ANTIRETROVIRAL DRUG DETECTION IN URINE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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DECLARATION

I, Margerite Potgieter hereby declare that this dissertation represents my own work and that I have not submitted it to this or any other institution in application for admission to a degree, diploma or any other qualification.

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Signature Date

ABSTRACT

Antiretroviral drug detection in urine using high performance liquid chromatography

Background: Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) was first recognised in 1981. The explosive worldwide pandemic has become the leading cause of death in South Africa. An estimated 10.2% of the South African population is currently infected with HIV/AIDS and more than 2500 new infections occur on a daily basis, revealing South Africa as the country with the highest infection rate worldwide. A devastating fifteen percent of new infections worldwide occur in South Africa alone despite extensive roll out programs implemented by the department of health.

Extensive research and drug development was conducted since its discovery. Many antiretroviral drugs (ARVs) have been developed and various treatment protocols have been implemented to combat the epidemic, but no cure has been discovered yet. When a patient is first diagnosed, an aggressive treatment regimen of 3 or more antiretrovirals is prescribed to reduce the viral load and increase the life expectancy of a positive patient.

The ARVs are well absorbed with good bioavailability after oral administration. The drugs are metabolised by the liver and mainly excreted in the urine. Unfortunately all ARVs pose the threat of toxicity. The roll out program implemented by the department of health now reaches more infected patients than ever before. The increased availability of these drugs increases the likelihood of misuse or accidental toxicity. Treatment of such a patient can be problematic and very costly if a correct diagnosis cannot be made.

Increased requests for therapeutic drug monitoring of the ARVs to establish patient compliance and to monitor pharmacokinetic interactions with other drugs was also a key motivator for the development of a method for ARV determination in our area. The current high performance liquid chromatography (HPLC) method developed by the Department of Pharmacology/Toxicology can identify the presence of lamivudine, abacavir, zidovudine, nevirapine and efavirenz in urine. Continued research in the field of drug development launches new drugs on the market on a regular basis. Treatment

regimens change as the new drugs become available, and older line ARVs become outdated. This dynamic and TDM requests warrant the necessity to extend the application of the method to accommodate new ARVs. It is also important as part of method validation to analyse samples from patients using antiretroviral drugs to see if the method that was developed complies with the analytical criteria required.

Methods: Lamivudine, abacavir, zidovudine, nevirapine, efavirenz, stavudine, lopinavir and tenofovir were extracted from urine using a liquid-liquid extraction and analysed with a Hewlett-Packard (HP) 1090 Liquid Chromatograph. The eluent was monitored and full spectra of each peak were recorded at 210 nanometre (nm) with an ultraviolet-visible spectrophotometry (UV/VIS) Diode Array detector.

Results: Spectra recorded for each peak showed excellent reproducibility and agreement to database recorded spectra. The calibration curves of the ARVs were linear and reproducible. Recovery exceeded 74%. Validation data for control samples show inter – assay precision with a coefficient of variation (CV) of < 16.9%. Short-term stability testing indicated that concentrations remained stable at room temperature and refrigeration, but freezing of samples is not advised.

Conclusion: A qualitative HPLC method for the identification of ARVs in urine was developed and validated. This analysis can be used as a component of an emergency toxicological screening in suspected overdoses. It requires minimal sample pre–treatment, small sample volume, minimal expertise, and provides results within 30 minutes.

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LIST OF ABBREVIATIONS

3TC	Lamivudine
α	Column selectivity
k’	Capacity
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
AIVR	Acquired immunodeficiency virus-associated retrovirus
ARV	Antiretroviral
ARVs	Antiretroviral drugs
ATV	Atazanavir
ATV/r	Atazanavir/Ritonavir
AUC	Area under the curve
AZT	Zidovudine
BMI	Body mass index
CC-CKR- 5	Chemokine receptor gene 5
CCR5	Chemokine receptor 5
CD4	Cluster of differentiation 4
CDC	Centre for disease control
CH_2Cl_2	Dichloromethane
$CH_3CH(OH)CH_3$	Isopropanol
Cmax	Maximum concentration of the drug reached in the plasma
CNS	Central nervous system
CSF	Cerebrospinal fluid
CUT	Central University of Technology
CV	Coefficient of variation
CXCR4	Chemokine (C-X-C Motif) Receptor 4
d4T	Stavudine
ddI	Didanosine
DLV	Delavirdine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRV	Darunavir
EFV	Efavirenz

ENF	Enfuvirtide
ESI-MS/MS	Electrospray tandem mass spectrometry
ETR	Etravirine
EVG	Elvitegravir
FDC	Fixed-dose combination
FPV	Fosamprenavir
FTC	Emtricitabine
GC/MS	Gas chromatography/Mass spectrometry
GC/MS-SIM	Gas chromatography-mass spectroscopy in selected ion monitoring mode
GCS	Glascow coma scale
g/dl	Gram per decilitre
GI	Gastrointestinal
GLP	Good laboratory practice
gp41	Glycoprotein 41
H_3PO_4	Phosphoric Acid
Hb	Haemoglobin
HETP	Highest equivalent of theoretical plates
HIV	Human Immunodeficiency virus
HP	Hewlett-Packard
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography-mass spectrometry
HR1	Heptad repeat 1
HR2	Heptad repeat 2
HTLV –III	Human T cell lymphotropic virus type III
ICH	International conference on harmonisation
ID	Identification
IDV	Indinavir
IV	Intravenous
LAV	Lymphadenopathy associated virus
LC	Liquid chromatography
LC/MS	Liquid chromatography-mass spectrometry
L/kg	Litres per kilogram

LPV	Lopinavir
LPV/r	Lopinavir/ritonavir
K_2CO_3	Potassium bicarbonate
KS	Kaposi's sarcoma
MIC	Minimum inhibitory concentration
ml/min	Millilitres per minute
mm^3	Cubic millimetre
MMWR	Morbidity and mortality weekly report
MS	Mass spectrometry
MVC	Maraviroc
N	Quantity of theoretical plates
$NaHCO_3$	Sodium hydroxy carbonate
nm	Nanometre
NNRTI	Non-nucleoside reverse transcriptase inhibitors
nRTI	Nucleoside and nucleotide reverse transcriptase inhibitors
NSAIDs	Non-steroidal anti-inflammatory drugs
NVF	Nelfinavir
NVP	Nevirapine
P	Statistical significance
PIs	Protease Inhibitors
R	Resolution
r	Correlation coefficient
r^2	Coefficient of determination
RAL	Raltegravir
RAM	Restricted access material
rpm	Revolutions per minute
RPV	Rilpivirine
Rx	Treatment
SQV	Saquinavir
TB	Tuberculosis
TDF	Tenofovir
TDM	Therapeutic drug monitoring
TEAP	Tetraethylammonium phosphate
TOF	Time of flight mass spectrometry

TPV	Tipranavir
^{t}R1	Retention time of peak 1
^{t}R2	Retention time of peak 2
^{t}m	Retention time of solvent front
µg/ml	Micrograms per millilitre
µl	micro litre
u	Flow rate
UGT1A1	Uridine diphosphate glucuronyl transferse 1A1
UGT1A3	UDP glucuronosyltransferase 1 family, polypeptide A3
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
UV/VIS	Ultraviolet-visible spectrophotometry
V3 loop	third variable loop
^{W}B1	Peak width at base of peak 1
^{W}B2	Peak width at base of peak 2

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1. BACKGROUND

1.1 INTRODUCTION

Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) have become a leading cause of death in South Africa. South Africa has one of the most explosive HIV epidemics in the world with about 2500 new infections every day; it is estimated that 10.2% of the South African population is infected with the virus (Infographic, 2014). Fifteen percent of all the estimated HIV infections in the world occur in South Africa (5.51 million), the highest infection rate in the world, while South Africa constitutes only 0.68% of the world population (Statistics South Africa 2014).

Since AIDS was first recognized in 1981, development of drugs for the treatment and prophylaxis of HIV have been researched extensively, but no successful cure for the pandemic has been discovered yet. Many antiretroviral drugs (ARVs) have been developed since, increasing the quality of life and the life expectancy of infected patients (Rang, Dale & Ritter 1999).

After a patient is diagnosed with HIV, treatment commences with a combination of three or more antiretroviral agents before immunodeficiency becomes evident, and the aim of treatment is to reduce the plasma viral concentration as much as possible, and for as long as possible (Denelsbeck, 2006). The ARVs are well absorbed after oral administration, with relatively good bioavailability. The ARVs are metabolized by the liver and excreted in the urine (Rang *et al*. 1999).

Of importance with the use of ARVs is therapeutic drug monitoring (TDM) related. Poor compliance and pharmacokinetic problems also warrants the monitoring of these drugs in patients using ARV treatment (Slish, 2008). Unfortunately all of these drugs pose the threat of toxicity, and where the use of ARVs become ever increasing in the population, so does the tendency in using these ARVs in overdose (Holtt & Ju, 2006). Diagnosis and treatment of patients with a suspected overdose are cumbersome and extremely costly.

Because of new drugs that become available and an increase in requests for a method to identify ARVs, it was necessary to develop and evaluate this method. Although the purpose of this method is only the identification of ARVs in urine, it is also important as part of method validation and good laboratory practice (GLP) to analyse samples from patients using

antiretroviral drugs to see if the method that was developed complies with the analytical criteria required.

1.2 RATIONALE

South Africa has the highest HIV infection rate in the world. Extensive rollout of ARV treatment has been mobilized to treat the growing HIV positive population of South Africa. The current HPLC method developed by the Department of Pharmacology/Toxicology, University of the Free State in Bloemfontein, South Africa, can identify the presence of lamivudine, abacavir, zidovudine, nevirapine and efavirenz in urine. Because of new drugs that become available and requests for TDM it is necessary to extend the application of the method. It is also important as part of method validation and GLP to analyse samples from patients using ARVs to see if the method that was developed complies with the analytical criteria required (Micromedex, 2013).

1.3 AIM

The aim of the study is to detect the presence of antiretroviral drugs in urine using high performance liquid chromatography.

1.4 OBJECTIVES

Further method development and investigation was necessary to identify and include stavudine, didanosine, lopinavir, ritonavir and tenofovir in the spectrum of antiretroviral drugs detectable by the method used by Pharmacology which only included the identification of lamivudine, abacavir, zidovudine, nevirapine and efavirenz in urine.

1.4.1 Modification of a qualitative HPLC method for the identification of antiretroviral drugs in urine by means of diode array detection

1.4.2 Expanding the spectrum of antiretroviral drugs detectable by the current method

1.4.3 Record peak retention time and spectra in a computerized library

1.4.4 Identify ARV in patient urine samples

2 LITERATURE REVIEW

2.1 INTRODUCTION

Antiretroviral drugs are medications used in the treatment of retroviral infections, primarily HIV. ARVs are classified by the phase of retrovirus lifecycle that the drug inhibits. Nucleoside and nucleotide reverse transcriptase inhibitors (nRTI) inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its elongation. Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase directly by binding the enzyme and disrupting its function. Protease Inhibitors (PIs) inhibit the activity of protease, an enzyme used by HIV to cleave proteins for final assembly of new virions. Integrase inhibitors inhibit the enzyme integrase which is responsible for the integration of viral DNA into the DNA of the infected cell. Fusion inhibitors (or entry inhibitors) disrupts binding, fusion and entry of HIV to the host cell by blocking of targets on the cell. Maturation inhibitors block the conversion of the viral polyprotein into the mature capsid protein, thereby releasing defective and non-infectious virions. Broad spectrum inhibitors are natural antivirals such as extracts from certain mushrooms that contain multiple pharmacologically active compounds which attack the virus at different stages in its lifecycle (Rang *et al*. 1999).

2.2 HIV AND AIDS IN SOUTH AFRICA

According to the World health organization (WHO), there are approximately 35 million people living with HIV/AIDS worldwide of which 3.2 million are children. Sub-Saharan Africa accounts for almost 70% of the global total of new HIV infections with 24.7 million [23.5 million – 26.1 million] people living with HIV in sub-Saharan Africa. Women account for 58% of the total number of people living with HIV in sub-Saharan Africa. An estimated 2.1 million new infections occur annually of which 240 000 are children. Most of these children live in sub Saharan Africa and were infected by their HIV-positive mothers during pregnancy, childbirth or breastfeeding (UNAIDS 2013).

South Africa has the highest infection rate globally with 5.51 million people of the global 35 million people infected. This is an alarming 15% of all HIV infections worldwide occurring in South Africa. Nigeria rates second with 3.3 million people infected and India third with 2.4 million people living with HIV/AIDS. HIV prevalence continues to vary by gender and race. In South Africa, females aged 30–34 years (36%) and males aged 35–39 years (28.8%) have

been found to have the highest HIV prevalence, which is probably a function of both treatment and new infections in this group. Among adults aged 15‑49 years, the number of new infections was 1.7 times higher in females than in males. The incidence rates among young females remain concerning. The HIV-incidence rate among female youth aged 15‑24 was over four times higher than the incidence rate found in males in this age group (2.5% vs 0.6%). Almost a quarter (24.1%) of all new HIV infections occurred in young females aged 15‑24 years. Black Africans, especially females, continue to be disproportionately affected by HIV and AIDS, followed by Coloureds. The highest levels of HIV prevalence are observed among female black African adults aged 20–34 years. The probable reasons for the high prevalence of HIV in this group, above and beyond the implementation of ART, are both biological susceptibility and the socio-economic conditions in which these women live which may give rise to risky sexual behaviour. The high prevalence observed among black Africans made it necessary to interrogate the results further. It was found that the high HIV prevalence in the black African population is associated with low prevalence of marriage, low socio-economic status, and other behavioural and social factors that affect this group (Shisana *et al.* 2014; Statistics South Africa 2014).

The prevalence for Indians, Asians and whites is less than 1%. However, the figures for whites are considered unreliable because of the low response rate. In attempting to clarify the possible reasons for differential racial HIV prevalence, the findings suggest that black Africans (39.1%) were less likely than all other races (>85%) to live in urban formal areas. Urban informal areas are generally under-resourced and lack some of the basic necessities such as formal housing, water, sanitation, and access to preventive health services. The other distinguishing factor among races is marital status. Black Africans are less likely to report being married than whites and Indians or Asians. HIV prevalence was found to be higher in the unmarried, co-habiting population than in the married population (UNAIDS 2013, Shisana *et al.* 2014).

HIV prevalence is estimated to be 28 times higher among people who inject drugs, 12 times higher among sex workers, 19 times higher among gay men and other men who have sex with men and up to 49 times higher among transgender women than among the rest of the adult population. In sub-Saharan Africa, adolescent girls and young women account for one in four new HIV infections (UNAIDS 2013).

2.3 HUMAN IMMUNODEFICIENCY VIRUS AND ACQUIRED IMMUNODEFICIENCY SYNDROME

Kaposi's sarcoma (KS) was a rare form of relatively benign cancer that tended to occur in older people. But by March 1981 at least eight cases of a more aggressive form of KS had occurred amongst young gay men in New York (Hymes *et al*. 1981). The United States' Centre for disease control (CDC) published a morbidity and mortality weekly report (MMWR) on the 5th of June 1981 describing peculiar cases of a lung infection *Pneumocystis carinii* pneumonia in five young, previously healthy, gay men in Los Angeles. All the men suffered from other unusual infections as well, indicating that their immune systems did not function properly. By the time the report was published, two of the men already passed away. This edition of the MMWR marked the first official reporting of what will become known as the AIDS epidemic. Within days of this report, the CDC was flooded with reports of similar cases across America, and within six months 270 cases have been reported of which 121 have died (MMWR, 1981). In June of 1982 report of a group of cases amongst gay men in Southern California suggested that the disease might be caused by a virus that was sexually transmitted. By the beginning of July 1982 a total of 452 cases, from 23 states, had been reported to the CDC (CDC, 1982). Later that month the first reports appeared that the disease was occurring in Haitians, as well as hemophiliacs, heterosexual males and females, and similar cases was reported abroad in the United Kingdom and Africa (MMWR, 1982). The acronym AIDS was suggested at a meeting in Washington, D.C., and extensive research programs were launched to investigate the causative agent.

A transmissible retrovirus that was isolated by three different laboratories and named human T cell lymphotropic virus type III (HTLV–III), lymphadenopathy associated virus (LAV) and acquired immunodeficiency virus associated retrovirus (ARV) has been renamed the human immunodeficiency virus (HIV) (Victor & Daniels, 1987). Two closely related viruses, HIV-1 and HIV-2 have been identified as causing acquired immunodeficiency syndrome (AIDS). HIV-1 causes most cases of AIDS in the Western Hemisphere, Europe, and Central, South, and East Africa: HIV-2, which appears less virulent than HIV-1, is the principle agent of AIDS in West Africa (Harvey & Mycek, 2000).

Retroviruses contain the reverse transcriptase enzyme that converts viral ribonucleic acid (RNA) into a proviral deoxyribonucleic acid (DNA) copy that becomes integrated into the host cell DNA, where they duplicate with normal cellular genes during each cell division. Multiple copies of the infectious virus may be produced, causing other cells to be infected. The virus

destroys helper T cells, thus depressing cell mediated immunity. Although B cells and T cells initially mount a vigorous response to viral exposure, in time a profound deficit of antibodies develops and the cytotoxic cells become nonresponsive to viral cues, resulting in the increased susceptibility of patients to opportunistic infections, malignancies, neurological dysfunction and a variety of other syndromes (Berkow, Fletcher & Beers, 1992).

The virus multiplies steadily in the lymph nodes throughout most of the chronic asymptomatic period. Symptomatic AIDS gradually appears a few months to ten years later when the lymph nodes are destroyed and can no longer contain the virus. The infectious specificity of HIV reflects the fact that the cluster of differentiation 4 (CD4) proteins, through which immune cells exchange information, provide the avenue for HIV's attack. A particular harpoon-like HIV coat glycoprotein (gp120) fits into the CD4 receptor (Marieb, 1997). HIV also needs a co-receptor that changes with the stage of infection. The protein chemokine receptor gene 5 (CC-CKR- 5) is the CD4's partner during the early stage, whereas the fusin receptor aids HIV entry during later stages of infection. Once inside, the virus uses the enzyme reverse transcriptase to produce DNA from the information encoded in its viral RNA. This DNA copy or provirus then inserts itself in the host cell to produce new copies of viral RNA and proteins so that the virus can multiply and infect other cells (Marieb, 1997; Bradshaw, 2008).

Unfortunately, no cure has been discovered for HIV and AIDS yet. The introduction of antiretroviral (ARV) drugs changed the prospects of an HIV/AIDS patient dramatically. The aim of current treatment regimens is to reduce the plasma viral concentration as much as possible, and for as long as possible. Although the ARVs cannot cure HIV/AIDS, they can increase the life expectancy and support the general health of an HIV positive patient. The national antiretroviral treatment (ART) programme in South Africa was launched in April 2004. Patients were considered eligible for ART if they had a stage IV illness (excluding extra pulmonary tuberculosis) or a CD4 count less than 200 cells/µl. The adult regimens consisted of two nucleotide reverse transcriptase inhibitors (NRTIs) and one non-nucleotide reverse transcriptase inhibitor (NNRTI). Initially, the NRTI backbone consisted of zidovudine and lamivudine, but was later changed to stavudine and lamivudine. Paediatric regimens varied, with NNRTIs and protease inhibitors being variously used with the NRTI backbone. Six-monthly CD4 counts and viral-load testing were provided in the programme, together with safety monitoring according to the specific regimens. Today a standardised national line of ART regimens are in place specifically designed for South Africa's unique challenges facing the pandemic (Table 1)

Table 1: Standardised national ART regimens for adults and adolescents

Population	1st line	Comments
All new patients needing treatment, including pregnant women	TDF + FTC (or 3TC) + EFV FDC preferred	Replace EFV with NVP in patients with significant psychiatric co-morbidity or intolerance to EFV and where the neuropsychiatric toxicity of EFV may impair daily functioning, e.g. shift workers.
Contraindications to EFV	TDF + FTC (or 3TC) + NVP	Use NVP based regimen: In patients with significant psychiatric co morbidity or intolerance to EFV and where the neuropsychiatric toxicity of EFV may impair daily functioning, e.g. shift workers.
Contraindication to TDF	AZT+ 3TC + EFV (or NVP)	Renal disease or the use of other nephrotoxic drugs e.g. aminoglycosides
Contraindication to TDF and AZT	d4T + 3TC+ EFV (or NVP)	Renal disease and anaemia or the use of other nephrotoxic drugs, aminoglycosides
Contraindication to TDF, AZT and d4T	ABC + 3TC + EFV (or NVP)	Renal disease, anaemia, peripheral neuropathy, the use of other nephrotoxic drugs
Currently on d4T-based regimen	TDF + FTC (or 3TC) + EFV FDC preferred	Mandatory if patients experience toxicity and patients who are at high risk of toxicity (high BMI or pregnant). Switch to TDF if virally suppressed and the patient has normal creatinine clearance, even if well tolerated
Who	**2nd line**	**Action**
Management of virological failure		If plasma HIV RNA >1000 copies, Check for adherence, compliance, tolerability and drug-drug interaction and assess psychological issues. Repeat VL test 2 months later
Failing on a TDF-based 1st line regimen	AZT+3TC+ LPV/r	Patients with anaemia and renal failure switch to ABC
Failing on a d4T-based 1st line regimen	TDF+3TC (or FTC) and LPV/r	
Dyslipidaemia or diarrhoea associated with LPV/r	Switch LPV/r to ATV/r	
Who	**3rd line**	**Action**
Failing any 2nd line regimen	Specialist referral	
Should be expert and genotype resistance testing based decision and supervised care Patients failing on second line therapy will be managed by an expert panel. The drugs for third line will be managed centrally. More discussion is required to deal with the modalities	Most likely regimen would be Raltegravir/Darunavir//Etravirine adjusted according to genotype interpretation. Should be by expert and take into account prior exposure and predictable mutations	

(The national department of health antiretroviral treatment guidelines, 2015)

List of abbreviations used in table 1

3TC,	lamivudine	FDC	fixed-dose combination
ABC	abacavir	FTC	emtricitabine
ATV/r	atazanavir/ritonavir	LPV/r	lopinavir/ritonavir
AZT	zidovudine	NVP	Nevirapine
d4T	stavudine	TDF	Tenofovir
EFV	efavirenz		

2.4 ANTIRETROVIRAL DRUGS

There are currently six classes (table 2) of ARV drugs available, each classified according to their mechanism of action: nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (IIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CRAs). Each class of ARV drug targets a different step in the life cycle of the virus (figure 1) during infection of a $CD4^+$ T lymphocyte or other target cells (Arts & Hazuda, 2012; Rathbun, 2013).

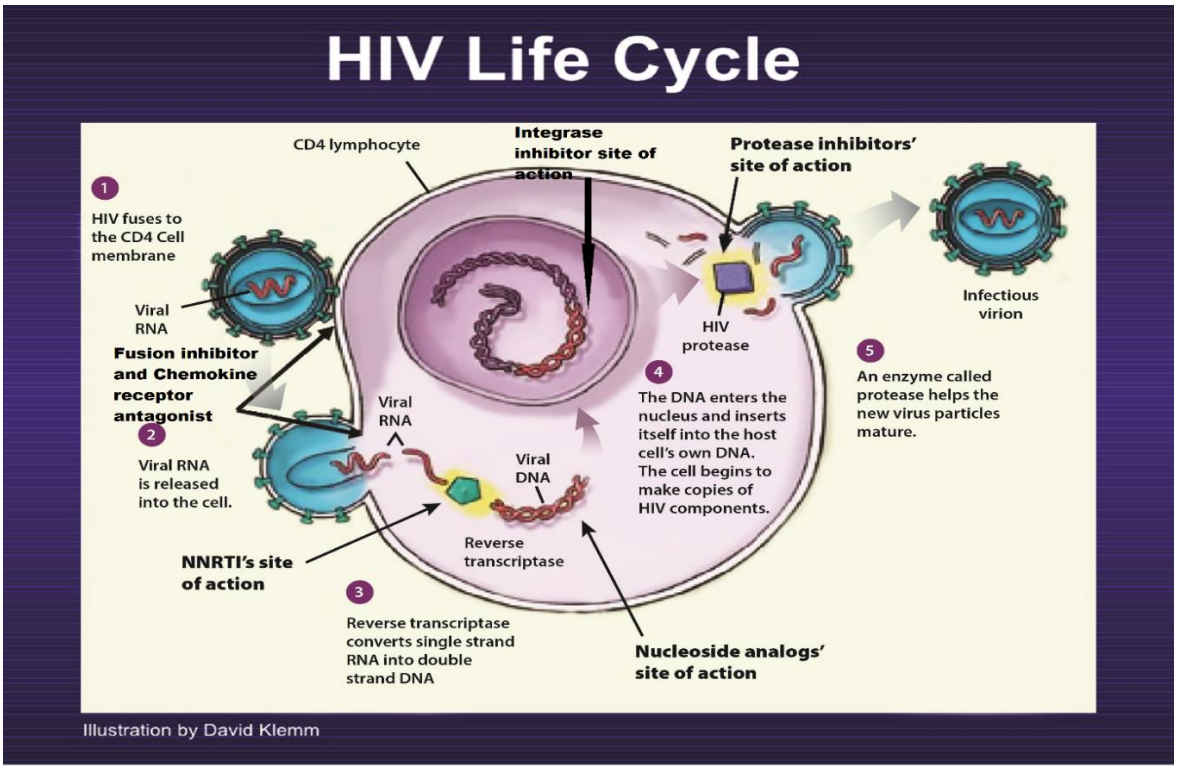
Table 2: Classes of ARVs

NRTIs	NNRTIs	PIs	IIs	FIs	CRAs
abacavir didanosine lamivudine tenofovir zidovudine emtricitabine stavudine	efavirenz nevirapine delavirdine rilpivirine etravirine	atazanavir darunavir fosamprenavir indinavir nelfinavir saquinavir tipranavir lopinavir/ritonavir	raltegravir elvitegravir	enfuvirtide	maraviroc

List of abbreviations used in table 2

3TC	lamivudine	FPV	fosamprenavir
ABC	abacavir	FTC	emtricitabine
ATV	atazanavir	IDV	indinavir
AZT	zidovudine	LPV/r	lopinavir/ritonavir
d4T	stavudine	MVC	maraviroc
ddI	didanosine	NVF	nelfinavir
DLV	delavirdine	NVP	nevirapine
DRV	darunavir	RAL	raltegravir
EFV	efavirenz	RPV	rilpivirine
ENF	enfuvirtide	SQV	saquinavir
ETR	etravirine	TDF	tenofovir
EVG	elvitegravir	TPV	tipranavir

Figure 1: Site of action of ARVs in the HIV life cycle



(AIDS approach patients, 2010)

2.4.1 NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIS)

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first antiretroviral drugs available for HIV management. The NRTIs remain part of the current standard care although they are less potent than nonnucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). They show activity against both HIV-1 and HIV-2. The NRTIs include abacavir, didanosine, lamivudine, tenofovir and zidovudine described in the study (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.1.1 Mechanism of action

NRTIs disrupt HIV replication through competitive inhibition of HIV reverse transcriptase and DNA chain termination. The HIV-specific DNA polymerase, reverse transcriptase, allows the HIV RNA to be transcribed into single strand and double-strand proviral DNA and incorporation into the host-cell genome. NRTIs are structurally similar to DNA nucleoside bases and become incorporated into the proviral DNA chain, resulting in termination of the proviral DNA chain formation (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.1.2 Pharmacokinetics

NRTIs must undergo phosphorylation by intracellular kinase in order to exert their activity. Oral bioavailability ranges from 25%-93% with tenofovir and didanosine at the lowest end of the spectrum of bioavailability. Most of the NRTIs' absorption is not affected by food, except didanosine which must be taken on an empty stomach to achieve optimal absorption. Most NRTIs are eliminated by the kidneys and require dose adjustments in patients with renal insufficiency. Drug interactions are minimal, mainly because of the fact that NRTIs are not metabolized by the cytochrome P450 system. In the case of didanosine, some significant drug interactions have been noted. When didanosine is taken in combination with tenofovir, lower doses of didanosine are given to avoid serious adverse effects (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.1.3 Adverse events

Mitochondrial toxicities such as lactic acidosis, pancreatitis, peripheral neuropathy, lipoatrophy and neuropathy can occur. This is due to NRTI binding to mitochondrial DNA polymerase-y enzyme which impairs cellular respiration. Hypersensitivity reactions may occur with abacavir, and bone marrow suppression, myopathy and headaches with zidovudine. Increased risk for adverse cardiovascular events has been associated with abacavir and didanosine. Bone turnover increases with bone loss from the spine and hip, with an average 6% loss in bone mass density within a year of commencing treatment (McNicholl, 2012; Rathbun, 2013).

2.4.2 NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIS)

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) have been used in the treatment of HIV since 1996 with the introduction of nevirapine onto the market. NNRTIs form part of the initial regimens and they exhibit potent activity against HIV-1. First generation NNRTIs include efavirenz, nevirapine and delavirdine. Second generation NNRTIs currently include rilpivirine and etravirine (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.2.1 Mechanism of action

NNRTIs bind the p66 subunit of HIV reverse transcriptase. They induce the formation of a hydrophobic pocket proximal to the active site which changes the spatial conformation of the substrate-binding site and reduces polymerase activity. All NNRTIs show activity against

HIV-1 isolates, and in vitro studies have shown that etravirine are also active against HIV-2 (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.2.2 Pharmacokinetics

NNRTIs are metabolized by utilizing the cytochrome P450 system and exert either induction or inhibition effects on the isoenzymes CYP3A4 or CYP2C9. This results in a significant potential for drug-drug interactions. Etravirine is a substrate of 3A4, 2C9 and 2C19. Nevirapine is metabolized by 3A4 and 2B6 to some degree. Delavirdine primarily uses the 3A4 isoenzyme for metabolism (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.2.3 Adverse events

NNRTIs are associated with severe allergic reactions of the skin and may cause Stevens-Johnson syndrome. Elevation in liver function tests, hepatitis, nausea and diarrhoea is also common in patients. All NNRTIs may have significant interactions with other drugs. Dosage adjustment of interacting agents may be required to eliminate severe adverse reactions. Delavirdine and efavirenz may increase transaminase levels, while nevirapine can cause severe toxicity, including liver necrosis in patients with CD4 counts above 250 cells/µL. CNS effects such as insomnia, vivid dreaming, hallucinations and confusion may be caused by efavirenz. Side effects can be minimized by avoiding food at the time of administration and by taking the agent at bedtime (McNicholl, 2012; Rathbun, 2013).

2.4.3 PROTEASE INHIBITORS

HIV protease inhibitors (PIs) forms an integral part of treatment of HIV infection and was first introduced in 1995. The group consists of 8 compounds: atazanavir, darunavir, fosamprenavir, indinavir, nelfinavir, saquinavir, tipranavir and lopinavir/ritonavir. Lopinavir/ritonavir was investigated in this study. All the protease inhibitors exhibit the same mode of action, although some differences in pharmacokinetics, efficacy and side effects exist (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.3.1 Mechanism of action

HIV protease inhibitors (PIs) are competitive inhibitors that bind to HIV protease and prevent cleavage of polypeptides. The aspartic acid protein, HIV protease, is a 99-amino-acid protein responsible for maturation of virus particles in the later viral life cycle. HIV protease cleaves individual proteins from the gag and gag-pol precursors into functional subunits of viral capsid formation during or after viral budding from infected cells. While HIV can still replicate in the

presence of protease inhibitors, the resulting virions are immature and unable to infect new cells (Rathbun, 2013).

2.4.3.2 Pharmacokinetics

Great variability exists in the pharmacokinetics of patients on PIs. First-pass metabolism by cytochrome P450 (CYP) 3A4 and 3A5 is significant and intestinal efflux by p-glycoprotein occurs. Great potential exists for drug-drug interaction due to the reliance on metabolism *via* CYP3A4, a pathway relied on by many medications for clearance. Protease inhibitors are mainly protein bound (97-99%) to albumin and alpha 1 acid glycoprotein with the exception of indinavir. A low dose of ritonavir is frequently co administered to block hepatic 3A and intestinal metabolism. This co administration improves pharmacokinetic variability and consistency in serum concentrations (Rathbun, 2013).

2.4.3.3 Adverse events

Metabolic complications such as insulin resistance, lipodystrophy and dyslipidaemia are most commonly experienced by patients on PIs. Dyslipidaemia occurs in 70% of patients and requires lipid lowering therapy. Genetics and lifestyle are important contributing factors to the severity of lipid abnormalities. Modest effects on glucose metabolism have been observed with fosamprenavir, tipranavir, nelfinavir and lopinavir/ritonavir, while indinavir shows the greatest potential for glucose metabolism abnormalities. Fat redistribution is observed in 40-50% of patients on combinations of PIs and NRTIs. Cardiac abnormalities may develop in 5% of patients taking atazanavir, ritonavir, lopinavir/ritonavir and nelfinavir. Intracranial bleeding and gastrointestinal side effects can also occur (Rathbun, 2013).

2.4.4 INTEGRASE INHIBITORS

HIV's RNA is converted into DNA by the reverse transcriptase enzyme. After this process has taken place, the HIV DNA must then be incorporated into the DNA of a CD4 cell. This is known as integration. Integrase was the most recent HIV-1 enzyme successfully targeted for potential drug development with the FDA approval of raltegravir in 2007 and other integrase inhibitors including elvitegravir. Integrase catalyses 3' end processing and viral DNA and strand transfer. Integration inhibitors block this process (Arts & Hazuda, 2012; Hicks, 2013).

2.4.4.1 Mechanism of action

HIV integrase is responsible for proviral DNA attachment and transportation to host-cell chromosomes. It allows the transcription of viral proteins and subsequent assembly of virus

particles involving two catalytic reactions: 3'-processing in the host-cell cytoplasm to prepare proviral strands for attachment and strand transfer where proviral DNA is covalently linked to cellular DNA. The integrase inhibitors inhibit the strand transfer reaction by binding metallic ions in the active site (Arts & Hazuda, 2012; Hicks, 2013).

2.4.4.2 Pharmacokinetics

Raltegravir is rapidly absorbed and may be taken with or without food. The terminal half-life is 10-12 hours and is administered twice a day. Raltegravir is 83% bound to plasma proteins and is a substrate for P-glycoprotein. Its pharmacokinetics demonstrates a sex-related variation due to the fact that the half-life is slightly longer in women than in men. Antacids may decrease absorption, but no interaction with proton pump inhibitors and H2 antagonists are expected. Uridine diphosphate glucuronyl transferse 1A1 (UGT1A1) is responsible for its metabolism and dosage adjustment is not required in patients with renal insufficiency or hepatic impairment. Low-dose ritonavir is co administered with elvitegravir to reduce its first-pass metabolism. This results in a 20-fold increase in systemic exposure and a half-life of 10-13 hours. Elvitegravir is metabolized *via* CYP3A4 and UGT1A1/ UDP glucuronosyltransferase 1 family, polypeptide A3 (UGT1A3). Less than 7% is eliminated *via* the kidneys and dosage adjustment is unlikely in patients with renal insufficiency. The co administration of ritonavir is likely to cause drug-drug interactions (Arts & Hazuda, 2012; Hicks, 2013).

2.4.4.3 Adverse events

Cases of myopathy, rhabdomyolysis and elevated creatinine kinase levels have been reported. More common adverse effects include headaches and gastrointestinal effects such as nausea and diarrhoea. Alanine aminotransferase and aspartate aminotransferase, serum cholesterol, triglycerides, amylase and lipase may increase. A relative risk of malignancy of 1.2 cases per 100 patient-years has been reported with the use of raltegravir in phase II and phase III clinical studies and requires continued surveillance (Arts & Hazuda, 2012; Hicks, 2013).

2.4.5 FUSION INHIBITORS

Fusion inhibitors (FIs) were the first class of antiretroviral drugs to target the replication cycle of HIV with the approval of enfuvirtide in 2003. The development of enfuvirtide emerged from a serendipitous observation made during epitope-mapping experiments in the race to find an

HIV vaccine. Synthetic peptides derived from the HIV envelope glycoprotein 41 (gp41) produced an antiviral effect when incubated with HIV virus and human T cells. Subsequent insight and comprehension of the fusion process, and how envelope glycoproteins interact, led to an appreciation of how these peptides inhibit the fusion of HIV with the human cell membrane, and interrupt the HIV life cycle. Fusion inhibitors have a unique mechanism of action that provides additional treatment options for patients who are highly treatment resistant (Boyd & Pett, 2008; Rathbun, 2013).

2.4.5.1 Mechanism of action

Fusion inhibitor act extracellularly to prevent the fusion of HIV to CD4 cells or other target cells by blocking the second step in the fusion pathway by binding to the HR1 region of gp41. This mechanism prevents conformational change of gp41 required to complete the final step in the fusion process by preventing HR1 and HR2 to fold properly (Rathbun, 2013).

2.4.5.2 Pharmacokinetics

Enfuvirtide cannot be administered orally as it is a large peptide which is broken down in the digestive tract before absorption. It requires a twice-daily subcutaneous injection. As a peptide it is catabolised and does not rely on hepatic metabolism. It does not show any influence on the metabolism of other drugs utilizing the cytochrome P450 which limits the potential for meaningful drug-drug interactions (Boyd & Pett, 2008; Rathbun, 2013).

2.4.5.3 Adverse events

Enfuvirtide requires extensive patient counselling on management of possible side effects, adherence and injection technique. Injection site reactions are common, especially erythema, cysts and nodules may occur. Hypersensitivity reactions include rash, fever, chills, nausea and vomiting. Neutropenia may encourage an increased frequency of pneumonia (Boyd & Pett, 2008; McNicholl, 2012).

2.4.6 CHEMOKINE RECEPTOR ANTAGONISTS (CRAS)

Maraviroc was introduced to the market in 2007. It was the first drug in a novel class of ARVs known as the chemokine receptor 5 (CCR5) antagonists. Chemokine receptor antagonists inhibit the entry of HIV into the host cell. Two chemokine receptors, CXCR4 and CCR5, are necessary for the virus to enter the cell, so by inhibiting these chemokine receptors the disease can be suppressed (Arts & Hazuda, 2012; Rathbun, 2013).

2.4.6.1 Mechanism of action

HIV binds to CD4 cells and ultimately fuses with the host cell. The gp120 HIV surface protein binds to the CD4 receptor which induces a structural change that reveals the third variable loop (V3 loop) of the protein. The V3 loop binds with either CCR5 or CXCR4, allowing glycoprotein 41 (gp41) to insert itself into the host cell leading to cell membrane fusion. Maraviroc is a small molecule that reversibly and selectively binds the CCR5 coreceptor, blocking interaction of the V3 loop, inhibiting fusion of the cellular membranes (Rathbun, 2013).

2.4.6.2 Pharmacokinetics

Maraviroc is extensively metabolized by CYP3A4, with renal clearance accounting for approximately 23% of total clearance. It is also a substrate for the efflux pump p-glycoprotein. The half-life of maraviroc is approximately 15-30 hours. It is approximately 75% protein bound, mainly to albumin and alpha$_1$ acid glycoprotein. Maraviroc does not inhibit any of the major CYP450 enzymes at clinically significant doses and it has not shown any clinically relevant effects on plasma concentrations of other agents; therefore no dose adjustments of co-administered drugs are required. Maraviroc exposure is altered by agents that modulate the activity of CYP3A4 and, in some circumstances; maraviroc dose adjustment is required (Abel, Back & Vourvahis, 2009; Rathbun, 2013).

2.4.6.3 Adverse events

Maraviroc may cause gastrointestinal disturbances including diarrhoea and nausea. An increased incidence of upper respiratory tract infections and persistent cough has been reported. Some patients may experience elevations in liver function tests, hepatitis, joint and muscle pain, fatigue, dizziness and headaches. Caution is advised in patients with hepatic impairment. Postural hypotension may occur in patients receiving in excess of 600mg/day (McNicholl, 2012; Rathbun, 2013).

Abacavir, didanosine efavirenz, lamivudine, lopinavir/ritonavir, nevirapine, stavudine, tenofovir and zidovudine are the most popular ARVs considered in current HIV/AIDS treatment regimens (Shisana *et al.* 2014).

2.4.7 ABACAVIR

2.4.7.1 Therapeutic use

Abacavir (Trizivir®) is a drug indicated for the treatment of HIV infections in adults and children older than 12 years (Mallal *et al.* 2008; Micromedex, 2009).

2.4.7.2 Contra-indications

Abacavir is contra-indicated in patients with hepatic impairment, neutropenia and patients with low haemoglobin (Hb). Abacavir is not recommended in breastfeeding mothers, and safety during pregnancy is not established (Micromedex, 2009).

