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Development and validation of anti-retroviral drug levels for therapeutic drug monitoring using dried blood spots

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Development and validation of anti-retroviral drug levels for therapeutic drug monitoring using dried blood spots

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A dissertation submitted to the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine in Chemical Pathology – Johannesburg, Parktown 2020

Declaration

I, Simon Modiba (Student number: 1790096) declare that this Dissertation is my own, unaided work, unless otherwise specified. It is being submitted for the degree of Master of Science in medicine the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university

Signature:  Date: 02-11-2021

Abstract

Background: Plasma concentrations of antiretroviral (ARV) drugs are frequently used for therapeutic drug monitoring of antiretroviral drugs. Dried blood spot (DBS) and dried plasma spots (DBS) sampling offers a patient-friendly and easy alternative to plasma sampling. Therefore the aim was to develop and validate a method for the measurement of ARVs in DBS and DPS from whole blood spotted onto filter paper and onto plasma separation cards (PSC) using ultra-pressure liquid chromatography in tandem with mass spectrometry (UHPLC-MS/MS).

Method: DBS and DPS were prepared by spotting whole blood onto the collection cards and PSC respectively. The cards were extracted and subsequently analysed using UHPLC-MS/MS. The method for measurement of ARVs from DBS and DPS was validated by determining linearity, accuracy, precision, recovery and limit of detection (LOD), limit of quantification (LOQ) and stability for each of the drugs. ARV Drugs in DBS (Lamivudine (3TC), Abacavir (ABC), Efavirenz (EFV), Ritonavir (RTV) and Lopinavir (LPV)) ARVs were compared plasma concentrations. Effect of Haematocrit on DBS was also investigated. ARVs in DPS (3TC, ABC, EFV, RTV, LPV, Nevirapine (NVP), Emtracitabine (FTC), Raltegravir (RAL), Atazanivir (ATV), and Darunavir (TMC114)) were compared for plasma and DPS.

Results: The assay had an acceptable linear regression over the concentration ranges tested in DBS for all the analytes, while in DPS it was acceptable for ranges tested for all the analytes except for 3TC, NVP, ATV. Accuracy was within acceptable deviation of 15% for all the analytes in both DBS and DPS. Inter- and intra-day precision gave a coefficient of variation that was within acceptable deviation of 15%, except for 3TC and LPV, while in DPS coefficient of variation was within acceptable deviation of 15%, except for EFV, RTV, LPV, ATV and TMC114. All drugs had acceptable recoveries in both DBS and DPS. In DBS, The LOD ranged from 0.006 µg/ml for RTV to 0.110 µg/ml for LPV and the LOQ ranged from 0.021 µg/ml for RTV to 0.367 µg/ml for LPV. In DPS the LOD ranged from 0.002 µg/ml for 3TC to 0.423 µg/ml for LPV and the LOQ ranged from 0.008 µg/ml for 3TC to 1.409 µg/ml for LPV. All the drugs in DBS were stable at bench top for 30days, while in DPS only ABC and RTV were stable at bench-top at day 7. Correction for haematocrit in patients with low haematocrit improved the agreement between plasma and DBS.

Conclusion: A method for the measurement of several ARVs from DBS and DPS was developed and validated. Accurate quantification of drugs in DBS was not satisfactory. However, the results suggest that DBS can be used to determine patient's adherence.

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Abbreviations

°C	Degree Celsius
µg/ml	Micrograms per milliliter
µL	Microlitre
3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immunodeficiency Dyndrome
AC	Assigned concentration
ALT	Alanine transaminase
ART	Antiretroviral therapy
ARV	Antiretroviral
AZT	Zidovudine
BMI	Body mass index
Conc	Concentration
CV	Coefficient of variation
DBS	Dried Blood Spots
dH_2O	Deionized water
DPS	Dried plasma spots
EFV	Efavirenz
et al	And others
Fbpp	Fraction of drug bound to plasma protein
FTC	Emtracitabine
HCT	Haematocrit
HQC	High quality control
HIV	Human immunodeficiency virus
InSTI	Integrase strand transfer inhibitor
IQR	Interquartile range
Kg/m^2	Kilograms is divided by height in meters squared
MC	Measured concentration
MRM	Multiple reaction monitoring
M/Z	Mass divided by charge number
LC-MS/MS	Liquid chromatography in tandem with mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Low quality control
NA	Not applicable
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PI	Protease inhibitor
PSC	Plasma separation card

QC	Quality Control
R^2	Correlation coefficient
RAL	Raltegravir
RTV	Ritonivir
SD	Standard deviation
S/N	Signal to noise ratio
STD	Standard
TDF	Tenofovir disoproxil fumarate
TDM	Therapeutic drug monitoring
TMC114	Darunavir
UHPLC	Ultra high-performance liquid chromatography
U/L	Units per liter
VL	Viral load
WHO	World Health Organization

Definition of terms

Accuracy is how close the measured value is to an accepted reference value. Accuracy was evaluated by replicate analysis of a known amount of sample (i.e., QCs) including internal standard. It was measured using a minimum of five determinations per concentration.

Adherence is the extent to which a person's behaviour – taking medication, following a diet and/or changing lifestyle – corresponds with agreed recommendations from a healthcare worker

Limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected by an analytical method, but not necessarily quantitated as an exact value.

Limit of quantification (LOQ) is the lowest concentration of analyte in a sample which can be quantitatively determined by analytical procedure with suitable precision and accuracy.

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample.

Precision is the closeness of agreement among a series of measurements obtained from the same sample under the same conditions.

Recovery describes extraction efficiency of an analytical method within the limits of variability.

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal use.

Chapter 1: Introduction

1.1 Epidemiology of HIV

Despite global efforts to halt the human immunodeficiency virus (HIV) epidemic, the number of individuals living with HIV continues to rise (Dwyer-Lindgren *et al.*, 2019). Sub-Saharan Africa has been shown to be the region with a leading burden of HIV/AIDS worldwide (Dwyer-Lindgren *et al*., 2019). However, effective antiretroviral (ARV) therapy has transformed the course of HIV infection from an acute illness to a chronic disease (Punyawudho *et al*., 2016).

1.2 Antiretroviral therapies

Antiretroviral therapy combines three or more drugs to minimize the risk of drug resistance (SADOH, 2020). At the time of data collection (2018), a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs, e.g. Tenofovir Disoproxil Fumarate (TDF) or Lamivudine (3TC)) with the addition of either a non-nucleoside reverse transcriptase inhibitor (NNRTI, e.g. Nevirapine (NVP) or Efavirenz (EFV)) made up the first-line treatment regimen. A backbone of two NRTIs (e.g. Zidovudine (ZDV) or Lamivudine (3TC)) with the addition of a protease inhibitor (PI) (e.g. Lopinavir (LPV) boosted with Ritonavir (RTV) made up the second-line treatment regimen (Meintjes *et al*., 2017) . The 2020 National Consolidated Guidelines for the management of HIV in adults, adolescents, children and infants and prevention of mother-to-child transmission recommended that integrase inhibitor, Dolutegravir, be included in the first line-treatment in place of the NNRTI (e.g. TNF, 3TC and DTG). However, Dolutegravir it is not recommended in women or adolescent girls with the potential to bear children, because it has been shown to cause neural tube defects in unborn babies, hence these women remain on TNF, 3TC and EFV (SADOH, 2020).

Guidelines recommend that PIs be coupled with a ritonavir in small doses (RTV) as this enhances bioavailability. In patients on salvage therapy, either a NRTI, (e.g. TDF) or a fusion inhibitor may be included as add-on therapy (Kredo *et al*., 2009). The drug regimen may be altered according to patient response, toxicity and the development of resistance. The ARVs are typically administered in fixed doses. There is often no dose adjustment for sex, diet, genetic polymorphisms or altered pharmacokinetics (i.e. drug absorption, distribution, metabolism, and elimination) (Kredo *et al*., 2009), all of which can affect drug concentrations. Therefore, it is important to monitor the treatment.

1.3 Monitoring ARV therapy

All HIV positive patients who have initiated ART treatment should be monitored for its safety and efficacy throughout the treatment duration. While there are various tools to determine a patient's compliance to treatment, the laboratory methods of preference include: Viral load count, CD4 count, therapeutic drug monitoring, or pill count (Meintjes *et al*., 2017, Okatch *et al*., 2016).

1.4 Measuring success in HIV drug therapy

The introduction of highly active antiretroviral therapy in the treatment of HIV has shown good outcomes, leading to a decrease in mortality rates and enhancement of the quality of life of people living with HIV/AIDS (Saha *et al*., 2014). Viral loads less than 50 copies/mL are considered indicative of virological suppression and treatment success (Kapiamba *et al*., 2016). HIV viral loads greater than this, indicate virological failure. Maximal suppression of HIV viral load is associated with a lower risk of virological failure over time (Kapiamba *et al*., 2016) and hence becomes the goal of ART (Saha *et al*., 2014).

1.5 Virological failure

There are various causes of virological failure, including noncompliance; drug toxicity; factors relating to the action of the drugs in the body (pharmacodynamics) and the emergence of viral resistance (Moneti *et al*., 2012, Hawkins *et al*., 2016). Poor compliance with ARV treatment can cause the development of drug-resistant viral strains with the transmission of resistant strains from person to person and thus increase mortality rates (Nachega *et al*., 2011, Chesney, 2000).

There is an increasing number of patients with resistance mutations; this is likely to be the single most important determining factor for long term success of treatment programs (Aghokeng *et al*., 2011).

Several studies have been conducted in South Africa to assess the prevalence of HIV drug resistant mutations in patients failing their 1st line treatment regimen (van Zyl *et al*., 2011, Sigaloff *et al*., 2012). Despite good virological suppression in the majority of the patients, Etta *et al*, (2017) showed that 84% of patients failing 1st line regimens in rural areas developed at least one drug-resistant mutation during treatment. One reason for this is that patients are not fully compliant (van Zyl *et al*., 2011).

1.6 ARV therapy compliance

ARV compliance is often a problem among HIV patients and this can impact the patient, as well as the health care system. Poor compliance leads to exacerbation of disease and thus increased treatment

costs (Jimmy and Jose, 2011). Patient medication compliance is very important to ensure virological suppression in HIV patients (Iacob *et al*., 2017).

High levels of motivation and compliance are required from the patient in order to prevent the development of resistant viral strains and thus maintain the effectiveness of highly active antiretroviral therapy (Nachega *et al*., 2011, Martin *et al*., 2008, Leyva-Moral *et al*., 2019). It has been shown that adherence of more than 75% is required for optimized virological outcomes (Cheng *et al*., 2018). HIV treatment regimen compliance is characterized by taking all the prescribed pills at the correct time, in the correct doses and in the correct way (Nischal *et al*., 2005). Poor compliance causes low therapeutic drug levels that lead to elevated HIV viral concentrations and the development of resistance to ARVs (Saha *et al*., 2014). Therapeutic drug monitoring (TDM) of ARVs can prove to be useful in assessing patient compliance.

1.7 Definition of TDM

TDM is a branch of clinical chemistry and clinical pharmacology that specializes in monitoring of drug concentrations and adjusting the dosage regimen on the basis of these concentrations. It focuses on the concentration of the drug at which the drug is expected to be effective without causing toxicity. This will directly influence drug prescribing procedures in order to address treatment failure. TDM is also referred to as the individualization of drug dosage by maintaining plasma or blood drug concentrations within a targeted therapeutic range or window (Perrone *et al*., 2014).

1.7.1 Indicators for TDM

The indications for drug monitoring include toxicity, efficacy, compliance, drug-drug interactions, and drugs with narrow therapeutic ranges. The data obtained may correlate better with the drugs' concentrations than they do with standardized dosing (Bochner and Tonkin, 1993, Acosta, 1999). The contribution of pharmacokinetic variability to differences in dose requirements can be identified by measuring the drug concentration at the steady state and modifying the dose in order to accomplish the desired concentration known to be related to efficacy. Nevertheless, there is substantial inter-individual variability at a given plasma concentration, hence a range of concentrations rather than a single level is usually targeted (Levy, 1994).

1.7.2 TDM of ARVs

The aim of monitoring ARVs is to improve efficacy and safety by maintaining individual patients ARV plasma concentrations within the therapeutic range. Previous research has indicated that monitoring ARV concentrations may improve outcomes in HIV-infected individuals (Aarnoutse *et al*., 2003, Acosta

et al., 2002, Burger *et al*., 2003, Fletcher *et al*., 2002, Back *et al*., 2001, Back *et al*., 2002). It is suggested that TDM of ARVs can potentially prevent patients from being exposed to toxic or sub-therapeutic drug concentrations. ARV TDM is therefore potentially a tool to optimize efficacy and minimize the toxicity of ARV therapy.

1.7.3 TDM drug characteristics

For a drug to be considered for TDM, it must possess necessary characteristics. The most important of these is that the plasma concentration must correlate with drug efficacy or toxicity. Pharmacokinetic data of a drug must be available. High inter-patient variability must be notable, as well as low intra-patient variability in plasma concentrations. The drug must have a narrow therapeutic range. It should also have pharmacological effects that cannot be determined clinically or by common laboratory tests. A reliable drug assay with high accuracy, sensitivity and a rapid turnaround time must be available (Acosta, 1999). Additionally, the drug should be able to be adjusted to achieve target levels, and ultimately improve response to therapy (Back *et al.*, 2002). A number of ARV drugs such as protease inhibitors and NNRTIs are suitable for drug monitoring, while the NRTIs are metabolized intracellularly and so may not be suitable. These are discussed further below.

1.7.4 Protease inhibitors as candidates for TDM

There is strong retrospective evidence on PI-based therapy that supports the association between drug exposure and virological suppression (Estebanez and Arribas, 2012). This association is observed across different patients, ranging from those starting treatment for the first time, to those on salvage therapy. The association is less clear in patients who have multiple resistance mutations, because they have been pre-treated with ARVs. In such cases, the therapeutic range for drug concentrations is poorly defined (Boffito *et al*., 2005, Burger *et al*., 2003).

Although TDM has been shown to be useful, there is still a question about its value in patients harboring resistance mutations. There are studies that have shown that using PI plasma concentrations with resistance test results had a positive impact on virological outcomes (Durant *et al*., 2000, Baxter *et al*., 2002). Nonetheless, in some studies with treatment-experienced patients, TDM failed to show any benefit (Durant *et al*., 2000, Bossi *et al*., 2004). The drawbacks of these studies were that the target plasma PI concentration was probably insufficient, given that the target PI plasma concentrations were available only in wild-type HIV-1 (Boffito, 2006).

Toxicity that is linked with PI plasma concentrations has been described in small retrospective observational studies (Boffito *et al*., 2005, Back *et al*., 2002). Diarrhea and nausea are well-known

short-term adverse effects of the PIs; these effects may be associated with higher peak plasma concentrations (Boffito *et al*., 2005). Antiviral efficacy has been correlated with RTV dosage and its plasma levels (Danner *et al*., 1995) and the risk of adverse effects (Gatti *et al*., 1999). There is slow emergence of resistance-associated mutations at higher plasma concentrations. A study conducted by Gatti *et al,* (1999) reported that high plasma RTV concentrations are associated with increased risks of neurological and gastrointestinal adverse events. Therefore, TDM may be useful in adjusting the dose to avoid adverse effects, while maintaining therapeutic concentrations. PIs may be given with a small therapeutic dose of RTV, where RTV is added to inhibit the metabolic breakdown of the primary PI, in this manner increasing the bioavailability and therapeutic drug concentration. For example, LPV is unsuitable for clinical use, because it has a very short half-life of not more than one hour. However, the addition of low-dose RTV effectively enhances the pharmacokinetics of LPV (Zeldin and Petruschke, 2004, Bierman *et al*., 2009). When RTV is used as a booster of other classes of antiretroviral drugs in highly active antiretroviral therapy that do not contain a PI, a sub therapeutic dose of RTV may accelerate the emergence of HIV-1 variants resistant to PIs (Xu *et al*., 2010).

Therapeutic drug monitoring has shown clinical value in preventing virological failure in NNRTIs and PIs (Buzibye *et al*., 2019). A study evaluating the pharmacokinetics of LPV and RTV in tuberculosis-HIV co-infected African adult patients receiving Rifabutin, showed reduced concentrations of LPV and RTV (Ouedraogo *et al*., 2020), suggesting that TDM may be useful for such cases. Furthermore, boosted drug concentrations have in some studies been found to exceed the necessary therapeutic concentrations (Murphy *et al*., 2001).

The inter-individual variability of LPV trough concentrations has been shown to be moderately high and therefore TDM may also be of value in detecting sub-therapeutic concentrations in patients. In a recent report LPV trough concentrations varied 11-fold (Poirier *et al*., 2001). Therefore, it seems rational to assume that a certain percentage of patients treated with LPV may have sub-therapeutic concentrations; mainly PI-experienced patients who carry the virus with reduced susceptibility to this agent (Kempf *et al*., 2000).

1.7.5 NNRTIs as candidates for TDM

Currently Dolutegravir based regimens are preferred for people initiating treatment, however EFV is still used widely. It is a safe and effective NNRTI recommended by the World Health Organisation (WHO, 2016). Its correlation with virological outcomes has been supported by studies that looked at drug levels versus viral loads. In a subgroup analysis in the 2NN study, participants with EFV trough

plasma concentrations greater than 1.1 µg/ml had an 89% likelihood of not failing virologically (Leth *et al*., 2006). High concentrations of EFV are associated with neuropsychiatric adverse effects such as, dizziness, headache and insomnia (Gounden *et al*., 2010, Keegan *et al*., 2019) making it a good candidate for TDM.

1.7.6 NRTIs as candidates for TDM

According to the 2020 National Consolidated Guidelines for the management of HIV in adults, adolescents, children and infants and prevention of mother-to-child transmission, preferred first-line NRTIs in adults should be: TDF + 3TC/FTC or ZDV/ABC + 3TC (SADOH, 2020). The NRTIs are pro-drugs that require intracellular activation to their active form. The process of converting them to their active triphosphate form takes place in peripheral blood mononuclear cells hence they are recommended to be quantified in peripheral blood mononuclear cells (Xiao *et al*., 2018). The procedure of measuring intracellular concentrations of the NRTIs is both difficult and expensive. Even though early small prospective studies proposed a concentration-effect relationship for NRTIs (Fletcher *et al*., 2000), these agents are generally not considered suitable candidates of TDM, because plasma levels may not be a good indicator of intracellular triphosphate concentrations (Burger, 2010). They may be at low or undetectable levels in plasma, but at suppressive concentrations within cells.

1.7.7 The potential role of TDM in the management of HIV

The benefits of monitoring ARV drug concentrations in adults has been described by several investigators (Clevenbergh *et al*., 2002, Fletcher *et al*., 2002, Burger *et al*., 2003, Bossi *et al*., 2004, Crommentuyn *et al*., 2005, Torti *et al*., 2005, Khoo *et al*., 2006, Best *et al*., 2007). These studies suggest that TDM of ARVs can potentially identify patients with sub-therapeutic, toxic or appropriate drug concentrations. Similar to previous studies, a large study with 573 samples by Buzibye *et al*, (2019) supports the use of TDM for HIV management.

The controversy regarding the value of ARV TDM is reflected by the contradictory HIV management guidelines internationally. Western Europe and the United Kingdom centers approve the use of TDM in NNRTIs and PIs to determine the cause of treatment failure and to check for toxicity, as well as to adjust dosing of PIs when they are used in combinations with drugs that may induce drug interactions (Delfraissy *et al*., 2000, Carosi *et al*., 2006, Gazzard *et al*., 2006, Liu *et al*., 2010). The British HIV Association supports the practice of TDM in pregnancy, pediatric patients, management of drug-interactions and in salvage therapy when TDM can be coupled with genotyping of the viral DNA to evaluate resistance. In all these cases, drug concentrations may be difficult to predict (Gazzard *et al*.,

2006). These guidelines also support TDM in renal and hepatic dysfunction; in cases with ARV toxicity and with new regimens whose efficacy and safety are not yet well defined.

Guidelines from the United States do not provide specific indications for TDM, but are supportive (Gazzard *et al*., 2006). There is evidence that demonstrates the impact of TDM on clinical outcomes of patients. A real-life study in a large cohort of patients showed that TDM of ARV drugs had a positive association with higher adherence to therapy, reduced length of hospital stay and reduced cost of illness (Perrone *et al*., 2014). However, the South African National guidelines (Africa, 2020) and the World Health Organization (WHO, 2016) guidelines for adults and adolescents of resource-limited settings do not support the use of TDM.

1.8 Monitoring concentrations of ARVs

There is increasing numbers of patients on ARV therapy in resource-limited settings. However, the key major challenge of HIV treatment programs in these settings is ART monitoring (Stevens and Marshall, 2010, Roberts *et al*., 2012). Traditionally monitoring concentrations of ARVs is done in plasma; however, these samples have to be transported to central laboratories on ice and within a limited time period, because of sample stability. Despite progress made in ART therapy, people in resource-limited settings still lack access to standard care (Roberts *et al*., 2016). Moreover, many people who are infected with HIV live in resource-limited settings (Schmitz *et al*., 2017). Therefore, there is a need in resource-limited settings to investigate the use of alternative sample types which allow for improved sample stability and transport.

1.9 Alternate monitoring of ARV concentrations

Dried blood spots (DBS) are an alternative sample type compared to plasma. They have been extensively used in resource-limited countries for storing specimens for viral load analysis (Johannessen *et al*., 2009). DBS can be stored and shipped at ambient temperature. Drug stability on DBS has shown to be superior compared to wet matrices, thus avoiding the requirement for a freezer and transport on ice. Lower biohazard risks associated with shipment and handling may be an advantage of DBS (Edelbroek *et al*., 2009). Drug concentrations from DBS have been shown to correlate well with plasma concentrations (Meesters *et al*., 2010).

Other advantages of using DBS for TDM include: (1) the patient can administer the finger prick at home and no phlebotomist is necessary (2) only a small volume of blood is required and (3) monitoring at any desired sampling time can be undertaken conveniently. However, DBS are not

without limitations, these include (i) the small volumes available require a sensitive analytical technique for analysis (ii) sampling is not always successful since the patients conduct the sampling on their own and (iii) capillary concentrations can be different from venous concentrations, because the capillary blood is diluted by interstitial fluid (Wilhelm *et al*., 2014). Furthermore, the qualitative analysis of DBS may be affected by haematocrit (HCT).

1.10 Effect of HCT on DBS

HCT is the ratio of the volume of red blood cells to the total volume of blood and it has an effect on blood viscosity. HCT is currently identified as the single most important parameter influencing the spread of blood on DBS cards, and therefore the concentration. Blood with a high HCT tends to disseminate to a lesser degree across the filter paper (smaller blood spot diameter), hence the target analyte diffusion distance is shorter (De Kesel *et al*., 2013, Timmerman *et al*., 2011). The opposite occurs for a lower HCT. As a result, the determined concentration compared with the "normal" HCT sample would be over or under-recovered, because of the differences in the analytes distribution and infusion pattern across the blood spot (O'Mara *et al*., 2011, Vu *et al*., 2011).

The HCT concentration level below 34% in females and 39% in males is considered to be low and the HCT concentration above 48% in females and 51% in males is considered to be high (Lawrie *et al*., 2009). These distinctions in HCT will influence the relative plasma level in the spot. This is very important for analytes that are generally found in plasma/serum. Comparison of concentrations of EFV and LPV between plasma and DBS has shown pronounced differences of 47.4% and 48.1%, respectively. However, correction for HCT improved the agreement between plasma and DBS so that the percentage difference was within the ±20% allowable limit (Duthaler *et al*., 2018). Kromdijk *et al*, (2012) showed that DBS concentrations of NVP and EFV were equal to paired plasma concentrations after correction for HCT.

Physical properties of the drug may cause it to undergo a non-homogeneous distribution within the DBS sample (O'Mara *et al*., 2011, Ren *et al*., 2010). Non-homogeneous distribution of the drug is also seen with high blood loading volumes (Fan *et al*., 2011). Thus HCT could impact the validity of the results generated by DBS methods by affecting the spot formation, spot size, drying time, homogeneity, and ultimately, the robustness and reproducibility of the assays (Timmerman *et al*., 2011). Therefore, for accurate drug quantification, HCT must be carefully assessed. The effect of HCT can be reduced by analyzing the entire DBS which gets rid of variation caused by spreading and non-homogeneity. It requires that spots be made by trained personnel (Fan and Lee, 2012). Alternatively,

pre-cut DBS can be used. There is less variation (<3.1%) observed in drug concentrations obtained from pre-cut DBS compared to punched DBS (Youhnovski *et al*., 2011).

1.10.1 Possible causes of HCT differences

There are a number of causes for low HCT resource-limited setting. One of these is anaemia. Anaemia has been shown to be the most common haematological abnormality in HIV-infected patients globally (Bhardwaj *et al*., 2020). Anaemia is common among HIV infected patients, because they are more likely to be malnourished, have advanced immunosuppression, and have high rates of comorbidities such as tuberculosis (Semba and Gray, 2001, Russell *et al*., 2010). Another important cause is malaria. Globally there were an estimated 229 million malaria cases in 2019 of which, the African region was estimated to account for 215 millon cases (WHO, 2020). Malaria leads to haematological abnormalities that include anaemia, the destruction of red blood cells which will ultimately lead to low HCT and hence cause high drug recoveries in such patients (Chaudry *et al*., 2015).

Other causes for low HCT are iron deficiency (du Plessis *et al*., 2019, Pornprasert *et al*., 2008, Weatherall, 2008), pregnancy (Tunkyi and Moodley, 2015) and chronic infection such as tuberculosis and HIV (Schapkaitz *et al*., 2015). In contrast, tobacco smoking (Anandha *et al*., 2014, Guedes *et al*., 2007) and water scarcity (Dill and Costill, 1974, Holsworth *et al*., 2013) may cause high HCT; consequently, drug levels of these patients may be over or under-estimated.

1.11 Dried plasma spots (DPS)

Innovations that allow the rapid separation of cells from plasma could assist in overcoming the limitations of DBS that arise from the HCT effect. Dried plasma spots (DPS) are shown to be useful tools for monitoring ARV treatment efficacy and pharmacokinetics (Calcagno et al., 2015, D'Avolio et al., 2010).

The Cobas® plasma separation card (PSC) shares many features of DBS, but collects plasma rather than whole blood. The device is spotted with whole blood samples obtained from fingerpricks or venous blood. A porous membrane allows only plasma to pass through and be collected on an underlying polyester fleece (Carmona *et al*., 2019). These cards have the advantages of DBS with regards to sample collection, while providing plasma as a matrix for laboratory analysis. A number of investigations have shown that viral loads measured by DPS correlated well with measurement by plasma (Carmona *et al*., 2019, Vubil *et al*., 2020).

1.12 Quantification of ARVs

Normally, immunoassays have been the technology of preference for the detection and quantitation of biomolecules. Although immunoassays are more widely available, selectivity can be affected by autoantibodies (Spencer *et al*., 1998) which can lead to false results with serious outcomes (Rotmensch and Cole, 2000) and an immunoassay such as Enzyme-linked immunoassay which typically requires 100-200 µl of a sample which is a problem with samples with limited quantity (Cross and Hornshaw, 2016). However, over the past two decades, alternative technologies have emerged to provide a complementary role to immunoassays. Liquid chromatography-mass spectrometry (LC-MS/MS) is one such technology, because of its high sensitivity and accuracy. it has shown to offer a dominant alternative to immunoassays (Cross and Hornshaw, 2016).

LC-MS/MS is currently a routine technique with the advancement of electrospray ionization offering a simple and vigorous interface (Cross and Hornshaw, 2016). It can be used for a variety of biological molecules. Furthermore, the use of tandem MS and stable isotope internal standards allows highly sensitive and accurate assays to be developed. There are different kinds of ion sources which can be utilized as part of LC-MS/MS frameworks inside clinical research centers. Electrospray ionization works well with polar molecules and is thus well suited to the analysis of many metabolites including drug metabolites. Liquid samples are pumped through a capillary and nebulized at the tip to form a fine spray of charged droplets. With the application of heat and dry nitrogen, the droplets are quickly evaporated and the residue of electrical charge on the droplets is transferred to the analyte. The ionized analytes are then transferred into the high vacuum of the mass spectrometer (Pitt, 2009).

LC-MS/MS is liquid chromatography (usually high-performance liquid chromatography or ultra-performance (UHPLC)) combined with tandem mass spectrometry (Cross and Hornshaw, 2016). Chromatography is a technique used to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase (column) and carries the components of the mixture with it (sample in a liquid form). Sample components that display stronger affinity to the stationary phase will move more slowly through the column as compared to components with a lower affinity for the stationary phase. This difference in affinity causes the separation of various components. Mass spectrometers operate by converting the analyte molecules to a charged (ionized) state, with subsequent analysis of the ions

and fragment ions that are produced during the ionization process using their mass to charge ratio (m/z) to identify the analyte of interest (Cross and Hornshaw, 2016).

UHPLC-MS/MS has been utilized, because it has the functionality to perform multi-compound analysis, thereby enabling the measurement of all the ARV drugs at once. Furthermore, UHPLC-MS/MS can handle the low sample volumes acquired from DBS. It has been shown to have high specificity and accuracy at low volumes (<5 µl) therefore making it the method of choice (Cross and Hornshaw, 2016). While there are many benefits of using UHPLC-MS/MS, it has not replaced Immunoassays in routine analysis. This implies that it has some limitations. Some strengths of UHPLC-MS/MS and its limitations are summarized in table 1 below (Cross and Hornshaw, 2016).

Table 1: Qualities and limitations of UHPLC-MS/MS

Strength of UHPLC-MS/MS	Limitations of UHPLC-MS/MS
• Day to day reagent costs are low • Few manual steps hence the results are reproducible • Can identify very structurally similar biomolecules • High sample throughput • Only small sample volumes are required	• Instrumentation is expensive • Mass spectrometers are thought to be difficult to operate and there is a lack of standardized methodology

Taken together, the advantages that DBS and DPS offer over conventional sampling could help establish TDM as an important tool in the management of HIV, particularly in remote areas.

1.13 STUDY AIMS AND OBJECTIVES

Aim:

To develop and validate a method for the measurement of ARVs in DBS and DPS from whole blood spotted onto filter paper and onto plasma separation cards as compared to the plasma levels from blood obtained by venipuncture.

Objectives:

- Develop and validate a method for the measurement of ARVs in DBS from whole blood spotted onto filter paper.
- Evaluate the effect of HCT on ARV concentrations in DBS.
- Develop and validate a method for the measurement of ARVs in plasma from DPS.
- Correlate anti-retroviral levels from DBS and DPS with plasma levels obtained by venepuncture in two treatment groups.

Chapter 2: Methodology

2.1 Research design

This study was a sub-study of a larger study "the Analysis of antiretroviral in HIV positive patients as an indication of patient compliance" (Ethics certificate number M151003 (Appendix: B.1)).

This research was carried out in two phases. During the first phase an UHPLC–MS/MS method that has previously been reported (Koal *et al*., 2005) to quantify ARV concentrations in plasma, was validated for use in DBS and DPS. This was done using commercially available standards and controls and the protocol established by the Food and Drug Administration guidelines (Food and Drug Administration, 2018)

The second phase was carried out once the validation was complete.

- First line and second line treatment regimen ARVs were quantified in 231 patient samples using DBS and plasma (122 patients on 1st line treatment and 109 patients on 2nd line treatment). Samples were acquired from patients who presented at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) for routine check-up at the HIV clinic. Anthropometric data including age, gender, height, viral load, and drug regimen were recorded on a spread sheet and numerical data was captured.
- ARVs were quantified in 211 HIV-infected patients' remnant samples collected from the Haematology Lab using DBS, plasma and DPS (Ethics certificate number M190689 (Appendix B.2)). The anthropometric data and drug regimen are not available for this data.

2.2 Sample collection

231 Samples were collected from the HIV clinic for verification of drug concentrations in DBS.

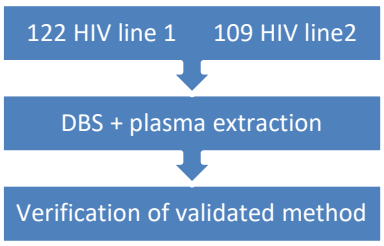


Figure 1: Flow diagram of sample collection for DBS

211 Samples were collected from the Haematology Laboratory for verification of drug concentrations in DPS and DBS.

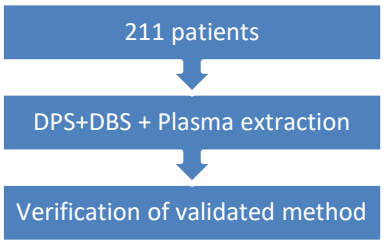


Figure 2: Flow diagram of sample collection for DBS

2.3 Site of study

- The participants in this study were adult male and female patients attending the HIV clinic at CMJAH.
- Random remnant Ethylenediaminetetraacetic acid samples were received from the Haematology Laboratory at CMJAH.

2.4 Sample size

The sample size required for method comparison was calculated to be a minimum of 80 samples. This is in accordance with Clinical and Laboratory Standards Institute guidelines and EP9 method comparison (Tholen *et al*., 2003). The samples from the HIV clinic were divided into two groups according to the treatment regimen, that is, a minimum of 40 patients on line 1 regimen and a minimum of 40 patients on line 2 regimens.

According to the Department of Health annual report published in 2016, 3100 000 South Africans with HIV were being treated with ARV's. Non-compliance statistics have been estimated to be as high as 20% in studies carried out in Brazil, Ethiopia and Nigeria (Monreal *et al*., 2002). Therefore (P) based on the general population was 20%. The additional variables are precision of 5% (d) and a confidence interval of 95% (Z)

$n= Z^2P\ (1-P)/\ d^2$

$= 1.96^2 0.2(1-0.2)/\ 0.05^2$

$=246$

The minimum sample number required is 246.

A total of 251 patients were recruited, however only 231 samples were used in analysis due to exclusion criteria.

The inclusion and exclusion criteria used are stated as follows:

2.5 Inclusion criteria

- HIV seropositive and on either first line or second line treatment
- Consent to participate in the study
- Subjects must be >18 years
- Essential demographic and clinical data must be available (VL, Alanine aminotransferase (ALT) and current drug regimen)

2.6 Exclusion criteria

The following exclusion criteria were applied:

- ALT more than three times the upper reference limits.

This was in order to exclude patients with liver disease, because it affects drug clearance by reducing the hepatic clearance of drugs and thus makes drug measurement inaccurate.

2.7 Sampling

Samples (venous and capillary blood) were collected in the morning from the patients attending the adult HIV clinic. Most samples were collected in the mid-dose interval (12-18 hours after dosing). Whole blood samples were obtained by venipuncture using Ethylenediaminetetraacetic acid tubes for measurements of plasma ARVs, HCT and preparation of venous blood and DBS cards. Whole blood (30 µL) was spotted on the DBS cards. After sterile cleaning of the skin, capillary blood was obtained from the same patients via lancet puncture. Exactly 30 µL was sampled using a pipette and spotted on to DBS cards.

DPSs were prepared using remnant samples received from HIV-infected patients whose samples had been sent for CD4 analysis to the Haematology laboratory. The samples were prepared by pipetting whole blood (140 µL) on to the DPSs. Both DBS and DPS cards were left to dry at room temperature (± 24°C) overnight prior to extraction overnight prior to extraction(Koal *et al*., 2005). To ensure exclusion criteria were met, serum ALT levels were analysed on a Cobas 8000. Yellow top, serum separator tubes were collected prior to centrifugation. Patient's whole blood from Ethylenediaminetetraacetic acid samples were measured for HCT on a Haematokrit 210 (Heraeus Christ). Plasma was obtained

from the remaining whole blood samples by centrifugation at 3500 revolutions per minute for 10 minutes at 4°C using an Allegra X-22R centrifuge (Beckman Coulter, Brea, California, USA) and decanted in Eppendorf tubes and stored at −80°C for further analysis.

2.8 Materials and Methods

2.8.1 Materials

All reagents were purchased from Merck (Amsterdam, Netherlands). Matrix matched ARV plasma standards and controls consisting of Atazanivir, Elvitegravir, Etravirine, Lopinavir, Ritonavir, Tipranavir, Amprenavir, Darunavir, Delavirdine, Maraviroc, Nevirapine, Raltegravir, Efavirenz, Indinavir, Nelfinavir, M8-Nelfinavir, Rilpivirine and Saquinavir were purchased from Chromsystems (Munich, Germany). These standards were used to establish a 6-point standard curve that was used in the analysis along with deuterated internal standards. Where a drug was not available in the Chromsystems standards and controls, certified reference standards were purchased from LGC (Teddington, Middlesex, UK) and spiked into the matrix matched curve as described below.

2.8.2 Analytical methods

Stock solutions of 3TC, ABC and FTC were diluted in methanol: water (50:50) to produce standard solutions. Six mixed calibration standards were then prepared by spiking the commercially available plasma standards with standard solutions (50:50, v/v). Spiked commercially available plasma standards were then added to whole blood (50:50, v/v) to give the following final concentrations in µg/ml (see Tables 2 and 3).

Table 2: Standard curve concentrations for each drug in DBS

Drug	STD 0	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6
	µg/ml						
3TC	0	0.119	0.238	0.357	0.595	0.714	0.833
ABC	0	0.119	0.238	0.357	0.595	0.714	0.833
EFV	0	0.323	0.725	1.122	1.556	1.974	2.360
RTV	0	0.104	1.512	2.831	4.156	5.440	6.640
LPV	0	0.370	1.453	2.491	3.616	4.652	5.685
NVP	0	0.925	1.458	2.051	2.634	3.172	3.676
FTC	0	0.060	0.119	0.179	0.298	0.298	0.417
RAL	0	0.012	0.061	0.129	0.192	0.250	0.307
ATV	0	0.028	0.310	0.617	0.924	1.210	1.505
TMC114	0	0.025	0.557	1.168	1.738	2.312	2.862

STD = standard

Table 3: Standard curve concentrations for each drug in plasma and DPS

Drug	STD 0	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6
	µg/ml						
3TC	0	0.119	0.238	0.357	0.595	0.714	0.833
ABC	0	0.119	0.238	0.357	0.595	0.714	0.833
EFV	0	0.625	1.400	2.229	3.087	3.871	4.650
RTV	0	0.194	2.252	5.278	7.877	10.434	12.717
LPV	0	0.686	2.710	4.810	6.965	9.007	10.891
NVP	0	1.850	2.916	4.102	5.268	6.344	7.352
FTC	0	0.060	0.119	0.179	0.298	0.298	0.417
RAL	0	0.026	0.122	0.258	0.384	0.500	0.614
ATV	0	0.051	1.114	2.335	3.475	4.623	5.723
TMC114	0	0.051	1.169	2.253	3.333	4.544	5.612

STD = standard

Two quality control (QC) samples, namely; low quality control (LQC) and high quality control (HQC) were prepared. Stock solutions of 3TC, ABC and FTC were diluted in methanol: water (50:50) to produce standard solutions. To prepare the controls with all the necessary drugs, the two commercially purchased controls were spiked with of 3TC, ABC and FTC at concentrations equivalent to STD 1 and STD 6 in µg/ml. Due to the volume differences between plasma, DBS and DPS samples, in-house reference ranges were established using mean and standard deviation as reported in Tables 4 and 5.

