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**The Plasma, Whole Blood and Intracellular Concentrations of
Antiretroviral Agents in South African Children Receiving
Combination Antiretroviral Therapy with and without
Concomitant Antitubercular Treatment**

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Doctor of Philosophy in Pharmacology

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DECLARATION

I, Yuan Ren, declare that the work represented in this thesis is my own unaided work, both in concept and execution, and that apart from the normal guidance from my supervisors. Neither the substance nor any part of the thesis has been submitted in the past, or is being, or is to be submitted for a degree at this university or at any other university.

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Abstract

Background: Tuberculosis (TB) is the most common opportunistic infection in children with human immunodeficiency virus (HIV) infection in developing countries, and co-treatment for HIV infection and TB is frequently indicated. Efavirenz and lopinavir/ritonavir (ratio 1:1) as part of antiretroviral therapy are used in combination with rifampicin-based antitubercular treatment in South African TB/HIV co-infected children. Adult studies show that concomitant rifampicin significantly reduces efavirenz and lopinavir plasma concentrations. However, the pharmacokinetics (PK) of efavirenz and lopinavir/ritonavir are poorly characterized in children, especially African children and no study has evaluated the effect of rifampicin-based antitubercular treatment on efavirenz and lopinavir/ritonavir plasma concentrations in children. Although therapeutic drug monitoring (TDM) is recommended in selected patients (including young children and patients receiving concomitant antitubercular treatment), TDM is seldom available in resource-constrained countries. There is an urgent need to develop a field friendly method which requires small volumes of blood, and inexpensive processing and storage conditions. Furthermore, because HIV replicates in the cells, efavirenz and lopinavir need to penetrate into these infected cells to inhibit viral replication. Therefore, directly measurement of intracellular concentrations of these drugs in HIV-infected children could provide better understanding of drug exposure at the action site. It is also important to evaluate the effects of frequently co-administered drugs on intracellular accumulation of efavirenz and lopinavir.

Objectives: 1) To evaluate efavirenz and lopinavir/ritonavir plasma concentrations and determine the effects of rifampicin on efavirenz and lopinavir/ritonavir PK in HIV-infected African children with and without rifampicin-based antitubercular treatment. 2) To develop and validate the dried blood spot (DBS) method as an alternative to conventional plasma methods of drug concentration measurement in TDM. 3) To evaluate *in vivo* intracellular concentrations of efavirenz and lopinavir/ritonavir in HIV-infected children with and without concomitant antitubercular treatment. 4) To determine the *in vitro* modulation effects on the intracellular accumulation of efavirenz

and lopinavir in human peripheral blood mononuclear cells (PBMCs) by drug efflux protein inhibitors, as well as frequently co-administered rifampicin and ritonavir (at low dose; as pharmacoenhancer).

Methods: 1) Plasma efavirenz and lopinavir/ritonavir concentrations were measured by validated liquid chromatography/tandem mass spectrometry (LC/MS/MS) method in TB/HIV co-infected children during and after rifampicin-based antitubercular treatment as well as in a group of controls (HIV-infected children without TB). Children in the efavirenz study (n= 30) were receiving standard doses of efavirenz as part of antiretroviral treatment. Trough concentrations (C_{\min}) of efavirenz were estimated by extrapolation of the log-linear concentration-time line to 24 hours after the previous dose. Children in the lopinavir/ritonavir study were receiving additional ritonavir (lopinavir: ritonavir ratio 1:1) during antitubercular treatment (n= 15), and standard doses of lopinavir/ritonavir (LPV/r; ratio 4:1) after antitubercular treatment, and in controls (n= 15). The PK of lopinavir and ritonavir were characterized from concentration-time curves using WinNonlin version 4.1 by non-compartmental analysis.

2) Aliquots of 50 μ L of whole blood from the efavirenz and lopinavir/ritonavir studies were dried onto filter paper. The drug concentrations were analyzed using validated LC/MS/MS method. The effects of high temperature and direct sunlight on the stabilities of these antiretroviral drugs in DBS samples were tested.

3) Intracellular concentrations of efavirenz, lopinavir and ritonavir were measured in trough concentrations of 11 TB/HIV co-infected children using a validated LC/MS/MS method. Six children were receiving double dose of LPV/r (4:1) with concomitant rifampicin; 5 children were receiving standard doses of efavirenz with rifampicin-based antitubercular treatment, 3 of them had intracellular concentrations measured again after completing rifampicin-based antitubercular treatment.

4) *in vitro* intracellular accumulation of efavirenz and lopinavir were measured in human PBMCs in the absence and presence of P-glycoprotein inhibitors (verapamil at 50 μ M,

furosemide at 50 μM and cyclosporine A at 20 μM) and frequently co-administered drugs at levels representing the average concentrations found in patients (ritonavir at 5 mg/L and rifampicin at 4 mg/L). The concentrations of efavirenz and lopinavir in PBMCs were determined by LC/MS/MS.

Results and Conclusions: 1) The co-administration of rifampicin did not significantly reduce efavirenz estimated C_{\min} concentrations. A high proportion of children with and without concomitant antitubercular treatment had sub-therapeutic efavirenz concentrations despite being correctly dosed according to the manufacturer's instructions, raising concerns about the adequacy of current efavirenz dosing recommendations in children. The lopinavir key PK parameter, C_{\min} , was not significantly different in same group of children during and after rifampicin-based antitubercular treatment or compared to HIV-infected children without tuberculosis. The recommended minimum therapeutic concentration was achieved in 87% of children during antitubercular treatment and in 92% without concomitant antitubercular treatment. Therefore, in the context of limited options, LPV/r with additional ritonavir (ratio 1:1) is an acceptable approach to treat young children receiving concomitant rifampicin-based antitubercular treatment, although safety remains a concern and hepatic alanine transaminase levels should be monitored regularly.

2) Plasma and DBS concentrations of efavirenz, lopinavir and ritonavir were strongly correlated. The median (interquartile range, IQR) DBS/plasma concentration ratios for efavirenz, lopinavir and ritonavir were 0.93 (IQR 0.83, 1.08), 0.73 (IQR 0.61, 0.90) and 1.05 (IQR 0.74, 1.21), respectively. PK parameters of efavirenz and ritonavir were closely similar between DBS and plasma; whereas lopinavir pre-dose and C_{\min} (at 12 hours after lopinavir intake) concentrations were 16% lower in DBS samples. The 3 antiretroviral drugs in DBS samples were stable at 37 °C for 7 days and with exposure to direct sunlight for 2 hours. DBS can be used as an alternative field-friendly method for efavirenz, lopinavir and ritonavir concentration monitoring. However, pre-dose and C_{\min} concentrations of lopinavir in DBS samples need to be increased by 16% when used to predict plasma concentrations.

3) *In vivo* median intracellular/plasma concentration ratios for efavirenz, lopinavir and ritonavir amongst 11 TB/HIV co-infected children during antitubercular treatment were 0.91 (IQR 0.54, 1.19), 0.22 (IQR 0.09, 0.31) and 4.17 (IQR 1.30, 7.33), respectively. Two children had efavirenz intracellular/plasma concentration ratios during vs. after antitubercular treatment: 1.00 vs. 0.61 and 0.27 vs. 0.79.

4) Furosemide significantly increased efavirenz and lopinavir accumulation in healthy human PBMC samples by 1.2- 1.5 fold. Whereas, neither verapamil nor cyclosporin A had significant effects on efavirenz or lopinavir intracellular accumulation. Despite being an inducer of P-glycoprotein, rifampicin increased the accumulation of both efavirenz and lopinavir to different extents in all 3 PBMC samples. The low-dose ritonavir (at the concentration found in HIV-infected patients) had no effect on intracellular accumulation of efavirenz and lopinavir at therapeutic concentrations.

Research Output

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ALB	Albumin
ALT	Alanine Transaminase
ARV	Antiretroviral
AUC	Area under the Curve
AUC ₀₋₁₂	Area under the Curve to 12 Hours
BSA	Body Surface Area
BMI	Body Mass Index
cART	Combination Antiretroviral Therapy
CI	Confidence Interval
CL/F	Apparent Elimination Clearance
C _{max}	Maximum Concentration
C _{min}	Trough Concentration
C _{pre-dose}	Pre-dose Concentration
CYP	Cytochrome P450
DBS	Dried Blood Spot
FCS	Foetal Calf Serum
HB	Haemoglobin
HIV	Human Immunodeficiency Virus
IQR	Interquartile Range
k	Elimination Rate Constant

LC/MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLOQ	Lower Limit of Quantification
LPV/r	Lopinavir co-formulated with ritonavir in a ratio of 4:1
MDI	Mid-Dosing Interval
MDR	Multidrug Resistance
MTCT	Mother-to-Child Transmission
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
One-way ANOVA	One-way Analysis of Variance
OIs	Opportunistic Infections
PACTG	Pediatric AIDS Clinical Trials Group
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
P-gp	P-glycoprotein
PI	Protease Inhibitor
PK	Pharmacokinetics
RS	Wilcoxon Ranksum Test
SR	Wilcoxon Matched-Pairs Signed-Ranks Test
TB	Tuberculosis
T _{max}	Time to Maximum Concentration
TDM	Therapeutic Drug Monitoring
UNL	Upper Normal Limit
WHO	World Health Organization

Chapter 1

Introduction

1.1 HIV/AIDS

1.1.1 HIV Prevalence

The human immunodeficiency virus (HIV) is a retrovirus that infects cells of the human immune system, destroying or impairing their function. The most advanced stage of HIV infection is acquired immunodeficiency syndrome (AIDS). AIDS was first reported in the United States in 1981. Since then, it has become a global health problem affecting all regions, countries and communities. In 2007, there were estimated 33.2 million people living with HIV worldwide, two third lived in sub-Saharan Africa, a total of 22.5 million [World Health Statistics 2008, WHO].