2.4.7.3 Mechanism of action

Abacavir is a carbocyclic nucleoside with reverse transcriptase inhibitory properties. Abacavir is an "indirect" prodrug of carbovir. Carbovir triphosphate metabolite is active intracellularly, although the initial activation step is the phosphorylation to abacavir monophosphate (Mallal *et al.* 2008; Micromedex, 2009).

2.4.7.4 Absorption, Distribution, Metabolism and Excretion

Abacavir has a high bioavailability of 83% and its first-pass metabolism is calculated to be approximately 17%. Abacavir may be taken with or without food, and no effect on bioavailability was noted. Fifty percent of Abacavir binds to plasma proteins and protein binding is independent of the concentration. Abacavir levels in cerebrospinal fluid were 18-33% of the concentrations reached in the plasma. Abacavir is extensively metabolized by the liver. The primary routes of metabolism are alcohol dehydrogenase and glucoronyl transferase. Excretion of abacavir is mainly *via* the kidneys. Only 1% of the unchanged compound can be retrieved from urine.

Recovery for abacavir's metabolites from the urine range from 36% for the 5-glucuronide metabolite; 30% as the carboxylic acid metabolite and approximately 15% of unidentified metabolites. Sixteen percent of abacavir is excreted in the faeces (Micromedex, 2009).

2.4.7.5 Common side effects

Side effects experienced with abacavir treatment include blood discrasias, myelosuppression, pancreatitis, muscle disorders, CNS effects, cough, skin rash, nail and skin pigmentation, urticaria, pruritus, sweat, fever, lethargy, fatigue, malaise, urinary frequency, chest pain and gynecomastia in male patients (Mallal *et al.* 2008; Micromedex, 2009).

2.4.7.6 Toxicological adverse effects

Toxicological effects of abacavir include nausea, vomiting, fatigue, headache, diarrhoea, and loss of appetite. Fatal lactic acidosis and severe hepatomegaly with steatosis have been reported. Fatal hypersensitivity reactions have also been reported and symptoms may include skin rash, fever, fatigue, nausea, vomiting, diarrhoea, abdominal pain, pharyngitis, dyspnoea, or cough (Micromedex, 2009).

2.4.7.7 Pharmacokinetic drug interactions of Abacavir

The AUC of abacavir is increased by ethanol, methadone clearance increases and trimethoprim increases lamivudine plasma levels. Renal excretion of zidovudine is reduced by probenecid, rifampicin decreases AUC of zidovudine and inhibition of microsomal metabolism may alter zidovudine metabolism. Nephrotoxic and myelosuppressant drugs may increase the risk of zidovudine adverse reactions (Berkow *et al*. 1992).

2.4.8 DIDANOSINE

2.4.8.1 Therapeutic use

Didanosine (Videx®) is a nucleoside reverse transcriptase inhibitor used in combination with other antiretroviral agents for the palliative treatment of advanced HIV in adults and children with progressive immunodeficiency (Brinkman *et al*. 1998; Micromedex, 2010).

2.4.8.2 Contra-indications

Didanosine safety in pregnancy and lactation has not been established. Didanosine is contra–indicated in children and in patients with impaired renal and hepatic function. Concomitant treatment with allopurinol and ribavirin is not advised.

2.4.8.3 Mechanism of action

Didanosine is a purine nucleoside analogue. The deamination product of dideoxyadenosine inhibits HIV replication in both T cells and monocytes. Didanosine is converted intracellularly to the mono-, di- and triphosphates of dideoxyadenosine. These triphosphates inhibit HIV reverse transcriptase thereby blocking viral DNA synthesis and suppression of HIV replication (Brinkman *et al*. 1998; Micromedex, 2010).

2.4.8.4 Absorption, Distribution, Metabolism, and Excretion

Didanosine bioavailability ranges from 38% to 43% when taken with an antacid orally. The fraction of didanosine absorbed after oral administration is dependent upon the dosage and

the buffering capacity of the antacid administered concomitantly. Absorption of didanosine is greatly decreased when taken with a meal and it is therefore advised to take didanosine at least 30 minutes before, or 2 hours after a meal. Less than 5% of didanosine is bound to plasma proteins. The cerebrospinal fluid (CSF) to plasma concentration ratio is 21%. Didanosine is metabolized *via* the same pathways responsible for the elimination of endogenous purines. Eighteen percent of didanosine is excreted *via* the kidneys (Micromedex, 2010).

2.4.8.5 Common side effects

Side effects commonly experienced by patients include cardiovascular effects such as dysrhythmia, heart failure, myocardial infarction, palpitations and thrombophlebitis. Rash, alopecia, Steven-Johnson syndrome, myalgia and rhabdomyolysis can also occur. Fat maldistribution, diabetes mellitus, hypoglycaemia, electrolyte disturbances, abdominal pain, diarrhoea, nausea, vomiting, pancreatitis and xerostomia are common side effects associated with didanosine. Bilateral gynecomastia is seen in males on didanosine. Didanosine may cause headaches, peripheral neuropathy, insomnia and asthenia, and is known to induce seizures in patients with electrolyte disturbances. Haematological adverse effects include anaemia, leukopenia, thrombocytopenia and myelosuppression (Brinkman *et al*. 1998; Micromedex, 2010).

2.4.8.6 Toxicological adverse effects

Overdose may lead to fatal lactic acidosis, fatal pancreatitis and extensive hepatocellular necrosis with collapse-fibrosis and Steven-Johnson syndrome. Heart failure and myocardial infarction can also occur in overdose (Brinkman *et al*. 1998; Micromedex, 2010).

2.4.8.7 Pharmacokinetic drug interactions of Didanosine

Concomitant treatment with allopurinol increases didanosine AUC 4-fold and should not be used together. The bioavailability of didanosine is enhanced when taken with oral antacids. All didanosine formulations contain an antacid already, and by concomitant use of antacids such as aluminium carbonate and aluminium hydroxide will increase the risk of adverse effects from the antacids. Didanosine reduces the effect of indinavir, itraconazole, amprenavir, cinoxacin, enoxacin, gatifloxacin delavirdine and atevirdine. Atazanavir decreases the solubility of the didanosine formulation. Didanosine reduces the bioavailability of ciprofloxacin. Ganciclovir and hydroxyurea increases the risk of didanosine toxicity and

fatal pancreatitis and hepatotoxicity may develop. Concomitant treatment with zalcitabine causes peripheral neuropathy (Micromedex, 2010).

2.4.9 EFAVIRENZ

2.4.9.1 Therapeutic use

Efavirenz (Stocrin®) is a non-nucleoside reverse transcriptase inhibitor used in combination with other ARVs, indicated for HIV-1 infection in adults, adolescents and children (Denelsbeck, 2006; Micromedex, 2009).

2.4.9.2 Contra-Indications

Efavirenz is contra-indicated in pregnancy because of its teratogenic effect on the foetus. Safety in lactation and children under three years of age, or a bodyweight below 13 kg, is not established. It is not recommended to use efavirenz with terfenadine, astemizole, cisapride, midazolam, triazolam, ergot derivatives or St John's Wort (Micromedex, 2009).

2.4.9.3 Mechanism of action

The action of efavirenz is through non-competitive inhibition of HIV-1 reverse transcriptase. Efavirenz has no inhibitory effect on human immunodeficiency virus type-2 (HIV-2) reverse transcriptase or human cellular DNA polymerases alpha, beta, gamma, or delta (Micromedex, 2009).

2.4.9.4 Absorption, Distribution, Metabolism and Excretion

Animal studies show a bioavailability of approximately 42%. Oral absorption of efavirenz can be increased by 17-22% when taken with a fatty meal. Efavirenz is highly protein bound, 99.5% to 99.75%, to plasma protein, primarily albumin. Cerebrospinal fluid values ranged from 0.26% to 1.19% of the corresponding plasma concentration in HIV-1 patients treated for at least one month with doses of 200mg to 600mg daily of efavirenz. The volume of distribution for intravenous administration has been reported to be 2 to 4 litres per kilogram (L/kg). Efavirenz is metabolized by the liver *via* the cytochrome P450 system, and efavirenz is also known to induce the cytochrome P450 system, thereby inducing its own metabolism. Approximately 14-34% of efavirenz is excreted in the urine, mainly as metabolites. Approximately 16-61% of the efavirenz mother compound is excreted in the faeces (Micromedex, 2009, Patel *et al*. 2014).

2.4.9.5 Common side effects

Side effects commonly experienced are central nervous system (CNS) effects such as psychosis, severe acute depression and suicide, Steven Johnson syndrome, abnormal co-ordination, hot flushes, flu–like symptoms, syncope, malaise, peripheral neuropathy, speech-, visual-, and taste disorders, paraesthesia, hepatitis and GI disturbances (Denelsbeck, 2006; Micromedex, 2009).

2.4.9.6 Toxicological adverse effects

Toxicological adverse effects include significant CNS effects such as psychiatric effects, rash (some with blistering, desquamation, and mucosal involvement), fever, and liver function test abnormalities occur (Micromedex, 2009).

2.4.9.7 Pharmacokinetic drug interactions of Efavirenz

Drug interactions associated with efavirenz treatment include CNS effects which are increased with alcohol and psychoactive drug use. Plasma concentration of compounds that use the CYP3A4 substrate for metabolism is decreased. The area under the curve (AUC) of plasma concentration/time is a common measure of the extent of bioavailability for a drug given by a particular route and is expressed as a percentage of the AUC when the drug is administered intravenously (100% absorption). The AUC and maximum concentration of the drug reached in the plasma (Cmax) of indinavir, saquinavir and clarithromycin decrease. Methadone plasma levels also decrease in patients, resulting in withdrawal symptoms in HIV infected intravenous (IV) drug users (Patel *et al*. 2014). It is not recommended to use efavirenz with terfenadine, astemizole, cisapride, midazolam, triazolam, Ergot derivatives or St John's Wort (Micromedex, 2009).

2.4.10 LAMIVUDINE

2.4.10.1 Therapeutic use

Lamivudine (Combivir®) is a drug used in combination with other antiretroviral agents for the treatment of HIV in adults and children with progressive immunodeficiency (Parkin *et al*. 2000; Micromedex, 2009).

2.4.10.2 Contra-indications

Lamivudine is contra-indicated in patients with neutropenia, and low haemoglobin levels. Lamivudine safety in pregnancy and lactation has not been established.

2.4.10.3 Mechanism of action

Lamivudine is a synthetic nucleoside used for the treatment of HIV and AIDS. The mechanism of antiviral activity is related to the intracellular conversion to the 5'-triphosphate derivative, which inhibits HIV reverse transcriptase. This metabolite also serves as a chain terminator (Micromedex, 2009).

2.4.10.4 Absorption, Distribution, Metabolism, and Excretion

Lamivudine has an excellent bioavailability ranging from 82% to 87%, and bioavailability is not affected by the intake of food. Less than 36% of lamivudine is bound to plasma proteins. Lamivudine concentration in cerebrospinal fluid is approximately 54% of the concentration found in the plasma. CSF levels increase proportionally to the dosage of lamivudine. Lamivudine has free passage over the placenta. Metabolism of lamivudine is the minor route of elimination. Approximately 4.2% of lamivudine is converted to its trans-sulfoxide metabolite. Lamivudine is mainly excreted by the kidneys and 70% of the mother compound can be recovered in the urine (Micromedex, 2009).

2.4.10.5 Common side effects

Side effects commonly experienced by patients include pancreatitis, lactic acidosis, hepatomegaly, malaise, headache, musculoskeletal pain, peripheral neuropathy, blood discrasias and paraesthesia. CNS effects such as convulsions and cerebral events, sweat, chest pain, chills, urticaria, pruritus, and flu like symptoms may occur (Parkin *et al*. 2000; Micromedex, 2009).

2.4.10.6 Toxicological adverse effects

Based on toxicities seen with chronic (therapeutic) administration, acute overdoses may be expected to result in bone marrow suppression, peripheral neuropathies, and gastrointestinal effects (Micromedex, 2009).

2.4.10.7 Pharmacokinetic drug interactions of Lamivudine

Lamivudine treatment can interfere with the metabolism of other drugs taken by the patient. Trimethoprim increases plasma levels, and an increased incidence of neutropenia with paracetamol use is observed. Inhibition of hepatic microsomal metabolism causes an increased risk for toxicity of nephrotoxic and myelosuppressant drugs. Probenecid increases lamivudine half-life and the AUC of zidovudine (Micromedex, 2009).

2.4.11 LOPINAVIR/RITONAVIR

2.4.11.1 Therapeutic use

Lopinavir/Ritonavir (Kaletra®, Aluvia®) are drugs used in combination with other dual-NRTI component antiretroviral agents for the treatment of HIV in adults and children with progressive immunodeficiency (Micromedex, 2010).

2.4.11.2 Contra-indications

Lopinavir/Ritonavir safety in pregnancy and lactation has not been established. Concomitant use of lopinavir/ritonavir together with drugs highly dependent on CYP3A for clearance is not advised. Lopinavir/ritonavir should be used with caution in patients at risk for cardiac conduction abnormalities (Delfraissy *et al*. 2008).

2.4.11.3 Mechanism of action

Lopinavir is a protease inhibitor of the human immunodeficiency virus. It prevents the cleavage of the Gag-Pol polyprotein, thereby reducing the probability of viral particles reaching a mature, infectious state. Ritonavir is administered solely to increase lopinavir levels by inhibiting the CYP3A mediated metabolism of lopinavir.

2.4.11.4 Absorption, Distribution, Metabolism, and Excretion

Lopinavir has a low bioavailability due to its rapid metabolism after oral administration. The AUC of lopinavir increases significantly when given with ritonavir due to its inhibition of lopinavir's CYP3A mediated metabolism. The AUC and Cmax of lopinavir increases when taken with a fatty meal. Lopinavir is highly protein bound. Approximately 98% to 99% of the drug is bound primarily to alpha-1-acid glycoprotein and albumin. Lopinavir is extensively metabolized in the liver *via* cytochrome P450 CYP3A. Thirteen metabolites of lopinavir have been identified. The major metabolites are 4-oxo-lopinavir and 4-hydroxy-lopinavir. Less than 3% of lopinavir is excreted unchanged in the urine and approximately 20% in faeces (Micromedex, 2010).

2.4.11.5 Common side effects

Side effects commonly experienced by patients include cardiovascular abnormalities such as PR interval prolongation, prolonged QT interval and Torsades de pointes. Diabetes mellitus, dyslipidaemia, fat maldistribution, impaired glucose tolerance, nausea, diarrhoea, vomiting, abdominal pain with flatulence, pancreatitis can also occur. Patients with haemophilia type A and B often experience bleeding, spontaneous skin hematomas and hemarthrosis. A

musculoskeletal abnormality such as osteonecrosis of the femoral head was also reported. Headaches, insomnia and asthenia are also common. Lopinavir has a teratogenic and embryocidal effect in pregnancy and should be avoided unless the potential benefit justifies the potential risk to the foetus (Delfraissy *et al*. 2008; Micromedex, 2010).

2.4.11.6 Toxicological adverse effects

Based on toxicities seen with chronic (therapeutic) administration, acute overdoses may be expected to result in atrioventricular block and myocardial infarction, hyperglycaemia, pancreatitis and fatal hepatic dysfunction (Micromedex, 2010).

2.4.11.7 Pharmacokinetic drug interactions of Lopinavir/Ritonavir

Concomitant use of lopinavir with potent CYP3A inducers such as cisapride, ergotamine, St. John's Wort, lovastatin, simvastatin and benzodiazepines will result in the increased metabolism of lopinavir and reduced plasma concentrations of the drug. This will cause a loss of therapeutic efficacy and increase the possibility of resistance to lopinavir (Micromedex, 2010).

2.4.12 NEVIRAPINE

2.4.12.1 Therapeutic use

Nevirapine (Viramune®) is a drug used in combination with other antiretroviral agents, and is indicated for HIV-1 infection and to reduce the risk of mother to child transmission (Denelsbeck, 2006; Micromedex, 2009).

2.4.12.2 Contra-indications

Nevirapine is contra-indicated in patients with severe hepatic impairment and in patients with end-stage renal failure when the patient is not being dialyzed. Safety and efficacy in neonates with a bodyweight less than 2.5 kg is not established. Nevirapine should not be used together with Ketoconazole, Rifampicin and St. John's Wort (Micromedex, 2009).

2.4.12.3 Mechanism of action

Nevirapine is a nonnucleoside antiretroviral agent. Nevirapine is a dipyrodiazepinone derivative which selectively inhibits reverse transcriptase activity and replication of HIV-1. It has no activity against reverse transcriptase of other retroviruses such as HIV-2, and is inactive against human DNA polymerases. Unlike nucleoside analogues, nevirapine does

not need intracellular phosphorylation for antiviral activity; it reacts directly with reverse transcriptase (Micromedex, 2009).

2.4.12.4 Absorption, Distribution, Metabolism and Excretion

Nevirapine has a good bioavailability of over 90% after oral ingestion. The intake of food has no significant effect on nevirapine's absorption, and can be taken with or without food. Nevirapine is approximately 60% bound to plasma proteins. Nevirapine concentrations in cerebrospinal fluid were 45% of the concentration of the plasma, with the ratio approximately equivalent to the unbound fraction. Nevirapine is extensively metabolized by the liver, and biotransformation of nevirapine occurs *via* auto induction of the cytochrome P450 CYP3A and CYP2B6 isoenzymes. Nevirapine is known to induce these isoenzymes by as much as 20%. The excretion of nevirapine is 81.3% as metabolites, while the mother compound only comprises of 5% of the 81.3% that can be recovered from the urine. Nevirapine is also excreted in the faeces and accounts for 10.1% of the total excretion (Micromedex, 2009).

2.4.12.5 Common side effects

Side effects found with nevirapine treatment include fatigue, headache, insomnia, lymphadenopathy, myalgia, arthralgia, angioedema, fever and blood discrasias (Holtt & Ju, 2006; Micromedex, 2009).

2.4.12.6 Toxicological adverse effects

Overdose information regarding nevirapine is minimal. Nevirapine appears to have minimal toxicity when taken in higher than normal doses. Toxicological effects found with nevirapine overdose include severe and life threatening skin reactions such as Steven Johnson syndrome, toxic epidermal necrolysis, and severe hepatotoxicity (Micromedex, 2009).

2.4.12.7 Pharmacokinetic drug interactions of Nevirapine

Nevirapine can cause drug interactions with other medications used by the patient. Low plasma concentrations of drugs metabolized by CYP3A4 or CYP2B, decreased enzymes and methadone levels, lower efavirenz levels, and increased levels with ketoconazole are found. The AUC and Cmax of nevirapine are reduced by Rifampicin (Berkow *et al*. 1992).

2.4.13 STAVUDINE

2.4.13.1 Therapeutic use

Stavudine (Stavir®, Zerit®) is a nucleoside reverse transcriptase inhibitor used in combination with other antiretroviral agents for the treatment of HIV in adults and children (Micromedex, 2010).

2.4.13.2 Contra-indications

Stavudine safety in pregnancy and lactation has not been established. Stavudine is contra-indicated in children under the age of 6 months, and concomitant use with didanosine and zidovudine should be avoided (Carr & Cooper 2000).

2.4.13.3 Mechanism of action

Stavudine is a synthetic thymidine nucleoside analogue. Stavudine is phosphorylated by cellular enzymes to its monophosphate, diphosphate and triphosphate form of which the triphosphate form is responsible for its antiviral effects through selective inhibition of HIV reverse transcriptase.

2.4.13.4 Absorption, Distribution, Metabolism, and Excretion

Stavudine has good bioavailability of 77% to 86% after oral ingestion. Stavudine may be taken with or without food. Protein binding of the drug is negligible and mean CSF to plasma concentration ratio is 59%. Metabolism of stavudine occurs in the liver although metabolism plays a minor role in the clearance of the drug. Unchanged stavudine is the major circulating component in the plasma while metabolites such as oxidized stavudine, glucoronide conjugates and an N-acetylcysteine conjugate is the minor component in the plasma. Approximately 40% of the unchanged drug is excreted *via* the kidneys, and 3% is excreted in the faeces (Micromedex, 2010).

2.4.13.5 Common side effects

Side effects commonly experienced by patients include rash, diabetes mellitus, fat maldistribution, abdominal pain diarrhoea, nausea, vomiting and a loss of appetite. Lactic acidosis, hepatomegaly, steatosis of the liver and liver failure may occur. Muscle weakness and myalgia are common. Haematological adverse effects include anaemia, leukopenia, macrocytosis, neutropenia and thrombocytopenia. CNS effects such as Guillain-Barre syndrome, headaches, peripheral neuropathy and cramping has also been reported. Stavudine is ototoxic and may cause a bilateral neurosensorial hearing deficit. Stavudine

may also cause nervousness and sleep disorders with mania and anxiety (Carr & Cooper 2000; Micromedex, 2010).

2.4.13.6 Toxicological adverse effects

Acute overdoses may result in fatal lactic acidosis, bone marrow suppression, peripheral neuropathies, respiratory failure, liver failure, steatosis of the liver and manic episodes (Micromedex, 2010).

2.4.13.7 Pharmacokinetic drug interactions of Stavudine

Didanosine increases stavudine plasma concentrations and the combination may cause severe lactic acidosis and hepatotoxicity. Doxorubicin hydrochloride, zidovudine, ribavirin and methadone decreases stavudine efficacy. The hepatotoxic effects of stavudine are increased by hydroxyurea (Carr & Cooper 2000; Micromedex, 2010).

2.4.14 TENOFOVIR

2.4.14.1 Therapeutic use

Tenofovir (Truvada®, Viread®) is a drug used in combination with other antiretroviral agents for the treatment of HIV in adults and children and for the treatment of chronic Type B Hepatitis in the HIV positive patient (Gallant & Deresinski, 2003; Micromedex, 2010).

2.4.14.2 Contra-indications

Tenofovir safety in pregnant and lactating woman, and children younger than 18 has not been established. Tenofovir is contra-indicated in patients with renal failure, and should not be used in Hepatitis B virus co-infections unless an appropriate antiretroviral combination regimen is in place to avoid HIV-1 resistance.

2.4.14.3 Mechanism of action

Tenofovir disoproxil fumarate is an acyclic nucleoside phosphonate nucleotide. It does not require intracellular phosphorylation and is rapidly converted to its corresponding active diphosphate form. The active diphosphate form of tenofovir inhibits retroviral reverse transcriptase intracellularly (Micromedex, 2010).

2.4.14.4 Absorption, Distribution, Metabolism, and Excretion

Tenofovir has an approximate bioavailability of 25% to 40% after oral administration. Tenofovir AUC and bioavailability increases when administered with food to HIV patients. Less than 7.2% of the drug is protein bound and is extensively distributed in lymphocytes where it is converted to its active diphosphate form. Tenofovir undergoes minimal systemic metabolism. Tenofovir disoproxil fumarate is rapidly hydrolysed to tenofovir by endogenous plasma esterases after oral absorption. Tenofovir is excreted through both glomerular filtration and tubular secretion *via* the kidneys and approximately 70%-80% of the compound is excreted unchanged (Micromedex, 2010)

2.4.14.5 Common side effects

Side effects commonly experienced by patients include headaches, dizziness, depression, peripheral neuropathy, nausea, diarrhoea, vomiting, flatulence, neutropenia, pruritus, maculopapular rash, pustular rash and urticaria. Common metabolic disturbances such as antiretroviral associated fat maldistribution and lipodystrophies including central obesity, dorsocervical fat enlargement, peripheral and facial wasting, breast enlargement, hypophosphatemia, renal failure, hepatotoxicity and lactic acidosis can also occur. Disorders of the muscles and bones such as osteomalacia, osteopenia with associated increase in bone fragility, muscle weakness and rhabdomyolysis is a common phenomenon in patients on tenofovir treatment. Tenofovir may cause Fanconi syndrome, allergic reactions and immune reconstitution syndrome where the patient may have an inflammatory response to indolent or residual opportunistic infections (Gallant & Deresinski, 2003; Micromedex, 2010).

2.4.14.6 Toxicological adverse effects

Severe and fatal lactic acidosis has been reported in acute and chronic overdoses of tenofovir. Acute toxicological events such as severe allergic reactions, renal failure, hepatotoxicity and hepatic steatosis may occur in overdose (Micromedex, 2010).

2.4.14.7 Pharmacokinetic drug interactions of Tenofovir

Tenofovir treatment can interfere with the metabolism of other drugs taken by the patient. Tenofovir increases plasma levels of other drugs that are eliminated by active tubular secretion. Tenofovir increases the Cmax and AUC of didanosine while a decrease in atazanavir AUC and Cmin may occur (Micromedex, 2010).

2.4.15 ZIDOVUDINE

2.4.15.1 Therapeutic use

Zidovudine (Retrovir®) is a drug used in combination with other ARVs, indicated for HIV in adults and children where the T helper cell count is below 500 per cubic millimetre (mm^3) (Denelsbeck, 2006). Zidovudine is indicated in pregnant, HIV positive women after 14 weeks of gestation and new born infants to reduce the risk of maternal-foetal transmission. Zidovudine is also indicated in advanced disease states of HIV e.g. AIDS and ARC, as well as post exposure prophylaxis (Delfraissy *et al*. 2008 Micromedex, 2009).

2.4.15.2 Contra-indications

Zidovudine is contra-indicated in patients with Neutropenia and low Hb levels less than 7.5 gram per decilitre (g/dl). The safety of zidovudine during the first trimester of pregnancy is not established, and data available for use in children under 3 months is insufficient to propose a specific dose recommendation (Micromedex, 2009).

2.4.15.3 Mechanism of action

Zidovudine is a synthetic Thymidine analogue with antiretroviral properties. Zidovudine is a potent inhibitor of the *in vitro* replication and cytopathic effect of HIV. Zidovudine interferes with retroviral DNA polymerase (reverse transcriptase) and inhibits viral replication. Zidovudine is intracellularly converted to zidovudine monophosphate. It is further converted into a diphosphate and a triphosphate form. Zidovudine triphosphate interferes with reverse transcriptase thus inhibiting viral replication. The azido group substitution makes phosphadiester linkages impossible and terminates chain synthesis of DNA. HIV is 100 times more susceptible to inhibition by zidovudine than the DNA polymerase of human cells (Micromedex, 2009).

2.4.15.4 Absorption, Distribution, Metabolism and Excretion

Zidovudine has good bioavailability of 64%, and ingestion together with food does not affect the bioavailability, nor does gastric hypoacidity which occurs in 20% of patients with HIV. Less than 38% of zidovudine is plasma bound. Cerebrospinal fluid concentrations are 50% to 70% of plasma levels. Zidovudine crosses the placenta, and the foetal blood concentration is equivalent to concentrations in the maternal blood. Zidovudine and its glucoronide metabolite are highly distributed into the semen with semen/serum ratio levels ranging from 1.3 to 20.4, which are above the *in vitro* minimum inhibitory concentration (MIC) for HIV-1.

Zidovudine is extensively metabolized in the liver with significant first-pass metabolism and is mainly excreted *via* the kidneys, and urinary recovery of the drug is between fourteen to eighteen percent (14–18%). The urinary recovery for the glucoronized metabolite is 74% (Delfraissy *et al*. 2008; Micromedex, 2009).

2.4.15.5 Common side effects

Side effects commonly experienced are central nervous system (CNS) effects such as psychosis, severe acute depression and suicide, Steven Johnson syndrome, abnormal co-ordination, hot flushes, syncope, malaise, peripheral neuropathy, speech-, visual-, and taste disorders, paraesthesia, hepatitis and gastrointestinal (GI) disturbances. Side effects commonly experienced with zidovudine treatment include bone marrow depression, hepatomegaly, pancreatitis, lactic acidosis, convulsions, flu–like symptoms with cough, nail and skin pigmentations, chest pain, chills and gynecomastia in male patients (Holtt & Ju, 2006; Micromedex, 2009).

2.4.15.6 Toxicological adverse effects

Several cases of acute overdose with zidovudine have been reported in the literature with minimal toxicological effects. Overdose effects may include bone marrow suppression, lethargy, fatigue, and possibly seizures (Delfraissy *et al*. 2008; Micromedex, 2009).

2.4.15.7 Pharmacokinetic drug interactions of Zidovudine

Zidovudine treatment poses several drug interactions with other medication. Increased incidents of neutropenia occur with the use of paracetamol. Inhibition of hepatic microsomal metabolism causes an increased toxic risk with potent nephrotoxic and myelosuppressant drugs (Micromedex, 2009).

2.5 ANALYTICAL METHODS USED IN THE DETECTION OF METABOLITES IN URINE

The identification of drug metabolites in biological matrixes such as urine, plasma, and bile, as well as in in vitro systems, is an important step in drug discovery and development. Mass spectrometry, particularly when combined with high-performance liquid chromatography, can enable detailed structural information to be obtained on the metabolites of a drug as a result of metabolism. The successful identification of drug metabolites by high performance liquid chromatography-mass spectrometry (HPLC-MS) based methods require careful optimization

of a number of factors. First, the chromatographic separation should provide good resolution of the individual xenobiotic metabolites present in the sample. It is also required to minimize the interference caused by the presence of endogenous metabolites, which may interfere with mass spectrometry (MS) detection. Ideally, untreated samples should be profiled to reduce the likelihood of missing important metabolites due to losses during sample preparation, but, depending upon the matrix, some degree of sample extraction and concentration of the metabolites may be required using liquid–liquid or liquid–solid extraction. Second, the MS conditions must be carefully selected in order to maximize the potential of detecting the separated metabolites, which may have a very different character to the parent compound. The use of radiolabeled drugs in metabolism experiments greatly aids in the detection and quantification of metabolites, directing the investigator towards peaks that need to be characterized by MS. The presence of characteristic isotope patterns from either the incorporation of stable isotopes (e.g. ^{13}C, ^{15}N) or naturally occurring isotope patterns from substituents on the molecule (e.g. $^{35/37}Cl$, $^{79/81}Br$) can also provide a useful handle on the drug and its metabolites for the purposes of detection and spectrometric interpretation (Wilson, 2010).

The basic rule of thumb is that most metabolites are more hydrophilic when compared to the parent compound, and also more acidic. The most used and versatile analytical methods for metabolite profiling are based on combining liquid chromatography with mass spectrometry (LC/MS), providing both qualitative and quantitative information simultaneously. Use of mass spectrometry as a detection method provides also high detection specificity, as the mass selective detection decreases the need for complete chromatographic separation for compounds with different molecular weight.

The most efficient approaches in metabolite identification are those using time-of-flight mass (TOF) spectrometry or Orbitrap mass spectrometry. The high mass resolving power of these instruments provides high specificity of detection, whereas the high mass accuracy enables identification of biotransformations in the detected metabolites with very high certainty by elucidation of changes in molecular formula with respect to the parent compound (Admescope, 2014).

Ter Heine *et al*. (2009) described liquid chromatography coupled to linear ion trap mass spectrometry and liquid chromatography coupled to triple quadrupole mass spectrometry to identify atazanavir metabolites. Glucoronidase treatment and a liquid chromatography-

tandem mass spectrometry (LC/MS/MS) method for the determination of nevirapine metabolites was proposed by Cammett *et al*. (2009).

An LC/MS/MS method equipped with a triple-quadrupole mass spectrometer and an electrospray ion source, operated in positive-selective-reaction monitoring mode, was used by Chen *et al*. (2007).

2.6 ANALYTICAL METHODS USED IN THE DETECTION OF ARV DRUGS IN URINE

Several studies describe different methods for the detection of ARV drugs in biological matrixes. A two-dimensional reversed-phase liquid chromatography – tandem mass spectrometric method to determine antiretroviral drugs abacavir, nevirapine and indinavir in rat serum and urine was proposed by Rao & Shinde (2009). The analytes were extracted on-line from rat serum and urine by a restricted access material (RAM) column and back-flushed into the reversed-phase C18 column for separation by LC. Detection was carried out by electrospray tandem mass spectrometry (ESI-MS/MS).

Morris & Selinger (1994) have described a high-pressure liquid chromatographic method for the determination of lamivudine in urine which allows direct injection of urine with column switching. The method requires two columns, one for removal of unwanted urine constituents, and the other for elution of lamivudine. Supriya, Ashish & Meena (2012) proposed a high performance liquid chromatography tandem mass spectrometric method for the estimation of emtricitabine in human urine by utilizing solid phase extraction. The samples were chromatographed on Hypurity Advance, 50 x 2.1, 5µ column using a mobile phase consisting 5mM ammonium acetate: acetonitrile: methanol: (30:30:40 v/v).

Elens *et al*. (2009) described an ultra-performance liquid chromatography (UPLC) method with a solid-phase extraction method used for plasma. A linear gradient of 50 mmol/L ammonium acetate and 50 mmol/L formic acid in water versus acetonitrile was used to achieve chromatographic separations. Direct injection of diluted urine (1:10/1:50) and analysis with a 150-mm column with UV detection was used by Agibothu *et al*. (2006). The HPLC system used comprised of two pumps, a diode array detector, and a rheodyne manual injector attached with a 20 microliter sample loop for sample injection.

Remmel *et al*. (2000) proposed high performance liquid chromatography with a liquid-liquid extraction for sample preparation using methanol as a solvent. Lemmer *et al*. (2005) described a gas chromatography-mass spectroscopy in selected ion monitoring mode (GC/MS-SIM) detection method together with solid-phase extraction for determination of antiretrovirals in human plasma.

A reverse-phase high performance liquid chromatography method utilizing ultraviolet detection was proposed by de Oliveira *et al*. (2005) for the determination in didanosine tablets. They proposed a pre-packed Lichrospher 100 Rp-8 column using 0.01 M sodium acetate solution: methanol (85:15 v/v). Acetic acid was used as mobile phase at a flow rate of 1.5 ml/min and a 248 nm detection. Fletcher *et al*. (2000) used a reverse-phase high pressure liquid chromatography method to quantify indinavir in plasma. A 35:65 (v/v) solution of acetonitrile in a 50 mM phosphoric acid o-phosphoric acid buffer adjusted to a pH of 3.1 was used. A liquid-liquid extraction was used to separate compounds of interest from the plasma matrix. A reverse-phase high performance liquid chromatography method utilizing a diode array detector and liquid-liquid extraction was used to develop the current HPLC method used by the Department of Pharmacology/Toxicology, University of the Free State in Bloemfontein, South Africa. Various GC/MS and HPLC methods and conditions, as well as extraction methods have been investigated.

2.6.1 CHROMATOGRAPHY

Chromatography refers to a physical separation method used to separate components. The components which need to be separated are distributed in two phases, a stationary phase and a mobile phase. During the chromatographical process a continuing sorbsion and desorpsion of particles take place as the components move through the stationary bed. Separation is achieved because of the difference in distribution coefficients of each component (Dinunzio, Hutchison, & Yost 1985).

2.6.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography refers to high speed, high resolution separation of substances. Separation of substances is achieved by the extraction of the components from the urine and reconstituting the precipitate into a mobile phase compatible with the selected HPLC system. The extraction is then injected into a liquid mobile phase which acts as a carrier for the different components. The extraction is then carried through a

chromatographical column *via* the mobile phase. The column consists of a cylindrical bed, tightly packed with small sorbsion particles with a diameter of 10µm or smaller (Dinunzio *et al*. 1985).

The HPLC system is selected so that every component in the extraction has a unique and characteristic speed through which it will move through the column. Sorbsion of the components causes its retention, and retention differences are a result of the chemical selectivity of the system. Separation of the components takes place and the components form thin chromatographic bands which elutes at the end of the column. The mobile phase flows through a detector which records the chromatographic bands and then sends a signal to a register which interprets the chromatographic bands as a series of peaks on a chromatogram (Dinunzio *et al*. 1985).

2.6.3 REVERSED PHASE CHROMATOGRAPHY

Reversed phase chromatography is the most popular form of HPLC because it can handle the greatest variety of sample types. In reversed phase a non-polar stationary phase and polar mobile phase is used. The introduction of alkyl chains bonded covalently to the silica support surface enables the polar compounds to elute first while the non-polar compounds are retained in the column. The retention time of a compound can be increased by adding more water to the mobile phase. This will increase the affinity of the hydrophobic compound for the hydrophobic stationary phase relative to the now more hydrophilic mobile phase. Retention times of compounds can be decreased by adding more organic solvent to the mobile phase mixture (Guzzetta, 2001).

2.6.4 GRADIENT ELUTION

Gradient elusion refers to a separation in which the mobile phase composition is changed during the separation process. Gradient elusion decreases retention times of later-eluting components so that they elute faster, giving narrower and taller peaks for most compounds. This also improves the peak shape for tailed peaks. A gradient elution method was used where the mobile phase composition was changed during the analysis time of 30 minutes. At the beginning of the run Solvent B (organic solvent) is at 0% strength and Solvent A (aqueous solvent) at 100% (10% Acetonitrile (ACN) preservative added) strength resulting in the resolution of the early components. As the separation progresses, the ratio of Solvent B

to Solvent A is gradually increased so that the subsequent components elute within a reasonable time (Du Plessis, 1994).

2.7 COLUMN THEORY

Chromatography separates a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation. A successful chromatographical separation requires a delicate balance between resolution, sample capacity and analysis time as described in 2.5.1 to 2.5.5 below:

2.7.1 RESOLUTION (R)

Resolution reveres to the physical separation between two components (Figure 1). For optimal quantitative and qualitative analysis, the resolution value must be equal to or greater than 1.25 (Dinunzio *et al*. 1985). Resolution can be quantified by the following formula:

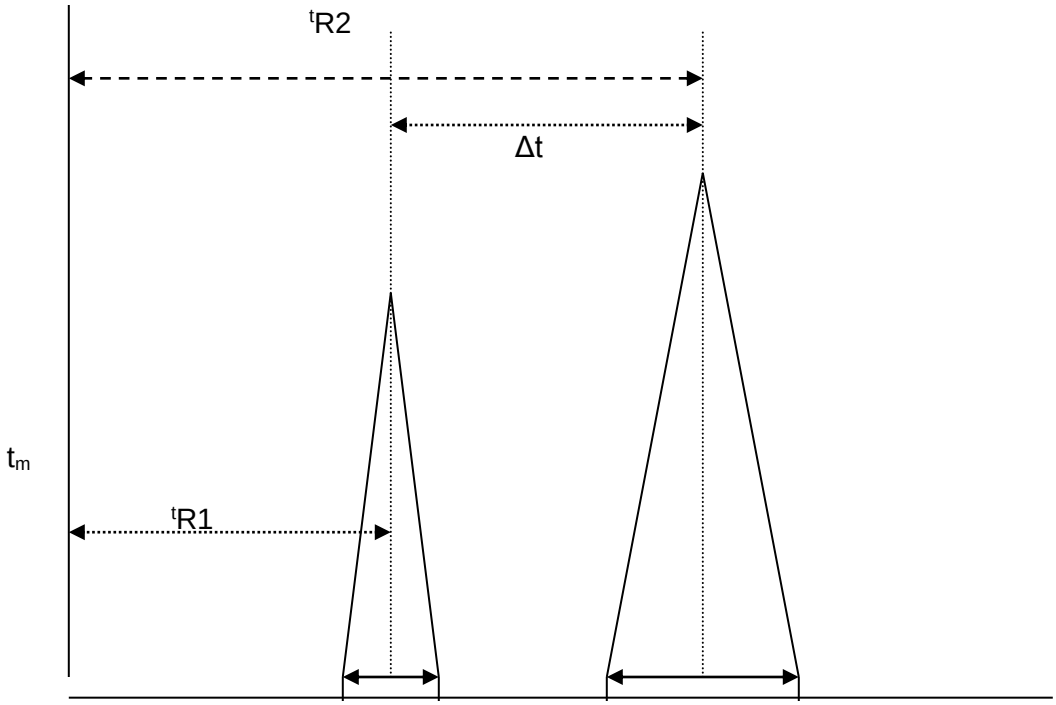
$$R = \frac{{}^{t}R2 - {}^{t}R1}{\frac{W_2 + W_1}{2}} = \frac{2\Delta t}{W_2 + W_1}$$

R = resolution

${}^{t}R_2$ and ${}^{t}R_1$ = retention times of components

W_2 and W_1 = peak width at the base of the peak

Δt = difference in time



$^{t}R_1$ = retention time of component 1

$^{t}R_2$ = retention time of component 2

$^{w}B1$ = peak width at base of peak 1

$^{w}B2$ = peak width at base of peak 2

Δt = difference in time

Figure 2: Resolution of the physical separation between two components

For optimal quantitative and qualitative analysis: R ≥ 1.25 (Dinunzio *et al*. 1985).