Table 4: Established concentrations of the internal controls for each analyte in DBS

Analyte	LQC (range) (µg/ml)	HQC (range) (µg/ml)
3TC	0.121 (0.097-0.145)	0.768 (0.596-0.941)
ABC	0.135 (0.116-0.155)	0.889 (0.715-1.064)
EFV	0.597 (0.481-0.713)	1.725 (1.146-2.304)
RTV	0.744 (0.560-0.928)	4.494 (3.296-5.691)
LPV	0.987 (0.658-1.316)	3.788 (2.852-4.723)
NVP	1.259 (0.9565-1.552)	2.395 (1.883-2.907)
FTC	0.050 (0.033-0.068)	0.328 (0.237-0.418)
RAL	0.036 (0.026-0.047)	0.182 (0.136-0.229)
ATV	0.139 (0.101-0.176)	0.897 (0.676-1.119)
TMC114	0.227 (0.169-0.284)	1.684 (1.261-2.107)

LQC = Lower level quality control

HQC= Higher level quality control

Table 5: Established concentrations of the internal controls for each analyte in DPS

Analyte	LQC (range) (µg/ml)	HQC (range) (µg/ml)
3TC	0.121 (0.103-0.138)	0.843 (0.768-0.918)
ABC	0.127 (0.105-0.148)	0.755 (0.618-0.893)
EFV	1.486 (1.158-1.814)	3.533 (2.536-4.530)
RTV	1.212 (0.858-1.565)	11.397 (9.226-13.569)
LPV	2.129 (1.666-2.592)	11.085 (8.324-13.846)
NVP	3.090 (2.560-3.620)	5.939 (5.151-6.726)
FTC	0.054 (0.046-0.062)	0.418 (0.379-0.458)
RAL	0.073(0.063-0.083)	0.464 (0.403-0.525)
ATV	0.619 (0.482-0.755)	6.044 (4.806-7.283)
TMC114	0.604 (0.416-0.792)	4.837 (3.993-5.680)

A Shimadzu Nexera 8060 UHPLC-MS/MS (Shimadzu, Japan) was used for the validation and analysis of the ARV drug levels in DBS and DPS.

Plasma, DBS and DPS samples were extracted and run in batches of approximately 81 samples. Low and high QC samples were run with every 20 samples. For DBS, the following drug levels were validated: 3TC, ABC, EFV, RTV and LPV. Due to the commercially available standards and matrix-matched spiked controls, the following additional drugs were also validated: NVP, FTC, ATV, RAL and TMC114 the results of the validation of are presented in the appendix (A: 1.6., A: 1.7, A: 1.8, A: 1.9 and A: 1.10). These were not included as they were not part of the main focus at the onset of the DBS study. For DPS, remnant samples were received from haematology Laboratory and did not know what drug regimen patients were on therefore the following drug were validated: 3TC, ABC, EFV, RTV, LPV, NVP, FTC, RAL, ATV and TMC114.

2.8.3 Sample extraction and chromatographic conditions

2.8.2.1 Plasma samples

For plasma samples the extraction procedure was as follows:

Samples were extracted utilising protein precipitation. Acetonitrile (400 µL) and internal standard (10 µL) were added to 50 µL of plasma sample and vortex mixed for 30 seconds. This was followed by centrifugation for 10 minutes at 4°C at 14000 revolutions per minute. Two hundred microliters of the supernatant was aliquoted into amber sample vials and 2 µL was injected onto an Acquity UPLC BEH C18 1.7 µm (2.1X 100 mm) column on UHPLC-MS/MS for analysis. HPLC grade reagents were used, Mobile phase A consisted of dH_2O + 0.1% (v/v) formic acid and mobile phase B consisted of acetonitrile + 0.1% (v/v) formic acid.

2.8.2.2 Dried blood spots

A previously validated extraction method for PIs and NNRTIs in DBS described by Koal *et al*. (2005) was initially used for the extractions of the analytes. However, lower recoveries of the analytes were observed. Therefore the method was modified as follows: Pre-cut DBS were removed from filter cards into 4 ml stoppered glass tubes and treated with 600 µL extraction reagent consisting of (v/v) 0.1% formic acid in methanol/0.2 M zinc sulfate heptahydrate (Manallack *et al*., 2013). Ten microliters of internal standard was added, the sample was mixed and left at room temperature for 30 minutes. Samples were then vortex mixed for 30 seconds and centrifuged for 10 minutes at 4500 revolutions per minute at 4°C. A volume of 10 µL supernatant was analyzed on the UHPLC-MS/MS.

2.8.2.3 Dried plasma spots

The top layer of the PSC (the 'spotting layer') together with the membrane, were manually removed by pulling on a removable flap on the PSC and discarded. A single spot was removed and transferred into a glass tube to which 500 µL methanol and 30 µL internal standard were added and allowed to stand for 30 minutes. Samples were then vortex mixed for 30 seconds and the DPS was removed. Lastly the supernatant was dried down under nitrogen gas and reconstituted with 100 µL of methanol: water (50:50). A volume of 10 µL supernatant was injected into UHPLC-MS/MS.

2.8.4 UHPLC conditions

The liquid chromatographic separation was performed using on an Acquity UPLC BEH C18 1.7 µm (2.1X 100 mm) column (Waters, Massachusetts USA) and a stepwise gradient using mobile phase A (dH2O + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid). The final run time was

6.01 minutes per sample at a flow rate of 400 µL/minute. The column and auto sampler were maintained at 40°C and 8°C, respectively.

2.8.5 Mass spectrometry conditions

The drugs were separated by UHPLC and detected with tandem mass spectrometry. A triple quadrupole mass spectrometer utilizing Electrospray ionization in positive ionization mode was used for the identification and quantitation of the ARV drug levels. Nitrogen gas was used as the nebulizer, auxiliary and curtain gases, while Argon was used as a collision gas. Analytes were detected using multiple reaction monitoring (MRM) transitions as listed in Table 6.

Table 6: MRM transitions (Legg-E'Silva & Snyman, unpublished)

Parent drug	MRM transitions	Dwell time (msec)	Collision energy
3TC	230.03 > 112.00	4.0	-12.0
ABC	287.00 >191.05	19.0	-12.0
EFV	315.99 > 243.94	8.0	15.0
RTV	721.20 > 170.91	19.0	-20.0
LPV	629.50 > 447.10	19.0	-35.0
NVP	267.00 > 226.00	47.0	-20.0
FTC	248.25 > 213.25	4.0	-10.0
RAL	445.00 > 109.00	74.0	-45.0
ATV	705.40 >168.20	47.0	-10.0
TMC114	548.10 > 392.30	47.0	-20.0

2.9 Method validation of DBS and DPS

Validation for the determination of plasma ARV levels was done previously (Legg-E'Silva & Snyman, unpublished). This data is available as supplementary data (A: 3).

2.9.1 Validation criteria in DBS and DPS.

Linearity was evaluated using six different concentrations of each drug to form a standard curve. Reference standard solution was prepared and analyzed in triplicate. A calibration curve was plotted using a least square regression analysis. The linear regression between the peak height ratios and drug concentrations was used to generate a calibration curve. Notably, the linearity of the analytical

method is deemed to be acceptable if the regression co-efficient (R^2) is greater than 0.98 (Shabir, 2005).

Accuracy was measured using a minimum of six determinations per concentration. The mean value should be within 15% of the actual value except at the calculated limit of detection (LOD) and limit of quantification (LOQ), where it should not -deviate by more than 20% (Food and Drug Administration, 2018).

The precision test comprises the intra-day and the inter-day analysis. Intra-day analysis was analyzed in one day and inter-day analysis was analyzed over the course of five days. Relative standard deviation (RSD) was determined at each concentration and should not exceed 15% of RSD except at concentrations equivalent to the LOD and LOQ, where it should not exceed 20% (Food and Drug Administration, 2018).

The recovery of the method was determined by comparing mean measured concentrations of extracted samples (QCs) with actual (expected) concentrations that represent 100% recovery. The mean recovery value should lie between 80-120% (Food and Drug Administration, 2018).

The LOD was determined from the lowest concentration peak response to the background noise ratio. A signal-to-noise ratio greater than a value of three is generally considered acceptable for estimating the detection limit (Food and Drug Administration, 2018).

The LOQ was determined from the lowest concentration peak response to the background noise ratio and has been described as the concentration that gives a signal-to-noise ratio greater than a value of ten (Food and Drug Administration, 2018, Shabir, 2005).

The stability of the drugs on DBS was determined by comparing the concentrations of the HQC and LQC samples of freshly prepared samples from stock solutions at room temperature and DBS which had been stored on the bench for 1 day, 2, 7 and 30 days (drying time) prior to extraction.

Method robustness was tested by deliberately changing the length and functional types of the column, as well the column temperature, injection volume and the composition of the mobile phase.

2.10 Haematocrit determination in DBS

Haematocrit effects on DBS were determined by assessing drug recovery at different percentages of HCT. Whole blood was centrifuged at 3500 rpm for 10 minutes at 4°C to separate the plasma and red

blood cells. Increasing volumes of plasma were added to whole blood to "dilute" it, providing decreasing HCT levels of 80%, 40%, and 20%, respectively. The non-centrifuged whole blood served as 100% control. After the HCTs were measured using a Haematokrit 210 (Heraeus Christ), the blood was subsequently spiked with the lowest and the highest drug concentrations of each analyte providing two concentrations (STD 1 and STD 6) (Table 4) at each HCT level. Spiked samples were spotted on the DBS cards for drug extraction and drug levels were then compared with their corresponding HCT levels. The effect of HCT on spots formation, spots size and homogeneity were avoided by analyzing the entire DBS.

Given that low HCTs due to anaemia and tuberculosis infection are common among HIV patients, the effect of low HCT on ARV drugs using DBS was also addressed (Redig and Berliner, 2013, Kerkhoff *et al*., 2014). The experiment performed at HCT concentrations of 20%, 40%, 80% and 100% (non-centrifuged blood) are equivalent to the HCT of 9%, 18%, 36% and 45%, respectively (Amara *et al*., 2015). These true values will be used to describe the experiments further. The HCT of 9% and 18% levels are infrequent in the normal population for this study therefore they will not be discussed further. In the studied patient population, HCT concentration below 34% in females, and 39% in males, is considered to be low. These HCTs concentrations correspond to 36% and 45% HCTs concentrations in the experiments. Therefore these concentrations were used to reflect drug levels.

2.11 Laboratory tests:

For the routine workup on patients, liver function and renal function (for diabetic patients) tests, ALT, and viral load values were obtained from the laboratory information system. Where ALT results were unavailable, these patient samples were subsequently analysed on a ROCHE Cobas 8000 (Roche, USA), using stored serum to ascertain the ALT value. Less than 20% of all patients had recent CD4 cell counts available, so this data was not included. The practice at the CMJAH regard viral load of <100 copies/ml as viral suppression, therefore this cut off was used in this study.

2.12 Anonymity and confidentiality

Anonymity was ensured by using research identifiers and the names of the participants deleted. To ensure confidentiality the clinical and laboratory information and health status of the participants was not linked to their names. The study obtained ethical approval from the Wits Research Ethics Committee at the University of the Witwatersrand (M1706102 (Appendix B.3)).

2.13 Data analysis

2.13.1 Statistics

Normally distributed variables were described as means (± SD) and analyzed with parametric testing and non-normally distributed variables as medians (interquartile ranges) and analyzed with non-parametric tests. Categorical variables were described as numbers (percentages). Bland-Altman plots were used to assess correlations between plasma and DBS methods. To determine the difference between plasma compared to DBS and DPS, the formula (%difference = concentration in fluid 1-concentration in fluid 2/mean concentration * 100) as proposed by Duthaler *et al*, (2018) was utilized.

Theoretical plasma concentration of patients with lower HCTs were obtained by adjusting DBS concentration with the patient's HCT and with the fraction of drug bound to plasma protein (fbpp) as proposed by Kromdijk *et al*. (2012) using the formula : [DBS[analyte]/(1-HCT)]* fbpp = plasma [analyte]. The fbpp used for 3TC, ABC, EFV, RTV and LPV was 0.29, 0.50, 0.99, 0.98 and 0.98, respectively (Boffito *et al*., 2003, Duthaler *et al*., 2018).

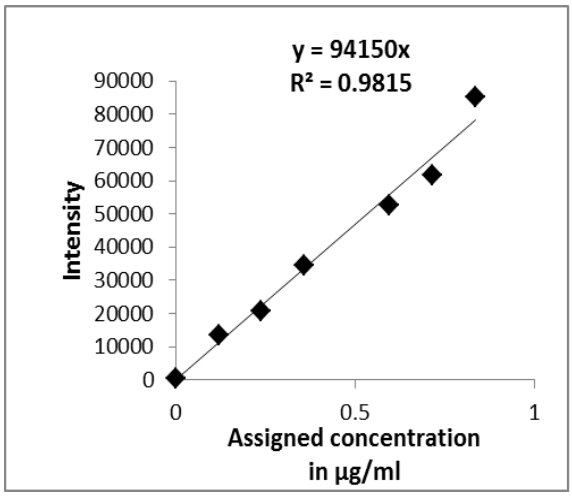
Plasma is considered a gold standard hence plasma ARV drug concentrations were used to reference the therapeutic ranges based on previously published data (Duong *et al*., 2004, Moyer *et al*., 1999, Donnerer *et al*., 2003, Alexander *et al*., 2003, Kaufman, 2013, Schoenenberger *et al*., 2013) and they are as follows: 0.100-1.000 µg/ml for 3TC, 0.119-833 µg/ml for ABC, 1.100-4.000 µg/ml for EFV, >1.110 µg/ml for RTV and 3.000-7.000 µg/ml for LPV, 3.400-6.000 µg/ml, 0.060-0.417 µg/ml and 0.150-0.850 µg/ml ATV.

Chapter 3: Results

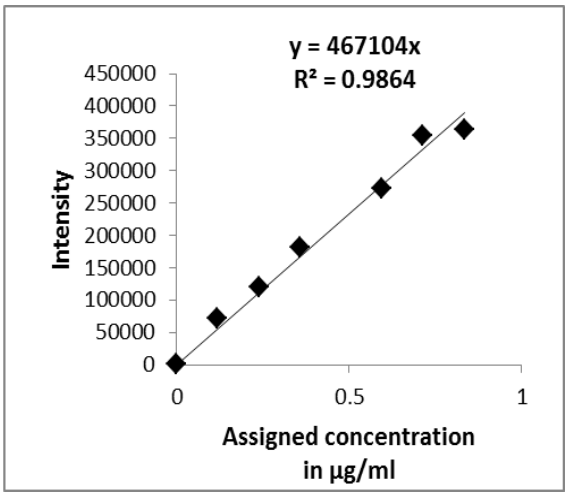
3.1 DBS Assay validation

3.1.1 Linearity

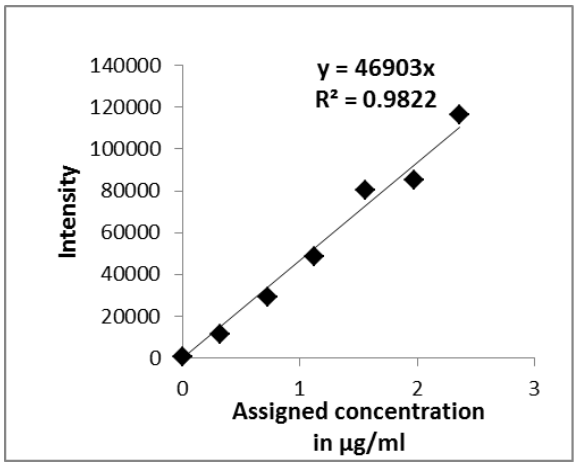
The calibration curves were fit using a least-square linear regression model and found to be linear for all drugs over their respective concentration ranges (see figure 3(a-e)). The drugs had the acceptable regression co-efficient (R^2) of ≥ 0.98. The linearity ranged from 0.9815 for 3TC to 0.9983 for LPV.



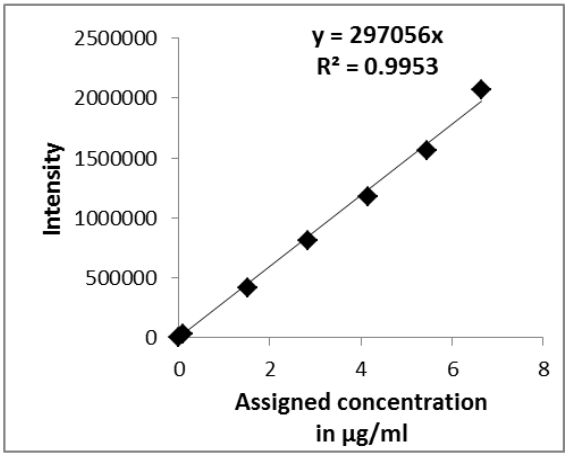
3(a) 3TC



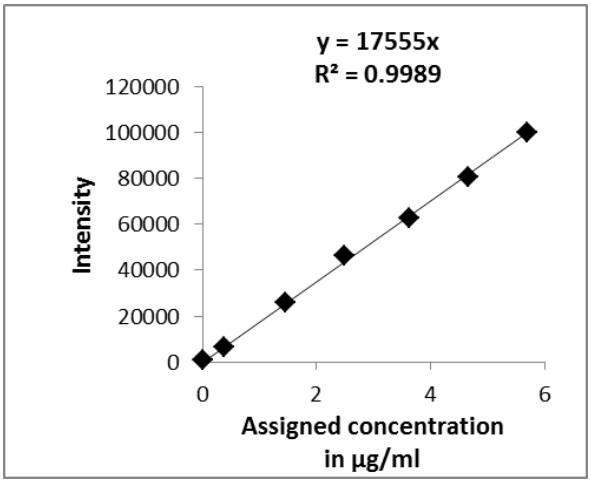
3(b) ABC



3(c) EFV



3(d) RTV



3(e) LPV

Figure 3 (a-e): Linear regression between the mean peak height and assigned drug concentration over the course of 5 days

3.1.2 Accuracy for DBS

Table 7 shows the accuracy of the drugs over the course of 5 days. Using QC samples the % difference between the mean and assigned concentrations were within acceptable deviation of 15%. The % difference for the LQC ranged from -12% for LPV to 2% for ABC and for HQC it ranged from -3% to 4%, for LPV and 3TC, respectively.

Table 7: Accuracy of the drugs in DBS

Drug	Mean concentration (SD) (µg/ml)	Assigned concentration (µg/ml)	% difference
3TC			
LQC	0.119 (±0.010)	0.121	-1
HQC	0.800 (±0.109)	0.768	4
ABC			
LQC	0.138 (±0.009)	0.135	2
HQC	0.913 (±0.097)	0.889	3
EFV			
LQC	0.552 (±0.064)	0.597	-8
HQC	1.761 (0.165)	1.725	2
RTV			
LQC	0.673 (±0.083)	0.744	-10
HQC	4.410 (±0.524)	4.494	-2
LPV			
LQC	0.873 (±0.098)	0.987	-12
HQC	3.686 (±0.323)	3.788	-3

LQC = Lower quality control; HQC = High quality control; SD = Standard deviation.

The percentage differences using the 6-point standard curve were within acceptable deviation of 15% with the exception of 3TC where a percentage difference of 18% at STD 6 was observed. This data is available supplementary data (A 1.1.2)

3.1.3 Precision for DBS

Intra-day precision and Inter-day precision was found to be acceptable for ABC, EFV and RTV as % CV values of the drugs were within acceptable deviation of 15%. 3TC showed unacceptably high CV (17%) at HQC concentration and LPV at LQC concentration (16%) (Table 8).

Table 8: **Inter-day and Intra-day precision for DBS at LQC and HQC for all drugs**

		Intra-day			Inter-day		
Drugs		**Mean (µg/ml)**	**SD**	**CV**	**Mean (µg/ml)**	**SD**	**CV**
3TC	LQC	0.101	0.005	5	0.119	0.010	9
	HQC	0.743	0.129	17	0.800	0.109	14
ABC	LQC	0.131	0.017	13	0.138	0.009	7
	HQC	0.813	0.059	7	0.913	0.097	11
EFV	LQC	0.588	0.084	14	0.552	0.064	12
	HQC	1.532	0.063	4	1.761	0.165	9
RTV	LQC	0.684	0.088	13	0.673	0.083	12
	HQC	4.268	0.528	12	4.410	0.524	12
LPV	LQC	0.887	0.138	16	0.873	0.098	11
	HQC	3.622	0.457	13	3.686	0.323	9

LQC = Lower quality control; HQC = High quality control; SD = Standard deviation; CV = Coefficient of variation

Intra-day precision = analysis over the course of 5 days

Intra-day precision = analysis within one day

3.1.4 Recovery (extraction efficiency) for DBS

Table 9 shows the mean value percentage recoveries of the drugs at LQC and HQC. All drugs had acceptable recoveries that were between 80-120%. The % recovery for the LQC ranged from 88%, to 102% and for HQC it ranged from 97% to 104%.

3.1.5 LOD and LOQ for DBS

The LOD and LOQ values for the spiked DBS samples are shown in Table 10. The LOD ranged from 0.006 µg/ml for RTV to 0.110 µg/ml for LPV and at the LOQ ranged from 0.021 µg/ml for RTV to 0.367 µg/ml for LPV.

Table 9: Mean percentage recovery for DBS over the course of 5 days

Drugs		% Recovery
3TC	LQC	99
	HQC	104
ABC	LQC	102
	HQC	103
EFV	LQC	92
	HQC	102
RTV	LQC	90
	HQC	98
LPV	LQC	88
	HQC	97

LQC= Lower quality control; HQC= High quality control

Table 10: Limit of detection and limit of quantification at LQC and HQC for the drugs in DBS

Drugs	LOD (µg/ml) S/N > 3	LOQ (µg/ml) S/N > 10
3TC	0.010	0.034
ABC	0.007	0.025
EFV	0.053	0.178
RTV	0.006	0.021
LPV	0.110	0.367

LOD= Limit of detection; LOQ= Limit of quantification; S/N = Signal to noise ratio.

3.1.6 Stability studies (effect of drying time)

Stability of the drugs was determined for a period of 30 days prior to extraction of the samples. The stability was assessed using the LQC and HQC samples, shown in table 11. The concentrations of measured drugs were not affected by any time delays between spotting and extraction. The largest % differences were observed on day 2 and 7 for LPV where it ranged from -20% at LQC to 16% at HQL.

Table 11: The % difference of LQC and HQC versus the assigned concentration for days 1, 2, 7 and 30

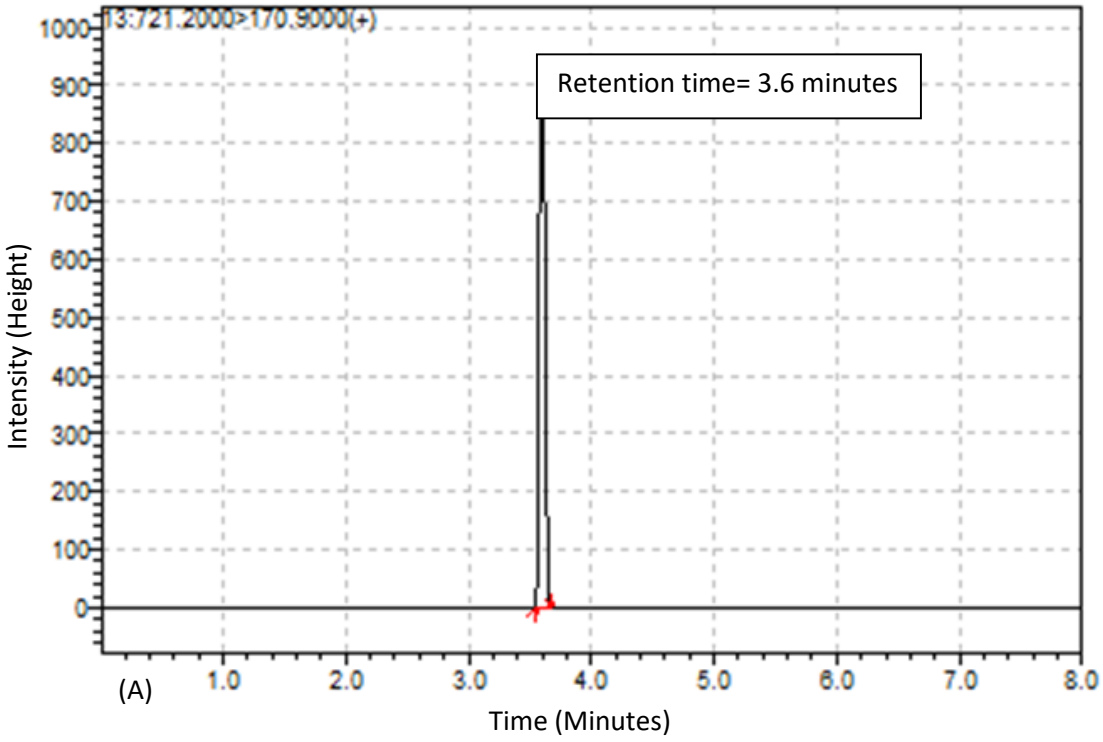
Drug	AC (µg/ml)	MC (µg/ml)	% D	MC (µg/ml)	% D	MC (µg/ml)	% D	MC (µg/ml)	% D
		Day 1		Day 2		Day 7		Day 30	
3TC									
LQC	0.121	0.108	-11	0.132	9	0.131	8	0.127	5
HQC	0.768	0.715	-7	0.885	15	0.806	5	0.765	0
ABC									
LQC	0.135	0.130	-4	0.130	-4	0.124	-8	0.134	-1
HQC	0.889	0.766	14	0.862	-3	0.872	-2	0.763	-14
EFV									
LQC	0.597	0.607	2	0.668	12	0.612	3	0.649	9
HQC	1.725	1.545	-10	1.686	-2	1.861	8	1.543	-11
RTV									
LQC	0.744	0.773	4	0.619	-7	0.688	-8	0.833	12
HQC	4.494	5.073	13	4.520	1	4.057	-10	4.854	8
LPV									
LQC	0.987	0.796	-11	0.969	-2	0.787	-20	0.972	-2
HQC	3.788	3.659	-5	4.398	16	3.680	-3	4.304	14

LQC= Lower quality control; HQC= High quality control; AC = Assigned concentration; MC = Measured concentration; %D = % difference concentration

3.1.7 Robustness for DBS

Method robustness was tested by changing the length, type and temperature of the column, injection volume, extraction solution and the composition of the mobile phase. The chromatogram obtained for a sample containing representative impurities, when using modified parameter(s), was compared to the chromatogram obtained using the target parameters. Changing the column type influenced the retention times and the intensities of all the drugs whereas the peak shape remained the same. Figure 4 shows the chromatogram of RTV obtained from different columns for illustration purposes only. Shifts in retention times (3.2 to 3.6 minutes for RTV (short retentions times on C18 column)) were noted. Alterations of the injection volume, ranged from 2 µL to 10 µL, where 10 µL gave a better

response on all drugs. Acetonitrile was used as an alternative extraction solution, however, this caused under recovery of LPV and EFV, except for the remaining drugs. Ammonium acetate was used as an alternative additive to the buffer, all the drugs were detected under these running conditions, except for EFV. At a high column temperature (40°C) clearer chromatograms were obtained for all the drugs.



(A)

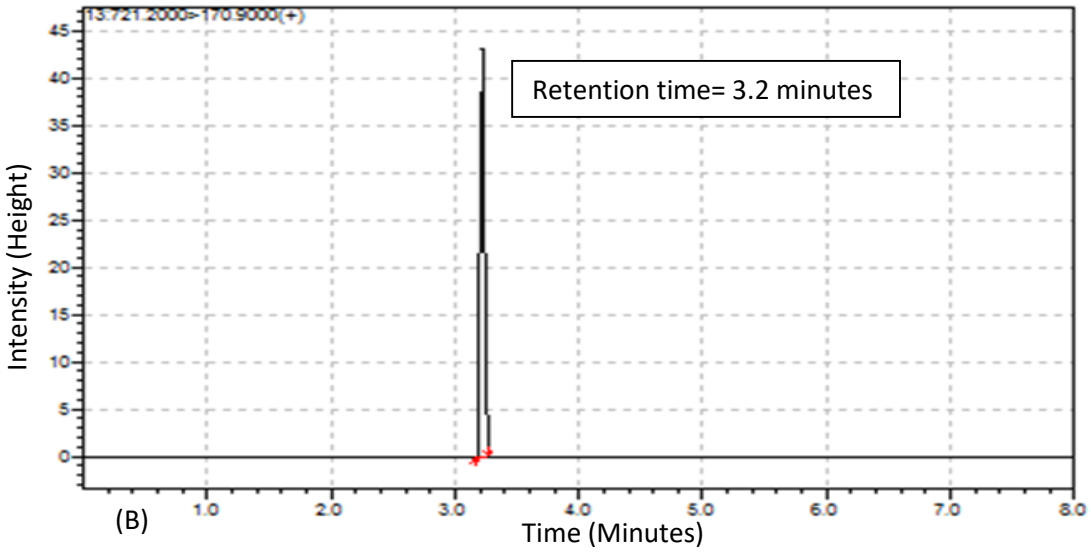
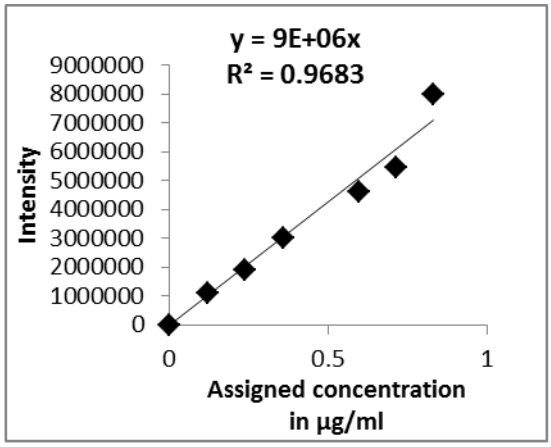


Figure 4: UHPLC chromatogram of (A) RTV standard 1 using a T3 column for separation and (B) RTV standard 1 using a C18 column for separation

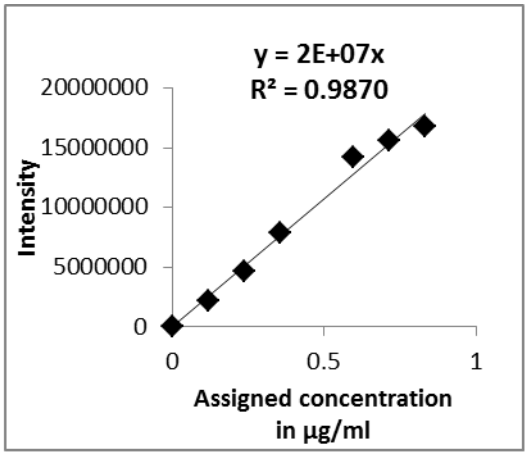
3.2 DPS Assay validation

3.2.1 Linearity for DPS

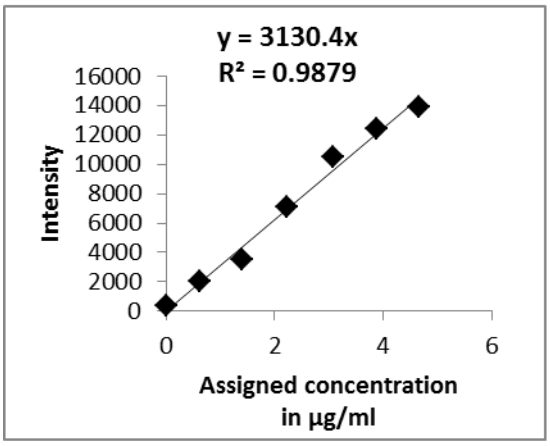
The calibration curves were found to be linear for the drugs over their respective concentration ranges (see figure 5 (a-i)). The drugs had acceptable regression co-efficient (R^2) of ≥ 0.98 except for 3TC, NVP, ATV and TMC114. The linearity ranged from 0.9298 for ATV to 0.9953 for RAL.



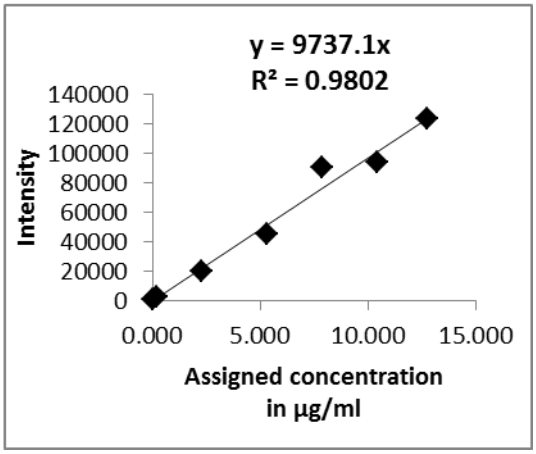
5(a) 3TC



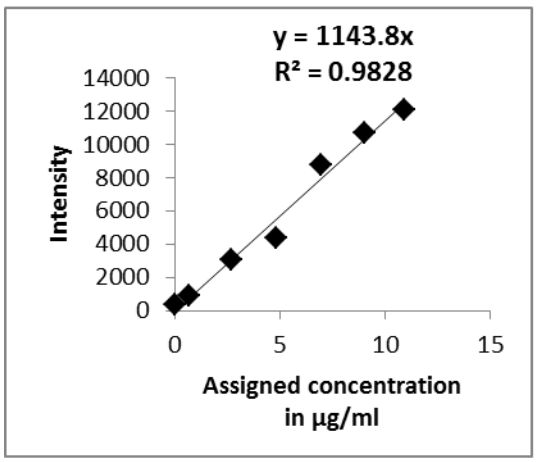
5(b) ABC



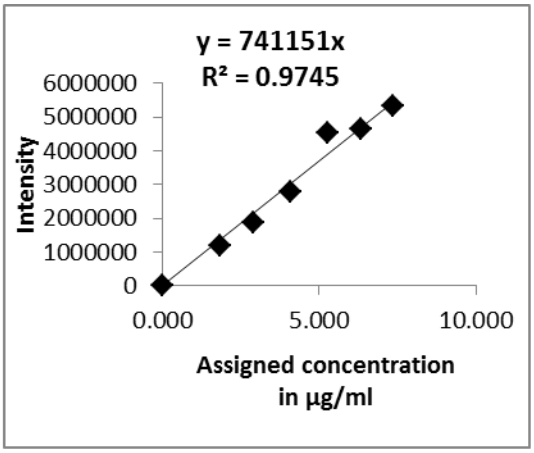
5(c) EFV



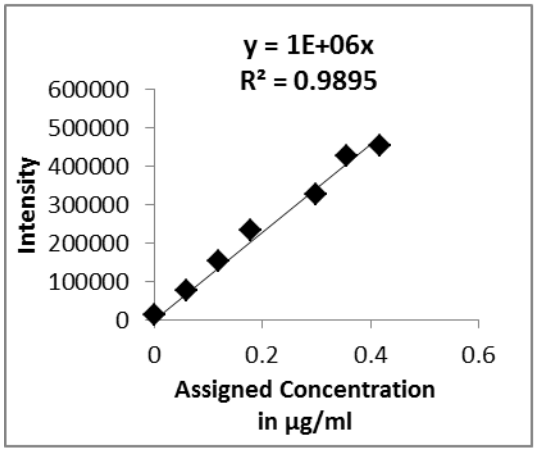
5(d) RTV



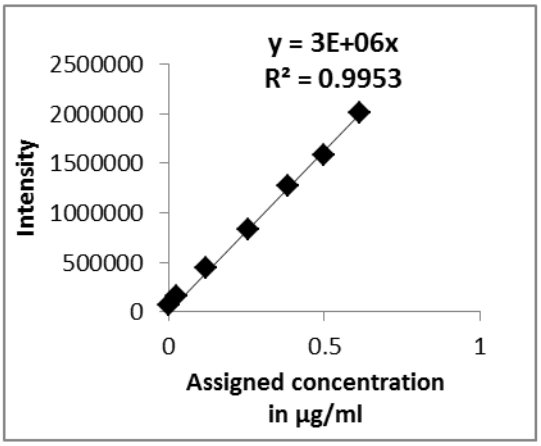
5(e) LPV



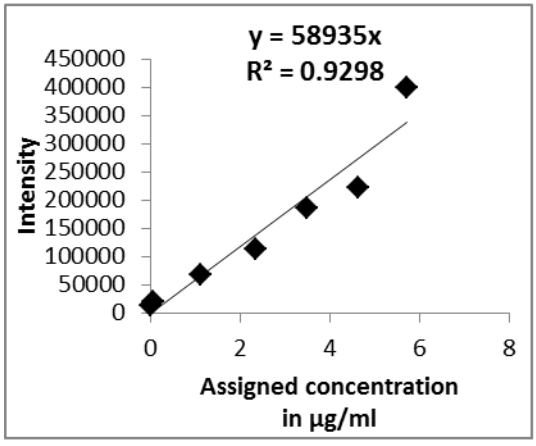
5(f) NVP



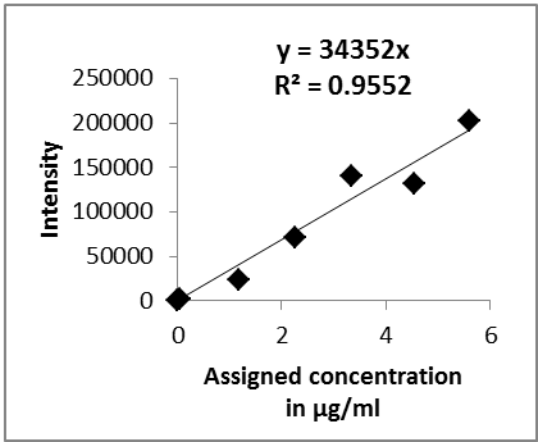
5(g) FTC



5(h) RAL



5(i) ATV



5(j) TMC114

Figure 5(a-j): Linear regression curves between the mean peak height and assigned drug concentration over the course of 5 days

3.2.2 Accuracy for DPS

Table 12 shows the accuracy of the drugs over the course of 5 days. Using QC samples, the % difference was within acceptable deviation of 15%. The % difference for the LQC ranged from -12% for RTV to 6% for RAL and for HQC it ranged from -6% to 6% for RTV and RAL, respectively.

Table 12: Accuracy of the drugs on DPS

Drug		Mean Concentration (µg/ml (±SD))	Assigned Concentration (µg/ml)	% Difference
3TC	LQC	0.126 (±0.010)	0.121	4
	HQC	0.815 (±0.048)	0.843	-3
ABC	LQC	0.113 (±0.020)	0.127	-11
	HQC	0.762 (±0.062)	0.775	1
EFV	LQC	1.398 (±0.139)	1.486	-6
	HQC	3.481 (±0.605)	3.533	-1
RTV	LQC	1.068 (±0.124)	1.212	-12
	HQC	10.768 (±1.266)	11.397	-6
LPV	LQC	2.063 (±0.219)	2.050	1
	HQC	10.885 (±1.023)	11.085	-2
NVP	LQC	3.209 (±0.328)	3.090	4
	HQC	5.812 (±0.562)	5.939	-2
FTC	LQC	0.048 (±0.003)	0.054	-11
	HQC	0.407 (±0.024)	0.418	-3
RAL	LQC	0.077 (±0.004)	0.073	6
	HQC	0.491 (±0.017)	0.464	6
ATV	LQC	0.624 (±0.065)	0.619	1
	HQC	6.003 (±0.760)	6.044	-1
TMC114	LQC	0.567 (±0.108)	0.604	-6
	HQC	4.627 (±0.377)	4.837	-4

LQC = Lower quality control; HQC = High quality control; SD = Standard deviation.