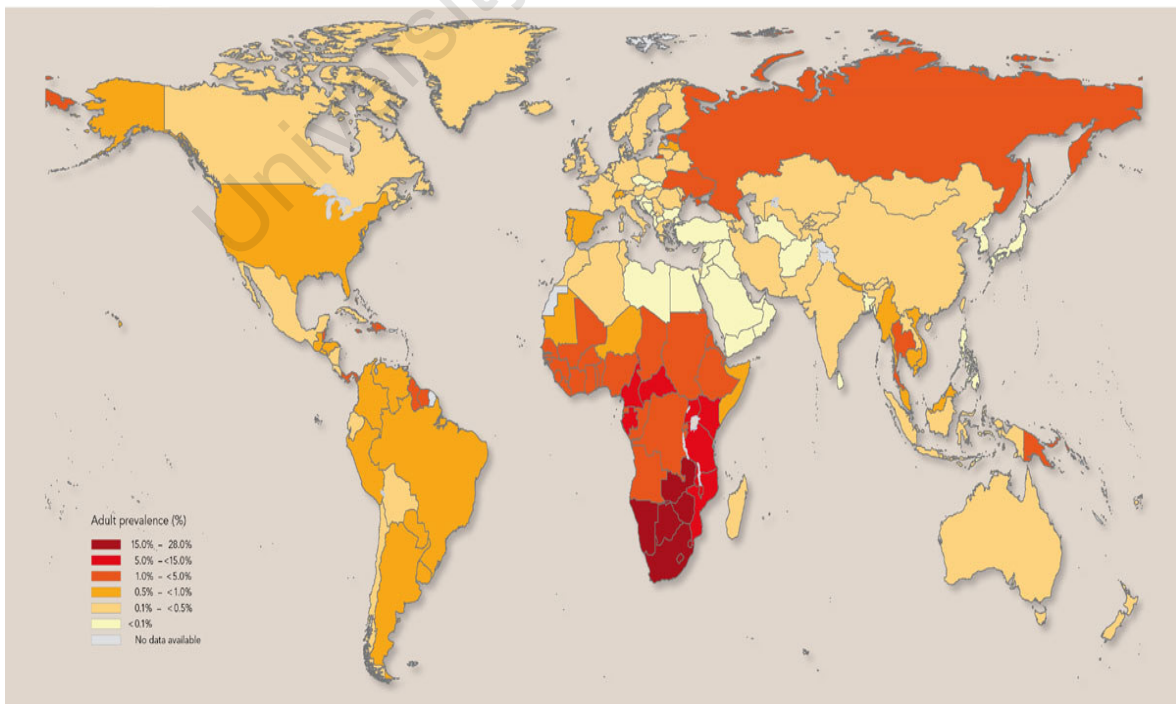


Figure 1.1 A global view of prevalence of HIV infection among population aged 15- 49 years (2007). Image taken from http://www.who.int/hiv/facts/global_HIV_map_2007_fullscr.jpg (Last accessed 08/08/2009).

1.1.2 Mother-to-Child Transmission

An estimated 2.1 million children younger than 15 years are living with HIV in 2007 worldwide. About 90% of children living with HIV are in sub-Saharan Africa [World Health Statistics 2008, WHO]. Although children could be infected through blood transfusions and infected tools, more than 90% of them acquire HIV infection via mother-to-child transmission (MTCT). Women in the reproductive age are 2.5 times more likely to be infected than men in the same age group, and the incidence of MTCT is on the rise [Moodley *et al.* 2005]. In the absence of any intervention, the rate of MTCT varies between 15 and 45% (5- 10% during pregnancy, 10- 20% during labour and delivery and 5- 20% through breastfeeding) [de Cock *et al.* 2000].

1.2 Opportunistic Infections and HIV-related Tuberculosis

1.2.1 Opportunistic Infections

In the early stages of HIV infection, a person may not have symptoms for many years but can still transmit the disease to others. The virus multiplies in the body and destroys the immune system, as the infection progresses, immune system becomes weaker, and the person becomes more susceptible to infections or malignancies. AIDS represents the terminal stage of HIV infection when patients suffer from opportunistic infections (OIs). Approximately 50% of HIV-infected persons will develop AIDS after 7 to 10 years of infection. The average survival time for a person with AIDS may be only 6 months in developing countries and 1 to 3 years in developed countries.

1.2.2 HIV-related Tuberculosis

Amongst the 9.27 million incident cases of tuberculosis (TB) in 2007 worldwide, an estimated 1.37 million (14.8%) were HIV-positive. Deaths from TB among HIV-positive people account for 23% of the estimated 2 million HIV deaths that occurred in 2007

[Global tuberculosis control: epidemiology, strategy, financing: WHO report 2009]. TB is the leading HIV-related OI in developing countries, particularly in sub-Saharan Africa. In South Africa, the prevalence of HIV in incident TB cases was 73%, which is the second highest in the world [Global tuberculosis control: epidemiology, strategy, financing: WHO report 2009]. There is a 7- 10% annual risk of developing TB disease among HIV-infected patients with latent TB infection, which is significantly higher than those in HIV-negative persons [Girardi *et al.* 2000]. Furthermore, patients with TB disease tend to have higher HIV viral loads and a more rapid progression of their HIV illness than HIV-infected patients without TB [Whalen *et al.* 1995].

There is an extremely high incidence (48%) of TB among HIV-infected South African children, especially prior to antiretroviral (ARV) treatment [Walters *et al.* 2008]. In the absence of ARV therapy, HIV-infected children with confirmed TB have poor outcomes on antitubercular therapy, and are at high risk of death during or after completion of antitubercular therapy [Hesseling *et al.* 2005]. Co-treatment with ARV therapy is likely to reduce TB related morbidity and mortality in TB/HIV co-infected children [Walters *et al.* 2008].

1.3 Treatment in HIV-infected Children with TB

1.3.1 Combination Antiretroviral Therapy in Children

Effective antiretroviral therapy includes at least three drugs from two different ARV classes among nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Since the introduction of combination antiretroviral therapy (cART), a reduction of 60- 90% in HIV-related deaths and OIs has been observed among adults living in the developed countries [Mocroft *et al.* 1998; de Martino *et al.* 2000]. The effectiveness of cART in infants and children to reduce HIV-1-related death is similar, or even greater than, that observed in adults [Bolton-Moore *et al.* 2007].

The South African national paediatric antiretroviral treatment guidelines recommended regimens to treat children with HIV infection are shown in Table 1.1.

Table 1.1 Paediatric first-line and second-line treatments. Lopinavir co-formulated with ritonavir in a lopinavir: ritonavir ratio of 4:1 (LPV/r) as Kaletra[®] is used in South African children aged between 6 months to 3 years, who have failed nevirapine-based prevention of MTCT. [National Antiretroviral Treatment Guidelines, 2004]

	6 months- 3 years old	>3 years old or >10 Kg
First-line	<ul style="list-style-type: none"> • Stavudine -NRTIs • Lamivudine -NRTIs • Lopinavir/ritonavir -PIs 	<ul style="list-style-type: none"> • Stavudine -NRTIs • Lamivudine -NRTIs • Efavirenz -NNRTIs
Second-line	<ul style="list-style-type: none"> • Zidovudine -NRTIs • Didanosine -NRTIs • Nevirapine -NNRTIs 	<ul style="list-style-type: none"> • Zidovudine -NRTIs • Didanosine -NRTIs • Lopinavir/ritonavir -PIs

1.3.2 Antitubercular Treatment in Children

Treatment regimens for drug susceptible TB have an initial (or intensive) phase lasting 2 months and a continuation phase usually lasting 4 months. During the intensive phase, treatment comprises 3 or more drugs (isoniazid, rifampicin, pyrazinamide and, in children with extensive disease, ethambutol), in order to rapidly kill tubercle bacilli. During the continuation phase, treatment consists of fewer drugs (isoniazid and rifampicin) but lasts for a longer time. The sterilizing effect of the drugs eliminates the remaining bacilli and prevents subsequent relapse. Antitubercular treatment is the same for HIV infected and HIV negative children with TB. [South African National Tuberculosis Control Programme Practical Guidelines, 2004].

1.3.3 cART with Concomitant Antitubercular Treatment in TB/HIV Co-infected Children

As TB is a common OI in HIV-infected children in developing countries, co-treatment with antiretroviral and antitubercular therapy is frequently indicated. The current South African antiretroviral treatment guidelines for management of children who are receiving

rifampicin-based antitubercular treatment recommend standard doses of efavirenz plus 2 NRTIs for children over 3 years old or over 10 kg, and additional ritonavir with LPV/r (lopinavir: ritonavir ratio 1:1) plus 2 NRTIs for children under 3 years or under 10 kg [National Antiretroviral Treatment Guidelines, 2004].

1.3.4 Pharmacokinetics of Efavirenz and Lopinavir in Children

(Summarized in Appendix Table A.1 and A.2)

1.3.4.1 Pharmacokinetics of Efavirenz in Children

Efavirenz is highly protein bound (99%), mainly to albumin. Efavirenz is metabolized by cytochrome P450 (CYP) isoenzyme 2B6 and to a lesser extent CYP3A4 [Adkins *et al.* 1998; Smith *et al.* 2001; Ward *et al.* 2003]. The genetic polymorphisms on CYP2B6 have been investigated in several studies. Allele *6 presents at high frequency (up to 30%) in African-American population [Klein *et al.* 2005; Haas *et al.* 2004; Wang *et al.* 2006]. The effect of CYP2B6-516-G>T polymorphism is associated with high efavirenz plasma concentrations, high efavirenz drug exposure and reduced oral clearance rates [Haas *et al.* 2004; Tsuchiya *et al.* 2004; Saitoh *et al.* 2007; Puthanakit *et al.* 2009; ter Heine *et al.* 2008; Cohen *et al.* 2009]. The recommended therapeutic range for efavirenz trough concentrations (C_{\min}) at 24 hours after dose is 1 to 4 mg/L [la Porte *et al.* 2006; www.hivpharmacology.com].