2.7.2 COLUMN EFFICIENCY

To obtain optimal separations of compounds, sharp, symmetrical chromatographic peaks must be obtained and peak broadening must be limited. The efficiency of a column is expressed by the quantity of theoretical plates (N).

Column efficiency increases as the quantity of theoretical plates increases (Dinunzio *et al*. 1985). The value of N is independent from the retention time and can be calculated by the following formula:

$$N = [{}^{t}R/\alpha]^2$$

N = quantity of theoretical plates

${}^{t}R$ = retention time

α = column selectivity

Column length is a variable factor. Because of this it is necessary to standardize the formula for efficiency which is independent from the column length. The highest equivalent of theoretical plates (HETP) is used to compare columns with different lengths:

$$HETP = \frac{L}{N}$$

HETP = highest equivalent of theoretical plates

L = length of the column

N = quantity of theoretical plates

2.7.3 COLUMN SELECTIVITY (A)

Column selectivity describes the relative position of two adjacent peaks.

$$\alpha = \frac{{}^{t}R2-{}^{t}m}{{}^{t}R1-{}^{t}m}$$

$$= \frac{{}^{t}R2'}{{}^{t}R1'}$$

α = column selectivity

$^{t}R1$ = retention time of peak 1

$^{t}R2$ = retention time of peak 2

^{t}m = retention time of solvent front

When α = 1, the two peaks have the same retention time and therefore no resolution. Better resolution can be achieved by changing α by changing either the mobile phase or the stationary phase (Dinunzio *et al*. 1985).

2.7.4 FLOW RATE (U)

Component molecules move through the column *via* a mobile phase. The flow rate of the mobile phase influences the chromatographic process. Flow rate can be determined with the following formula:

$$u = L\ (^{t}m)$$

u = flow rate

L = column length in mm

^{t}m = retention time of solvent front

An optimal flow rate exists where the column is most effective in the separation of components. If the flow rate is to slow, component molecules will diffuse in the mobile phase which will result in broad peaks. If the flow rate is too fast, component molecules will elute with the solvent front and no separation will be achieved. Most HPLC determinations use a flow rate of 0.5 – 2.0 ml/min. (Guzzetta, 2001).

2.7.5 CAPACITY (K')

Resolution is a function of the relationship of the volumes of the mobile phase and the stationary phase in the column. This relationship is known as the capacity factor. The capacity factor indicates how long each compound is retained by the stationary phase, and the time the component spends in the mobile phase. This relationship can be expressed by the following formula:

$$k' = \frac{^{t}R}{^{t}m}$$

^{t}R = retention time

k' = capacity

^{t}m = retention time of solvent front

Small values for k' indicates that the component is minimally retained in the column and results in low resolution of the component. An ideal value for k' would be between 2 and 7 (Dinunzio *et al*. 1985).

The relationship of abovementioned characteristics can be expressed in the following formula:

$$R = \frac{1}{4}\sqrt{N} \quad (\alpha - 1/\alpha) \quad (k'/k' + 1)$$

a **b** **c**

a = column efficiency

b = column selectivity

c = capacity

▪ Resolution can be optimized by either changing **a, b** or **c**.

To obtain optimal separations of compounds, sharp, symmetrical chromatographic peaks must be obtained and peak broadening must be limited. This can be achieved by manipulating the different factors in the column theory:

- For optimal quantitative and qualitative analysis, resolution (R) must be equal to, or higher than 1.25
- Column efficiency can be optimized by the addition of stationary phase. If this is not possible, another column can be attached which in turn will increase the quantity of the theoretical plates
- Increase resolution by changing column selectivity (α)
- Changing column selectivity (α) by changing either the mobile phase or the stationary phase in order achieve good separation of peaks
- By manipulation of the flow rate (u)
- Capacity (k') can be optimized by manipulating the flow rate (u), or by changing the mobile phase or stationary phase to obtain a value between 2 and 7

- By changing the gradient elution

The selectivity of the HPLC system can be increased by manipulation of the different factors involved. Highly selective HPLC systems will ensure good quality separations of substances and easy identification and quantification of these substances (Snyder, 1997).

2.8 DIODE ARRAY DETECTION

Chemical compounds have characteristic spectra which can assist in their identification. The diode array detector offers detection over a wide range of ultraviolet (UV) wavelength. The most popular wavelengths range from 210 nanometre (nm) to 330nm. Ultraviolet light from a deuterium lamp passes through a flow cell after which it is dispersed into its component wavelengths by a fixed grating. The intensity at each wavelength is simultaneously measured by an array of several hundred photodiodes. The output from each diode is processed, stored and displayed continuously as the run progresses. The results can then be used to construct absorption spectra that can be compared with standard spectra for identification purposes (Scott, 2010).

A special feature of some variable wavelength UV detectors is the ability to perform spectroscopic scanning and precise absorbance readings at a variety of wavelengths while the peak is passing though the flow cell. Diode array adds a new dimension of analytical capability to liquid chromatography because it permits qualitative information to be obtained beyond simple identification by retention time. Highly selective HPLC systems with diode array detection will ensure easy identification and quantification of these substances.

3 MATERIALS AND METHODS

3.1 INTRODUCTION

The development of a qualitative HPLC method for the identification of ARVs in urine will be presented in this chapter. The identification, standardization and validation of this method included the recording of retention time and spectra of the known antiretroviral pure compounds, setting up of a calibration curve at different concentrations of each compound, accuracy, precision, recovery of the different antiretroviral compounds and the identification of ARV metabolites in a urine sample.

3.2 STUDY LOCATION

The study was conducted at the Department of Pharmacology/Toxicology of the University of the Free State in Bloemfontein, South Africa.

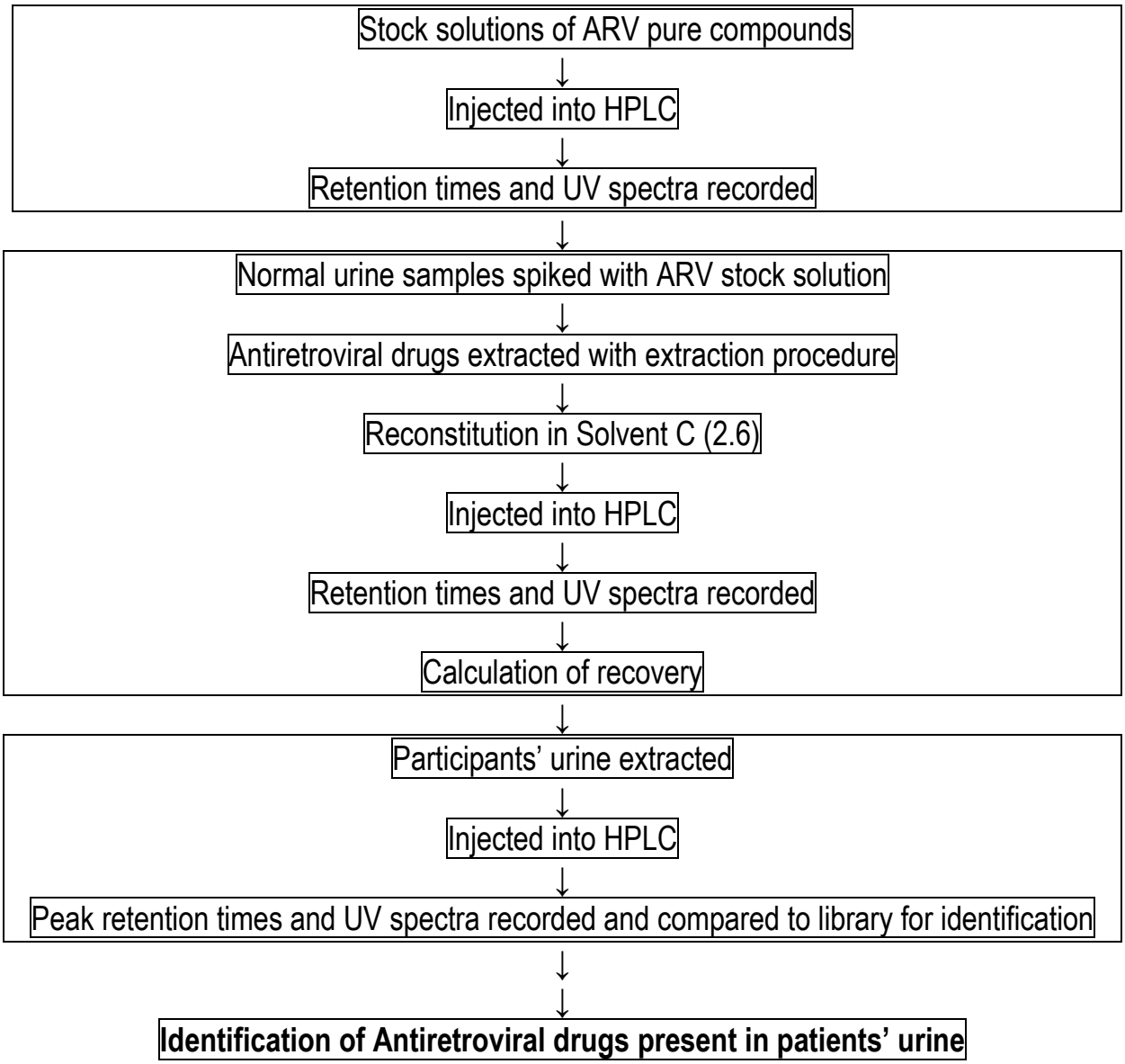
3.3 STUDY DESIGN

It is an experimental, explorative descriptive study

3.4 STUDY LAYOUT

The first step in the development of an HPLC method was to identify a suitable solvent in which the pure compounds to be investigated can dissolve in. A solvent (Solvent C, a mixture of Tetraethylammonium phosphate buffer, Acetonitrile and H_3PO_4) was selected and Solvent C was used to prepare stock solutions and to reconstitute residues of urine samples after extraction method was completed. A stock solution of each ARV, lamivudine, abacavir, zidovudine, nevirapine, efavirenz and the new additions, stavudine, didanosine, lopinavir, ritonavir and tenofovir was prepared by dissolving the pure compound in solvent C. Initial trial runs were performed to assess the required concentration for each stock solution. Each individual ARV stock solution was injected into the HPLC. After the analysis was completed, retention times (Figure 4.11) and UV spectra (Appendix 7.25 to 7.32) of each ARV was recorded and stored in a computerised library. Normal urine samples were spiked with the stock solutions and the pure compounds was extracted with the extraction procedure described in 3.10. The precipitate was then reconstituted in Solvent C and injected into the HPLC to analyse the retention times and UV spectra after the extraction procedure was

performed on the samples. The recovery of each compound was calculated to establish the efficacy of the extraction procedure. Participants on ARV treatment donated urine samples for analysis of the method. The patient urine samples were subjected to the same extraction procedure described in 3.10, injected into the HPLC and investigated. Peaks and UV spectra detected in patient samples were compared to the stored database of ARVs in the computerised library. Successful identifications of the ARVs could be made in the donor patient urine.



3.5 ETHICAL ASPECTS

Ethical approval was granted by the ethics committee, UFS. ETOVS no: 2010/100. Permission for the study was granted by Internal Medicine at Pelonomi Hospital in Bloemfontein, South Africa and the department of Pharmacology/Toxicology at the University of the Free State in Bloemfontein, South Africa.

3.6 STUDY POPULATION

The study population was HIV positive patients on ARV treatment. Subjects were recruited from internal medicine at Pelonomi hospital, Bloemfontein. The subjects were identified with a PM number supplied by Pelonomi hospital. Thirty seven individuals were identified by clinicians from Internal medicine according to the ARV regimen of each patient in order to cover the spectrum of the antiretroviral drugs applicable to the study. The clinicians introduced the subjects to the study by supplying them with an information document and explained the procedures and confidentiality of their participation. They assisted the subjects with completion of the informed consent forms. Urine samples were collected during four consecutive routine follow-up visits to the HIV clinic of Internal medicine. The study was explorative in nature and did not require a large sample population.

The donor of the urine samples that was spiked with pure ARV compounds was a healthy individual that has never taken antiretroviral drugs. Some commonly used acidic drugs such as paracetamol, salicylic acid and the non-steroidal anti-inflammatory drugs (NSAIDs) can be detected by HPLC. Therefore the donor was free from any medication for at least 24 hours prior to donation. The donor also received an information document and was required to complete informed consent forms.

3.6.1 INCLUSION CRITERIA

- HIV positive male and female patients
- Patients above the age of 18 years
- Subjects must be on the following ARV treatment applicable to the study: Abacavir, Didanosine, Efavirenz, Lamivudine, Lopinavir/Ritonavir, Nevirapine, Stavudine, Tenofovir and Zidovudine.
- Subjects must be on ARV treatment for at least 6 weeks in order to participate in the study

- Subjects who have given informed consent to participate in the study

3.6.2 EXCLUSION CRITERIA

- HIV negative patients
- Patients not on ARV treatment
- Patients younger than 18 years
- Pregnant women

3.6.3 FINANCIAL IMPLICATION FOR THE PATIENTS

Participants did not receive any payment for their participation in the study, nor was any payment received from the patients. Sample containers were supplied by the researcher at no cost to the patients.

3.6.4 PATIENT SAFETY

One urine sample was collected over 4 consecutive routine weekly follow-up visits to the HIV clinic. Urine samples contained at least 20 ml of urine. Samples were collected according to prescribed sterile sample collection techniques to insure patient safety.

3.6.5 WITHDRAWAL CRITERIA

Participants could withdraw from the study at any time, irrespective of the reason, without any penalty or effect on the current or future care and treatment. No patients withdrew from the study and all participating patients adhered to their scheduled follow-up visits for a full medical check-up and sample collection.

3.7 STORAGE AND DISPOSAL OF HUMAN TISSUE

Urine samples were refrigerated until processed within 48 hours after donation. Urine samples were then frozen at a temperature of -20˚C until the study has been completed. Samples were stored in a confidential manner. No patient details were on the sample containers. Samples were identified by a PM number supplied by Internal medicine at Pelonomi Hospital. Biological samples were discarded in biological waste containers supplied by the Department of Pharmacology for safe removal after the study was completed.

Specimens were not used for any other tests than that mentioned to the patient and approved by the ethics committee.

3.8 APPARATUS

3.8.1 APPARATUS

A precision balance from Mettler Toledo Instruments (Switzerland) was used to weigh the pure compounds of the individual stock solutions and the constituents of the mobile phases. The precision balance was calibrated daily and has a minimum weight capacity of 0.01g. Pipettes from Finnipipettes® and their consumables were used for the accurate pipetting of blood and urine samples, chemicals and solutions. Pipettes were calibrated annually by AEC Amersham as determined by the manufacturer. For the spiking of the stock solutions, 10µL and 100µL syringes from Hamilton (Switzerland) were used. A horizontal shaker from Gesellschaft Labortechnik (Germany) was used for the mixing of the extraction samples. Samples were centrifuged with a Labofuge 400 from Haraeus Instruments (Germany). All centrifuges were calibrated annually during the yearly service specified by the manufacturer. The organic layer of the extraction was evaporated to dryness by using an evaporator with a nitrogen gas supply manufactured by the Electronics division of the University of the Free State in Bloemfontein, South Africa (Du Plessis, 1994).

HP 1090 Liquid Chromatograph by Hewlett Packard equipped with Chemetrix System Software and a solvent delivery system and auto sampler was used. A synergi 4U Fusion – RP 80 Column with a size of 150 x 4.6 mm and a UV/VIS Diode array detector by Agilent Technologies was used.

3.8.2 CHEMICALS AND REAGENTS

3.8.2.1 Antiretroviral pure compounds

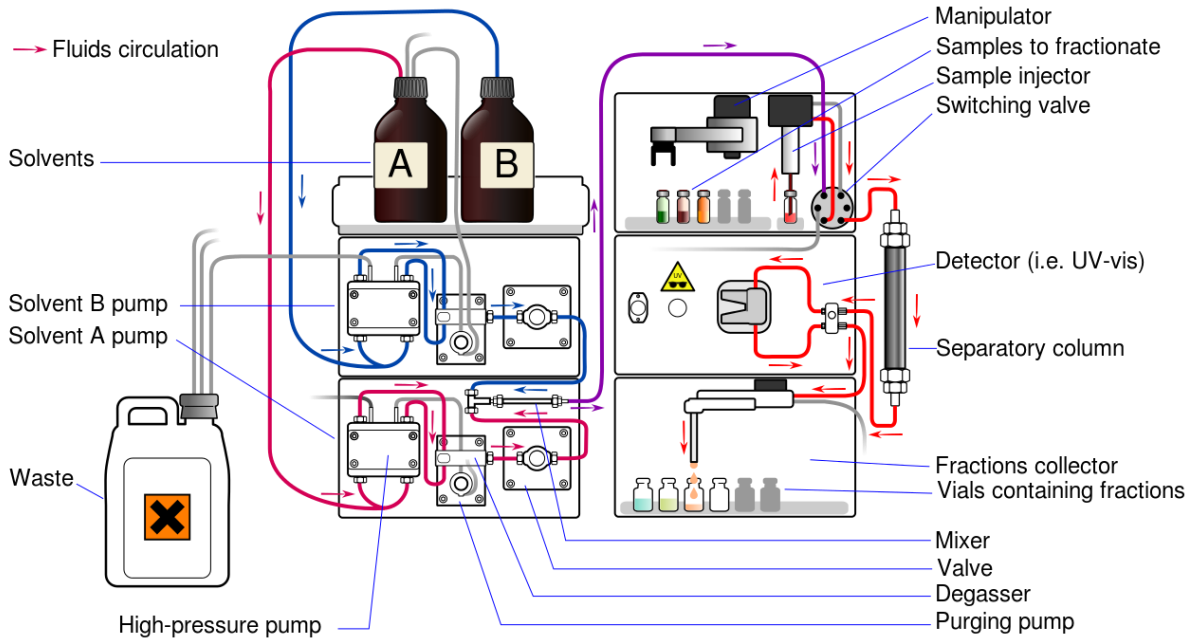
Pure compounds of zidovudine (reference number 1736), abacavir (reference number 2067) and lamivudine (reference number 535) were donated by GlaxoSmithKline. Nevirapine pure compound (reference number B1P1-0053) was obtained from Boehringer & Ingelheim, and efavirenz pure compound (reference number 050301) was obtained from Xiamen Mchem. Pure compounds of clomipramine (internal standard), didanosine, stavudine, tenofovir and lopinavir were obtained from the Pharmacology reference stock library.

3.8.2.2 Chemicals

HPLC grade acetonitrile, isopropyl alcohol, and dichloromethane were purchased from Burdick & Jackson (USA). Potassium bicarbonate (K_2CO_3), sodium hydroxy carbonate ($NaHCO_3$), phosphoric acid (H_3PO_4) and Tetraethylammonium phosphate formula was purchased from Merck (SA).

3.8.2.3 Solvents

A gradient mobile phase was used in the HPLC analysis which consisted of two solvents, solvent A and solvent B. The mobile phase solvents acts as carriers of the compounds of interest from the extraction through the HPLC column and are loaded on to the HPLC before the run commences. This process will be clarified in section 3.11 Chromatographic systems and conditions discussed in this chapter.



(Chromatography, 2008)

Figure 3: The HPLC system

Three different solvents were used in the study:

1. Solvent A: Tetraethylammonium phosphate (TEAP) buffer + Acetonitrile (9:1). Solvent A was used as a mobile phase in the gradient elution and loaded on to the HPLC

2. Solvent B: Acetonitrile + H_3PO_4. Solvent B was used as a mobile phase in the gradient elution and loaded on to the HPLC

3. Solvent C was prepared by adding 150ml of Solvent A to 30ml of Solvent B (5:1). Solvent C was used to reconstitute the evaporated extraction for injection into the HPLC.

3.9 PREPARATION OF MOBILE PHASES

A gradient mobile phase was used in the HPLC analysis which consisted of two solvents, solvent A and solvent B. The mobile phase solvents acts as carriers of the compounds through the HPLC column.

Solvent A (Aqueous solvent)

Solvent A comprised of 450ml tetraethylammonium phosphate (TEAP) buffer and 50ml of acetonitrile (ACN). It was prepared by adding 15.54g tetraethylammonium hydroxide to 400ml of deionised water, adding 50ml of ACN, and reconstituting it to 500 ml by using deionised water.

Solvent B (Organic solvent)

Solvent B was prepared by adding 100µl H_3PO_4 to 950ml of ACN and reconstituting it to 1L with ACN.

3.9.1 PREPARATIONS OF OTHER SOLVENTS

K_2CO_3 / $NaHCO_3$ Buffer

The K_2CO_3 / $NaHCO_3$ Buffer (pH 9.6) was prepared by adding 20g K_2CO_3 and 20g $NaHCO_3$ to 160ml deionised water. The pH was gradually lowered to 9.6 by adding 20% $NaHCO_3$.

Dichloromethane isopropanol

Dichloromethane isopropanol (CH_2Cl_2 / CH_3CH (OH) CH_3) in a 9:1 ratio was prepared by adding 100ml of isopropanol to 900ml dichloromethane. Dichloromethane isopropanol was used to extract the compounds of interest from urine. The compounds migrate from the urine matrix into the Dichloromethane isopropanol during mixing and centrifugation. This is referred to as the organic layer.

3.9.2 PREPARATION OF STANDARD SOLUTIONS

The current HPLC method developed by the Department of Pharmacology/Toxicology, University of the Free State in Bloemfontein, South Africa, can identify the presence of lamivudine, abacavir, zidovudine, nevirapine and efavirenz in urine. Further method development and investigation was necessary to identify and include stavudine, didanosine, lopinavir, ritonavir and tenofovir in the spectrum of antiretroviral drugs detectable by the current method. In the current method being used, stock solutions of ARVs were made up with methanol (Potgieter *et al*. 2009). Unfortunately not all ARVs, particularly those added in this study, dissolved in methanol. Other solvents such as ethanol, acetonitrile, tetraethylammonium phosphate (TEAP) buffer, dichloromethane, isopropanol, dimethyl sulfoxide (DMSO) and combinations of these solvents were explored to find a common solvent in which all ARVs for this study could dissolve. A mixture of solvent A and solvent B (5:1) proved to be the best combination in which all the ARVs could dissolve. This mixture was named solvent C. The standard solutions were prepared weekly and refrigerated. Calibration curves were compared from day to day and indicated that stock solutions remained stable over 3 consecutive days it was required for.

Solvent C

Solvent C was prepared by adding 150ml of Solvent A to 30ml of Solvent B.

Solvent C was used to prepare stock solutions and to reconstitute residues of urine samples after extraction method was completed. A stock solution of each ARV was prepared by dissolving the pure compound in solvent C. Initial trial runs were performed to assess the required concentration for each stock solution.

The following concentrations of stock solutions were decided on:

Abacavir: 400.00 µg/ml
Efavirenz: 1443.75 µg/ml
Lamivudine: 253.00 µg/ml
Lopinavir: 643.50 µg/ml
Nevirapine: 790.00 µg/ml
Stavudine: 562.00 µg/ml
Tenofovir: 1162.50 µg/ml
Zidovudine: 832.00 µg/ml

Different volumes of the stock solutions were spiked to urine to obtain the different concentrations required for the construction of each ARV's calibration curve (Vibhuti *et al*. 2009).

The following concentrations were used (Table 3):

Table 3.1: Concentrations of ARVs used to construct calibration curve

Name	Concentration (µg/ml urine)	Volume spiked (µl)	Stock Concentration (µg/ml)
Abacavir 1	6.40	16.00	400.00
Abacavir 2	18.00	45.00	400.00
Abacavir 3	32.00	80.00	400.00
Abacavir 4	48.00	120.00	400.00
Efavirenz 1	49.70	35.00	1443.75
Efavirenz 2	71.00	50.00	1443.75
Efavirenz 3	142.00	100.00	1443.75
Efavirenz 4	231.00	160.00	1443.75
Lamivudine 1	5.06	20.00	253.00
Lamivudine 2	10.11	40.00	253.00
Lamivudine 3	20.22	80.00	253.00
Lamivudine 4	25.30	100.00	253.00
Lopinavir 1	19.80	40.00	643.50
Lopinavir 2	34.65	70.00	643.50
Lopinavir 3	49.50	100.00	643.50
Lopinavir 4	64.35	130.00	643.50
Nevirapine 1	19.75	25.00	790.00
Nevirapine 2	39.50	50.00	790.00
Nevirapine 3	59.25	75.00	790.00
Nevirapine 4	79.00	100.00	790.00
Stavudine 1	14.05	25.00	562.00
Stavudine 2	28.40	50.00	562.00
Stavudine 3	42.15	75.00	562.00
Stavudine 4	56.20	100.00	562.00
Tenofovir 1	69.75	60.00	1162.50
Tenofovir 2	93.00	80.00	1162.50
Tenofovir 3	186.00	160.00	1162.50
Tenofovir 4	232.50	200.00	1162.50
Zidovudine 1	10.40	12.50	832.00
Zidovudine 2	20.80	25.00	832.00
Zidovudine 3	41.60	50.00	832.00
Zidovudine 4	83.20	100.00	832.00

3.10 EXTRACTION PROCEDURE FOR SAMPLES AND STANDARDS

The current HPLC method developed by the Department of Pharmacology/Toxicology, University of the Free State in Bloemfontein, South Africa, used 200µl methanol to reconstitute the evaporated residue after extraction was performed. As previously discussed, methanol was not a common solvent for all the ARVs. Solvent C proved to be the most appropriate solvent to use in reconstitution of the residues. Solvent C was also found to be more compatible with the mobile phases used in the gradient elusion.

The extraction procedure was applied to urine standards spiked with ARV stock solutions, as well as patient urine samples.

The following procedure was used for the extraction of the ARVs from urine. To 1ml of urine (from patients and urine standards spiked with stock solution), the internal standard (100 µl clomipramine), 500µl buffer and 5ml of dichloromethane isopropanol (9:1) was added. The tubes were vortexed and shaken horizontally on a horizontal shaker for 5 minutes. The tubes were then centrifuged at 2500 revolutions per minute (rpm) for 5 minutes.

The organic phase containing the compounds of interest was situated beneath the water phase after centrifugation. The water phase was removed and the organic layer was transferred to an ampoule and evaporated to dryness at 50˚C under a stream of nitrogen. The residue was reconstituted in 200µl of Solvent C, vortexed and transferred to a glass vial for injection into the HPLC.

3.11 CHROMATOGRAPHIC SYSTEM AND CONDITIONS

The HPLC method developed by the Department of Pharmacology/Toxicology, University of the Free State in Bloemfontein, South Africa, used a gradient elution method where the eluent composition was changed during the analysis time of 25 minutes. At the beginning of the run Solvent B was at 10% strength and Solvent A at 90% strength resulting in the resolution of the early components. As the separation progressed, Solvent B's strength was increased and Solvent A's strength was decreased gradually over 30 minutes so that the subsequent components elute within a reasonable time. Chromatography was performed at ambient temperature using a flow rate of 0.8ml/min. for the first two minutes, after which the flow rate was raised to 2ml/min for the remaining 23 minutes of the run.

Various gradient elusion methods with different mobile phase composition strengths were explored. Constant flow rates as well as varying flow rates were investigated to find the most suitable chromatographic conditions to separate and elute all components within a reasonable time.

A gradient elution method was used where the mobile phase composition was changed during the analysis time of 30 minutes. At the beginning of the run Solvent B was at 0% strength and Solvent A at 100% strength resulting in the resolution of the early components. As the separation progressed, Solvent B's strength was gradually increased to 10% over the first 9 minutes and Solvent A's strength was decreased gradually over the first 9 minutes to 90%. Solvent B was gradually increased to 50% from the 9th minute to the 20th minute after which the Solvent A to B ratio remained 1:1 for the last 10 minutes so that the subsequent components elute within a reasonable time.

Separation was performed on a HP 1090 liquid chromatograph system with an auto sampler and an Agilent 1100 diode array detector. Data was collected by using Chem Station Software.

Detection was recorded at 210nm. Chromatographic separations were performed with a Synergi 4U Fusion – RP 80 Column with a size of 150mm x 4.6mm, coupled to a Phenomenex C18 precolumn (4mm x 3mm). Chromatography was performed at ambient temperature (±21ºC) using an initial flow rate of 0.8 ml/min which was increased to 2 ml/min after 9 minutes for optimal elution of each compound. A post run of 5 minutes was added to ensure adequate equilibrium of the column before the next run.

3.12 VALIDATION METHODOLOGY

3.12.1 CALIBRATION CURVE

Calibration of each individual ARV was achieved by constructing a 4 point calibration curve for each ARV compound (Kaiser, 2000). General guidelines suggest the use of 5 and more calibration points for the construction of a calibration curve to adequately define the relationship between concentration and response. A 5 point calibration was attempted, but outliers and certain points of influence were noted, particularly in the higher concentration ranges. All patient samples produced results within the first four calibration points. Taking this into account and adhering to guidelines stated by Kaiser, it was decided to discard

outliers in the higher concentration ranges. The inconsistency noted in the higher concentration ranges may be due to on board instability which may be caused by time lapsed in between injections, and precipitation of compounds. Since the main objective was not to develop a quantitative method, further investigation is required. Each ARV concentration was repeated 5 times and all samples were prepared as described in the extraction procedure (3.10). During the course of the study it was decided to spike calibration samples with more than one ARV stock solution at a time because of time and cost limitations. The different concentrations to combine in the calibrations were carefully considered to ensure that the quality of the calibrations will not be compromised. With limited funding available, this ensured extensive savings on time expenditure, the use of chemicals and pure compounds. The bulk calibration with compounds grouped together cut down on pure compound usage which ensured the completion of the study. The concentrations used to construct the calibration curve can be viewed in table 3.

The peak area-ratio of each drug concentration to the internal standard was plotted on a linear regression curve for the investigation of linearity and reproducibility, and for the determination and identification of patient samples. Data acquired from the Chem Station program was used to calculate the ratios by using the following formula: (Area of ARV/Area of internal standard). The ratio results were plotted against its respective spiked concentrations to obtain a calibration curve. The procedure was repeated on three different days to determine accuracy and reproducibility.

Mean values of the calculated ratios of the calibrations for the different concentrations were used to construct a calibration curve by plotting the mean ratios against the respective spiked concentrations.

3.12.2 ACCURACY

Two different concentrations, one high, and one low concentration internal quality control samples were extracted and injected in duplicate. The sample preparation procedure was followed as described in 3.10 and applied to the internal quality controls as well as the patient samples. This procedure was repeated over three consecutive days.

Values of the internal quality controls were read from the calibration curve. The internal quality controls' values were known and were compared to the values obtained from the calibration curve. Results obtained over a period of three consecutive days were used to calculate the coefficient of variation by using the following formula:

(standard deviation/mean) x 100.

3.12.3 RECOVERY

Recovery describes the extraction efficiency of a methodology within the limits of variability. The following procedure was used to determine the recovery of this method. Three different concentrations for each ARV were spiked into 1ml of urine. The samples were prepared according to the sample preparation procedure described in 3.10. The same three different concentrations for each ARV were spiked directly into an ampoule and the internal standard (Clomipramine) was added. These reference samples were evaporated to dryness by using a steady stream of nitrogen gas at a temperature of 50˚C. The precipitate was reconstituted with 200µl of Solvent C, vortexed and injected. The areas obtained from the samples extracted were compared to the reference samples to determine each ARV drug's recovery. Using the ratio obtained for the directly spiked samples as the 100% reference point and comparing the ratio obtained for the samples spiked in urine, the percentage of recovery was determined. The following formula was used to determine the recovery:

(Area of extracted compound/Area of unextracted compound) x 100.

3.12.4 SHORT TERM STABILITY

Short term stability of abacavir, efavirenz, lamivudine, lopinavir, nevirapine, stavudine, tenofovir and zidovudine was determined after storage at different temperature conditions to evaluate the stability of these compounds in urine. Stability was investigated using low and high quality control (QC) samples for each ARV. The calibration curves were demonstrated to be linear. Calibration curves were constructed everyday over three consecutive days and short term stability samples analysed. Each sample was prepared in duplicate by following

the extraction procedure described in 3.10 and stored at different temperatures. Fresh urine samples were spiked with stock solutions of each ARV on day one. The first duplicate samples spiked with a low QC and high QC respectively was stored at room temperature; second samples were stored at 2-8˚C for 24 hours and 48 hours respectively and the third samples were stored at -20˚C for 24 hours and 48 hours respectively. This procedure was applied to each individual ARV. Duplicate samples were run on day one at room temperature. Results were obtained by calculating the ratios by using the following formula: (Area of ARV/Area of internal standard), and comparing it to the calibration curve. Duplicate samples stored at 2-8˚C and at -20˚C for 24 hours, and 48 hours respectively, were allowed to thaw and reach room temperature before extraction procedure was applied. Freshly prepared samples was extracted together with 24 hour and 48 hour samples and injected for comparison purposes. Stability was tested over a period of 48 hours because emergency overdose samples rarely take longer than 24 hours to reach the Pharmacology laboratory. Variations in ARV concentrations were observed over a period of 48 hours. The short-term stability studies are shown in Tables 4.26 to 4.33, and results have been plotted in graphs and shown in Figures 4.20 to 4.35.

The following concentrations were used:

Table 3.2: Concentrations of ARVs used for short term stability

ARV	CONCENTRATIONS (µg/ml)	
Abacavir	19.20	38.40
Efavirenz	85.20	170.40
Lamivudine	15.17	30.33
Lopinavir	19.80	44.55
Nevirapine	31.60	63.20
Stavudine	11.24	16.86
Tenofovir	37.20	83.70
Zidovudine	31.20	72.80

3.12.5 INTERFERENCE

Interference was investigated for all the ARV drugs in the study. Interference among the individual ARVs was investigated, as well as interference from other drugs such as common

drugs used for the treatment of Tuberculosis (TB) which is often found in HIV positive patients. During initial method development it was noted that peaks from lamivudine and abacavir overlapped. Peaks from lopinavir and efavirenz also interfered with each other due to their similar retention times. Interference can be eliminated by changing the chromatographical conditions such as the gradient elusion ratio of the mobile phases or the flow rate in the column until optimal separations of the drugs can be achieved. Good separation with high quality peaks was achieved by reducing the initial flow rate and by modification of the extraction procedure. Current HIV/TB co-infection rates exceed 70% with TB being the most prevalent opportunistic infection in HIV positive patients in South Africa. Due to this fact it was necessary to investigate interference from the most common TB drugs as well since patients on ARVs are likely to have TB drugs on board. Trimethoprim and isoniazide, two commonly used drugs for the treatment of TB was detected with the method. Not all TB drugs could be detected by the method. Pure compound stock solutions of 100µg/ml trimethoprim and isoniazide was injected to determine their retention times and spectra and the data was stored in a computerised library. Effective differentiation and identification of the substances was achieved and no interference with the ARVs was observed.

3.12.6 LIMIT OF DETECTION (LOD)

A limit of detection was established for each ARV drug investigated in the study. There are several terms that have been used to define LOD. In general, the LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the analysis (Shrivastava & Gupta 2011).

The International Conference on Harmonisation (ICH) guidelines for detection limit parameters of analytical method validation was used. By using the signal-to-noise method, the peak-to-peak noise around the analyte retention time is measured, and subsequently, the concentration of the analyte that would give a signal equal to certain value of noise to signal ratio is estimated. The noise magnitude can be measured either by auto integrator of the instrument, or manually on the chromatogram printout. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD. This method is commonly applied to analytical methods that exhibit baseline noise (Shrivastava & Gupta 2011).

3.13 STATISTICAL ANALYSIS

All statistical analysis was revised by a qualified biostatistician. Data was captured by the researcher on a data sheet using Microsoft Excel. Any further analysis was done by a biostatistician using SAS Version 90.1.3. Descriptive statistics namely means and standard deviations or medians and percentiles were calculated for numerical data. Frequencies and percentages were calculated for categorical data.

4 RESULTS

4.1 RESULTS

After the extraction procedure was followed as described in 3.10, the extraction was injected into the HPLC for analysis. Each ARV was repeated 5 times to investigate reproducibility of the results. The retention time and spectra for each ARV was recorded and stored. Since it was established that there was no interference from the different ARV profiles, more than one ARV pure compound was spiked to urine samples at a time. The specificity is indicated in the UV spectra in figure 7.25 to 7.32. The red line indicates the spectra of the pure compounds and the blue line indicates the spectra captured from the extractions. The computer library matches the spectra of the extractions to the spectra of the pure compounds as a percentage match. The spectra matched between 92 and 100%.

The interference of other compounds is visible in all the chromatograms. The peaks would overlap if there was interference. This was eliminated by changing the flow rate and gradient elution resulting in chromatograms that shows no overlapping of peaks, and therefore can be concluded that no interference was experienced after method development was concluded. The chromatograms have been selected to demonstrate the relevant peaks as clearly as possible.

4.1.1 CHROMATOGRAPHIC PERFORMANCE

A blank urine sample was run to determine any chromatographic interference. Figure 4.1 shows the chromatogram of a blank urine sample that was subjected to the extraction procedure described in 3.10. Each ARV was repeated 5 times and all samples were prepared as described in the extraction procedure (3.10). No interference from the blank urine sample, or any other components used in the extraction procedure was detected.

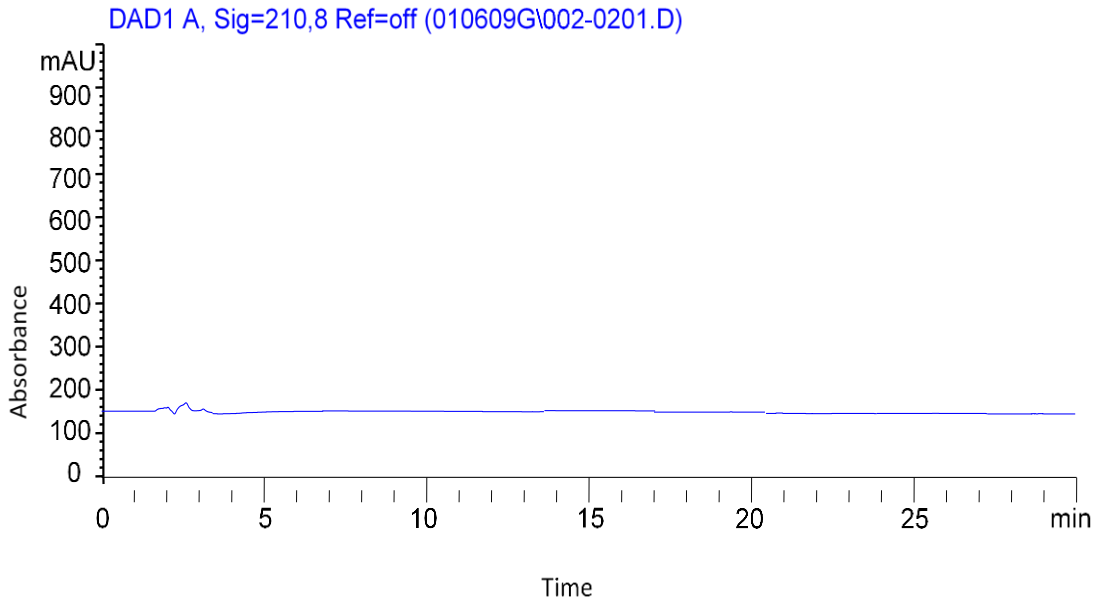


Figure 4.1: Chromatogram of blank urine sample

A sample of the internal standard (clomipramine) was run to determine its retention time and spectrum. The internal standard was repeated 5 times and was prepared as described in the extraction procedure (3.10). Figure 4.2 illustrates the high resolution quality peak of the internal standard Clomipramine and its retention time of approximately 16.8 minutes, after it has been subjected to the extraction procedure and injected into the HPLC.

