regimens

Females (n=167, 77.0%) had slightly higher median nevirapine plasma levels of 5.699 [IQR 4.707, 7.415] μg/mL compared to 5.573 [4.299, 6.890] μg/mL median levels in males (n=50, 23.0%) as shown in **Table 4.7.** The difference in nevirapine plasma levels with sex was not significant (p=0.501).

Table 4.7: Nevirapine plasma levels with sex, BMI and ART regimens in the study population

Variable	Characteristic	n (%)	Nevirapine levels (µg/mL)		P Value
			Median	IQR	
Sex	Females	167 (77.0)	5.699	4.707, 7.415	0.501
	Males	50 (23.0)	5.573	4.299, 6.890	
BMI	<18.5	12 (5.5)	5.139	3.619, 7.391	0.179
	18.5-24	130 (59.9)	5.956	4.843, 7.623	
	25-29	54 (24.9)	5.562	4.097, 6.668	
	≥30	21 (9.7)	5.549	4.957, 6.196	
Current ART regimen	TDF/3TC/NVP	132 (60.8)	5.978	4.958, 7.752	**0.046**
	AZT/3TC/NVP	77 (35.5)	5.607	4.017, 6.570	
	D4T/3TC/NVP	10 (3.7)	5.016	4.066, 6.276	
Initial ART regimen	TDF/3TC/NVP	62	5.822	4.404, 7.415	0.059
	AZT/3TC/NVP	74	5.611	4.215, 6.392	
	D4T/3TC/NVP	77	6.073	4.843, 8.212	
	AZT/3TC/EFV	1	4.816	4.816, 4.816	
	TDF/3TC/EFV	3	8.577	6.181, 16.67	
Switched regimen (tenofovir)	No	136	5.619	4.207, 6.865	**0.032**
	Yes	80	6.027	4.853, 8.311	
Previous use of efavirenz	Not used	213	5.660	4.493, 7.205	0.204
	Used	4	7.379	5.499, 12.62	
Total		**217 (100)**	**5.689**	**4.506, 7.224**	

The variability of nevirapine plasma levels with BMI categories was not significant. Nevirapine plasma levels varied with the type of regimen the participant was on **(Table 4.7).**

There was slight significant variation in nevirapine plasma levels depending on the type of treatment regimen the patients were initiated on (p=0.059). Participants on tenofovir based regimens (n=132, 60.8%) had significantly higher median nevirapine plasma levels of 5.978 µg/mL compared to those on zidovudine and stavudine based therapy (p=0.046). Participants who changed regimens, mainly from stavudine (n=74) to tenofovir based regimens, had significantly higher median nevirapine plasma levels of 6.027 µg/mL compared to 5.619 µg/mL for those who did not change regimens (p=0.032). Participants who had previously used efavirenz, although not significant, had higher median levels of 7.379 µg/mL compared to 5.66 µg/mL for those who had not previously used the drug (p=0.204).

4.3.2.2.3 Nevirapine plasma levels in relation to CYP2B6 genotypes and phenotypes

Participants with homozygous CYP2B6 983CC (n=2, 1.0%) genotype had significantly higher median nevirapine plasma levels of 10.60 µg/mL compared to heterozygous 983TC and wild type 983TT genotypes (p=0.001). Furthermore, participants who had homozygous CYP2B6 516TT (n=32, 16.6%) had significantly higher median nevirapine plasma levels of 8.450 µg/mL compared to heterozygous 516 GT and wild type 516 GG genotypes (p<0.001). **Table 4.8** shows nevirapine plasma levels for different CYP2B6 genotypes and phenotypes.

Table 4.8: Nevirapine plasma levels with CYP2B6 genotypes and phenotypes in the study population

Characteristic	n (%)	Nevirapine plasma levels (µg/mL)		P value
		Median	IQR	
CYP2B6 983T>C genotype				0.001
Wild type (TT)	182 (89.7)	5.533	4.299, 6.919	
Heterozygous (TC)	19 (9.3)	6.858	6.000, 8.322	
Homozygous (CC)	2 (1.0)	10.60	8.267, 12.95	
Total	203 (100)	5.652	4.437, 7.224	
CYP2B6 516G>T genotype				<0.001
Wild type (GG)	88 (45.6)	5.575	4.285, 6.762	
Heterozygous (GT)	73 (37.8)	5.518	4.568, 6.392	
Homozygous (TT)	32 (16.6)	8.450	6.346, 9.784	
Total	193 (100)	5.652	4.506, 7.415	
CYP2B6 Phenotypes				<0.001
Extensive metabolizers	70 (37.2)	5.029	4.017, 6.196	
Intermediate metabolizers	83 44.2)	5.690	4.954, 6.852	
Slow metabolizers	35 (18.6)	8.667	.516, 10.03	
Total	**188 (100)**	**5.675**	**4.465, 7.530**	

Nevirapine plasma levels variability with CYP2B6 genotypes is represented in **Figure 4.2**. Participants with homozygous CYP2B6 983 CC and 516 TT genotypes had significantly higher median nevirapine levels compared to those with heterozygous and wild type genotypes.

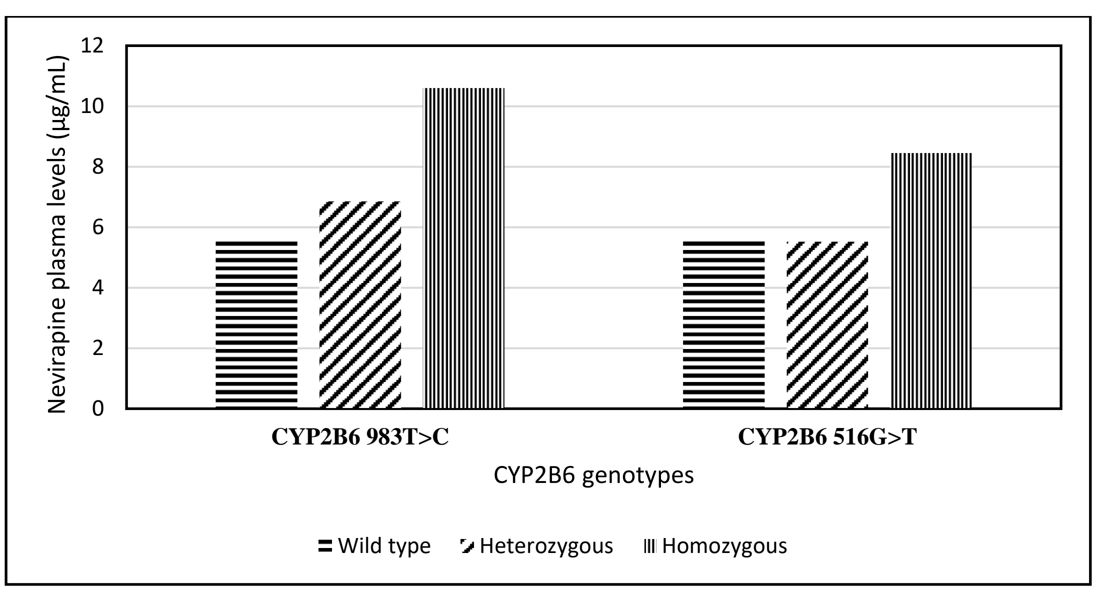


Figure 4.2: Nevirapine plasma levels with CYP2B6 genotypes in the study population

Nevirapine plasma levels varied significantly with metabolic phenotypes assigned by combined CYP2B6 983T>C and 516G>T genotypes. CYP2B6 phenotypic slow metabolizers (n=35) had higher median nevirapine plasma levels of 8.667 µg/mL compared to extensive (5.029 µg/mL) and intermediate (5.690 µg/mL) phenotypes (p=0.001) as shown in **Table 4.8**. The variability of Nevirapine plasma levels with metabolic phenotypes is also shown in **Figure 4.3**.

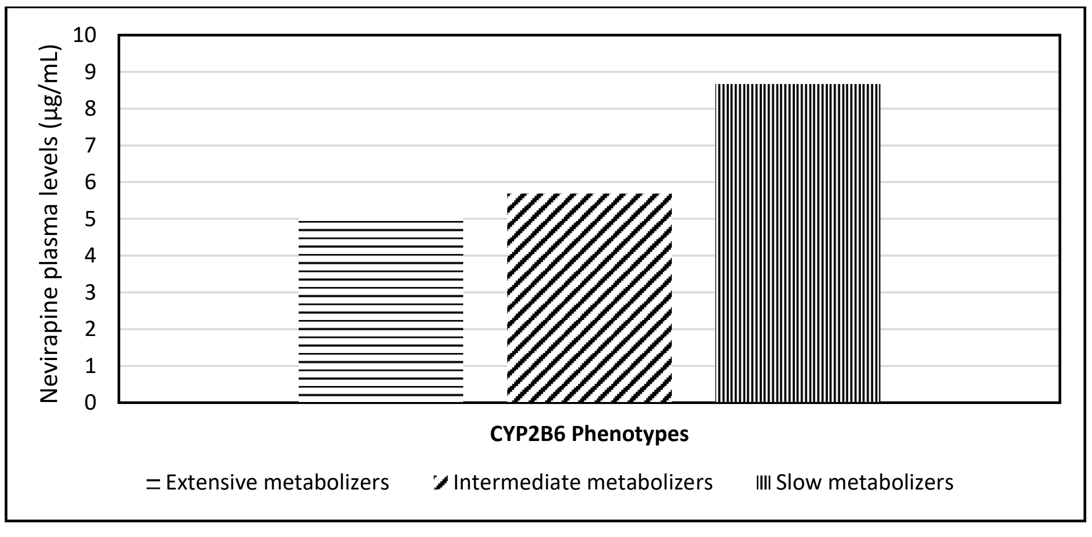


Figure 4.3: Nevirapine plasma levels with CYP2B6 phenotypes in the study population

4.3.3 Factors affecting nevirapine plasma concentrations in the study population

4.3.3.1 Bivariable analysis of predictors of steady-state nevirapine plasma levels

Differences in median nevirapine plasma concentration across categorical predictors and the correlation between nevirapine concentration and continuous predictors are presented in **Tables 4.9 and 4.10**. Significant predictors of nevirapine plasma concentration were subsequently used in linear regression analysis.