Pediatric AIDS Clinical Trials Group (PACTG) 382 study [Starr *et al.* 1999] has determined the efavirenz pharmacokinetics (PK) in children (n=50; median age 8.0 years, range 3.8 to 16.8) taking efavirenz capsules with the average dose of 11.7 mg/kg daily. The mean 24-hour area under the curve (AUC), AUC_{0-24} , was 218 $\mu\text{mol}\cdot\text{h/L}$ (68.8 mg·h/L) at week 2. Close to half of the children (44%) had their efavirenz doses increased based on PK assessment of the AUC_{0-24} at 2 weeks after starting the study regimen whereas 22% had efavirenz C_{\min} concentrations < 1 mg/L (personal communication, Professor Coutney V. Fletcher, University of Colorado Health Sciences Centre, Denver, CO, 2006). The mean doses of efavirenz increased to 369 mg/day (14.2 mg/kg daily) at week 6, resulting in an AUC_{0-24} of 244 $\mu\text{mol}\cdot\text{h/L}$ (77.0 mg·h/L).

The efavirenz 24-hour concentration profiles were evaluated in 11 HIV-infected children at the age of 4 to 10 years receiving efavirenz doses of 10-15 mg/kg body weight daily. The mean efavirenz plasma C_{\min} was 1.29 mg/L (95% confidence interval, CI 0.89, 1.70); mean maximum concentration (C_{\max}) was 5.55 mg/L (95% CI 3.95, 7.15) and mean AUC was 63.6 mg·h/L (95% CI 44.2, 83.0). Seven of these 11 (63.6%) children had efavirenz concentrations below target range (1- 4 mg/L) [von Hentig *et al.* 2006].

In the International Maternal Paediatric Adolescent AIDS Clinical Trial P1058 study, children were receiving efavirenz doses of 350 mg/m² (median 10.7 mg/kg, range 3.4, 13.7) in combination with lopinavir/ritonavir had median efavirenz C_{\max} of 3.57 mg/L (range 1.4, 7.6); T_{\max} 3.0 hours (range 2.0, 11.9); half-life 9.94 hours (range 4.9, 27.8); estimated C_{\min} 0.77 mg/L (range 0.2, 2.5) and estimated AUC₀₋₂₄ 40.87 mg·h/L (range 13.7, 86.2). Sub-therapeutic efavirenz estimated C_{\min} and AUC₀₋₂₄ were observed in 53% and 14% of children, respectively [King *et al.* 2009].

A recent study by Hirt *et al.* (ANRS 12103) evaluated efavirenz PK in 48 West African children using a population PK model. The mean C_{\min} was 1.64 mg/L; C_{\max} 3.71 mg/L; AUC 65.2 mg·h/L; apparent elimination clearance (CL/F) 0.21 L/h/kg and volume of distribution 4.48 L/kg. It was found that 19% children had efavirenz C_{\min} below therapeutic concentration (1 mg/L), and 89% of them (8 of 9 children) weighed less than 15 kg. They concluded that children weighing less than 15 kg were more likely to have sub-therapeutic concentrations [Hirt *et al.* 2009].

Wintergerst *et al.* reported the median efavirenz plasma concentration during the mid-dosing interval (MDI) to be 2.8 mg/L (range 0.13, 11.6) with 8.8% samples < 1 mg/L in 33 HIV-infected children (median age 8.2 years; range 2.1, 16.7). The sampling time was between 8 and 20 hours in two thirds of these children [Wintergerst *et al.* 2008].

A recent study in 63 Thai children (median age 12.3 years; range 3.1, 18.7) found mean± standard deviation efavirenz plasma MDI concentration of 3.14± 3.31 mg/L. Sub-therapeutic efavirenz concentrations were found in 13% children. The mean duration

between blood sampling and the prior dose in their study was 14.8 ± 0.8 hours [Puthanakit *et al.* 2009].

Differences in the sampling strategies, study populations and age may contribute to the discrepant findings.

Liquid formulation of efavirenz had low bioavailability [Starr *et al.* 2002] and is not available in South Africa.

1.3.4.2 PK of Lopinavir in Children

Lopinavir is highly protein bound (98-99%) to both α_1 -acid glycoprotein (AAG) and albumin. Lopinavir is primarily metabolized by CYP3A4 isoenzyme and is also a substrate of P-glycoprotein (P-gp) [Sham *et al.* 2001; Vishnuvardhan *et al.* 2003; Agarwal *et al.* 2007]. The PK of lopinavir is characterized by food effect, poor bioavailability due to efflux by P-gp, short half-life due to rapid first-pass metabolism by CYP3A4 and high inter-individual variability, which precludes its use as a single PI. Ritonavir is a substrate and inhibitor of CYP3A4 and P-gp, which presents a favourable drug-drug interaction with lopinavir. Because lopinavir plasma concentrations are important for ARV treatment outcomes [Masquelier *et al.* 2002], ritonavir was co-administered with lopinavir at a low dose to increase lopinavir plasma concentrations [Sham *et al.* 1998]. Multiple dose studies showed that co-administration of lopinavir with ritonavir in a ratio of 4:1 (LPV/r) is the optimal combination to overcome the PK shortcomings of lopinavir as the single agent [Lal *et al.* 1998; Bertz *et al.* 1999]. The recommended minimum therapeutic concentration for lopinavir C_{\min} at 12 hours after dose is 1 mg/L [la Porte *et al.* 2006; www.hivpharmacology.com].

The PK of lopinavir was evaluated in children aged 6 months to 12 years ($n=12$) receiving LPV/r at $230/57.5$ mg/m² twice daily. The mean \pm standard deviation AUC_{0-12} was 72.6 ± 31.1 mg·h/L; C_{\max} 8.16 ± 2.94 mg/L; T_{\max} 3.8 ± 1.6 hours; C_{\min} 3.35 ± 2.14 mg/L and half-life 5.8 ± 3.0 hours [Sáez-Llorens *et al.* 2003].

Verweel *et al.* reported comparable lopinavir plasma concentrations in 23 children with a median age of 5.6 years (range 0.4, 13.2). The mean \pm standard deviation AUC_{0-12} was 75.3 ± 33.7 mg·h/L; C_{max} 9.33 ± 3.27 mg/L; T_{max} 2.61 ± 2.09 hours; C_{min} 3.68 ± 2.48 mg/L and half-life 6.59 ± 4.19 hours [Verweel *et al.* 2007]. However, suboptimal lopinavir C_{min} concentrations (< 1 mg/L) were found in 30.4% of children. These children had significantly higher clearance than children with adequate C_{min} . It was also found that children younger than 2 years were more likely to have inadequate lopinavir C_{min} . An escalated dose of LPV/r ($300/75$ mg/m²) was recommended for children less than 2 years of age.

PACTG P1030 Study in 18 infants less than 6 months of age treated with LPV/r at $300/75$ mg/m² twice daily reported that the apparent clearance was slightly higher than that in the older children [Sáez-Llorens *et al.* 2003]. Lopinavir PK parameters were: median plasma pre-dose concentration 2.37 mg/L (range <0.1 , 8.44); AUC 67.52 mg·h/L (range 23.66, 164.04); apparent clearance 0.187 L/h/kg (range 0.073, 0.610); volume of distribution 0.92 L/kg (range 0.34, 3.97) and half-life 3.68 hours (range 1.2, 13.11). The mean lopinavir C_{min} and C_{max} were 2.0 mg/L and 9.4 mg/L, respectively. The pre-dose concentrations stabilized at a higher level after the first sampling at 2 weeks, indicating the tolerance to the oral solution may play a role as it putatively has a horrid taste. Infants with viral suppression had higher lopinavir exposure than those who did not achieve suppression, but the difference was not statistically significant [Chadwick *et al.* 2008].

In the International Maternal Paediatric Adolescent AIDS Clinical Trial P1030 study, lopinavir plasma PK was evaluated in 9 infants less than 6 weeks of age treated with LPV/r at $300/75$ mg/m². Due to growth, the median actual dose of lopinavir received on the day of PK sampling was 267 mg/m² (range 246, 305) twice daily. Lopinavir PK parameters were: median pre-dose concentration 2.22 mg/L (range 0.99, 4.87); AUC 36.6 mg·h/L (range 27.9, 62.6); apparent clearance 0.37 L/h/kg (range 0.15, 0.75); volume of distribution 1.86 L/kg (range 0.80, 3.79) and half-life 3.51 hours (range 2.06, 5.80). The mean lopinavir C_{min} and C_{max} were 1.4 mg/L and 5.2 mg/L, respectively [Chadwick *et al.* 2009]. Age-related factors may alter the absorption, protein binding and clearance. As the

result, lopinavir exposure was lower in young infants than that in infants > 6 weeks [Chadwick *et al.* 2008] (median AUC 36.6 vs. 67.52 mg-h/L and mean C_{\min} 2.0 vs. 1.4 mg/L).