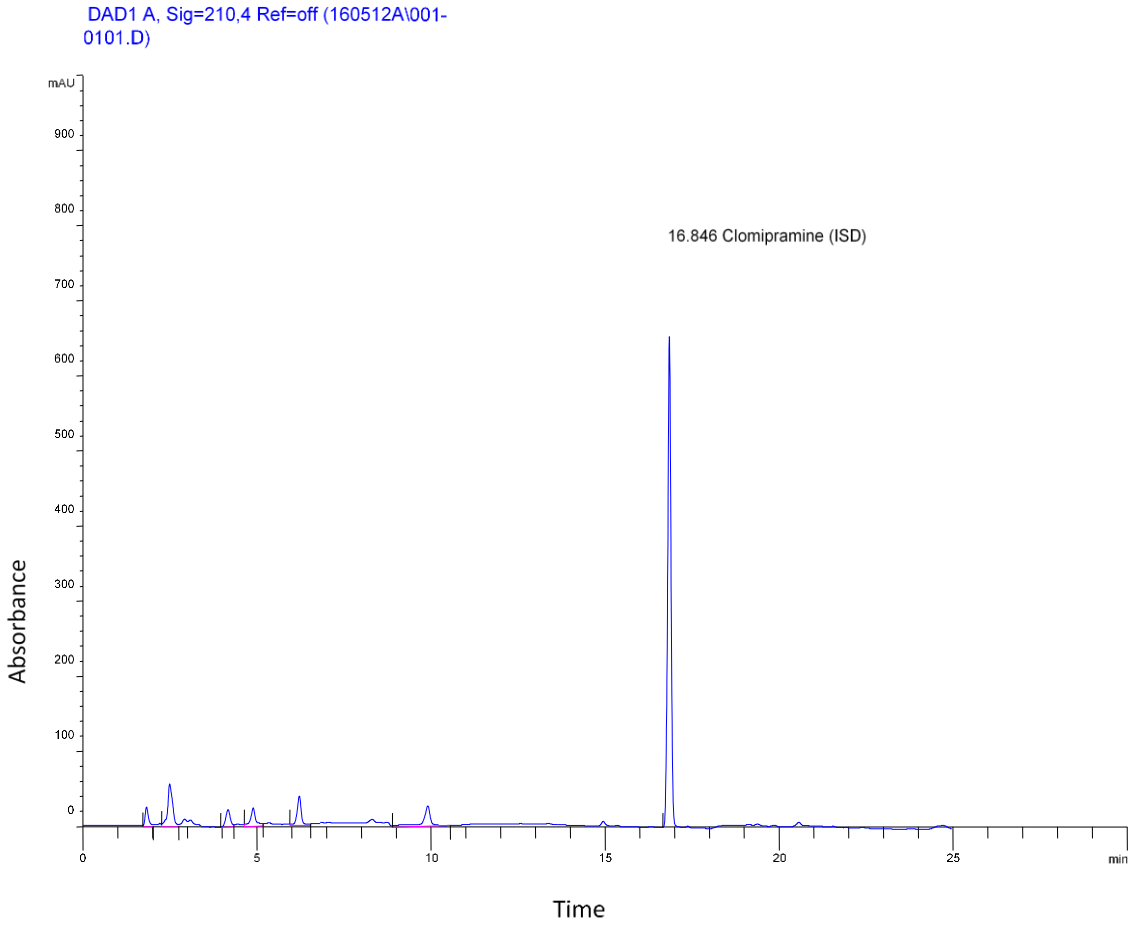


Figure 4.2: Chromatogram of internal standard Clomipramine

A urine sample was spiked with both abacavir (16 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.3 indicates the high resolution quality peaks and retention times of abacavir and clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances

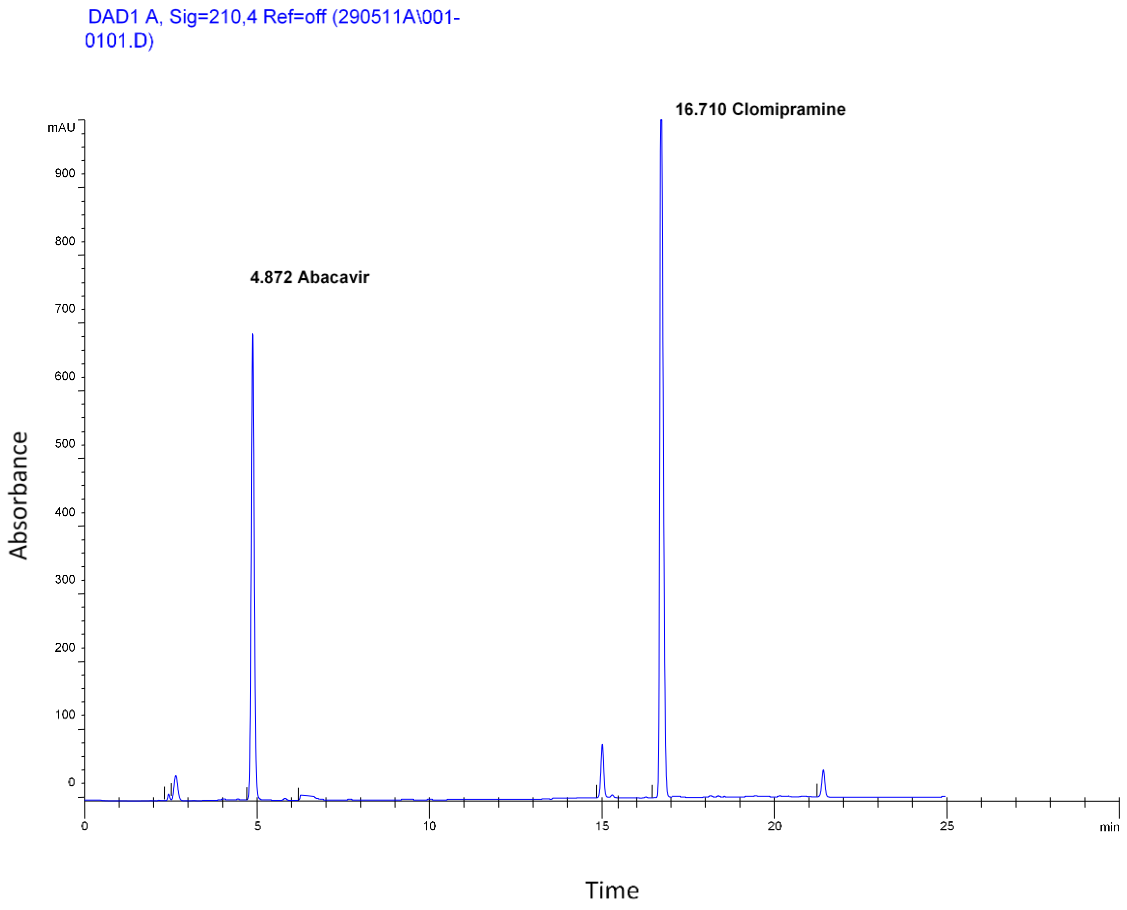


Figure 4.3: Chromatogram of Abacavir and Clomipramine

A urine sample was spiked with both efavirenz (142 µg/ml) and the internal standard clomipramine (71.4 µg/ml). According to figure 4.4, the high resolution quality peaks and retention times of efavirenz and clomipramine are indicated after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances.

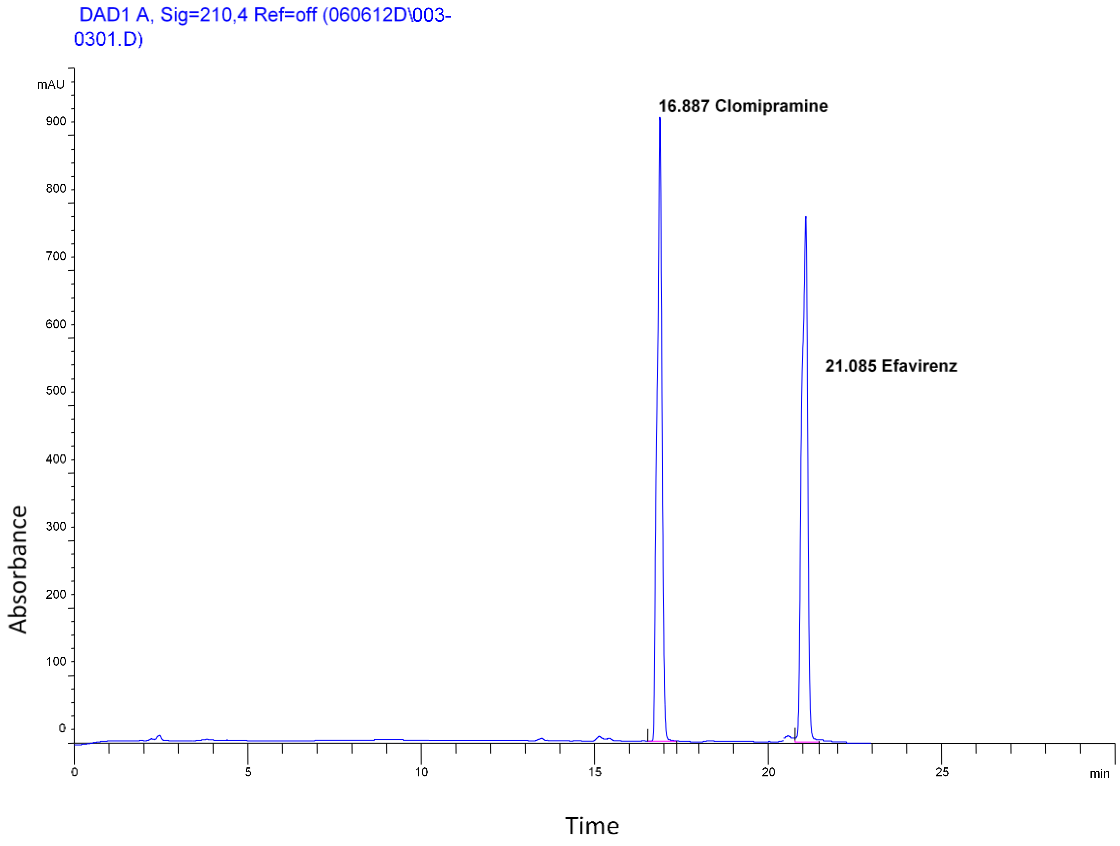


Figure 4.4: Chromatogram of Efavirenz and Clomipramine

A urine sample was spiked with both lamivudine (20.22 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.5 demonstrates the high resolution quality peaks and retention times of Lamivudine and Clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances, nor any additional ARV spiked for the purposes of bulk calibration.

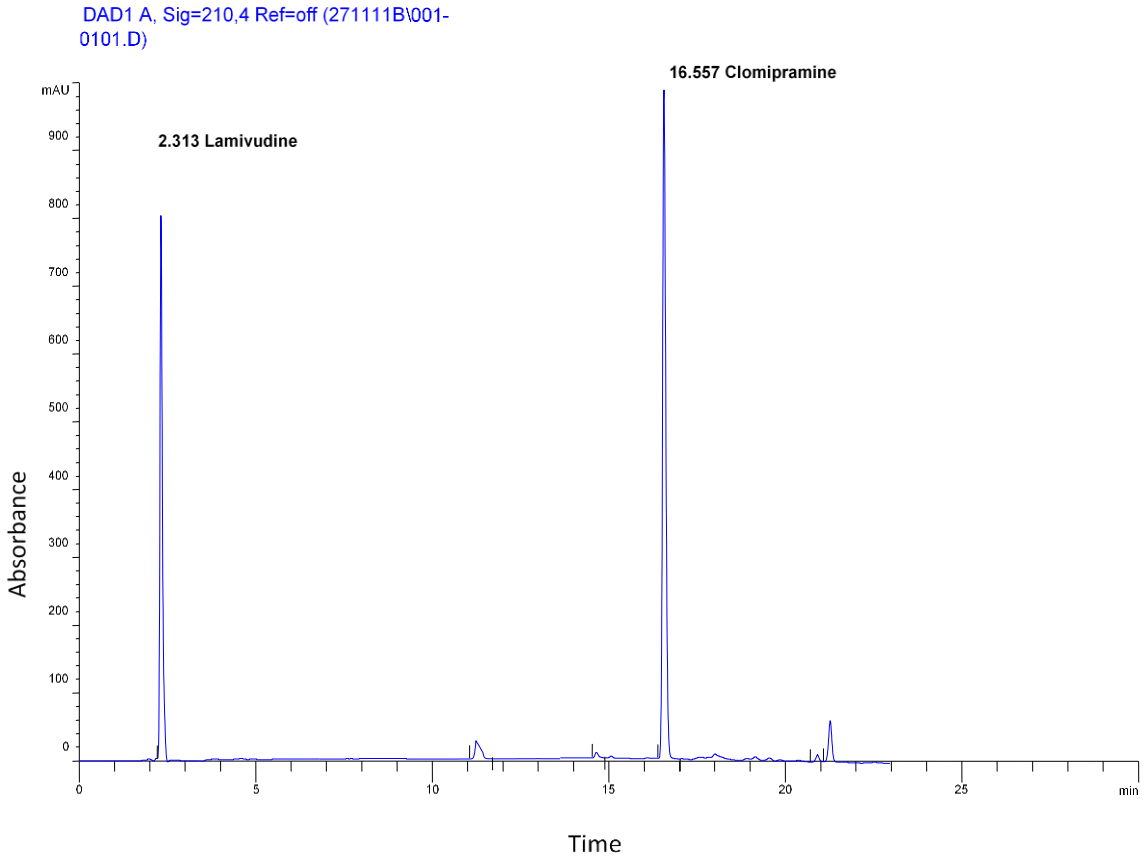


Figure 4.5: Chromatogram of Lamivudine and Internal standard (Clomipramine)

A urine sample was spiked with both lopinavir (49.5 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.6 shows the high resolution quality peaks and retention times of lopinavir and clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances.

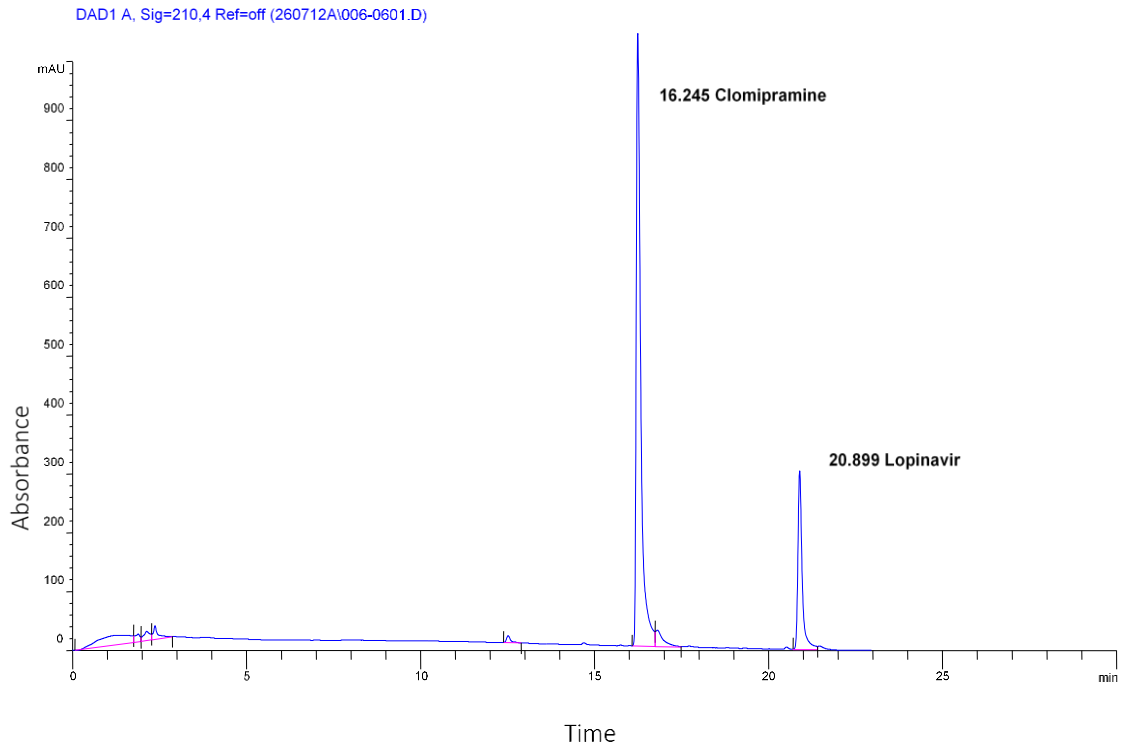


Figure 4.6: Chromatogram of Lopinavir and Clomipramine

A urine sample was spiked with both nevirapine (79.0 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.7 illustrates the high resolution quality peaks and retention times of nevirapine and clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances.

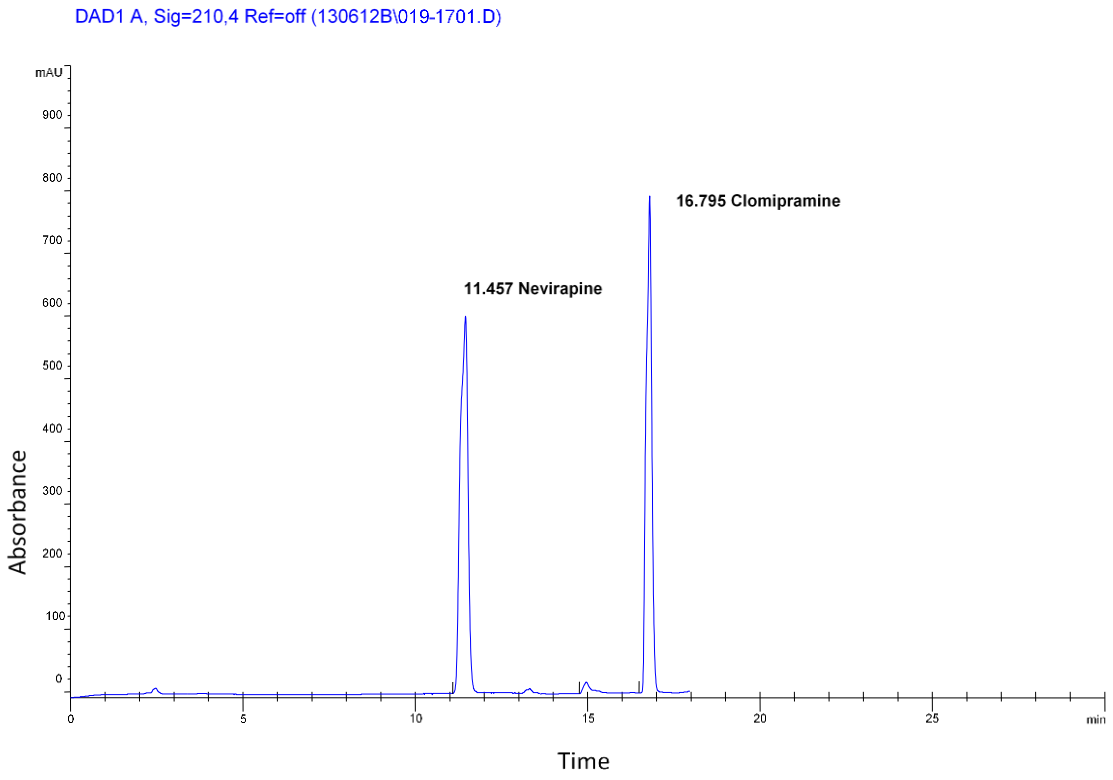


Figure 4.7: Chromatogram of Nevirapine and Clomipramine

A urine sample was spiked with both stavudine (28.1 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.8 illustrates the high resolution quality peaks and retention times of stavudine and clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances.

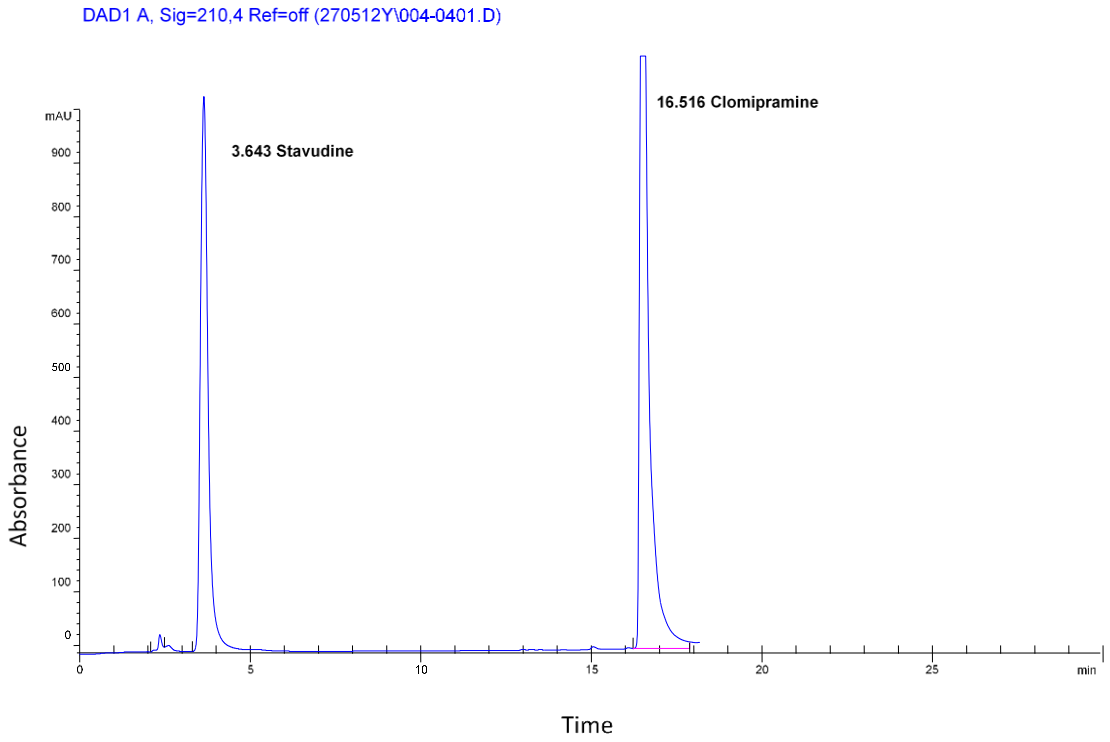


Figure 4.8: Chromatogram of Stavudine and Clomipramine

A urine sample was spiked with both tenofovir (28.1 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.9 illustrates the high resolution quality peaks and retention times of tenofovir and clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances, and no interference was observed with other ARVs spiked for the purposes of bulk calibration.

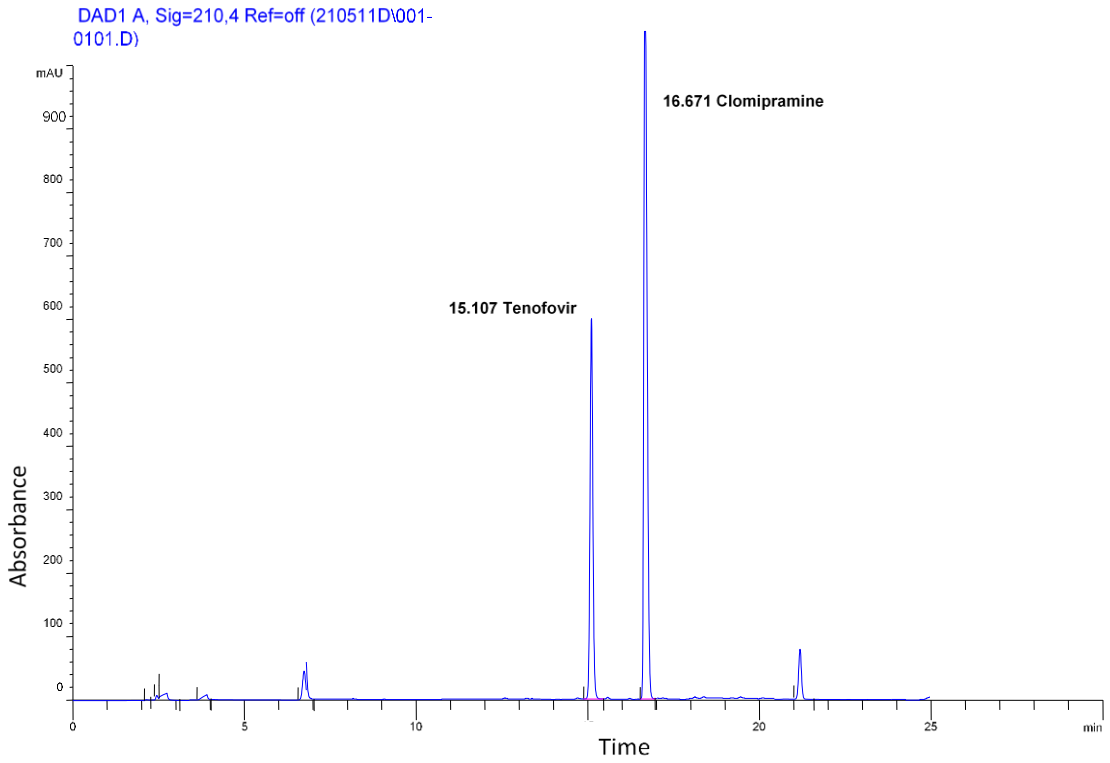


Figure 4.9: Chromatogram of Tenofovir and Clomipramine

A urine sample was spiked with both zidovudine (20.8 µg/ml) and the internal standard clomipramine (71.4 µg/ml). Figure 4.10 shows the high resolution quality peaks and retention times of zidovudine and clomipramine after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. The chromatogram confirms that no interference exists between the two substances.

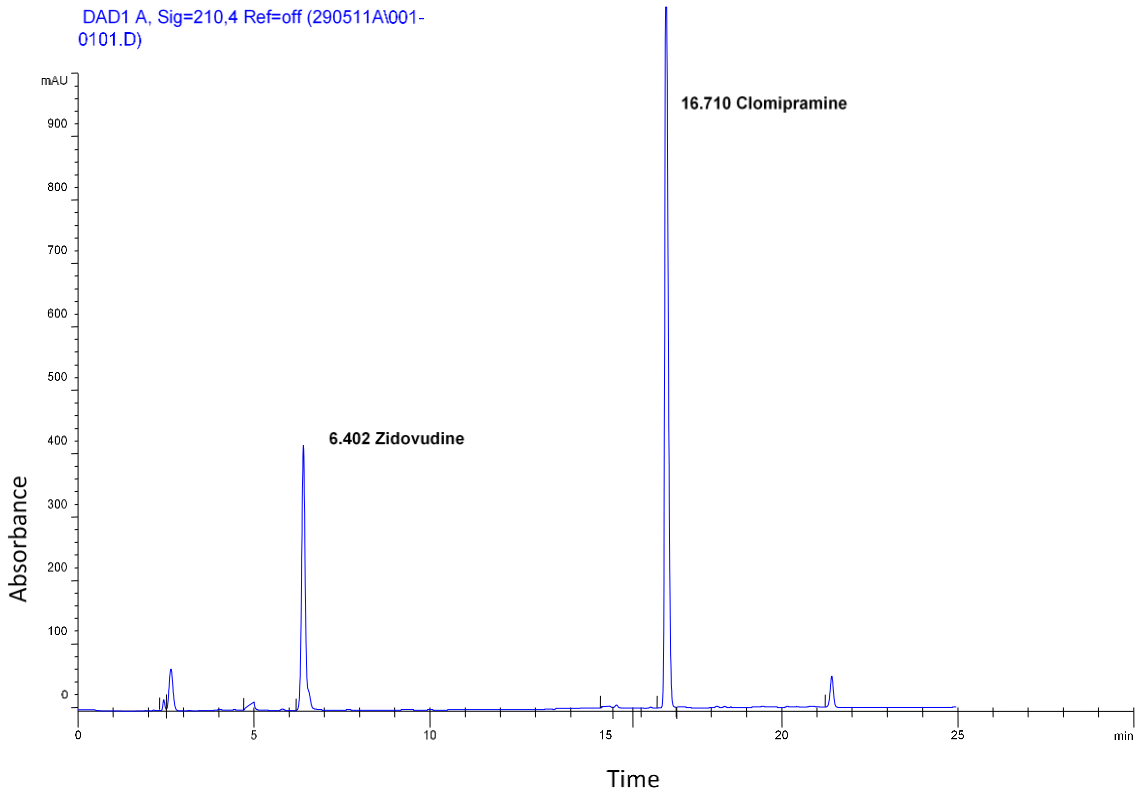


Figure 4.10: Chromatogram of Zidovudine and Clomipramine

A urine sample was spiked with abacavir, efavirenz, lamivudine, lopinavir, nevirapine, stavudine, tenofovir, zidovudine and the internal standard (clomipramine). Figure 4.11 summarizes the high resolution quality peaks and retention times after the sample was subjected to the extraction procedure described in 3.10 and injected into the HPLC. Optimal separation of each substance was achieved and the chromatogram confirms that no interference exists between any of the substances.

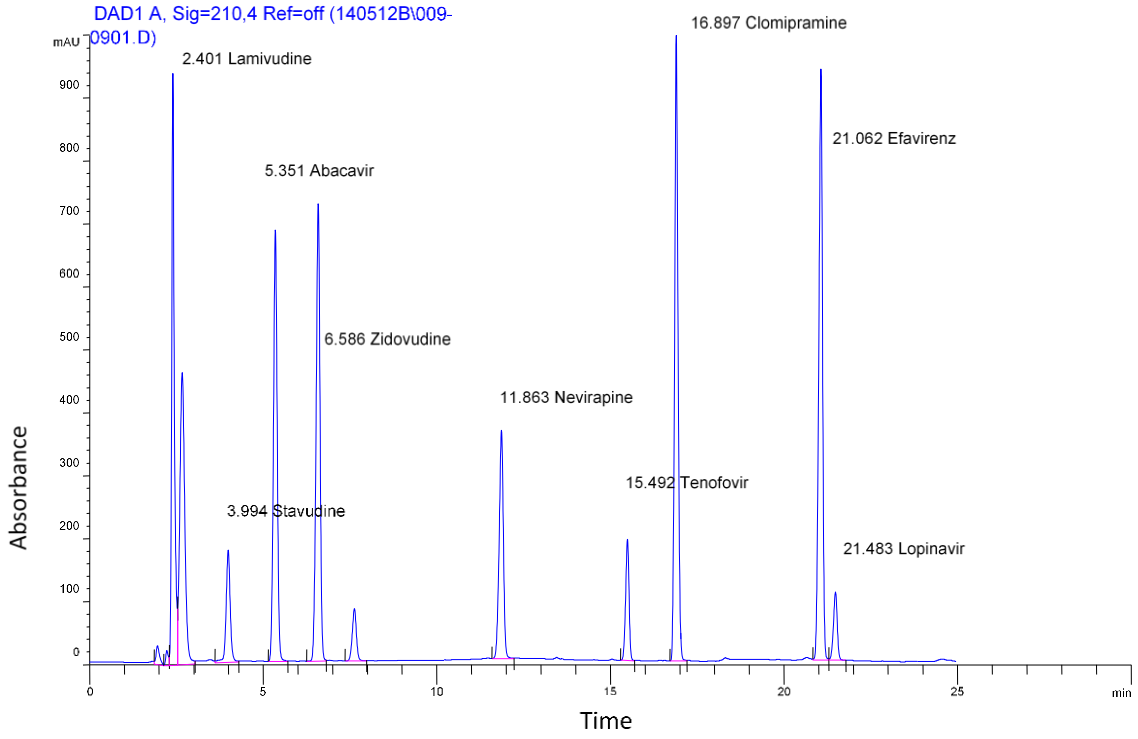


Figure 4.11: Chromatogram of Abacavir, Efavirenz, Lamivudine, Lopinavir, Nevirapine, Stavudine, Tenofovir, Zidovudine and Clomipramine (Extracted)

4.1.2 CALIBRATION CURVE

Calibration of each individual ARV was achieved by constructing a calibration curve for each ARV compound. Each ARV concentration was repeated five times and over three consecutive days. All samples were prepared daily as described in the extraction procedure. The peak area-ratio of each drug concentration to the internal standard was plotted on a linear regression curve for the investigation of linearity and reproducibility. The data for the calibration curves is shown in Tables 4.1 to 4.8. The average curve for each ARV is demonstrated in Figures 4.12 to 4.19.

4.1.2.1 HPLC calibrations for Abacavir

Tables 4.1 represent the HPLC calibrations for abacavir, and are summarized in figure 4.12:

r = 0.9989

r^2 = 0.9979

P < 0.0001

Table 4.1 HPLC calibrations for Abacavir

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
6.40	0.100	0.103	0.100
6.40	0.101	0.104	0.100
6.40	0.100	0.096	0.100
6.40	0.100	0.098	0.100
6.40	0.100	0.100	0.100
Mean	**0.100**	**0.100**	**0.100**
18.00	0.304	0.269	0.266
18.00	0.294	0.276	0.302
18.00	0.272	0.294	0.274
18.00	0.271	0.271	0.293
18.00	0.277	0.305	0.269
Mean	**0.273**	**0.283**	**0.281**
32.00	0.538	0.599	0.544
32.00	0.528	0.556	0.542
32.00	0.551	0.539	0.542
32.00	0.534	0.537	0.604
32.00	0.594	0.542	0.557
Mean	**0.549**	**0.555**	**0.558**
48.00	0.806	0.793	0.815
48.00	0.784	0.842	0.792
48.00	0.833	0.792	0.820
48.00	0.811	0.820	0.797
48.00	0.796	0.812	0.846
Mean	**0.806**	**0.811**	**0.814**

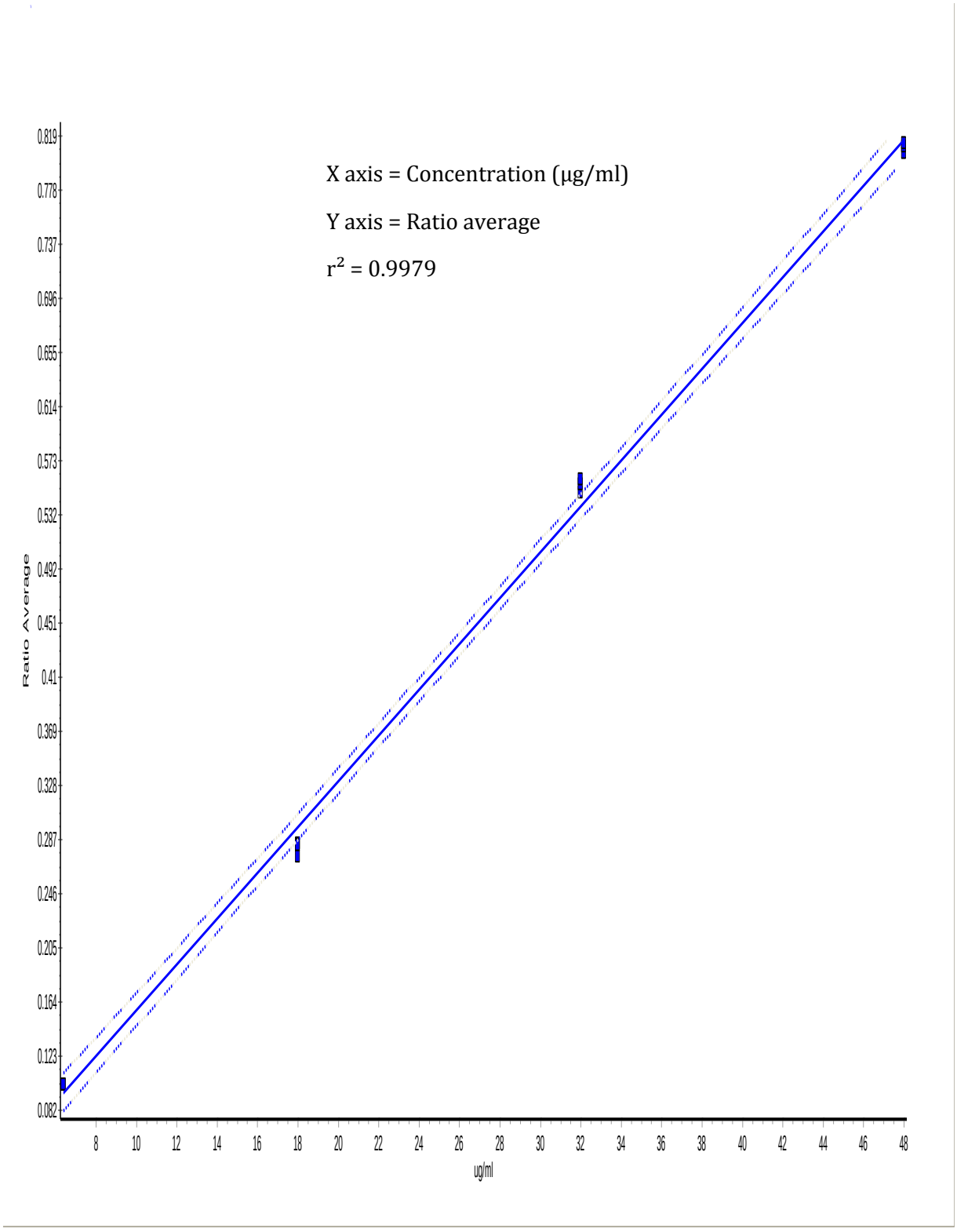


Figure 4.12: Average calibration curve of Abacavir for the daily calibrations

4.1.2.2 HPLC calibrations for Efavirenz

Tables 4.2 represent the HPLC calibrations for efavirenz, and are summarized in figure 4.13:

r = 0.9959

r^2 = 0.9919

P < 0.0001

Table 4.2 HPLC calibrations for Efavirenz

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
49.70	0.232	0.230	0.211
49.70	0.229	0.202	0.228
49.70	0.209	0.223	0.223
49.70	0.205	0.197	0.202
49.70	0.199	0.210	0.197
Mean	**0.215**	**0.212**	**0.212**
71.00	0.288	0.273	0.240
71.00	0.302	0.286	0.261
71.00	0.287	0.263	0.224
71.00	0.263	0.302	0.312
71.00	0.274	0.288	0.236
Mean	**0.283**	**0.282**	**0.255**
142.00	0.519	0.531	0.528
142.00	0.536	0.543	0.537
142.00	0.555	0.540	0.543
142.00	0.533	0.531	0.528
142.00	0.541	0.527	0.522
Mean	**0.537**	**0.534**	**0.532**
213.00	0.687	0.683	0.676
213.00	0.753	0.703	0.776
213.00	0.770	0.785	0.739
213.00	0.758	0.733	0.662
213.00	0.744	0.702	0.659
Mean	**0.742**	**0.721**	**0.702**

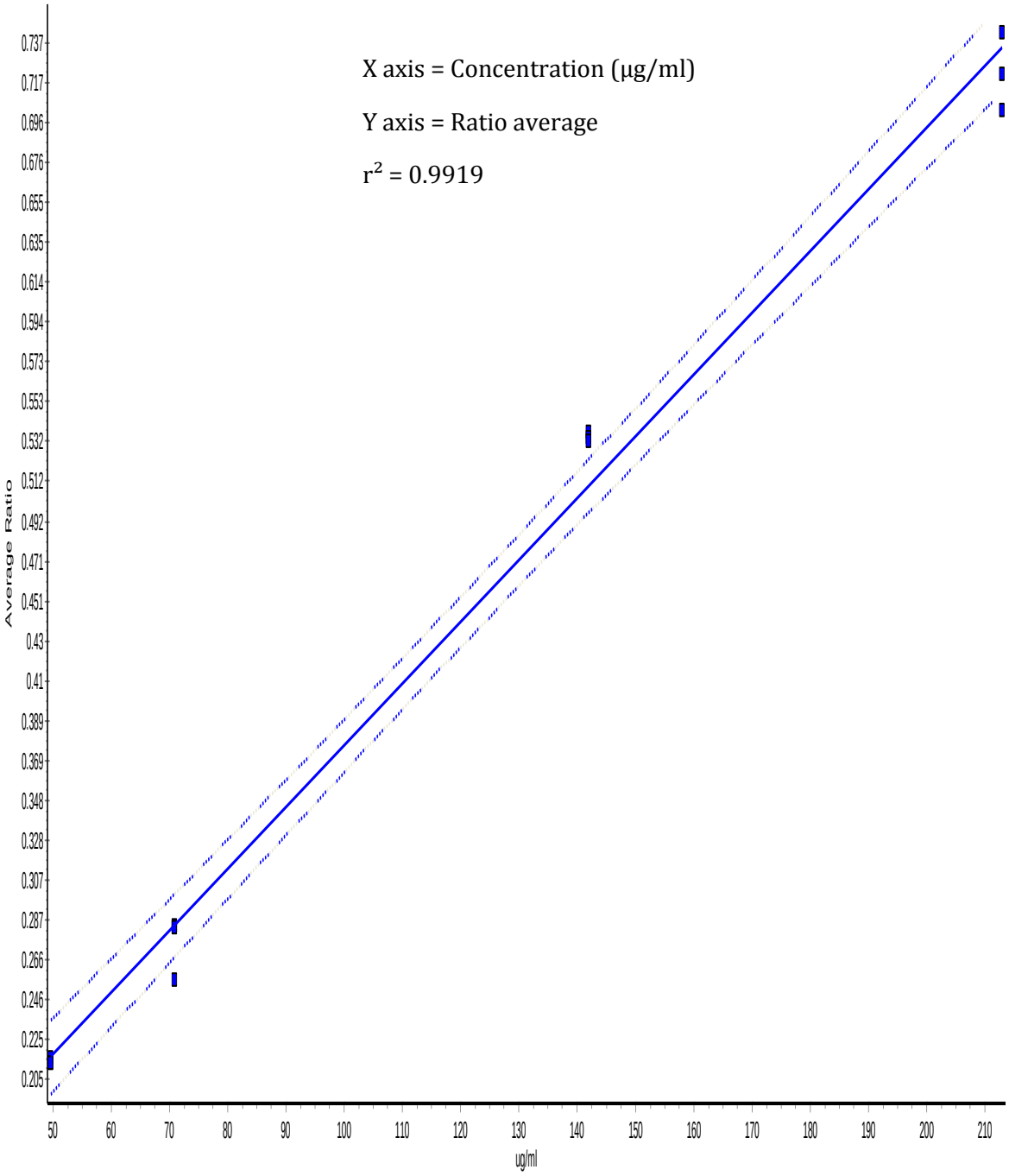


Figure 4.13: Average calibration curve of Efavirenz for the daily calibrations

4.1.2.3 HPLC calibrations for Lamivudine

Tables 4.3 represent the HPLC calibrations for lamivudine, and are summarized in figure 4.14:

r = 0.9993

r^2 = 0.9985

P <0.0001

Table 4.3 HPLC calibrations for Lamivudine

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
5.06	0.100	0.097	0.098
5.06	0.123	0.123	0.100
5.06	0.105	0.103	0.125
5.06	0.106	0.103	0.098
5.06	0.099	0.099	0.136
Mean	**0.107**	**0.105**	**0.111**
10.11	0.202	0.201	0.210
10.11	0.203	0.196	0.195
10.11	0.203	0.201	0.196
10.11	0.198	0.202	0.195
10.11	0.212	0.211	0.190
Mean	**0.204**	**0.202**	**0.197**
20.22	0.440	0.412	0.413
20.22	0.387	0.432	0.433
20.22	0.395	0.390	0.389
20.22	0.431	0.442	0.397
20.22	0.414	0.398	0.442
Mean	**0.413**	**0.415**	**0.415**
25.30	0.499	0.496	0.496
25.30	0.484	0.489	0.496
25.30	0.490	0.480	0.543
25.30	0.533	0.531	0.503
25.30	0.497	0.496	0.511
Mean	**0.501**	**0.498**	**0.510**