Although not significant, nevirapine plasma concentration increased as the level of education increased with degree holders (n=17) having the highest median concentrations of 7.719 (p=0.114). Likewise, levels of nevirapine were not significantly higher in employed participants compared to those who were unemployed (p=0.932). There was no major variation in nevirapine levels with marital status.

Smokers had lower plasma nevirapine levels compared to nonsmokers although the difference was not significant (p=0.480). There was no significant difference in nevirapine plasma levels between alcohol drinkers and non-drinkers (p=0.911).

In the ethnic linguistic groups, differences in nevirapine plasma levels was not significant (p=0.966). Among the Bantus, Kikuyus had the highest median levels of nevirapine (5.925 µg/mL) while the Kambas had the lowest median concentrations at 5.615 µg/mL. The variation in nevirapine plasma levels across ethnic groups of participants (p=0.983) and ethno-linguistic groups (p=0.966) was not significant. Participants who had diabetes and anemia had higher nevirapine plasma concentrations compared to those with other illnesses.

Table 4.9: Predictors of nevirapine plasma concentration in the study population

Variable	Characteristic	n	NVP levels (µg/mL), Median [IQR]	P value
Marital status	Married	138	5.675 [4.383, 7.205]	0.976
	Single	53	6.063 [4.999, 7.543]	
	Divorced	3	5.518 [3.398, 13.71]	
	Widowed	22	5.633 [4.784, 7.181]	
Education	Primary	42	5.033 [4.094, 6.890]	0.114
	High school	108	5.671 [4.402, 6.916]	
	Diploma	50	5.810 [4.967, 7.515]	
	Degree	17	7.719 [5.512, 8.322]	
Employment status	Unemployed	17	5.342 [4.038, 8.323]	0.932
	Employed	98	5.633 [4.383, 7.719]	
	Self employed	101	5.738 [4.863, 6.890]	
Smoker	No	212	5.694 [4.499, 7.319]	0.480
	Yes	5	5.024 [4.954, 6.073]	
Alcohol use	No	155	5.738 [4.568, 7.201)	0.911
	Yes	62	5.619 [4.383, 7.543]	
Ethno-linguistic groups	Bantu	172	5.679 [4.420, 7.465]	0.966
	Nilotes	39	5.689 [4.816, 7.719]	
	Cushite	1	5.462 [5.462, 5.462]	
	Other	5	6.314 [5.513, 6.376]	
Ethnicity	Kikuyu	87	5.922 [4.215, 7.205]	0.983
	Luo	37	5.689 [4.954, 7.181]	
	Kamba	33	5.614 [4.437, 6.976]	
	Luhya	23	5.699 [4.009, 7.820]	
	Meru/Embu	5	5.643 [5.342, 13.71]	
	Kisii	16	5.848 [4.824, 9.047]	
	Kalenjin	2	8.127 [3.055, 13.19]	
	Cushite	1	5.462 [5.462, 5.462]	
Comorbidities	Non	163	5.660 [4.568, 7.415]	0.291
	Hypertension	32	5.488 [3.872, 7.428]	
	Diabetes	2	8.292 [6.890, 9.695]	
	PUD	4	6.202 [6.089, 10.77]	
Renal disease	Absent	201	5.738 [4.568, 7.415]	0.839
	Present	12	5.465 [4.607, 7.214]	
Delay in taking the dose (>2hrs)	Most of the time	3	3.768 [0.268, 7.201]	0.421
	Rarely	130	5.656 [4.819, 6.890]	
	Never	84	5.954 [4.327, 7.763]	
Adherence to treatment	Poor	41	5.512 [4.215, 6.063]	**0.055**
	Good	176	5.939 [4.663, 7.618]	

Participants who adhered to treatment had higher median nevirapine levels of 5.939 µg/mL compared to 5.512 µg/mL for those with did not adhere (p=0.055). Those who never delayed taking the drug had higher median levels of 5.954 µg/mL compared to 5.656 and 3.768 µg/mL for those who rarely delayed and those who delayed most of the time respectively (p=0.421). In addition, there was a negative correlation between the days since the last missed dose and nevirapine levels (rho=-0.097, p=0.154). The number of days in the previous week the participants missed taking drugs significantly affected nevirapine plasma concentration (rho=-0.149, p=0.028). There was a slightly significant positive correlation between the CASE adherence index score and the nevirapine plasma concentration (rho=0.132; p=0.053) as shown in **Table 4.10**.

Table 4.10: Correlation between potential predictors and nevirapine plasma levels

Variable (n=217)	Correlation coefficient	P value
Age (years)	-0.055	0.416
Initial weight (kg)	-0.073	0.284
Height (cm)	-0.042	0.536
Initial BMI	-0054	0.429
Last missed dose	-0.097	0.154
Days missed dose	-0.149	**0.028**
Adherence score	0.132	**0.053**

There was a negative correlation between levels of nevirapine and age, initial weight, BMI and height although not significant.

Therefore, the significant predictors ($P \leq 0.05$) of nevirapine plasma levels from the bivariable analysis were CYP2B6 983 T>C genotype (p=0.001), CYP2B6 516 G>T genotype ($P<0.001$), switching of ART regimens (p=0.032), initiation ART regimen (p=0.059), use of tenofovir based ART regimens (p=0.046), adherence (p=0.055), and number of days missed to take the drug (p=0.028).

4.3.3.2 Sub-group analysis of sub-populations with high and low nevirapine plasma levels

Sub-groups of participants with low levels of nevirapine below 3.0 µg/mL (n=14, 6.5%) and high levels above 12.5 µg/mL (n=14/5.8%) were analyzed to determine whether they were systematically different from the rest of the study population. Participants with low levels of nevirapine were not systematically different from the rest of the study population. It was noted that among the Bantu ethnolinguistic group, Kambas had lower levels compared to other ethnic groups (p=0.039).

When the sub–group of the population with high nevirapine plasma concentrations above 12.5 µg/mL was analyzed, the prevalence of CYP2B6 983CC and 516TT homozygous variants were high than the rest of the population. In addition, the number of patients who switched regimens was

higher than the rest of the study population as shown in **Table 4.11**. Therefore, the sub-population with high Nevirapine levels may be different from the rest of the study population.

Table 4.11: Characteristics of the sub-group with high nevirapine plasma levels

Variable	Prevalence (%)	
	High levels sub-group	Study population
CYP2B6 983T>C genotypes		
TT	69.2	89.7
TC	23.1	9.3
CC	7.7	2.0
CYP2B6 516G>T genotypes		
GG	38.5	45.6
GT	15.4	37.8
TT	46.1	16.6
Switched regimens		
Yes	64.3	37.0
No	35.7	630

4.3.3.3 Linear regression analysis of steady-state nevirapine plasma levels

Linear regression analysis was performed using nevirapine plasma concentration as the outcome variable and the results are presented in **Table 4.12**. CYP2B6 983T>C genotype was found to have the greatest effect on nevirapine plasma levels with a crude β coefficient of 2.139 (95% CI 0.793, 3.486; p=0.002). CYP2B6 983T>C accounted for 6.4% of the variability in nevirapine plasma levels. On adjusting for confounding, the influence of 983T>C SNP remained significant with an adjusted β coefficient of 2.619 (95% CI 1.337, 3.901; P<0.001).

Table 4.12: Factors affecting nevirapine plasma levels in the study population

Variable	**Crude β coefficient (95% Confidence Interval)**	**P value**	**Adjusted β coefficient (95% CI))**	**P value**
Sex	--0.018 (-1.025, 0.989)	0.972	-	-
Age	0.524 (-0.007, 0.111)	0.081	-	-
Initial weight	-0.018 (-0.052, 0.17)	0.312	-	-
Height	0.447 (-5.281, 6.174)	0.878	-	-
Initial BMI	-0.054 (-0.154, 0.046)	0.287	-	-
Marital status	-0.072 (-3.981, 0.254)	0.663	-	-
Education	0.448 (-0.170, 0.913)	0.059	-	-
Occupation	-0.126 (-0.695, 0.443)	0.663	-	-
Smoker	-0.931 (-2.426, 0.565)	0.221	-	-
Alcohol use	0.002 (-0.805, 0.808)	0.997	-	-
Ethno-linguistic groups	0.111 (-0.388, 0.610)	0.661	-	-
Ethnicity	0.043 (-0.094, 0.179)	0.537	-	-
Comorbidities	0.007 (-0.175, 0.189)	0.940	-	-
Renal disease	-0.340 (-1.491, 0.811)	0.561	-	-
Use of tenofovir	-0.803 (-1.423, -1.841)	**0.011**	-	-
Initial regimen	0.395 (-0.111, 0.901)	0.126	-	-
Switched regimen (tenofovir)	1.038 (0.191, 1.886)	**0.017**	1.202 (0.402, 2.003)	**0.003**
Previous efavirenz use	2.870 (-1.697, 7.437)	0.217	-	-
Adherence	0.870 (-0.054, 1.793)	0.065	**-**	**-**
Adherence score	0.169 (-0.066, 0.403)	0.157	-	-
Delay dose	0.374 (-0.417, 1.166)	0.352	-	-
Last missed dose	-0.118 (-0.188, 0.423)	0.449	-	-
Days dose missed	0.939 (0.222, 1.657)	**0.011**	0.836 (0.084, 1.587)	**0.024**
CYP2B6 983T>C	2.139 (0.793, 3.486)	**0.002**	2.619 (1.337, 3.901)	**<0.001**
CYP2B6 516G>T	0.981 (0.295, 1.667)	**0.005**	1.332 (0.665, 1.999)	**<0.001**

CYP2B6 516G>T accounted for 5.8% of the variability in nevirapine plasma levels with a crude β coefficient of variation of 0.981 (95 CI 0.295, 1.667; p=0.005). On adjusting for confounding, 516G>T SNP significantly influenced nevirapine plasma levels with an adjusted β coefficient of 1.332 (95% CI 0.665, 1.999; p=0.002). Both CYP2B6 SNPs collectively accounted for 16.3% of the variance in nevirapine plasma levels.