1.3.5 PK Drug-Drug Interactions between ARV Drugs and Antitubercular Drugs

1.3.5.1 Plasma Drug-Drug Interactions of Efavirenz with Rifampicin

Rifampicin is one of the most potent inducers of hepatic CYP3A4, CYP2B6 and P-gp in humans [Miguet *et al.* 1977; Rae *et al.* 2001; Loboz *et al.* 2006; Fromm *et al.* 2000; Greiner *et al.* 1999], thereby rifampicin has significant PK drug-drug interactions with NNRTIs and PIs which are substrates of these enzymes and P-gp.

In a study amongst healthy adult volunteers, concomitant rifampicin reduced the efavirenz AUC and C_{\min} by 26% and 20%, respectively [Benedek *et al.* 1998]. Similar findings were reported in TB/HIV co-infected adults: the mean C_{\max} , C_{\min} and AUC decreased by 24%, 25% and 22%, respectively, in the presence of rifampicin. However, the differences did not reach statistical significance due to large inter-individual variability [Lopez-Cortes *et al.* 2002]. When the efavirenz dose was increased from 600 mg to 800 mg daily in patients receiving concomitant rifampicin-based antitubercular treatment, efavirenz concentrations with concomitant rifampicin were similar to those observed after antitubercular treatment and decreasing the efavirenz dose to 600 mg daily [Lopez-Cortes *et al.* 2006]. Another study found a 35% reduction (95% CI -56.2%, -2.1%) in efavirenz TDM sample concentrations amongst 339 patients (17% on rifampicin) after adjustment for weight, ethnicity and concomitant zidovudine [Stöhr *et al.* 2008]. However, a study in Thai patients showed that the median efavirenz MDI concentrations were comparable amongst patients receiving efavirenz at 600 mg or at 800 mg daily with concomitant rifampicin. Racial differences and the lower body mass index (BMI) of Thai patients could explain the different findings [Manosuthi *et al.* 2005]. The virological and immunological outcomes were similar at 48 weeks in both groups [Manosuthi *et al.* 2006]. An efficacy study in South African HIV-infected adults also reported the comparable virological outcomes in patients receiving standard 600 mg daily dose of efavirenz with and without concomitant antitubercular treatment [Boulle *et al.* 2008].

Two studies in South African HIV-infected adults on standard dose of efavirenz showed that there was no significant reduction in efavirenz concentrations during compared to after rifampicin-based antitubercular therapy [Friedland *et al.* 2006; Cohen *et al.* 2009].

Although, some authorities recommend increasing the efavirenz dose by 25% when given with concomitant rifampicin to compensate the reduction in efavirenz concentrations [Panel on Antiretroviral Guidelines for Adult and Adolescents, 2008], there is currently not consensus on optimal dosing of efavirenz with rifampicin. No studies have evaluated efavirenz concentrations in children receiving concomitant rifampicin.

1.3.5.2 Plasma Drug-Drug Interactions of Lopinavir with Rifampicin

The interaction between rifampicin and lopinavir (in combination with ritonavir, LPV/r, 400/100 mg twice daily) was evaluated in a study amongst healthy adult volunteers. Rifampicin reduced lopinavir plasma AUC and C_{\min} by 75 and 99%, respectively [Bertz *et al.* 2000]. For this reason, co-administration of rifampicin and LPV/r is not recommended. A further study in healthy adult volunteers [la Porte *et al.* 2004] showed that adding ritonavir to LPV/r (lopinavir: ritonavir ratio of 1:1) with concomitant rifampicin resulted in acceptable peak and trough lopinavir concentrations, suggesting this adjusted dose regimens of LPV/r could be used with rifampicin-based antitubercular treatment in TB/HIV co-infected patients. However, this approach has not been evaluated in TB/HIV co-infected children receiving concomitant rifampicin-based antitubercular treatment.

1.3.5.3 Drug-Drug Interactions between ARV Drugs with Isoniazid

Isoniazid, which is given in combination with rifampicin in antitubercular treatment, has been shown in *in vitro* studies to inhibit CYP3A4 and CYP 2A6 amongst other enzymes [Wen *et al.* 2002].

When CYP2B6 function is impaired, accessory metabolic pathways may control the rate of efavirenz metabolism [di Lulio *et al.* 2008]. The major isoenzymes involved in the

accessory pathways are CYP3A and CYP2A6. Thus, there is potential for drug-drug interactions between isoniazid and the ARV drugs, including efavirenz and lopinavir.

1.3.5.4 Drug-Drug Interactions between ARV Drugs and Rifampicin at Cellular Level

P-gp belongs to a family of plasma membrane proteins encoded by the multidrug resistance (MDR) genes and functions as an energy-dependent efflux pump which removes drugs from the cell membrane and cytoplasm. Many substrates metabolized by CYP3A4 are also substrates of P-gp, including ARV drugs. Furthermore, P-gp is expressed in the blood-brain barrier, liver, kidney, as well as peripheral blood mononuclear cells (PBMCs) [Coon *et al.* 1991]. Multidrug resistance-associated protein (MRP) is another energy-dependent transmembrane efflux pump, which is also expressed on PBMCs [Abbaszadegan *et al.* 1994; Oselin *et al.* 2003]. Rifampicin is the inducer of P-gp. Efavirenz is a substrate for P-gp [Chandler *et al.* 2003], and PIs are not only a substrate for P-gp [Profit *et al.* 1999; Washington *et al.* 1998; Lee *et al.* 1998; Gutmann *et al.* 1999] but also for MRP [Gutmann *et al.* 1999; Srinivas *et al.* 1998; Agarwal *et al.* 2007]. Concomitant rifampicin may also affect the concentrations of these ARV drugs at the cellular level through actions on these transmembrane drug transporters.

1.4 The Importance of Measurement of Efavirenz and Lopinavir Plasma Concentrations in HIV-infected Children with or without TB

There is great importance to evaluate efavirenz and lopinavir plasma concentrations in children, particularly in African children, because:

1.4.1 Children Are Different from Adults

There is limited information on PK of ARV drugs in children. In the absence of complete PK data or established dosing guidelines, the doses of ARV drugs in children were extrapolated based on the established dose for adults. The PK of these drugs may differ greatly between children and adults because of age-associated changes in body composition and organ function are dynamic and can be discordant during the first decade of life. For example, elevated intragastric pH, increased gastric emptying and

intestinal motility can affect on the absorption of drugs. Relatively large extracellular and total-body water spaces, changes in the composition and amount of circulating plasma proteins, such as albumin and AAG, influence the distribution of highly bound drugs. Difference in expression of drug transporting protein such as P-gp also can alter the drug distribution. Maturation of drug-metabolizing enzyme activity may account for differences in drug metabolism and clearance. In particular, hepatic enzyme activity changes with age. It increases to adult levels during infancy, and then exceeds adult levels at 1 to 4 years of age, and then returns back to adult levels after puberty. Age-associated factors can also affect renal function and therefore drug elimination [Kearns *et al.* 2003; King *et al.* 2002].

1.4.2 Disease States and Concomitant Treatment May Alter the PK of ARV drugs

It is important to measure drug concentrations in diseased patients rather than in healthy volunteers. TB/HIV co-infection may induce biochemical changes, including elevated AAG level, altered P-gp expression and drug metabolizing enzyme activity in patients [Kremer *et al.* 1988; Andreana *et al.* 1996]. Disease states may also cause physiological changes, for example wasting, which have important impact on drug distribution.

The major concerns of concomitant antitubercular treatment are the PK drug-drug interactions between rifampicin and efavirenz and lopinavir, respectively. However, there are no studies evaluating the effect of rifampicin-based antitubercular treatment on the ARV drug plasma concentrations in children.

1.4.3 Drug Concentrations Affect treatment Tolerance and Response

Studies have shown associations between treatment efficacy and toxicity with plasma concentrations of efavirenz and lopinavir [Marzolini *et al.* 2001; Csajka *et al.* 2003; Joshi *et al.* 1999; van Leth *et al.* 2006; Ståhle *et al.* 2004; González de Requena *et al.* 2004; Masquelier *et al.* 2002; van Rossum *et al.* 2002]. Maintaining efavirenz and lopinavir plasma concentrations above the minimum therapeutic ranges throughout the dosing interval is crucial for delaying the development of drug-resistant mutants and ensuring treatment success. Furthermore, both efavirenz and lopinavir present marked

inter-individual variability. It is important to evaluate plasma concentrations of these drugs in children dosed according to current treatment guidelines.

1.4.4 Lack of Paediatric PK Information from High Burden Countries

African children are a population vulnerable to HIV-infection. Although majority of the HIV-infected children are living in Africa, there is no data regarding efavirenz and lopinavir PK in African paediatric populations. Children in Africa are subject to different genomics and dietary structure than children in America or Europe, which can alter the PK measurements of ARV drugs [Klein *et al.* 2005; Haas *et al.* 2004; Wang *et al.* 2006; Saitoh *et al.* 2007; Bhardwaj *et al.* 2002; Yeh *et al.* 1998].

1.5 The Urgency of New Method Development and Validation for ARV drug Concentration Assay

Serum and plasma are the common matrices for ARV drug concentration testing. Conventional methods require extensive sample collection and expensive processing techniques, storage and transportation conditions which are difficult or unavailable in resource-limited settings. Furthermore, paediatric PK studies are often limited by the volume of blood required. There is an urgent need to develop and validate a field friendly method which requires small volumes of blood, and inexpensive processing and storage conditions. A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed for ARV concentration measurement in dried blood spot (DBS) samples by Koal *et al.* This study showed a linear correlation between DBS and plasma concentrations in 70 samples with total of 6 ARV drugs [Koal *et al.* 2005]. However, the use of DBS method has not been validated in a paediatric study or amongst patients.