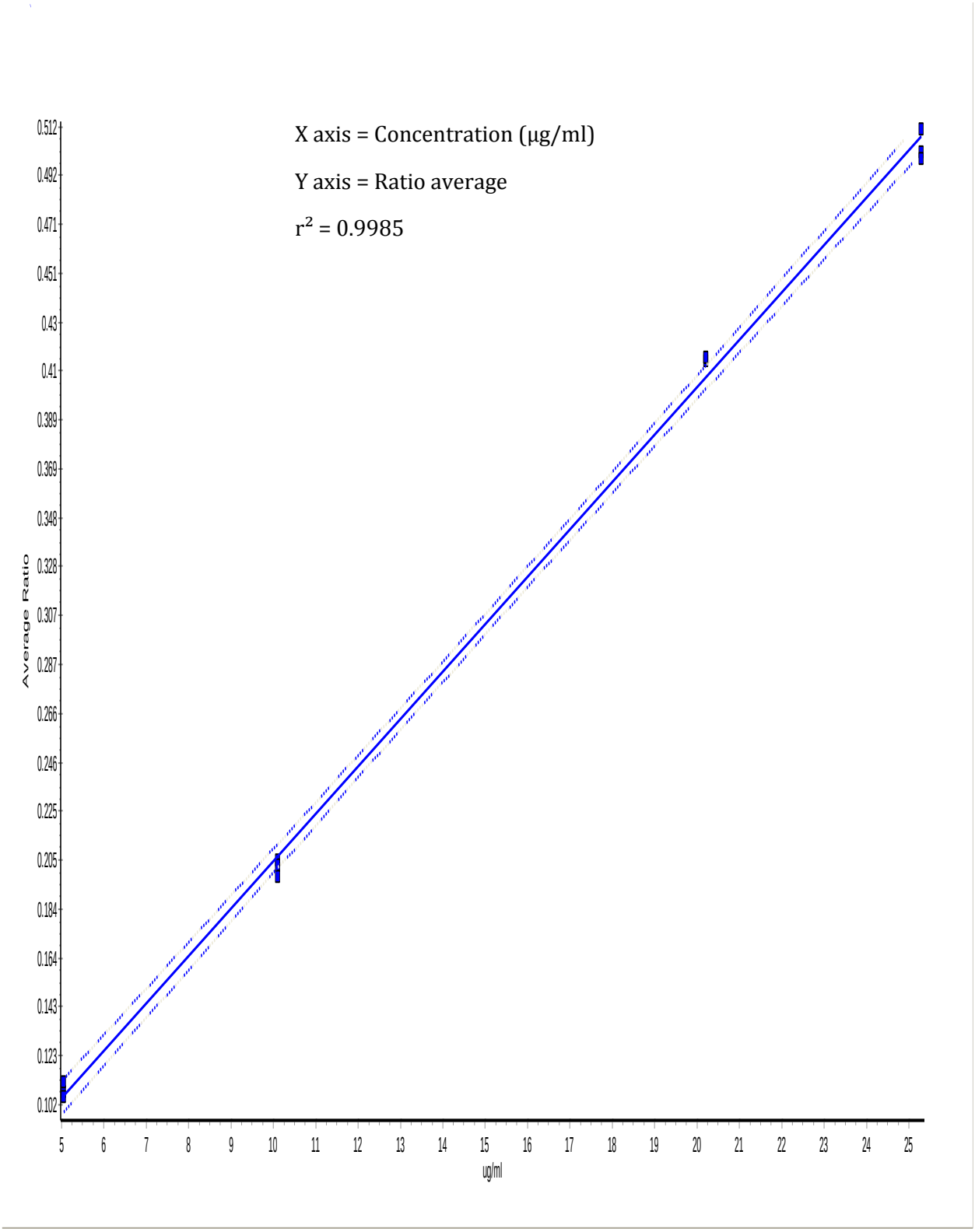


Figure 4.14: Average calibration curve of Lamivudine for the daily calibrations

4.1.2.4 HPLC calibrations for Lopinavir

Tables 4.4 represent the HPLC calibrations for lopinavir, and are summarized in figure 4.15:

r = 0.9992

r^2 = 0.9983

P <0.0001

Table 4.4 HPLC calibrations for Lopinavir

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
19.80	0.046	0.052	0.058
19.80	0.040	0.050	0.053
19.80	0.053	0.054	0.050
19.80	0.051	0.052	0.059
19.80	0.053	0.050	0.051
Mean	**0.049**	**0.052**	**0.054**
34.65	0.110	0.109	0.105
34.65	0.100	0.101	0.110
34.65	0.104	0.103	0.107
34.65	0.107	0.108	0.104
34.65	0.105	0.108	0.100
Mean	**0.105**	**0.106**	**0.105**
49.50	0.151	0.147	0.153
49.50	0.155	0.147	0.156
49.50	0.158	0.152	0.154
49.50	0.153	0.150	0.160
49.50	0.150	0.154	0.152
Mean	**0.153**	**0.150**	**0.155**
64.35	0.211	0.201	0.198
64.35	0.204	0.194	0.200
64.35	0.202	0.199	0.207
64.35	0.206	0.205	0.203
64.35	0.201	0.204	0.203
Mean	**0.205**	**0.200**	**0.202**

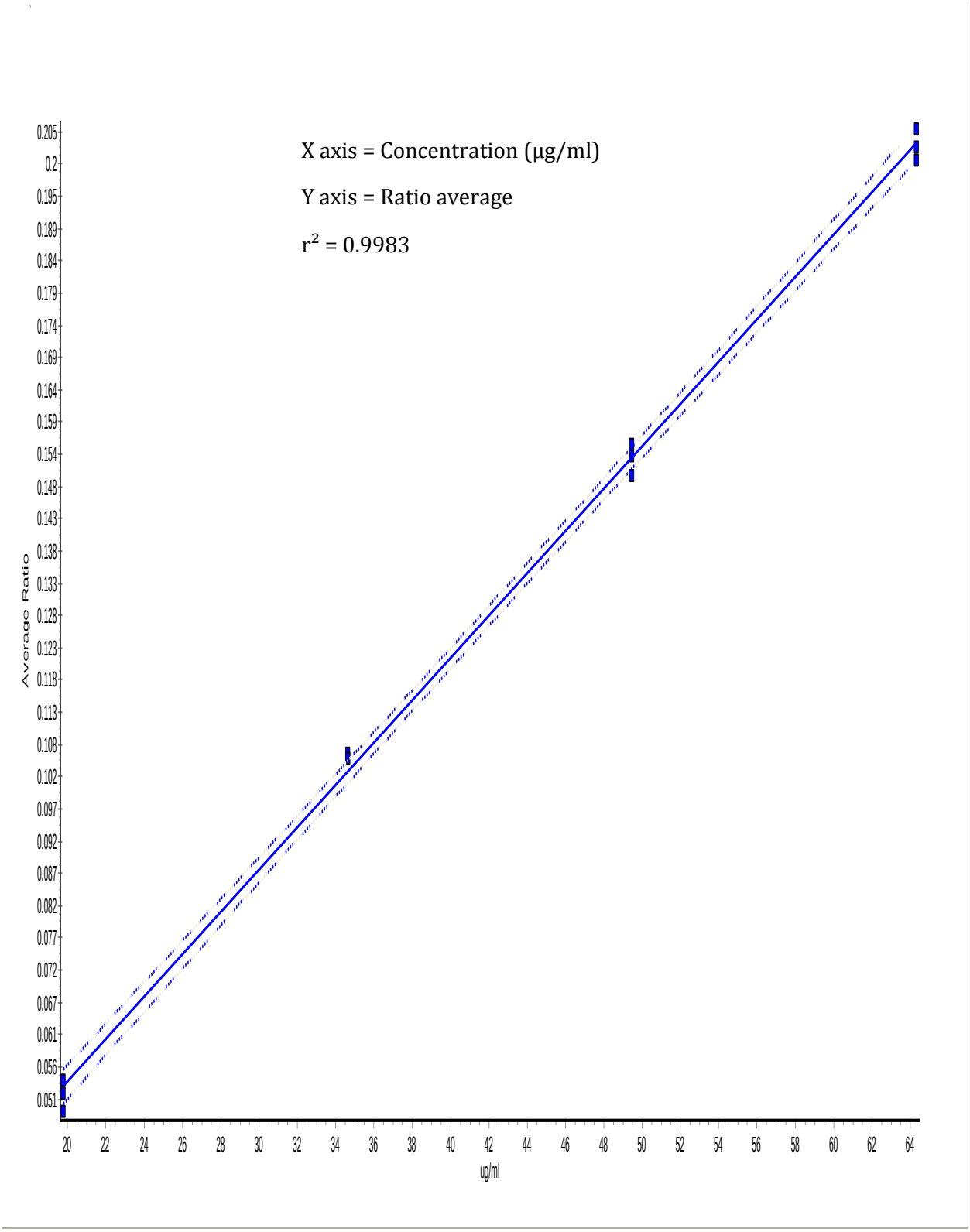


Figure 4.15: Average calibration curve of Lopinavir for the daily calibrations

4.1.2.5 HPLC calibrations for Nevirapine

Tables 4.5 represent the HPLC calibrations for nevirapine, and are summarized in figure 4.16:

r = 0.9996

r^2 = 0.9993

P < 0.0001

Table 4.5 HPLC calibrations for Nevirapine

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
19.75	0.103	0.133	0.142
19.75	0.132	0.118	0.124
19.75	0.132	0.103	0.148
19.75	0.118	0.122	0.114
19.75	0.121	0.097	0.123
Mean	**0.121**	**0.115**	**0.130**
39.50	0.294	0.300	0.267
39.50	0.258	0.261	0.261
39.50	0.298	0.267	0.287
39.50	0.286	0.294	0.297
39.50	0.265	0.287	0.293
Mean	**0.280**	**0.282**	**0.281**
59.25	0.470	0.460	0.430
59.25	0.438	0.433	0.435
59.25	0.433	0.471	0.439
59.25	0.428	0.430	0.458
59.25	0.458	0.439	0.471
Mean	**0.445**	**0.447**	**0.447**
79.00	0.640	0.584	0.641
79.00	0.660	0.662	0.601
79.00	0.624	0.581	0.630
79.00	0.580	0.626	0.598
79.00	0.584	0.641	0.557
Mean	**0.618**	**0.619**	**0.605**

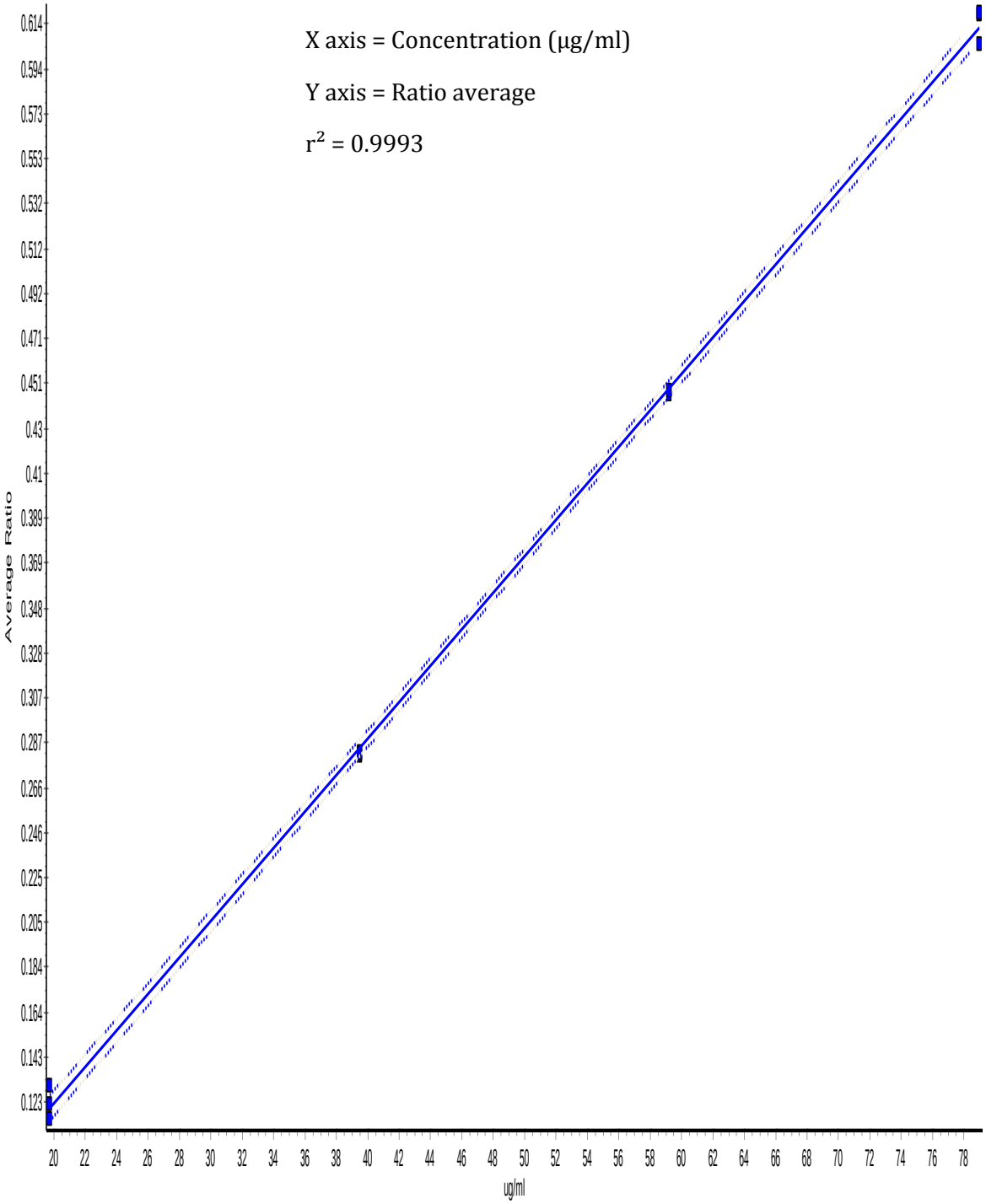


Figure 4.16: Average calibration curve of Nevirapine for the daily calibrations

4.1.2.6 HPLC calibrations for Stavudine

Tables 4.6 represent the HPLC calibrations for stavudine, and are summarized in figure 4.17:

r = 0.9957

r^2 = 0.9914

P < 0.0001

Table 4.6 HPLC calibrations for Stavudine

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
14.05	0.168	0.175	0.178
14.05	0.169	0.172	0.171
14.05	0.179	0.161	0.161
14.05	0.176	0.180	0.175
14.05	0.161	0.184	0.173
Mean	**0.171**	**0.174**	**0.172**
28.40	0.389	0.377	0.415
28.40	0.415	0.416	0.407
28.40	0.415	0.405	0.407
28.40	0.404	0.406	0.378
28.40	0.379	0.419	0.415
Mean	**0.400**	**0.405**	**0.404**
42.15	0.593	0.613	0.632
42.15	0.650	0.613	0.560
42.15	0.646	0.563	0.707
42.15	0.622	0.610	0.667
42.15	0.573	0.622	0.789
Mean	**0.617**	**0.605**	**0.671**
56.20	0.853	0.822	0.812
56.20	0.811	0.778	0.782
56.20	0.893	0.792	0.764
56.20	0.894	0.820	0.817
56.20	0.881	0.800	0.848
Mean	**0.866**	**0.802**	**0.805**

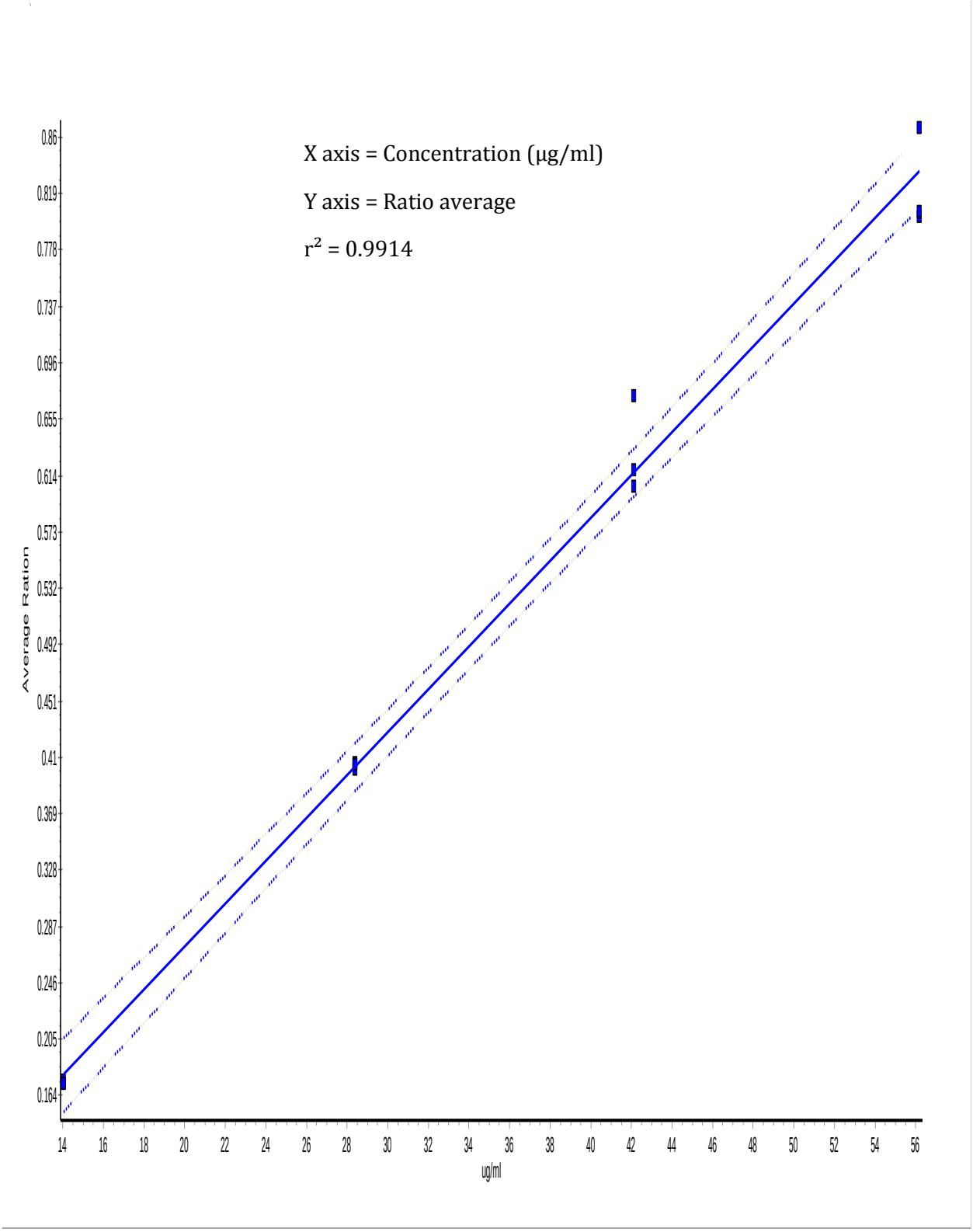


Figure 4.17: Average calibration curve of Stavudine for the daily calibrations

4.1.2.7 HPLC calibrations for Tenofovir

Tables 4.7 represent the HPLC calibrations for tenofovir, and are summarized in figure 4.18:

r = 0.9962

r^2 = 0.9924

P < 0.0001

Table 4.7 HPLC calibrations for Tenofovir

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
69.75	0.217	0.205	0.204
69.75	0.181	0.180	0.181
69.75	0.194	0.196	0.198
69.75	0.196	0.193	0.195
69.75	0.183	0.183	0.185
Mean	**0.194**	**0.191**	**0.193**
93.00	0.252	0.250	0.250
93.00	0.213	0.211	0.211
93.00	0.229	0.212	0.234
93.00	0.234	0.234	0.233
93.00	0.227	0.228	0.229
Mean	**0.231**	**0.227**	**0.231**
186.00	0.450	0.458	0.460
186.00	0.520	0.525	0.525
186.00	0.361	0.366	0.367
186.00	0.469	0.471	0.469
186.00	0.442	0.472	0.447
Mean	**0.448**	**0.458**	**0.454**
232.50	0.588	0.590	0.559
232.50	0.613	0.601	0.640
232.50	0.648	0.647	0.630
232.50	0.562	0.629	0.586
232.50	0.597	0.566	0.608
Mean	**0.602**	**0.607**	**0.605**

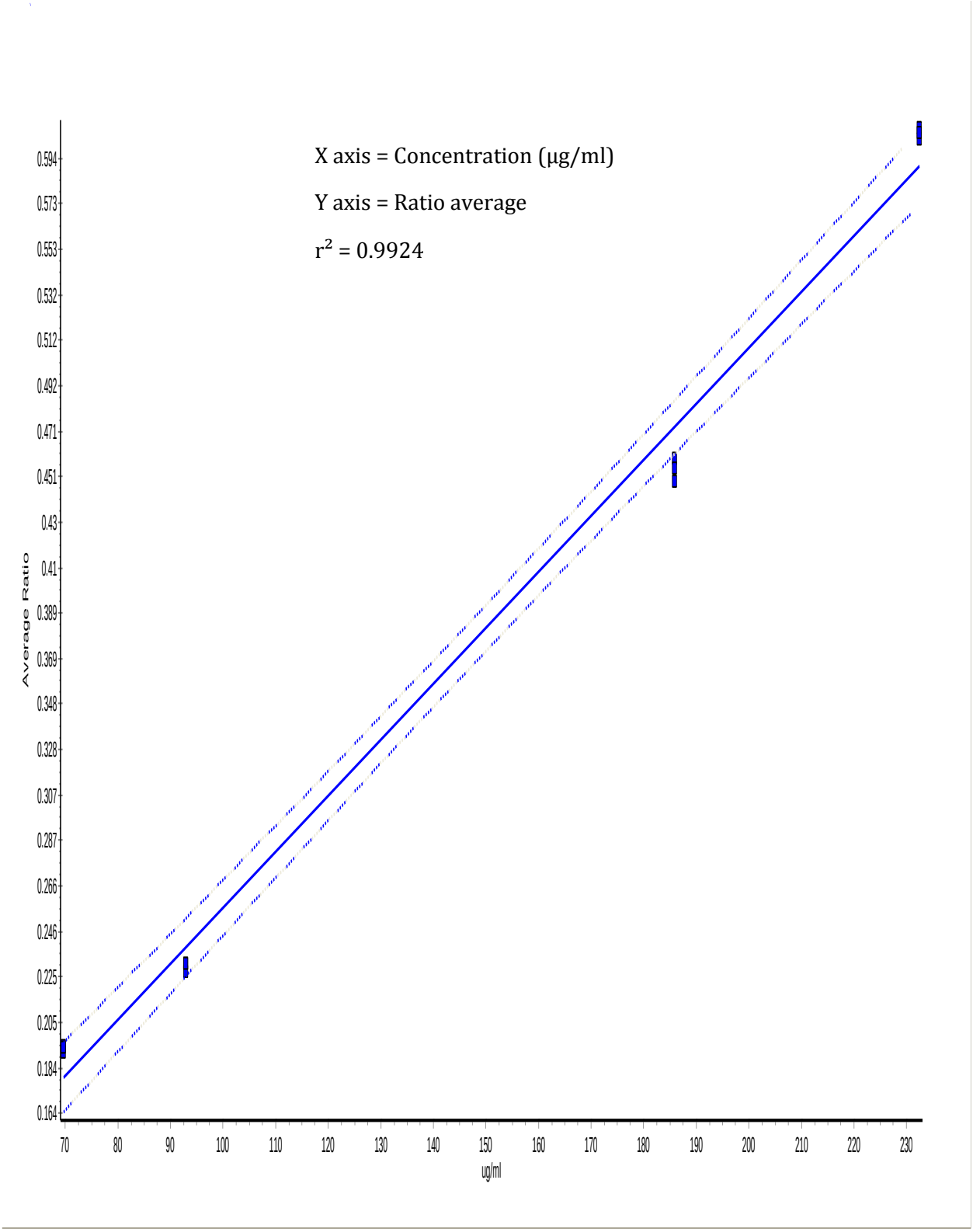


Figure 4.18: Average calibration curve of Tenofovir for the daily calibrations

4.1.2.8 HPLC calibrations for Zidovudine

Tables 4.8 represent the HPLC calibrations for zidovudine, and are summarized in figure 4.19:

r = 0.9990

r^2 = 0.9981

P < 0.001

Table 4.8 HPLC calibrations for Zidovudine

Concentration (µg/ml)	Calibration day 1 Ratio	Calibration day 2 Ratio	Calibration day 3 Ratio
10.40	0.081	0.082	0.090
10.40	0.089	0.090	0.070
10.40	0.070	0.072	0.080
10.40	0.080	0.083	0.080
10.40	0.090	0.090	0.090
Mean	**0.082**	**0.083**	**0.082**
20.80	0.230	0.224	0.224
20.80	0.217	0.200	0.231
20.80	0.211	0.218	0.199
20.80	0.225	0.211	0.217
20.80	0.200	0.230	0.224
Mean	**0.217**	**0.217**	**0.219**
41.60	0.412	0.423	0.407
41.60	0.397	0.414	0.409
41.60	0.413	0.406	0.402
41.60	0.404	0.400	0.427
41.60	0.421	0.406	0.413
Mean	**0.409**	**0.410**	**0.412**
83.20	0.816	0.801	0.822
83.20	0.789	0.817	0.798
83.20	0.806	0.798	0.836
83.20	0.825	0.836	0.806
83.20	0.803	0.822	0.820
Mean	**0.808**	**0.815**	**0.816**

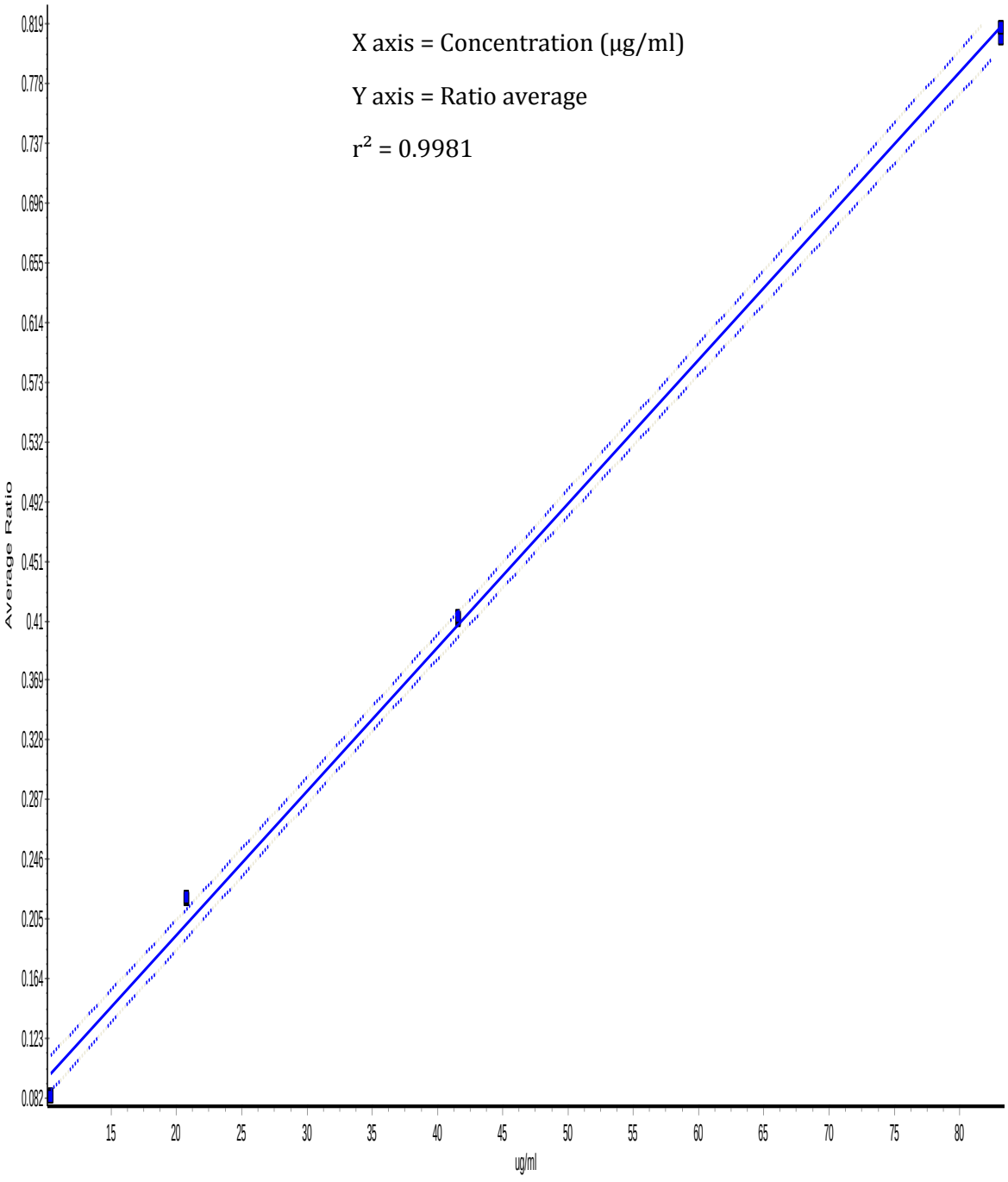


Figure 4.19: Average calibration curve of Zidovudine for the daily calibrations

4.2 PRECISION, ACCURACY AND RECOVERY

Acceptable levels of accuracy require that the method consistently reproduce average concentration results that range between 80 percent and 120 percent of the actual concentration (Shabir, 2004). The optimal is 100 percent, which means that the average concentration matches exactly with the actual concentration in the sample. While this eventuality is rare, a deviation of less than 20 percent in either direction is still considered acceptable. Recovery percentages close to 100 percent are desirable, which would clearly indicate that the extraction procedure is capable of removing the entire ARV fraction from the urine matrix. In practice however, results as low as 50 percent are acceptable. A summary of the precision (CV%), accuracy (%), recovery, average concentration, actual concentration and standard deviation is presented in the tables 4.9 to 4.24 below for the ARVs over a period of 3 days.

4.2.1 ABACAVIR

4.2.1.1 Values for spiked concentrations obtained

Table 4.9 Summary of spiked concentrations obtained for Abacavir

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (19.2 µg/ml)	0.308	**18.73**	0.278	**16.98**	0.308	**18.73**
High (38.4 µg/ml)	0.583	**34.59**	0.564	**33.49**	0.591	**35.05**

4.2.1.2 Precision, accuracy and recovery

Table 4.10 Summary of the precision, accuracy and recovery for Abacavir

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
19.2	18.73	16.98	19.88	18.53	1.46	7.88	96.50	96.51
38.4	34.59	33.49	35.05	34.38	0.80	2.33	89.53	89.53

4.2.2 EFAVIRENZ

4.2.2.1 Values for spiked concentrations obtained

Table 4.11 Summary of spiked concentrations obtained for Efavirenz

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (85.20 µg/ml)	0.300	**76.29**	0.286	**71.88**	0.293	**74.09**
High (170.40 µg/ml)	0.674	**193.90**	0.667	**191.70**	0.676	**194.53**

4.2.2.2 Precision, accuracy and recovery

Table 4.12 Summary of the precision, accuracy and recovery for Efavirenz

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
85.20	76.29	71.88	74.09	74.09	2.21	2.98	86.96	86.96
170.40	193.90	191.70	194.53	193.38	1.49	0.77	113.49	113.49

4.2.3 LAMIVUDINE

4.2.3.1 Values for spiked concentrations obtained

Table 4.13 Summary of spiked concentrations obtained for Lamivudine

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (15.17 µg/ml)	0.263	**12.98**	0.263	**12.98**	0.266	**13.13**
High (30.33 µg/ml)	0.592	**29.56**	0.513	**25.58**	0.488	**24.32**

4.2.3.2 Precision, accuracy and recovery

Table 4.14 Summary of the precision, accuracy and recovery for Lamivudine

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
15.17	12.91	12.91	13.13	12.98	0.09	0.69	85.56	85.89
30.33	29.56	25.58	24.32	26.49	2.74	10.34	87.34	87.34

4.2.4 LOPINAVIR

4.2.4.1 Values for spiked concentrations obtained

Table 4.15 Summary of spiked concentrations obtained for Lopinavir

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (19.80 µg/ml)	0.032	**13.53**	0.041	**16.20**	0.035	**14.42**
High (44.55 µg/ml)	0.128	**42.08**	0.118	**39.10**	0.114	**37.91**

4.2.4.2 Precision, accuracy and recovery

Table 4.16 Summary of the precision, accuracy and recovery for Lopinavir

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
19.80	13.53	16.20	14.42	14.72	1.36	9.24	74.34	74.34
44.55	42.08	39.10	37.91	39.70	2.15	5.42	89.11	89.11

4.2.5 NEVIRAPINE

4.2.5.1 Values for spiked concentrations obtained

Table 4.17 Summary of spiked concentrations obtained for Nevirapine

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (31.60 µg/ml)	0.210	**30.62**	0.219	**31.71**	0.205	**30.02**
High (63.20 µg/ml)	0.489	**64.20**	0.509	**66.60**	0.504	**66.00**

4.2.5.2 Precision and accuracy

Table 4.18 Summary of the precision, accuracy and recovery for Nevirapine

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
31.60	30.62	31.71	30.02	30.78	0.86	2.79	97.41	97.40
63.20	64.20	66.60	66.00	65.6	1.25	1.91	103.80	103.80

4.2.6 STAVUDINE

4.2.6.1 Values for spiked concentrations obtained

Table 4.19 Summary of spiked concentrations obtained for Stavudine

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (25.30 µg/ml)	0.081	**15.79**	0.077	**15.04**	0.084	**16.36**
High (39.35 µg/ml)	0.161	**30.91**	0.171	**32.80**	0.165	**31.67**

4.2.6.2 Precision, accuracy and recovery

Table 4.20 Summary of the precision, accuracy and recovery for Stavudine

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
11.24	7.81	7.55	8.00	7.79	0.23	2.95	69.31	69.30
16.86	12.95	13.59	13.20	13.24	0.32	2.42	78.53	78.59

4.2.7 TENOFOVIR

4.2.7.1 Values for spiked concentrations obtained

Table 4.21 Summary of spiked concentrations obtained for Tenofovir

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (37.20 µg/ml)	0.92	**34.91**	0.117	**44.84**	0.128	**49.20**
High (83.70 µg/ml)	0.198	**77.00**	0.223	**86.92**	0.213	**82.95**

4.2.7.2 Precision, accuracy and recovery

Table 4.22 Summary of the precision, accuracy and recovery for Tenofovir

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
37.20	34.9	44.84	49.20	42.98	7.32	17.03	115.54	115.54
83.70	77.00	86.92	82.95	82.29	4.99	6.06	98.32	98.32

4.2.8 ZIDOVUDINE

4.2.8.1 Values for spiked concentrations obtained

Table 4.23 Summary of spiked concentrations obtained for Zidovudine

Stock solution	Day 1 Average Ratio	Concentration from calibration curve	Day 2 Average Ratio	Concentration from calibration curve	Day 3 Average Ratio	Concentration from calibration curve
Low (31.20 µg/ml)	0.287	**29.49**	0.279	**28.68**	0.296	**30.40**
High (72.80 µg/ml)	0.644	**65.67**	0.643	**65.57**	0.662	**67.49**

4.2.8.2 Precision, accuracy and recovery

Table 4.24 Summary of the precision, accuracy and recovery for Zidovudine

Concentration µg/ml	Day 1	Day 2	Day 3	Mean	SD	CV%	Accuracy (%)	Recovery (%)
31.20	29.49	28.68	30.40	29.52	0.86	2.91	94.61	94.62
72.80	65.67	65.57	67.49	66.24	1.08	1.63	90.98	90.99

Accuracy of the method was found to be within required levels for abacavir, efavirenz, lamivudine, nevirapine, tenofovir, and zidovudine, showing that the results obtained by the method closely reflects the true value of the ARV. For lopinavir and stavudine however, accuracy within the acceptable range could not be achieved and further investigation is needed. The method however, will not be used for quantification of these drugs, but only for identification purposes in suspected overdose cases. Quantification needs further investigation. During the precision determination of the method, it was found that both concentration ranges of the ARVs yielded a coefficient of variation that did not exceed 10% in most cases. In the case of lamivudine high control samples and tenofovir low control samples, a smaller degree of precision was found. The recovery achieved by the analytical method was good for all the ARVs. The recovery achieved was not optimal for stavudine and lopinavir, but values within these ranges are considered to be acceptable, provided that the method has been proved to be precise and accurate. In the case of stavudine and lopinavir, precision was achieved, but accuracy and recovery was not optimal for the lower extracted concentrations. Although accuracy is not achieved for all the ARVs, the method however, proves precision and reproducibility, and for the purposes and aim of the study which required a qualitative method development, the method is of great value considering the fact that all the ARVs evaluated by this method can be identified.

4.3 SHORT-TERM STABILITY

Stability was determined for two different concentrations of each ARV. The following concentrations were used as summarised in table 4.25:

Table 4.25 ARV concentrations used in the determination of short-term stability

ARV	Low concentration (µg/ml)	High concentration (µg/ml)
Abacavir	19.20	38.40
Efavirenz	85.20	170.40
Lamivudine	15.17	30.33
Lopinavir	19.80	44.55
Nevirapine	31.60	63.20
Stavudine	11.24	16.86
Tenofovir	37.20	83.70
Zidovudine	31.20	72.80

Each sample was prepared in duplicate by following the procedure described in 3.10 and stored at different temperatures. The first was stored at room temperature; second samples were stored at 2-8˚C for 24 hours and 48 hours respectively and the third samples were stored at -20˚C for 24 hours and 48 hours respectively. Duplicate samples were run on day one at room temperature. Duplicate samples stored at 2-8˚C and at -20˚C were run after 24 hours, and after 48 hours. Stability was tested over a period of 48 hours because emergency overdose samples rarely take longer than 24 hours to reach the Pharmacology laboratory. Variations in ARV concentrations were observed over a period of 48 hours. The short-term stability studies are shown in Tables 4.58 to 4.73, and results have been plotted in graphs and shown in Figures 4.20 to 4.35.