The percentage difference using the 6-point standard curve was within the accepted range for all the drugs except for EFV at STD 2, LPV at STD 3, ATV at STD 2, 3 and 6 and TMC114 at STD 2, 3, 4 and 5 with percentage differences exceeding 15%. This data is available as supplementary (A: 2.3.2, A: 2.5.2, S: 2.9.2 and A: 2.10.2).

3.2.3 Precision on DPS

Intra-day precision and Inter-day precision was found to be acceptable for 3TC NVP, FTC and RAL as % CV values of all the analytes were within acceptable deviation of 15%. ABC showed unacceptably high CV values at HQC and LQC concentrations whereas RTV, LPV and TMC114 showed unacceptably high CV values only at LQC concentrations. Lastly EFV showed unacceptably high CV value only at HQC concentrations Inter-day precision whereas ATV showed unacceptably high CV value only at HQC concentrations Intra-day precision (Table 13).

Table 13: Intra-day and inter-day precision in DPS

		Intra-day			Inter-day		
Drug		**Mean (µg/ml)**	**SD**	**CV**	**Mean (µg/ml)**	**SD**	**CV**
3TC	LQC	0.105	0.005	5	0.126	0.010	8
	HQC	0.825	0.063	8	0.815	0.048	6
ABC	LQC	0.128	0.015	12	0.113	0.020	17
	HQC	0.591	0.143	24	0.762	0.062	9
EFV	LQC	1.366	0.066	5	1.398	0.139	10
	HQC	3.398	0.264	8	3.481	0.605	17
RTV	LQC	1.520	0.299	20	1.0.68	0.124	12
	HQC	11.804	0.544	5	10.768	1.266	12
LPV	LQC	2.384	0.434	18	2.063	0.219	11
	HQC	12.794	0.454	4	10.885	1.023	9
NVP	LQC	2.593	0.027	1	3.209	0.328	10
	HQC	5.727	0.535	9	5.812	0.562	10
FTC	LQC	0.051	0.005	9	0.048	0.003	5
	HQC	0.400	0.009	2	0.407	0.024	6
RAL	LQC	0.068	0.007	10	0.077	0.004	6
	HQC	0.401	0.022	5	0.491	0.017	3
ATV	LQC	0.485	0.041	8	0.624	0.065	10
	HQC	5.524	1.307	24	6.003	0.760	13
TMC114	LQC	0.678	0.033	5	0.567	0.108	19
	HQC	4.754	0.340	7	4.627	0.377	8

LQC = Lower quality control; HQC = High quality control; SD = Standard deviation; CV = Coefficient of variation

(CV after excluding not more than two outliers)

3.2.4 Recovery (extraction efficiency) for DPS

The drugs had acceptable recoveries that were between 80-120%. Table 14 shows the mean value percentage recovery of the drugs at LQC and HQC over the course of 5 days. The % recovery for the LQC ranged from 88% for RTV to 106% for RAL and for HQC it ranged from 94% for RTV to 106% for RAL, respectively.

Table 14: Mean percentage recovery of the analytes at LQC and HQC in DPS

Drug	% recovery	
3TC	LQC	104
	HQC	97
ABC	LQC	89
	HQC	101
EFV	LQC	94
	HQC	99
RTV	LQC	88
	HQC	94
LPV	LQC	101
	HQC	98
NVP	LQC	104
	HQC	98
FTC	LQC	89
	HQC	97
RAL	LQC	106
	HQC	106
ATV	LQC	101
	HQC	99
TMC114	LQC	94
	HQC	96

LQC= Lower quality control; HQC= High quality control

3.2.5 LOD and LOQ for DPS

The limit of detection and limit of quantification values for the spiked DPS samples are shown in Table 15. The LOD ranged from 0.002 µg/ml for 3TC to 0.423 µg/ml for LPV and the LOQ ranged from 0.008 µg/ml for 3TC to 1.409 µg/ml for LPV.

Table 15: Limit of detection and limit of quantification for the drugs in DPS

Drug	LOD (µg/ml) S/N > 3	LOQ (µg/ml) S/N > 10
3TC	0.002	0.008
ABC	0.003	0.009
EFV	0.320	1.068
RTV	0.188	0.625
LPV	0.423	1.409
NVP	0.008	0.027
FTC	0.007	0.024
RAL	0.024	0.080
ATV	0.099	0.330
TMC114	0.083	0.277

LOD= Limit of detection, LOQ= Limit of quantification S/N = signal -to- noise ratio.

3.2.6 Stability for DPS

Stability of the drugs was determined for a period of 30 days prior to extraction of the samples. It was noted that RTV and TMC114 exhibited poor recovery of the LQC at day 2. At day 7 ABC and RTV showed good recovery for both LQC and HQC, while NVP, RAL and ATV had poor recovery for both LQC and HQC. LPV, NVP and FTC had poor recovery of the LQC, while 3TC and TMC114 had poor recovery of the HQC.

3.3 Haematocrit determination in DBS

The results of varying HCT show that there is no effect at HCT concentration of 45% in samples spiked with lower (STD 1) and higher (STD 6) drug concentrations (Table 17). However, over-recovery was shown in the samples spiked with the lower standard (HCT concentrations of 36%, 18% and 9%) for all

the drugs. Recovery of the samples spiked with the higher standard (STD 6) was acceptable for the drugs except for 3TC and ABC where recovery was over the limit of 20% at the HCT concentrations of 36%, 18% and 9%.

Table 16: Differences between assigned and mean concentrations in DPS expressed as % for days 1 and 7

Drug		Assigned concentration (µg/ml)	Mean concentration (µg/ml)	% Difference	Mean concentration (µg/ml)	% Difference
			Day 1		Day 7	
3TC	**LQC**	0.121	0.122	-4	0.143	13
	HQC	0.843	0.824	-3	0.683	-19
ABC	**LQC**	0.127	0.130	3	0.120	-6
	HQC	0.775	0.785	4	0.754	0
RTV	**LQC**	1.212	1.017	-16	1.181	-3
	HQC	11.397	11.019	-3	11.158	-2
LPV	**LQC**	2.050	2.063	1	1.298	-37
	HQC	11.085	10.594	-4	11.582	4
NVP	**LQC**	3.090	3.341	8	2.434	-21
	HQC	5.939	5.877	-1	6.901	16
FTC	**LQC**	0.054	0.056	3	0.077	43
	HQC	0.418	0.423	1	0.438	5
RAL	**LQC**	0.070	0.072	3	0.104	49
	HQC	0.458	0.480	5	0.351	-23
ATV	**HQC**	0.581	0.591	2	0.978	68
	LQC	6.044	5.679	-6	3.702	-39
TMC114	**LQC**	0.604	0.491	-19	0.570	-6
	HQC	4.837	4.847	0	4.050	-16

Table 17: The effect of varying HCT on drug recovery in DBS

Drug		%HCT							
		45% HCT		36% HCT		18% HCT		9% HCT	
	AC	MC	% recovery	MC	% recovery	MC	% recovery	MC	% recovery
	µg/ml								
3TC									
STD 1	0.119	0.130 ±0.015	109	0.290 ±0.079	244	0.219 ±0.040	184	0.251 ±0.012	211
STD 6	0.833	0.892 ±0.219	107	1.088 ±0.188	131	1.254 ±0.523	151	1.207 ±0.214	145
ABC									
STD 1	0.119	0.119 ±0.008	100	0.563 ±0.051	473	0.592 ±0.130	497	0.532 ±0.113	447
STD 6	0.833	0.853 ±0.080	102	2.176 ±0.383	261	2.349 ±0.318	282	2.073 ±0.236	249
EFV									
STD 1	0.323	0.344 ±0.050	107	0.581 ±0.046	180	0.522 ±0.051	162	0.588 ±0.053	182
STD 6	2.360	2.413 ±0.335	102	2.726 ±.390	116	2.670 ±0.331	113	2.117 ±0.302	90
RTV									
STD 1	0.104	0.120 ±0.004	116	1.418 ±0.236	1364	1.463 ±0.305	1406	1.577 ±0.369	1517
STD 6	6.640	6.207 ±0.099	93	7.640 ±1.861	106	1.712 ±0.806	106	7.093 ±0.792	107
LPV									
STD 1	0.370	0.359 ±0.037	97	0.946 ±0.149	256	0.939 ±0.261	254	0.936 ±0.089	253
STD 6	5.685	5.532 ±0.785	97	5.832 ±0.363	103	4.673 ±1.079	82	4.600 ±0.204	81

AC = Assigned concentration; MC = Measured concentration; STD = Standard concentration

3.4 Patients results

Two hundred and thirty-one patients on 1st and 2nd line treatment consented to participate in this study. The characteristics of all the patients included are summarized in Table 18. The mean age of participants on first line treatment was 45 ± 10 year and 44 ± 11 years for those on second line treatment. Most patients were female (58% and 68% for 1st and 2nd line treatment, respectively) where 90% and 76% on 1st and 2nd line treatment, respectively, had achieved viral suppression (<100 copies/ml). The average HCT was the same (42% ±7) for 1ST and 2nd line treatment. Low HCT concentrations were seen in 5% and 6% of males and females, respectively, whereas high HCT concentrations were seen in 6% and 5% of males and females, respectively. The proportions of patients who were underweight, normal weight, overweight and obese were similar in both groups.

Table 18: Patient characteristics of all Patients

Characteristics	**1st line (n=122)**	**2nd line (n=109)**
Age, yrs. (mean(±SD))	45 (±10)	44 (±11)
Gender		
Female N (%)	71 (58)	74 (68)
HIV RNA N (%)		
<100 copies/ml	110 (90)	76 (70)
>100 copies/ml	12 (10)	33 (30)
Mean ALT (U/L (±SD)	28 (±29**)**	24 (±15**)**
Mean BMI (kg/m^2) (N)		
BMI ≤18.5 kg/m^2	15.9 (1)	18.1 (1)
BMI ≥18.5 and ≤25 kg/m^2	21.9 (43)	21.9 (46)
BMI ≤25 and ≤30 kg/m^2	27.4 (47)	26.9 (30)
BMI ≥30 kg/m^2	35.5 (31)	36.2 (32)
Mean haematocrit (% (±SD))	42 (±7)	42 (±7)
Mean haematocrit for males (%(±SD))	44 (±7)	46 (±7)
Mean haematocrit for female (%(±SD))	40 (±6)	40 (±5)

3.4.1 Agreement between plasma compared to venous DBS, plasma compared to capillary DBS and venous compared to capillary DBS

Figure 6 shows correlation coefficients and Bland-Altman plots for 3TC (6a), ABC (6b), EFV (6c), RTV (6d) and LPV (6e) between plasma and venous DBS, plasma and capillary DBS and venous DBS and capillary DBS. Most of the differences in concentration between the two assays fell within ±2 standard deviations (95% confidence interval [95% CI]).

Biases of +55% for 3TC, +14% for ABC, +49% for EFV, +33% for RTV and +49% and for LPV between plasma compared to venous DBS were observed. For comparison of plasma with capillary DBS a bias of +48% for 3TC, +40% for ABC, +66% for EFV, +41% for RTV and +57% LPV was observed. In contrast, comparison between venous DBS and capillary DBS concentrations showed acceptable bias that was +8%, +3%, +16%, +7%, and +9% for 3TC, ABC, EFV, RTV and LPV, respectively.

3.4.2 Method Comparison: plasma compared to venous DBS, plasma compared to capillary DBS and venous DBS compared to capillary DBS

Table 19 shows the number of patients who had allowable 20% difference for each drug when comparing plasma to venous DBS, plasma to capillary DBS and venous DBS to capillary DBS. The percentage of patients with allowable % difference ranged from 7% for LPV to 79% for ABC between plasma and venous DBS, 3% for LPV to 75% for ABC between plasma and capillary DBS and 22% for LPV to 93% for RTV between venous DBS and capillary DBS.

Lamivudine (3TC)

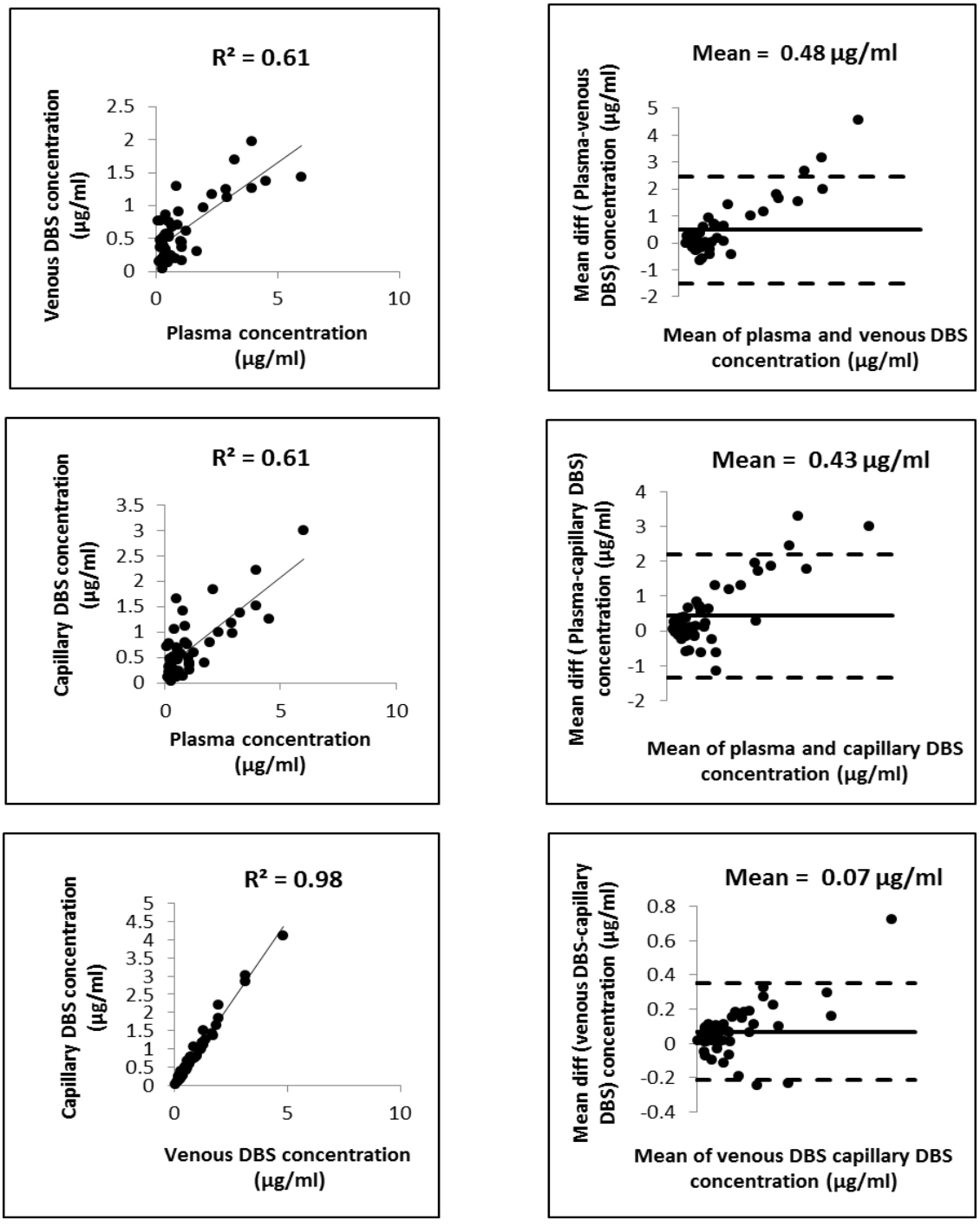


Figure 6 (a): 3TC correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Abacavir (ABC)

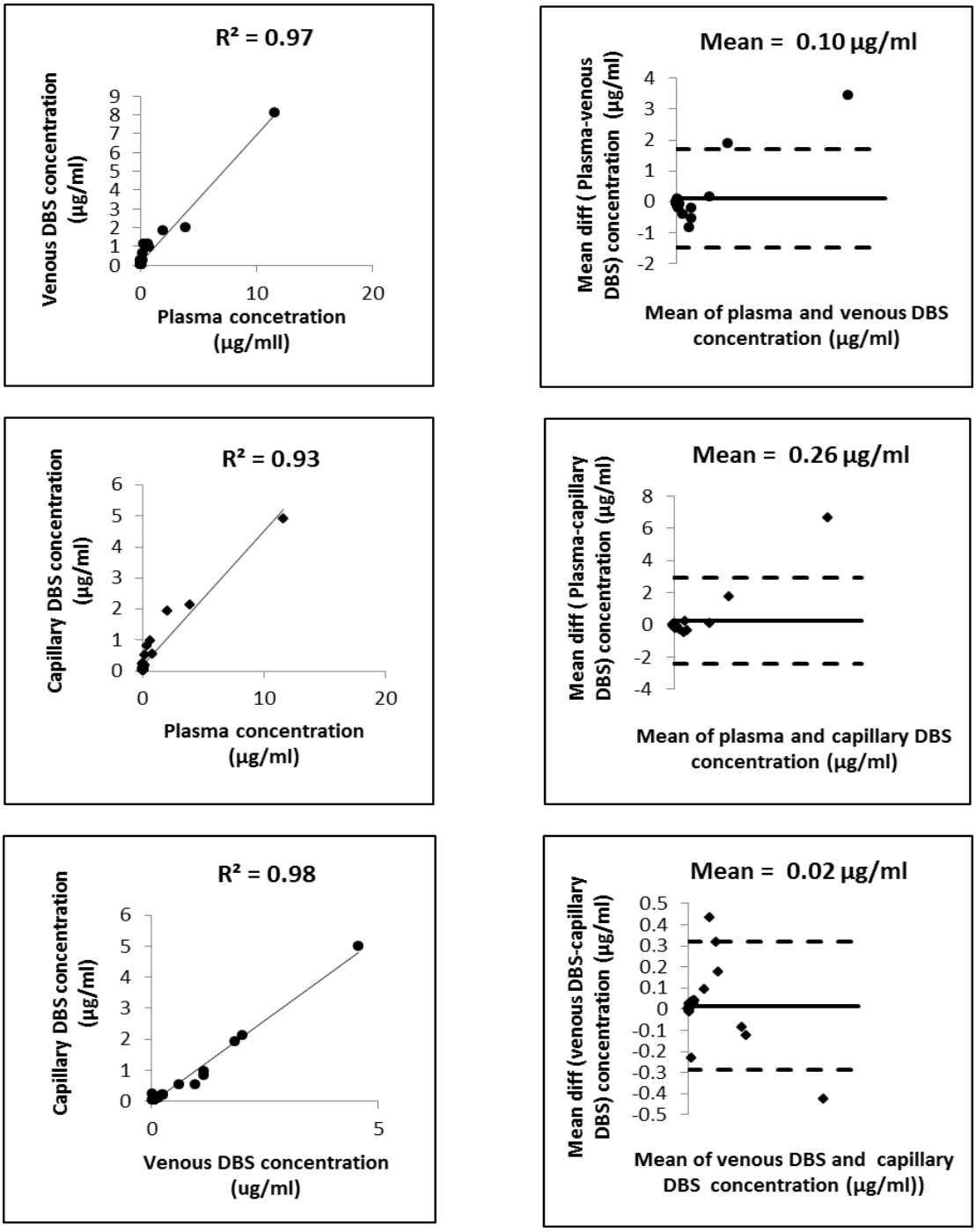


Figure 6 (b): ABC correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Efavirenz (EFV)

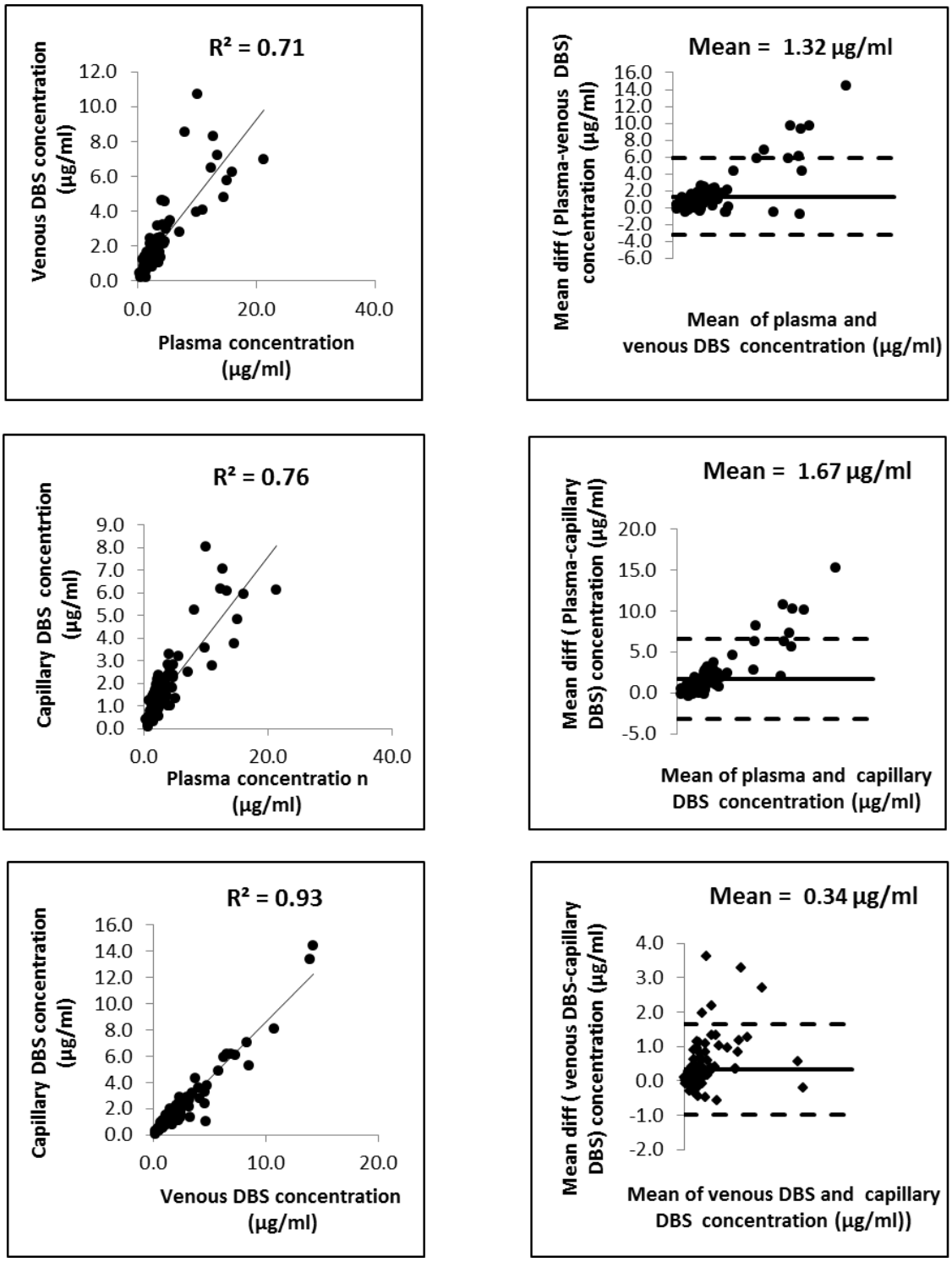


Figure 6 (c): EFV correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Ritonavir (RTV)

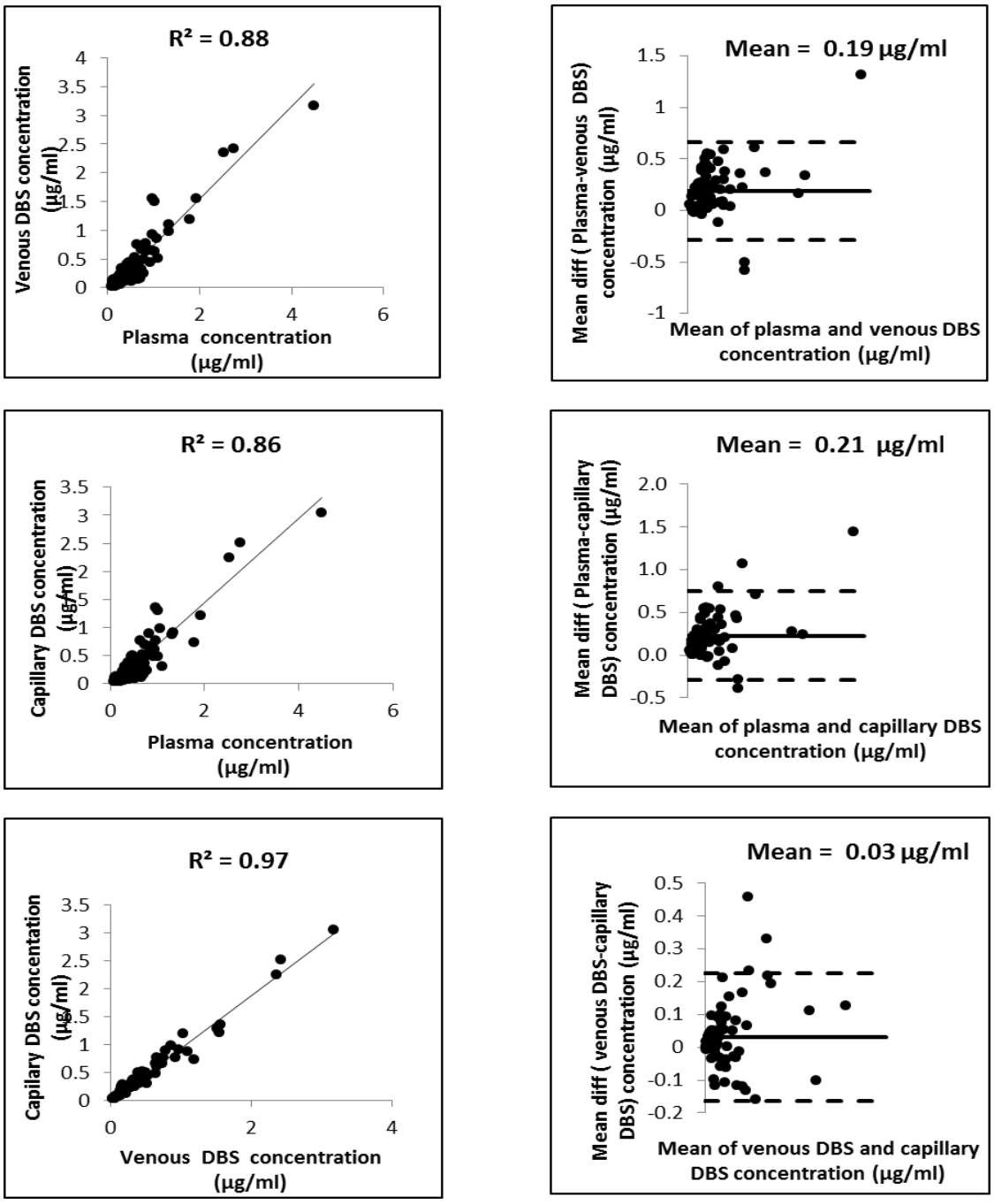


Figure 6 (d): RTV correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Lopinavir (LPV)

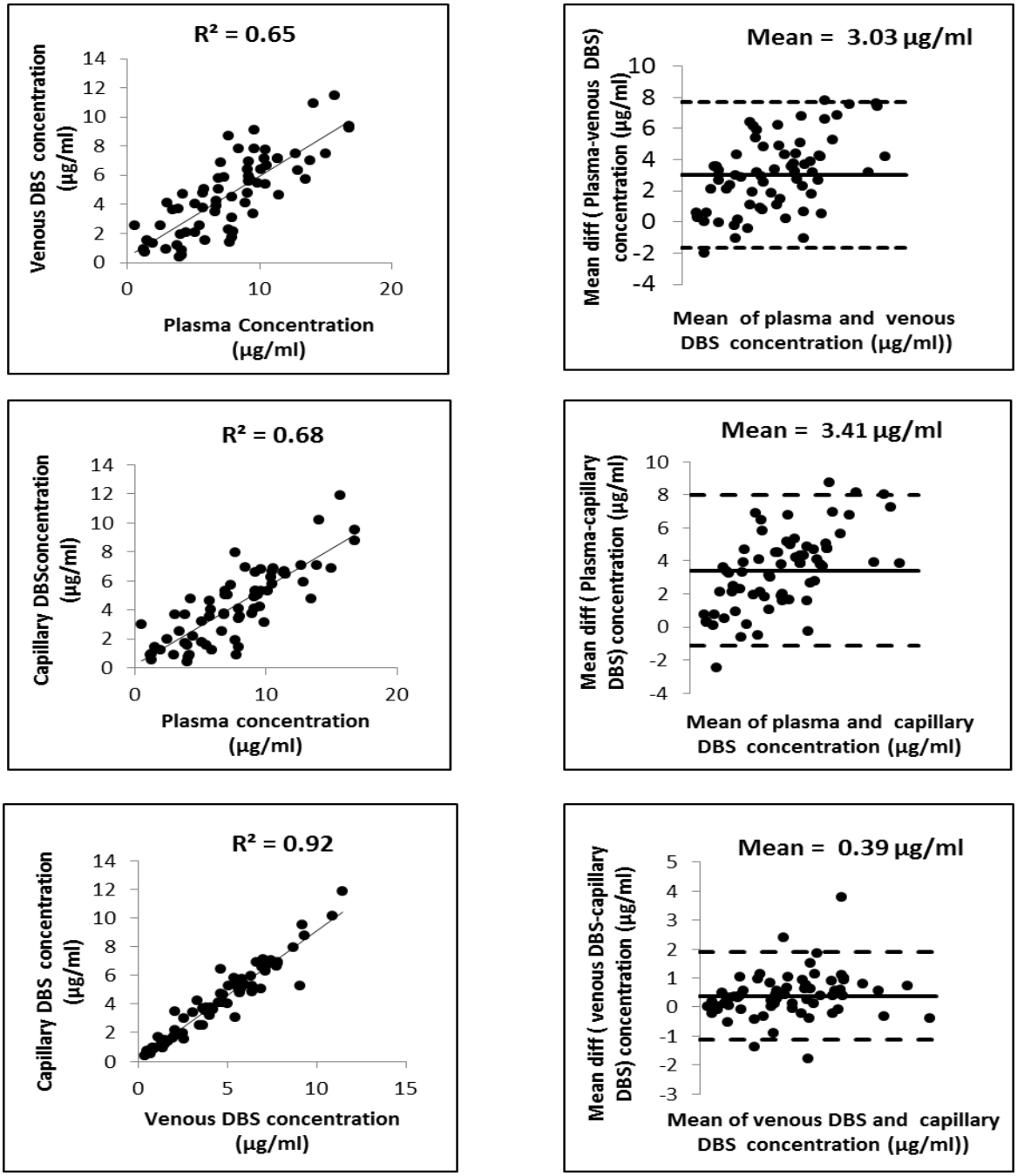


Figure 6 (e): LPV correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Figure 6 (a-e): Correlation coefficients and Bland-Altman plots for drugs between plasma and venous DBS, plasma and capillary DBS and venous DBS and capillary DBS.

Table 19: The number of patients with acceptable percentage difference when plasma is compared to venous DBS and capillary DBS and when venous DBS is compared to capillary DBS

	Plasma and Venous DBS	
Drug (Expected No)	**No. detected in both plasma and venous DBS**	**No. within ±20% limits** (%)
3TC (62**)**	48	11 (23)
ABC (29)	26	4 (15)
EFV (118)	107	24 (22)
RTV (109)	74	21 (28)
LPV (83)	68	21 (18)
	Plasma and capillary DBS	
Drug (Expected No)	**No. detected in both plasma and capillary DBS**	**No. within ±20% limits**
3TC (62)	51	7 (14)
ABC (29)	26	6 (23)
EFV (118)	107	10 (9)
RTV (109)	74	13 (18)
LPV (83)	68	7 (10)
	Venous DBS and capillary DBS	
Drug (Expected No)	**No. detected in both venous DBS and capillary DBS**	**No. within ±20% limits**
3TC (62)	53	37 (70)
ABC (29)	26	16 (62)
EFV (118)	112	73 (65)
RTV (109)	76	53 (70)
LPV (83)	68	48 (71)

Table 20 shows the percentage of patients who had undetectable drug levels in both plasma and DBS or only had detectable drug levels in plasma. Where drug levels were detected in plasma, but not DBS, the plasma drug levels were found to be low.

Table 20: Patient's with undetectable drug levels

Expected (N)	VL copies/ml <100		VL copies/ml >100	
	% Not detected in plasma and DBS (N)	% Detected in plasma only (N)	% Not detected in plasma and DBS (N)	% Detected in plasma only (N)
3TC (62)	11 (7)	0	8 (5)	0
ABC (29)	0	0	7 (2)	0
EFV (118)	2 (3)	1 (1)	0	0
RTV (109)	3 (3)	4 (4)	8 (9)	0
LPV (83)	2 (2)	0	10 (8)	0

Amongst patients with undetectable VLs there was good concordance with the detection of the drugs tested in plasma and DBS. These drugs were detected in plasma, but not in DBS. For those patients with VL > 100 copies/ml there was good concordance with the detection of the drugs in plasma and DBS.

Plasma, venous DBS and capillary DBS drug concentrations for all the patients were analyzed and are shown in table 21. More patients were on EFV than on any other drug. The highest concentrations of drugs were observed in plasma, followed by venous DBS and the lowest concentrations were observed in capillary DBS. This is with the exception of ABC, where the highest concentrations were seen in capillary DBS followed by venous DBS and the lowest concentrations in plasma. Drugs concentrations do not differ significantly when plasma is compared to venous DBS and capillary DBS, except for LPV with the p-value of 0.011 and 0.001, respectively. Thirty-two percent (75) of the patients had drug concentrations in the toxic range. Of these, seventy-six percent (57) of patients achieved viral suppression. Twenty-one percent (49) of patients had sub-therapeutic drug concentration amongst which eighty-six percent (42) patient's achieved viral suppression. Thirty-nine

percent (91) of the patient's had therapeutic drug concentrations amongst which eighty-nine percent (81) of the patient's achieved viral suppression and eleven percent of patient's (10) being non-virally suppressed (>100 copies/ml). Seven percent (16) had no drugs detected in their samples. However, forty-four percent (7) achieved viral suppression.

Table 21: Patients drug concentration levels

Drugs (N)	Plasma concentration (µg/ml)	Venous DBS concentration (µg/ml)	Capillary DBS concentration (µg/ml)
	Median (IQR)		
3TC (56)	0.561 (0.330-1.254)	0.516 (0.271-1.119)	0.479 (0.241-0.992)
ABC (28)	0.052 (0.033-0.263)	0.091 (0.055-0.795)	0.085 (0.040-0.531)
EFV (115)	2.199 (1.455-3.812)	1.432 (1.029-2.428)	1.248 (0.894-2.136)
RTV (81)	0.508 (0.310-0.813)	0.284 (0.145-0.631)	0.267 (0.122-0.530)
LPV (71)	7.770(4.429-10.130)	4.680 (2.219-6.765)*	4.107 (2.062-6.097)**

IQR = Interquartile Range

*P =0.011

** P=0.001

3.4.3 The effect of HCT on drug levels

The effects of low HCT on drug levels in patients was assessed and are reported in table 22. Plasma drug concentrations are considered a gold standard and they are compared with capillary DBS and adjusted capillary DBS with patients HCT. The patients with lower HCT in this study had an average HCT of 30% and 34% for females and males, respectively. For extrapolation of data these concentrations correspond to the HCT concentration of 36% in the experiment on HCT effect. Therefore, reflecting at this HCT in the experiment, at a lower HCT concentration and lower drug concentration there is a possibility for overestimation of drug concentrations. However, the drug concentrations of patients at lower HCT levels (30% and 34%) were within normal reference limits of the plasma with the exception of RTV. Correction of the DBS concentrations for the HCT improved the agreement between plasma and DBS samples except for ABC and 3TC where lower drug

concentrations in capillary DBS were observed when compared to plasma. None of the patients had high drug concentrations in the low HCT subgroup, however, it can be state that an effect was observed in patients with low HCT concentrations.

3.5 DPS Patients

ARVs were quantified in 211 remnant HIV-infected patient samples collected from the Haematology Laboratory. This was done measuring the drugs in DBS, plasma and plasma using DPS. The clinical data and drug regimens were not available for this data.

3.5.1 Method comparisons of plasma to DBS and plasma to DPS

Correlation coefficients and Bland-Altman plots for 3TC (7a), ABC (7b), EFV (7c), RTV (7d), LPV (7e) and FTC (7f) between plasma compared to venous DBS and plasma compared to DPS are shown in figure 7. Most of the differences in concentration between the two assays fell within ±2 standard deviations (95% confidence interval [95% CI]). Due to an insignificant number of patients, NVP and ATV are not included.

Plasma compared to venous DBS showed pronounced biases of +33% for 3TC, -107% for ABC, +32% for EFV, +25% for RTV, +30% for LPV, -4% for NVP, -83% for FTC, +74% and -96% for ATV. When plasma is compared to DPS, the following biases were observed: -21% for 3TC, -164% for ABC, -84% for EFV, -164% for RTV, -188% for LPV, -142% for NVP, -125% for FTC and -96% for ATV.

3.5.2 Method comparison

Table 23 shows the number of patients who had allowable percentage differences (±20%) for each drug between plasma and venous DBS and between plasma and DPS. More patients had drug concentrations between the allowable limits using DBS compared to DPS. DBS and DPS sample numbers do not match, because fewer drugs were detected in DPS compared to those detected in DBS. The drug regimens for this data were not available and so the numbers of patients on each drug are not known. However, what was detected in each sample has been reported.

Plasma, venous DBS and DPS drug concentrations for the patients were analyzed and are shown in table 24. The highest concentrations of drugs were observed in DPS, followed by plasma and the lowest in venous DBS. The exception was that of ABC and FTC, where the highest concentrations were observed in DPS, followed by venous DBS and the lowest drug concentrations observed in plasma. The

drug concentrations were reported as median (IQR). RAL and TMC114 are not included, because they could not be detect in any of the patient samples.