Participants who switched regimens, mainly from stavudine based to tenofovir containing therapy, had higher plasma levels than those who were still on their first regimen [β=1.038 (95% CI 0.191-1.886); p=0.017]. This observation remained significant even after adjusting for confounding by genotype and adherence [adjusted β=1.202 (95% CI 0.402-1.587); p=0.003].

The number of days the patient adhered to taking medication measured using the CASE adherence tool, caused an increase in nevirapine levels [β=0.939 (95% CI 0.222-1.657; p=0.011]. Adherence measured by the number of days the patient took medication on time remained significant after adjusting for confounding by genotype and participants switching regimens [adjusted β=0.836 (95% CI 0.084-1.587); p=0.029].

The current ART regimen [[crude β =-0.803 (95% CI -1.423—1.841); p=0.011] although significant on linear regression, became insignificant on adjusting for confounding. Hence factors that affected nevirapine plasma levels were: CYP2B6 genotypes, participants switching regimens to tenofovir based ART and adherence.

4.3.4 Impact of nevirapine plasma levels on clinical outcomes

The maximum CD4 cell counts attained varied with the therapeutic nevirapine plasma levels as show in **Table 4.13**. Participants with nevirapine plasma levels within the therapeutic range attained higher maximum CD4 cell counts 639 [IQR 447, 831] compared to those with sub-therapeutic levels 470 [403, 642]. Participants with nevirapine plasma levels above 4.3 µg/mL attained higher CD4 cell counts compared to those with mutant selection levels 481 [375, 764].

Table 4.13: Maximum CD4 cell counts attained at different nevirapine therapeutic levels

Nevirapine plasma levels (µg/mL)	n (%)	Median [IQR] µg/mL	CD4 cell counts [IQR] (10^9/L)
Therapeutic range (3.0 – 8.0)	162 (74.7)	5.518 [4.568, 6.290]	639 [447, 831]
Sub-therapeutic (<3.0)	14 (6.5)	2.111 [0, 2.512]	470 [403, 642]
Supra-therapeutic (>8.0)	41 (18.9)	9.695 [8.504, 13.047]	593 [440,800]
Durable viral suppression (>4.3)	168 (77.4)	6.196 [5.449, 7.808]	640 [473, 831]
Mutant selection (3.0 - 4.3)	35 (16.1)	3.868 [3.633, 4.069]	481 [375, 764]

Nevirapine plasma levels were positively correlated to maximum CD4 cell counts attained during treatment (rho=0.196; p=0.004). In addition, nevirapine levels were higher in participants with severe skin reactions with median concentrations of 8.322 µg/mL compared to 5.401 µg/mL and 5.78 µg/mL for those with mild and no adverse reaction respectively (p=0.085). There was no significant difference in levels of nevirapine in participants with hepatotoxicity or not (p=0.348) as presented in **Table 4.14**.

Table 4.14: Bivariable analysis of nevirapine plasma levels and clinical outcomes

Variable	Characteristic	n	NVP levels (µg/mL), Median [IQR]	P value
Hepatotoxicity	No	215	5.699 [4.568, 7.415]	0.348
	Yes	1	4.383 [4.383, 4.383]	
Grade of skin reactions	No reaction	82	5.796 [4.819, 6.914]	0.085
	Mild reaction	10	5.401 [4.383, 7.674]	
	Severe reaction	5	8.322 [8.050, 9.931]	

The results of regression analysis performed between some clinical outcomes and nevirapine plasma levels is presented in **Table 4.15**.

Table 4.15: Influence of nevirapine plasma levels on clinical outcomes in the study population

Outcome	Crude β coefficient (95% CI)	P value
Skin reactions	0.028 (-0.015, 0.072)	0.201
Hepatotoxicity	-0.00 1(-0.003, 0.001)	0.323
ALT elevation	1.176 (1.001, 1.381)	**0.049**[a]
Maximum CD4	14.50 4(0.498,28.51)	**0.042**

[a] - Logistic regression of ALT levels with more than one fold increase

Nevirapine plasma levels had a significant impact on the maximum CD4 cell counts attained by patients on linear regression [β=14.504 (95% CI 0.498-28.51); p=0.042]. The nevirapine plasma levels did not affect the occurrence nor the severity of skin reactions. However, there was a positive association between nevirapine plasma levels and elevation of ALT levels [odds ratio= 1.176].

4.4 Discussion

Nevirapine plasma levels of the study population varied widely ranging from 0.454 to 19.821 µg/mL with inter- and intra-patient variability of 46% and 28% respectively. The high variability in nevirapine plasma levels and the presence of a sub-group with high levels (above 12.5 µg/mL) may indicate differences in pharmacokinetic profiles among the patients. Furthermore, 14 (6.5%) patients had sub-therapeutic levels (less than 3.0 µg/mL) while 41 (18.9%) patients had supra-therapeutic levels (above 8.0 µg/mL) and may indicate differences in distribution of determinants of nevirapine plasma levels. Nevirapine plasma levels above 4.3 µg/mL have been reported to offer lasting viral suppression and 168 (77.4%) patients had levels above cut-off point. About 35 (16.1%) patients had nevirapine plasma levels between 3.0 and 4.3 µg/ reported to result in mutant selection and might not have adequate viral suppression.

Female participants (n=167, 77.0%) had slightly higher nevirapine plasma levels with a median of 5.699 (IQR 4.707, 7.415) µg/mL compared to males (n=50, 23.0%) 5.573 (IQR 4.299, 6.890) µg/mL (p=0.501). This difference among between sexes in nevirapine plasma levels was not significant and was comparable to that reported in other studies (Kappelhoff *et al.*, 2005; Zhou *et al.*, 1999). The difference might be attributed to differences nevirapine clearance between males and females. In addition, there was no significant difference in nevirapine concentration with age as reported by Wyen *et al.,* 2008.

Nevirapine levels were higher in nonsmokers than in smokers. This might be attributed to enzyme induction in smokers, hence higher metabolism and reduced nevirapine levels in plasma. There was no difference in nevirapine plasma levels between patients who took alcohol and those who did not. Long term use of alcohol might reduce metabolism of nevirapine by decreasing the concentrations of metabolic enzymes concentrations due to reduced liver function (Thorn *et al.*, 2010).

Patients who had previously used efavirenz had high nevirapine plasma levels compared to those who had not used efavirenz previously (p=0.204). This may be attributed to the fact that the two

drugs are metabolized by the same enzymes hence changing from efavirenz because of toxicity to nevirapine may also result in higher nevirapine plasma levels.

Nevirapine plasma levels influence the maximum CD4 cell counts achieved during treatment ($p=0.04$). Patients with therapeutic nevirapine plasma levels had greater viral suppression and better immunological recovery (attained higher CD4 cell counts) than those with sub-therapeutic levels. Nevirapine plasma levels did not significantly influence the occurrence and severity of skin reactions. Nevirapine plasma levels led to mild elevation of ALT levels and hence may influence occurrence of hepatotoxicity.

4.5 Conclusion

In conclusion, factors that affected nevirapine plasma levels on bivariable analysis were: CYP2B6 genotypes, switching to tenofovir based ART and adherence. On multivariable analysis and adjustment for confounding, the significant predictors of nevirapine plasma levels were CYP2B6 genotypes, adherence and use of tenofovir based ART regimens. Nevirapine plasma levels influenced immunological response with mild elevation of ALT levels but not the occurrence of skin reactions.

CHAPTER FIVE

GENERAL DISCUSSION

This study was able to improve and validate a HPLC-UV method for determination of nevirapine in plasma. Nevirapine plasma levels varied within and between patients in the study population. Nevirapine plasma levels were influenced by adherence measured by the number of days the patient misses a dose. This finding has not been previously described in the population studied. In addition, this study found that patients on tenofovir based regimens, had higher nevirapine levels compared to those who were on stavudine or zidovudine based therapy. This is a new finding in the study population. The study found an association between CYP2B6 983T>C and 516G>T genotypes and nevirapine plasma levels accounting for about 16.3% variance in nevirapine concentrations. CYP2B6 983 T>C had greater influence on nevirapine levels than 516 G>T. Nevirapine plasma levels influenced the maximum CD4 cell counts attained by the patients.