The short term stability determination for two different concentrations of abacavir, 19.2 µg/ml and 38.4 µg/ml are summarized in Tables 4.58 and 4.59, and demonstrated in figures 4.20 and 4.21.

Table 4.26 Summary of the short-term stability determination of 19.20 µg/ml Abacavir and 38.40 µg/ml Abacavir

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
19.20	**Ambient temp**	18.73	17.00	19.88	18.54	1.45	7.82
19.20	**2-8˚C**		17.23	17.63	17.43	0.28	1.61
19.20	**-20˚C**		16.48	16.54	16.51	0.04	0.24
38.40	**Ambient temp**	34.59	33.49	35.05	34.38	0.80	2.33
38.40	**2-8˚C**		34.82	34.53	34.68	0.21	0.61
38.40	**-20˚C**		33.84	34.53	34.19	0.49	1.43

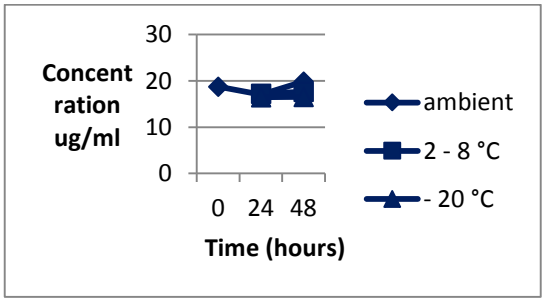


Figure 4.20: Short-term stability of 19.20 µg/ml Abacavir

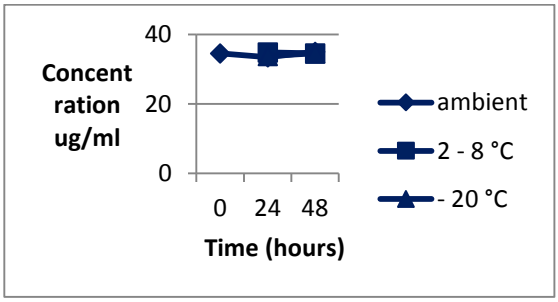


Figure 4.21: Short-term stability of 38.40 µg/ml Abacavir

The short term stability determination for two different concentrations of efavirenz, 85.2 µg/ml and 170.4 µg/ml are summarized in Tables 4.60 and 4.61, and demonstrated in figures 4.22 and 4.23.

Table 4.27 Summary of the short-term stability determination of 85.20 µg/ml Efavirenz and 170.40 µg/ml Efavirenz

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
85.20	**Ambient temp**	76.29	71.88	74.09	74.09	2.21	2.98
85.20	**2-8˚C**		75.34	75.03	75.19	0.22	0.30
85.20	**-20˚C**		75.03	72.20	73.62	2.00	2.72
170.40	**Ambient temp**	193.90	191.70	194.53	193.38	1.49	0.77
170.40	**2-8˚C**		179.44	153.96	166.70	18.09	10.85
170.40	**-20˚C**		179.44	176.61	178.03	2.00	1.12

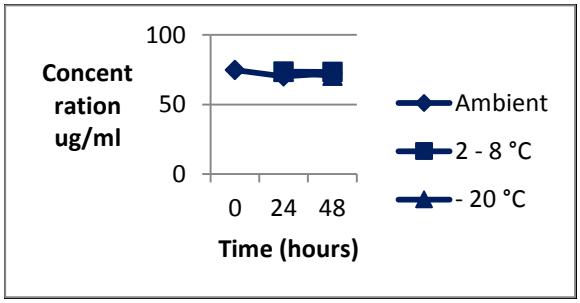


Figure 4.22: Short-term stability of 85.20 µg/ml Efavirenz

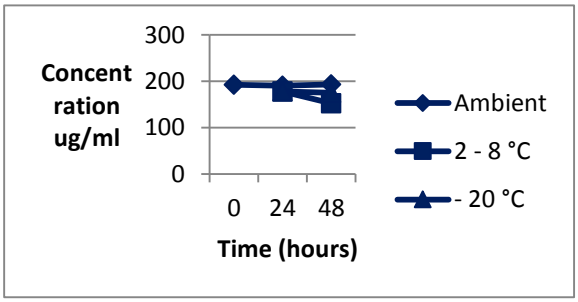


Figure 4.23: Short-term stability of 170.40 µg/ml Efavirenz

The short term stability determination for two different concentrations of lamivudine, 15.17 µg/ml and 30.33 µg/ml are summarized in Tables 4.62 and 4.63, and demonstrated in figures 4.24 and 4.25.

Table 4.28 Summary of the short-term stability determination of 15.17 µg/ml Lamivudine and 30.33 µg/ml Lamivudine

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
15.17	**Ambient temp**		12.98	13.13	13.0	0.09	0.70
15.17	**2-8˚C**		13.13	13.33	13.23	0.14	1.1
15.17	**-20˚C**		13.64	13.69	13.67	0.04	0.30
30.33	**Ambient temp**	29.56	25.58	28.30	28.81	2.03	7.05
30.33	**2-8˚C**		25.38	25.58	25.48	0.14	0.55
30.33	**-20˚C**		24.17	24.92	24.55	0.53	2.16

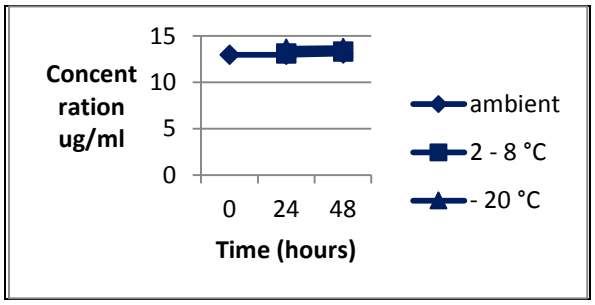


Figure 4.24: Short-term stability of of15.17 µg/ml Lamivudine

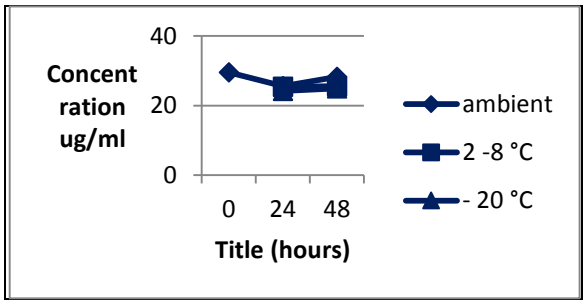


Figure 4.25: Short-term stability 30.33 µg/ml lamivudine

The short term stability determination for two different concentrations of lopinavir, 19.80 µg/ml and 44.55 µg/ml are summarized in Tables 4.64 and 4.65, and demonstrated in figures 4.26 and 4.27.

Table 4.29 Summary of the short-term stability determination of 19.80 µg/ml Lopinavir and 44.55 µg/ml Lopinavir

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
19.80	**Ambient temp**	13.53	16.20	14.42	14.72	1.36	9.24
19.80	**2-8˚C**		15.61	15.31	15.46	0.21	1.36
19.80	**-20˚C**		14.12	14.12	14.12	0.00	0.00
44.55	**Ambient temp**	42.08	39.10	37.91	39.70	2.15	5.42
44.55	**2-8˚C**		38.51	37.91	38.21	0.40	1.05
44.55	**-20˚C**		37.61	37.32	37.47	0.21	0.56

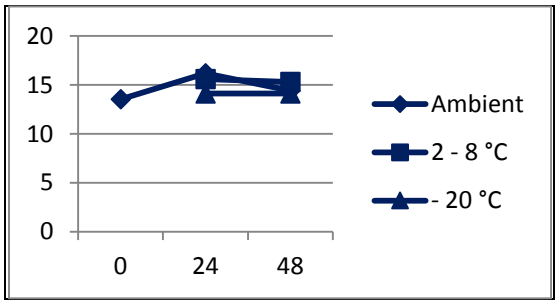


Figure 4.26: Short-term stability of 19.80 µg/ml Lopinavir

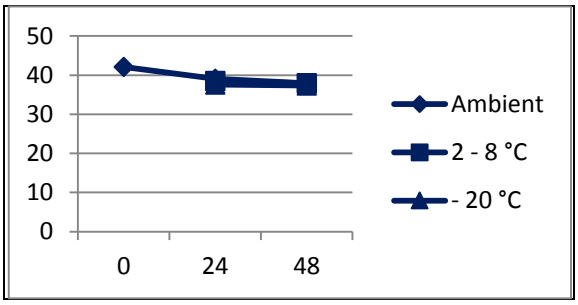


Figure 4.27: Short-term stability of 44.55 µg/ml Lopinavir

The short term stability determination for two different concentrations of nevirapine, 31.60 µg/ml and 63.20 µg/ml are summarized in Tables 4.66 and 4.67, and demonstrated in figures 4.28 and 4.29.

Table 4.30 Summary of the short-term stability determination of 31.60 µg/ml Nevirapine and 63.20 µg/ml Nevirapine

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
31.60	**Ambient temp**	30.62	31.71	30.02	30.78	0.86	2.79
31.60	**2-8˚C**		32.55	32.31	32.43	0.17	0.52
31.60	**-20˚C**		32.67	32.67	32.67	0	0
63.20	**Ambient temp**	64.20	66.60	66.00	65.6	1.25	1.91
63.20	**2-8˚C**		63.96	64.08	64.02	0.08	0.12
63.20	**-20˚C**		63.23	64.92	64.08	1.20	1.87

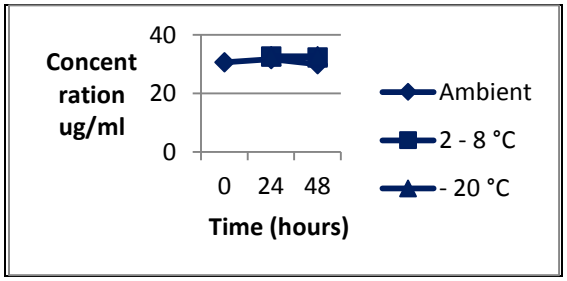


Figure 4.28: Short-term stability of 31.60 µg/ml Nevirapine

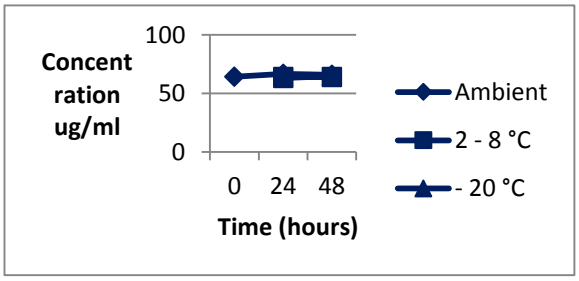


Figure 4.29: Short-term stability of 63.20 µg/ml Nevirapine

The short term stability determination for two different concentrations of stavudine, 11.24 µg/ml and 16.86 µg/ml are summarized in Tables 4.68 and 4.69, and demonstrated in figures 4.30 and 4.31.

Table 4.31 Summary of the short-term stability determination of 11.24 µg/ml Stavudine and 16.86 µg/ml Stavudine

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
11.24	**Ambient temp**	7.81	7.55	8.00	7.79	0.225	2.89
11.24	**2-8˚C**		7.43	8.00	7.72	0.403	5.22
11.24	**-20˚C**		8.33	8.45	8.39	0.085	1.01
16.86	**Ambient temp**	12.88	12.75	12.95	12.86	0.101	0.79
16.86	**2-8˚C**		13.59	13.20	13.40	0.276	2.06
16.86	**-20˚C**		13.33	12.82	13.08	0.360	2.75

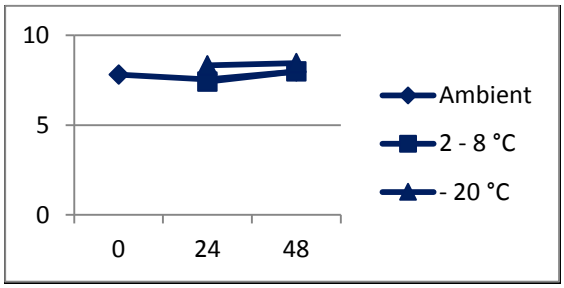


Figure 4.30: Short-term stability of 11.24 µg/ml Stavudine

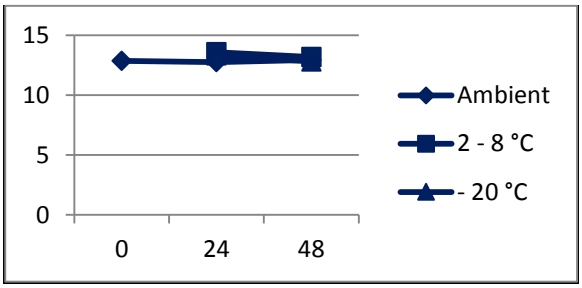


Figure 4.31: Short-term stability of 16.86 µg/ml Stavudine

The short term stability determination for two different concentrations of tenofovir, 37.20 µg/ml and 83.70 µg/ml are summarized in Tables 4.70 and 4.71, and demonstrated in figures 4.32 and 4.33.

Table 4.32 Summary of the short-term stability determination of 37.20 µg/ml Tenofovir and 83.70 µg/ml Tenofovir

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
37.20	**Ambient temp**	34.91	44.84	49.20	42.98	7.28	16.9
37.20	**2-8˚C**		43.64	44.44	44.04	0.57	1.29
37.20	**-20˚C**		40.47	39.67	40.07	0.57	1.42
83.70	**Ambient temp**	76.99	86.92	82.95	82.29	5.0	6.07
83.70	**2-8˚C**		89.30	88.11	88.71	0.84	0.95
83.70	**-20˚C**		126.22	127.81	127.02	1.12	0.88

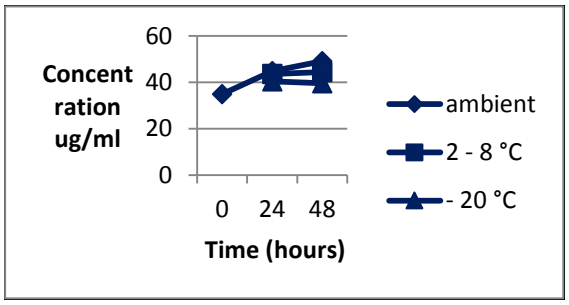


Figure 4.32: Short-term stability of 37.20 µg/ml Tenofovir

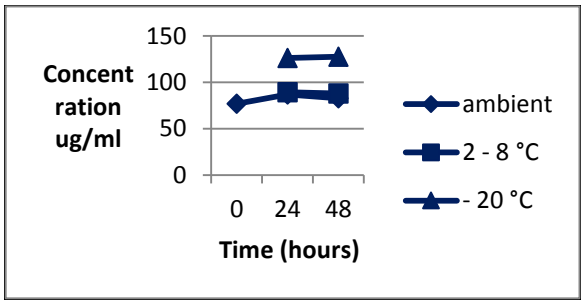


Figure 4.33: Short-term stability of 83.70 µg/ml Tenofovir

The short term stability determination for two different concentrations of zidovudine, 31.20 µg/ml and 72.80 µg/ml are summarized in Tables 4.72 and 4.73, and demonstrated in figures 4.34 and 4.35.

Table 4.33 Summary of the short-term stability determination of 31.20 µg/ml Zidovudine and 72.80 µg/ml Zidovudine

Concentration µg/ml	Temperature	stability 0 hours	stability 24 hours	stability 48 hours	mean	SD	CV%
31.20	**Ambient temp**	29.69	28.68	30.40	29.59	0.86	2.91
31.20	**2-8˚C**		28.38	29.19	28.79	0.57	1.98
31.20	**-20˚C**		27.57	29.19	28.38	1.14	4.02
72.80	**Ambient temp**	65.67	65.57	67.49	66.24	1.08	1.63
72.80	**2-8˚C**		64.76	64.25	64.51	0.36	0.56
72.80	**-20˚C**		62.53	63.95	63.24	1.00	1.58

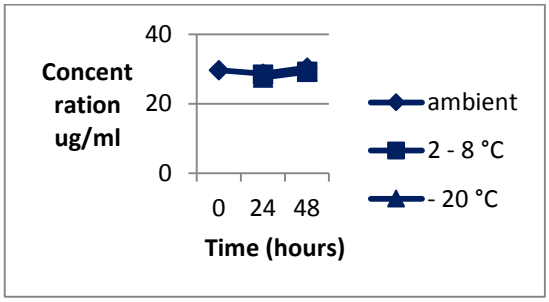


Figure 4.34: Short-term stability of 31.20 µg/ml Zidovudine

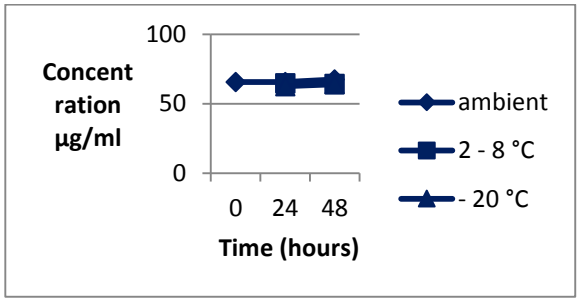


Figure 4.35: Short-term stability of 72.80 µg/ml Zidovudine

The short term stability of a drug in urine can be influenced by many factors including physiochemical properties of the drug, tendency to conjugate/deconjugate, specimen collection procedure, degree of microorganism contamination, container selection and the use of preservatives or other additives. In general, drug instability in any toxicological specimen is due to metabolic degradation, chemical transformation, or a combination of both. Most ARVs remained stable at room temperature and refrigeration over 48 hours. Freezing of samples rendered variable results. Long term stability of the ARVs was not determined. It is therefore concluded that the samples should be refrigerated to limit bacterial degradation of the sample and be evaluated within 48 hours.

4.4 LIMIT OF DETECTION

A limit of detection was established for each ARV drug investigated in the study. The LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the analysis.

The International Conference on Harmonisation (ICH) guidelines for detection limit parameters was applied in the validation of the analytical method. The signal to noise method was used whereby the peak-to-peak noise around the analyte retention time was measured, and subsequently, the concentration of the analyte that would give a signal equal to a specific value of noise to signal ratio was assessed. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD and was applied in the validation. The limit of detection is represented in Table 4.74.

Table 4.34 Limit of detection

ARV drug	Limit of detection (µg/ml)
Abacavir	4.40
Efavirenz	22.49
Lamivudine	5.06
Lopinavir	11.80
Nevirapine	14.20
Stavudine	11.13
Tenofovir	58.75
Zidovudine	7.84

4.5 INTERFERENCE

Interference was tested individually for each of the ARV drugs. Interference among the different ARV drugs, as well as interference from other drugs was investigated. Interference was eliminated by reducing or increasing the initial flow rate until all ARV compounds achieved excellent separation, or by modification of the extraction procedure. Overlapping of peaks from lamivudine and abacavir was detected as they both had a similar retention times. Overlapping of peaks from lopinavir and efavirenz was also noted. The overlapping was eliminated by reducing the initial flow rate to 0.8ml/min for the first two minutes, after which it was adjusted to 2ml/min for the rest of the run period. A gradient elution method was also

implemented where the mobile phase composition was changed during the analysis time of 30 minutes. At the beginning of the run Solvent B (organic solvent) was at 0% strength and Solvent A (aqueous solvent) at 100% (10% ACN preservative added) strength resulting in the resolution of the early components. As the separation progressed, Solvent B's strength was gradually increased to 10% over the first 9 minutes and Solvent A's strength was decreased gradually over the first 9 minutes to 80%. Solvent B was gradually increased to 50% from the 9th minute to the 20th minute after which the Solvent A to B ratio remained 1:1 for the remainder of the run so that the subsequent components elute within a reasonable time and overlapping of peaks were eliminated. All compounds achieved excellent separation after flow rate adjustments. No further interferences occurred. Current HIV/Tuberculosis (TB) co-infection rates exceed 70% with TB being the most common opportunistic infection in South Africa. It was therefore necessary to investigate interference from TB drugs as well. Not all TB drugs are detectable by the method. Trimethoprim and isoniazide, two commonly used drugs for the treatment of TB was detected with the method. Pure compound stock solutions of trimethoprim (100 µg/ml) and isoniazide (100 µg/ml) was injected to determine retention times and spectra. Data was stored in a computerized library. Samples were spiked with ARVs, trimethoprim and isoniazide, extracted and injected. No overlapping of peaks were observed, and effective differentiation and identification among substances were achieved. Neither of these drugs interfered with any of the ARVs.

4.6 PATIENT RESULTS

Thirty seven participants were identified to take part in the study. The participants were HIV positive patients being treated with ARVs by the Pelonomi Hospital HIV centre of excellence as part of the ARV rollout program in the Free State.

Each participating patient received an ARV regimen as established according to the CD4 count and viral load of each patient, and a detailed medical history of previous failed regimens, nephropathy and psychiatric conditions. All identified participants were being treated by combination regimens of the required ARVs in the study. Each participant's regimen was known, and extracted sample results were compared to the known regimen of each patient as illustrated in Table 4.75. Each participant's HPLC results were compared over four consecutive follow up visits to the clinic.

Table 4.35 Patient Results

Patient no	Abacavir 600 mg		Efavirenz 600 mg		Lamivudine 300 mg		Lopinavir 400 mg		Nevirapine 400 mg		Stavudine 80 mg		Tenofovir 300 mg		Zidovudine 600 mg	
	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID
1							√	X							√	√
2	√	X					√	X							√	X
3					√	X			√	X	√	X				
4			√	X	√	X							√	X		
5			√	√	√	√									√	√
6			√	√	√	√					√	√				
7			√	√	√	√									√	√
8					√	√	√	√								
9			√	√	√	√									√	√
10			√	√	√	√							√	X		
11			√	√	√	√							√	√		
12			√	√	√	√							√	√		
13							√	√							√	√
14			√	√	√	√									√	√
15			√	X	√	√							√	X		
16			√	√	√	√							√	√		
17			√	√	√	√							√	√		
18					√	√			√	√	√	√				

Patient no	Abacavir 600 mg		Efavirenz 600 mg		Lamivudine 300 mg		Lopinavir 400 mg		Nevirapine 400 mg		Stavudine 80 mg		Tenofovir 300 mg		Zidovudine 600 mg	
	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID	Rx	ID
19					√	√			√	√	√	√				
20			√	X	√	√							√	X		
21					√	√			√	√	√	√				
22					√	√	√	√							√	√
23			√	X	√	√							√	X		
24	√	X					√	√							√	X
25			√	X	√	√							√	X		
26			√	√	√	√							√	√		
27					√	√			√	√					√	√
28			√	√	√	√					√	√				
29			√	X	√	X							√	X		
30			√	√	√	√							√	√		
31	√	√			√	√							√	√		
32	√	X					√	√							√	X
33					√	√	√	√							√	√
34					√	√			√	√	√	√				
35	√	√			√	√							√	√		
36							√	X							√	√
37	√	X					√	√							√	X
% Compliance	33.33		68.42		90.00		70.00		83.33		85.71		53.33		64.29	

Rx	treatment
ID	identification
√	on treatment
√	identified in urine
X	not identified in urine
	patient compliant
	Patient non-compliant

4.7 DISCUSSION AND CONCLUSION

The HPLC method developed by the Department of Pharmacology/Toxicology, University of the Free State in Bloemfontein, South Africa, could only identify the presence of lamivudine, abacavir, zidovudine, nevirapine and efavirenz in urine. However further method development/modification and investigation was necessary to identify and include stavudine, didanosine, lopinavir, ritonavir and tenofovir in the spectrum of antiretroviral drugs detectable by the HPLC method.

A fast and effective high performance liquid chromatography method using UV detection and a liquid-liquid extraction for the qualitative identification of lamivudine, abacavir, zidovudine, nevirapine, efavirenz, stavudine, lopinavir and tenofovir was modified for the toxicology laboratory of the University of the Free State. Good separations of the ARVs with high quality peaks were obtained, and no interference occurred among the ARVs, the tuberculosis drugs investigated or from the blank urine samples used in the study. The linear regression curves constructed proved linearity and reproducibility of results obtained by the method.

Accuracy of the method was found to be within required levels of 80 to 120% for abacavir, efavirenz, lamivudine, nevirapine, tenofovir, and zidovudine, showing that the results obtained by the method closely reflects the true value of the ARV. For lopinavir and stavudine however, accuracy within the acceptable range could not be achieved and further investigation is needed. Accuracy of the method for lopinavir and stavudine can be improved by exploring potential systematic errors such as faults in the analytical procedure or the HPLC system that may have occurred during the course of the study. The possibility exists that lopinavir and stavudine may not be as soluble in the selected solvents as the rest of the ARVs included in the study.

In the method being used, stock solutions of ARVs were made up with methanol to the following concentrations:

Lamivudine = 1017.00 µg/ml

Abacavir = 1005.00 µg/ml

Zidovudine = 1053 µg/ml

Nevirapine = 1040.75 µg/ml

Efavirenz = 1014.00 µg/ml

Unfortunately not all ARVs, particularly those included in this study, dissolved in methanol. Other solvents such as ethanol, acetonitrile, tetraethylammonium phosphate (TEAP) buffer, dichloromethane, isopropanol, dimethyl sulfoxide (DMSO) and combinations of these solvents were explored in this study to find a common solvent in which all ARVs for this study could dissolve.

Most of the combinations used by other researchers were successful for the extraction of one or two different ARVs. Elens *et al*. (2009) described an ultra-performance liquid chromatography (UPLC) method with a solid-phase extraction method used for plasma. A linear gradient of 50 mmol/L ammonium acetate and 50 mmol/L formic acid in water versus acetonitrile was used to achieve chromatographic separations. Oliveira *et al*. (2005) proposed 0.01M sodium acetate solution: methanol (85:15 v/v) and acetic acid as a mobile phase for the determination of didanosine. Supriya, Ashish & Meena (2012) proposed a high performance liquid chromatography tandem mass spectrometric method for the estimation of emtricitabine in using a mobile phase consisting 5mM ammonium acetate: acetonitrile: methanol: (30:30:40 v/v). These combinations were not optimal for the simultaneous extraction of 8 different ARVs. A universal solvent (Solvent C) was prepared by adding 150ml of Solvent A [Tetraethylammonium phosphate (TEAP) buffer + Acetonitrile (9:1)] to 30ml of Solvent B (100µl H_3PO_4 added to 950ml of ACN and reconstituted it to 1L with ACN) in a ratio of 5:1 and used to dissolve pure compounds in. Solvent C proved to be the combination of solvents in which all ARVs explored in this study could dissolve. A gradient elution method consisting of an aqueous solvent (Solvent A) containing 450ml tetraethylammonium phosphate (TEAP) buffer and 50ml of acetonitrile (ACN), and an organic solvent (Solvent B) consisting of acetonitrile and H_3PO_4 proved to be the most effective method to elude all the ARVs in this study.

Didanosine could not be detected by the method. Attempts were made to dissolve pure compound of didanosine in water, ACN, TEAP buffer, DMSO, methanol, ethanol, dichloromethane, isopropanol and combinations of different strengths of before mentioned chemicals. Didanosine was only partially dissolvable in DMSO. It was therefore decided to exclude didanosine from the study to preserve the integrity of the method without compromising the quality of extraction of the other ARVs. DMSO is also a highly toxic agent. Ritonavir is an ARV combined with lopinavir for the treatment of HIV and AIDS. Ritonavir is administered solely to increase lopinavir levels by inhibiting the CYP3A mediated metabolism

of lopinavir. It was therefore removed from the study as ritonavir is only produced concomitantly bound to lopinavir and potential risk of overdose is minimal.

The method developed did unfortunately not achieve accuracy and precision for all the ARVs. This method however, will not be used for quantification of these drugs, but only for qualitative identification purposes. Quantification was not achieved. Although quantification did not form part of the envisaged outcomes, it could have been achieved if two or more calibration points were included. Limited quantities of pure compounds were available during the course of the study. Had larger quantities of pure compounds been available, more calibration points (six to eight points are generally recommended) could have been included during calibration, which would have rendered an increase in precision of the method.

The recovery achieved by the analytical method was good for all the ARVs. The recovery achieved was not optimal for stavudine and lopinavir, but values within these ranges are considered to be acceptable, provided that the method has been proved to be precise and accurate. The recovery for lopinavir ranged between 74% to 89%, and for stavudine between 69% and 78%. In the case of stavudine and lopinavir, precision was achieved, but accuracy and recovery was not optimal for the lower extracted concentrations. The lower level of recovery may be attributed to various factors such as the method not extracting the pure compound completely from the urine matrix, or it might be that the compounds are not completely soluble in the universal solvent selected in the study. The recovery of stavudine and lopinavir may be increased if a more suitable solvent is used. It must however be taken into consideration that a universal solvent must be found that will dissolve all the ARVs required in this study. Many solvents have been investigated and considered for the purposes of this study. The solvents selected proved to be the most appropriate for the combination of ARVs explored in this study, and one must consider the integrity of the method and the purpose of the method in its entirety. This method can now be used in conjunction with other emergency toxicology screenings such as GC/MS and rapid identification tests of individual substances, such as paracetamol and salicylic acid, on analysers to identify potential ARVs taken in overdose. The method developed can identify the ARVs included in the study, and may serve as a helpful tool in the race to make a diagnosis in an overdose patient.

The stability of a drug in a biological matrix is dependent on the chemical properties of the drug, the sample storage conditions, the container used and the matrix itself. The stability of

the drug should be evaluated under conditions that would likely be encountered during analysis and sample handling (Vibuthi *et al* 2009). Stability was tested over a period of 48 hours because emergency overdose samples rarely take longer than 24 hours to reach the Pharmacology laboratory. ARV concentrations remained stable at room temperature and refrigeration over 48 hours. Freezing of samples rendered variable results. Long term stability of the ARVs was not determined. It is therefore advisable to analyse the urine as soon as possible, and if samples cannot be analysed immediately, storage at room temperature or 2-8˚C (to limit bacterial degradation) is required.

The method was successfully applied to participating patient samples. Compliance and non-compliance were identified in the participants. Twenty three patients (62%) were compliant on their ARV regimens. The HPLC extraction results of these patients proved similar over 4 consecutive follow-up visits to the clinic. Fourteen patients (38%) did not comply. ARVs were detected in 10 of the non-compliant patients, but not all ARVs that should have been detected according to their treatment (Rx) regimens, were detected. Patient compliance was good for efavirenz, lopinavir, nevirapine, stavudine and zidovudine, with lamivudine showing the highest patient compliance of all the ARVs in the study. Four non-compliant patients' urine samples were negative for any ARVs. High resolution peaks and spectra were observed in compliant patients.

Low patient compliance was identified for abacavir and tenofovir. Low patient compliance may be attributed to demographics and psychosocial factors such as unemployment and alcohol use, medication-related issues and other patient-related matters. Medication-related adverse effects, such as neuropathy, headache, nausea, loss of memory, diarrhoea, fatigue and a heavy pill burden are probably the main reason for poor adherence (McNicholl, 2012; Rathbun, 2013). Poor adherence to treatment is an important concern relating to HIV management in our setting and needs to be addressed. Therapy selection proved to be a valuable tool to increase patient adherence. Patient specific factors should be considered when selecting a treatment regimen. The factors to consider include potential for pregnancy, associated comorbidities, treatment convenience, potential drug-drug and food-drug interactions (Rathbun, 2013). Ongoing efforts to educate and eradicate the stigma associated with HIV/AIDS will contribute in the reduction of new infections and encourage patients to adhere to their treatment regimens.

Another threat to the integrity of the treatment campaign against HIV and AIDS emerged. Some of the ARVs for example efavirenz are used as a recreational drug. Efavirenz are crushed and the powder is snorted or mixed and inhaled together with other substances of abuse to increase the euphoric effect. Due to this fact, lifesaving stock of the ARVs are stolen and sold as recreational drugs, or are being abused by patients on ARV treatment. This contributes to the enormous challenges faced by our department of health that are already strained by financial, demographical and logistical factors in the fight against HIV and AIDS (Gatch *et al* 2013).

Another pitfall in the developed method is the practical application with regards to therapeutic drug monitoring of the ARVs. Some of the ARVs were quantifiable by the method, but further method development and research is required to increase quantification across a broad spectrum of ARVs. The method may aid therapeutic drug monitoring, but only due to possible identification of the drugs the patient are taking or not. Once again quantification becomes necessary to establish the degree of compliance by the patient by investigating levels of the ARVs and whether or not they comply with established therapeutic ranges of the drugs. Another potential pitfall with quantification is that the work load may simply be too big once levels can be determined. Thousands of patients are on ARV treatment, keeping this in mind, therapeutic drug monitoring with this method may overload the Pharmacology laboratory since each sample requires extensive and time consuming extraction procedures and the sample load is limited to only one sample at a time on HPLC, and each sample will have a processing time of 30 minutes. It is clearly evident that the application of the method is limited to toxicology only, and can only deliver a significant contribution in the identification of potential overdoses, or the elimination thereof. Other methods must be considered for quantification and therapeutic drug monitoring purposes.

The method developed needs further investigation, especially with regards to quantification of the ARVs, and to include new drugs being used in HIV and AIDS management. New emerging regimes include emtricitabine, atazanavir, darunavir, fosamprenavir and raltegravir in fixed dose combinations. The method developed is reliable, cost effective and relatively user friendly. The method however can only detect a limited number of the ARVs available on the market. With newer and more effective drugs being researched and marketed every day, the method is likely to become outdated. It is therefore essential to expand the spectrum of ARVs detectable by the method continuously to ensure positive identification of newer drugs to assess patient compliance or that may potentially be used in overdose cases.

A higher survival rate can also be achieved due to the reduction in organ damage. Further method development is therefore necessary to include other ARVs in the screen, to enhance the current accuracy of the method, to quantify the ARVs and to lower the limit of detection. Further investigations with regards to the interferences of other drugs are also recommended.

ARV determination on blood was also explored by applying the same method developed for urine on blood samples. The method proved not to be successful on blood samples and other extraction methods such as solid phase, derivatisation and LC/MS can be considered for future research on blood samples.

After the method was developed, it was applied to patients with suspected overdoses where severe lactic acidosis could not be explained by other diagnostic methods such as GC/MS screening and rapid identification assays for individual substances. These patients' chromatograms showed broad and erratic peaks with large areas under the curve. When compared to chromatograms of patients compliant on ARVs, the broad and erratic peaks with large AUCs indicate overload of the urine matrix which translates into higher than normal quantities of ARVs present. The assumption can be made that an overdose is present. Suspected overdose patients' results were compared to results of compliant patients. The comparison clearly indicated whether or not an overdose was present. These results were successfully applied as a diagnostic tool and aided in the management of the patients. In most suspected overdose cases, activated charcoal would be administered, a specific antidote can be used or in extreme overdose cases dialysis would be required. Continued laboratory investigation to monitor improvement in reduction or elimination of a substance taken in overdose will assist and guide clinicians in the treatment of such a patient.

Further investigation is essential for the identification of suspected metabolites since all of the ARVs are metabolized in the body to produce a number of different metabolites. Positive identification and perhaps quantification of the metabolites may aid in diagnosis by identifying a substance were the parent compound can no longer be detected in the urine, thereby indicate the extent of an overdose, time after ingestion, contribute to the management of an overdose patient, and hopefully a better outcome. The most used and versatile analytical methods for metabolite profiling are based on combining liquid chromatography with mass spectrometry (LC/MS), providing both qualitative and quantitative information simultaneously. The most efficient approaches in metabolite identification are those using time-of-flight mass (TOF) spectrometry or Orbitrap mass spectrometry. The high mass resolving power of these

instruments provides high specificity of detection, whereas the high mass accuracy enables identification of biotransformations in the detected metabolites with very high certainty by elucidation of changes in molecular formula with respect to the parent compound. Unfortunately LC/MS and TOF facilities were not available were the study was conducted, and other avenues explored for the purposes of metabolite identification had failed.

The method can only identify a limited number of the ARVs currently being used in treatment regimens. The method developed has been implemented successfully as part of a range of diagnostic tools being used to aid in the identification of unknown substances in patients with a suspected overdose. Since HIV and AIDS became more prevalent in our community, more of these drugs are currently in circulation in the general population than before. The increase of ARV availability increased the risk of potential overdose in suicide attempts. The method developed will aid in the identification or elimination of unknown substances, if present in the urine of patients with a suspected overdose. The method covers another category of drugs that could not be identified previously by the Pharmacology department and may contribute to patient outcomes after overdose. The method can also be used as a platform for future method development in the field of ARV investigation with numerous potential add on and modification capabilities. The method expands the spectrum of identifiable substances of toxicology screens used by the toxicology laboratory of the University of the Free State and can make a valuable contribution to service delivery in future.