1.6 Intracellular ARV Drug Concentrations in HIV-infected Patients (*in vivo*)

Therapeutic drug monitoring (TDM) is recommended to optimize efficacy and to reduce toxicity of ARV treatment by maintaining drug plasma concentrations within their therapeutic ranges. However, the site of action of ARV drugs is inside the HIV-infected cells. Therefore, the measurement of intracellular drug concentrations can provide a better indication of ARV exposure and may contribute to the improvement of TDM.

To date, several studies have investigated the intracellular concentrations of efavirenz, lopinavir and ritonavir in HIV-infected adults. The previously reported intracellular/plasma concentration ratios for efavirenz were: median 0.9 [Rotger *et al.* 2005]; geometric mean 0.69 (geometric coefficient of variation 101) [Colombo *et al.* 2006]; and median 1.3 (range 0.7- 3.3) [Almond *et al.* 2005 a].

When lopinavir and ritonavir were given in a combination (LPV/r; 400/100 mg twice daily), the reported intracellular/plasma concentration ratios of lopinavir from previous studies were: median 1.18 (interquartile range, IQR 0.74, 2.06) [Crommentuyn *et al.* 2004]; median 1.55 (range 0.67, 3.80) [Hoggard *et al.* 2002]; and mean 0.65 (standard error 0.12) [Colombo *et al.* 2004]. Whereas, the intracellular/plasma concentration ratios of ritonavir in the combination with lopinavir were median 4.59 (IQR 3.20, 7.70) [Crommentuyn *et al.* 2004]; median 5.28 [Hoggard *et al.* 2002]; and mean 0.94 (standard error 0.18) [Colombo *et al.* 2004]. The median intracellular/plasma concentration ratios of ritonavir when taken alone at a dose of 500- 600 mg twice daily was 1.00 (range 0.60, 2.28) [Khoo *et al.* 2002]. Breilh *et al.* reported much higher intracellular/plasma concentration ratios of lopinavir C_{\min} in the virological success group than those in the failure group: median 3.2 (IQR 2.6, 3.8) vs. 2.3 (IQR 1.9, 2.8) and median 2.4 (IQR 1.6, 3.1) vs. 1.4 (IQR 1.3, 1.7) at 1 month and 6 months after LPV/r administration (400/100 mg or 533/133 mg, twice daily), respectively. The intracellular lopinavir C_{\min} efficacy threshold was defined at 8 mg/L [Breilh *et al.* 2004]. (Summarized in Appendix Table A.3)

To our knowledge, there is no data available on intracellular accumulation (intracellular/plasma concentration ratios) of these ARV drugs in HIV-infected children with or without TB and antitubercular treatment. As mentioned earlier, age-related factors may induce the biological and physiological changes and therefore influence intracellular accumulation. Co-infection with TB and rifampicin-based antitubercular treatment may alter the protein binding of efavirenz and lopinavir. For these highly protein bound ARV drugs, a small change in degree of binding may have a significant impact on the amount

of free drug available for entering the cells. Furthermore, efavirenz, lopinavir and ritonavir are substrates of several drug efflux transporters. The induction effect of concomitant rifampicin on these drug efflux transporters may also interfere with the intracellular concentrations of these 3 ARV drugs.

1.7 *In vitro* Intracellular ARV Drug Accumulation

Intracellular accumulation of ARV drugs is a result of influx and efflux processes. A study by Janneh *et al.* demonstrated the effects of several efflux transport protein inhibitors on the intracellular accumulation of saquinavir in human PBMCs [Janneh *et al.* 2005]. However, the study may have used much higher drug concentrations than those found in patients. Moreover, ritonavir and rifampicin are known inhibitor and inducer of energy-dependent drug efflux proteins, respectively, and they are frequently co-administered with efavirenz and lopinavir. Therefore, investigations on the *in vitro* interactions between low-dose ritonavir (as pharmacoenhancer, in combination with lopinavir) and rifampicin with efavirenz and lopinavir in human PBMCs at the concentrations representing average drug concentrations in patients could give some insights into the effects of drug-drug interactions at the cellular level.

1.8 The Objectives of This Study

1. To define efavirenz and lopinavir/ritonavir plasma concentrations in African children receiving cART with and without concomitant rifampicin-based antitubercular treatment
 - To develop and validate a simple and rapid ARV drug concentration testing method requiring small volumes of plasma using LC/MS/MS
 - To evaluate the effect of concomitant rifampicin-based antitubercular treatment on efavirenz concentrations as well as the concentrations of lopinavir/ritonavir in the adjusted dose regimens (lopinavir: ritonavir ratio of 1:1)
2. To develop and validate the use of the DBS method to facilitate the measurement of ARV drug concentrations in research and TDM practice in resource-limited settings

- To develop and validate LC/MS/MS method for ARV concentration assay in DBS sample
 - To define the correlation between DBS and plasma concentrations for efavirenz, lopinavir and ritonavir
 - To identify factors that affecting the correlation between DBS and plasma concentrations
 - To test the stability of efavirenz, lopinavir and ritonavir in DBS sample
3. To determine intracellular efavirenz, lopinavir and ritonavir trough concentrations in HIV-infected children with and without concomitant rifampicin-based antitubercular treatment (*in vivo*)
 - To develop and validate LC/MS/MS method for intracellular ARV concentration assay
 4. To evaluate the effects of rifampicin and ritonavir on efavirenz and lopinavir intracellular accumulation in human PBMCs (*in vitro*)

Chapter 2

Materials and Methods

2.1 Plasma Sample Preparation and Concentration Assay

2.1.1 Preparation of Plasma Sample

Whole blood samples (1.2 mL of each sample) from the efavirenz and lopinavir-ritonavir study were collected in lithium-heparin tubes at each time point of PK visit. An aliquot of 50 μ L whole blood was removed from each sample to make a dried blood spot, which is described in Section 2.2.1. The rest of the blood was centrifuged (within 30 minutes after blood taken) at 700 \times g for 5 minutes and the plasma was stored immediately at -80 $^{\circ}$ C while awaiting quantification of concentrations.

Efavirenz, lopinavir and ritonavir plasma concentrations were determined simultaneously by LC/MS/MS (API 4000 triple quadrupole MS/MS Applied Biosystems, South Africa) using a modification of the method by Chi *et al.* [Chi *et al.* 2002], in the laboratory of the Division of Clinical Pharmacology, University of Cape Town. An aliquot of 500 μ L of precipitation reagent (80% methanol: 20% 0.2M ZnSO₄ v/v) (methanol from Merck KGaA, Darmstadt, Germany; ZnSO₄ from Sigma Chemical Co.) with reserpine (Sigma Chemical Co.) at 0.4 μ g/mL as internal standard was added to 50 μ L aliquot of plasma and vortexed at high speed for 15 seconds. The suspension was then sonicated for 10 minutes and centrifuged at 12000 \times g for 5 minutes to pellet the precipitated proteins and give a clear supernatant. These clear extracts were transferred to vial inserts and placed in the autosampler tray for injection onto LC/MS/MS column (Phenomenex Gemini C18, 50 \times 2.00 mm, with 5 micron particle size packing).

2.1.2 Plasma ARV Concentration Assay by LC/MS/MS

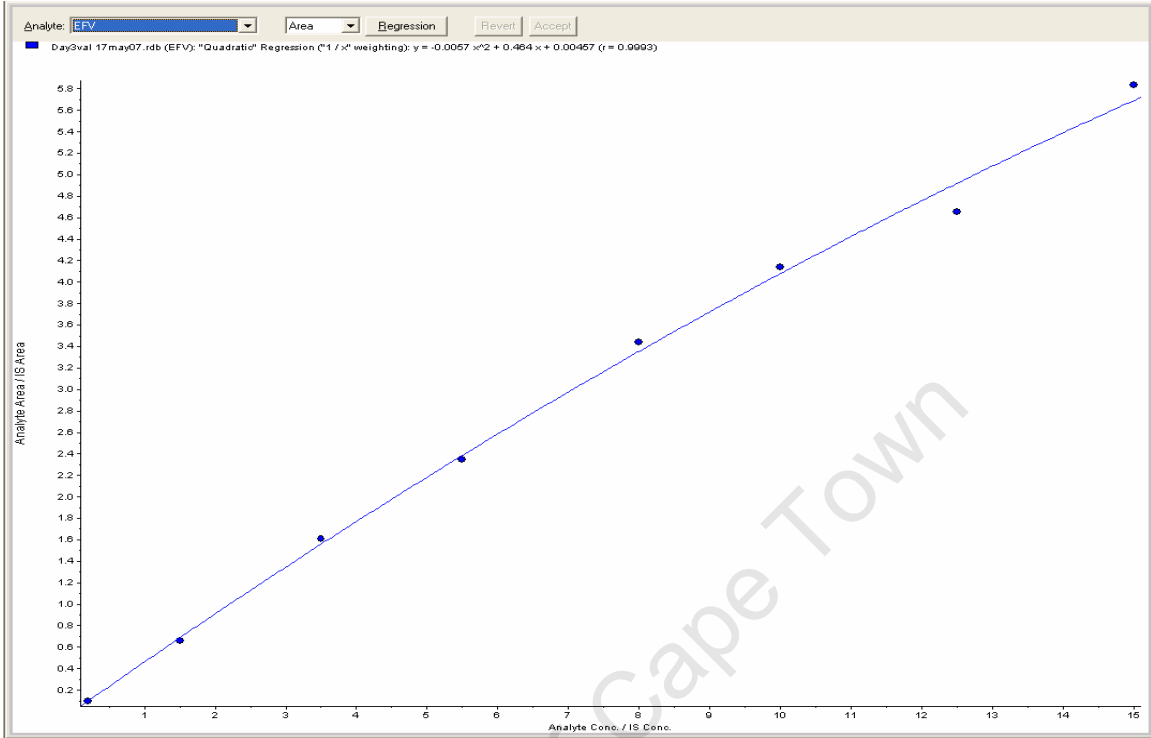
Mobile phase A was 10% methanol and mobile phase B was 90% methanol: 10% 10mM of ammonium acetate in 0.1% acetic acid v/v. The flow gradient was initially 100% of A for 0.20 minute, switched to 100% B from 0.21 minutes, held at 100% B for 2.79 minutes. Then returned to 100% A over 0.10 minute, held for a further 3.90 minutes prior to the injection of another sample. The total run time for each sample was 7.00 minutes. The flow rate of mobile phase was kept constantly at 500 $\mu\text{L}/\text{minute}$. The volume of injection was 5 μL .