Table 22: The effect of low HCT on drug levels (µg/ml) in patients

Females																
Hct	Drug	P RTV	C RTV	RTV Adj	P LPV	C LPV	LPV Adj	P EFV	C EFV	EFV Adj	P ABC	C ABC	ABC Adj	P 3TC	C 3TC	3TC Adj
0.29	EFV							1.575	0.748	1.277						
0.26	EFV							1.909	1.942	3.315						
0.33	EFV,ABC,3TC							5.538	3.178	5.425	0.203	0.524	0.452	0.329	0.411	0.206
0.27	EFV							3.433	2.098	3.581						
0.30	EFV							0.692	0.186	0.317						
0.20	EFV, ABC, 3TC							1.818	1.686	2.878	0.033	0.204	0.176	0.540	0.672	0.336
0.30	RTV and LPV	0.141	0.112	0.189	7.058	5.036	8.509									
0.33	RTV and LPV	0.099	0.072	0.122	2.487	1.982	3.349									
0.31	RTV, LPV, ABC and 3TC	0.458	0.313	0.529	9.108	4.875	8.237				0.033	0.057	0.049	0.394	0.508	0.254
0.32	RTV and LPV	0.650	0.109	0.184	7.680	1.890	3.193									
0.31	RTV and LPV	0.720	0.347	0.586	16.810	9.536	16.113									
0.33	RTV,LPV and 3TC	0.820	0.897	1.516	10.360	6.273	10.599							2.310	0.992	0.496
0.32	RTV,LPV and 3TC	1.099	0.294	0.497	9.840	3.097	5.233							0.427	1.059	0.530
0.30	RTV	2.526	2.247	3.797												
Males																
Hct	Drugs	P RTV	C RTV	RTV Adj	P LPV	C LPV	LPV Adj	P EFV	C EFV	EFV Adj	P ABC	C ABC	ABC Adj	P 3TC	C 3TC	3TC Adj
0.35	EFV							2.150	1.333	2.275						
0.37	EFV,ABC,3TC							1.704	1.009	1.722	0.073	0.055	0.047	0.463	0.479	0.240
0.37	EFV,ABC,3TC							3.627	1.425	2.432	3.901	2.131	1.837	1.973	0.793	0.397
0.30	EFV							2.987	1.236	2.110						
0.26	EFV,ABC							4.403	1.838	3.138	0.777	0.537	0.463			
0.23	EFV							2.230	2.174	3.711						
0.38	ABC and 3TC										0.015	0.032	0.028	0.260	0.188	0.094
0.37	EFV,ABC,3TC							1.631	1.551	2.647	0.008	0.109	0.094	0.500	1.656	0.828
0.38	RTV	1.010	0.476	0.804												
0.34	LPV and 3TC	.			1.950	1.209	2.043							0.960	0.748	0.374
0.36	LPV				4.429	2.141	3.618									
0.31	LPV,EFV and 3TC				2.120	1.436	2.426	0.286	0.384	0.380				1.710	0.398	0.199

HCT= Haematocrit; P= plasma; C= capillary; Adj= Adjusted DBS with patients HCT

Lamivudine (3TC)

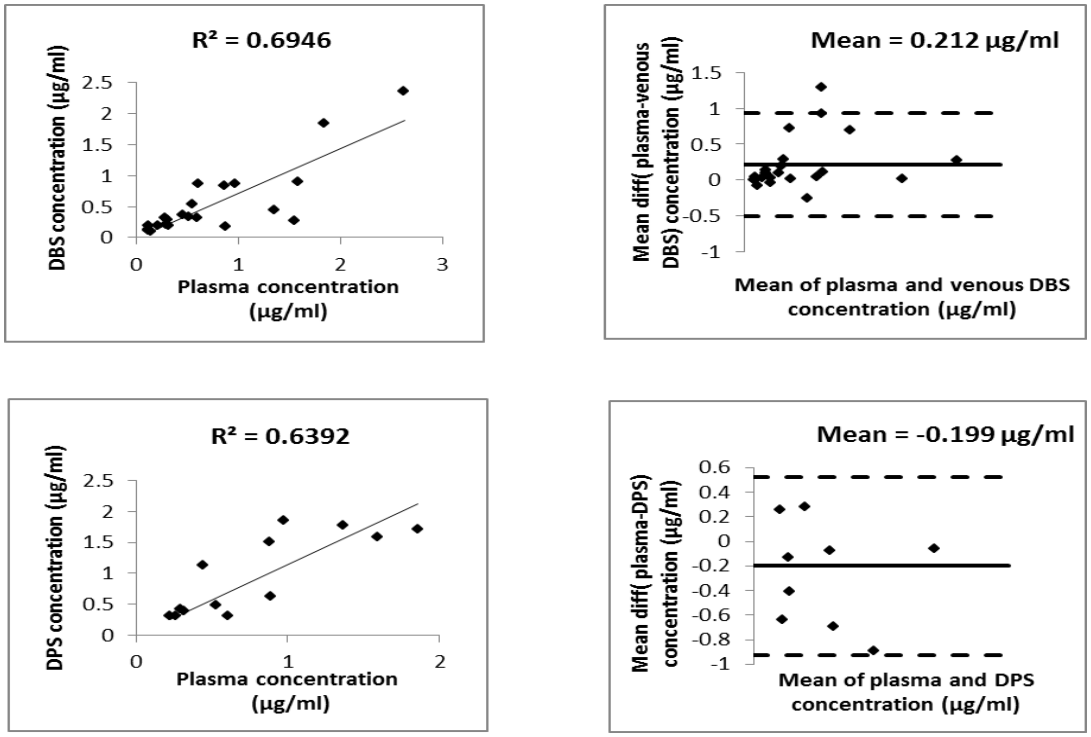


Figure 7 (a): 3TC correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Abacavir (ABC)

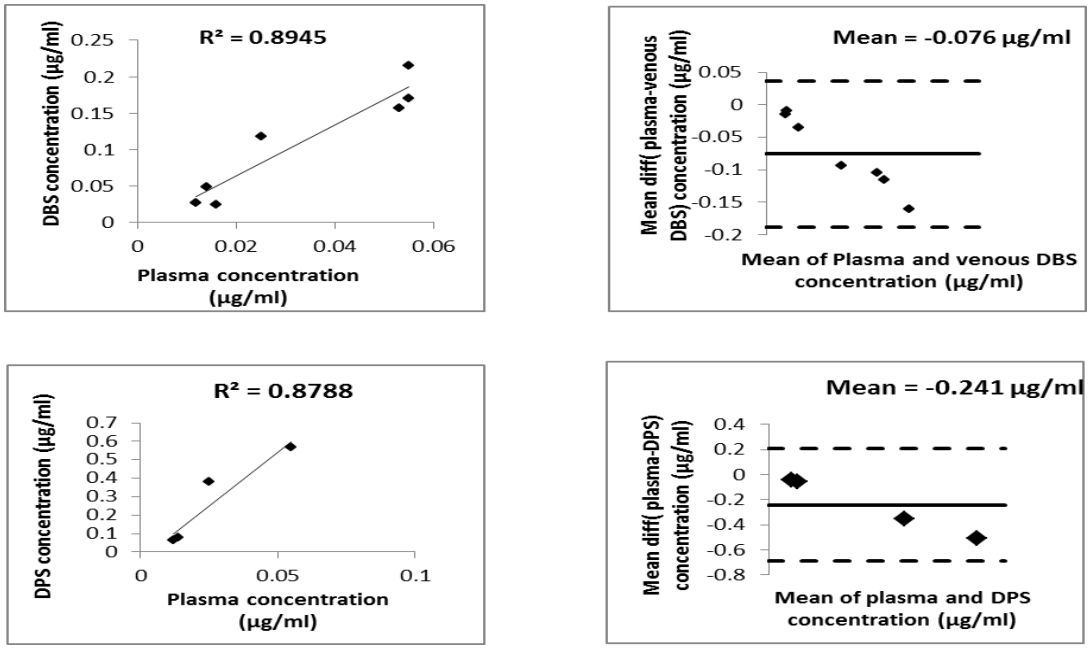


Figure 7 (b): ABC correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Efavirenz (EFV)

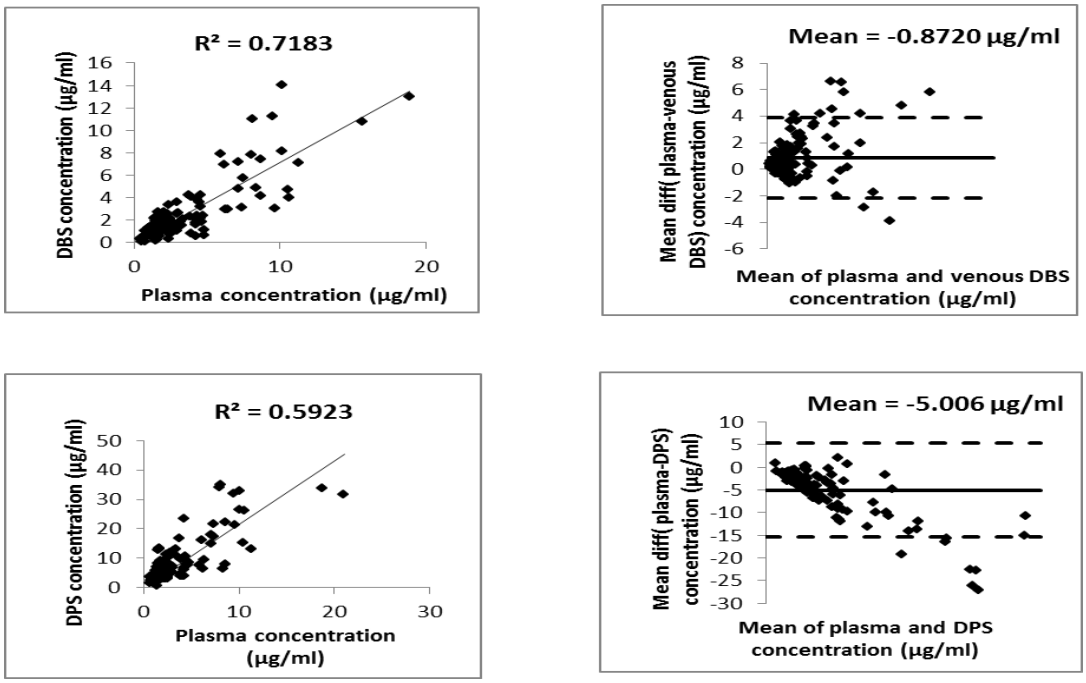


Figure 7 (c): EFV correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Ritonavir (RTV)

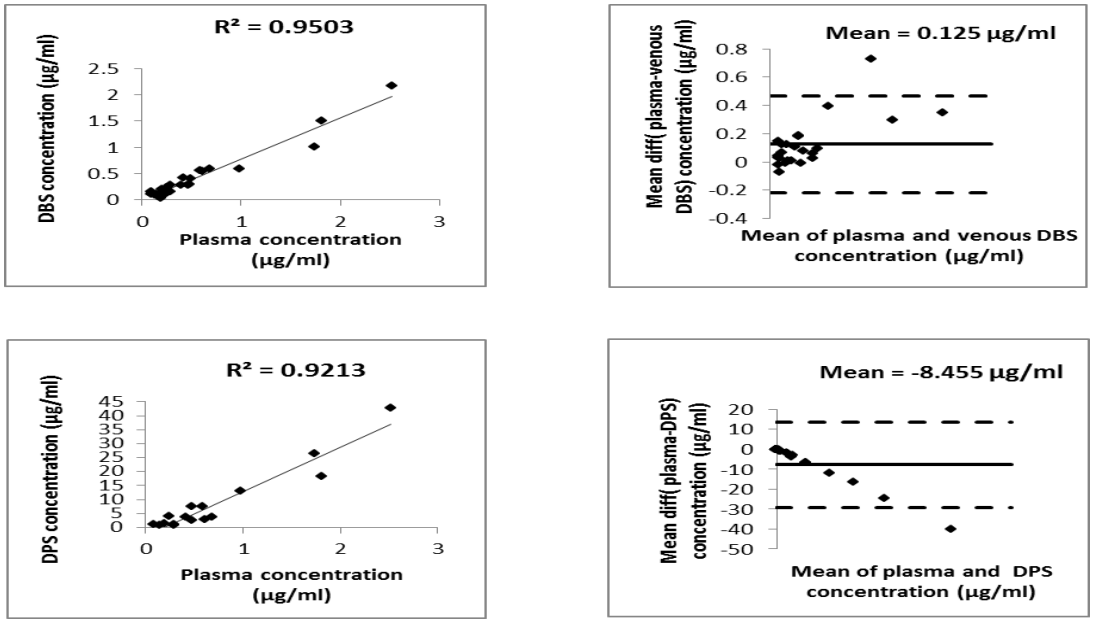


Figure 7 (d): RTV correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Lopinovir (LPV)

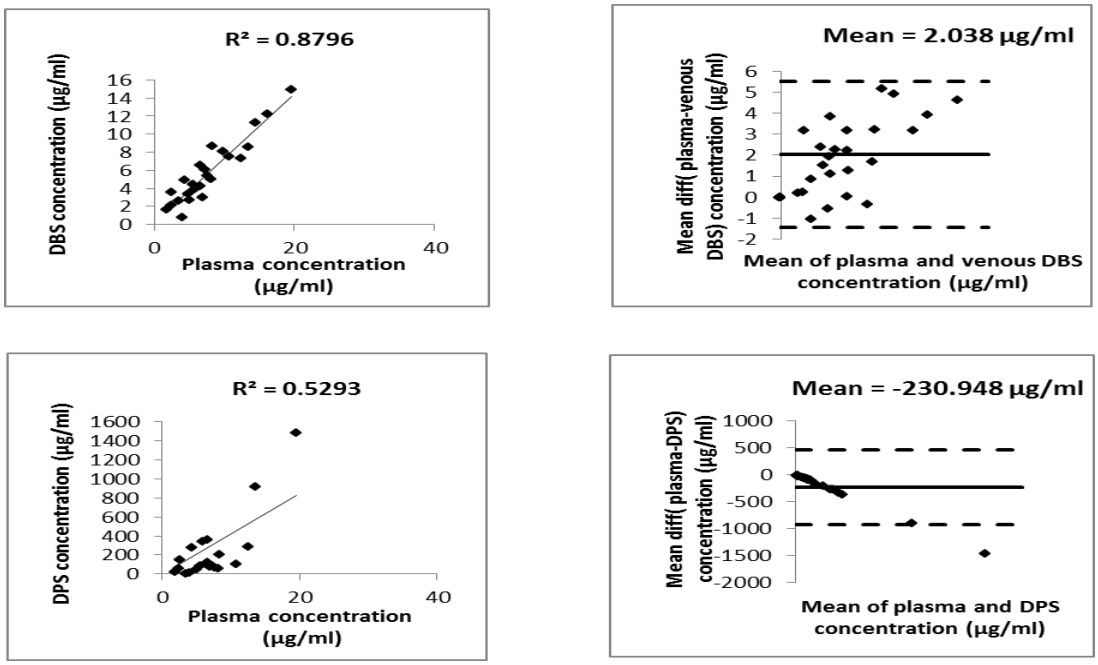


Figure 7 (e): LPV correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Emtricitabine (FTC)

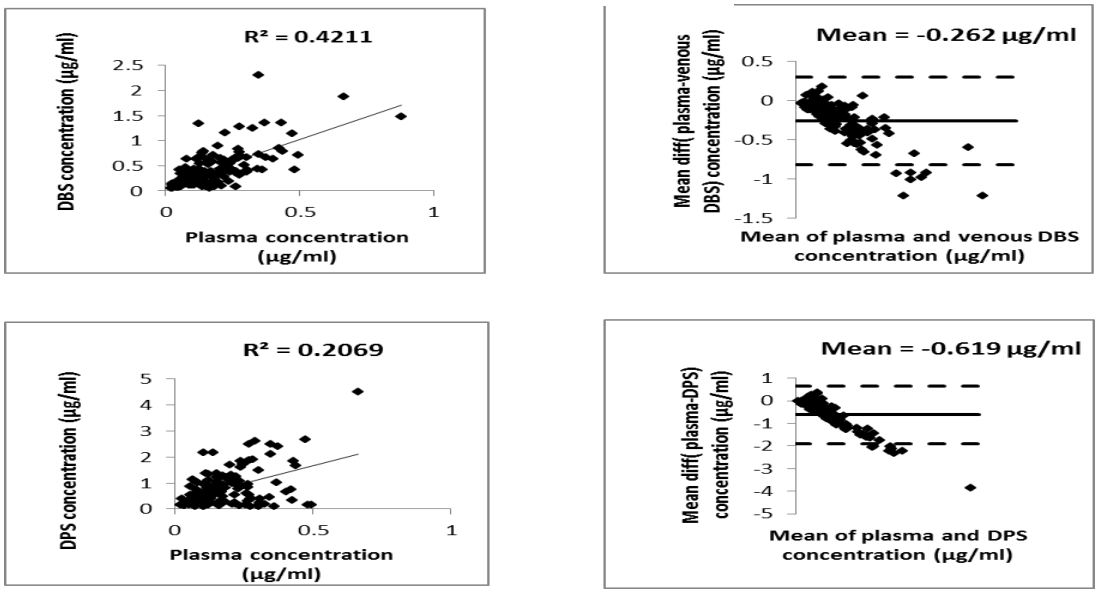


Figure 7 (f): FTC correlation coefficients (the sold line is the line of identity) and Bland-Altman plots (the continuous line is the mean and the broken lines represent the 95% CI (±2 SD)) between two different matrixes.

Figure 7 (a-f): Correlation coefficients and Bland-Altman plots for drugs between plasma compared to venous DBS and plasma compared to DPS

Table 23: Agreements between plasma compared to venous DBS and plasma compared to DPS (95% limits of agreement)

Plasma and Venous DBS		
Drug	**No. detected in both plasma and venous DBS**	**No. within ±20% limits** (%)
3TC	22	10 (45)
ABC	7	0 (0)
EFV	150	37 (25)
RTV	24	12 (50)
LPV	24	7 (29)
NVP	3	1 (33)
FTC	148	12 (8)
ATV	2	0 (0)
Plasma and DPS		
Drug	**No. detected in both plasma and DPS**	**No. within ±20% limits**
3TC	14	5 (36)
ABC	4	0 (0)
EFV	128	9 (7)
RTV	13	0 (0)
LPV	20	0 (0)
NVP	3	0 (0)
FTC	137	9 (7)
ATV	2	0 (0)

Table 24: Plasma, venous DBS and DPS drug concentrations for the patients

Drug (N)	Plasma concentration (µg/ml)	DBS concentration (µg/ml)	DPS concentration (µg/ml)
	Median (IQR)		
3TC (23)	0.498 (0.286-0.912)	0.327 (0.198-0.850)	1.324 (0.413-1.793)
ABC (7)	0.025 (0.015-0.054)	0.084 (0.042-0.160)	0.117 (0.065-0.312)
EFV (157)	2.151 (1.435-3.912)	1.503 (1.069-2.564)	5.827 (3.931-9.894)
RTV (24)	0.338 (0.191-0.589)	0.281 (0.125-0.549)	3.773 (2.352-11.441)
LPV (24)	6.834(4.913-9.793)	4.932 (3.310-7.687)	93.129 (62.030-277.822)
NVP (3)	5.896 (4.473-7.007)	4.159 (3.292-5.523)	7.865 (6.477-16.218)
FTC (152)	0.153 (0.098-0.239)	0.372 (0.227-0.578)	0.591 (0.280-1.066)
ATV (2)	0.767 (0.435-1.098)	0.339 (0.205-0.472)	2.174 (1.403-2.944)

Chapter 4: Discussion and conclusion

4.1 Main findings of the study

The method for the measurement of ARVs in DBS and DPS from whole blood spotted onto filter paper and onto PSC was successfully developed and validated. It has been noted that drug concentrations from DBS were affected by low HCT concentrations (Table 21). Dried blood spot drug concentrations were also shown to be lower compared to plasma concentrations. In contrast DPS ARV concentrations were higher compared to plasma. Both DBS and DPS did not perform well for quantification of drugs as shown by proportion of patients within allowable errors (Table 19 and 23). However, the results suggest that DBS could possibly be used for determining non-compliance in patients failing to achieve viral suppression (VL > 100 copies/ml) (Table 20). Although the DPS validation passed using standards and controls as samples, much higher drug concentrations were obtained compared to venous blood and to DBS (Table 24). This highlights the need to test methods using patient samples or matrix matched standards, as well as look at alternative plasma sampling devices. The main findings will now be discussed below.

4.2 Development and method validation

DBS

Ter heine *et al*. (2011) have shown that DBS can be used to measure multiple ARVs in DBS using UHPLC–MS/MS. The ARV drugs included in their study were in their study were TMC114, Etravirine, RAL and RTV. Here a simple method that allows the simultaneous detection of nine antiretroviral agents using mass spectrometry was developed and validated. This study included South African drug regimens and are as follows: four PI (LPV, RTV, ATV, and TMC114), two NNRTIs (EFV and NVP), one integrase inhibitor (RAL) and three NRTIs (3TC, ABC and FTC).

A previously validated extraction method for PIs and NNRTIs in DBS described by Koal *et al*. (2005) was initially used for the extractions of the analytes. The recovery of the analytes was poor (< 60%) when the DBS cards were treated with methanol and 0.2M zinc sulphate. A slight modification of this method with 0.1% formic acid improved the extraction efficacy of the method to within the FDA guidelines. Addition of dilute acid such as formic acid to methanol makes compounds containing basic groups to form soluble salt and becomes more water soluble hence improve extraction (Manallack *et al*., 2013). Therefore, a final mixture of 0.1% formic acid in methanol and 0.2 M zinc Sulfate heptahydrate (50:50, v/v) was used in order to optimize the extraction.

The LOQs for DBS are about 1 fold higher compared to plasma levels (0.001-0.223 µg/ml) determined at the Chemical Pathology Laboratory of CMJAH (Legg-E'Silva & Snyman, unpublished). A similar trend was found by Koal and colleagues (Koal *et al*., 2005) when they looked at PIs and NNRTIs and found LOQ in DBS to be 2 fold higher compared LOQ using the plasma standard samples. This is probably caused by endogenous interferences from salts and lipids that are not removed when methanol plus zinc Sulfate are used for protein precipitation. It can also be the effect of HCT on DBS and hence plasma is considered the gold standard.

Using plasma spiked with standards, acceptable recoveries were shown for all the drugs. This is in line with the study by Villanelli *et al*, (2015) who reported recoveries that were within 80-120% when they quantified anticonvulsants in DBS using UHPLC–MS/MS. Similar to the method for this study, they also included 0.1% formic acid to their extraction procedure (as explained above). Koal and colleagues reported that RTV and LPV performed poorly with recoveries of 77% and 68%, respectively (Koal *et al*., 2005). Modification of their method as stated above showed improved recovery of these drugs with recovery ranges of 88-97% for both RTV and LPV. Additionally, the extraction efficiency of the modified method for all drugs was also found to be good with recoveries (88-104%) that were within the acceptable range of 80-120%.

The short term stability of drugs from DBS in this study is in line with a number of other studies (Duthaler *et al*., 2018, Kromdijk *et al*., 2012, Malm *et al*., 2009). The only exception was LPV with poor recoveries noted on days 2 and 7 may have been caused by the instrument malfunction or pipetting errors. The study that has evaluated the stability of ABC in DBS and commentary on its performance on this platform is somewhat limited. However, in plasma it was shown to be stable for up to 7 days at room temperature (Verweij-van Wissen *et al*., 2005). Therefore, this study suggests that it is more stable in DBS compared to wet samples.

DPS

The validation of the DPS showed good linearity, accuracy and recovery using standards spiked into plasma. However, the LOQ values in DPS were found to be very high compared to plasma levels (Legg-E'Silva & Snyman, unpublished). Stability at day 7 was also poor. This is the first report of the use of DPS for the measurement of ARVs. Alternatives to DBS include glass paper filters (D'Avolio *et al*., 2010) or the use of dried plasma spots (Baietto *et al*., 2013, Baietto *et al*., 2014). D'Avolio quantified nine ARV drugs in dried plasma spots using a glass filter with good recoveries that were above 80%,

except for ETV, with a recovery of 64% (D'Avolio *et al*., 2010). Two studies showed poor recoveries when quantifying antibiotics using dried plasma spots (Baietto *et al*., 2013, Baietto *et al*., 2014). This shows that drugs concentrations and recovery can be affected by the medium used for spotting.

Similar to DBS samples, the bench top stability of the drugs was tested using DPS (Table 16). Only ABC and RTV were found to be stable for up to 7 days. Similar to this study, Baietto *et al*, (2014) found Daptomycin (antibiotic) from dried plasma spots to be stable for only up to 7 days at room temperature. Conversely, most ARVs in DPS using glass filter spots were found to be more stable (>90 days) at room temperature when they were stored after thermal inactivation (58◦C for 35 min). These results suggest that measured concentrations may be affected by the material used to separate cells and plasma (D'Avolio *et al*., 2010). DPS were designed to measure the viral loads from the cells and the original design was not meant to use the plasma.

Other possible reasons for analytical variations could be due to analytical errors, such as: (1) instrument malfunction (2) pipetting errors (3) dilution/calculation errors (4) quality control errors, as they were observed to be the cause of variations in clinical chemistry laboratories (Teshome *et al*., 2021, Sakyi *et al*., 2015). However, in this study, failures observed in validation of ARVs in both DBS and DPS could be assigned to pipetting errors.

4.3 Effect of HCT on ARV concentrations in DBS

In the HCT experiment, the samples with lower HCT concentration (36%) spiked with the highest drug concentrations showed no over recoveries observed except for 3TC and ABC where over recovery is observed (Table 17). Literature to explain this could not be found, however, it was hypothesized that this may be because 3TC and ABC were spiked into the commercially available matrix matched calibration standards. For this reason It may be feasible that they are easily extracted (as they are unbound) compared to the manufacturers calibration standards. This is not the effect of HCT on the drugs therefore, it cannot be seen in patient's samples.

Haematocrit affects DBS concentrations and correction of the DBS concentrations for the HCT has been shown to improve the agreement between plasma and DBS samples (Duthaler *et al*., 2018, Kromdijk *et al*., 2012). Similarly, this study showed that agreement between plasma and DBS samples was improved after correcting for the HCT in the patients who had low HCT, as described above (Table 22). The only exception was with ABC and 3TC where lower drug concentrations in capillary DBS were observed when compared to plasma concentration after the correction of DBS sample. These lower

concentrations may be attributed by the fact that NRTIs accumulate intracellularly hence they are recommended to be quantified in peripheral blood mononuclear cells rather than whole blood (Xiao *et al*., 2018).

There are a number of proposed methods that have been used to correct for the effect of HCT induced biased in quantitative DBS analysis. One approach includes adjusting quantitative analysis by determining the volume of blood in the DBS (Abu-Rabie *et al*., 2015). Another approach includes determining the HCT of the blood spotted on to the card and subsequently using it to translate DBS concentrations into theoretical plasma concentrations (Velghe *et al*., 2019). A study by *Abu-Rabie et al, (2015)* has also shown that overall HCT can be circumvented by the analysis of whole blood spots using the strategies that minimize HCT-based recoveries. This includes: (1) addition of internal standard into blood prior to blood spotting, (2) spray addition of the internal standard onto DBS samples prior to sample extraction, (3) spray addition of the internal standard onto the DBS card prior to blood spotting. Deuterated internal standard has been shown to eradicate recovery bias (Abu-Rabie *et al*., 2015) and so was utilised in this study. Addition of internal standard into blood prior to blood spotting has also been shown to eradicate recovery bias when an analyte is extracted with an internal standard. This strategy is able to allow quantitative co-extraction of the analyte and internal standard which will then eradicate the recovery bias (Abu-Rabie *et al*., 2015). These strategies are able to allow quantitative co-extraction of the analyte and internal standard which will then eradicate the recovery bias.

4.4 Application of the method

The method described in this study was applied to 231 patients' samples to investigate their drug levels in DBS. Three patients were excluded, because they had an ALT measurement of three times the upper reference limit. The main finding of this study is that dried blood spot concentrations correlate well with plasma concentrations however the measured DBS drug concentrations are not equal, but rather proportional to plasma concentrations. Both DBS and DPS did not perform well for quantification of drugs as shown by the proportion of patient's results within allowable errors (Table 19 and 23). However, the results of this study suggest that it could possibly be used for determining non-compliance (Table 20). This can be helpful among patients who are on ART in South Africa since ARV compliance is often a problem among HIV patients (Yu *et al*., 2018). It can also offer advantages in resource-limited countries where equipment for plasma sampling is often not available and samples have to be transported to central laboratories.

The highest drug concentrations were observed in plasma and the lowest in DBS (venous and capillary). This is in agreement with what has been found in literature. A study by Amara *et al*, (2015) reported that EFV concentrations in DBS were approximately 41.9% lower compared to that in plasma. Furthermore, Duthaler *et al*, (2018) found DBS concentrations of EFV and LPV to be lower by 52% and 72%, respectively compared to plasma. Both studies reported similar findings to those of this study. As shown by Li and Tse, (2010) this difference between plasma and DBS could be due to compound-specific plasma protein binding and HCT. For this study, in order to minimize the effect of HCT on DBS drug concentrations, the entire blood spot was used, and all samples with low HCT underwent correction.

Most of the patients in this study were virally suppressed; their measured drug levels were also found to be within normal reference ranges that were used in this study for plasma as a gold standard. Adherence to ART is one of the most important considerations in the prevention of resistance. Yager *et al,* (2019) showed moderately high Tenofovir concentrations defined as 700-1249 fmol/punch with high viraemia (> 20 copies/ml) and was suggestive of ART resistance. Patients with VL < 100 copies per ml and had undetectable drugs levels in plasma were noted to be between 2 and 11%, while in DBS were between 2 and 9%. Drug concentrations reported in this study are single plasma drug levels and thus only reflect exposure of a drug for a short period of time. In line with this, 11% of patients in this study had normal to high drug concentrations observed with non-viral suppression. This may suggest resistance to the drugs the patient is on. It could also be due to sampling trough levels.

An unexpected finding was that in seven percent (16) of the patients in this study did not have drugs detected in both plasma and DBS. A possible reason for this is non-adherence. Other possible reason could be that these drugs were below the limit of quantification for DBS. However, forty-four percent (7) of these patients were virally suppressed. One explanation for this is delayed viral rebound. This is in line with the finding in the study conducted by Li *et al*, (2016) who reported that patients have varying times to viral rebound and noted that a significant number of participants were able to maintain undetectable viral loads for months after stopping of ART.

In some instances, drugs that patients said they were taking were detected either in plasma or DBS, but not in both plasma and DBS, as expected. It was found that drugs at low concentrations could be detected in plasma, but not in DBS and this may be explained by the volume differences during the

extraction or differences in the limit of detection. In a few cases drugs were found in DBS, but not in plasma. This could be due to cross-contamination. Instruments used to excise the discs have the potential to cause cross-contamination between samples (Mitchell *et al*., 2010). However, for this study pre-cut discs were used because they reduce the chances of cross-contamination.

The Cobas® PSC was used to investigate the drug concentrations in DPS in 211 patients, but was found not to correlate well with plasma concentrations. Although the DPS validation passed using standard and control samples, much higher drug concentrations were obtained when compared to those measured in venous blood and plasma (Table 24). This suggests that there could be something in the PSC membrane that interferes with recoveries of drugs, because the card was not intended for drug measurement, but rather to measure viral loads. No study in literature could be found that has attempted to quantify ARVs using the Roche PSC. Other studies used this device to measure the baseline HIV-1 viral loads (Vubil *et al*., 2020, Carmona *et al*., 2019).

When plasma concentrations were compared to those obtained by either DBS or DPS, a number of results for both DBS and DPS were not within the allowable 20% difference for each drug (Table 23). This is, because the majority of these samples had drug concentrations that were very low or below the therapeutic range. At the very low concentrations, the accuracy was shown to be poor and hence there are larger measurement discrepancies.

4.5 Clinical implications

DBS are easy to collect and they offer a useful strategy for storage and shipment of samples to the TDM laboratory at a cost that would be lower compared to wet matrices. This offers advantages in resource-limited countries. The samples are stable at room temperature for seven days, within which time samples should arrive at a central laboratory.

TDM requires regular blood sampling by clinicians for the analysis and this poses a significant burden on patients regarding trips to the clinic (Klak *et al*., 2019). Another advantage of DBS is the possibility of self-sampling. Patients can prepare their own samples and send them to the laboratory for analysis (Cheung *et al*., 2008, McShane *et al*., 2016). There are three studies that reported the possibility of capillary sampling by patients (self-sampling), one study reported self-sampling in a hospital setting and two in a home setting. Unsuitability of self-sampling was reported in 1.8% (n = 108) of patients for self-sampling in a hospital setting (Cheung *et al*., 2008) and 3.8% (n = 26) and 11.6% (n = 216) in a

home setting (van Boekel *et al*., 2015, Al-Uzri *et al*., 2017). In addition there was no significant difference between DBS made by patients and those from trained personnel (Koster *et al*., 2013).

There is no gold standard for adherence measurements (Garrison and Haberer, 2017). Self-reported adherence remains the most commonly used method by researchers and clinicians to assess adherence particularly in developing countries. However, self-reported adherence has some limitations including recall bias and tendency of patients to give answers that will favored by the clinician (Alcaide *et al*., 2017).

Medication event monitoring systems (MEMs) could also provide a better picture of adherence over a period of time (Thirumurthy *et al*., 2012). However MEMs data can only be acquired during a clinic visit and reflects adherence that would have occurred long before the data can be evaluated, thus limiting its usefulness (Liu *et al*., 2001). Directly observed therapy could be a gold standard when it is executed well, however, it is rarely utilized to monitor ARV therapy due to cost (Winchester *et al*., 2020). Though there is no gold standard adherence measurements, DBS can be of use since it was shown to be superior to self-reported measures (Castillo-Mancilla *et al.,* 2015).

Viral loads are also used as a measure of adherence (Morrow *et al*., 2019) however, it takes a long period of poor adherence to be noticeable (Ford *et al*., 2010). Rosenblum *et al*. reported that some individuals on the most potent antiretroviral regimens can achieve virologic suppression with adherence as low as 50% (Rosenblum *et al*., 2009), thus making an undetectable HIV VL an incomplete marker for adherence in HIV infection. Therefore, TDM of ARVs to identify poor adherence could prevent the development of viremia and worsening of the disease (Gardner *et al*., 2009, Cohen *et al*., 2016). Additionally, HIV viral load as a marker of adherence is not relevant to pre-exposure prophylaxis and so in this case TDM using DBS can be useful.

In patients who were not virologically suppressed, good concordance was observed as drugs measured in plasma were also detected in DBS. As the measurement of drug concentrations is much cheaper when compared to resistance testing, clinicians could start with this and for those for whom drugs are undetectable only, offer resistance testing. Carmona *et al,* (2019) have shown that the use of DBS increases access to VL testing. The same can be expected for measurement of drug levels.

An extension of this study would be to compare the numbers of patients with mutations in those with detectable versus low drug levels.

Possible future work will need to include looking at other plasma separation devices. It has been shown that DPS ARVs on a glass filter were stable for longer time when viral thermal inactivation using a glass filter and this has been shown to perform well (D'Avolio *et al*., 2010). Another possibility is the use of a Mitra micro sampling device with inclusion of a sonication step within sample extraction procedure. This can overcome volumetric Hct effects, while maintaining benefits associated with DBS (Velghe *et al*., 2019, Mano *et al*., 2015).

4.6 Strength and weakness of this study

The strengths of this study include a large sample size and detectable drug levels using DBS. The limitations of the study are as follows: The NRTs could not be measured accurately at intracellular levels, because the procedure is both difficult and expensive. Furthermore, patient's treatment compliance could not be distinguished from non-compliance. Therefore, an accurate indication of actual patient drug levels could not be identified. Compliance can be overestimated, as patients may take their medication prior to clinic visits particularly when they are aware that drug levels are going to be measured. Remnant HIV-positive patient samples collected from the Haematology laboratory have no demographics and clinical data. For this reason drug concentrations obtained are inconclusive since a clear picture of drug metabolism could not be established. Drug regimens were also unavailable therefore; where drugs are not detected it's not clear if that is, because of non-compliance or whether or not the patient is not on a particular drug regimen.

Similar to quantification of ARV parent drug concentrations in DBS, future studies must evaluate measurement of intracellular concentrations for NRTs in red blood cells and PBMCs. Electronic pill containers that provide immediate information on dosing events should also be considered in order to ovoid compliance over or under-estimation. These methods can actively monitor treatment compliance in "real-time" between clinical or study visits. For clear correlations between plasma and DPS, a large sample size that consists of patients' demographics, clinical data and a known drug regime are required. It will also be important to determine if the use of DBSs for monitoring compliance in people living with HIV improves compliance.

4.7 Conclusion

In summary a method for the measurement of several ARVs from DBS was established. Although, it was noted that drug concentrations in DBS are not equal to plasma concentrations, good concordance was observed as drugs measured in plasma were also detected in DBS. Therefore, this method can be

used to determine the presence or absence of drugs. This work can be extended to determine the failure to detect drugs from DBS as predictive of future viraemia as was shown for Tenofovir by Morrow *et al,* (2019).