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6 APPENDIXES

Appendix 7.1: Precision and accuracy testing of HPLC method for Abacavir,

Day 1

Table 7.1: PRECISION AND ACCURACY DATA FOR ABACAVIR, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
19.20	18.92
19.20	17.99
19.20	19.28
	Average= 18.73

µg/ml (Spiked)	µg/ml (Extracted)
38.40	33.25
38.40	33.41
38.40	37.11
	Average= 34.59

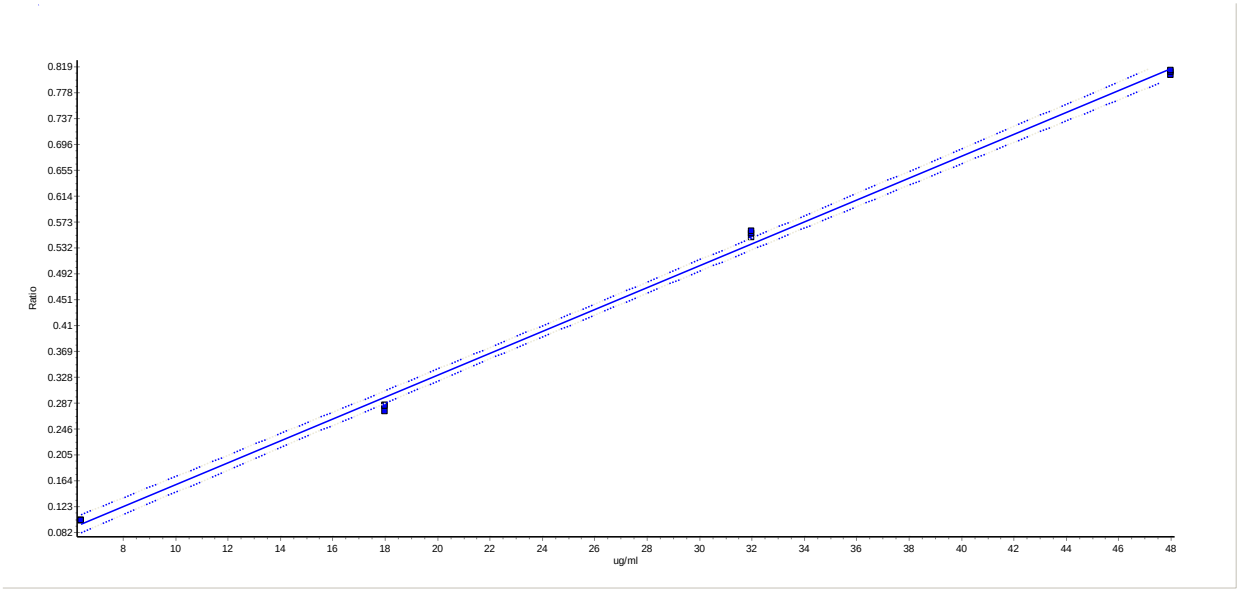


Figure 7.1: Calibration graph of Abacavir used in precision and accuracy testing, Day 1

Appendix 7.2: Precision and accuracy testing of HPLC method for Abacavir,

Day 2

Table 7.2: PRECISION AND ACCURACY DATA FOR ABACAVIR, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
19.20	18.12
19.20	17.55
19.20	15.27
	Average= 16.98

µg/ml (Spiked)	µg/ml (Extracted)
38.40	30.63
38.40	31.58
38.40	38.26
	Average= 33.49

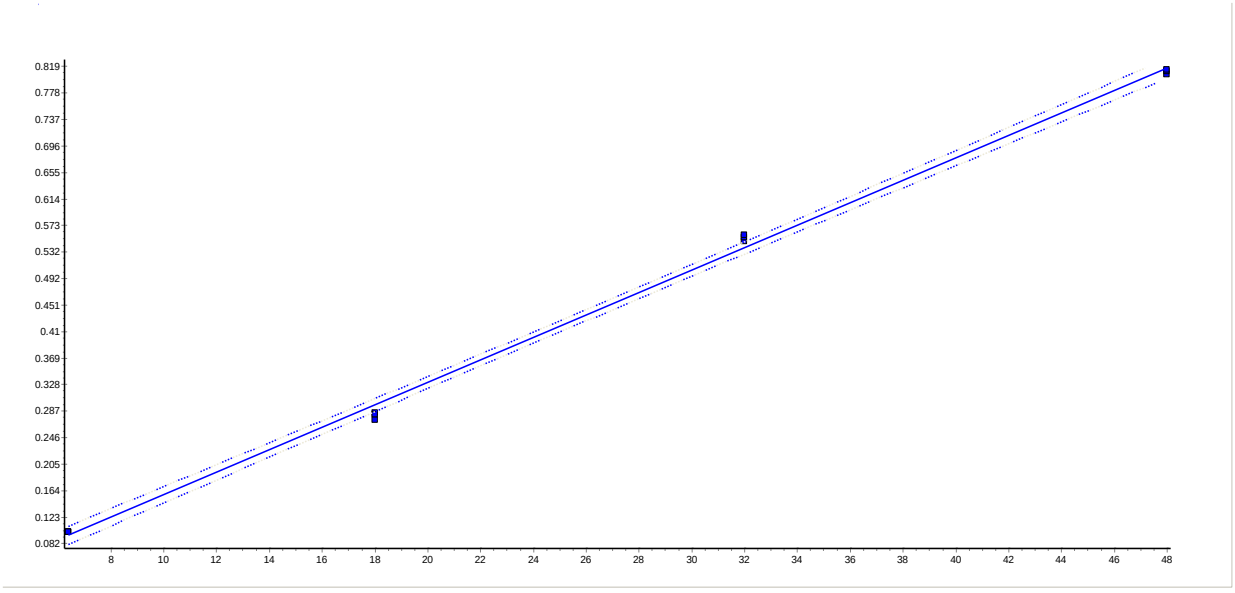


Figure 7.2: Calibration graph of Abacavir used in precision and accuracy testing, Day 2

Appendix 7.3: Precision and accuracy testing of HPLC method for Abacavir,

Day 3

Table 7.3: PRECISION AND ACCURACY DATA FOR ABACAVIR, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
19.20	21.15
19.20	18.62
19.20	19.87
	Average= 19.88

µg/ml (Spiked)	µg/ml (Extracted)
38.40	36.34
38.40	33.79
38.40	35.02
	Average= 35.05

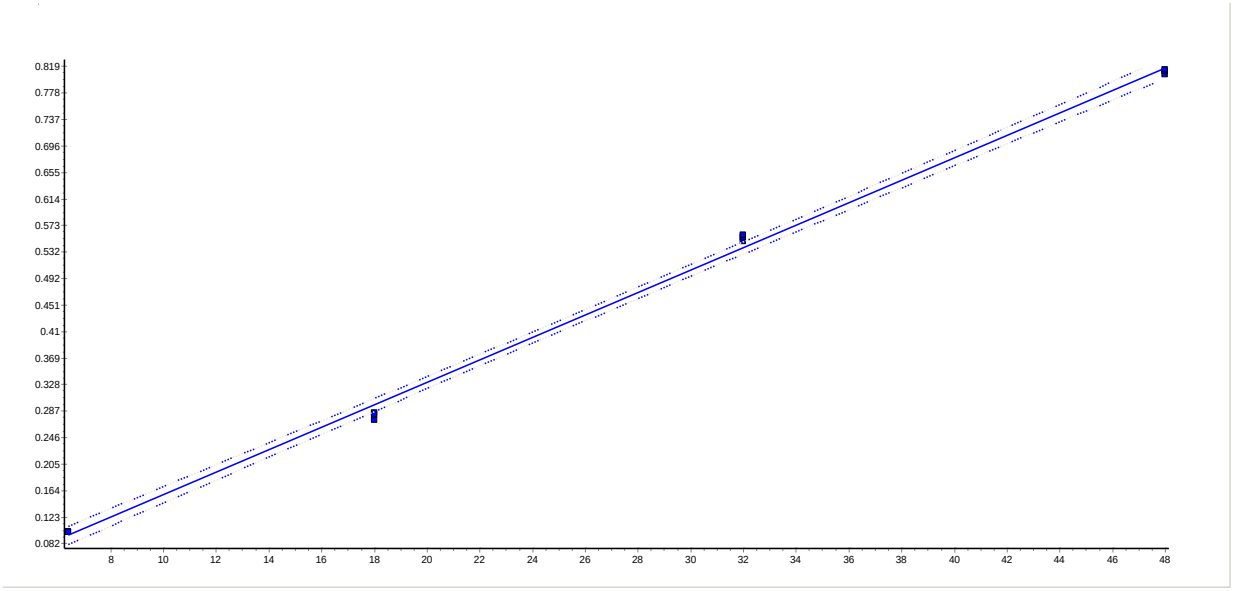


Figure 7.3: Calibration graph of Abacavir used in precision and accuracy testing, Day 3

Appendix 7.4: Precision and accuracy testing of HPLC method for Efavirenz,

Day 1

Table 7.4: PRECISION AND ACCURACY DATA FOR EFAVIRENZ, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
85.20	73.45
85.20	81.78
85.20	73.64
	Average= 76.29

µg/ml (Spiked)	µg/ml (Extracted)
170.40	196.25
170.40	191.94
170.40	193.51
	Average= 193.90

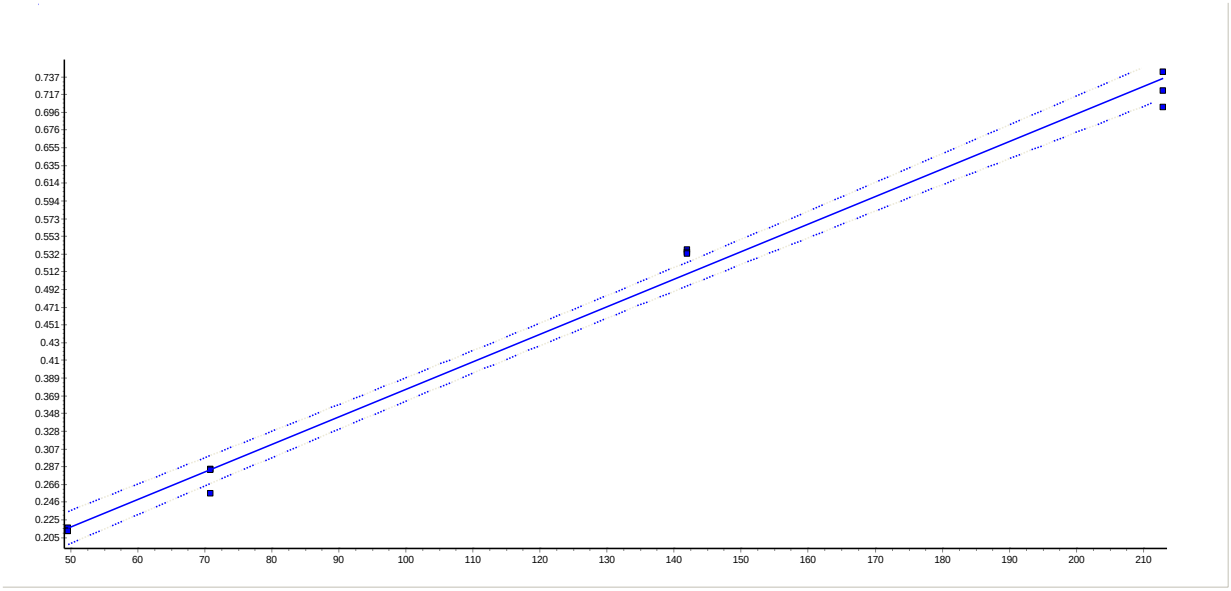


Figure 7.4: Calibration graph of Efavirenz used in precision and accuracy testing, Day 1

Appendix 7.5: Precision and accuracy testing of HPLC method for Efavirenz,

Day 2

Table 7.5: PRECISION AND ACCURACY DATA FOR EFAVIRENZ, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
85.20	73.00
85.20	68.24
85.20	74.40
	Average= 71.88

µg/ml (Spiked)	µg/ml (Extracted)
170.40	189.58
170.40	190.89
170.40	194.63
	Average= 191.70

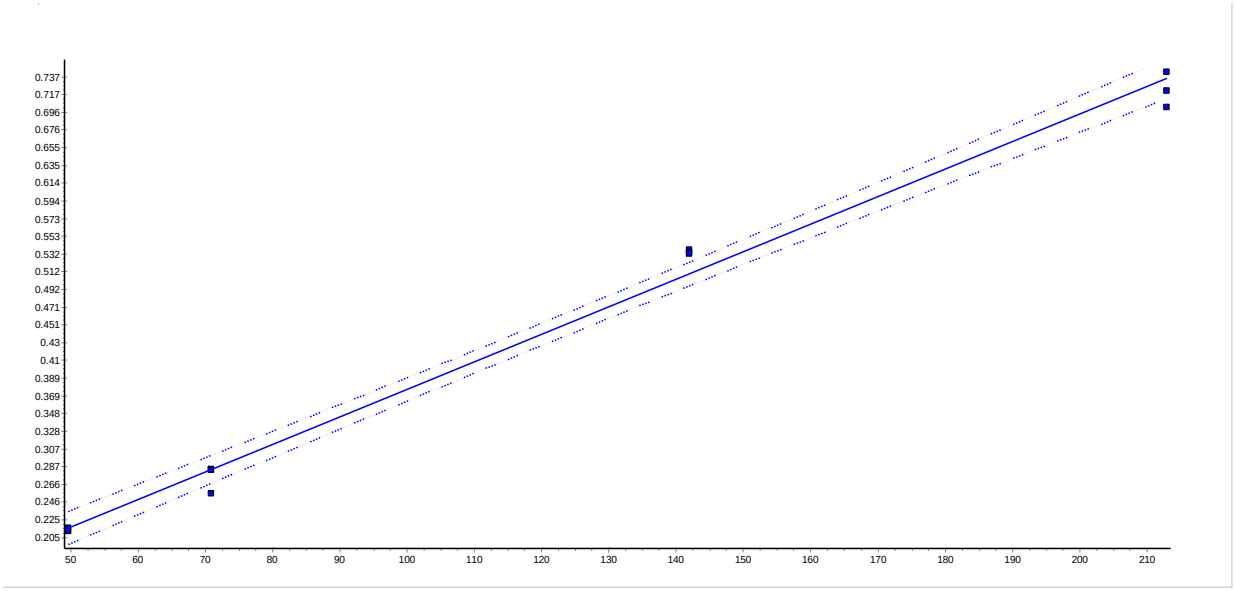


Figure 7.5: Calibration graph of Efavirenz used in precision and accuracy testing, Day 2

Appendix 7.6: Precision and accuracy testing of HPLC method for Efavirenz,

Day 3

Table 7.6: PRECISION AND ACCURACY DATA FOR EFAVIRENZ, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
85.20	71.84
85.20	71.93
85.20	78.50
	Average= 74.09

µg/ml (Spiked)	µg/ml (Extracted)
170.40	195.36
170.40	196.16
170.40	192.07
	Average= 194.53

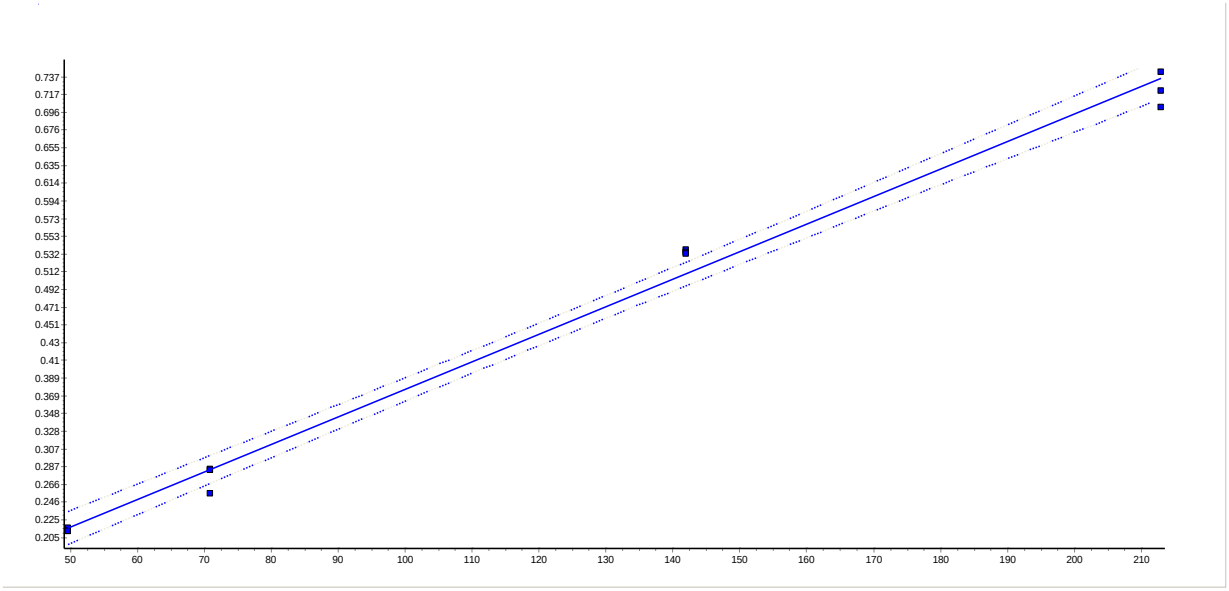


Figure 7.6: Calibration graph of Efavirenz used in precision and accuracy testing, Day 3

Appendix 7.7: Precision and accuracy testing of HPLC method for Lamivudine,

Day 1

Table 7.7: PRECISION AND ACCURACY DATA FOR LAMIVUDINE, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
15.17	13.34
15.17	11.88
15.17	13.51
	Average= 12.91

µg/ml (Spiked)	µg/ml (Extracted)
30.33	28.31
30.33	31.27
30.33	29.10
	Average= 29.56

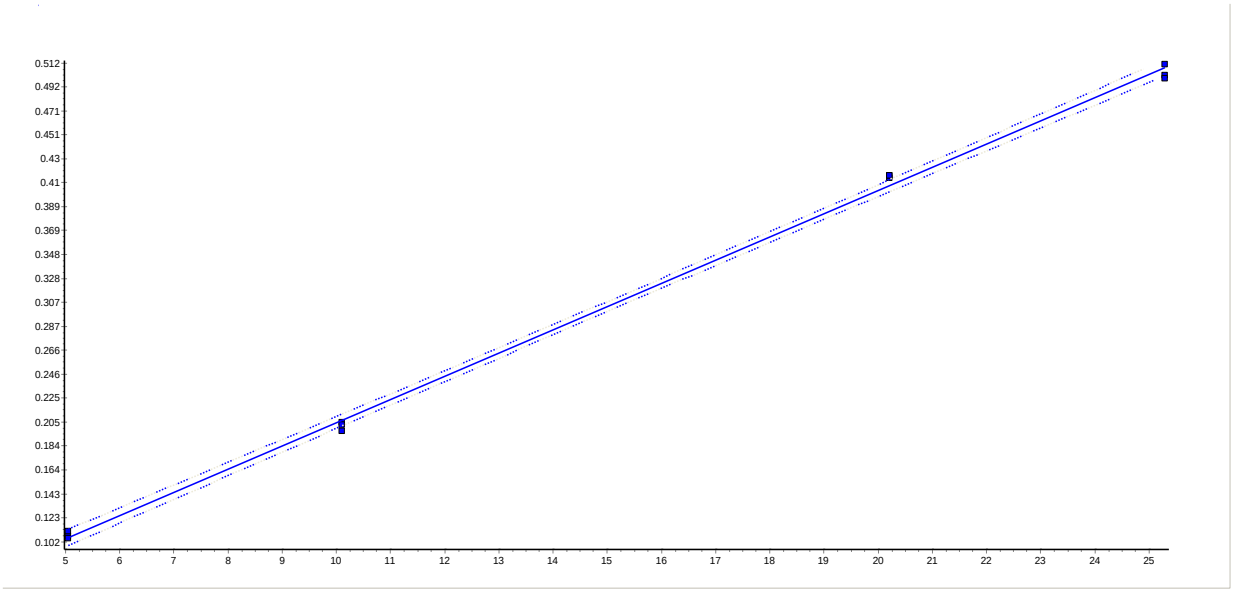


Figure 7.7: Calibration graph of Lamivudine used in precision and accuracy testing, Day 1

Appendix 7.8: Precision and accuracy testing of HPLC method for Lamivudine,

Day 2

Table 7.8: PRECISION AND ACCURACY DATA FOR LAMIVUDINE, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
15.17	12.64
15.17	12.04
15.17	14.05
	Average=12.91

µg/ml (Spiked)	µg/ml (Extracted)
30.33	28.19
30.33	26.03
30.33	22.52
	Average= 25.58

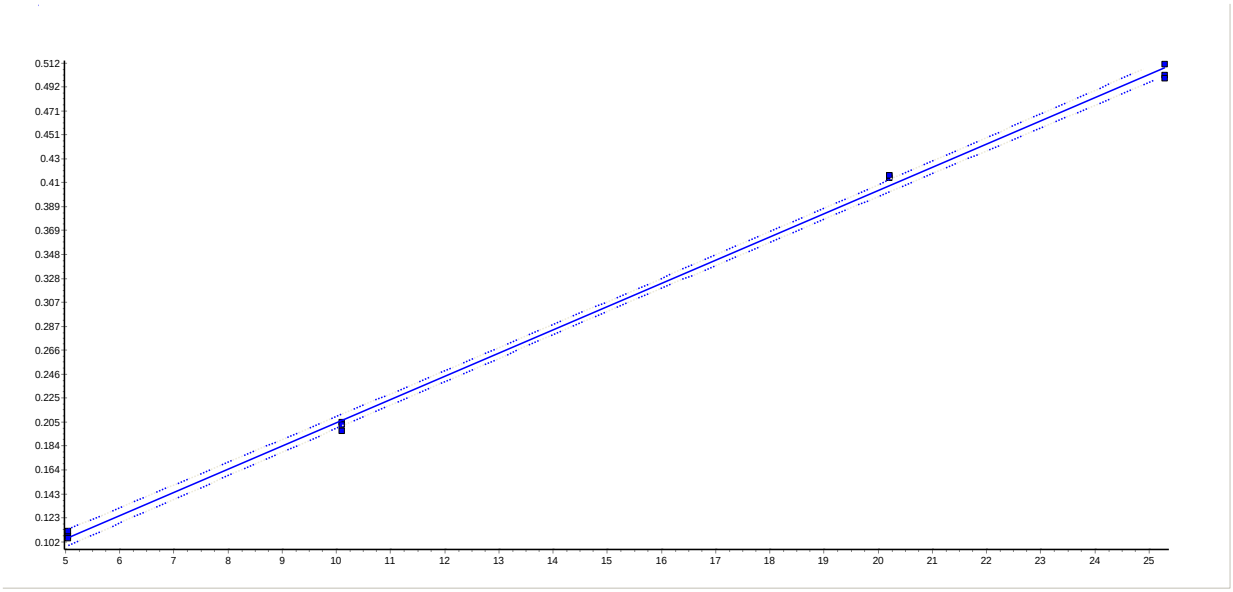


Figure 7.8: Calibration graph of Lamivudine used in precision and accuracy testing, Day 2

Appendix 7.9: Precision and accuracy testing of HPLC method for Lamivudine,

Day 3

Table 7.9: PRECISION AND ACCURACY DATA FOR LAMIVUDINE, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
15.17	15.01
15.17	13.34
15.17	11.04
	Average= 13.13

µg/ml (Spiked)	µg/ml (Extracted)
30.33	26.59
30.33	22.96
30.33	23.41
	Average= 24.32

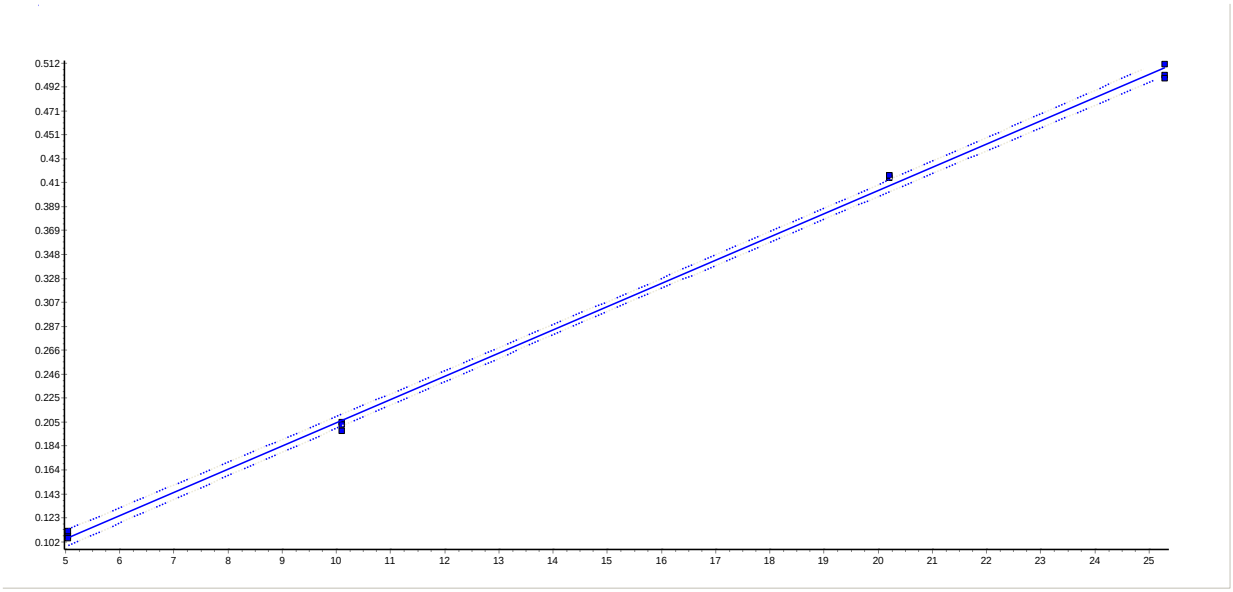


Figure 7.9: Calibration graph of Lamivudine used in precision and accuracy testing, Day 3

Appendix 7.10: Precision and accuracy testing of HPLC method for Lopinavir,

Day 1

Table 7.10: PRECISION AND ACCURACY DATA FOR LOPINAVIR, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
19.80	14.12
19.80	12.87
19.80	13.60
	Average= 13.53

µg/ml (Spiked)	µg/ml (Extracted)
44.55	43.52
44.55	40.27
44.55	42.45
	Average= 42.08

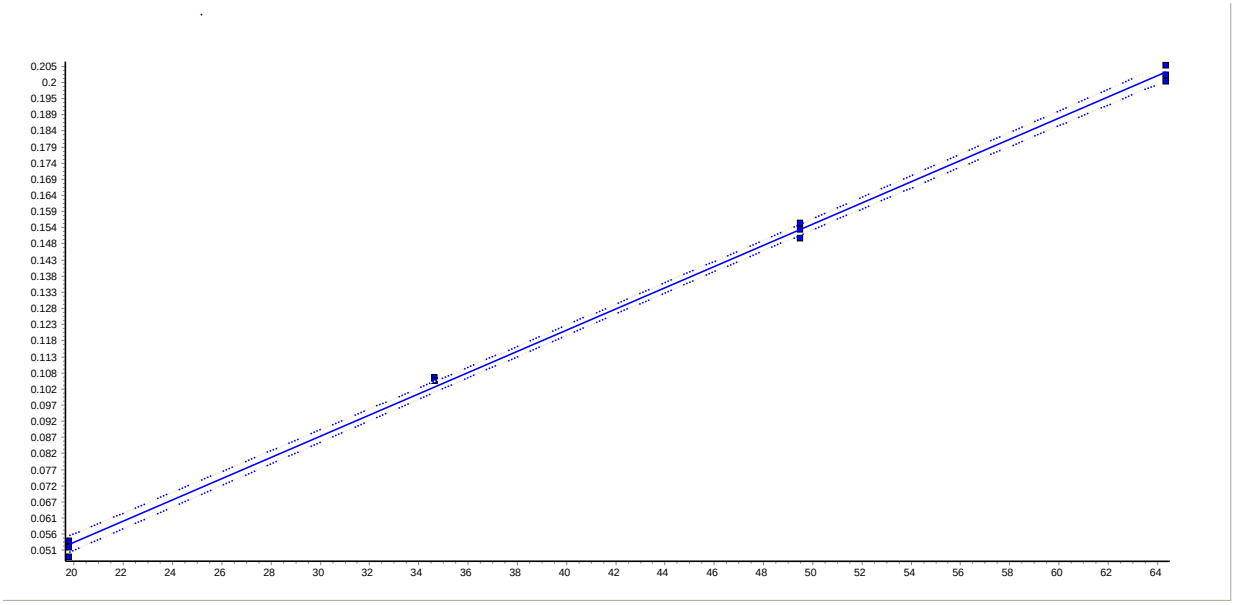


Figure 7.10: Calibration graph of Lopinavir used in precision and accuracy testing, Day 1

Appendix 7.11: Precision and accuracy testing of HPLC method for Lopinavir,

Day 2

Table 7.11: PRECISION AND ACCURACY DATA FOR LOPINAVIR, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
19.80	15.30
19.80	16.92
19.80	16.83
	Average= 16.20

µg/ml (Spiked)	µg/ml (Extracted)
44.55	37.44
44.55	38.90
44.55	40.96
	Average= 39.10

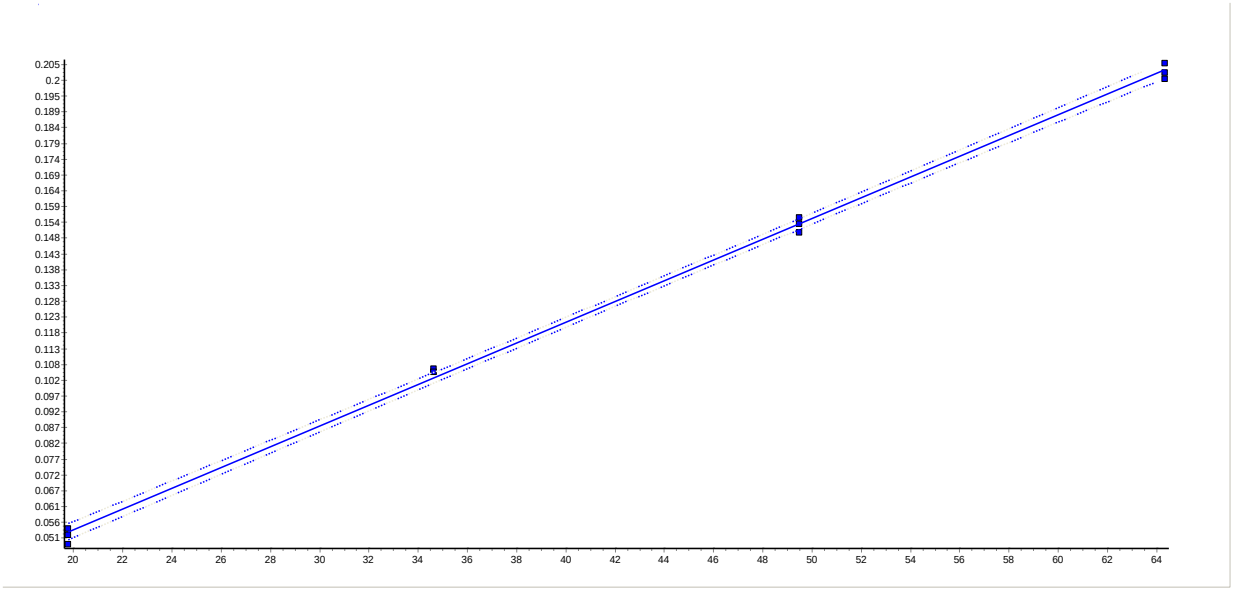


Figure 7.11: Calibration graph of Lopinavir used in precision and accuracy testing, Day 2

Appendix 7.12: Precision and accuracy testing of HPLC method for Lopinavir,

Day 3

Table 7.12: PRECISION AND ACCURACY DATA FOR LOPINAVIR, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
19.80	12.68
19.80	15.48
19.80	15.10
	Average= 14.42

µg/ml (Spiked)	µg/ml (Extracted)
44.55	37.68
44.55	40.21
44.55	35.84
	Average= 37.91

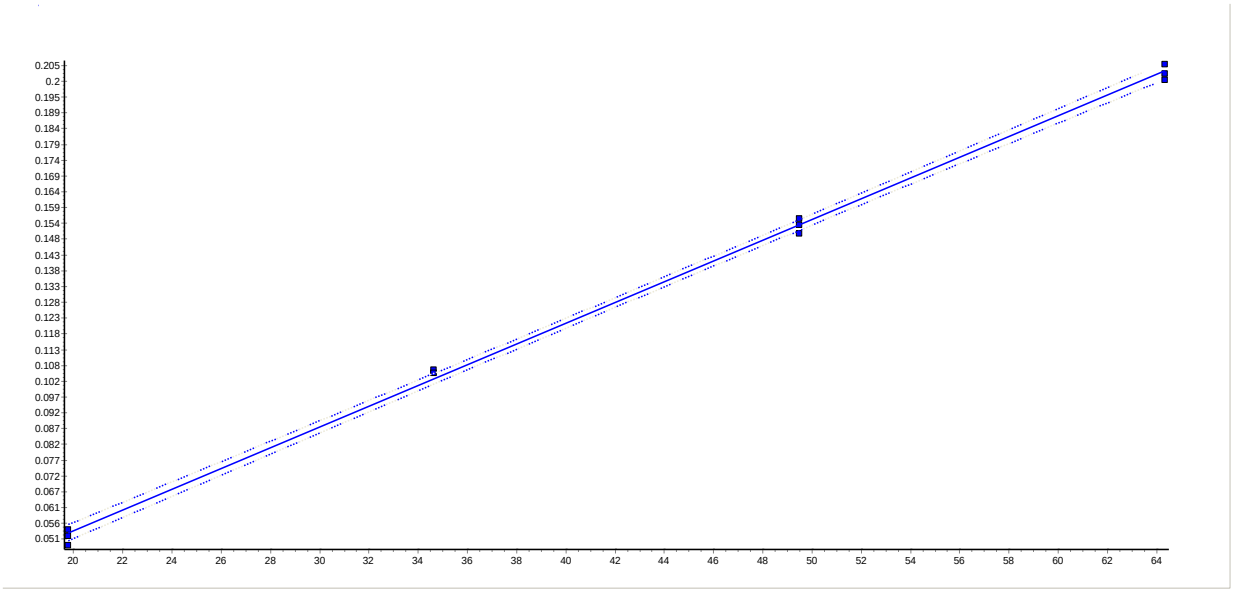


Figure 7.12: Calibration graph of Lopinavir used in precision and accuracy testing, Day 3

Appendix 7.13: Precision and accuracy testing of HPLC method for Nevirapine, Day 1

Table 7.13: PRECISION AND ACCURACY DATA FOR NEVIRAPINE, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
31.60	30.79
31.60	29.87
31.60	31.20
	Average= 30.62

µg/ml (Spiked)	µg/ml (Extracted)
63.20	65.32
63.20	61.56
63.20	65.72
	Average= 64.20

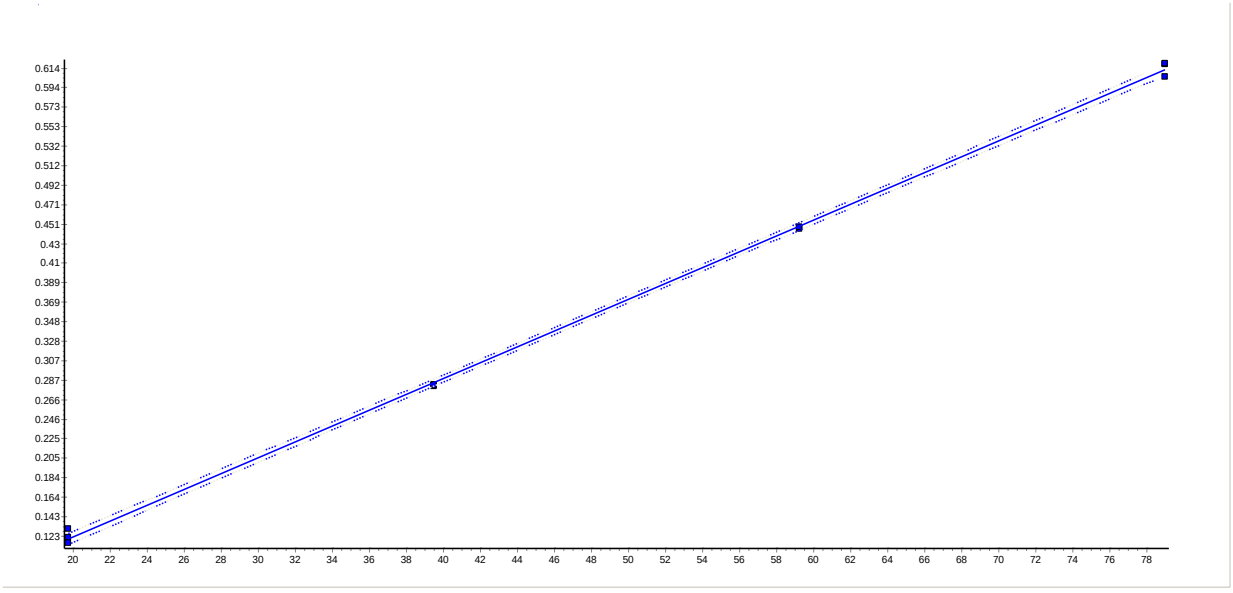


Figure 7.13: Calibration graph of Nevirapine used in precision and accuracy testing, Day 1

Appendix 7.14: Precision and accuracy testing of HPLC method for Nevirapine, Day 2

Table 7.14: PRECISION AND ACCURACY DATA FOR NEVIRAPINE, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
31.60	32.04
31.60	30.84
31.60	32.25
	Average= 31.71

µg/ml (Spiked)	µg/ml (Extracted)
63.20	64.30
63.20	67.81
63.20	67.69
	Average= 66.60

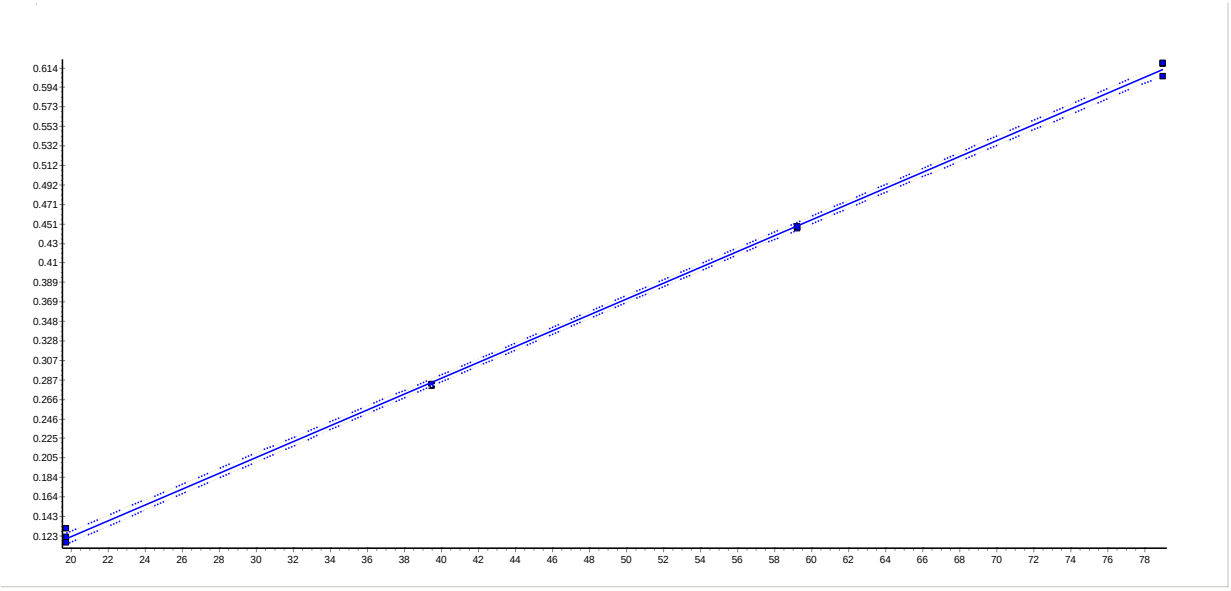


Figure 7.14: Calibration graph of Nevirapine used in precision and accuracy testing, Day 2

Appendix 7.15: Precision and accuracy testing of HPLC method for Nevirapine, Day 3

Table 7.15: PRECISION AND ACCURACY DATA FOR NEVIRAPINE, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
31.60	28.31
31.60	32.55
31.60	29.20
	Average= 30.02

µg/ml (Spiked)	µg/ml (Extracted)
63.20	63.40
63.20	67.62
63.20	66.98
	Average= 66.00

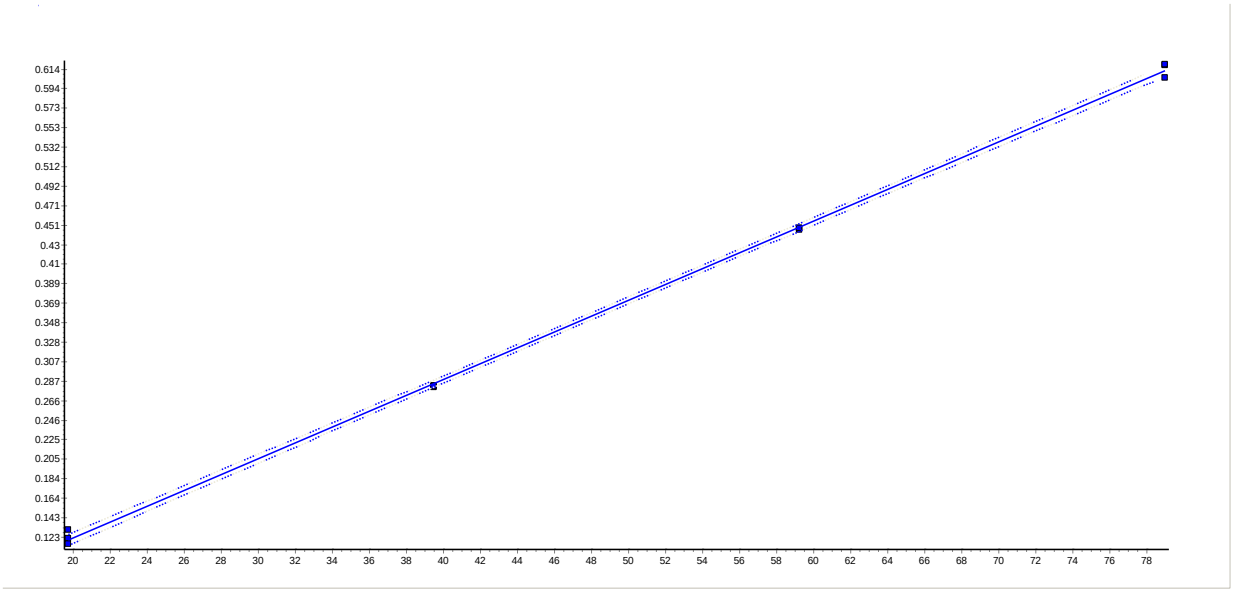


Figure 7.15: Calibration graph of Nevirapine used in precision and accuracy testing, Day 3

Appendix 7.16: Precision and accuracy testing of HPLC method for Stavudine,

Day 1

Table 7.16: PRECISION AND ACCURACY DATA FOR STAVUDINE, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
11.24	7.94
11.24	7.38
11.24	8.11
	Average= 7.81

µg/ml (Spiked)	µg/ml (Extracted)
16.86	13.13
16.86	13.64
16.86	12.08
	Average= 12.95