5.1 Monitoring nevirapine plasma levels in HIV patients

The HPLC-UV method was accurate, precise, and selective over wide range of concentrations (0.5-25.0 µg/mL) which was comparable to reported methods (Hamrapurkar *et al.*, 2010; Kabra *et al.*, 2009). The sensitivity of the method (0.5 µg/mL) was comparable with other reported methods for quantifying nevirapine in plasma although lower when compared to liquid chromatography with mass spectrometric detection gold standard (Ren *et al.*, 2010).

The sample work up by protein precipitation was simple and involved fewer and less tedious steps compared to other methods such as liquid-liquid extraction. This bioanalytical method can be used to analyze a wide range of concentrations and hence it can be used to assess samples obtained from populations with high variability in nevirapine plasma levels and in cases where there is suspected overdose of nevirapine. The method can be used in therapeutic drug monitoring of nevirapine in patients with poor compliance. The method can also be employed in resource-limited settings as the materials and reagents used are readily available in most analytical laboratories (Minzi *et al.*, 2010).

5.2 Variability in nevirapine plasma levels

Nevirapine plasma levels varied widely among the participants with a range of 0.454 to 19.821 µg/mL similar to that reported by Oluka, (2012) and Oluka *et al.*, (2015). Inter- and intra-patient variability in nevirapine plasma levels was 46% and 28% respectively and was slightly higher compared to that reported by Chou *et al.*, (2010) in the Cambodian population. The high variability may be due to wide distribution of the CYP2B6 genotype variants and differences in adherence or may be due to outliers with high nevirapine plasma levels above 12.5 µg/mL.

The median steady-state nevirapine plasma levels was 5.675 µg/mL and was within 4.0-6.5 µg/mL reported by Nellen *et al.*, (2008) and comparable to studies in Burundian and Cambodian populations (Bertrand *et al.*, 2012; Calcagno *et al.*, 2012). The median nevirapine plasma levels were however lower than 9.14 µg/mL (Oluka *et al.,* 2015) reported on an all-female cohort in a study carried out in Mombasa and higher than those reported by Mahungu *et al.*, (2009) in a Caucasian population. The difference may be attributed to lower clearance in women leading to higher nevirapine plasma levels compared to males (Zhou *et al.*, 1999).

5.3 Nevirapine plasma levels and viral suppression

The number of participants with nevirapine plasma concentrations above 3.0 µg/mL (93.5%) required for viral suppression and with sub-therapeutic levels (less than 3.0 µg/mL, 6.5%) was comparable to those reported by Bertrand *et al.*, 2012 in the Cambodian population. The proportion of participants with sub-therapeutic plasma levels was lower than that reported by Gunda *et al.*, 2013 in cohort of patients with immunological failure in a Tanzanian population. Forty three participants had supra-therapeutic nevirapine plasma levels (above 8.0 µg/mL) and may require monitoring as they may develop adverse reactions (Duong *et al.*, 2004). The sub-group of participants with high levels of nevirapine (above 12.5 µg/mL) had higher prevalence of CYP2B6 983T>C SNPs, CYP2B6 516G>T SNPs and participants who were on tenofovir based regimens compared to the rest of the study population.

The number of participants with nevirapine plasma levels above 4.3 µg/mL that is reported to offer longer lasting viral suppression was 168 (77.4%) was higher than 66% reported by Oluka, 2012 and similar to Calcagno *et al.*, 2012. About 22.6% of participants, lower than that reported by Oluka, 2012, had nevirapine plasma levels below 4.3 µg/mL and were prone to mutant selection that could result in resistance development and failure in virological suppression (Duong *et al.*, 2004).

5.4 Factors affecting nevirapine plasma levels in HIV patients

Factors that affected nevirapine plasma levels were: CYP2B6 genotypes, use of tenofovir based regimens and adherence.

CYP2B6 genotypes independently influenced nevirapine plasma levels and accounted for 16.3% variance. This finding may be attributed to independent influence on nevirapine clearance by CYP2B6 genotypes as reported by Bertrand *et al.*, 2012. CYP2B6 983T>C had a greater impact on nevirapine plasma levels (6.3%) compared to 516G>T (5.8%) similar to a study carried out by Wyen *et al.*, 2008 in a cohort of Caucasians and Blacks. Participants with homozygous CYP2B6 516 TT genotype had higher median nevirapine plasma levels of 8.450 µg/mL compared to heterozygous CYP2B6 516GT 5.518 µg/mL and CYP2B6 GG wild type 5.575 µg/mL genotypes similar to a study carried out by Calcagno *et al.*, 2012. This may be because CYP2B6 516 T variants have reduced expression of the enzyme due to defective splicing that lead to reduced substrate dependent or independent enzyme activity (Zanger *et al.*, 2013).

Participants with homozygous CYP2B6 983CC genotype had higher median nevirapine plasma concentration of 10.60 µg/mL compared to heterozygous CYP2B6 983 TC (6.858 µg/mL) and CYP2B6 983TT wild type (5.533 µg/mL) genotypes. This is similar to a study carried out in the Kenyan population by Oluka *et al.*, 2015 and another study by Wyen *et al.*, 2008. The higher concentrations in homozygous genotype may be attributed to reduced metabolism of nevirapine and hence the poor metabolizer phenotype. This is because CYP2B6 983 CC variants may not express the functional proteins resulting in reduced enzyme activity (Zanger *et al.*, 2013). This could also explain why CYP2B6 983 T>C SNP has a greater impact on nevirapine metabolism than 516 G>T as observed in other studies (Youle *et al.*, 2011).

The effect of CYP2B6 genotypes on nevirapine plasma levels was reflected in the observed metabolic phenotypes where those assigned slow metabolizing characteristics had higher plasma levels compared to intermediate and extensive metabolizers. The relationship between CYP2B6 genotypes and phenotypic characteristics has been reported by Zanger *et al.*, 2013.

Participants who were on switched to tenofovir based ART regimens had higher median nevirapine plasma levels of 6.027 µg/mL compared to those on stavudine and zidovudine based therapy. The influence of tenofovir on nevirapine plasma levels has been reported by Stöhr *et al.*, 2008 although studies carried out to assess drug interactions have reported no relationship (Droste *et al.*, 2006). The observation may as a result of confounding by the number of patients who changed regimens

from stavudine based regimens due to toxicity to tenofovir based therapy. Furthermore, although tenofovir is reported to cause renal toxicity that may reduce renal clearance, the effect on nevirapine may be minimal as less than 3% of nevirapine parent drug is excreted in urine.

Adherence measure by the number of days a participant missed to take the dose in the previous significantly affected nevirapine plasma levels. Participants who never missed to take the drug had higher median nevirapine plasma levels than those who missed the dose regularly. This observation is similar to that reported by Wyen *et al.*, 2008 on the influence of the duration of missing the dose. This variance in plasma levels may be attributed to unstable steady-state nevirapine plasma concentrations in those who never adhered to treatment. Patients who missed there doses had lower levels of nevirapine than those who never missed. Silveira *et al.*, 2015 and Gunda *et al.*, 2013 reported an associated between adherence and success of HAART treatment.

5.5 Nevirapine plasma levels and clinical outcomes in HIV patients.

Nevirapine plasma levels influence the maximum CD4 cell counts achieved during treatment. This finding is dissimilar to one of by Oluka, 2012 who found no association between plasma levels of nevirapine and CD4 change during treatment. Oluka *et al.,* 2015 and Wang *et al.*, 2011 found an association between nevirapine plasma levels and CD4 cell counts. The observation may be explained reported high virological response with high nevirapine levels and better immunological recovery (Veldkamp *et al.*, 2001).

Nevirapine plasma levels did not significantly influence the occurrence and severity of skin and liver adverse reactions. Kappelhoff *et al.*, 2005 and Oluka *et al.,* 2015 also found no association between nevirapine plasma levels and occurrence of toxicity. On the other hand, Dong *et al.*, 2011 reported an association between nevirapine plasma levels and development of rash. The ALT levels on multivariate analysis were affected by nevirapine plasma levels. This observation may be as a result of other confounders such as gender as Oluka *et al.,* 2015 and Kappelhoff *et al.*, 2005 reported no association.

5.6 Study strengths and limitations

A large sample size of 240 participants was studied as compared to previous studies in this population and this was a better representation of the study population. The participants had varied backgrounds with different ethnic origins hence covering a large scope of the population. This is

the first study to evaluate the influence of CYP2B6 983 T>C on nevirapine on both sexes in this population.

The main limitation of this study is that other genotypes that affect nevirapine disposition such as CYP3A4, CYP3A5, CYP2D6, ABCB1 and ABCC1 were not considered that could help in explaining the unaccounted for 80% variance in levels of nevirapine in this population. There were some ethno-linguistic groups that had a few participants such as Kalenjins and Cushites and the nevirapine plasma levels analyzed may be different. Fewer second samples (n=10) were used in assessing intra-patient variability of levels of nevirapine.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Nevirapine plasma levels varied widely in this study population with inter- and intra-patient variability of 46% and 28% respectively. The nevirapine plasma levels were influenced by adherence, use of tenofovir based regimens and CYP2B6 516 G>T and 983 T>C genotypes. The maximum CD4 cell counts attained by patients was associated with nevirapine plasma levels. This implies that genetic and other factors that may influence nevirapine plasma levels affect the maximum therapeutic benefit of HAART. A HPLC-UV method was improved for determination of nevirapine plasma levels with validation parameters within FDA, 2001 standards.