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Appendix A (Supplemntary data)

1.Validation of Dry Blood Spots

1.1 lamuvidine (3TC)

Table A1.1.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	387		251	518		385	134	0
STD 1	10062	11965		16330	15730	13522	3009	0.119
STD 2	26166	16885		20412	19021	20621	3971	0.238
STD 3	32937	26166	21212	48705	44373	34679	11700	0.357
STD 4	50989	62662	26421	55257	67570	52580	15971	0.595
STD 5	53348	76234	42575	74559	62078	61759	14250	0.714
STD 6	93551	83373	62849	84522	1E+05	85289	14665	0.833

Table A1.1.2 Accuracy

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.004		0.005	0.005		0.005	0.000	
STD 1	0.106	0.126		0.153	0.147	0.133	0.119	12
STD 2	0.275	0.177		0.191	0.178	0.205	0.238	-14
STD 3	0.346	0.275	0.439	0.456	0.415	0.386	0.357	8
STD 4	0.535	0.658	0.547	0.517	0.632	0.578	0.595	-3
STD 5	0.560	0.800	0.881	0.697	0.581	0.704	0.714	-1
STD 6	0.982	0.875	1.300	0.791	0.955	0.981	0.833	18
CTRL 1	0.108	0.127	0.117	0.111	0.132	0.119	0.121	-1
CTRL 2	0.715	0.900	0.883	0.655	0.847	0.800	0.768	4

Table A1.1.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.004		0.008			0.006	0.003	
STD1	0.106		0.126			0.116	0.014	12
STD2	0.275		0.177			0.226	0.069	31
STD3	0.346		0.275			0.311	0.050	16
STD4	0.535		0.658			0.597	0.087	15
STD5	0.56		0.8			0.680	0.170	25
STD6	0.982		0.875			0.929	0.076	8
CTRL 1	0.109	0.108	0.098	0.100	0.102	0.103	0.005	5
CTRL 2	0.952	0.715	0.766	0.627	0.653	0.743	0.129	17

Table A1.1.4 Inter-run precision

	Run 1	Run 2	Run3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.004		0.005	0.005		0.005	0.001	12
STD1	0.106	0.126		0.153	0.147	0.133	0.021	16
STD2	0.275	0.177		0.191	0.178	0.205	0.047	23
STD3	0.346	0.275	0.439	0.456	0.415	0.386	0.075	19
STD4	0.535	0.658	0.547	0.517	0.632	0.578	0.063	11
STD5	0.560	0.800	0.881	0.697	0.581	0.704	0.138	20
STD6	0.982	0.875	1.300	0.791	0.955	0.981	0.194	20
CTRL 1	0.108	0.127	0.117	0.111	0.132	0.119	0.010	9
CTRL 2	0.715	0.900	0.883	0.655	0.847	0.800	0.109	14

Table A1.1.5 Mean percentage Recovery

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.004		0.005	0.005		0.005	0.000	
STD 1	0.106	0.126		0.153	0.147	0.133	0.119	112
STD 2	0.275	0.177		0.191	0.178	0.205	0.238	86
STD 3	0.346	0.275	0.439	0.456	0.415	0.386	0.357	108
STD 4	0.535	0.658	0.547	0.517	0.632	0.578	0.595	97
STD 5	0.560	0.800	0.881	0.697	0.581	0.704	0.714	99
STD 6	0.982	0.875	1.300	0.791	0.955	0.981	0.833	118
CTRL 1	0.108	0.127	0.117	0.111	0.132	0.119	0.121	99
CTRL 2	0.715	0.900	0.883	0.655	0.847	0.800	0.768	104

Table A1.1.6 LOD and LOQ

LOD AND LOQ						
	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	387		251	518		385.3333
Signal lowest STD	10062	11965		16330	15730	13521.75
S/N	35.091					
Actual Concentration	0.119					
LOD [actual]/[S/N]*3	0.010					
LOQ [actual]/[S/N]*10	0.034					

1.2 Abacavir

Table A1.2.1 Linearity was determined from mean height aganist assigned concentration

RUN1	day 1	day 2	day 3	day 4	day 5	Mean	SD	Assigned
STD 0		1423	1423	1613		1486	110	0
STD1		77551	77551	58735		71279	10863	0.119
STD2	54662	133933	133933	131554	149932	120803	37695	0.238
STD3	86246	207749	207749	176369	227764	181175	56164	0.357
STD4	121423	283495	283495	368266	308093	272954	91519	0.595
STD5	176199	392946	392946	274001	532968	353812	135163	0.714
STD6	163005	374744	374744	347312	560572	364075	141022	0.833

Table A1.2.2 Accuracy

Days	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%Diff
STD 0		0.000	0.000	0.000		0.000	0.000	
STD 1		0.144	0.143	0.124		0.137	0.119	15
STD 2	0.301	0.252	0.250	0.282	0.212	0.259	0.238	9
STD 3	0.475	0.392	0.389	0.379	0.329	0.393	0.357	10
STD 4	0.669	0.536	0.532		0.438	0.544	0.595	-9
STD 5	0.971	0.744	0.739	0.591	0.762	0.761	0.714	7
STD 6	0.898	0.710	0.704	0.750	0.796	0.772	0.833	-7
CLTR 1	0.134	0.149	0.147	0.130	0.130	0.138	0.135	2
CLTR 2	1.000	0.972	0.965	0.766	0.862	0.913	0.889	3

Table A1.2.3 Intra-run precision

Run	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.000	-0.001	0.001	0.000	0.000	0.000	0.001	
STD1	0.117	0.133	0.112	0.147	0.114	0.125	0.015	12
STD2	0.240	0.206	0.196	0.244	0.200	0.217	0.023	11
STD3	0.371	0.296	0.293	0.305	0.224	0.298	0.052	18
STD4	0.680	0.674	0.589	0.586	0.486	0.603	0.079	13
STD5	0.758	0.821	0.652	0.742		0.743	0.070	9
STD6	0.857	0.927	0.745	0.723	0.804	0.811	0.083	10
CLTR 1	0.155		0.120	0.130	0.120	0.131	0.017	13
CLTR 2	0.809	0.898	0.738	0.828	0.789	0.813	0.059	7

Table A1.2.4 Inter-run precision

Days	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD 0		0.000	0.000	0.000		0.000	0.000	
STD1		0.144	0.143	0.124		0.137	0.012	8
STD2	0.301	0.252	0.250	0.282	0.212	0.259	0.034	13
STD3	0.475	0.392	0.389	0.379	0.329	0.393	0.053	13
STD4	0.669	0.536	0.532		0.438	0.544	0.095	17
STD5	0.971	0.744	0.739	0.591	0.762	0.761	0.136	18
STD6	0.898	0.710	0.704	0.750	0.796	0.772	0.080	10
CLTR 1	0.134	0.149	0.147	0.130	0.130	0.138	0.009	7
CLTR 2	1.000	0.972	0.965	0.766	0.862	0.913	0.097	11

Table A1.2.5 Mean percentage Recovery

Days	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0		0.000	0.000	0.000		0.000	0.000	
STD 1		0.144	0.143	0.124		0.137	0.119	115
STD 2	0.301	0.252	0.250	0.282	0.212	0.259	0.238	109
STD 3	0.475	0.392	0.389	0.379	0.329	0.393	0.357	110
STD 4	0.669	0.536	0.532		0.438	0.544	0.595	91
STD 5	0.971	0.744	0.739	0.591	0.762	0.761	0.714	107
STD 6	0.898	0.710	0.704	0.750	0.796	0.772	0.833	93
CLTR 1	0.134	0.149	0.147	0.130	0.130	0.138	0.135	102
CLTR 2	1.000	0.972	0.965	0.766	0.862	0.913	0.889	103

Table A1.2.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank		1423	1423	1613		1486
Signal lowest STD		77551	77551	58735		71279
S/N	47.956					
Actual Conc	0.119					
LOD [actual]/[S/N]*3	0.007					
LOQ [actual]/[S/N]*10	0.025					

1.3 Efavirenz (EFV)

Table A1.3.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	486	708	708			634	128	0.000
STD 1	7655	10955	10955	16445		11503	3644	0.323
STD 2	17091		22235	39804	38294	29356	11404	0.728
STD 3	28951	47029	47029	58796	59979	48357	12492	1.122
STD 4	39023	61939	61939	137140	100495	80107	38783	1.556
STD 5	36478	82399	82399	64510	159804	85118	45780	1.974
STD 6	57483	107545	107545	120368	188279	116244	46931	2.360

Table A1.3.2 Accuracy

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.000	0.002	0.002			0.001	0.000	
STD 1	0.333	0.217	0.228	0.324		0.276	0.323	-15
STD 2	0.774		0.476	0.805	0.513	0.642	0.728	-12
STD 3	1.329	0.977	1.023	1.197	0.808	1.067	1.122	-5
STD 4	1.801	1.290	1.351	2.812	1.359	1.723	1.556	11
STD 5	1.682	1.721	1.802	1.315	2.167	1.737	1.974	-12
STD 6	2.664	2.250	2.355	2.466	2.554	2.458	2.360	4
CLTR 1	0.512	0.492	0.515	0.634	0.607	0.552	0.597	-8
CLTR 2	1.635	1.856	1.943	1.829	1.545	1.761	1.725	2

Table A1.3.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.000		0.003	0.000	0.000	0.001	0.001	
STD1	0.323		0.301	0.322	0.428	0.343	0.057	17
STD2	0.858	0.563		0.741	0.832	0.748	0.134	18
STD3	1.329	1.130	1.195	0.835	1.370	1.172	0.212	18
STD4	1.872	1.774	1.486	1.511	2.058	1.740	0.243	14
STD5	1.843	1.565	1.711	2.047	1.682	1.770	0.184	10
STD6	2.859	1.894	2.026	2.308	2.228	2.263	0.371	16
CLTR 1	0.735	0.535	0.532	0.571	0.564	0.588	0.084	14
CLTR 2	1.614	1.522	1.466	1.577	1.479	1.532	0.063	4

Table A1.3.4 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.000	0.002	0.002			0.001	0.001	
STD1	0.333	0.217	0.228	0.324		0.276	0.061	22
STD2	0.774		0.476	0.805	0.513	0.642	0.172	27
STD3	1.329	0.977	1.023	1.197	0.808	1.067	0.202	19
STD4	1.801	1.290	1.351	2.812	1.359	1.723	0.642	37
STD5	1.682	1.721	1.802	1.315	2.167	1.737	0.304	18
STD6	2.664	2.250	2.355	2.466	2.554	2.458	0.163	7
CLTR 1	0.512	0.492	0.515	0.634	0.607	0.552	0.064	12
CLTR 2	1.635	1.856	1.943	1.829	1.545	1.761	0.165	9

Table A1.3.5 Mean percentage Recovery

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0	0.000	0.002	0.002			0.001	0.000	
STD 1	0.333	0.217	0.228	0.324		0.276	0.323	85
STD 2	0.774		0.476	0.805	0.513	0.642	0.728	88
STD 3	1.329	0.977	1.023	1.197	0.808	1.067	1.122	95
STD 4	1.801	1.290	1.351	2.812	1.359	1.723	1.556	111
STD 5	1.682	1.721	1.802	1.315	2.167	1.737	1.974	88
STD 6	2.664	2.250	2.355	2.466	2.554	2.458	2.360	104
CLTR 1	0.512	0.492	0.515	0.634	0.607	0.552	0.597	92
CLTR 2	1.635	1.856	1.943	1.829	1.545	1.761	1.725	102

Table A:1.3.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	486	708	708			634
Signal lowest STD	7655	10955	10955	16445		11503
S/N	18.143					
Actual Conc	0.323					
LOD [actual]/[S/N]*3	0.053					
LOQ [actual]/[S/N]*10	0.178					

1.4 Ritonavir (RTV)

Table A:1.4.1 Linearity was determined from mean height aganist assigned concentration

RUN	day 1	day 2	day 3	day 4	day 5	Mean	SD	Assigned
STD 0	445	998	603			682	285	0.000
STD 1		31834	36347	34920		34367	2307	0.104
STD 2	299698	396535	386721	556593		409887	107050	1.512
STD 3	523567	846487	811276	951292	907249	807974	167911	2.832
STD 4	712258	1091734	1107429		1827986	1184852	466059	4.157
STD 5	915080	1527469	1385760	1055664	2786080	1534011	741888	5.441
STD 6	1138485	1816276	1570970	2237174	3340088	2020599	838152	6.640

Table A:1.4.2 Accuracy

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	-0.137	0.269	-0.001			0.044	0.000	
STD 1		0.094	0.144	0.108		0.116	0.104	11
STD 2	1.847	1.204	1.570	1.809		1.608	1.512	6
STD 3	3.229	2.574	3.296	3.096		3.049	2.832	8
STD 4	4.394	3.321	4.501		3.455	3.918	4.157	-6
STD 5	5.646	4.648	5.633	3.436	5.267	4.926	5.441	-9
STD 6	7.025	5.527	6.386	7.288	6.315	6.508	6.640	-2
CLTR 1	0.711	0.607	0.704	0.773	0.570	0.673	0.744	-10
CLTR 2	4.212	4.229	4.791	5.073	3.747	4.410	4.494	-2

Table A:1.4.2 Intra-run precision

	Run 1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.002	-0.002	0.000	0.000	0.001	-0.001	0.001	
STD1	0.135	0.101	0.107	0.140	0.132	0.123	0.018	14
STD2	1.758		1.270	1.604	1.513	1.536	0.204	13
STD3	3.080	2.811	2.795	2.278	3.041	2.801	0.320	11
STD4	4.840	4.775	4.232	3.640	4.214	4.340	0.489	11
STD5	5.240	4.536	5.264	5.988	4.139	5.033	0.717	14
STD6	8.117	6.103	5.963	6.305	5.833	6.464	0.940	15
CLTR 1	0.820	0.623	0.718	0.660	0.600	0.684	0.088	13
CLTR 2	4.158	4.569	3.910	5.011	3.692	4.268	0.528	12

Table A:1.4.4 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.137	0.269	-0.001			0.044	0.207	
STD1		0.094	0.144	0.108		0.116	0.026	22
STD2	1.847	1.204	1.570	1.809		1.608	0.295	18
STD3	3.229	2.574	3.296	3.096		3.049	0.327	11
STD4	4.394	3.321	4.501		3.455	3.918	0.616	16
STD5	5.646	4.648	5.633	3.436	5.267	4.926	0.926	19
STD6	7.025	5.527	6.386	7.288	6.315	6.508	0.687	11
CLTR 1	0.711	0.607	0.704	0.773	0.570	0.673	0.083	12
CLTR 2	4.212	4.229	4.791	5.073	3.747	4.410	0.524	12

Table A:1.4.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0	-0.137	0.269	-0.001			0.044	0.000	
STD 1		0.094	0.144	0.108		0.116	0.104	111
STD 2	1.847	1.204	1.570	1.809		1.608	1.512	106
STD 3	3.229	2.574	3.296	3.096		3.049	2.832	108
STD 4	4.394	3.321	4.501		3.455	3.918	4.157	94
STD 5	5.646	4.648	5.633	3.436	5.267	4.926	5.441	91
STD 6	7.025	5.527	6.386	7.288	6.315	6.508	6.640	98
CLTR 1	0.711	0.607	0.704	0.773	0.570	0.673	0.744	90
CLTR 2	4.212	4.229	4.791	5.073	3.747	4.410	4.494	98

Table A:1.4.6 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	445	998	603			682
Signal lowest STD		31834	36347	34920		34367
S/N	50.391					
Actual Conc	0.104					
LOD [actual]/[S/N]*3	0.006					
LOQ [actual]/[S/N]*10	0.021					

1.5 Lopinavir (LPV)

Table A:1.5.1 Linearity was determined from mean height aganist assigned concentration

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	636		816	439		630	189	0.000
STD 1		6257	6257	7564	6684	6691	616	0.370
STD 2	15569	23556	23556		40318	25750	10416	1.453
STD 3	28973	48595	48595	45446	60096	46341	11201	2.492
STD 4	36118	56920	56920		100186	62536	26948	3.617
STD 5	50763	75809	75809	48856	152049	80657	41979	4.652
STD 6	55088	87148	87148	99524	171174	100016	43051	5.685

Table A:1.5.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.002		0.003	-0.002		0.001	0.000	
STD 1		0.305	0.309	0.407		0.340	0.370	-8
STD 2	1.597	1.264	1.282		1.276	1.355	1.453	-7
STD 3	2.973	2.653	2.691	2.579	1.908	2.561	2.492	3
STD 4	3.706	3.115	3.159		3.190	3.292	3.617	-9
STD 5	5.208	4.163	4.221	2.775	4.848	4.243	4.652	-9
STD 6	5.652	4.792	4.859	5.681	5.460	5.289	5.685	-7
CLTR 1	0.876	0.821	0.832	1.041	0.796	0.873	0.987	-12
CLTR 2	3.605	3.437	3.485	4.241	3.660	3.686	3.788	-3

Table A:1.5.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.000	0.000		0.000		0.000	0.000	
STD1	0.328	0.328	0.346	0.487	0.413	0.380	0.069	18
STD2	1.620	1.291	1.318	1.546	1.592	1.473	0.157	11
STD3	2.767	2.461	2.662	2.041	2.731	2.532	0.299	12
STD4	3.951	4.056	3.903	3.336	3.877	3.824	0.282	7
STD5	4.401	3.945	4.866	5.153	3.163	4.306	0.787	18
STD6	6.757	5.099	5.201	5.236	4.774	5.413	0.773	14
CLTR 1	0.977	0.730	1.073	0.860	0.796	0.887	0.138	16
CLTR 2	3.685	3.619	3.509	4.287	3.008	3.622	0.457	13

Table A:1.5.4 Inter-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.002		0.003	-0.002		0.001	0.003	
STD1		0.305	0.309	0.407		0.340	0.058	17
STD2	1.597	1.264	1.282		1.276	1.355	0.162	12
STD3	2.973	2.653	2.691	2.579	1.908	2.561	0.394	15
STD4	3.706	3.115	3.159		3.190	3.292	0.277	8
STD5	5.208	4.163	4.221	2.775	4.848	4.243	0.930	22
STD6	5.652	4.792	4.859	5.681	5.460	5.289	0.432	8
CLTR 1	0.876	0.821	0.832	1.041	0.796	0.873	0.098	11
CLTR 2	3.605	3.437	3.485	4.241	3.660	3.686	0.323	9

Table A:1.5.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.002		0.003	-0.002		0.001	0.000	
STD 1		0.305	0.309	0.407		0.340	0.370	92
STD 2	1.597	1.264	1.282		1.276	1.355	1.453	93
STD 3	2.973	2.653	2.691	2.579	1.908	2.561	2.492	103
STD 4	3.706	3.115	3.159		3.190	3.292	3.617	91
STD 5	5.208	4.163	4.221	2.775	4.848	4.243	4.652	91
STD 6	5.652	4.792	4.859	5.681	5.460	5.289	5.685	93
CLTR 1	0.876	0.821	0.832	1.041	0.796	0.873	0.987	88
CLTR 2	3.605	3.437	3.485	4.241	3.660	3.686	3.788	97

Table A:1.5.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	636		816	439		630
Signal lowest STD		6257	6257	7564	6684	6691
S/N	10.614					
Actual Conc	0.370					
LOD [actual]/[S/N]*3	0.105					
LOQ [actual]/[S/N]*10	0.349					

1.6 Nevirapine (NVP)

Figure A1.6.1 Linearity of NVP in DBS

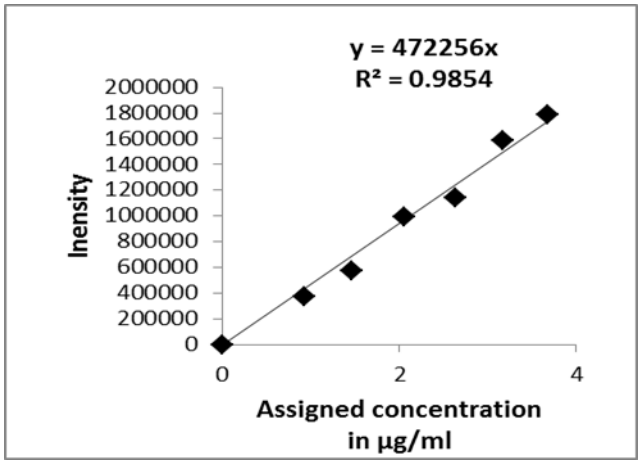


Figure 1.6.1 Linear regression curve between the mean peak height and assigned drug concentration over the course of 5 days

Table A:1.6.2 Linearity was determined from mean height aganist assigned concentration

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	563	634	538	668	428	566	93	0
STD 1	331163	422286		475112	280598	377290	87694	0.925
STD 2	562852	583796		676109	483606	576591	79143	1.458
STD 3	825051	966357	978644	1183798		988463	147698	2.051
STD 4	1024738	1128401	1109663	1340192	1107576	1142114	117735	2.634
STD 5	1290215	1673141	1540293	1865243	1547821	1583343	210101	3.172
STD 6	1397026	1912048	1745707	2002958	1860381	1783624	235259	3.676

Table A:1.6.3 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%Diff
STD 0	0.001	0.001	0.002	0.001	0.001	0.001	0.000	
STD 1	0.761	0.970		0.970	0.573	0.819	0.925	-12
STD 2	1.293	1.341		1.380	0.987	1.250	1.458	-14
STD 3	1.895	2.220	2.921	2.417		2.363	2.051	15
STD 4	2.354	2.592	3.312	2.736	2.261	2.651	2.634	1
STD 5	2.964	3.843	4.598	3.808	3.160	3.675	3.172	16
STD 6	3.209	4.392	5.211	4.089	3.798	4.140	3.676	13
CLTR 1	1.309	1.412	1.275		1.446	1.361	1.259	8
CLTR 2	2.654	2.583	2.711	2.683	2.668	2.660	2.395	11

Table A:1.6.4 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0		0.001	0.001			0.001	0.000	
STD1		0.97	0.573			0.772	0.281	36
STD2		1.38	0.987			1.184	0.278	23
STD3		2.417	1.655			2.036	0.539	26
STD4		2.736	2.261			2.499	0.336	13
STD5		3.808	3.16			3.484	0.458	13
STD6		4.089	3.798			3.944	0.206	5
CLTR 1		1.451	1.286	1.446	1.305	1.372	0.089	6
CLTR 2		2.558	2.574	2.668	2.545	2.586	0.056	2

Table A:1.6.5 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.001	0.001	0.002	0.001	0.001	0.001	0.000	
STD1	0.761	0.970		0.970	0.573	0.819	0.191	23
STD2	1.293	1.341		1.380	0.987	1.250	0.179	14
STD3	1.895	2.220	2.921	2.417		2.363	0.430	18
STD4	2.354	2.592	3.312	2.736	2.261	2.651	0.415	16
STD5	2.964	3.843	4.598	3.808	3.160	3.675	0.646	18
STD6	3.209	4.392	5.211	4.089	3.798	4.140	0.741	18
CLTR 1	1.309	1.412	1.275		1.446	1.361	0.081	6
CLTR 2	2.654	2.583	2.711	2.683	2.668	2.660	0.048	2

Table A:1.6.6 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.001	0.001	0.002	0.001	0.001	0.001	0.000	
STD 1	0.761	0.970		0.970	0.573	0.819	0.925	88
STD 2	1.293	1.341		1.380	0.987	1.250	1.458	86
STD 3	1.895	2.220	2.921	2.417		2.363	2.051	115
STD 4	2.354	2.592	3.312	2.736	2.261	2.651	2.634	101
STD 5	2.964	3.843	4.598	3.808	3.160	3.675	3.172	116
STD 6	3.209	4.392	5.211	4.089	3.798	4.140	3.676	113
CLTR 1	1.309	1.412	1.275		1.446	1.361	1.259	108
CLTR 2	2.654	2.583	2.711	2.683	2.668	2.660	2.395	111

Table A:1.6.7 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	day 6	MEAN
Signal Blank	563	634	538	668	428	566
Signal lowest STD	331163	422286		475112	280598	377290
S/N	666.354					
Actual Conc	0.925					
LOD [actual]/[S/N]*3	0.004					
LOQ [actual]/[S/N]*10	0.014					

1.7 Emtracitabine (FTC)

Figure A1.7.1 Linearity of FTC in DBS

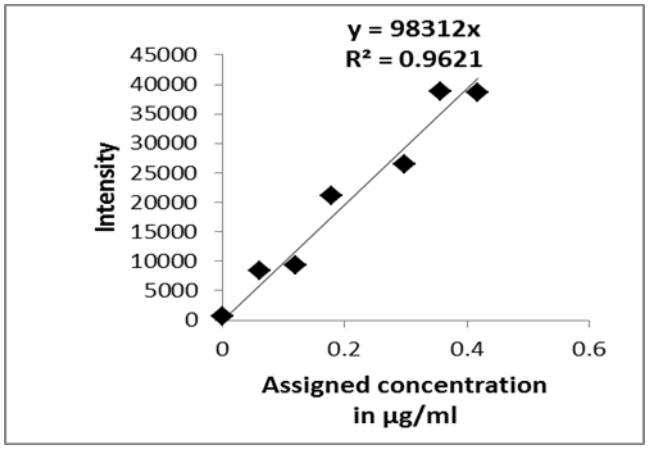


Figure 1.7.1 Linear regression curve between the mean peak height and assigned drug concentration over the course of 5 days

Table A:1.7.2 Linearity was determined from mean height aganist assigned concentration

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0		750	501	450	878	645	203	0.000
STD 1		8352	7636	9178		8389	772	0.060
STD 2	10478	10954	7494	8852	8504	9256	1433	0.119
STD 3	22818	23711	19131	22407	17149	21043	2782	0.179
STD 4	32776	32025		25635	15282	26430	8093	0.298
STD 5		42593	39537	40050	33156	38834	4014	0.357
STD 6	43028	39531	35231	37036		38707	3378	0.417

Table A:1.7.3 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0		0.007	0.007	0.005	0.010	0.007	0.000	
STD 1		0.076	0.101	0.104		0.094	0.060	56
STD 2	0.095	0.099	0.099	0.101	0.097	0.098	0.119	-17
STD 3	0.207	0.215	0.253	0.255	0.195	0.225	0.179	26
STD 4	0.298	0.291		0.292	0.174	0.264	0.298	-11
STD 5		0.387	0.522	0.456	0.377	0.436	0.357	22
STD 6	0.391	0.359	0.465	0.421		0.409	0.417	-2
CLTR 1	0.058	0.054	0.067		0.050	0.057	0.050	14
CLTR 2	0.313	0.325		0.440		0.359	0.328	10

Table A:1.7.4 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.007		0.004			0.006	0.002	
STD1	0.101		0.034			0.068	0.047	70
STD2	0.099		0.034			0.067	0.046	69
STD3	0.253		0.072			0.163	0.128	79
STD4	0.461		0.119			0.290	0.242	83
STD5	0.522		0.17			0.346	0.249	72
STD6	0.465		0.159			0.312	0.216	69
CLTR 1	0.067	0.058	0.055			0.060	0.006	10
CLTR 2	0.454	0.369	0.353	0.277		0.363	0.073	20

Table A:1.7.5 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0		0.007	0.007	0.005	0.010	0.007	0.002	
STD1		0.076	0.101	0.104		0.094	0.015	16
STD2	0.095	0.099	0.099	0.101	0.097	0.098	0.002	2
STD3	0.207	0.215	0.253	0.255	0.195	0.225	0.027	12
STD4	0.298	0.291		0.292	0.174	0.264	0.060	23
STD5		0.387	0.522	0.456	0.377	0.436	0.068	16
STD6	0.391	0.359	0.465	0.421		0.409	0.045	11
CLTR 1	0.058	0.054	0.067		0.050	0.057	0.007	13
CLTR 2	0.313	0.325		0.440		0.359	0.070	20

Table A:1.7.6 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0		0.007	0.007	0.005	0.010	0.007	0.000	
STD 1		0.076	0.101	0.104		0.094	0.060	156
STD 2	0.095	0.099	0.099	0.101	0.097	0.098	0.119	83
STD 3	0.207	0.215	0.253	0.255	0.195	0.225	0.179	126
STD 4	0.298	0.291		0.292	0.174	0.264	0.298	89
STD 5		0.387	0.522	0.456	0.377	0.436	0.357	122
STD 6	0.391	0.359	0.465	0.421		0.409	0.417	98
CLTR 1	0.058	0.054	0.067		0.050	0.057	0.050	114
CLTR 2	0.313	0.325		0.440		0.359	0.328	110

Table A:1.7.7 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank		750	501	450	878	645
Signal lowest STD		8352	7636	9178		8389
S/N	13.011					
Actual Conc	0.060					
LOD [actual]/[S/N]*3	0.014					
LOQ [actual]/[S/N]*10	0.046					

1.8 Raltagravir (RAL)

Figure A1.8.1 Linearity of RAL in DBS

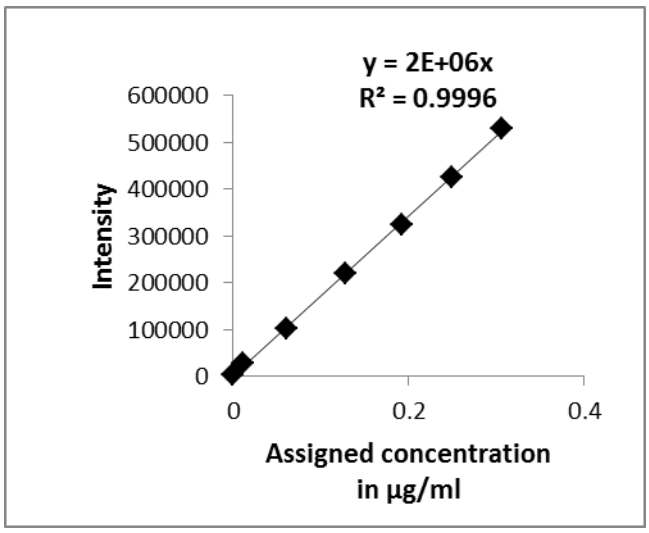


Figure 1.8.1 Linear regression curve between the mean peak height and assigned drug concentration over the course of 5 days

Table A:1.8.2 Linearity was determined from mean height aganist assigned concentration

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0		5160	4085		2015	3753	1599	0.000
STD 1	21971	28267	23509	33413		26790	5165	0.012
STD 2	121098	106932		96513	80895	101360	16960	0.061
STD 3	222108	235151		238149	182538	219487	25598	0.129
STD 4	358664	327698	327894	322059	285941	324451	25892	0.192
STD 5	344690	480787		478318	400624	426105	65813	0.250
STD 6	434596	634507	485908	567535	525341	529577	76481	0.307

Table A:1.8.3 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%Diff
STD 0		0.003	0.003		0.001	0.002	0.000	
STD 1	0.013	0.016	0.019	0.020		0.017	0.012	42
STD 2	0.069	0.061		0.057	0.048	0.059	0.061	-4
STD 3	0.127	0.135		0.141	0.108	0.128	0.129	-1
STD 4	0.206	0.188	0.271	0.190	0.169	0.205	0.192	7
STD 5	0.198	0.276		0.282	0.236	0.248	0.250	-1
STD 6	0.249	0.364	0.402	0.335	0.310	0.332	0.307	8
CLTR 1	0.044	0.040	0.034			0.039	0.036	9
CLTR 2	0.191	0.202	0.202	0.218	0.211	0.205	0.182	13

Table A:1.8.4 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.004			0.003		0.004	0.001	20
STD1	0.013			0.016		0.015	0.002	15
STD2	0.069			0.061		0.065	0.006	9
STD3	0.127			0.135		0.131	0.006	4
STD4	0.206			0.188		0.197	0.013	6
STD5	0.198			0.276		0.237	0.055	23
STD6	0.249			0.364		0.307	0.081	27
CLTR 1	0.028	0.032	0.030	0.040		0.033	0.005	16
CLTR 2	0.152		0.140	0.202	0.191	0.171	0.030	17

Table A:1.8.5 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0		0.003	0.003		0.001	0.002	0.001	
STD1	0.013	0.016	0.019	0.020		0.017	0.003	19
STD2	0.069	0.061		0.057	0.048	0.059	0.009	15
STD3	0.127	0.135		0.141	0.108	0.128	0.014	11
STD4	0.206	0.188	0.271	0.190	0.169	0.205	0.039	19
STD5	0.198	0.276		0.282	0.236	0.248	0.039	16
STD6	0.249	0.364	0.402	0.335	0.310	0.332	0.058	17
CLTR 1	0.044	0.040	0.034			0.039	0.005	13
CLTR 2	0.191	0.202	0.202	0.218	0.211	0.205	0.010	5

Table A:1.8.6 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0		0.003	0.003		0.001	0.002	0.000	
STD 1	0.013	0.016	0.019	0.020		0.017	0.012	142
STD 2	0.069	0.061		0.057	0.048	0.059	0.061	96
STD 3	0.127	0.135		0.141	0.108	0.128	0.129	99
STD 4	0.206	0.188	0.271	0.190	0.169	0.205	0.192	107
STD 5	0.198	0.276		0.282	0.236	0.248	0.250	99
STD 6	0.249	0.364	0.402	0.335	0.310	0.332	0.307	108
CLTR 1	0.044	0.040	0.034			0.039	0.036	109
CLTR 2	0.191	0.202	0.202	0.218	0.211	0.205	0.182	113

Table A:1.8.7 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank		5160	4085		2015	3753
Signal lowest STD	21971	28267	23509	33413		26790
S/N	7.138					
Actual Conc	0.012					
LOD [actual]/[S/N]*3	0.005					
LOQ [actual]/[S/N]*10	0.017					

1.9 Atazanavir (ATV)

Figure A1.9.1 Linearity ofATV in DBS

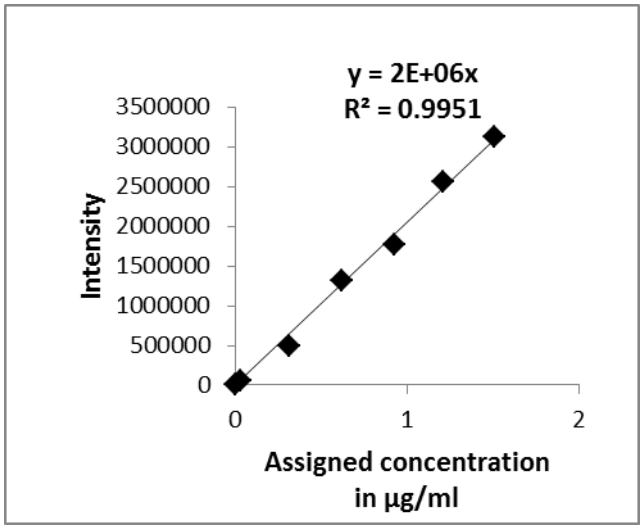


Figure 1.9.1 Linear regression curve between the mean peak height and assigned drug concentration over the course of 5 days

Table A:1.9.2 Linearity was determined from mean height aganist assigned concentration

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	assigned
STD 0	12195	12001			15481	13226	1956	0.000
STD 1	49427	45105		73344	44074	52988	13768	0.028
STD 2	534196	422570		546632	494737	499534	55875	0.310
STD 3	1169930	902456	1777748	1593237	1144172	1317509	357750	0.617
STD 4	1788730	1503493	2302568	1665075	1620782	1776130	311486	0.924
STD 5	1882371	2380364	3161803	3109319	2283006	2563373	554986	1.210
STD 6	2337968	3025871	3830428	3749393	2681470	3125026	654496	1.505

Table A:1.9.3 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%Diff
STD 0	0.007	0.007			0.007	0.007	0.000	
STD 1	0.028	0.026			0.021	0.025	0.028	-11
STD 2	0.306	0.242		0.264	0.239	0.263	0.310	-15
STD 3	0.670	0.517		0.770	0.553	0.628	0.617	2
STD 4	1.024	0.861		0.805	0.783	0.868	0.924	-6
STD 5	1.078	1.363	1.763	1.503		1.427	1.210	18
STD 6	1.339	1.733	2.136	1.812		1.755	1.505	17
CLTR 1	0.134	0.146		0.150	0.135	0.141	0.139	2
CLTR 2		0.976		0.919	0.850	0.915	0.897	2

Table A:1.9.4 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.018		0.007			0.013	0.008	
STD1	0.035		0.021			0.028	0.010	35
STD2	0.264		0.239			0.252	0.018	7
STD3	0.77		0.553			0.662	0.153	23
STD4	0.805		0.783			0.794	0.016	2
STD5	1.503		1.103			1.303	0.283	22
STD6	1.812		1.296			1.554	0.365	23
CLTR 1	0.152	0.150	0.147	0.135	0.138	0.144	0.008	5
CLTR 2	0.887	0.919	1.099	0.850	0.802	0.911	0.114	12

Table A:1.9.5 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.007	0.007			0.007	0.007	0.000	
STD1	0.028	0.026			0.021	0.025	0.004	14
STD2	0.306	0.242		0.264	0.239	0.263	0.031	12
STD3	0.670	0.517		0.770	0.553	0.628	0.115	18
STD4	1.024	0.861		0.805	0.783	0.868	0.109	13
STD5	1.078	1.363	1.763	1.503		1.427	0.286	20
STD6	1.339	1.733	2.136	1.812		1.755	0.328	19
CLTR 1	0.134	0.146		0.150	0.135	0.141	0.008	6
CLTR 2		0.976		0.919	0.850	0.915	0.063	7

Table A:1.9.6 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0	0.007	0.007			0.007	0.007	0.000	
STD 1	0.028	0.026			0.021	0.025	0.028	89
STD 2	0.306	0.242		0.264	0.239	0.263	0.310	85
STD 3	0.670	0.517		0.770	0.553	0.628	0.617	102
STD 4	1.024	0.861		0.805	0.783	0.868	0.924	94
STD 5	1.078	1.363	1.763	1.503		1.427	1.210	118
STD 6	1.339	1.733	2.136	1.812		1.755	1.505	117
CLTR 1	0.134	0.146		0.150	0.135	0.141	0.139	102
CLTR 2		0.976		0.919	0.850	0.915	0.897	102

Table A:1.9.7 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	12195	12001			15481	13226
Signal lowest STD	49427	45105		73344	44074	52988
S/N	4.006					
Actual Conc	0.028					
LOD [actual]/[S/N]*3	0.021					
LOQ [actual]/[S/N]*10	0.070					

1.10 Darunavir (TMC114)

Figure A1.10.1 Linearity of TMC114

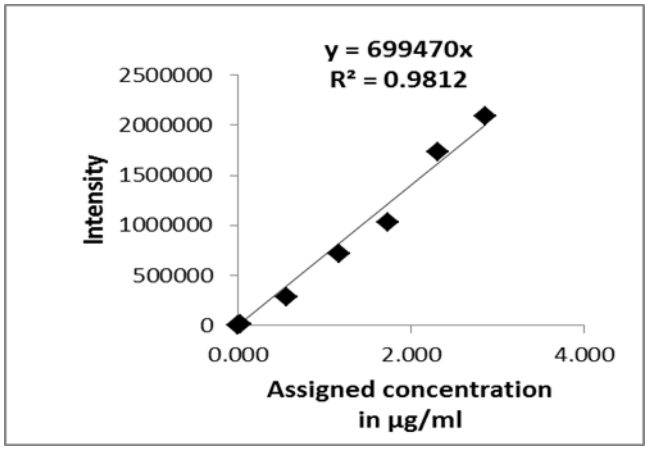


Figure 1.10.1 Linear regression curve between the mean peak height and assigned drug concentration over the course of 5 days

Table A:1.10.2 Linearity was determined from mean height aganist assigned concentration

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	assigned
STD 0	372	842	335		739	572	256	0.000
STD 1	15547	15484	15317	15510	14268	15225	542	0.025
STD 2	352689	285735		310115	207984	289131	60763	0.557
STD 3	819149	672234		785083	577594	713515	110241	1.168
STD 4	1143492	1056918	1101876	918633	908001	1025784	107202	1.738
STD 5	1613118	2046411		1656483	1603393	1729851	212298	2.312
STD 6	1870521	2419041	2173521	2112902	1858871	2086971	233085	2.862

Table A:1.10.3 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%Diff
STD 0	0.001	0.001	0.001	0.002	0.001	0.001	0.000	
STD 1	0.022	0.022	0.030	0.024	0.023	0.024	0.025	-3
STD 2	0.496	0.402		0.490	0.328	0.429	0.557	-23
STD 3	1.152	0.946		1.240	0.912	1.063	1.168	-9
STD 4	1.609	1.487	2.135	1.451	1.434	1.623	1.738	-7
STD 5	2.270	2.879		2.616	2.532	2.574	2.312	11
STD 6	2.632	3.403	4.211	3.336	2.935	3.303	2.862	15
CLTR 1	0.215	0.255	0.260	0.257	0.235	0.244	0.227	8
CLTR 2	1.528	1.820	1.872	1.859	1.845	1.785	1.684	6

Table A:1.10.4 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.001		0.001			0.001	0.000	
STD1	0.022		0.022			0.022	0.000	0
STD2	0.496		0.402			0.449	0.066	15
STD3	1.152		0.946			1.049	0.146	14
STD4	1.609		1.487			1.548	0.086	6
STD5	2.27		2.879			2.575	0.431	17
STD6	2.632		3.403			3.018	0.545	18
CLTR 1	0.215	0.223	0.202	0.255	0.250	0.229	0.023	10
CLTR 2	1.528	1.671	1.367	1.820	1.713	1.620	0.176	11