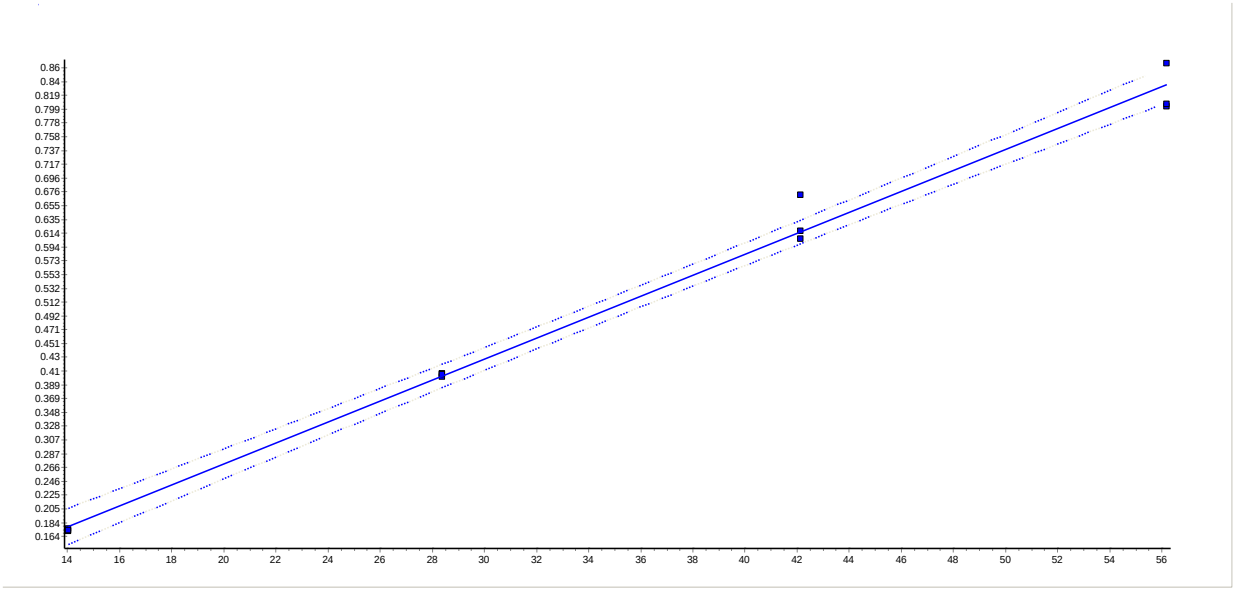


Figure 7.16: Calibration graph of Stavudine used in precision and accuracy testing, Day 1

Appendix 7.17: Precision and accuracy testing of HPLC method for Stavudine,

Day 2

Table 7.17: PRECISION AND ACCURACY DATA FOR STAVUDINE, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
11.24	7.62
11.24	8.02
11.24	7.01
	Average= 7.55

µg/ml (Spiked)	µg/ml (Extracted)
16.86	13.97
16.86	12.53
16.86	14.27
	Average= 13.59

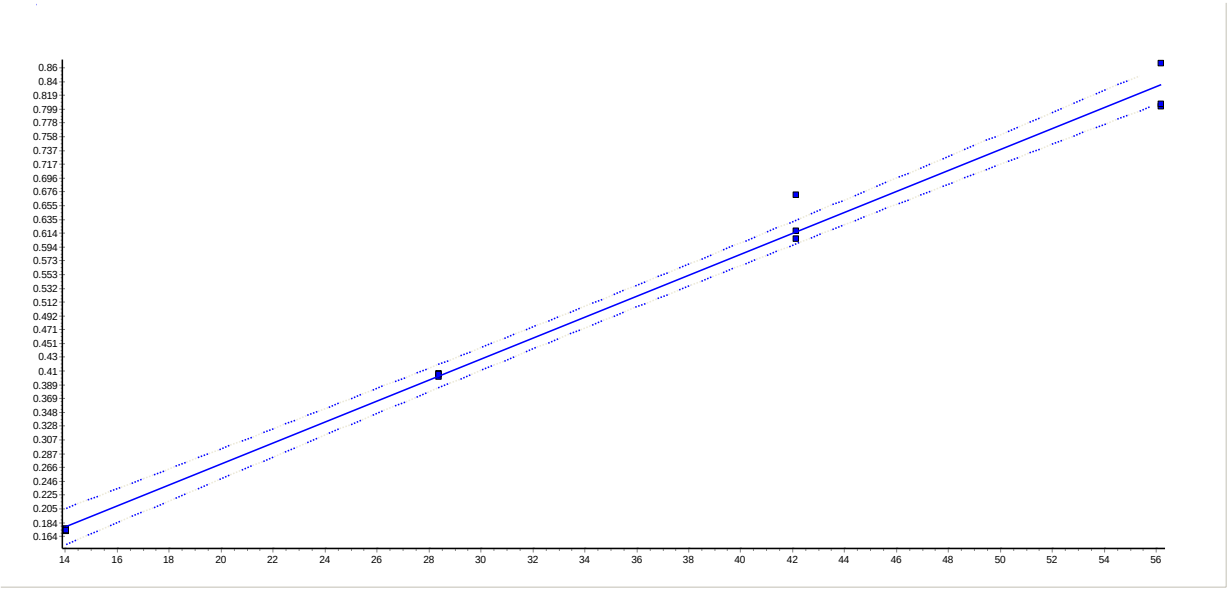


Figure 7.17: Calibration graph of Stavudine used in precision and accuracy testing, Day 2

Appendix 7.18: Precision and accuracy testing of HPLC method for Stavudine, Day 3

Table 7.18: PRECISION AND ACCURACY DATA FOR STAVUDINE, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
11.24	9.23
11.24	7.95
11.24	6.82
	Average=8.00

µg/ml (Spiked)	µg/ml (Extracted)
16.86	13.82
16.86	13.35
16.86	12.43
	Average= 13.20

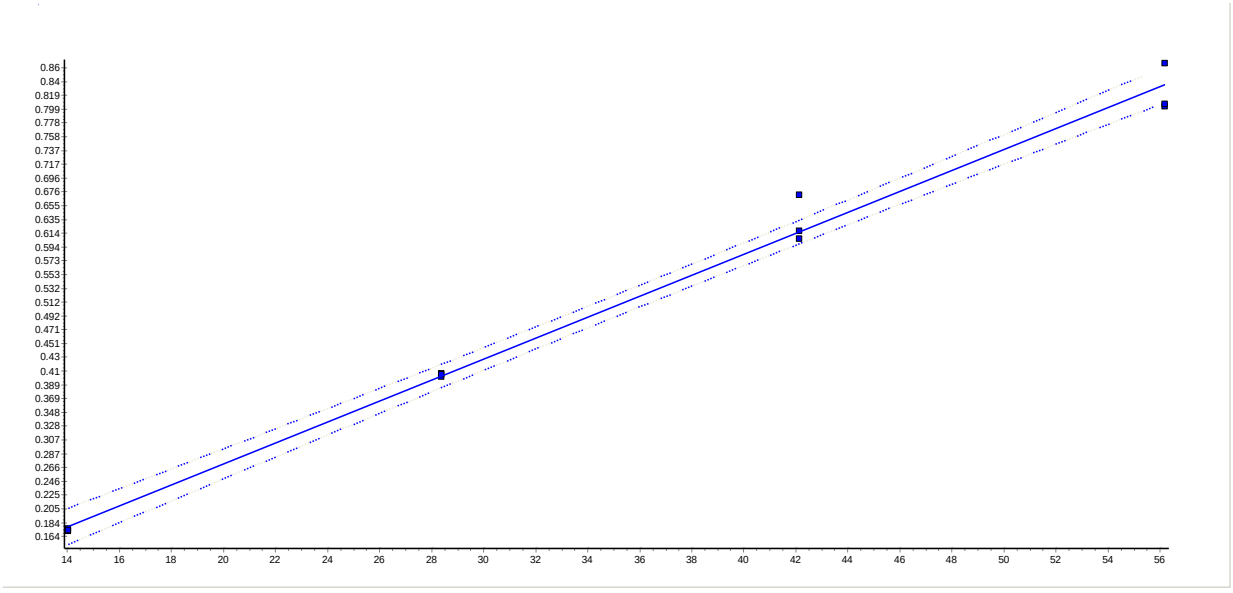


Figure 7.18: Calibration graph of Stavudine used in precision and accuracy testing, Day 3

Appendix 7.19: Precision and accuracy testing of HPLC method for Tenofovir,

Day 1

Table 7.19: PRECISION AND ACCURACY DATA FOR TENOFOVIR, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
37.20	33.34
37.20	37.15
37.20	34.21
	Average=34.90

µg/ml (Spiked)	µg/ml (Extracted)
83.70	77.15
83.70	75.81
83.70	78.04
	Average= 77.00

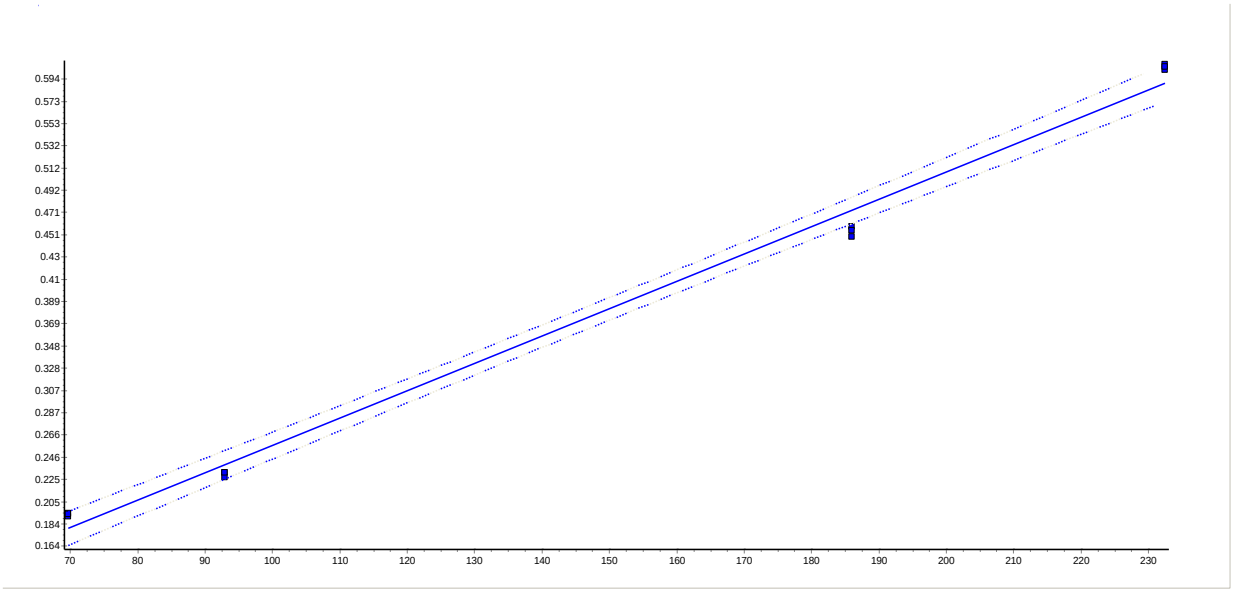


Figure 7.19: Calibration graph of Tenofovir used in precision and accuracy testing, Day 1

Appendix 7.20: Precision and accuracy testing of HPLC method for Tenofovir, Day 2

Table 7.20: PRECISION AND ACCURACY DATA FOR TENOFOVIR, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
37.20	46.11
37.20	45.91
37.20	42.50
	Average= 44.84

µg/ml (Spiked)	µg/ml (Extracted)
83.70	84.32
83.70	84.98
83.70	91.46
	Average= 86.92

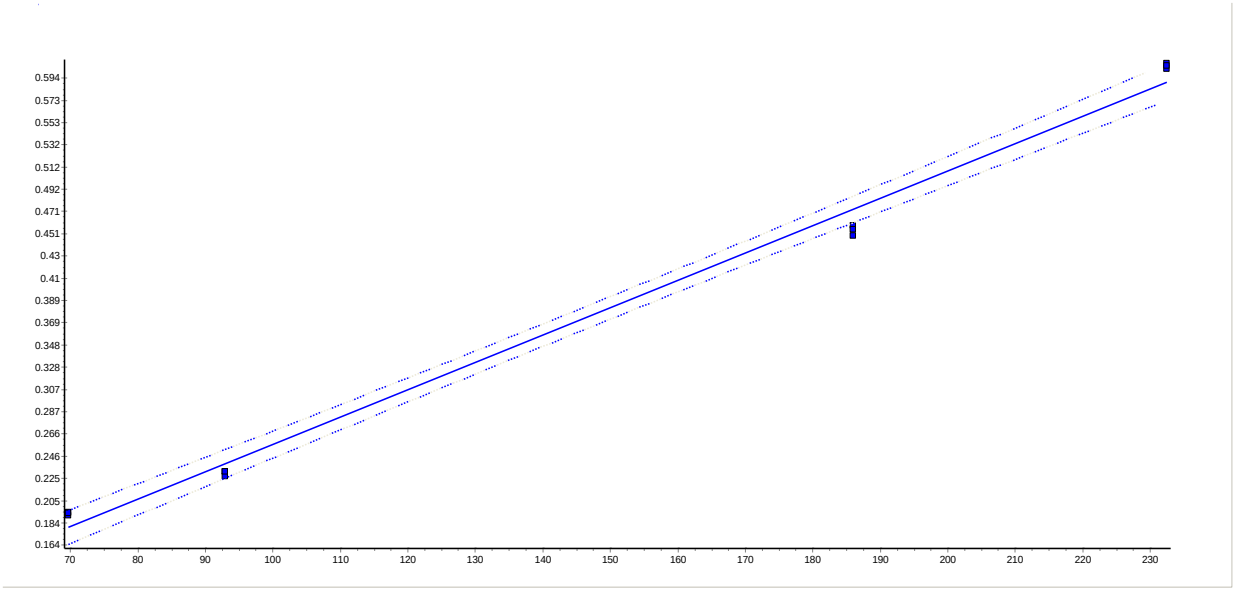


Figure 7.20: Calibration graph of Tenofovir used in precision and accuracy testing, Day 2

Appendix 7.21: Precision and accuracy testing of HPLC method for Tenofovir, Day 3

Table 7.21: PRECISION AND ACCURACY DATA FOR TENOFOVIR, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
37.20	50.29
37.20	49.95
37.20	47.36
	Average= 49.20

µg/ml (Spiked)	µg/ml (Extracted)
83.70	83.88
83.70	82.78
83.70	82.19
	Average= 82.95

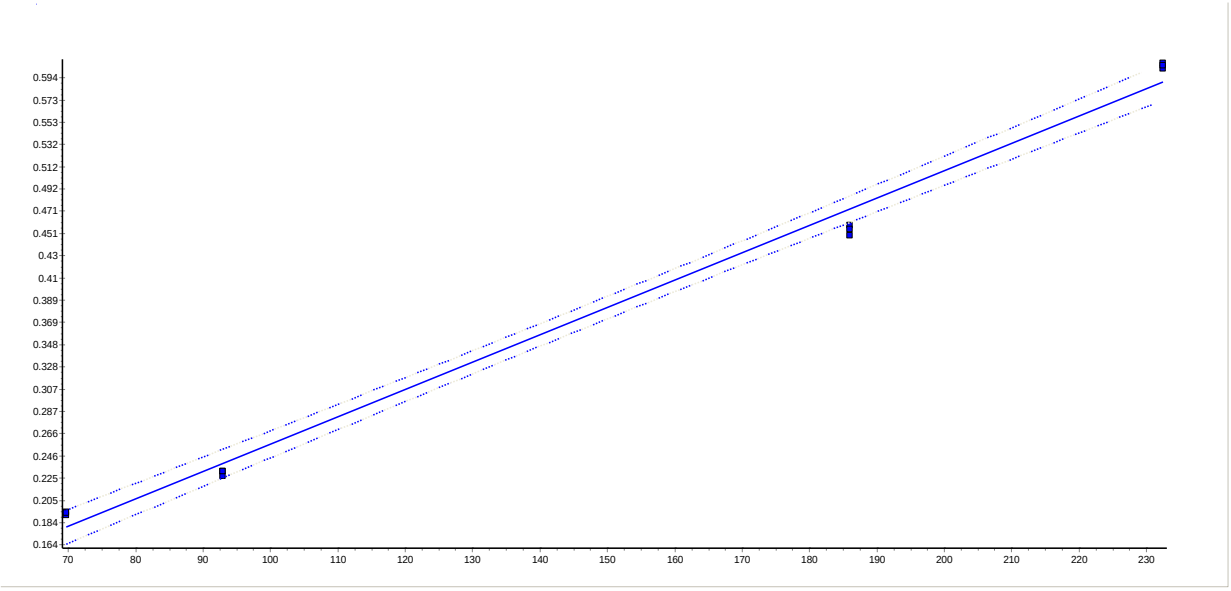


Figure 7.21: Calibration graph of Tenofovir used in precision and accuracy testing, Day 3

Appendix 7.22: Precision and accuracy testing of HPLC method for Zidovudine, Day 1

Table 7.22: PRECISION AND ACCURACY DATA FOR ZIDOVUDINE, DAY 1

µg/ml (Spiked)	µg/ml (Extracted)
31.20	29.87
31.20	29.10
31.20	29.50
	Average= 29.49

µg/ml (Spiked)	µg/ml (Extracted)
72.80	62.97
72.80	67.42
72.80	66.62
	Average=65.67

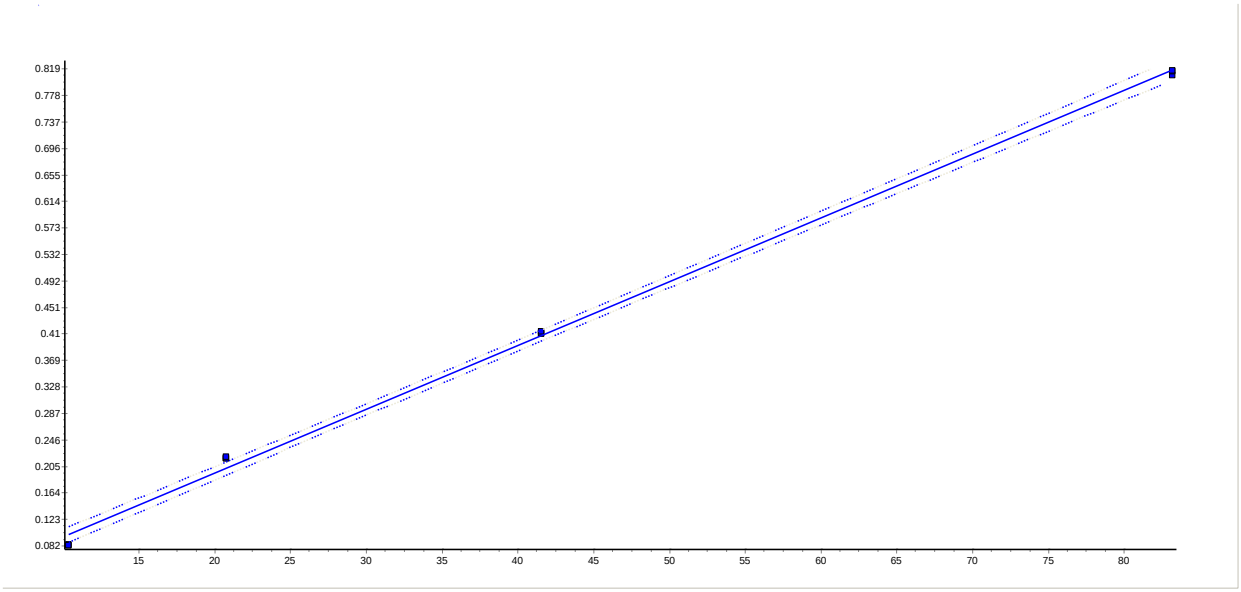


Figure 7.22: Calibration graph of Zidovudine used in precision and accuracy testing, Day 1

Appendix 7.23: Precision and accuracy testing of HPLC method for Zidovudine, Day 2

Table 7.23: PRECISION AND ACCURACY DATA FOR ZIDOVUDINE, DAY 2

µg/ml (Spiked)	µg/ml (Extracted)
31.20	29.10
31.20	28.95
31.20	27.99
	Average= 28.68

µg/ml (Spiked)	µg/ml (Extracted)
72.80	66.41
72.80	64.18
72.80	66.12
	Average=65.57

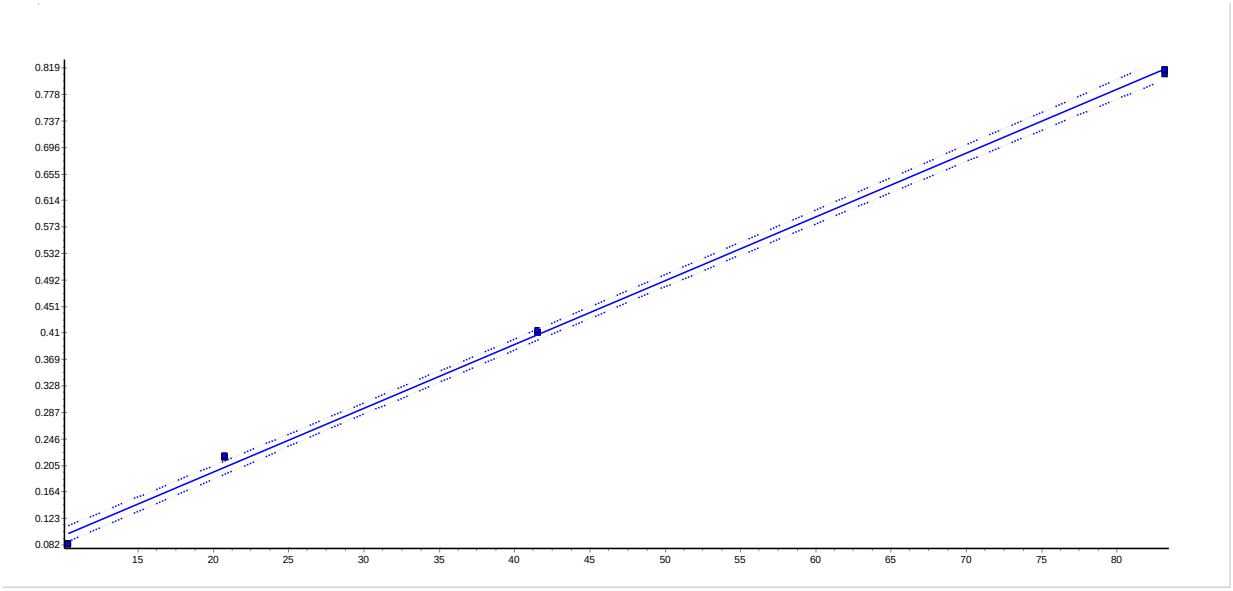


Figure 7.23: Calibration graph of Zidovudine used in precision and accuracy testing, Day 2

Appendix 7.24: Precision and accuracy testing of HPLC method for Zidovudine, Day 3

Table 7.24: PRECISION AND ACCURACY DATA FOR ZIDOVUDINE, DAY 3

µg/ml (Spiked)	µg/ml (Extracted)
31.20	30.72
31.20	29.01
31.20	31.47
	Average= 30.40

µg/ml (Spiked)	µg/ml (Extracted)
72.80	67.24
72.80	66.21
72.80	69.02
	Average= 67.49

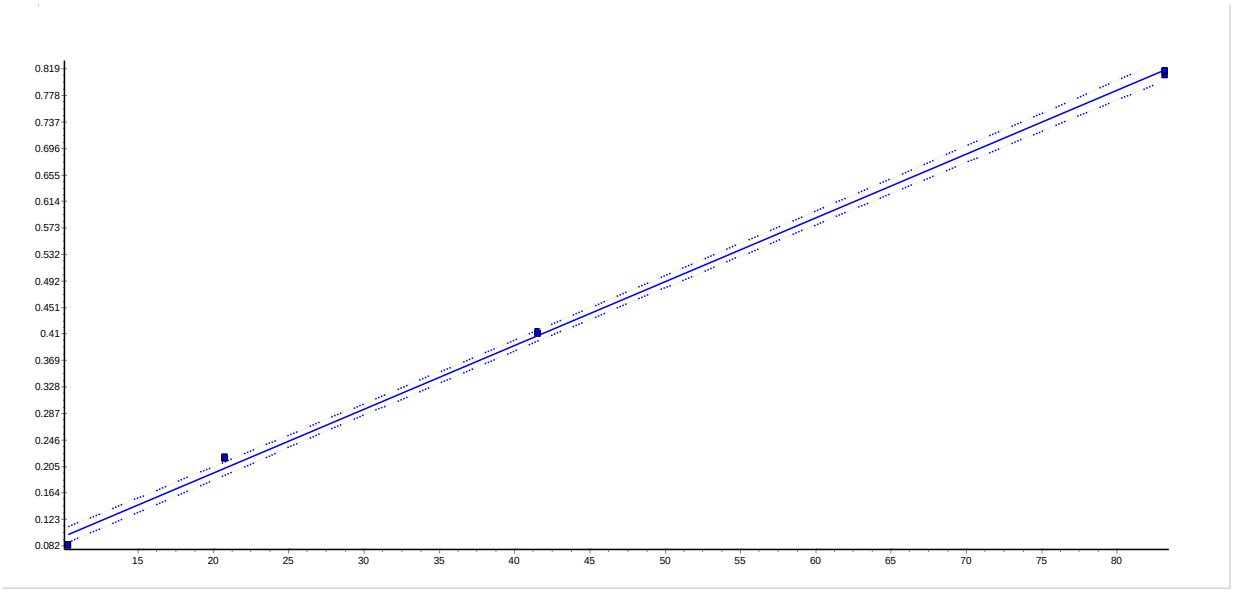
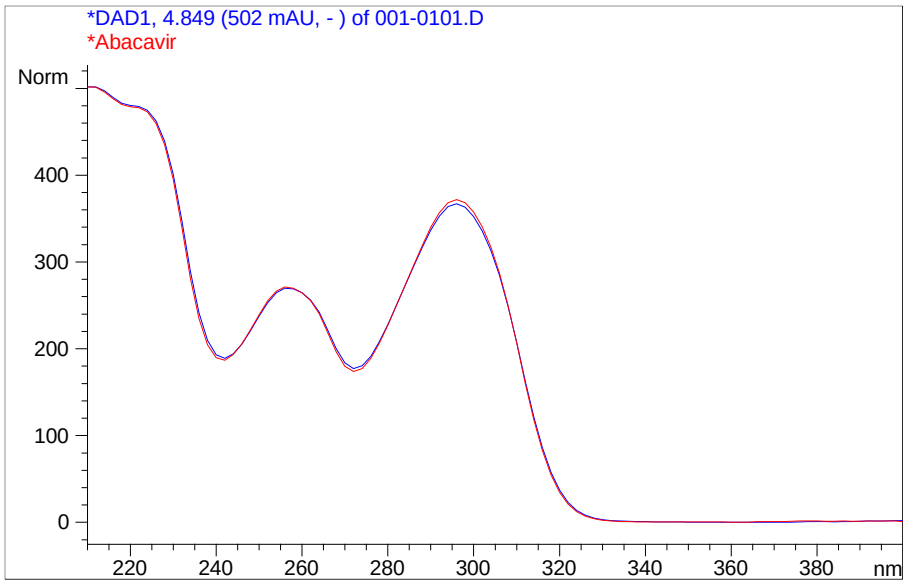
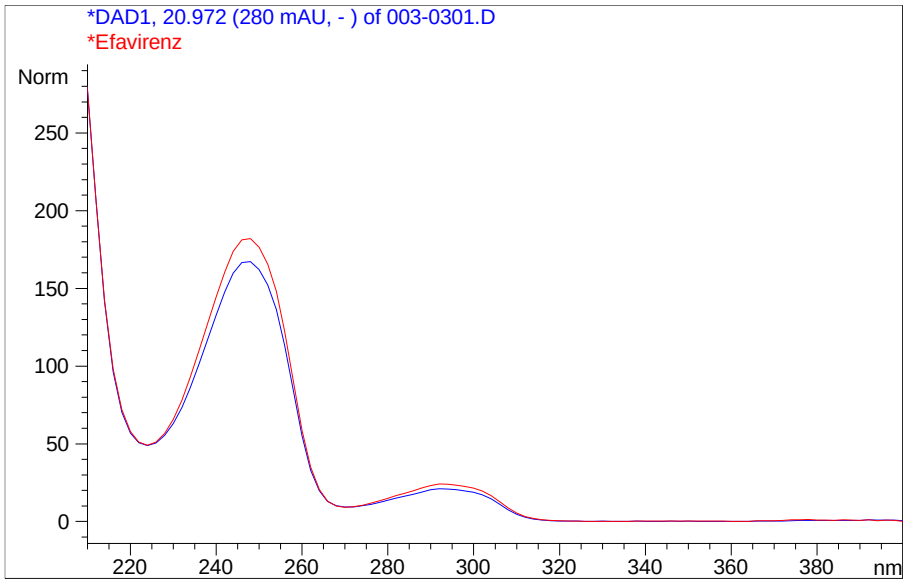


Figure 7.24: Calibration graph of Zidovudine used in precision and accuracy testing, Day 3

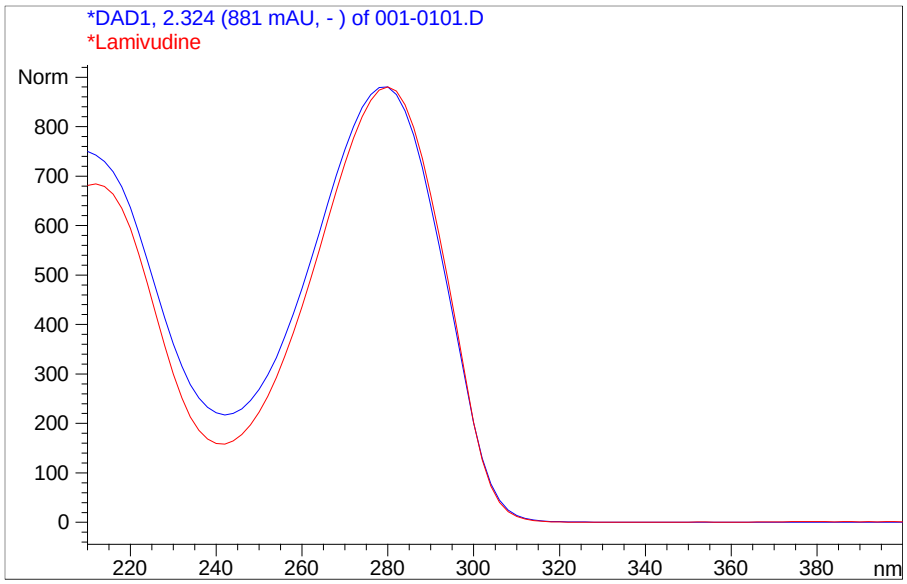
Appendix 7.25: UV spectrum of Abacavir



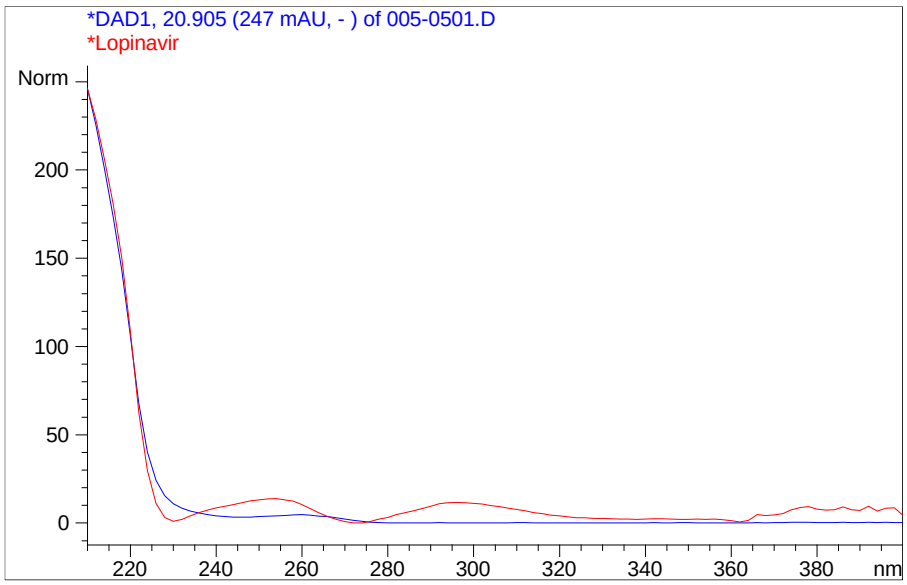
Appendix 7.26: UV spectrum of Efavirenz



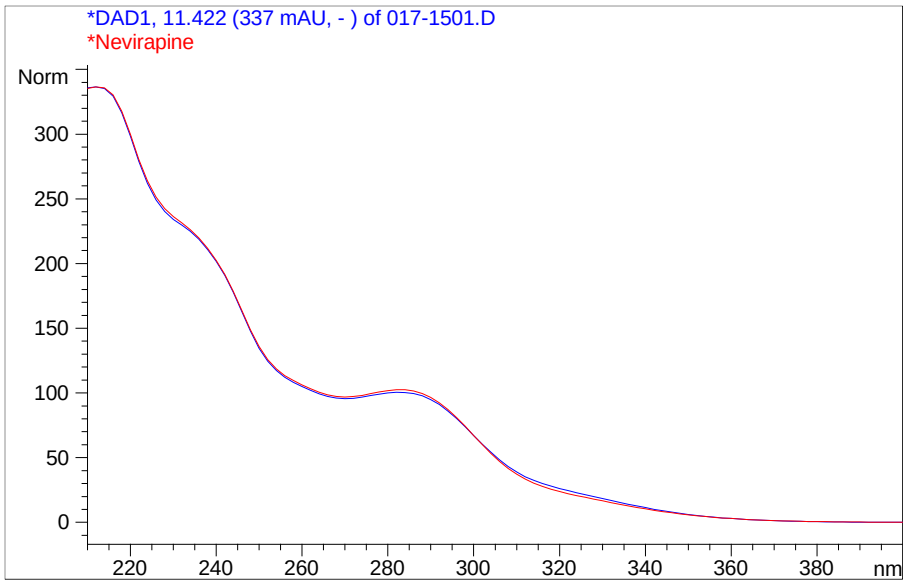
Appendix 7.27: UV spectrum of Lamivudine



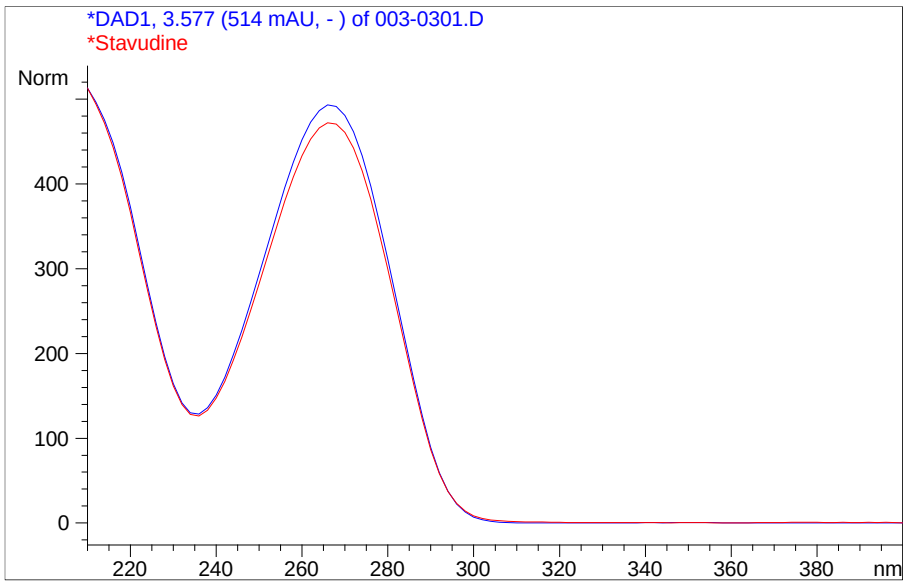
Appendix 7.28: UV spectrum of Lopinavir



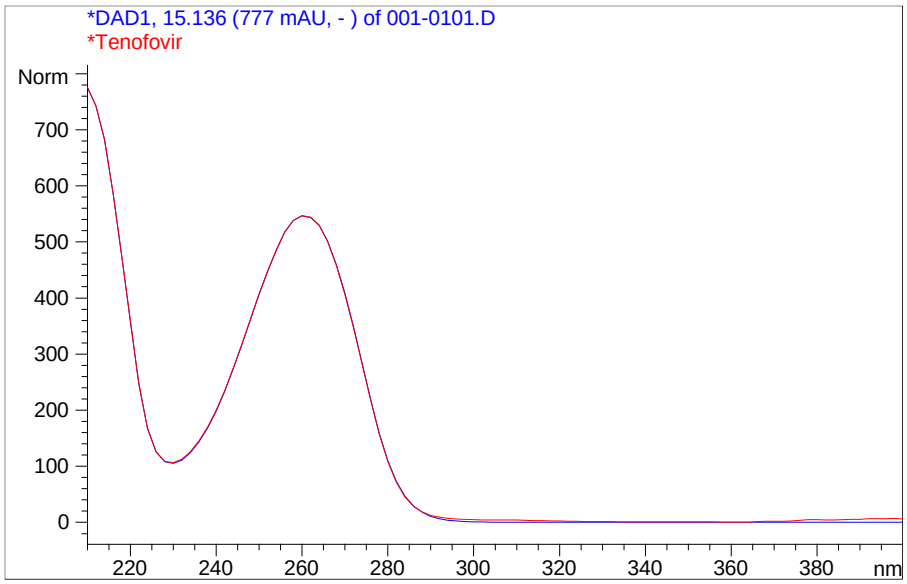
Appendix 7.29: UV spectrum of Nevirapine



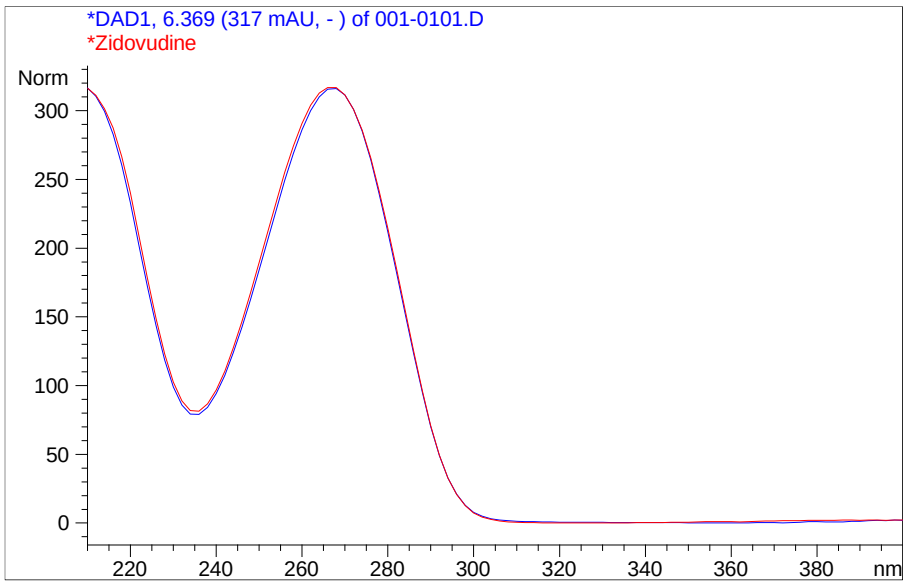
Appendix 7.30: UV spectrum of Stavudine



Appendix 7.31: UV spectrum of Tenofovir



Appendix 7.32: UV spectrum of Zidovudine



Appendix 7.33: Example of consent form

CONSENT TO PARTICIPATE IN RESEARCH

You have been asked to participate in a research study regarding the development of an HPLC method for the detection of antiretroviral drugs and their metabolites in urine.

You have been informed about the study by your attending clinician.

You may contact Margerite Potgieter at 072 384 3123 any time if you have questions about the research or if you are injured as a result of the research.

You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

_____ _____

Signature of Participant Date

_____ _____

Signature of Witness Date

(Where applicable)

_____ _____

Signature of Translator Date

(Where applicable)

Appendix 7.34: Example of information document

INFORMATION DOCUMENT

Antiretroviral drug detection in urine using high performance liquid chromatography

A research study is being undertaken by Margerite Potgieter in collaboration with the Departments of Pharmacology and Internal medicine, University of the Free State. The study aims to develop a High Performance Liquid Chromatography (HPLC) method for the identification and determination of ARV drugs and their metabolites in urine.

We are inviting you to participate in a research study by donating urine samples. If you are HIV positive and undergoing ARV treatment, you qualify for the study. A urine sample of at least 10ml will initially be collected, urine will be screened for any drugs and medication, and participants will be required to provide further urine samples for four consecutive weeks after initial donation. Biological samples collected will be stored for the duration of the study. Only twenty participants who adhere to the inclusion criteria will be selected.

There are no risks associated with the donation of urine samples. While involved in the project and after the results are available, the subject will be given relevant information about the study. Every effort will be made to keep personal information confidential, although absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Participation is voluntary, and refusal to participate will not involve a penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty. If results are published, this may lead to individual/cohort identification.

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