The plasma calibration curves of efavirenz (Merck Research Laboratories, Rahway N.J., USA) lopinavir and ritonavir (both from Abbott Laboratories, North Chicago, IL 60064, USA) were linear over the range of 0.20-15.00 mg/L, 0.05-20 mg/L and 0.025- 5 mg/L, respectively (Figure 2.1). Plasma calibration curves were prepared by spiking efavirenz, lopinavir and ritonavir in drug-free plasma at each calibration concentration. Three quality controls were prepared in the same manner (0.3 mg/L, 4 mg/L and 11 mg/L for efavirenz; 0.15mg/L, 8 mg/L and 16 mg/L for lopinavir; 0.075 mg/L, 1.5 mg/L and 3.5 mg/L for ritonavir). The lower limit of quantification (LLOQ) was 0.20 mg/L, 0.05 mg/L and 0.025 mg/L for efavirenz, lopinavir and ritonavir, respectively (Figure 2.2). Any sample with plasma concentration below the LLOQ was treated as 40% of LLOQ concentration in the data analysis. Any sample with plasma concentration higher than the upper limits of quantification was diluted with drug-free plasma and reanalyzed.

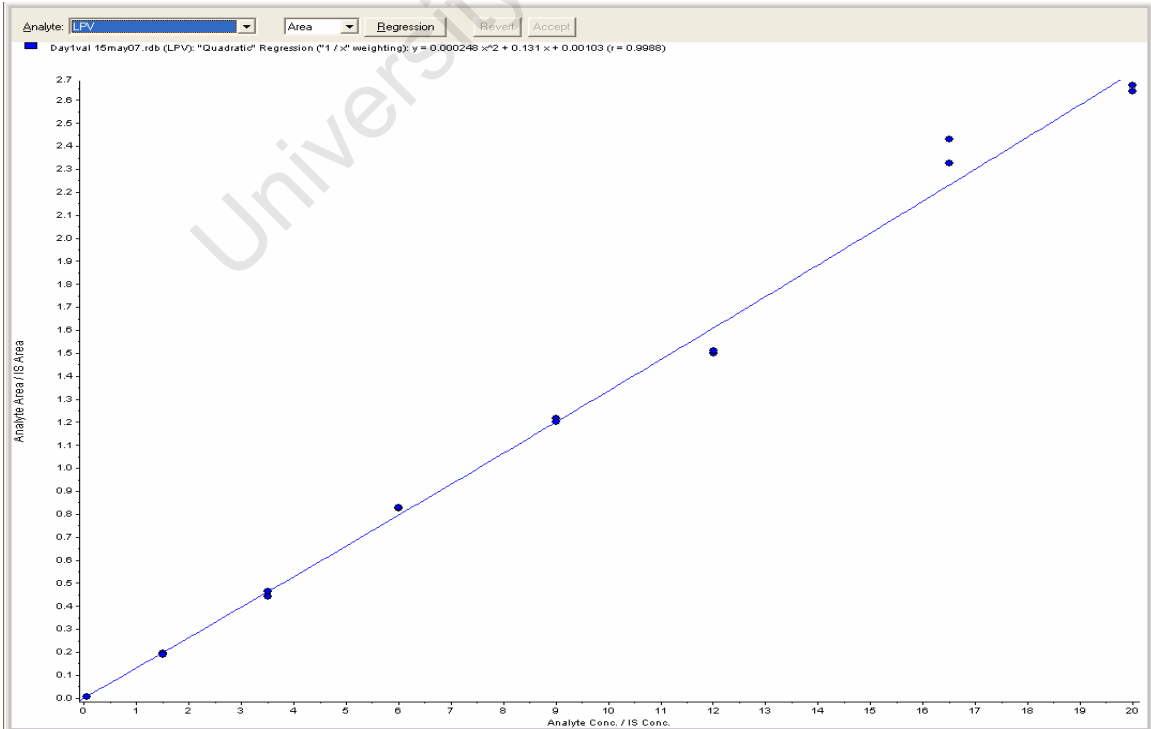
For efavirenz plasma assay: Accuracy ranged from 90.55% to 109.55%. The intra-day and inter-day precisions ranged from 1.34% to 9.61% and from 2.23% to 7.04%, respectively. For lopinavir assay: Accuracy ranged from 90.57% to 109.78%. The intra-day and inter-day precisions ranged from 0.36% to 5.47% and from 0.73% to 5.43%, respectively. For ritonavir assay: Accuracy ranged from 93.26% to 108.50%. The intra-day and inter-day precisions ranged from 1.10% to 8.33% and from 0.94% to 5.75%, respectively. The laboratory participates in the International Interlaboratory Control Programme of Stichting Kwaliteitsbewaking Klinische Geneesmiddelenanalyse en Toxicologie (KKGTT; Hague, the Netherlands) on an ongoing basis.

Figure 2.1 Plasma calibration curves

a) Efavirenz plasma calibration curve



b) Lopinavir plasma calibration curve



c) Ritonavir plasma calibration curve

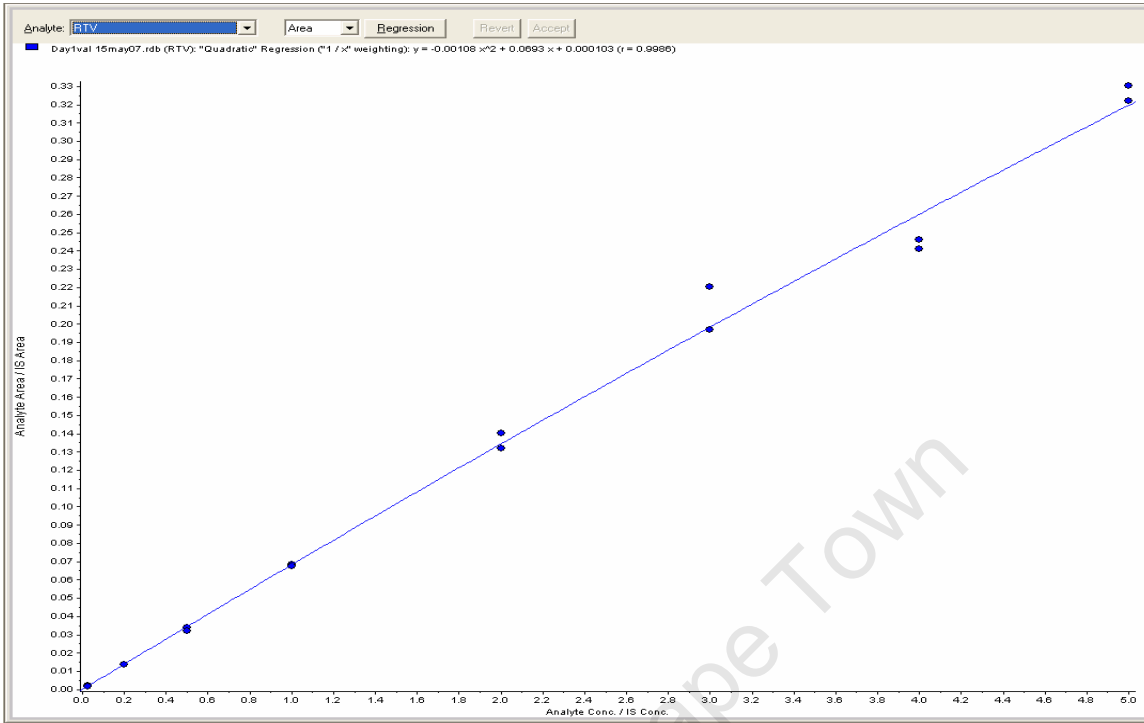
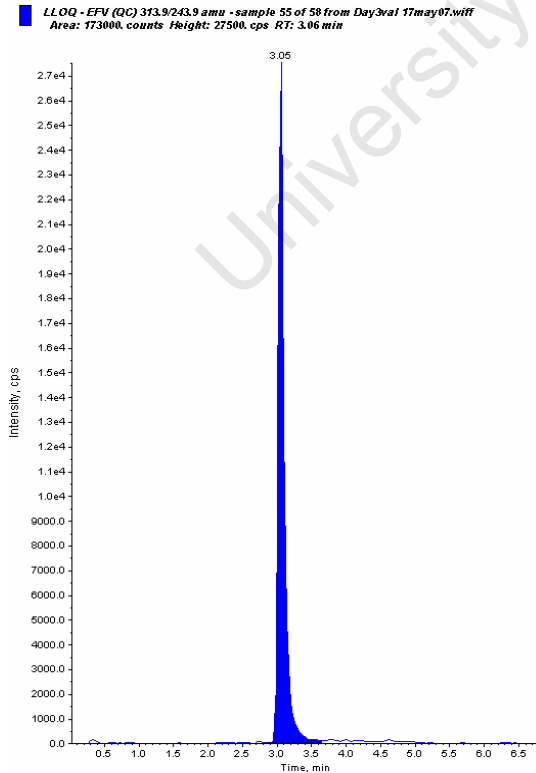
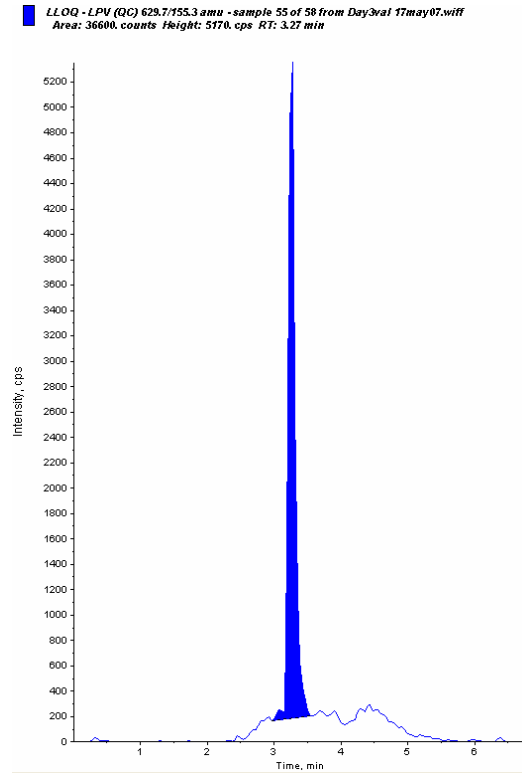