Table A:1.10.5 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.001	0.001	0.001	0.002	0.001	0.001	0.000	
STD1	0.022	0.022	0.030	0.024	0.023	0.024	0.003	14
STD2	0.496	0.402		0.490	0.328	0.429	0.080	19
STD3	1.152	0.946		1.240	0.912	1.063	0.159	15
STD4	1.609	1.487	2.135	1.451	1.434	1.623	0.294	18
STD5	2.270	2.879		2.616	2.532	2.574	0.251	10
STD6	2.632	3.403	4.211	3.336	2.935	3.303	0.596	18
CLTR 1	0.215	0.255	0.260	0.257	0.235	0.244	0.019	8
CLTR 2	1.528	1.820	1.872	1.859	1.845	1.785	0.145	8

Table A:1.10.6 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0	0.001	0.001	0.001	0.002	0.001	0.001	0.000	
STD 1	0.022	0.022	0.030	0.024	0.023	0.024	0.025	97
STD 2	0.496	0.402		0.490	0.328	0.429	0.557	77
STD 3	1.152	0.946		1.240	0.912	1.063	1.168	91
STD 4	1.609	1.487	2.135	1.451	1.434	1.623	1.738	93
STD 5	2.270	2.879		2.616	2.532	2.574	2.312	111
STD 6	2.632	3.403	4.211	3.336	2.935	3.303	2.862	115
CLTR 1	0.215	0.255	0.260	0.257	0.235	0.244	0.227	108
CLTR 2	1.528	1.820	1.872	1.859	1.845	1.785	1.684	106

Table A:1.10.7 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	372	842	335		739	572
Signal lowest STD	15547	15484	15317	15510	14268	15225
S/N	26.617					
Actual Conc	0.025					
LOD [actual]/[S/N]*3	0.003					
LOQ [actual]/[S/N]*10	0.009					

2.Validation of PSC (Missing values in the tables are the outliers)

2.1 lamuvidine (3TC)

Table A:2.1.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0		9660	13230	2427	2104	6855	5498	0.000
STD 1	4052742	424142	454425	250624	242506	1084888	1661914	0.119
STD 2	6457396	1028212	1074999	510406	488687	1911940	2556001	0.238
STD 3	9504975	1723224	1804508	1010647	939412	2996553	3659834	0.357
STD 4	14859892	2485784	2972012	1370744	1326788	4603044	5777707	0.595
STD 5	18221044	3245833	3489311	1232307	1124356	5462570	7216328	0.714
STD 6	28665182	3898148	4007198	1778783	1723485	8014559	11596492	0.833

Table A:2.1.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0		0.026	0.025	0.015	-0.024	0.011	0.000	
STD 1	0.149	0.114	0.111	0.122	0.133	0.126	0.119	6
STD 2	0.242	0.241	0.231	0.234	0.252	0.240	0.238	1
STD 3	0.307	0.387	0.372	0.448	0.339	0.371	0.357	4
STD 4	0.533	0.547	0.599	0.603	0.634	0.583	0.595	-2
STD 5	0.575	0.707	0.699	0.544	0.582	0.621	0.714	-13
STD 6	0.879	0.845	0.799	0.778	0.904	0.841	0.833	1
CLTR 1		0.126	0.136		0.116	0.126	0.121	4
CLTR 2		0.841		0.760	0.844	0.815	0.843	-3

Table A:2.1.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.005	0.005	0.006	0.006	0.006	0.006	0.001	
STD1	0.103	0.110	0.126	0.129	0.131	0.120	0.013	10
STD2	0.268	0.256	0.305	0.300	0.378	0.301	0.048	16
STD3	0.434	0.468	0.418	0.458	0.463	0.448	0.021	5
STD4	0.511	0.574	0.788	0.607	0.732	0.642	0.114	18
STD5	0.722	0.763	0.794	0.756	0.884	0.784	0.062	8
STD6	0.668	0.682	0.790	0.875	0.801	0.763	0.087	11
CLTR 1		0.101	0.110	0.104		0.105	0.005	4
CLTR 2		0.766		0.891	0.817	0.825	0.063	8

Table A:2.1.4 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0		0.026	0.025	0.015	-0.024	0.011	0.024	
STD1	0.149	0.114	0.111	0.122	0.133	0.126	0.016	12
STD2	0.242	0.241	0.231	0.234	0.252	0.240	0.008	3
STD3	0.307	0.387	0.372	0.448	0.339	0.371	0.053	14
STD4	0.533	0.547	0.599	0.603	0.634	0.583	0.042	7
STD5	0.575	0.707	0.699	0.544	0.582	0.621	0.076	12
STD6	0.879	0.845	0.799	0.778	0.904	0.841	0.053	6
CLTR 1		0.126	0.136		0.116	0.126	0.010	8
CLTR 2		0.841		0.760	0.844	0.815	0.048	6

Table A:2.1.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0		0.026	0.025	0.015	-0.024	0.011	0.000	
STD 1	0.149	0.114	0.111	0.122	0.133	0.126	0.119	106
STD 2	0.242	0.241	0.231	0.234	0.252	0.240	0.238	101
STD 3	0.307	0.387	0.372	0.448	0.339	0.371	0.357	104
STD 4	0.533	0.547	0.599	0.603	0.634	0.583	0.595	98
STD 5	0.575	0.707	0.699	0.544	0.582	0.621	0.714	87
STD 6	0.879	0.845	0.799	0.778	0.904	0.841	0.833	101
CLTR 1		0.126	0.136		0.116	0.126	0.121	104
CLTR 2		0.841		0.760	0.844	0.815	0.843	97

Table A:2.1.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank		9660	13230	2427	2104	6855
Signal lowest STD	4052742	424142	454425	250624	242506	1084888
S/N	158.256					
Actual Conc	0.119					
LOD [actual]/[S/N]*3	0.002					
LOQ [actual]/[S/N]*10	0.008					

2.2 Abacavir

Table A:2.2.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0	21480	11873		22717	18534	18651	4847	0.000
STD 1	2730497	2543962		2456091	2024110	2438665	299117	0.119
STD 2		4941206	2566375	5300736	5915039	4680839	1465864	0.238
STD 3		8435357	3264911	9025885	10533480	7814908	3159353	0.357
STD 4	14550922	11735352		15965655	14543377	14198827	1773234	0.595
STD 5	17775211	15497118	7446297	19195906	17887725	15560451	4727087	0.714
STD 6	18163097	15486310		15745461	17794277	16797286	1376520	0.833

Table A:2.2.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.003	0.008		-0.017	0.020	0.004	0.000	
STD 1	0.118	0.127		0.095	0.100	0.110	0.119	-8
STD 2		0.240	0.265	0.225	0.257	0.247	0.238	4
STD 3		0.404	0.337	0.395	0.443	0.395	0.357	11
STD 4	0.621	0.559		0.712	0.604	0.624	0.595	5
STD 5	0.758	0.736	0.764	0.860	0.739	0.771	0.714	8
STD 6	0.774	0.736		0.702	0.735	0.737	0.833	-12
CLTR 1	0.135	0.104	0.099			0.113	0.127	-11
CLTR 2	0.798	0.808	0.679			0.762	0.755	1

Table A:2.2.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.028	0.027	0.026	0.026	0.026	0.027	0.001	
STD1	0.102		0.119	0.087	0.098	0.102	0.013	13
STD2	0.197	0.194		0.210		0.200	0.009	4
STD3	0.442	0.543	0.435		0.561	0.495	0.066	13
STD4			0.368	0.296	0.465	0.376	0.085	23
STD5	0.503	0.735	0.570	0.465	0.504	0.555	0.107	19
STD6	1.219	1.203	1.228		0.910	1.140	0.154	13
CLTR 1	0.146	0.122	0.132	0.111		0.128	0.015	12
CLTR 2	0.668		0.679	0.426		0.591	0.143	24

Table A:2.2.4 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.003	0.008		-0.017	0.020	0.004	0.015	
STD1	0.118	0.127	0.123	0.095	0.100	0.113	0.014	13
STD2		0.240	0.265	0.225	0.257	0.247	0.018	7
STD3		0.404	0.337	0.395	0.443	0.395	0.044	11
STD4	0.621	0.559		0.712	0.604	0.624	0.064	10
STD5	0.758	0.736	0.764	0.860	0.739	0.771	0.051	7
STD6	0.774	0.736		0.702	0.735	0.737	0.029	4
CLTR 1	0.135	0.104	0.099			0.113	0.020	17
CLTR 2	0.798	0.808	0.679			0.762	0.072	9

Table A:2.2.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.003	0.008		-0.017	0.020	0.004	0.000	
STD 1	0.118	0.127	0.123	0.095	0.100	0.113	0.119	95
STD 2		0.240	0.265	0.225	0.257	0.247	0.238	104
STD 3		0.404	0.337	0.395	0.443	0.395	0.357	111
STD 4	0.621	0.559		0.712	0.604	0.624	0.595	105
STD 5	0.758	0.736	0.764	0.860	0.739	0.771	0.714	108
STD 6	0.774	0.736		0.702	0.735	0.737	0.833	88
CLTR 1	0.135	0.104	0.099			0.113	0.127	89
CLTR 2	0.798	0.808	0.679			0.762	0.755	101

Table A:2.2.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	21480	11873		22717	18534	18651
Signal lowest STD	2730497	2543962		2456091	2024110	2438665
S/N	130.753					
Actual Conc	0.119					
LOD [actual]/[S/N]*3	0.003					
LOQ [actual]/[S/N]*10	0.009					

2.3 Efavirenz (EFV)

Table A:2.3.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0	333	472		241		349	116	0.000
STD 1	1315		2726	1974	2150	2041	581	0.625
STD 2	2284	3179	3808	3027	5161	3492	1079	1.400
STD 3	6482	6972	9849	5946	6336	7117	1571	2.229
STD 4	7335	12193	11580	10276	11034	10484	1896	3.087
STD 5	7895	13770	14908	7157	18372	12420	4786	3.871
STD 6	11206	17614	11599	10356	18977	13950	4021	4.650

Table A:2.3.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.116	0.165		0.082		0.121	0.000	
STD 1	0.458		0.928	0.672	0.634	0.673	0.625	8
STD 2	0.796	1.108	1.296	1.030	1.522	1.150	1.400	-18
STD 3	2.259	2.430	3.352	2.024	1.868	2.387	2.229	7
STD 4	2.556	4.249	3.941	3.498	3.253	3.499	3.087	13
STD 5	2.751	4.799	5.074	2.436	5.417	4.095	3.871	6
STD 6	3.905	6.138	3.948	3.525	5.595	4.622	4.650	-1
CLTR 1	1.327	1.309			1.558	1.398	1.486	-6
CLTR 2		3.545	4.200	2.722	3.455	3.481	3.533	-1

Table A:2.3.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.116	0.165				0.141	0.035	25
STD1	0.458	0.276				0.367	0.129	35
STD2	0.796	1.108				0.952	0.221	23
STD3	2.259	2.430				2.345	0.121	5
STD4	2.556	4.249				3.403	1.197	35
STD5	2.751	4.799				3.775	1.448	38
STD6	3.905	6.138				5.022	1.579	31
CLTR 1	1.327	1.369		1.458	1.309	1.366	0.066	5
CLTR 2			3.093	3.557	3.545	3.398	0.264	8

(only two curves were available)

Table A:2.3.4 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.116	0.165		0.082		0.121	0.042	34
STD1	0.458		0.928	0.672	0.634	0.673	0.194	29
STD2	0.796	1.108	1.296	1.030	1.522	1.150	0.274	24
STD3	2.259	2.430	3.352	2.024	1.868	2.387	0.581	24
STD4	2.556	4.249	3.941	3.498	3.253	3.499	0.653	19
STD5	2.751	4.799	5.074	2.436	5.417	4.095	1.393	34
STD6	3.905	6.138	3.948	3.525	5.595	4.622	1.164	25
CLTR 1	1.327	1.309			1.558	1.398	0.139	10
CLTR 2		3.545	4.200	2.722	3.455	3.481	0.605	17

Table A:2.3.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.116	0.165		0.082		0.121	0.000	
STD 1	0.458		0.928	0.672	0.634	0.673	0.625	108
STD 2	0.796	1.108	1.296	1.030	1.522	1.150	1.400	82
STD 3	2.259	2.430	3.352	2.024	1.868	2.387	2.229	107
STD 4	2.556	4.249	3.941	3.498	3.253	3.499	3.087	113
STD 5	2.751	4.799	5.074	2.436	5.417	4.095	3.871	106
STD 6	3.905	6.138	3.948	3.525	5.595	4.622	4.650	99
CLTR 1	1.327	1.309			1.558	1.398	1.486	94
CLTR 2		3.545	4.200	2.722	3.455	3.481	3.533	99

Table A:2.3.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	333	472		241		349
Signal lowest STD	1315		2726	1974	2150	2041
S/N	5.854					
Actual Conc	0.625					
LOD [actual]/[S/N]*3	0.320					
LOQ [actual]/[S/N]*10	1.068					

2.4 Ritonavir (RTV)

Table A:2.4.1 Linearity was determined from mean height aganist assigned concentration

RUN2	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	937			758	1137	944	190	0.000
STD 1	4190	1916	2285		3327	2930	1031	0.194
STD 2	33829	14511	14279	8202	31499	20464	11450	2.252
STD 3	82855	29522	31097	22750	61839	45613	25703	5.278
STD 4	161257	69514	59491	43371	119156	90558	48608	7.877
STD 5		72865	50517	58286	194726	94099	67722	10.434
STD 6	179881	128240	86467	70403	155197	124038	45813	12.717

Table A:2.4.1 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	-0.009			0.092	0.031	0.038	0.000	
STD 1	0.221	0.179	0.211		0.199	0.203	0.194	4
STD 2	2.323	1.726	1.905	1.479	2.352	1.957	2.252	-13
STD 3	5.798	3.571	4.281	4.189	4.671	4.502	5.278	-15
STD 4	11.356	8.484	8.293	8.032	9.051	9.043	7.877	15
STD 5		8.896	7.025	10.811	14.827	10.390	10.434	0
STD 6	12.677	15.699	12.104	13.069	11.806	13.071	12.717	3
CLTR 1		1.048	1.099	1.212	0.912	1.068	1.212	-12
CLTR 2	9.316	11.342			11.645	10.768	11.397	-6

Table A:2.4.2 Intra-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.247	-0.194	0.125	0.264	0.215	0.131	0.190	
STD1	0.428	0.259	0.215	0.272	0.052	0.245	0.135	55
STD2	2.275	1.858	1.621	1.179	1.392	1.665	0.425	26
STD3	4.384	3.697	3.362	2.880	2.618	3.388	0.696	21
STD4	6.522	4.069	3.567	3.632	3.517	4.261	1.283	30
STD5	13.069	8.396	8.098	6.840	6.887	8.658	2.563	30
STD6	14.424	16.108	13.368	13.515	11.769	13.837	1.589	11
CLTR 1			1.784	1.580	1.196	1.520	0.299	20
CLTR 2		12.431	11.462		11.518	11.804	0.544	5

Table A:2.4.3 Inter-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.009			0.092	0.031	0.038	0.051	
STD1	0.221	0.179	0.211		0.199	0.203	0.018	9
STD2	2.323	1.726	1.905	1.479	2.352	1.957	0.379	19
STD3	5.798	3.571	4.281	4.189	4.671	4.502	0.825	18
STD4	11.356	8.484	8.293	8.032	9.051	9.043	1.346	15
STD5		8.896	7.025	10.811	14.827	10.390	3.338	32
STD6	12.677	15.699	12.104	13.069	11.806	13.071	1.549	12
CLTR 1		1.048	1.099	1.212	0.912	1.068	0.124	12
CLTR 2	9.316	11.342			11.645	10.768	1.266	12

Table A:2.4.4 Mean percentage Recovery

RUN1	day1	day 2	day 3	day 4	day 5	MEAN	Assigned	%recovery
STD 0	-0.009			0.092	0.031	0.038	0.000	
STD 1	0.221	0.179	0.211		0.199	0.203	0.194	104
STD 2	2.323	1.726	1.905	1.479	2.352	1.957	2.252	87
STD 3	5.798	3.571	4.281	4.189	4.671	4.502	5.278	85
STD 4	11.356	8.484	8.293	8.032	9.051	9.043	7.877	115
STD 5		8.896	7.025	10.811	14.827	10.390	10.434	100
STD 6	12.677	15.699	12.104	13.069	11.806	13.071	12.717	103
CLTR 1		1.048	1.099	1.212	0.912	1.068	1.212	88
CLTR 2	9.316	11.342			11.645	10.768	11.397	94

Table A:2.4.5 LOD and LOQ

LOD AND LOQ	day 1	day 2	day 3	day 4	day 5	MEAN
Signal Blank	937			758	1137	944
Signal lowest STD	4190	1916	2285		3327	2930
S/N	3.103					
Actual Conc	0.194					
LOD [actual]/[S/N]*3	0.188					
LOQ [actual]/[S/N]*10	0.625					

2.5 Lopinavir (LPV)

Table A:2.5.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	263	193	474		460	348	141	0.000
STD 1	798	674	1274	863		902	260	0.686
STD 2	2442	3053	3253	2127	4264	3028	827	2.710
STD 3	5212	3411	4990		3927	4385	858	4.809
STD 4	7606		12313		6454	8791	3104	6.965
STD 5	7864	10192	12847	12505	10072	10696	2035	9.007
STD 6	11610	9508	11572	12657	15190	12107	2067	10.891

Table A:2.5.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.263	0.028	0.286		0.338	0.229	0.000	
STD 1	0.729	0.516	0.790	0.580		0.654	0.686	-5
STD 2	2.163	2.929	2.038	1.444	3.260	2.367	2.710	-13
STD 3	4.578	3.292	3.132		3.001	3.501	4.809	-27
STD 4	6.666		7.747		4.942	6.452	6.965	-7
STD 5	6.891	10.167	8.084	8.541	7.721	8.281	9.007	-8
STD 6	10.158	9.474	7.280	8.646	11.652	9.442	10.891	-13
CLTR 1	2.338	2.021	2.111	2.115	1.731	2.063	2.050	1
CLTR 2	10.220		12.063		10.371	10.885	11.085	-2

Table A:2.5.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.443	0.402	0.576	0.290		0.428	0.118	
STD1	0.228	0.402		0.218		0.283	0.103	37
STD2	2.674	2.776	1.906		2.580	2.484	0.394	16
STD3	4.077	3.770	3.123	3.469	4.802	3.848	0.640	17
STD4	6.828	7.059	5.293	6.581	5.263	6.205	0.863	14
STD5	10.939	9.826	8.636	9.288	8.845	9.507	0.922	10
STD6	16.550	12.299	13.167	12.232	15.513	13.952	1.968	14
CLTR 1	2.980	2.397	1.969		2.190	2.384	0.434	18
CLTR 2		12.474	12.595	13.313		12.794	0.454	4

Table A:2.5.3 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.263	0.028	0.286		0.338	0.229	0.137	
STD1	0.729	0.516	0.790	0.580		0.654	0.127	19
STD2	2.163	2.929	2.038	1.444	3.260	2.367	0.727	31
STD3	4.578	3.292	3.132		3.001	3.501	0.728	21
STD4	6.666		7.747		4.942	6.452	1.415	22
STD5	6.891	10.167	8.084	8.541	7.721	8.281	1.215	15
STD6	10.158	9.474	7.280	8.646	11.652	9.442	1.636	17
CLTR 1	2.338	2.021	2.111	2.115	1.731	2.063	0.219	11
CLTR 2	10.220		12.063		10.371	10.885	1.023	9

Table A:2.5.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.263	0.028	0.286		0.338	0.229	0.000	
STD 1	0.729	0.516	0.790	0.580		0.654	0.686	95
STD 2	2.163	2.929	2.038	1.444	3.260	2.367	2.710	87
STD 3	4.578	3.292	3.132		3.001	3.501	4.809	73
STD 4	6.666		7.747		4.942	6.452	6.965	93
STD 5	6.891	10.167	8.084	8.541	7.721	8.281	9.007	92
STD 6	10.158	9.474	7.280	8.646	11.652	9.442	10.891	87
CLTR 1	2.338	2.021	2.111	2.115	1.731	2.063	2.050	101
CLTR 2	10.220		12.063		10.371	10.885	11.085	98

Table A:2.5.6 LOD and LOQ

LOD AND LOQ	day 1	day 2	day 3	day 4	day 5	MEAN
Signal Blank	211	253	158		164	194
Signal lowest STD	909	938	798	1274	863	956
S/N	4.868					
Actual Conc	0.686					
LOD [actual]/[S/N]*3	0.423					
LOQ [actual]/[S/N]*10	1.409					

2.6 Nevirapine (NVP)

Table A:2.6.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0	1184	3392	2189	1327	569	1732	1093	0.000
STD 1	1840110	1289704	579372	1264744	1070467	1208879	453746	1.850
STD 2	2429676	1989805	965584	2071384		1864112	628749	2.916
STD 3	2603727	2989587	1351129		4176389	2780208	1164270	4.102
STD 4	4407947	4744745	2156300	6036301	5400198	4549098	1476468	5.268
STD 5	4297258	5117722	2402533	6153654	5247273	4643688	1415257	6.344
STD 6	6051304	5836649	2627896	6407250	5781411	5340902	1536391	7.352

Table A:2.6.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	-0.107	0.092	0.067	0.211	-0.010	0.051	0.000	
STD 1	2.354	1.646	1.618	1.533	1.327	1.696	1.850	-8
STD 2	3.143	2.492	2.655	2.378		2.667	2.916	-9
STD 3	3.376	3.699	3.691		3.714	3.620	4.102	-12
STD 4	5.790	5.819	5.853	6.528	4.861	5.770	5.268	10
STD 5	5.642	6.269	6.514	6.651	7.675	6.550	6.344	3
STD 6	7.989	7.138	7.120	6.916	7.319	7.296	7.352	-1
CLTR 1	2.758	3.494	3.183	3.400		3.209	3.090	4
CLTR 2			6.274	5.977	5.186	5.812	5.939	-2

Table A:2.6. Intra-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.070	-0.070	-0.065			-0.068	0.003	
STD1	1.998	1.935	1.969	1.921	2.186	2.002	0.107	5
STD2	3.111	3.208	3.415	4.249	2.732	3.343	0.564	17
STD3	3.276	3.667	3.811	3.099	3.427	3.456	0.288	8
STD4	5.363	4.768	4.106	5.130	5.521	4.978	0.564	11
STD5	6.456	7.390	6.271	7.309	6.524	6.790	0.520	8
STD6	5.199	5.463	5.967	5.625	4.581	5.367	0.520	10
CLTR 1		2.615	2.601	2.562		2.593	0.027	1
CLTR 2		5.617		5.255	6.308	5.727	0.535	9

Table A:2.6.4 Inter-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.107	0.092	0.067	0.211	-0.010	0.051	0.119	
STD1	2.354	1.646	1.618	1.533	1.327	1.696	0.389	23
STD2	3.143	2.492	2.655	2.378		2.667	0.337	13
STD3	3.376	3.699	3.691		3.714	3.620	0.163	5
STD4	5.790	5.819	5.853	6.528	4.861	5.770	0.594	10
STD5	5.642	6.269	6.514	6.651	7.675	6.550	0.738	11
STD6	7.989	7.138	7.120	6.916	7.319	7.296	0.413	6
CLTR 1	2.758	3.494	3.183	3.400		3.209	0.328	10
CLTR 2			6.274	5.977	5.186	5.812	0.562	10

Table A:2.6.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	-0.107	0.092	0.067	0.211	-0.010	0.051	0.000	
STD 1	2.354	1.646	1.618	1.533	1.327	1.696	1.850	92
STD 2	3.143	2.492	2.655	2.378		2.667	2.916	91
STD 3	3.376	3.699	3.691		3.714	3.620	4.102	88
STD 4	5.790	5.819	5.853	6.528	4.861	5.770	5.268	110
STD 5	5.642	6.269	6.514	6.651	7.675	6.550	6.344	103
STD 6	7.989	7.138	7.120	6.916	7.319	7.296	7.352	99
CLTR 1	2.758	3.494	3.183	3.400		3.209	3.090	104
CLTR 2			6.274	5.977	5.186	5.812	5.939	98

Table A:2.6.6 LOD and LOQ

LOD AND LOQ	day 1	day 2	day 3	day 4	day 5	MEAN
Signal Blank	1184	3392	2189	1327	569	1732
Signal lowest STD	1840110	1289704	579372	1264744	1070467	1208879
S/N	697.887					
Actual Conc	1.850					
LOD [actual]/[S/N]*3	0.008					
LOQ [actual]/[S/N]*10	0.027					

2.7 Emtracitabine (FTC)

Table A:2.7.1 Linearity was determined from mean height aganist assigned concentration

RUN2	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0	13049		7791		11361	10734	2685	0.000
STD 1	70063		58143	101422	65299	73732	19099	0.060
STD 2	146685	86826	129092	186474	121037	134023	36510	0.119
STD 3		15872	165408	313435	176585	167825	121620	0.179
STD 4	333558	237589	284089	509804	278850	328778	106769	0.298
STD 5	433783	298769	387988	577032	320345	403583	110854	0.357
STD 6	443153	312858	402777	646328	333910	427805	132894	0.417

Table A:2.7.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0	0.005		0.012		-0.010	0.002	0.000	
STD 1	0.054		0.056	0.060	0.052	0.056	0.060	-8
STD 2	0.120	0.106	0.118	0.112	0.116	0.114	0.119	-4
STD 3		0.190	0.150	0.190	0.179	0.177	0.179	-1
STD 4	0.282	0.304	0.253	0.310	0.296	0.289	0.298	-3
STD 5	0.369	0.384	0.344	0.351	0.344	0.358	0.357	0
STD 6	0.377	0.402	0.357	0.394	0.359	0.378	0.417	-9
CLTR 1	0.046		0.051		0.048	0.048	0.054	-11
CLTR 2	0.440	0.383	0.397	0.407		0.407	0.418	-3

Table A:2.7.3 Intra-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.003	0.004	0.004	0.004	0.003	0.004	0.001	
STD1	0.059	0.061	0.057	0.062	0.059	0.060	0.002	3
STD2	0.147	0.152	0.164	0.147	0.133	0.149	0.011	8
STD3	0.192	0.184	0.183	0.195	0.192	0.189	0.005	3
STD4	0.293	0.323	0.267	0.230	0.260	0.275	0.035	13
STD5	0.408	0.443	0.404	0.378	0.367	0.400	0.030	7
STD6	0.300	0.346	0.304	0.286	0.290	0.305	0.024	8
CLTR 1	0.052	0.059	0.049	0.048	0.049	0.051	0.005	9
CLTR 2	0.400	0.406	0.408	0.387		0.400	0.009	2

Table A:2.7.4 Inter-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.005		0.012		-0.010	0.002	0.011	
STD1	0.054		0.056	0.060	0.052	0.056	0.003	6
STD2	0.120	0.106	0.118	0.112	0.116	0.114	0.006	5
STD3		0.190	0.150	0.190	0.179	0.177	0.019	11
STD4	0.282	0.304	0.253	0.310	0.296	0.289	0.023	8
STD5	0.369	0.384	0.344	0.351	0.344	0.358	0.018	5
STD6	0.377	0.402	0.357	0.394	0.359	0.378	0.020	5
CLTR 1	0.046		0.051		0.048	0.048	0.003	5
CLTR 2	0.440	0.383	0.397	0.407		0.407	0.024	6

Table A:2.7.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.005		0.012		-0.010	0.002	0.000	
STD 1	0.054		0.056	0.060	0.052	0.056	0.060	93
STD 2	0.120	0.106	0.118	0.112	0.116	0.114	0.119	96
STD 3		0.190	0.150	0.190	0.179	0.177	0.179	99
STD 4	0.282	0.304	0.253	0.310	0.296	0.289	0.298	97
STD 5	0.369	0.384	0.344	0.351	0.344	0.358	0.357	100
STD 6	0.377	0.402	0.357	0.394	0.359	0.378	0.417	91
CLTR 1	0.046		0.051		0.048	0.048	0.054	89
CLTR 2	0.440	0.383	0.397	0.407		0.407	0.418	97

Table A:2.7.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	1861	2249	2462	2203	1774	2110
Signal lowest STD	51678	54114	50336	54270	51641	52408
S/N	24.840					
Actual Conc	0.060					
LOD [actual]/[S/N]*3	0.007					
LOQ [actual]/[S/N]*10	0.024					

2.8 Raltagravir (RAL)

Table A:2.8.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	Assigned
STD 0	68273			68823	70347	69148	1074	0.000
STD 1	155673	173346	151278		164086	161096	9743	0.026
STD 2	442663	504285	444991	372466	425053	437892	47240	0.122
STD 3		800175	848720	795453	859781	826032	32951	0.258
STD 4	1277036	1280114	1248269	1259067	1260144	1264926	13341	0.384
STD 5	1681943	1598292	1403180		1620502	1575979	120510	0.500
STD 6	1955993	2238311	1899369		1955476	2012287	153007	0.614

Table A:2.8.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%Diff
STD 0	0.001	0.000	-0.003	-0.007	-0.002	-0.002	0.000	
STD 1	0.029	0.030	0.024	0.017	0.029	0.026	0.026	-1
STD 2	0.122	0.135	0.124	0.169	0.115	0.133	0.122	9
STD 3		0.228	0.261	0.255	0.258	0.251	0.258	-3
STD 4	0.391	0.380	0.397	0.414	0.390	0.394	0.384	3
STD 5	0.521	0.481	0.450		0.509	0.490	0.500	-2
STD 6	0.609	0.684	0.619		0.619	0.633	0.614	3
CLTR 1	0.074	0.083	0.074		0.078	0.077	0.073	6
CLTR 2	0.507		0.473		0.492	0.491	0.464	6

Table A:2.8.3 Intra-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.026	-0.021	-0.017	-0.027	-0.015	-0.021	0.005	
STD1		0.027	0.020		0.023	0.023	0.004	15
STD2	0.131	0.145	0.156	0.158	0.153	0.149	0.011	7
STD3	0.292	0.305	0.296	0.236	0.250	0.276	0.031	11
STD4	0.322	0.360	0.403	0.357	0.368	0.362	0.029	8
STD5	0.520	0.451	0.601	0.485	0.498	0.511	0.056	11
STD6	0.559	0.565	0.476	0.585	0.467	0.530	0.055	10
CLTR 1		0.064	0.064	0.076		0.068	0.007	10
CLTR 2	0.426		0.390	0.387		0.401	0.022	5

Table A:2.8.4 Inter-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.001	0.000	-0.003	-0.007	-0.002	-0.002	0.003	
STD1	0.029	0.030	0.024	0.017	0.029	0.026	0.005	21
STD2	0.122	0.135	0.124	0.169	0.115	0.133	0.021	16
STD3		0.228	0.261	0.255	0.258	0.251	0.015	6
STD4	0.391	0.380	0.397	0.414	0.390	0.394	0.013	3
STD5	0.521	0.481	0.450		0.509	0.490	0.032	6
STD6	0.609	0.684	0.619		0.619	0.633	0.034	5
CLTR 1	0.074	0.083	0.074		0.078	0.077	0.004	6
CLTR 2	0.507		0.473		0.492	0.491	0.017	3

Table A:2.8.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.001	0.000	-0.003	-0.007	-0.002	-0.002	0.000	
STD 1	0.029	0.030	0.024	0.017	0.029	0.026	0.026	99
STD 2	0.122	0.135	0.124	0.169	0.115	0.133	0.122	109
STD 3		0.228	0.261	0.255	0.258	0.251	0.258	97
STD 4	0.391	0.380	0.397	0.414	0.390	0.394	0.384	103
STD 5	0.521	0.481	0.450		0.509	0.490	0.500	98
STD 6	0.609	0.684	0.619		0.619	0.633	0.614	103
CLTR 1	0.074	0.083	0.074		0.078	0.077	0.073	106
CLTR 2	0.507		0.473		0.492	0.491	0.464	106

Table A:2.8.6 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank	55648	63797	64714	25016		52294
Signal lowest STD	173346		164086	181022	162847	170325
S/N	3.257					
Actual Conc	0.026					
LOD [actual]/[S/N]*3	0.024					
LOQ [actual]/[S/N]*10	0.080					

2.9 Atazanavir (ATV)

Table A:2.9.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0		15095	15215	11428		13913	2153	0.000
STD 1	21713		19293		23447	21484	2086	0.051
STD 2	56694	59362	64679	58004	101164	67981	18797	1.114
STD 3	109488	100193	109132	81735	162491	112608	30072	2.335
STD 4	198583	174320	174356	149268	228766	185059	30017	3.475
STD 5	239867	194828	191005	169870	318297	222773	59173	4.623
STD 6	449671	386089	361228			398996	45612	5.723

Table A:2.9.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0		-0.053	-0.029	0.024		-0.019	0.000	
STD 1	0.066		0.046		0.032	0.048	0.051	-6
STD 2	0.795	0.821	0.881		1.248	0.936	1.114	-16
STD 3	1.895	1.626	1.699	1.956	2.208	1.877	2.335	-20
STD 4	3.751	3.089	2.900	3.812	3.245	3.359	3.475	-3
STD 5	4.611	3.494	3.206	4.378	4.647	4.067	4.623	-12
STD 6	8.982	7.268	6.338			7.529	5.723	32
CLTR 1	0.550	0.621	0.618	0.708		0.624	0.619	1
CLTR 2		5.679	5.587	5.605	7.141	6.003	6.044	-1

Table A:2.9.3 Intra-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	-0.107	-0.022	-0.071	-0.210	-0.220	-0.126	0.087	
STD1	0.001		0.042		-0.023	0.007	0.033	493
STD2	0.989		0.968	0.977	1.057	0.998	0.040	4
STD3	0.985	1.487		1.744	1.540	1.439	0.322	22
STD4	3.249	2.984	3.622	3.528	3.456	3.368	0.255	8
STD5	5.395	4.349	5.679	5.400	5.817	5.328	0.577	11
STD6	5.593	7.971	6.256	8.850	6.253	6.985	1.365	20
CLTR 1		0.444		0.526	0.484	0.485	0.041	8
CLTR 2		6.448			4.600	5.524	1.307	24

Table A:2.9.4 Inter-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.041	-0.053	-0.029	0.024		-0.004	0.044	
STD1	0.066		0.046		0.032	0.048	0.017	36
STD2	0.795	0.821	0.881		1.248	0.936	0.211	23
STD3	1.895	1.626	1.699	1.956	2.208	1.877	0.230	12
STD4	3.751	3.089	2.900	3.812	3.245	3.359	0.405	12
STD5	4.611	3.494	3.206	4.378	4.647	4.067	0.671	16
STD6	8.982	7.268	6.338			7.529	1.341	18
CLTR 1	0.550	0.621	0.618	0.708		0.624	0.065	10
CLTR 2		5.679	5.587	5.605	7.141	6.003	0.760	13

Table A:2.9.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%recovery
STD 0	0.041	-0.053	-0.029	0.024		-0.004	0.000	
STD 1	0.066		0.046		0.032	0.048	0.051	94
STD 2	0.795	0.821	0.881		1.248	0.936	1.114	84
STD 3	1.895	1.626	1.699	1.956	2.208	1.877	2.335	80
STD 4	3.751	3.089	2.900	3.812	3.245	3.359	3.475	97
STD 5	4.611	3.494	3.206	4.378	4.647	4.067	4.623	88
STD 6	8.982	7.268	6.338			7.529	5.723	132
CLTR 1	0.550	0.621	0.618	0.708		0.624	0.619	101
CLTR 2		5.679	5.587	5.605	7.141	6.003	6.044	99

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Table A:2.9.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank		15095	15215	11428		13913
Signal lowest STD	21713		19293		23447	21484
S/N	1.544					
Actual Conc	0.051					
LOD [actual]/[S/N]*3	0.099					
LOQ [actual]/[S/N]*10	0.330					

2.10 Darunavir (TMC114)

Table A:2.10.1 Linearity was determined from mean height aganist assigned concentration

RUN	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	Assigned
STD 0		1222	1200		769	1064	255	0.000
STD 1	2211	2011	2274		1332	1957	431	0.051
STD 2		21728	27978	19261		22989	4493	1.169
STD 3	41258	56144	70483	44016	147022	71785	43618	2.253
STD 4	141525	136333	172795	110863		140379	25428	3.333
STD 5		128026	167174	101640		132280	32973	4.544
STD 6		226737	240093	140743		202524	53919	5.612

Table A:2.10.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	Assigned	%Diff
STD 0		0.055	0.031		-0.073	0.004	0.000	
STD 1	0.051	0.076	0.056		0.021	0.051	0.051	0
STD 2		0.608	0.647	0.736		0.664	1.169	-43
STD 3	1.483	1.537	1.624	1.688	1.292	1.525	2.253	-32
STD 4	5.158	3.699	3.977	4.259		4.273	3.333	28
STD 5		3.475	3.848	3.904		3.742	4.544	-18
STD 6		6.138	5.525	5.408		5.690	5.612	1
CLTR 1	0.632	0.433	0.673	0.530		0.567	0.604	-6
CLTR 2	4.731	4.144		5.052	4.581	4.627	4.837	-4

Table A:2.10.3 Intra-run precision

	Run 1	Run 2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0	0.037	0.030	0.055	0.024		0.037	0.013	
STD1	0.056	0.084		0.068	0.065	0.068	0.012	17
STD2	1.011	0.781	0.901	0.801	0.803	0.859	0.097	11
STD3	1.980	1.510	2.063	1.832	2.016	1.880	0.224	12
STD4	3.375	2.695	2.941	2.678	2.539	2.846	0.329	12
STD5	5.924	4.099	4.398	3.963	4.596	4.596	0.783	17
STD6	4.724	6.623	5.811	6.757	6.364	6.056	0.828	14
CLTR 1	0.671	0.705	0.686	0.705	0.624	0.678	0.033	5
CLTR 2	5.081	4.403	4.777			4.754	0.340	7

Table A:2.10.4 Inter-run precision

	Run1	Run2	Run 3	Run 4	Run 5	MEAN	SD	CV
STD 0		0.055	0.031		-0.073	0.004	0.068	
STD1	0.051	0.076	0.056		0.021	0.051	0.023	45
STD2		0.608	0.647	0.736		0.664	0.066	10
STD3	1.483	1.537	1.624	1.688	1.292	1.525	0.152	10
STD4	5.158	3.699	3.977	4.259		4.273	0.633	15
STD5		3.475	3.848	3.904		3.742	0.233	6
STD6		6.138	5.525	5.408		5.690	0.392	7
CLTR 1	0.632	0.433	0.673	0.530		0.567	0.108	19
CLTR 2	4.731	4.144		5.052	4.581	4.627	0.377	8

Table A:2.10.5 Mean percentage Recovery

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	assigned	%recovery
STD 0		0.055	0.031		-0.073	0.004	0.000	
STD 1	0.051	0.076	0.056		0.021	0.051	0.051	100
STD 2		0.608	0.647	0.736		0.664	1.169	57
STD 3	1.483	1.537	1.624	1.688	1.292	1.525	2.253	68
STD 4	5.158	3.699	3.977	4.259		4.273	3.333	128
STD 5		3.475	3.848	3.904		3.742	4.544	82
STD 6		6.138	5.525	5.408		5.690	5.612	101
CLTR 1	0.632	0.433	0.673	0.530		0.567	0.604	94
CLTR 2	4.731	4.144		5.052	4.581	4.627	4.837	96