6.2 Recommendations

There is need for assessing population pharmacokinetic parameters affecting nevirapine in this population due to the palpable effect of CYP2B6 genotypes on its levels in plasma. Studies to evaluate the possible pharmacokinetic interaction between tenofovir and nevirapine in this population are needed.

The improved HPLC-UV method described in this study can be validated for other drugs such as zidovudine, trimethoprim, sulphamethoxazole and tenofovir and used in determination of their levels in plasma in therapeutic drug monitoring.

REFERENCES

Angima, J. A. (2015). *The Effects of CYP2B6 Polymorphisms on CD4 Cell count in HIV Patients on Nevirapine Based Regimens at Kenyatta National Hospital*. Retrieved from http://erepository.uonbi.ac.ke

Anjali, J., & Moji, C. (2012). Reversed Phase LC-UV Method Development and Validation for Simultaneous determination of three antiretrovirals: Lamivudine, Zidovudine, Nevirapine and Possible Degradants in a Fixed Dose Pharmaceutical Product. *Journal of Pharmaceutical Technology and Drug Research*, *1*(1), 1–4.

Bertrand, J., Chou, M., Richardson, D. M., Verstuyft, C., Leger, P. D., Mentré, F., Taburet, A.-M., & Haas, D. W. (2012). Multiple genetic variants predict steady-state nevirapine clearance in HIV-infected Cambodians. *Pharmacogenetics and Genomics*, *22*(12), 868–876.

Boehringer Ingelheim Pharmaceuticals Inc. (2013). VIRAMUNE Product Monograph, 1–62.

Calcagno, A., D'Avolio, A., Simiele, M., Cusato, J., Rostagno, R., Libanore, V., Baietto, L., Siccardi, M., Bonora, S., & Di Perri, G. (2012). Influence of CYP2B6 and ABCB1 SNPs on nevirapine plasma concentrations in Burundese HIV-positive patients using dried sample spot devices. *British Journal of Clinical Pharmacology*, *74*(1), 134–140.

Cammett, A. M., MacGregor, T. R., Wruck, J. M., Felizarta, F., Miailhes, P., Mallolas, J., & Piliero, P. J. (2009). Pharmacokinetic assessment of nevirapine and metabolites in human immunodeficiency virus type 1-infected patients with hepatic fibrosis. *Antimicrobial Agents and Chemotherapyy*, *53*(10), 4147–4152.

Carr, D. F., Chaponda, M., Jorgensen, A. L., Castro, E. C., Van Oosterhout, J. J., Khoo, S. H., Lalloo, D. G., Heyderman, R. S., Alfirevic, A., & Pirmohamed, M. (2013). Association of human leukocyte antigen alleles and nevirapine hypersensitivity in a Malawian HIV-infected population. *Clinical Infectious Diseases*, *56*(9), 1330–1339.

Cheeseman, S. H., Mclaughlin, M. M., Koup, R. A., & Andrews, C. A. (1993). Pharmacokinetics of nevirapine : initial single- rising-dose study in humans. *Antimicrobial Agents and Chemotherapy*, *37*(2), 178–182.

Chou, M., Bertrand, J., Segeral, O., Verstuyft, C., Borand, L., Comets, E., Le, C., Becquemont, L., Ouk, V., Mentre, F., Taburet, A. M., Le Tiec, C., Becquemont, L., Ouk, V., Mentre, F., & Taburet, A. M. (2010). Population pharmacokinetic-pharmacogenetic study of nevirapine in HIV-infected Cambodian patients. *Antimicrobial Agents and Chemotherapy*, *54*(10), 4432–

4439.

Cohen, K., Hopkins, J., Ingraham, R., Pargellis, C., Wu, J., Palladino, D., Kinkade, P., Warren, T., Rogers, S., & Adams, J. (1991). Characterization of the binding site for nevirapine (BI-RG-587), a nonnucleoside inhibitor of human immunodeficiency virus type-1 reverse transcriptase. *Journal of Biological Chemistry*, *266*(22), 14670–14674.

Cooper, C. L., & van Heeswijk, R. P. G. (2007). Once-daily nevirapine dosing: A pharmacokinetics, efficacy and safety review. *HIV Medicine*, *8*(1), 1–7.

De Maat, M. M. R., Huitema, A. D. R., Mulder, J. W., Meenhorst, P. L., Van Gorp, E. C. M., & Beijnen, J. H. (2002). Population pharmacokinetics of nevirapine in an unselected cohort of HIV-1-infected individuals. *British Journal of Clinical Pharmacology*, *54*(4), 378–385.

Dong, B. J., Zheng, Y., Hughes, M. D., Frymoyer, A., Lizak, P., Sawe, F., Currier, J. S., & Lockman, S. (2012). Nevirapine (NVP) Pharmacokinetics (PK) and Risk of Rash and Hepatitis among HIV-Infected Sub-Saharan African Women. *AIDS*, *26*(7), 833–841.

Droste, J. H., Kearney, B. P., Hekster, Y. A., & Burger, D. M. (2006). Assessment of drug-drug interactions between tenofovir disoproxil fumarate and the nonnucleoside reverse transcriptase inhibitors nevirapine and efavirenz in HIV-infected patients. *Journal of Acquired Immune Deficiency Syndromes*, *41*(1), 37–43.

Drugbank. (2013). DrugBank Nevirapine (DB00238). Retrieved August 20, 2015, from http://www.drugbank.ca/drugs/DB00238

Duong, M., Golzi, A., Peytavin, G., Piroth, L., Froidure, M., Grappin, M., Buisson, M., Kohli, E., Chavanet, P., & Portier, H. (2004). Usefulness of therapeutic drug monitoring of antiretrovirals in routine clinical practice. *HIV Clinical Trials*, *5*(4), 216–223.

Fan, B., & Stewart, J. T. (2002). Determination of zidovudine/lamivudine/nevirapine in human plasma using ion-pair HPLC. *Journal of Pharmaceutical and Biomedical Analysis*, *28*(5), 903–908.

FDA. (2001). Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER).

Gatanaga, H., Hayashida, T., Tsuchiya, K., Yoshino, M., Kuwahara, T., Tsukada, H., Fujimoto, K., Sato, I., Ueda, M., Horiba, M., Hamaguchi, M., Yamamoto, M., Takata, N., Kimura, A., Koike, T., Gejyo, F., Matsushita, S., Shirasaka, T., Kimura, S., & Oka, S. (2007). Successful

efavirenz dose reduction in HIV type 1-infected individuals with cytochrome P450 2B6 *6 and *26. *Clinical Infectious Diseases*, *45*(9), 1230–1237.

Gunda, D. W., Kasang, C., Kidenya, B. R., Kabangila, R., Mshana, S. E., Kidola, J., Kalluvya, S. E., Kongola, G. W., & Klinker, H. (2013). Plasma Concentrations of Efavirenz and Nevirapine among HIV-Infected Patients with Immunological Failure Attending a Tertiary Hospital in North-Western Tanzania. *PLoS ONE*, *8*(9), 3–9.

Haas, D. W., Gebretsadik, T., Mayo, G., Menon, U. N., Acosta, E. P., Shintani, A., Floyd, M., Stein, C. M., & Wilkinson, G. R. (2009). Associations between CYP2B6 polymorphisms and pharmacokinetics after a single dose of nevirapine or efavirenz in African Americans. *The Journal of Infectious Diseases*, *199*(6), 872–880.

Hamrapurkar, P., Phale, M., Patil, P., & Shah, N. (2010). Determination of Nevirapine in Human Plasma by High Performance Liquid Chromatography with Ultraviolet Detection. *International Journal of PharmTech Research*, *2*(2), 1316–1324.

Hansana, V., Sanchaisuriya, P., Durham, J., Sychareun, V., Chaleunvong, K., Boonyaleepun, S., & Schelp, F. P. (2013). Adherence to antiretroviral therapy (ART) among people living with HIV (PLHIV): a cross-sectional survey to measure in Lao PDR. *BMC Public Health*, *13*(1), 617. http://doi.org/10.1186/1471-2458-13-617

International Pharmacopoeia. (2015). Monographs: Dosage forms: Specific monographs: Zidovudine , lamivudine and nevirapine tablets (Zidovudini , lamivudini et nevirapini compressi). In *The International Pharmacopoeia* (Fifth Edit). Retrieved from http://apps.who.int/phint/pdf/b/Jb.6.2.2.144.pdf

Kabra, V., Agrahari, V., Karthikeyan, C., & Trivedi, P. (2009). Simultaneous quantitative determination of zidovudine and nevirapine in human plasma using isocratic, reverse phase high performance liquid chromatography. *Tropical Journal of Pharmaceutical Research*, *8*(1), 79–86.

Kappelhoff, B. S., Rosing, H., Huitema, A. D. R., & Beijnen, J. H. (2003). Simple and rapid method for the simultaneous determination of the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma using liquid chromatography. *Journal of Chromatography B*, *792*(2), 353–362.

Kappelhoff, B. S., Van Leth, F., MacGregor, T. R., Lange, J. M. A., Beijnen, J. H., & Huitema, A. D. R. (2005). Nevirapine and efavirenz pharmacokinetics and covariate analysis in the 2NN study. *Antiviral Therapy*, *10*(1), 145–155.