Figure 2.2 Chromatograms of plasma LLOQ

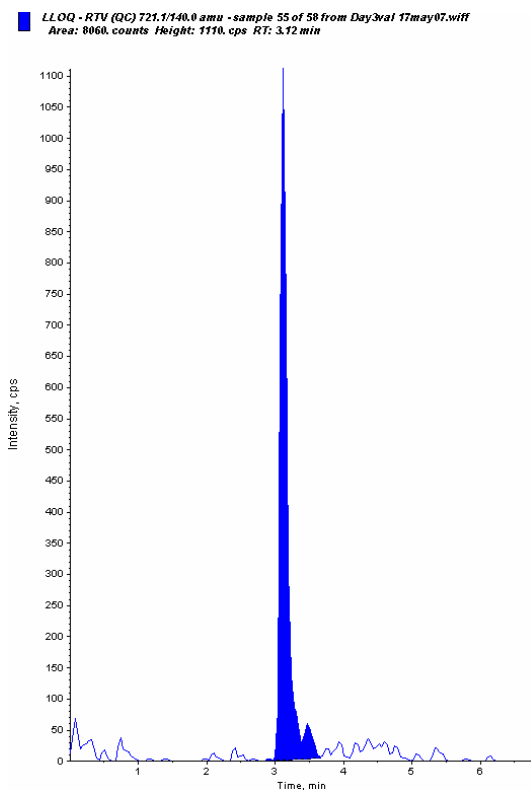
a) Chromatogram of efavirenz LLOQ



b) Chromatogram of lopinavir LLOQ



c) Chromatogram of ritonavir LLOQ



2.2 DBS Sample Preparation and Concentration Assay

2.2.1 Preparation of DBS Sample

DBS samples were obtained from efavirenz and lopinavir-ritonavir studies during plasma sampling. Exactly 50 μL from heparinized whole blood sample was spotted within 30 minutes after blood sampling onto 903 TFN filter paper card with pre-marked circles (Munktell, Niederschlag, Germany). The cards were dried at room temperature for 2 hours and stored in sealed plastic bags with desiccant at 4 $^{\circ}\text{C}$.

Each blood spot was cut into fine pieces and soaked in 100 μL of HPLC grade water (Merck KGaA, Darmstadt, Germany) for 10 minutes. An aliquot of 200 μL of precipitation reagent (50% methanol: 50% 0.2M ZnSO_4 v/v) with reserpine at 0.6 $\mu\text{g}/\text{mL}$ as internal standard was added to the sample and vortexed at high speed for 20 second. The mixture was then sonicated for further 20 minutes and centrifuged at 12000 $\times g$ for 10 minutes. The clear extracts were transferred to vial inserts and placed in the

autosampler tray for injection onto LC/MS/MS column [Koal *et al.* 2005].

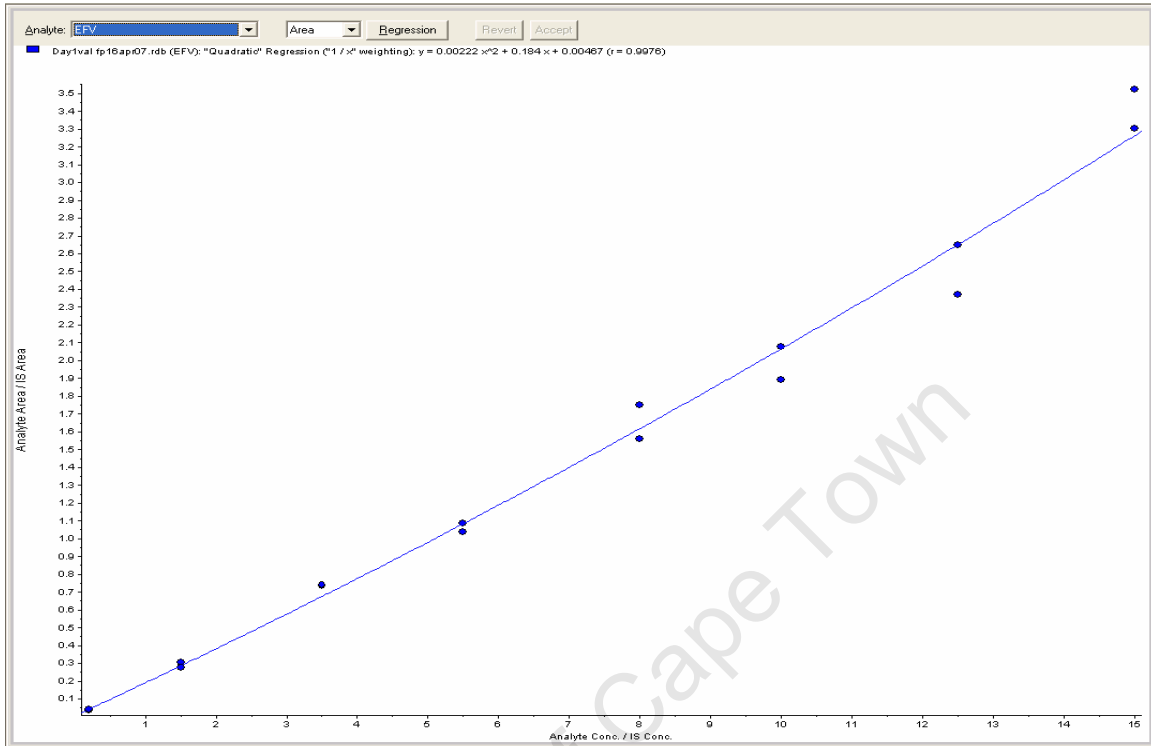
2.2.2 DBS ARV Concentration Assay by LC/MS/MS

DBS concentrations of efavirenz, lopinavir and ritonavir were quantified simultaneously by the same validated LC/MS/MS method used in plasma ARV drug concentration assay (Section 2.1.2). The DBS calibration curves were prepared by spiking ARV drugs in drug-free fresh whole blood at each calibration concentration, from which 50 μ L of blood was spotted on filter paper. Three quality controls were prepared in the same manner (0.3 mg/L, 4 mg/L and 11 mg/L for efavirenz; 0.15mg/L, 8 mg/L and 16 mg/L for lopinavir; 0.075 mg/L, 1.5 mg/L and 3.5 mg/L for ritonavir). The calibration curve was linear over the range of 0.2- 15 mg/L, 0.05- 20 mg/L, and 0.025- 5 mg/L for efavirenz, lopinavir and ritonavir, respectively (Figure 2.3). Any sample with concentration below LLOQ was treated as 40% of the LLOQ concentration in the data analysis. Any sample with the concentration above the upper limits of quantification was diluted with DBS precipitation reagent and reanalyzed. The laboratory participates in the International Interlaboratory Control Programme of Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie (KKGTT; Hague, the Netherlands) on an ongoing basis.