Table A:2.10.6 LOD and LOQ

LOD AND LOQ	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN
Signal Blank		1222	1200		769	1064
Signal lowest STD	2211	2011	2274		1332	1957
S/N	1.840					
Actual Conc	0.051					
LOD [actual]/[S/N]*3	0.083					
LOQ [actual]/[S/N]*10	0.277					

3.Validation of ARVs in plasma (Missing values in the tables are the outliers)

3.1 lamuvidine (3TC)

Table A:3.1.1 Linearity

	Day 1 Absolute response	Day 2	Day 3	Day 4	Day 5
STD0		61			
STD1		96	37	113	151
STD2	190	311	341	226	164
STD3		377	428	319	307
STD4	609	530		583	
STD5	820			826	869
STD6	833	906		777	

Mean	SD	CV
61		
99	47	48
246	76	31
358	56	16
574	40	7
839	27	3
839	65	8

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0			47		
STD1		117	55	150	97
STD2	190	238	183	134	211
STD3		281	563	382	358
STD4	609	496	624	563	706
STD5	820	891	598	607	651
STD6	833			1021	

Mean	SD	CV
47		
105	40	38
191	38	20
396	119	30
600	78	13
714	134	19
927	133	14

Table A:3.1.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0		0.061				0.061		
STD 1		0.096	0.037	0.113	0.151	0.099	0.047	48
STD 2	0.190	0.311	0.341	0.226	0.164	0.246	0.076	31
STD 3		0.377	0.428	0.319	0.307	0.358	0.056	16
STD 4	0.609	0.530		0.583		0.574	0.040	7
STD 5	0.820	0.000		0.826	0.869	0.629	0.420	67
STD 6	0.833	0.906		0.777		0.839	0.065	8

Table A:3.1.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3	Mean	SD	CV
STD0						
STD1		0.126	0.147	0.140	0.020	11
STD2	0.189	0.244	0.227	0.220	0.030	13
STD3		0.225	0.257	0.240	0.020	9
STD4	0.608	0.692		0.650	0.060	9
STD5	0.820	0.790	0.689	0.770	0.070	9
STD6	0.833		0.811	0.820	0.020	2

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3	Mean	SD	CV
STD0	0.060		0.061	0.060	0.00	1
STD1	0.096	0.090	0.088	0.090	0.000	4
STD2	0.310	0.170		0.240	0.100	41
STD3	0.377		0.271	0.320	0.070	23
STD4	0.529	0.578	0.634	0.580	0.050	9
STD5		0.529	0.807	0.670	0.200	29
STD6	0.905		0.850	0.88	0.040	4

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3	Mean	SD	CV
STD0						
STD1	0.036	0.189	0.113	0.110	0.080	67
STD2	0.340	0.252	0.199	0.260	0.070	27
STD3	0.428	0.335	0.271	0.350	0.080	22
STD4		0.567	0.620	0.590	0.040	6
STD5			0.804	0.800		
STD6		0.719		0.720		

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0		0.105		0.110		
STD1	0.113	0.063		0.090	0.030	39
STD2	0.226		0.196	0.210	0.020	9
STD3	0.319	0.289		0.300	0.020	6
STD4	0.583	0.611		0.600	0.020	3
STD5	0.826	0.810	0.888	0.840	0.040	4
STD6	0.776		0.748	0.760	0.020	2

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.151			0.150		
STD2	0.164	0.138	0.317	0.210	0.100	46
STD3	0.307		0.271	0.290	0.030	8
STD4		0.556		0.560		
STD5	0.869	0.744	0.630	0.750	0.120	15
STD6			0.967	0.970		

Table A:3.1.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	µg/ml		0.126	0.148
	Assigned	0.119	119.000	0.119
	% Recovery	0	0	124
2	µg/ml	0.190	0.244	0.228
	Assigned	0.238	0.238	0.238
	% Recovery	80	103	96
3	µg/ml	0.464	0.225	0.257
	Assigned	0.357	0.357	0.357
	% Recovery	130	63	72
4	µg/ml	0.609	0.693	0.413
	Assigned	0.595	0.595	0.595
	% Recovery	102	116	69
5	µg/ml	0.820	0.790	0.689
	Assigned	0.714	0.714	0.714
	% Recovery	115	111	96
6	µg/ml	0.833	0.103	0.811
	Assigned	0.833	0.833	0.833
	% Recovery	100	12	97

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.096	0.091	0.089
	Assigned	0.119	0.119	0.119
	% Recovery	81	76	74
2	µg/ml	0.311	0.170	0.083
	Assigned	0.238	0.238	0.238
	% Recovery	131	72	35
3	µg/ml	0.377	0.100	0.271
	Assigned	0.357	0.357	0.357
	% Recovery	106	28	76
4	µg/ml	0.530	0.579	0.635
	Assigned	0.595	0.595	0.595
	% Recovery	89	97	107
5	µg/ml	1.094	0.592	0.808
	Assigned	0.714	0.714	0.714
	% Recovery	153	83	113
6	µg/ml	0.906	0.593	0.851
	Assigned	0.833	0.833	0.833
	% Recovery	109	71	102

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.037	0.190	0.113
	Assigned	0.119	0.119	0.119
	% Recovery	31	159	95
2	µg/ml	0.341	0.253	0.199
	Assigned	238.000	238.000	238.000
	% Recovery	0	0	0
3	µg/ml	0.428	0.335	0.271
	Assigned	0.357	0.357	0.357
	% Recovery	120	94	76
4	µg/ml	0.265	0.568	0.620
	Assigned	0.595	0.595	0.595
	% Recovery	45	95	104
5	µg/ml	1.001	1.330	0.804
	Assigned	0.714	0.714	0.714
	% Recovery	140	186	113
6	µg/ml	1.008	0.720	0.986
	Assigned	0.833	0.833	0.833
	% Recovery	121	86	118

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.113	0.064	
	Assigned	0.119	0.119	0.119
	% Recovery	95	54	0
2	µg/ml	0.226		0.170
	Assigned	0.238	0.238	0.238
	% Recovery	95	0	71
3	µg/ml	0.319	0.290	0.180
	Assigned	0.357	0.357	0.357
	% Recovery	89	81	50
4	µg/ml	0.583	0.611	0.389
	Assigned	0.595	0.595	0.595
	% Recovery	98	103	65
5	µg/ml	0.826	0.810	0.889
	Assigned	0.714	0.714	0.714
	% Recovery	116	113	124
6	µg/ml	0.776	1.271	0.749
	Assigned	0.833	0.833	0.833
	% Recovery	93	153	90

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.151	0.085	
	Assigned	0.119	0.119	0.119
	% Recovery	127	72	0
2	µg/ml	0.164	0.138	0.317
	Assigned	0.238	0.238	0.238
	% Recovery	69	58	133
3	µg/ml	0.307	171.600	271.200
	Assigned	0.357	0.357	0.357
	% Recovery	86	48067	75966
4	µg/ml	0.447	0.557	0.357
	Assigned	0.595	0.595	0.595
	% Recovery	75	94	60
5	µg/ml	0.869	0.744	0.631
	Assigned	0.714	0.714	0.714
	% Recovery	122	104	88
6	µg/ml	1.256	1.307	0.968
	Assigned	0.833	0.833	0.833
	% Recovery	151	157	116

Table A:3.1.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	0	71	0	38	0	22
Signal lowest STD	159450	138	118000	98	116	55560
S/N	2549					
Actual	0.119					
LOD	0.001					
LOQ	0.001					

3.2 Abacavir

Table A:3.2.1 Linearity

	Day 1 Absolute response	Day 2	Day 3
STD 0	34657	2254	587
STD1	82641	81920	122239
STD2	111568	150951	263514
STD3			384323
STD4	334632	281820	609933
STD5	448018	338224	796520
STD6		364244	

Mean	SD	CV
12499	19207	154
95600	23073	24
175344	78855	45
384323		
408795	176181	43
527587	239285	45
364244		

Table A:3.2.2 Accuracy

RUN1	Day 1	Day 2	Day 3	MEAN	SD	CV
STD 0	34657	2254	587	12499	19207	154
STD 1	82641	81920	122239	95600	23073	24
STD 2	111568	150951	263514	175344	78855	45
STD 3			384323	384323		
STD 4	334632	281820	609933	408795	176181	43
STD 5	448018	338224	796520	527587	239285	45
STD 6		364244		364244		
RUN2	**Day 1**	**Day 2**	**Day 3**	**MEAN**	**SD**	**CV**
STD 0	3514			3514		
STD 1	70586	53033	139009	87543	45427	52
STD 2	211494		287140	249317	53490	21
STD 3	241870	285818		263844	31076	12
STD 4	546768	554675	830621	644021	161648	25
STD 5	600297	562793	931701	698264	203030	29
STD 6	726232	713265	1034632	824710	181914	22
RUN3	**Day 1**	**Day 2**	**Day 3**	**MEAN**	**SD**	**CV**
STD 0		767		767		
STD 1	98054	72497	111022	93858	19602	21
STD 2	194414	196062	348905	246460	88724	36
STD 3	317182	259394	412470	329682	77300	23
STD 4	249083	509371		379227	184051	49
STD 5	572520	672321	838490	694444	134358	19
STD 6	728119	732895	968561	809858	137461	17

Table A:3.2.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD 0	34657	2342	2037
STD1	82641	69368	66698
STD2	111568	205081	208164
STD3		239728	268558
STD4	334632	398550	471737
STD5	448018	536901	508182
STD6		511633	554586

Mean	SD	CV
13012	18746	144
72902	8539	12
174938	54901	31
254143	20386	8
401640	68605	17
497700	45359	9
533110	30372	6

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD 0	2254	495	80
STD1	81920	60033	64573
STD2	150951	151432	195203
STD3			280653
STD4	281820	367466	400720
STD5	338224	428507	533331
STD6	364244	482067	571665

Mean	SD	CV
943	1154	122
68842	11551	17
165862	25411	15
280653		
350002	61344	18
433354	97644	23
472659	104030	22

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD 0	587	602	433
STD1	122239	111168	128539
STD2	263514	311917	291478
STD3	384323	511548	510604
STD4	609933	751183	
STD5	796520	917166	927508
STD6			

Mean	SD	CV
541	94	17
120649	8794	7
288970	24299	8
468825	73182	16
680558	99879	15
880398	72824	8

Table A:3.2.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	µg/ml	0.140	0.100	0.09
	Assigned	0.120	0.120	0.12
	% Recovery	117	82	76
2	µg/ml	0.190	0.300	0.290
	Assigned	0.240	0.240	0.24
	% Recovery	79	125	120
3	µg/ml		0.350	0.370
	Assigned	0.360	0.360	0.360
	% Recovery		97	103
4	µg/ml	0.570	0.580	0.650
	Assigned	0.600	0.600	0.600
	% Recovery	96	97	109
5	µg/ml	0.770	0.780	0.698
	Assigned	0.710	0.710	0.710
	% Recovery	107	110	98
6	µg/ml		0.750	0.760
	Assigned	0.830	0.830	0.830
	% Recovery		89	91

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.100	0.100	0.090
	Assigned	0.120	0.120	0.120
	% Recovery	87	87	78
2	µg/ml	0.270	0.260	0.270
	Assigned	0.240	0.240	0.240
	% Recovery	115	107	115
3	µg/ml			0.390
	Assigned	0.360	0.360	0.36
	% Recovery			109.92
4	µg/ml	0.590	0.620	0.56
	Assigned	0.600	0.600	0.60
	% Recovery	100	103	94
5	µg/ml	0.730	0.720	0.74
	Assigned	0.710	0.710	0.71
	% Recovery	103	100	104
6	µg/ml	0.80	0.810	0.800
	Assigned	0.830	0.830	0.830
	% Recovery	95	97	95

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.120	0.090	0.1100
	Assigned	0.120	0.12	0.12
	% Recovery	98	80	88
2	µg/ml	0.250	0.250	0.230
	Assigned	0.240	0.240	0.240
	% Recovery	104	104	96
3	µg/ml	0.360	0.400	0.390
	Assigned	0.360	0.360	0.360
	% Recovery	100	111.41	110
4	µg/ml	0.570	0.580	
	Assigned	0.600	0.600	0.600
	% Recovery	95	97	
5	µg/ml	0.740	0.700	0.700
	Assigned	0.710	0.710	0.710
	% Recovery	103	98	99
6	µg/ml			
	Assigned	0.830	0.830	0.830
	% Recovery			

Table A:3.2.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	0	1007	196			401
Signal lowest STD	83760	69150	124090			92333
S/N	230.321					
Actual	0.060					
LOD	0.001					
LOQ	0.003					

3.3 Efavirenz (EFV)

Table A:3.3.1 linearity

	Day 1 Absolute. response	Day 2	Day 3	Day 4	Day 5
STD0				53	
STD1	385	748	667	787	814
STD2	1673	1932	1741	1890	1384
STD3	2137	2487	1912	3169	1888
STD4	3190	3244	3307	3569	2937
STD5	4085	3701	3521	3784	3832
STD6	4293	4468	4366	4591	4555

Mean	SD	CV
53		
680	174	26
1724	217	13
2319	532	23
3249	227	7
3785	205	5
4455	125	3

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0				159	219
STD1	207	430	738	321	485
STD2	896	1324	1304	1862	1387
STD3	1855	2569	2719	2713	2544
STD4	2550	3471	2938	2893	2379
STD5	2864	3878	3474	3628	3392
STD6	3316	4464	4963	6605	5950

Mean	SD	CV
189	43	23
436	199	46
1355	344	25
2480	358	14
2846	420	15
3447	375	11
5060	1283	25

Table A:3.3.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0				0.053		0.053		
STD 1	0.385	0.748	0.667	0.787	0.814	0.680	0.174	26
STD 2	1.673	1.932	1.741	1.890	1.384	1.724	0.217	13
STD 3	2.137	2.487	1.912	3.169	1.888	2.319	0.532	23
STD 4	3.190	3.244	3.307	3.569	2.937	3.249	0.227	7
STD 5	4.085	3.701	3.521	3.784	3.832	3.785	0.205	5
STD 6	4.293	4.468	4.366	4.591	4.555	4.455	0.125	3

Table A:3.3.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3	Mean	SD	CV
STD0	0.000	0.158	0.000	0.053	0.091	173
STD1	0.385	0.616	0.629	0.544	0.137	25
STD2	1.673	1.862	1.347	1.627	0.261	16
STD3	2.137	2.312	2.478	2.309	0.170	7
STD4	3.190		3.405	3.298	0.152	5
STD5	4.085	3.555	4.144	3.928	0.325	8
STD6	4.293	4.465		4.379	0.121	3

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3	Mean	SD	CV
STD0	0.000	0.000	0.000	0.000	0.000	0
STD1	0.748	0.000	0.562	0.437	0.390	89
STD2	1.932	1.352	0.000	1.095	0.991	91
STD3	2.487	0.000	1.987	1.491	1.315	88
STD4	3.244	3.145	3.520	3.303	0.194	6
STD5	3.701	3.759	3.708	3.723	0.032	1
STD6	4.468			4.468		

	DAY 3 run 1	Day 3 run 2	Day 3 run 3	Mean	SD	CV
STD0	0.000	0.000	0.000	0.000	0.000	0
STD1	0.667	0.606	0.741	0.671	0.068	10
STD2	1.741	2.128	1.269	1.713	0.430	25
STD3	1.912	2.306	2.099	2.106	0.197	9
STD4	3.307	2.550	2.509	2.789	0.450	16
STD5	3.521	4.920	4.182	4.208	0.700	17
STD6	4.366	4.904	4.679	4.650	0.270	6

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0	0.053	0.166	0.095	0.105	0.057	54
STD1	0.787	0.231	0.178	0.399	0.338	85
STD2	1.890	1.620	1.892	1.801	0.157	9
STD3	3.169	2.549	2.060	2.592	0.556	21
STD4	3.569	2.879	3.482	3.310	0.376	11
STD5	3.784	4.009	3.370	3.721	0.324	9
STD6	4.591	4.435	3.914	4.313	0.354	8

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.814	0.369	0.654	0.613	0.225	37
STD2	1.384	1.730	1.390	1.502	0.198	13
STD3	1.888	2.962	2.613	2.488	0.548	22
STD4	2.937	2.907	2.722	2.855	0.117	4
STD5	3.832			3.832		
STD6	4.555	5.033		4.794	0.338	7

Table A:3.3.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	µg/ml	0.385	0.616	0.629
	Assigned	0.656	0.656	0.656
	% Recovery	59	94	96
2	µg/ml	1.673	1.862	1.347
	Assigned	1.456	1.456	1.456
	% Recovery	115	128	92
3	µg/ml	2.137	2.312	2.478
	Assigned	2.244	2.244	2.244
	% Recovery	95	103	110
4	µg/ml	3.190		3.405
	Assigned	3.112	3.112	3.112
	% Recovery	103	0	109
5	µg/ml	4.085	3.555	4.144
	Assigned	3.948	3.948	3.948
	% Recovery	103	90	105
6	µg/ml	4.293	4.465	
	Assigned	4.720	4.720	4.720
	% Recovery	91	95	0

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.748		0.562
	Assigned	0.656	0.656	0.656
	% Recovery	114	0	86
2	µg/ml	1.932	1.351	
	Assigned	1.456	1.456	1.456
	% Recovery	133	93	0
3	µg/ml	2.487		1.987
	Assigned	2.244	2.244	2.244
	% Recovery	111	0	89
4	µg/ml	3.244	3.145	3.520
	Assigned	3.112	3.112	3.112
	% Recovery	104	101	113
5	µg/ml	3.701	3.759	3.708
	Assigned	3.948	3.948	3.948
	% Recovery	94	95	94
6	µg/ml	4.468		
	Assigned	4.720	4.720	4.720
	% Recovery	95	0	0

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.667	0.606	0.741
	Assigned	0.656	0.656	0.656
	% Recovery	102	92	113
2	µg/ml	1.741	2.128	1.269
	Assigned	1.456	1.456	1.456
	% Recovery	120	146	87
3	µg/ml	1.912	2.306	2.099
	Assigned	2.244	2.244	2.244
	% Recovery	85	103	94
4	µg/ml	3.307	2.550	2.509
	Assigned	3.112	3.112	3.112
	% Recovery	106	82	81
5	µg/ml	3.521	4.920	4.182
	Assigned	3.948	3.948	3.948
	% Recovery	89	125	106
6	µg/ml	4.366	4.904	4.679
	Assigned	4.720	4.720	4.720
	% Recovery	92	104	99

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.787	0.231	0.178
	Assigned	0.656	0.656	0.656
	% Recovery	120	35	27
2	µg/ml	1.890	1.620	1.892
	Assigned	1.456	1.456	1.456
	% Recovery	130	111	130
3	µg/ml	3.169	2.459	2.060
	Assigned	2.244	2.244	2.244
	% Recovery	141	110	92
4	µg/ml	3.569	2.879	3.482
	Assigned	3.112	3.112	3.112
	% Recovery	115	92	112
5	µg/ml	3.784	4.009	3.370
	Assigned	3.948	3.948	3.948
	% Recovery	96	102	85
6	µg/ml	4.591	4.435	3.914
	Assigned	4.720	4.720	4.720
	% Recovery	97	94	83

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.814	0.369	0.654
	Assigned	0.656	0.656	0.656
	% Recovery	124	56	100
2	µg/ml	1.384	1.730	1.390
	Assigned	1.456	1.456	1.456
	% Recovery	95	119	95
3	µg/ml	1.888	2.962	2.613
	Assigned	2.244	2.244	2.244
	% Recovery	84	132	116
4	µg/ml	2.937	2.907	2.722
	Assigned	3.112	3.112	3.112
	% Recovery	94	93	87
5	µg/ml	3.832		
	Assigned	3.948	3.948	3.948
	% Recovery	97	0	0
6	µg/ml	4.555	5.033	
	Assigned	4.720	4.720	4.720
	% Recovery	97	107	0

Table A:3.3.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	57	0	0		0	14
Signal lowest STD	486	549	442	281	324	416
S/N	29					
Actual	0.656					
LOD	0.067					
LOQ	0.223					

3.4 Ritonavir (RTV)

Table A:3.4.1 Linearity

	Day 1 Absolute. response	Day 2	Day 3	Day 4	Day 5
STD0					
STD1	223	276	335	311	307
STD2	3286	3106	3700	3532	3854
STD3		6478	5758	5901	6587
STD4	8092	8596	8264	8418	8547
STD5			11070	10167	10473
STD6		11930	10941	11231	12911

Mean	SD	CV
286	49	17
3406	263	8
6046	381	6
8342	215	3
10618	638	6
11367	508	4

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0					
STD1	223	249	274	241	271
STD2	3286	3520	3721	3666	3463
STD3		6492	6484	6578	6877
STD4	8092	8063	8118	7928	7424
STD5		10307	10019	9283	9364
STD6		12739	12753	13673	13970

Mean	SD	CV
255	17	7
3636	104	3
6518	52	1
8036	98	1
9870	528	5
13055	535	4

Table A:3.4.2 accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0								
STD 1	0.223	0.276	0.335	0.311	0.307	0.290	0.043	15
STD 2	3.286	3.106	3.700	3.532	3.854	3.496	0.303	9
STD 3		6.478	5.758	5.901	6.587	6.181	0.412	7
STD 4	8.092	8.596	8.264	8.418	8.547	8.383	0.207	2
STD 5			11.070	10.167	10.473	10.570	0.459	4
STD 6		11.930	10.941		12.911	11.927	0.985	8

Table A:2.4.3 precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD0	0.000	0.000	0.000
STD1	0.223	0.000	0.259
STD2	3.286	0.004	3.854
STD3	0.000	0.006	5.962
STD4	8.092	0.008	8.629
STD5		9.379	9.978
STD6			

Mean	SD	CV
0.000	0.000	86
0.161	0.140	87
2.381	2.079	87
1.989	3.441	173
5.576	4.830	87
9.679	0.423	4

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD0			
STD1	0.276	0.316	0.259
STD2	3.106	3.542	3.562
STD3	6.478	6.194	6.204
STD4	8.596	8.168	8.744
STD5		9.635	9.635
STD6	11.930		

Mean	SD	CV
0.284	0.029	10
3.403	0.258	8
6.292	0.161	3
8.503	0.299	4
9.635	0.000	0
11.930		

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD0			
STD1	0.335	0.179	0.257
STD2	3.700	3.688	3.544
STD3	5.758	6.053	5.464
STD4	8.264	8.625	7.207
STD5	11.070	10.589	10.151
STD6	10.941	10.919	11.558

Mean	SD	CV
0.257	0.078	30
3.644	0.087	2
5.758	0.294	5
8.032	0.737	9
10.603	0.460	4
11.139	0.363	3

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0						
STD1	0.311	0.239	0.240	0.263	0.041	16
STD2	3.532	3.920	3.716	3.723	0.194	5
STD3	5.901	5.268	5.469	5.546	0.324	6
STD4	8.418	9.059	8.665	8.714	0.323	4
STD5	10.167	10.192		10.180	0.018	0
STD6		11.596		11.596		

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.307	0.253	0.220	0.260	0.044	17
STD2	3.854	3.622	3.323	3.600	0.266	7
STD3	6.587	5.977	6.187	6.250	0.310	5
STD4	8.547	8.850	7.978	8.458	0.443	5
STD5	10.473	10.388	9.665	10.175	0.444	4
STD6	12.911	12.809	12.156	12.626	0.410	3

Table A:3.4.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	µg/ml	0.223	0.293	0.259
	Assigned	0.208	0.208	0.208
	% Recovery	107	141	124
2	µg/ml	3.286	3.882	3.854
	Assigned	3.024	3.024	3.024
	% Recovery	109	128	127
3	µg/ml		5.861	5.962
	Assigned	5.663	5.663	5.663
	% Recovery	0	103	105
4	µg/ml	8.092	8.024	8.629
	Assigned	8.313	8.313	8.313
	% Recovery	97	97	104
5	µg/ml		9.379	9.978
	Assigned	10.881	10.881	10.881
	% Recovery	0	86	92
6	µg/ml			
	Assigned	13.280	13.280	13.280
	% Recovery	0	0	0

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.276	0.316	0.259
	Assigned	0.208	0.208	0.208
	% Recovery	133	152	124
2	µg/ml	3.106	3.542	3.562
	Assigned	3.024	3.024	3.024
	% Recovery	103	117	118
3	µg/ml	0.647	6.194	6.204
	Assigned	5.663	5.663	5.663
	% Recovery	11	109	110
4	µg/ml	8.596	8.168	8.744
	Assigned	8.313	8.313	8.313
	% Recovery	103	98	105
5	µg/ml	9.066	9.635	9.657
	Assigned	10.881	10.881	10.881
	% Recovery	83	89	89
6	µg/ml	11.930	11.307	10.999
	Assigned	13.280	13.280	13.280
	% Recovery	90	85	83

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.335	0.179	0.257
	Assigned	0.208	0.208	0.208
	% Recovery	161	86	124
2	µg/ml	3.700	3.688	3.544
	Assigned	3.024	3.024	3.024
	% Recovery	122	122	117
3	µg/ml	5.785	6.053	5.464
	Assigned	5.663	5.663	5.663
	% Recovery	102	107	96
4	µg/ml	8.264	8.625	7.207
	Assigned	8.313	8.313	8.313
	% Recovery	99	104	87
5	µg/ml	11.070	10.589	10.151
	Assigned	10.881	10.881	10.881
	% Recovery	102	97	93
6	µg/ml	10.941	10.919	11.558
	Assigned	13.280	13.280	13.280
	% Recovery	82	82	87
		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.311	0.239	0.239
	Assigned	0.208	0.208	0.208
	% Recovery	150	115	115
2	µg/ml	3.532	3.920	3.716
	Assigned	3.024	3.024	3.024
	% Recovery	117	130	123
3	µg/ml	5.901	5.268	5.469
	Assigned	5.663	5.663	5.663
	% Recovery	104	93	97
4	µg/ml	8.418	9.059	8.665
	Assigned	8.313	8.313	8.313
	% Recovery	101	109	104
5	µg/ml	10.167	10.192	9.705
	Assigned	10.881	10.881	10.881
	% Recovery	93	94	89
6	µg/ml	11.231	11.569	10.768
	Assigned	13.280	13.280	13.280
	% Recovery	85	87	81

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.307	0.253	0.220
	Assigned	0.208	0.208	0.208
	% Recovery	147	122	106
2	µg/ml	3.854	3.622	3.323
	Assigned	3.024	3.024	3.024
	% Recovery	127	120	110
3	µg/ml	6.587	5.977	6.187
	Assigned	5.663	5.663	5.663
	% Recovery	116	106	109
4	µg/ml	8.547	8.850	7.978
	Assigned	8.313	8.313	8.313
	% Recovery	103	106	96
5	µg/ml	10.473	10.388	9.665
	Assigned	10.881	10.881	10.881
	% Recovery	96	95	89
6	µg/ml	12.911	12.809	12.156
	Assigned	13.280	13.280	13.280
	% Recovery	97	96	92

Table A:3.4.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	41	58	106	61	75	68
Signal lowest STD	5712	3897	3333	3384	3081	3882
S/N	57					
Actual	0.208					
LOD	0.011					
LOQ	0.037					

3.5 Lopinavir (LPV)

Table A:3.5.1 Linearity

	Day 1	Day 2	Day 3	Day 4	Day 5
STD0					
STD1	811	749	874	835	817
STD2	3176	3041	3638	3686	3384
STD3	4632	5764	5357	5051	5468
STD4	7206	7897	7855	7534	7796
STD5	9106	8635	10354	9199	8811
STD6	10253	11044	10937	10736	11159

Mean	SD	CV
817	45	6
3385	281	8
5254	431	8
7658	289	4
9221	673	7
10826	356	3

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0					
STD1	811	781	829	774	865
STD2	3176	3146	3249	2875	3069
STD3	4632	5473	5670	5717	5789
STD4	7206	7116	7137	6782	6703
STD5	9106	9120	8394	8223	7997
STD6	10253	10900	11257	12165	12114

Mean	SD	CV
812	37	5
3103	143	5
5456	475	9
6989	229	3
8568	517	6
11338	816	7

Table :3.5.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0								
STD 1	0.811	0.749	0.874	0.835	0.817	0.817	0.045	6
STD 2	3.176	3.041	3.638	3.686	3.384	3.385	0.281	8
STD 3	4.632	5.764	5.357	5.051	5.468	5.254	0.431	8
STD 4	7.206	7.897	7.855	7.534	7.796	7.658	0.289	4
STD 5	9.106	8.635	10.354	9.199	8.811	9.221	0.673	7
STD 6	10.253	11.044	10.937	10.736	11.159	10.826	0.356	3

Table A:2.5.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3	Mean	SD	CV
STD0						
STD1	0.811	0.821	0.780	0.800	0.020	3
STD2	3.176	3.587	3.639	3.470	0.250	7
STD3	4.632	5.354	5.388	5.120	0.430	8
STD4	7.206	7.667	7.845	7.570	0.330	4
STD5	9.106	9.076	9.755	9.310	0.380	4
STD6	10.253	10.214	10.293	10.250	0.040	1

0.001

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3	Mean	SD	CV
STD0						
STD1	0.749	0.724	0.866	0.780	0.070	10
STD2	3.041	3.100	3.182	3.110	0.070	3
STD3	5.764	5.449	5.291	5.500	0.240	4
STD4	7.897	7.358	7.822	7.690	0.290	4
STD5	8.635	8.197	8.598	8.480	0.240	3
STD6	11.044	11.009	10.879	10.980	0.090	1

	DAY 3 run 1	Day 3 run 2	Day 3 run 3	Mean	SD	CV
STD0						
STD1	0.874	0.764	0.724	0.790	0.080	10
STD2	3.638	3.349	3.307	3.430	0.180	5
STD3	5.357	5.110	4.872	5.110	0.240	5
STD4	7.855	6.882	6.909	7.220	0.550	8
STD5	10.354	9.648	9.061	9.690	0.650	7
STD6	10.937	10.118	9.8478	10.300	0.570	6

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV

STD0						
STD1	0.835	0.751	0.756	0.780	0.050	6
STD2	3.686	3.441	3.270	3.47	0.210	6
STD3	5.0521	5.077	4.849	4.990	0.120	3
STD4	7.5341	7.623	7.364	7.510	0.130	1
STD5	9.199	9.295	9.049	9.180	0.120	1
STD6	10.736	10.634	10.459	10.610	0.140	1

	DAY 5 run 1	**Day 5 run2**	**Day 5 run3**	**Mean**	**SD**	**CV**
STD0						
STD1	0.817	0.726	0.746	0.760	0.050	6
STD2	3.383	3.263	2.888	3.180	0.260	8
STD3	5.468	5.2927	4.99	5.250	0.240	5
STD4	7.796	7.611	6.697	7.370	0.590	8
STD5	8.811	8.733		8.770	0.050	1
STD6	11.159	11.103	10.815	11.030	0.180	2

Table A:3.5.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	µg/ml	0.811	0.823	0.780
	Assigned	0.740	0.740	0.740
	% Recovery	110	111	105
2	µg/ml	3.176	3.587	3.639
	Assigned	2.906	2.906	2.906
	% Recovery	109	123	125
3	µg/ml	4.632	5.354	5.388
	Assigned	4.983	4.983	4.983
	% Recovery	93	107	108
4	µg/ml	7.206	7.667	7.545
	Assigned	7.233	7.233	7.233
	% Recovery	100	106	104
5	µg/ml	9.106	9.076	9.755
	Assigned	9.304	9.304	9.304
	% Recovery	98	98	105
6	µg/ml	10.253	10.215	10.296
	Assigned	11.370	11.370	11.370
	% Recovery	90	90	91

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.749	0.727	0.866
	Assigned	0.740	0.740	0.740
	% Recovery	101	98	117
2	µg/ml	3.041	3.100	3.182
	Assigned	2.906	2.906	2.906
	% Recovery	105	107	109
3	µg/ml	5.764	5.449	5.291
	Assigned	4.983	4.983	4.983
	% Recovery	116	109	106
4	µg/ml	7.897	7.358	7.822
	Assigned	7.233	7.233	7.233
	% Recovery	109	102	108
5	µg/ml	8.635	8.197	8.598
	Assigned	9.304	9.304	9.304
	% Recovery	93	88	92
6	µg/ml	11.044	11.009	10.879
	Assigned	11.370	11.370	11.370
	% Recovery	97	97	96

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.874	0.765	0.724
	Assigned	0.740	0.740	0.740
	% Recovery	118	103	98
2	µg/ml	3.638	3.349	3.307
	Assigned	2.906	2.906	2.906
	% Recovery	125	115	114
3	µg/ml	5.357	5.110	4.872
	Assigned	4.983	4.983	4.983
	% Recovery	107	103	98
4	µg/ml	7.855	6.882	6.910
	Assigned	7.233	7.233	7.233
	% Recovery	109	95	96
5	µg/ml	10.354	9.648	9.061
	Assigned	9.304	9.304	9.304
	% Recovery	111	104	97
6	µg/ml	10.937	10.118	9.848
	Assigned	11.370	11.370	11.370
	% Recovery	96	89	87

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.835	0.751	0.756
	Assigned	0.740	0.740	0.740
	% Recovery	113	101	102
2	µg/ml	3.686	3.441	3.270
	Assigned	2.906	2.906	2.906
	% Recovery	127	118	113
3	µg/ml	5.051	5.077	4.849
	Assigned	4.983	4.983	4.983
	% Recovery	101	102	97
4	µg/ml	7.531	7.623	7.364
	Assigned	7.233	7.233	7.233
	% Recovery	104	105	102
5	µg/ml	9.199	9.295	9.049
	Assigned	9.304	9.304	9.304
	% Recovery	99	100	97
6	µg/ml	10.736	10.634	10.459
	Assigned	11.370	11.370	11.370
	% Recovery	94	94	92

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.817	0.726	0.746
	Assigned	0.740	0.740	0.740
	% Recovery	110	98	101
2	µg/ml	3.384	3.263	2.888
	Assigned	2.906	2.906	2.906
	% Recovery	116	112	99
3	µg/ml	5.468	5.293	4.994
	Assigned	4.983	4.983	4.983
	% Recovery	110	106	100
4	µg/ml	7.796	7.612	6.697
	Assigned	7.233	7.233	7.233
	% Recovery	108	105	93
5	µg/ml	8.811	8.733	8.236
	Assigned	9.304	9.304	9.304
	% Recovery	95	94	89
6	µg/ml	11.159	11.103	10.815
	Assigned	11.370	11.370	11.370
	% Recovery	98	98	95

Table A:3.5.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	111	73	55	70	153	92
Signal lowest STD	26289	15304	14182	12331	11021	15825
S/N	171					
Actual	0.740					
LOD	0.013					
LOQ	0.043					

3.6 Nevirapine (NVP)

Table A:3.6.1 Linearity

	Day 1 Absolute. response	Day 2	Day 3	Day 4	Day 5
STD0					
STD1	2300	2107	2390	2126	2215
STD2	3392	3475	3997	3745	3729
STD3	4284	4857	4746	4379	4698
STD4	5401	5394	5292	6092	5684
STD5	6430		6423	6442	6580
STD6		6494	7271	6763	7126

Mean	SD	CV
2230	137	6
3652	275	8
4567	278	6
5545	368	7
6432	10	0
6843	395	6

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0					
STD1	2300	2462	2411	2420	2333
STD2	3392	3480	3466	3133	3093
STD3	4284	4369	4266	4797	4838
STD4	5401	5320	5588	4951	5366
STD5	6430	6434	6066	5634	5506
STD6		7138	7405	8267	8066

Mean	SD	CV
2398	69	3
3368	161	5
4429	249	6
5315	267	5
6141	379	6
7603	590	8

Table A:3.6.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0								
STD 1	2.300	2.107	2.390	2.126	2.215	2.227	0.119	5
STD 2	3.392	3.475	3.997	3.745	3.729	3.668	0.240	7
STD 3	4.284	4.857	4.746	4.379	4.698	4.593	0.248	5
STD 4	5.401	5.394	5.292	6.092	5.684	5.572	0.325	6
STD 5	6.430		6.423	6.442	6.580	6.469	0.074	1
STD 6		6.494	7.271	6.763	7.126	6.913	0.352	5

Table A:3.6.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD0	0.000	0.000	0.000
STD1	2.300	2.330	2.041
STD2	3.392	3.524	3.465
STD3	4.284	3.972	4.416
STD4	5.401	5.338	5.197
STD5	6.430	6.104	6.374
STD6			

Mean	SD	CV
0.000	0.000	0
2.224	0.159	7
3.460	0.066	2
4.224	0.228	5
5.312	0.104	2
6.303	0.174	3

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD0	0.000	0.000	0.000
STD1	2.107	2.095	2.122
STD2	3.475	3.508	3.327
STD3	4.857	4.228	4.832
STD4	5.394	5.619	5.574
STD5			6.030
STD6	6.494		6.914

Mean	SD	CV
0.000	0.000	0
2.108	0.014	1
3.437	0.097	3
4.639	0.356	8
5.529	0.119	2
6.030		
6.704	0.297	4

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD0	0.000	0.000	0.000
STD1	2.390	2.349	2.029
STD2	3.997	3.713	3.596
STD3	4.746	4.392	4.540
STD4	5.292	5.053	5.150
STD5	6.423	6.598	6.616
STD6	7.271	6.868	6.582

Mean	SD	CV
0.000	0.000	0
2.256	0.197	9
3.769	0.206	5
4.559	0.178	4
5.165	0.120	2
6.546	0.107	2
6.907	0.346	5

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0	0.000	0.000	0.000	0.000	0.000	0
STD1	2.126	2.193	2.251	2.190	0.063	3
STD2	3.745	3.573	3.293	3.537	0.228	6
STD3	4.379	4.675	4.409	4.488	0.163	4
STD4	6.092	5.510	5.768	5.790	0.292	5
STD5	6.442	6.332	6.026	6.267	0.216	3
STD6	6.763	7.298	6.732	6.931	0.318	5

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0	0.000	0.000	0.000	0.000	0.000	0
STD1	2.215	2.053	2.303	2.190	0.127	6
STD2	3.729	3.176	3.582	3.496	0.287	8
STD3	4.698	4.433	4.105	4.412	0.297	7
STD4	5.684	5.455	5.390	5.509	0.154	3
STD5	6.580	6.128	6.208	6.305	0.241	4
STD6	7.126	7.609	7.135	7.290	0.277	4

Table A:3.6.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	**µg/ml**	2.300	2.330	2.041
	Assigned	1.909	1.909	1.909
	% Recovery	120	122	107
2	**µg/ml**	3.392	3.524	3.465
	Assigned	3.135	3.135	3.135
	% Recovery	108	112	111
3	**µg/ml**	4.284	3.972	4.416
	Assigned	4.274	4.274	4.274
	% Recovery	100	93	103
4	**µg/ml**	5.401	5.338	5.197
	Assigned	5.529	5.529	5.529
	% Recovery	98	97	94
5	**µg/ml**	6.430	6.104	6.374
	Assigned	6.675	6.675	6.675
	% Recovery	96	91	95
6	**µg/ml**			
	Assigned	7.680	7.680	7.680
	% Recovery	0	0	0