Kappelhoff, B. S., Van Leth, F., Robinson, P. A., MacGregor, T. R., Baraldi, E., Montella, F., Uip, D. E., Thompson, M. A., Russell, D. B., Lange, J. M. A., Beijnen, J. H., & Huitema, A. D. R. (2005). Are adverse events of nevirapine and efavirenz related to plasma concentrations ? *Antiviral Therapy*, *10*(4), 489–498.

Kesselring, A. M., Wit, F. W., Sabin, C. A., Lundgren, J. D., Gatell, J. M., Rauch, A., Montaner, J. S., Wolf, F. De, Reiss, P., Mocroft, A., & Gill, M. J. (2009). Risk factors for treatment-limiting toxicities in patients starting nevirapine-containing antiretroviral therapy. *AIDS*, *23*(13), 1689–1699.

Kondo, W., Carraro, E. A., Prandel, E., Dias, J. M., Perini, J., Macedo, R. L. De, Cornelsen, T. C., Sbalquiero, R., & Sasaki, M. das G. (2007). Nevirapine-induced side effects in pregnant women: experience of a Brazilian university hospital. *The Brazilian Journal of Infectious Diseases*, *11*(6), 544–548.

Kumar, D. A., Babu, M. V. N., Rao, J. V. L. N. S., & Rao, V. J. (2010). Simultaneous Determination of Lamivudine, Zidovudine and Nevirapine in Tablet Dosage Forms by RP-HPLC Method. *Journal of Chemistry*, *7*(1), 180–184.

Lam, Y. W. F., & Cavallari, L. H. (2013). *Principles of Pharmacogenomics: Pharmacokinetic, Pharmacodynamic, and Clinical Implications*. Elsevier. http://doi.org/10.1016/B978-0-12-391918-2.00001-9

Lamorde, M., Byakika-Kibwika, P., Okaba-Kayom, V., Ryan, M., Coakley, P., Boffito, M., Namakula, R., Kalemeera, F., Colebunders, R., Back, D., Khoo, S., & Merry, C. (2011). Nevirapine pharmacokinetics when initiated at 200 mg or 400 mg daily in HIV-1 and tuberculosis co-infected Ugandan adults on rifampicin. *Journal of Antimicrobial Chemotherapy*, *66*(1), 180–183.

Lipinski, C. A. (2000). Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological and Toxicological Methods*, *44*(1), 235–249.

Liptrott, N. J., Pushpakom, S., Wyen, C., Fätkenheuer, G., Hoffmann, C., Mauss, S., Knechten, H., Brockmeyer, N. H., Borge, E. H.-, Siccardi, M., Back, D. J., Khoo, S. H., Pirmohamed, M., & Owen, A. (2013). Association of ABCC10 polymorphisms with nevirapine plasma concentrations in the German Competence Network for HIV /AIDS. *Pharmacogenetics and Genomics*, *22*(1), 10–19.

Mahungu, T. W., Smith, C. J., Turner, F., Egan, D., Youle, M., Johnson, M. A., Khoo, S., Back, D., & Owen, A. (2009). Cytochrome P450 2B6 516G>T is associated with plasma

concentrations of nevirapine at both 200 mg twice daily and 400 mg once daily in an ethnically diverse population. *HIV Medicine*, *10*(5), 310–317.

Makori, J. O., Ambetsa, M. O., Osanjo, G. O., Oluka, M., Maitai, C. K., Guantai, A. N., Mcclelland, S., & Okalebo, F. A. (2015). Incidence and Risk Factors of Renal Dysfunction in Patients on Nevirapine-Based Regimens at a Referral Hospital in Kenya. *African Journal of Pharmacology and Therapeutics*, *4*(2), 48–58.

Mannheimer, S. B., Mukherjee, R., Hirschhorn, L. R., Dougherty, J., Celano, S. A., Ciccarone, D., Graham, K. K., Mantell, J. E., Mundy, L. M., Eldred, L., Botsko, M., & Finkelstein, R. (2006). The CASE adherence index: A novel method for measuring adherence to antiretroviral therapy. *AIDS Care*, *18*(7), 853–861.

Minzi, O. M. S., & Ngaimisi, E. (2010). Bioanalytical method for determination of Nevirapine in-vivo in resource constrained laboratories. *Journal of Chemical and Pharmaceutical Research*, *2*(2), 431–439.

Montaner, J. S., Reiss, P., Cooper, D., Vella, S., Harris, M., Conway, B., Wainberg, M. A., Smith, D., Robinson, P., Hall, D., Myers, M., & Lange, J. M. (1998). A randomized, double-blind trial comparing combinations of nevirapine, didanosine, and zidovudine for HIV-infected patients: the INCAS Trial. Italy, The Netherlands, Canada and Australia Study. *The Journal of the American Medical Association*, *279*(12), 930–937.

NASCOP. (2014). Kenya HIV Estimates.

NASCOP. (2016). *Guidelines on Use of Antiretroviral Drugs For Treating and Preventing HIV Infection*. Retrieved from http://www.nascop.or.ke/index.php/new-guidelines/#

Nellen, J. F. J. B., Damming, M., Godfried, M. H., Boer, K., Van Der Ende, M. E., Burger, D. M., De Wolf, F., Wit, F. W. N. M., & Prins, J. M. (2008). Steady-state nevirapine plasma concentrations are influenced by pregnancy. *HIV Medicine*, *9*(4), 234–238.

Oluka, M. N. (2012). *Pharmacogenetics of drug metabolizing enzymes and clinical implications in selected Kenyan populations*. Retrieved from http://erepository.uonbi.ac.ke/handle/11295/8988

Oluka, M. N., Okalebo, F. A., Guantai, A. N., McClelland, R. S., & Graham, S. M. (2015). Cytochrome P450 2B6 genetic variants are associated with plasma nevirapine levels and clinical response in HIV-1 infected Kenyan women: a prospective cohort study. *AIDS Research and Therapy*, *12*(10), 1–9.

Penzak, S. R., Kabuye, G., Mugyenyi, P., Mbamanya, F., Natarajan, V., Alfaro, R. M., Kityo, C., Formentini, E., & Masur, H. (2007). Cytochrome P450 2B6 (CYP2B6) G516T influences nevirapine plasma concentrations in HIV-infected patients in Uganda. *HIV Medicine*, *8*(2), 86–91.

PharmGKB.org. (2015). Nevirapine metabolic Pathway and CYP 2B6 haplotypes. Retrieved August 21, 2015, from https://www.pharmgkb.org/pathway/PA165950411

Ramachandran, G., Kumar, A. K. H., Vasantha, M., Shah, I., & Swaminathan, S. (2010). Plasma efavirenz in HIV infected children treated with generic antiretroviral drugs in India. *Indian Pediatrics*, *47*(10), 890–891.

Ranaware, P. S., Ingle, A. M., Ladke, A., Madgulkar, A. R., & Damle, M. C. (2012). Determination of nevirapine in human plasma by HPLC. *Journal of Chemical and Pharmaceutical Research*, *4*(6), 3003–3009.

Ratanasuwan, W., Jariyasethpong, T., Anekthananon, T., Intalapaporn, P., Kongpatanakul, S., Pongnarin, P., Wasinrapee, P., Chantharojwong, N., Raengsakulrach, B., Peters, P. J., McNicholl, J., McConnell, M. S., & Weidle, P. J. (2012). Association of Nevirapine Levels with Rash or Hepatotoxicity Among HIV-Infected Thai Women. *The Open AIDS Journal*, *6*(12), 266–73.

Ravisankar, P., & Rao, G. D. (2013). Development of a new RP-HPLC method for estimation of nevirapine in tablet dosage form. *International Journal of Pharmacy and Pharmaceutical Sciences*, *5*(3), 6–12.

Ren, C., Fan-Havard, P., Schlabritz-Loutsevitch, N., Ling, Y., Chan, K. K., & Liu, Z. (2010). A sensitive and specific liquid chromatography/tandem mass spectrometry method for quantification of nevirapine and its five metabolites and their pharmacokinetics in baboons. *Biomedical Chromatography*, *24*(7), 717–726.

Riska, P., Lamson, M., Macgregor, T., Sabo, J., Hattox, S., Pav, J., & Keirns, J. (1999). Disposition and biotransformation of the antiretroviral drug nevirapine in humans. *Drug Metabolism and Disposition*, *27*(8), 895–901.

Stöhr, W., Back, D., Dunn, D., Sabin, C., Winston, A., Gilson, R., Pillay, D., Hill, T., Ainsworth, J., Pozniak, A., Leen, C., Bansi, L., Fisher, M., Orkin, C., Anderson, J., Johnson, M., Easterbrook, P., Gibbons, S., & Khoo, S. H. (2008). Factors influencing efavirenz and nevirapine plasma concentration: Effect of ethnicity, weight and co-medication. *Antiviral Therapy*, *13*(5), 675–685.

Svensson, E., van der Walt, J.-S. S., Barnes, K. I., Cohen, K., Kredo, T., Huitema, A., Nachega, J. B., Karlsson, M. O., & Denti, P. (2012). Integration of data from multiple sources for simultaneous modelling analysis: experience from nevirapine population pharmacokinetics. *British Journal of Clinical Pharmacology*, *74*(3), 465–476.

Swaminathan, S., Ramachandran, G., Kumar, H., Kupparam, A., Mahalingam, V., Karunaianandham, R., Sikhamani, R., Kupparam, H. K. A., Mahalingam, V., Soundararajan, L., Kannabiran, B. P., Navaneethapandian, P. G. D., Shah, I., Karunaianandham, R., & Sikhamani, R. (2011). Factors influencing plasma nevirapine levels: A study in HIV-infected children on generic antiretroviral treatment in India. *Journal of Antimicrobial Chemotherapy*, *66*(6), 1354–1359.