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	2.107	2.095	2.122
	Assigned	1.909	1.909	1.909
	% Recovery	110	110	111
2	µg/ml	3.475	3.508	3.327
	Assigned	3.135	3.135	3.135
	% Recovery	111	112	106
3	µg/ml	4.857	4.228	4.833
	Assigned	4.274	4.274	4.274
	% Recovery	114	99	113
4	µg/ml	5.394	5.619	5.574
	Assigned	5.529	5.529	5.529
	% Recovery	98	102	101
5	µg/ml			6.030
	Assigned	6.675	6.675	6.675
	% Recovery	0	0	90
6	µg/ml	6.494		6.914
	Assigned	7.680	7.680	7.680
	% Recovery	85	0	90

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	2.389	2.349	2.029
	Assigned	1.909	1.909	1.909
	% Recovery	125	123	106
2	µg/ml	3.997	3.713	3.596
	Assigned	3.135	3.135	3.135
	% Recovery	127	118	115
3	µg/ml	4.746	4.392	4.540
	Assigned	4.274	4.274	4.274
	% Recovery	111	103	106
4	µg/ml	5.292	5.053	5.150
	Assigned	5.529	5.529	5.529
	% Recovery	96	91	93
5	µg/ml	6.423	6.598	6.616
	Assigned	6.675	6.675	6.675
	% Recovery	96	99	99
6	µg/ml	7.271	6.868	6.582
	Assigned	7.680	7.680	7.680
	% Recovery	95	89	86

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	2.126	2.193	2.251
	Assigned	1.909	1.909	1.909
	% Recovery	111	115	118
2	µg/ml	3.745	3.573	3.293
	Assigned	3.135	3.135	3.135
	% Recovery	119	114	105
3	µg/ml	437.809	4.675	4.409
	Assigned	4.274	4.274	4.274
	% Recovery	10244	109	103
4	µg/ml	6.092	5.510	5.768
	Assigned	5.529	5.529	5.529
	% Recovery	110	100	104
5	µg/ml	6.442	6.332	6.026
	Assigned	6.675	6.675	6.675
	% Recovery	97	95	90
6	µg/ml	6.763	7.298	6.732
	Assigned	7.680	7.680	7.680
	% Recovery	88	95	88

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	2.390	2.349	2.029
	Assigned	1.909	1.909	1.909
	% Recovery	125	123	106
2	µg/ml	3.997	3.713	3.596
	Assigned	3.135	3.135	3.135
	% Recovery	127	118	115
3	µg/ml	4.746	4.392	4.540
	Assigned	4.274	4.274	4.274
	% Recovery	111	103	106
4	µg/ml	5.292	5.053	5.150
	Assigned	5.529	5.529	5.529
	% Recovery	96	91	93
5	µg/ml	6.423	6.598	6.616
	Assigned	6.675	6.675	6.675
	% Recovery	96	99	99
6	µg/ml	7.271	6.868	6.582
	Assigned	7.680	7.680	7.680
	% Recovery	95	89	86

Table A:3.6.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	128	72	111	9	53	75
Signal lowest STD	39567	28354	25041	21798	20667	27086
S/N	363					
Actual	1.909					
LOD	0.016					
LOQ	0.053					

3.7 Emtracitabine (FTC)

Table A:3.7.1 Linearity

	Day 1	Day 2	Day 3	Day 4	Day 5
STD0		18			
STD1	43	66	56	82	69
STD2	87	125	128	104	96
STD3	154	151	244	191	169
STD4		316	344	387	298
STD5				415	
STD6		439	380	356	

Mean	SD	CV
18		
63	15	23
108	18	17
182	38	21
336	39	12
415		
392	43	11

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0		7		13	12
STD1	43	50	56	49	41
STD2	87	128	109	90	117
STD3	154		184	174	215
STD4		296		313	267
STD5		352	375	316	326
STD6		424	408	489	466

Mean	SD	CV
11	3	30
48	6	12
106	17	16
182	25	14
292	23	8
342	27	8
447	37	8

Table A:3.7.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0		0.018				0.018		
STD 1	0.043	0.066	0.056	0.082	0.069	0.063	0.015	23
STD 2	0.087	0.125	0.128	0.104	0.096	0.108	0.018	17
STD 3	0.154	0.151	0.244	0.191	0.169	0.182	0.038	21
STD 4		0.316	0.344	0.387	0.298	0.336	0.039	12
STD 5				0.415		0.415		
STD 6		0.439	0.380	0.356		0.392	0.043	11

Table A:2.7.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD0			
STD1	0.043	0.070	0.061
STD2	0.087	0.120	0.109
STD3	0.154	0.170	0.184
STD4		0.299	0.348
STD5		0.362	0.425
STD6			0.370

Mean	SD	CV
0.058	0.014	24
0.105	0.017	16
0.169	0.015	9
0.324	0.035	11
0.393	0.045	11
0.370		

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD0	0.018		
STD1	0.066	0.055	0.062
STD2	0.125	0.086	0.102
STD3	0.151	0.131	0.174
STD4	0.316	0.344	0.322
STD5		0.368	0.358
STD6	0.439		0.418

Mean	SD	CV
0.018		
0.061	0.005	9
0.104	0.020	19
0.152	0.022	14
0.327	0.015	4
0.363	0.007	2
0.429	0.015	3

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD0		0.013	
STD1	0.056	0.048	0.045
STD2	0.128	0.112	0.119
STD3	0.244	0.174	0.155
STD4	0.344	0.271	0.284
STD5		0.434	0.377
STD6	0.380	0.377	0.385

Mean	SD	CV
0.013		
0.050	0.006	12
0.120	0.008	7
0.191	0.047	25
0.300	0.039	13
0.405	0.040	10
0.381	0.004	1

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0						
STD1	0.082	0.044	0.048	0.058	0.021	36
STD2	0.104	0.087	0.104	0.098	0.010	10
STD3	0.191	0.128	0.168	0.162	0.032	20
STD4	0.387	0.408	0.271	0.355	0.074	21
STD5	0.415	0.380	0.321	0.372	0.048	13
STD6	0.356	0.432	0.364	0.384	0.041	11

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.069	0.058	0.060	0.062	0.006	9
STD2	0.096	0.112	0.077	0.095	0.018	19
STD3	0.169	0.184		0.177	0.011	6
STD4	0.298	0.308	0.327	0.311	0.015	5
STD5		0.368	0.392	0.380	0.017	4
STD6		0.426	0.394	0.410	0.023	6

Table A:3.7.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	**µg/ml**	0.043	0.070	0.061
	Assigned	0.060	0.060	0.060
	% Recovery	71	117	101
2	**µg/ml**	0.087	0.120	0.109
	Assigned	0.119	0.119	0.119
	% Recovery	73	101	91
3	**µg/ml**	0.154	0.170	0.184
	Assigned	0.179	0.179	0.179
	% Recovery	86	95	103
4	**µg/ml**	0.240	0.299	0.348
	Assigned	0.298	0.298	0.298
	% Recovery	81	100	117
5	**µg/ml**	0.299	0.362	0.425
	Assigned	0.357	0.357	0.357
	% Recovery	84	101	119
6	**µg/ml**	0.298	0.276	0.370
	Assigned	0.417	0.417	0.417
	% Recovery	72	66	89

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.066	0.055	0.062
	Assigned	0.060	0.060	0.060
	% Recovery	111	92	103
2	µg/ml	0.125	0.086	0.102
	Assigned	0.119	0.119	0.119
	% Recovery	105	72	86
3	µg/ml	0.151	0.131	0.174
	Assigned	0.179	0.179	0.179
	% Recovery	84	73	97
4	µg/ml	0.316	0.344	0.322
	Assigned	0.298	0.298	0.298
	% Recovery	106	115	108
5	µg/ml	0.475	0.368	0.358
	Assigned	0.357	0.357	0.357
	% Recovery	133	103	100
6	µg/ml	0.439	0.336	0.418
	Assigned	0.417	0.417	0.417
	% Recovery	105	81	100

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.056	0.048	0.045
	Assigned	0.060	0.060	0.060
	% Recovery	94	80	75
2	µg/ml	0.128	0.112	0.119
	Assigned	0.119	0.119	0.119
	% Recovery	108	94	100
3	µg/ml	0.244	0.174	0.155
	Assigned	0.179	0.179	0.179
	% Recovery	136	97	87
4	µg/ml	0.344	0.271	0.284
	Assigned	0.298	0.298	0.298
	% Recovery	116	91	95
5	µg/ml	0.541	0.434	0.377
	Assigned	0.357	0.357	0.357
	% Recovery	152	121	106
6	µg/ml	0.380	0.377	0.385
	Assigned	0.417	0.417	0.417
	% Recovery	91	90	92

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.082	0.044	0.048
	Assigned	0.060	0.060	0.060
	% Recovery	137	74	80
2	µg/ml	0.104	0.087	0.104
	Assigned	0.119	0.119	0.119
	% Recovery	87	73	87
3	µg/ml	0.191	0.128	0.168
	Assigned	0.179	0.179	0.179
	% Recovery	107	72	94
4	µg/ml	0.387	0.408	0.271
	Assigned	0.298	0.298	0.298
	% Recovery	130	137	91
5	µg/ml	0.415	0.380	0.321
	Assigned	0.357	0.357	0.357
	% Recovery	116	106	90
6	µg/ml	0.356	0.432	0.364
	Assigned	0.417	0.417	0.417
	% Recovery	85	104	87

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.069	0.058	0.060
	Assigned	0.060	0.060	0.060
	% Recovery	115	97	99
2	µg/ml	0.096	0.112	0.077
	Assigned	0.119	0.119	0.119
	% Recovery	81	94	65
3	µg/ml	0.169	0.184	0.112
	Assigned	0.179	0.179	0.179
	% Recovery	94	103	63
4	µg/ml	0.298	0.308	0.327
	Assigned	0.298	0.298	0.298
	% Recovery	100	103	110
5	µg/ml	0.325	0.368	0.392
	Assigned	0.357	0.357	0.357
	% Recovery	91	103	110
6	µg/ml	0.345	0.426	0.394
	Assigned	0.417	0.417	0.417
	% Recovery	83	102	94

Table A:3.7.5LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	0	36	21	19	0	15
Signal lowest STD	548	453	333	316	293	389
S/N	25					
Actual	0.060					
LOD	0.007					
LOQ	0.024					

3.8 Raltagravir (RAL)

Table A:3.8.1 Linearity

	Day 1 Absolute response	Day 2	Day 3	Day 4	Day 5
STD0			3		
STD1	35	30	14	31	28
STD2	124	132	131	142	172
STD3	233	261	295	263	232
STD4	428	367	381	416	348
STD5	500		525	463	480
STD6	612		696	573	602

Mean	SD	CV
3		
28	8	29
140	19	14
257	26	10
388	34	9
492	27	5
621	53	9

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0				10	
STD1	35	20	32	30	39
STD2	124	176	140	141	132
STD3	233	262	304	268	236
STD4	428	387	365	304	345
STD5	500	459	444		380
STD6	612	579	598	646	750

Mean	SD	CV
10		
31	7	23
143	20	14
260	29	11
366	46	13
446	50	11
637	68	11

Table A:3.8.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0			0.003			0.003		
STD 1	0.035	0.030	0.014	0.031	0.028	0.028	0.008	29
STD 2	0.124	0.132	0.131	0.142	0.172	0.140	0.019	14
STD 3	0.233	0.261	0.295	0.263	0.232	0.257	0.026	10
STD 4	0.428	0.367	0.381	0.416	0.348	0.388	0.034	9
STD 5	0.500		0.525	0.463	0.480	0.492	0.027	5
STD 6	0.612		0.696	0.573	0.602	0.621	0.053	9

Table A:3.8.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD0			
STD1	0.035	0.048	0.019
STD2	0.124	0.151	0.134
STD3	0.233	0.247	0.305
STD4	0.428	0.400	
STD5	0.500	0.464	
STD6	0.612	0.581	0.574

Mean	SD	CV
0.034	0.015	43
0.137	0.013	10
0.262	0.038	15
0.414	0.020	5
0.482	0.025	5
0.589	0.020	3

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD0			
STD1	0.030	0.018	0.027
STD2	0.132	0.170	0.167
STD3	0.261	0.261	0.294
STD4	0.367	0.375	0.365
STD5			
STD6		0.519	0.575

Mean	SD	CV
0.025	0.006	25
0.156	0.021	13
0.272	0.019	7
0.369	0.005	1
0.547	0.040	7

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD0	0.003		0.009
STD1	0.014	0.042	0.014
STD2	0.131	0.146	0.134
STD3	0.295	0.278	0.204
STD4	0.381	0.353	0.380
STD5	0.525	0.529	0.506
STD6	0.696	0.571	0.448

Mean	SD	CV
0.006	0.004	76
0.023	0.016	69
0.137	0.008	6
0.259	0.048	19
0.372	0.016	4
0.520	0.012	2
0.572	0.124	22

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0						
STD1	0.031	0.030	0.034	0.032	0.002	6
STD2	0.142	0.147	0.176	0.155	0.018	12
STD3	0.263	0.234	0.268	0.255	0.019	7
STD4	0.416	0.362	0.400	0.393	0.028	7
STD5	0.463		0.521	0.492	0.041	8
STD6	0.573	0.585	0.509	0.556	0.041	7

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.028	0.024	0.020	0.024	0.004	17
STD2	0.172	0.120	0.182	0.158	0.034	21
STD3	0.232	0.280	0.244	0.252	0.025	10
STD4	0.348	0.368	0.365	0.360	0.011	3
STD5	0.480	0.509		0.495	0.021	4
STD6	0.602	0.598	0.581	0.594	0.011	2

Table A:3.8.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	**µg/ml**	0.035	0.048	0.019
	Assigned	0.026	0.026	0.026
	% Recovery	138	189	74
2	**µg/ml**	0.124	0.151	0.134
	Assigned	0.139	0.139	0.139
	% Recovery	89	109	97
3	**µg/ml**	0.233	0.247	0.305
	Assigned	0.251	0.251	0.251
	% Recovery	93	98	121
4	**µg/ml**	0.428	0.400	0.446
	Assigned	0.368	0.368	0.368
	% Recovery	116	109	121
5	**µg/ml**	0.500	0.164	0.178
	Assigned	0.494	0.494	0.494
	% Recovery	101	33	36
6	**µg/ml**	0.612	0.581	0.574
	Assigned	0.605	0.605	0.605
	% Recovery	101	96	95

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.030	0.018	0.027
	Assigned	0.026	0.026	0.026
	% Recovery	115	69	105
2	µg/ml	0.132	0.168	0.167
	Assigned	0.139	0.139	0.139
	% Recovery	95	121	120
3	µg/ml	0.261	0.261	0.294
	Assigned	0.251	0.251	0.251
	% Recovery	104	104	117
4	µg/ml	0.367	0.375	0.365
	Assigned	0.368	0.368	0.368
	% Recovery	100	102	99
5	µg/ml	0.433	0.391	0.398
	Assigned	0.494	0.494	0.494
	% Recovery	88	79	81
6	µg/ml	0.490	0.519	0.575
	Assigned	0.605	0.605	0.605
	% Recovery	81	86	95

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.014	0.042	0.014
	Assigned	0.026	0.026	0.026
	% Recovery	54	163	55
2	µg/ml	0.131	0.146	0.134
	Assigned	0.139	0.139	0.139
	% Recovery	94	105	96
3	µg/ml	0.295	0.278	0.204
	Assigned	0.251	0.251	0.251
	% Recovery	117	111	81
4	µg/ml	0.381	0.353	0.380
	Assigned	0.368	0.368	0.368
	% Recovery	104	96	103
5	µg/ml	0.525	0.529	0.506
	Assigned	0.494	0.494	0.494
	% Recovery	106	107	102
6	µg/ml	0.696	0.571	0.448
	Assigned	0.605	0.605	0.605
	% Recovery	115	94	74

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.031	0.030	0.034
	Assigned	0.026	0.026	0.026
	% Recovery	120	118	132
2	µg/ml	0.142	0.147	0.176
	Assigned	0.139	0.139	0.139
	% Recovery	102	106	126
3	µg/ml	0.263	0.234	0.268
	Assigned	0.251	0.251	0.251
	% Recovery	105	93	107
4	µg/ml	0.416	0.362	0.400
	Assigned	0.368	0.368	0.368
	% Recovery	113	98	109
5	µg/ml	0.463	0.547	0.521
	Assigned	0.494	0.494	0.494
	% Recovery	94	111	105
6	µg/ml	0.573	0.585	0.509
	Assigned	0.605	0.605	0.605
	% Recovery	95	97	84

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.028	0.024	0.020
	Assigned	0.026	0.026	0.026
	% Recovery	111	94	78
2	µg/ml	0.172	0.120	0.182
	Assigned	0.139	0.139	0.139
	% Recovery	124	86	131
3	µg/ml	0.232	0.280	0.244
	Assigned	0.251	0.251	0.251
	% Recovery	93	111	97
4	µg/ml	0.348	0.368	0.365
	Assigned	0.368	0.368	0.368
	% Recovery	94	100	99
5	µg/ml	0.480	0.509	0.408
	Assigned	0.494	0.494	0.494
	% Recovery	97	103	83
6	µg/ml	0.602	0.598	0.581
	Assigned	0.605	0.605	0.605
	% Recovery	99	99	96

Table A:3.8.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	77	124	110	41	0	70
Signal lowest STD	804	544	399	618	356	544
S/N	8					
Actual	0.026					
LOD	0.010					
LOQ	0.034					

3.9 Atazanavir (ATV)

Table A:3.9.1 Linearity

	Day 1 Absolute response	Day 2	Day 3	Day 4	Day 5
STD0					
STD1	88	75	69	54	68
STD2	857	864	940	903	858
STD3	1393	1475	1484	1526	1404
STD4	2198	2204	1995	2238	2043
STD5	2434	2431	2962	2828	2506
STD6	2944	3209	3478	3084	3411

Mean	SD	CV
71	12	17
884	36	4
1456	56	4
2135	109	5
2632	246	9
3225	222	7

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0					
STD1	88	58	69	66	72
STD2	857	890	792	790	766
STD3	1393	1622	1499	1494	1620
STD4	2198	2117	2107	1817	1853
STD5	2434	2358	2451	2336	2312
STD6	2944	3153	3271	3695	3574

Mean	SD	CV
71	11	16
819	52	6
1526	97	6
2018	171	8
2378	61	3
3328	307	9

Table A:3.9.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0								
STD 1	0.088	0.075	0.069	0.054	0.068	0.071	0.012	17
STD 2	0.857	0.864	0.940	0.903	0.858	0.884	0.036	4
STD 3	1.393	1.475	1.484	1.526	1.404	1.456	0.056	4
STD 4	2.198	2.204	1.995	2.238	2.043	2.135	0.109	5
STD 5	2.434	2.431	2.962	2.828	2.506	2.632	0.246	9
STD 6	2.944	3.209	3.478	3.084	3.411	3.225	0.222	7

Table A:3.9.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD0			
STD1	0.088	0.098	0.042
STD2	0.857	0.796	0.897
STD3	1.393	1.428	1.558
STD4	2.198	2.150	2.252
STD5		2.616	2.703
STD6	2.944	2.966	2.954

Mean	SD	CV
0.076	0.030	39
0.850	0.051	6
1.460	0.087	6
2.200	0.051	2
2.660	0.061	2
2.955	0.011	0

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD0			
STD1	0.075	0.058	0.063
STD2	0.864	0.804	0.813
STD3	1.475	1.450	1.489
STD4	2.204	2.078	2.301
STD5	2.431	2.474	2.512
STD6	3.209	3.113	3.180

Mean	SD	CV
0.065	0.009	13
0.827	0.032	4
1.471	0.020	1
2.194	0.112	5
2.473	0.040	2
3.167	0.049	2

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD0			
STD1	0.069	0.056	0.066
STD2	0.940	0.857	0.819
STD3	1.484	1.420	1.441
STD4	1.995	2.000	1.767
STD5	2.962	2.647	2.721
STD6	3.478	3.002	2.869

Mean	SD	CV
0.064	0.007	11
0.872	0.062	7
1.448	0.032	2
1.920	0.133	7
2.777	0.165	6
3.117	0.320	10

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0						
STD1	0.054	0.065	0.065	0.061	0.006	10
STD2	0.903	0.881	0.779	0.854	0.066	8
STD3	1.526	1.362	1.204	1.364	0.161	12
STD4	2.238	2.179	2.052	2.156	0.095	4
STD5	2.828	2.566	2.682	2.692	0.132	5
STD6	3.084	3.218	2.908	3.070	0.155	5

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.069	0.097	0.063	0.077	0.018	24
STD2	0.940	0.858	0.808	0.868	0.067	8
STD3	1.484	1.391	1.418	1.431	0.048	3
STD4	1.995	2.073	1.973	2.013	0.052	3
STD5	2.962	2.601	2.336	2.633	0.314	12
STD6	3.478	3.323	3.361	3.387	0.081	2

Table A:3.9.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	**µg/ml**	0.088	0.098	0.042
	Assigned	0.067	0.067	0.067
	% Recovery	132	147	63
2	**µg/ml**	0.857	0.796	0.897
	Assigned	0.687	0.687	0.687
	% Recovery	125	116	131
3	**µg/ml**	1.393	1.428	1.558
	Assigned	1.281	1.281	1.281
	% Recovery	109	112	122
4	**µg/ml**	2.198	2.149	2.252
	Assigned	1.911	1.911	1.911
	% Recovery	115	112	118
5	**µg/ml**	2.434	2.616	2.703
	Assigned	2.504	2.504	2.504
	% Recovery	97	104	108
6	**µg/ml**	2.944	2.966	2.954
	Assigned	3.138	3.138	3.138
	% Recovery	94	95	94

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.075	0.058	0.063
	Assigned	0.067	0.067	0.067
	% Recovery	112	87	94
2	µg/ml	0.864	0.804	0.813
	Assigned	0.687	0.687	0.687
	% Recovery	126	117	118
3	µg/ml	1.475	1.450	1.489
	Assigned	1.281	1.281	1.281
	% Recovery	115	113	116
4	µg/ml	2.204	2.078	2.301
	Assigned	1.911	1.911	1.911
	% Recovery	115	109	120
5	µg/ml	2.431	2.474	2.512
	Assigned	2.504	2.504	2.504
	% Recovery	97	99	100
6	µg/ml	3.209	3.113	3.180
	Assigned	3.138	3.138	3.138
	% Recovery	102	99	101

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.069	0.056	0.066
	Assigned	0.067	0.067	0.067
	% Recovery	104	84	99
2	µg/ml	0.940	0.857	0.819
	Assigned	0.687	0.687	0.687
	% Recovery	137	125	119
3	µg/ml	1.484	1.420	1.441
	Assigned	1.281	1.281	1.281
	% Recovery	116	111	112
4	µg/ml	1.995	2.000	1.767
	Assigned	1.911	1.911	1.911
	% Recovery	104	105	92
5	µg/ml	2.962	2.647	2.721
	Assigned	2.504	2.504	2.504
	% Recovery	118	106	109
6	µg/ml	3.478	3.002	2.869
	Assigned	3.138	3.138	3.138
	% Recovery	111	96	91

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.054	0.065	0.065
	Assigned	0.067	0.067	0.067
	% Recovery	81	97	97
2	µg/ml	0.903	0.881	0.779
	Assigned	0.687	0.687	0.687
	% Recovery	131	128	113
3	µg/ml	1.526	1.362	1.204
	Assigned	1.281	1.281	1.281
	% Recovery	119	106	94
4	µg/ml	2.238	2.179	2.052
	Assigned	1.911	1.911	1.911
	% Recovery	117	114	107
5	µg/ml	2.828	2.566	2.682
	Assigned	2.504	2.504	2.504
	% Recovery	113	102	107
6	µg/ml	3.039	3.218	2.908
	Assigned	3.138	3.138	3.138
	% Recovery	97	103	93

		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.068	0.097	0.063
	Assigned	0.067	0.067	0.067
	% Recovery	102	146	95
2	µg/ml	0.585	0.858	0.808
	Assigned	0.687	0.687	0.687
	% Recovery	85	125	118
3	µg/ml	1.404	1.391	1.418
	Assigned	1.281	1.281	1.281
	% Recovery	110	109	111
4	µg/ml	2.043	2.073	1.973
	Assigned	1.911	1.911	1.911
	% Recovery	107	108	103
5	µg/ml	2.506	2.601	2.336
	Assigned	2.504	2.504	2.504
	% Recovery	100	104	93
6	µg/ml	3.411	3.323	3.361
	Assigned	3.138	3.138	3.138
	% Recovery	109	106	107

Table A:3.9.5LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	78	102	49	47	82	72
Signal lowest STD	3827	2358	2209	1913	2589	2579
S/N	36					
Actual	0.067					
LOD	0.006					
LOQ	0.019					

3.10 Darunavir (TMC114)

Table A:3.10.1 Linearity

	Day 1 Absolute response	Day 2	Day 3	Day 4	Day 5
STD0					
STD1	39	47	65	62	54
STD2	1170	1377	1449	1352	1217
STD3	2084	2440	2440	2182	2381
STD4	3224	3469	3380	3490	3514
STD5					
STD6	4599	5273	5578	5525	5311

Mean	SD	CV
53	11	20
1313	116	9
2305	163	7
3415	118	3
5257	391	7

	Day 1 curve 1	Day 2 curve 1	Day 3 curve 1	Day 4 curve 1	Day 5 curve 1
STD0					
STD1	39	61	44	44	52
STD2	1170	1290	1232	1100	1100
STD3	2084	2434	2408	2306	2426
STD4	3224	3221	3193	2852	2973
STD5					
STD6	4599	5414	5541	6117	5867

Mean	SD	CV
48	8	17
1178	83	7
2332	148	6
3093	170	6
5508	578	10

Table A:3.10.2 Accuracy

RUN1	Day 1	Day 2	Day 3	Day 4	Day 5	MEAN	SD	CV
STD0								
STD 1	0.039	0.047	0.065	0.062	0.054	0.053	0.011	20
STD 2	1.170	1.377	1.449	1.352	1.217	1.313	0.116	9
STD 3	2.084	2.440	2.440	2.182	2.381	2.305	0.163	7
STD 4	3.224	3.469	3.380	3.490	3.514	3.415	0.118	3
STD 5								
STD 6	4.599	5.273	5.578	5.525	5.311	5.257	0.391	7

Table A:3.10.3 Precision

	DAY 1 run 1	Day 1 run2	Day1 run3
STD0			
STD1	0.039	0.045	0.037
STD2	1.170	1.346	1.194
STD3	2.084	2.285	2.337
STD4	3.224	3.267	3.254
STD5			4.148
STD6	4.599	5.068	4.705

Mean	SD	CV
0.040	0.004	10
1.236	0.096	8
2.235	0.134	6
3.248	0.022	1
4.148		
4.791	0.246	5

Intraday

	DAY 2 run 1	Day 2 run2	Day 2 run3
STD0			
STD1	0.047	0.028	0.070
STD2	1.377	1.233	1.284
STD3	2.440	2.320	2.384
STD4	3.469	3.496	3.463
STD5			
STD6	5.273	5.096	5.317

Mean	SD	CV
0.048	0.021	44
1.298	0.073	6
2.381	0.060	3
3.476	0.018	1
5.229	0.117	2

Intraday

	DAY 3 run 1	Day 3 run 2	Day 3 run 3
STD0			
STD1	0.065	0.055	0.053
STD2	1.449	1.379	1.202
STD3	2.440	2.405	2.096
STD4	3.380	3.323	3.306
STD5			
STD6	5.578	5.112	5.412

Mean	SD	CV
0.058	0.006	10
1.343	0.127	9
2.314	0.189	8
3.336	0.039	1
5.367	0.236	4

	DAY 4 run 1	Day 4 run2	Day 4 run3	Mean	SD	CV
STD0						
STD1	0.062	0.052	0.037	0.050	0.012	25
STD2	1.352	1.380	1.347	1.360	0.018	1
STD3	2.182	2.231	2.165	2.193	0.034	2
STD4	3.490	3.561	3.560	3.537	0.041	1
STD5						
STD6	5.525	5.294	5.017	5.279	0.254	5

	DAY 5 run 1	Day 5 run2	Day 5 run3	Mean	SD	CV
STD0						
STD1	0.054	0.058	0.056	0.056	0.002	3
STD2	1.217	1.186	1.117	1.173	0.051	4
STD3	2.381	2.274	2.255	2.303	0.068	3
STD4	3.514	3.176	3.121	3.270	0.213	7
STD5						
STD6	5.311	5.830	5.678	5.606	0.267	5

Table A:3.10.4 Recovery

		DAY 1 run 1	Day 1 run2	Day1 run3
1	**µg/ml**	0.039	0.045	0.037
	Assigned	0.052	0.052	0.052
	% Recovery	76	87	71
2	**µg/ml**	1.170	1.346	1.194
	Assigned	1.169	1.169	1.169
	% Recovery	100	115	102
3	**µg/ml**	2.084	2.285	2.337
	Assigned	2.253	2.253	2.253
	% Recovery	93	101	104
4	**µg/ml**	3.224	3.267	3.254
	Assigned	3.333	3.333	3.333
	% Recovery	97	98	98
5	**µg/ml**	4.078	4.148	4.148
	Assigned	4.544	4.544	4.544
	% Recovery	90	91	91
6	**µg/ml**	4.599	5.068	4.705
	Assigned	5.612	5.612	5.612
	% Recovery	82	90	84

		DAY 2 run 1	Day 2 run2	Day 2 run3
1	µg/ml	0.047	0.028	0.070
	Assigned	0.052	0.052	0.052
	% Recovery	90	54	136
2	µg/ml	1.368	1.233	1.284
	Assigned	1.169	1.169	1.169
	% Recovery	117	106	110
3	µg/ml	2.440	2.320	2.384
	Assigned	2.253	2.253	2.253
	% Recovery	108	103	106
4	µg/ml	3.469	3.496	3.463
	Assigned	3.333	3.333	3.333
	% Recovery	104	105	104
5	µg/ml	4.107	3.856	4.382
	Assigned	4.544	4.544	4.544
	% Recovery	90	85	96
6	µg/ml	5.273	5.096	5.317
	Assigned	5.612	5.612	5.612
	% Recovery	94	91	95

		DAY 3 run 1	Day 3 run2	Day 3 run3
1	µg/ml	0.065		
	Assigned	0.052	0.052	0.052
	% Recovery	125	0	0
2	µg/ml	1.449		
	Assigned	1.169	1.169	1.169
	% Recovery	124	0	0
3	µg/ml	2.440		
	Assigned	2.253	2.253	2.253
	% Recovery	108	0	0
4	µg/ml	3.380		
	Assigned	3.333	3.333	3.333
	% Recovery	101	0	0
5	µg/ml	4.976	4.742	4.473
	Assigned	4.544	4.544	4.544
	% Recovery	110	104	98
6	µg/ml	5.578	5.112	5.412
	Assigned	5.612	5.612	5.612
	% Recovery	99	91	96

		DAY 4 run 1	Day 4 run2	Day 4 run3
1	µg/ml	0.062	0.052	0.037
	Assigned	0.052	0.052	0.052
	% Recovery	120	100	72
2	µg/ml	1.352	1.380	1.347
	Assigned	1.169	1.169	1.169
	% Recovery	116	118	115
3	µg/ml	2.182	2.231	2.165
	Assigned	2.253	2.253	2.253
	% Recovery	97	99	96
4	µg/ml	3.490	3.561	3.560
	Assigned	3.333	3.333	3.333
	% Recovery	105	107	107
5	µg/ml	4.643	4.672	4.603
	Assigned	4.544	4.544	4.544
	% Recovery	102	103	101
6	µg/ml	5.525	5.294	5.017
	Assigned	5.612	5.612	5.612
	% Recovery	98	94	89

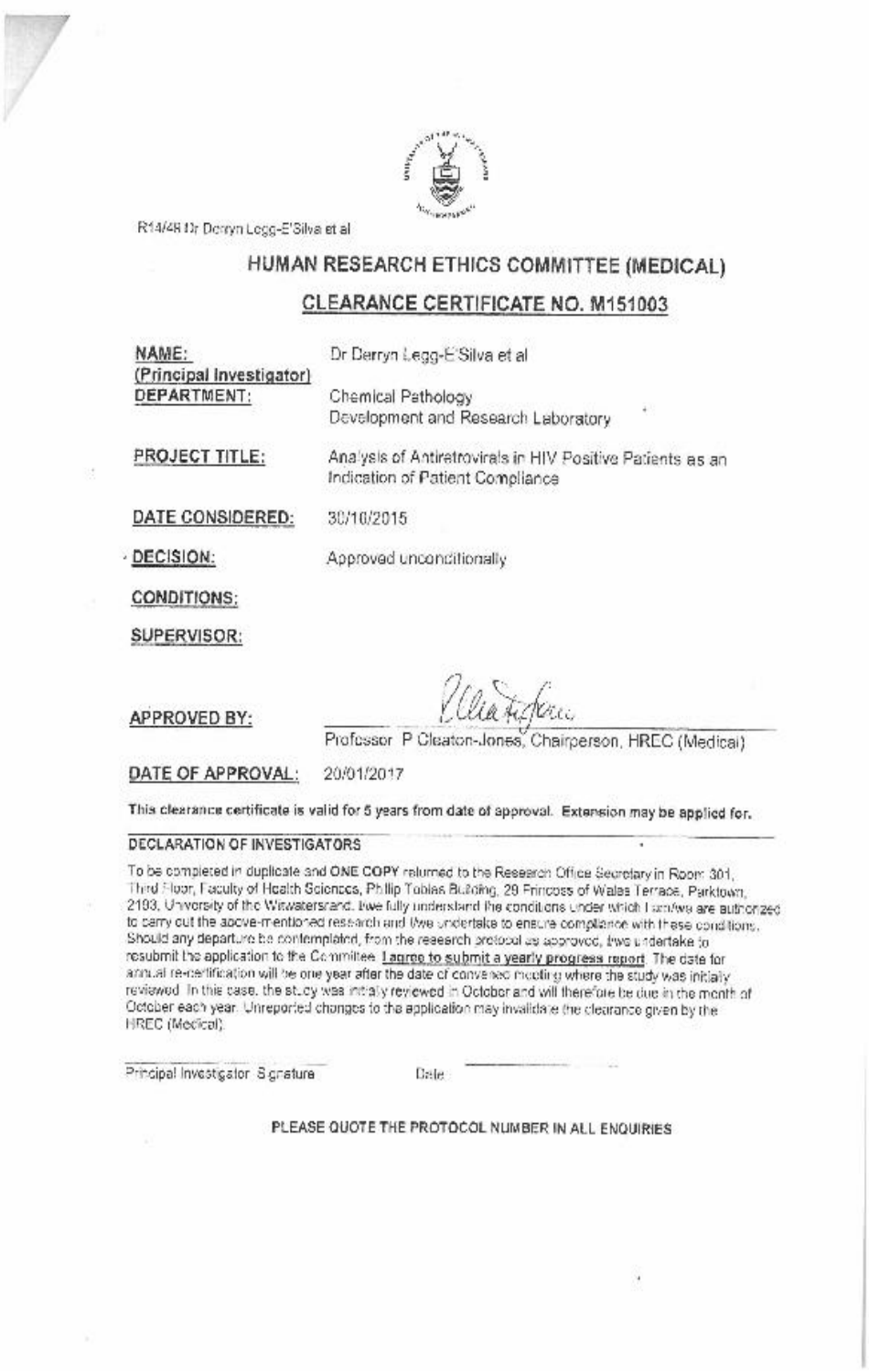
		DAY 5 run 1	Day 5 run2	Day 5 run3
1	µg/ml	0.054	0.058	0.056
	Assigned	0.052	0.052	0.052
	% Recovery	106	112	109
2	µg/ml	1.217	1.186	1.117
	Assigned	1.169	1.169	1.169
	% Recovery	104	101	96
3	µg/ml	1.217	1.186	1.117
	Assigned	2.253	2.253	2.253
	% Recovery	54	53	50
4	µg/ml	3.514	3.176	3.121
	Assigned	3.333	3.333	3.333
	% Recovery	105	95	94
5	µg/ml	4.234	4.251	4.294
	Assigned	4.544	4.544	4.544
	% Recovery	93	94	95
6	µg/ml	5.341	5.830	5.678
	Assigned	5.612	5.612	5.612
	% Recovery	95	104	101

Table A:3.10.5 LOD and LOQ

	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
Signal blank	51	28	60	15	29	36
Signal lowest STD	1122	28	1971	668	735	905
S/N	25					
Actual	0.051					
LOD	0.006					
LOQ	0.021					

Appendix B

1. Ethics certificate for the larger study



R14/48 Dr Derryn Legg-E'Silva et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M151003

NAME: (Principal Investigator)	Dr Derryn Legg-E'Silva et al
DEPARTMENT:	Chemical Pathology Development and Research Laboratory
PROJECT TITLE:	Analysis of Antiretrovirals in HIV Positive Patients as an Indication of Patient Compliance
DATE CONSIDERED:	30/10/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	20/01/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in October and will therefore be due in the month of October each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

2. Ethics certificate for PSC

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Prof Mpho Maphayi et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190689

NAME: (Principal Investigator)	Prof Mpho Maphayi et al
DEPARTMENT:	School of Pathology Department of Chemical Pathology Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Validation of plasma separation cards for the measurements of anti-retrovirals
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Lab Study
SUPERVISOR:	
APPROVED BY:	Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	08/07/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed June and will therefore be due in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

3. Ethics for this study

R14/49 Mr Simon Matlole Modiba and Dr Segio Carmona

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1706102

NAME: (Principal Investigator)	Mr Simon Matlole Modiba and Dr Segio Carmona
DEPARTMENT:	Chemical Pathology University of the Witwatersrand
PROJECT TITLE:	Development and Validation of Anti-retroviral Drug Levels for Therapeutic Drug Monitoring Using Dried Blood Spots
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-Study under Primary Study (M151003)
SUPERVISOR:	Prof Jaya George, Ms Tracy Snyman and Dr Derryn Legg-E'silva
APPROVED BY:	Professor P. Cleaton-Jones Chairperson, HREC (Medical)
DATE OF APPROVAL:	10/07/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certificaton will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially review June and will therefore be due in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

4. A letter to attend the HIV clinic

14 June 2017

CEO

Charlotte Maxeke Johannesburg Academic Hospital

To the CEO

I Simon Modiba, will be conducting a sub study using samples from the project entitled “Analysis of anti-retrovirals in HIV positive patients as an indication of patient compliance” conducted by Dr Legg-E’Silva (ethical clearance number M151003)

I will be using the samples to establish and validate a Dry blood spot (DBS) method to be used in Therapeutic drug monitoring. DBS can be used as alternative, simple, non-invasive sample collection method for therapeutic drug monitoring. Completing this project will allow us to quantitate ARV drug concentrations from DBS cards providing a more stable less invasive method for ARV drug levels in TDM. There would be no additional cost or sample collection.

Your support in this regard is highly appreciated.

Yours sincerely

Modiba Simon

Dept. Chemical Pathology

E-mail: modibasimon8@gmail.com