Thorn, C. F., Lamba, J. K., Lamba, V., Klein, T. E., & Altman, R. B. (2010). PharmGKB summary: very important pharmacogene information for CYP2B6. *Pharmacogenetics and Genomics*, *20*(8), 520–523.

Turpeinen, M., & Zanger, U. M. (2012). Cytochrome P450 2B6: Function, genetics, and clinical relevance. *Drug Metabolism and Drug Interactions*, *27*(4), 185–197.

UNAIDS. (2013). Global report: UNAIDS report on the global AIDS epidemic 2013. Retrieved from www.unaids.org/.../unaids/.../2013/gr2013/UNAIDS_Global_Report_2013

Veldkamp, A. I., Weverling, G. J., Lange, J. M., Montaner, J. S., Reiss, P., Cooper, D. A., Vella, S., Hall, D., Beijnen, J. H., & Hoetelmans, R. M. (2001). High exposure to nevirapine in plasma is associated with an improved virological response in HIV-1-infected individuals. *AIDS (London, England)*, *15*(9), 1089–1095.

Vogel, M., Bertram, N., Wasmuth, J. C., Emmelkamp, J., Rockstroh, J. K., & Reichel, C. (2010). Determination of nevirapine in plasma by GC-MS. *Journal of Chromatographic Science*, *48*(2), 91–94.

Wang, H., & Tompkins, L. M. (2008). CYP2B6: New insights into a historically overlooked cytochrome P450 isozyme. *Current Drug Metabolism*, *9*(7), 598–610.

Wang, J., Kou, H., Fu, Q., Han, Y., Qiu, Z., Zuo, L., Li, Y., Zhu, Z., Ye, M., Ma, Q., & Li, T. (2011). Nevirapine plasma concentrations are associated with virologic response and hepatotoxicity in Chinese patients with HIV infection. *PLoS ONE*, *6*(10), 1–7.

WHO. (2016). Consolidated Guidelines on HIV Prevention, Diagnosis, Treatment and Care for Key Populations-2016 Update. Retrieved from

http://www.who.int/hiv/pub/guidelines/keypopulations-2016/en/

Wyen, C., Hendra, H., Vogel, M., Hoffmann, C., Knechten, H., Brockmeyer, N. H., Bogner, J. R., Rockstroh, J., Esser, S., Jaeger, H., Harrer, T., Mauss, S., van lunzen, J., Skoetz, N., Jetter, A., Groneuer, C., Fätkenheuer, G., Khoo, S. H., Egan, D., Back, D. J., & Owen, A. (2008). Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. *Journal of Antimicrobial Chemotherapy*, *61*(4), 914–918.

Youle, M., Schipani, A., Wyen, C., Mahungu, T., Hendra, H., Egan, D., Siccardi, M., Davies, G., Khoo, S., Fätkenheuer, G., Youle, M., Rockstroh, J., Brockmeyer, N. H., Johnson, M. A., Owen, A., & Back, D. J. (2011). Integration of population pharmacokinetics and pharmacogenetics: An aid to optimal nevirapine dose selection in HIV-infected individuals. *Journal of Antimicrobial Chemotherapy*, *66*(6), 1332–1339.

Zanger, U. M., & Klein, K. (2013). Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): Advances on polymorphisms, mechanisms, and clinical relevance. *Frontiers in Genetics*, *4*(24), 1–12.

Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & Therapeutics*, *138*(1), 103–41.

Zhou, X. J., Sheiner, L. B., D'Aquila, R. T., Hughes, M. D., Hirsch, M. S., Fischl, M. A., Johnson, V. A., Myers, M., & Sommadossi, J. P. (1999). Population pharmacokinetics of nevirapine, zidovudine, and didanosine in human immunodeficiency virus-infected patients. The National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group Protocol 241 Investigators. *Antimicrobial Agents and Chemotherapy*, *43*(1), 121–128.

APPENDICES

APPENDIX A: KNH/UoN ethical approval for the study

KENYATTA NATIONAL HOSPITAL APPROVED
15 FEB 2016
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15th February, 2016

Titus Masai Shapaya
Reg. No. U52/74967/2014
Dept. of Pharmacology and Pharmacognosy
School of Pharmacy
College of Health Sciences
University of Nairobi

Dear Titus,

Revised research proposal: Evaluation of Genetic Polymorphism of CYP 2B6 on the Population Pharmacokinetics of Nevirapine in HIV Patients in Kenyatta National Hospital (P655/10/2015)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH-UoN ERC) has reviewed and **approved** your above proposal. The approval period is from 15th February 2016 – 14th February 2017.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study.
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

Yours sincerely,

PROF. M.L. CHINDIA
SECRETARY, KNH-UoN ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director, CS, KNH
The Chair, KNH-UoN ERC
The Assistant Director, Health Information, KNH
The Dean, School of Pharmacy, UoN
The Chair, Dept. of Pharmacology and Pharmacognosy, UoN
Supervisors: Dr. Margaret N. Oluka, Dr. Hezekiah Chepkwony, Dr. Timothy Kamanu Kuria

APPENDIX B: Initial KNH/UoN ethical approval - Makori *et al., 2015*

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Ref: KNH-ERC/A/122

Link:www.uonbi.ac.ke/activities/KNHUoN

7th May 2014

Dr. Makori Jones Obonyo
Dept of Pharmacology and Pharmacognosy
School of Pharmacy
University of Nairobi

Dear Dr. Obonyo

RESEARCH PROPOSAL: PREVALENCE AND RISK FACTORS FOR SYMPTOMS OF HEPATOTOXICITY IN PATIENTS ON NEVIRAPINE AT KENYATTA NATIONALHOSPITAL (P10/01/2014)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and approved your above proposal. The approval periods are 7th May 2014 to 6th May 2015.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

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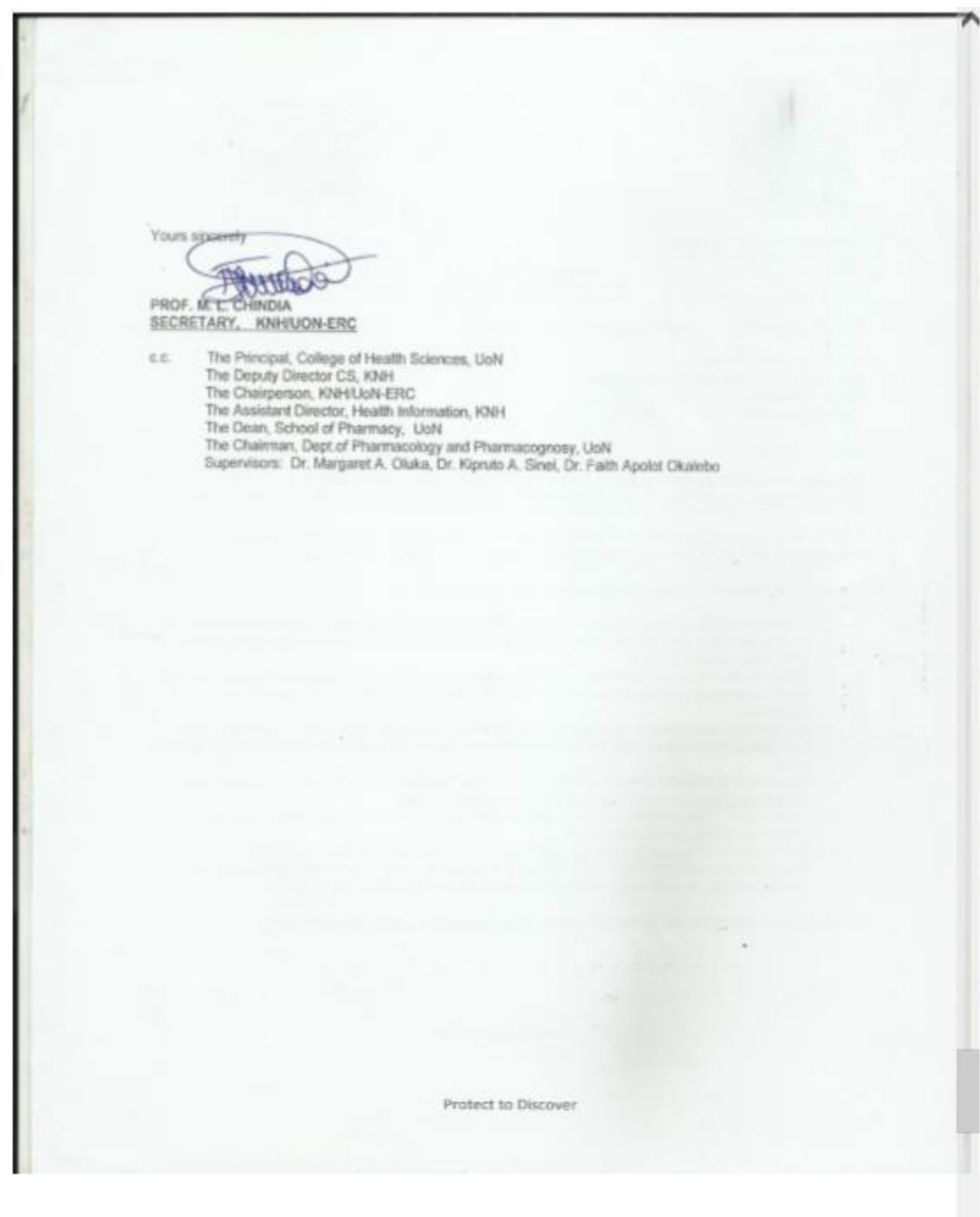

Yours sincerely

PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chairperson, KNH/UoN-ERC
The Assistant Director, Health Information, KNH
The Dean, School of Pharmacy, UoN
The Chairman, Dept.of Pharmacology and Pharmacognosy, UoN
Supervisors: Dr. Margaret A. Oluka, Dr. Kipruto A. Sinei, Dr. Faith Apolot Okalebo

Protect to Discover

APPENDIX C: Nevirapine concentrations and peak area ratios used for calibration curves

Concentrations for high nevirapine plasma levels calibration curve

Calibration standards (µg/mL)	**Peak area ratio (nevirapine/carbamazepine)**
2.5	0.1174
5	0.3798
7.5	0.6341
10	0.8535
15	1.3421
20	1.9172
25	2.3985

Concentrations for low nevirapine plasma levels calibration curve

Calibration standards (µg/mL)	**Peak area ratio (nevirapine/carbamazepine)**
0.5	0.0109
0.75	0.0187
1	0.0291
1.5	0.0500
2	0.0681
2.5	0.0922

APPENDIX D: **Shapiro-Wilk test for normality**

Summary of nevirapine plasma concentration (n=240)

Statistic	Concentration(µg/mL)		
		Smallest	**Highest**
1%	0.4537	0.4537	15.2673
5%	2.6502	0.4537	16.2875
10%	3.5781	0.4537	16.6732
25%	4.5374		19.8211
50% (median)	5.6751		
75%	7.2034		
90%	9.3451		
95%	12.9485		
99%	16.2875		
Mean	6.1847		
Std Dev	2.8470		
Variance	8.1059		
Skewness	1.4060		
Kurtosis	6.7131		

APPENDIX E: Bivariable analysis of potential predictors of nevirapine plasma levels

Variable	Characteristic	n	Nevirapine levels (µg/mL), Median [IQR]	P value
Sex	Female	167	5.699[4.7066, 7.4152]	0.5011
	Male	50	5.573[4.299, 6.89052]	
Alcohol use	No	155	5.738[4.5689, 7.2016)	0.9114
	Yes	62	5.6198[4.3837, 7.5433]	
Delay dose	Most of the time	3	3.7689[0.2684, 7.2016]	0.4210
	Rarely	130	5.6565[4.8194, 6.8905]	
	Never	84	5.9540[4.3275, 7.7636]	
Marital status	Married	138	5.6751[4.3837,7.2051]	0.9766
	Single	53	6.0639[4.9990, 7.5433]	
	Divorced	3	5.5187[3.3985, 13.7198]	
	Widowed	22	5.6336[4.7849, 7.1814]	
	Separated	1	5.5166[5.5166,5.5166]	
Education	Primary	42	5.03315[4.094131, 6.89052]	0.1140
	High school	108	5.67102[4.402922, 6.916676]	
	Diploma	50	5.810809[4.967195, 7.515927]	
	Degree	17	7.719555[5.512238, 8.322601]	
Occupation	Unemployed	17	5.342983[4.038748, 8.323254]	0.9328
	Employed	98	5.633525[4.383747, 7.719555]	
	Self employed	101	5.738416[4.863444, 6.89052]	
	Other	1	6.487601[6.487601, 6.487601]	
Ethnicity	Kikuyu	87	5.92254[4.215169, 7.205156]	0.9826
	Luo	37	5.689567[4.954152, 7.181443]	
	Kamba	33	5.614733[4.437263, 6.976283]	
	Luhya	23	5.699078[4.009455, 7.820507]	
	Meru/Embu	5	5.643402[5.342983, 13.71798]	
	Kisii	16	5.848176[4.824178, 9.047332]	
	Kalenjin	2	8.12716[3.055254, 13.19907]	
	Cushite	1	5.462118[5.462118, 5.462118]	
	Other	5	6.314197[5.51316, 6.37647]	
	Missing	7	5.518745[3.768951, 6.89052]	
Smoking	No	212	5.694322[4.499749, 7.31988]	0.4801
	Yes	5	5.024325[4.954152, 6.073194]	
Frequency of alcohol intake	Never	154	5.774778[4.568915, 7.181443]	0.4982
	Occasionally	62	5.619883[4.383747, 7.543366]	
	Regularly	1	8.300852[8.300852, 8.300852]	
Comorbidities	Non	163	5.660692[4.568915, 7.415228]	0.2910
	Hypertension	32	5.488806[3.872336, 7.428361]	
	Diabetes	2	8.292894[6.89052, 9.695268]	
	PUD	4	6.202572[6.089626, 10.77862]	
	Asthma	3	4.299765[4.017365, 5.139494]	
	Chronic pain	5	6.329865[6.053795, 6.570434]	
	Cancer	2	4.452228[3.398574, 5.505882]	
	Depression	1	7.201658[7.201658, 7.201658]	
	URTI	1	5.516653[5.516653, 5.516653]	
	Anemia	1	8.577377[8.577377, 8.577377]	
	Others	3	3.811362[3.055254, 8.790956]	
Allergies of patients	None	189	5.643402[4.437263, 7.201658]	0.8658
	Sulphur	14	5.835638[5.325964, 8.212808]	
	NSAIDS	3	6.570434[5.907196, 8.790956]	
	Proteins	3	7.415228[5.000363, 10.71371]	
	Quinine	3	6.000886[4.097415, 8.577377]	
	Stavudine	1	6.19643[6.19643, 6.19643]	
	Dust	2	5.991779[4.038748, 7.94481]	
	Efavirenz	1	4.383747[4.383747, 4.383747]	
	Dapsone	1	6.053795[6.053795 6.053795]	
Switched regimen	No	136	5.619883[4.207953, 6.865078]	0.0327
	Yes	80	6.02734[4.853414, 8.311727]	
Initiation regimen	TDF,3TC,NVP	62	5.822806[4.404148, 7.415228]	0.0591
	AZT,3TC,NVP	74	5.611026[4.215169, 6.392696]	
	D4T,3TC,NVP	77	6.073194[4.843384, 8.212808]	
	AZT,3TC,NVP	1	4.81636[4.81636, 4.81636]	

	TDF,3TC,EFV	3	8.577377[6.181641, 16.67327]	
Second regimen	TDF,3TC,NVP	73	6.053795[4.999078, 8.241851]	0.4719
	AZT,3TC,NVP	9	4.81636[3.744552, 12.93742]	
Baseline renal disease	No	193	5.660692[4.620921, 7.224532]	0.4304
	Yes	4	7.193299[4.984898, 8.56849]	
Presence of renal disease	No	201	5.738416[4.568915, 7.415228]	0.8395
	Yes	12	5.465244[4.607718, 7.214844]	
Previous use of efavirenz	Not used	213	5.660692[4.49342, 7.205156]	0.2041
	Used	4	7.379509[5.499001, 12.62532]	
CYP2B6 983T>C genotype	Wild type	182	5.533836[4.299765, 6.919045]	0.0008
	Heterozygous	19	6.858281[6.000886, 8.322601]	
	Homozygous	2	10.60891[8.267691, 12.95014]	
CYP2B6 983T>C phenotype	EM	180	5.57553[4.325878, 6.947664]	0.0009
	IM	19	6.858281[6.000886, 8.322601	
	Slow	2	10.60891[8.267691, 12.95014]	
CYP2B6 516G>T genotype	Wild type	88	5.57553[4.285596, 6.762543]	0.0001
	Heterozygous	73	5.518745[4.568915, 6.392696]	
	Homozygous	32	8.450315[6.346445, 9.784687]	
Ethno-linguistic groups	Bantu	172	5.679885[4.420705, 7.465578]	0.9664
	Nilotes	39	5.689567[4.81636, 7.719555]	
	Cushite	1	5.462118[5.462118, 5.462118]	
	Other	5	6.314197[5.51316, 6.37647]	
CYP2B6 516G>T phenotype	EM	87	5.548927[4.271427, 6.817093]	0.0001
	IM	73	5.518745[4.568915, 6.392696]	
	Slow	32	8.450315[6.346445, 9.784687]	
CYP2B6 genotypes	Wild type	88	5.57553[4.285596, 6.762543	0.0651
	With SNPs	105	5.956121[4.863444, 7.719555]	
Combined phenotype	EM	70	5.028522[4.017365, 6.19643]	0.0001
	IM	83	5.689567[4.954152, 6.851685]	
	Slow	35	8.667023[7.515927, 10.03117]	
Reported adherence	No	41	5.512238[4.215169, 6.063957]	0.0556
	Yes	176	5.939331[4.663768, 7.618651]	
Current regimen	TDF,3TC,NVP	132	5.978503[4.958443, 7.752004]	0.0462
	AZT,3TC,NVP	77	5.607318[4.017365, 6.570434]	
	D4T,3TC,NVP	8	5.016289[4.066439, 6.276244]	

Correlation between nevirapine plasma levels and potential predictors

Variable(n=217)	Correlation coefficient	P value
Age (years)	-0.0554	0.4164
Initial weight (kg)	-0.0728	0.2859
Height (cm)	-0.0423	0.5357
Initial BMI	-00540	0.4286
Days missed	0.1487	0.0285
Last missed dose	0.0971	0.1540
Index score	0.1317	0.0527
Duration of therapy	0.1626	0.0176
Days missed corrected	0.1487	0.0285
Adherence score	0.1317	0.0527