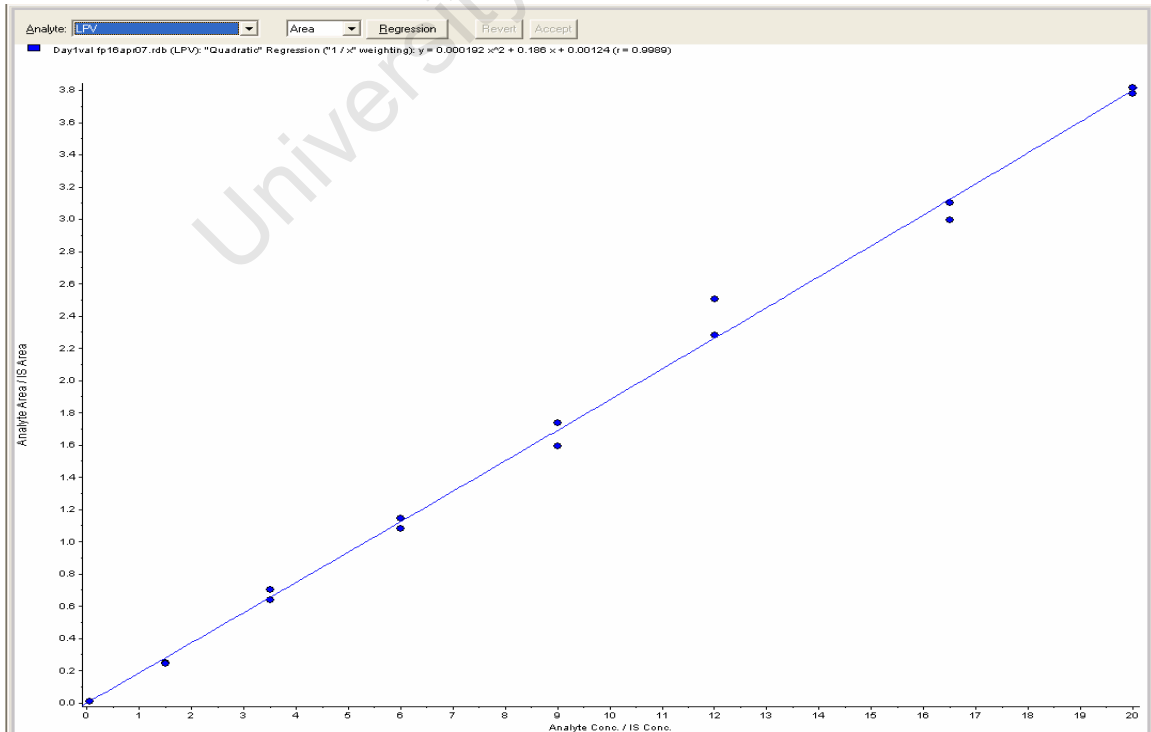
For efavirenz DBS assay: Accuracy ranged from 91.92% to 109.56%. The intra-day and inter-day precisions ranged from 0.34% to 7.57% and from 4.42% to 8.37%, respectively. For lopinavir assay: Accuracy ranged from 90.00% to 112.19%. The intra-day and inter-day precisions ranged from 0.65% to 7.24% and from 2.15% to 5.87%, respectively. For ritonavir assay: Accuracy ranged from 91.53% to 112.19%. The intra-day and inter-day precisions ranged from 1.04% to 11.22% and from 2.99% to 7.53%, respectively.

Figure 2.3 DBS calibration curves

a) Efavirenz DBS calibration curve



b) Lopinavir DBS calibration curve



c) Ritonavir DBS calibration curve

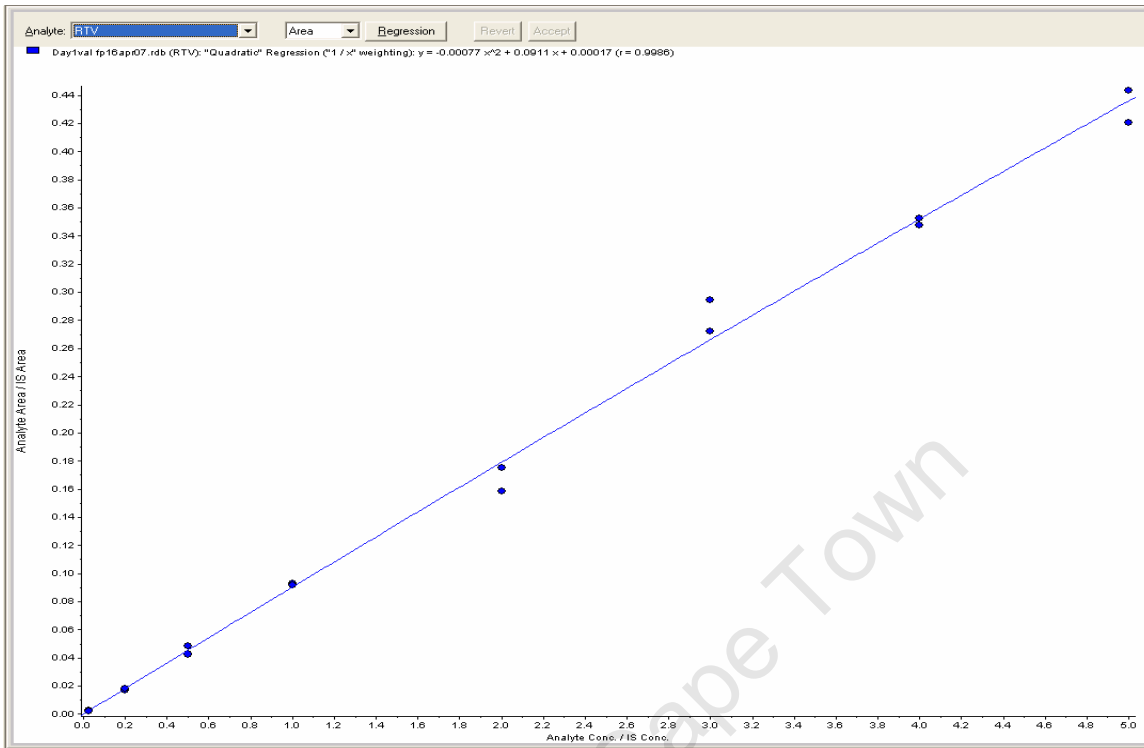
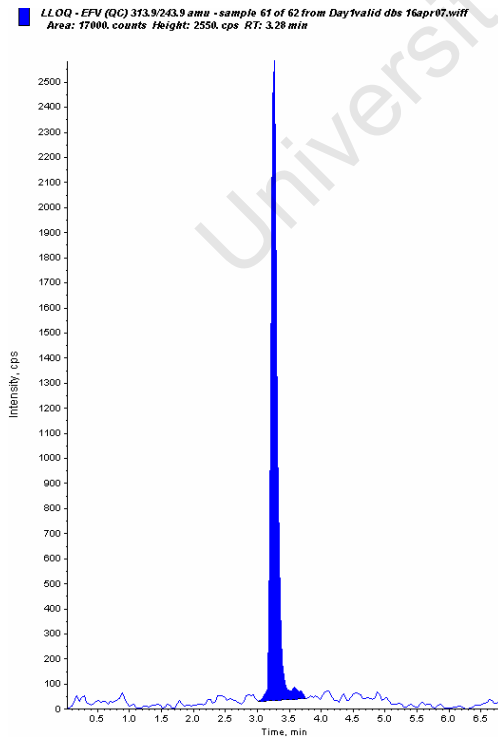
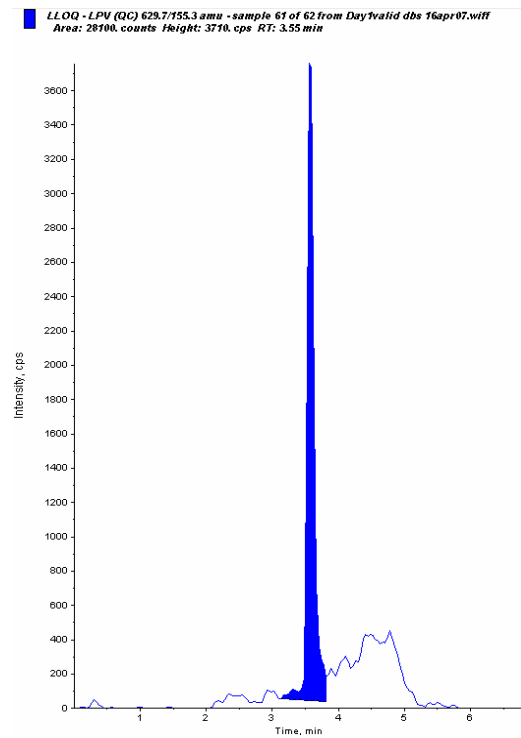


Figure 2.4 Chromatograms of DBS LLOQ

a) Chromatogram of efavirenz LLOQ

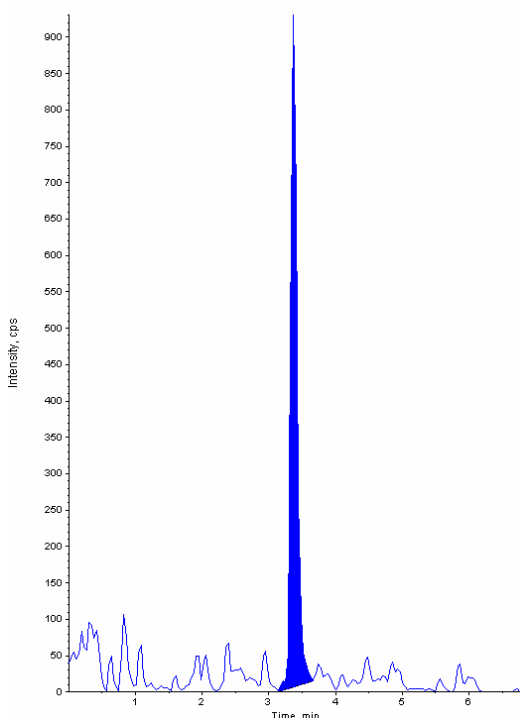


b) Chromatogram of lopinavir LLOQ



c) Chromatogram of ritonavir LLOQ

■ LLOQ - RTV (QC) 721.1/140.0 amu - sample 61 of 62 from Dayvalid dbs 16apr07.wiff
Area: 5806. counts Height: 923. cps RT: 3.37 min



2.2.3 Stabilities of ARV Drugs in DBS Samples

The stabilities of efavirenz, lopinavir and ritonavir in DBS samples were tested at 37 °C for 7 days. The stabilities of two drying methods were also tested: 1) drying under direct sunlight for 2 hours and 2) using hair drier at higher setting for 3 minutes.

2.3 *In vitro* ARV Drug Accumulation Assay

2.3.1 Isolation of Human PBMCs from Leukoreduction Chambers

The leukoreduction chambers were donated by Western Province Blood Transfusion Service Centre in Cape Town at the conclusion of the aphaeresis procedures. Tubing of leukoreduction chambers was sealed before removing the chamber from the aphaeresis instrument (Trima Accel[®], Gambro BCT, Inc. Lakewood, CO USA). Human PBMCs were isolated from leukoreduction chamber within 1 hour after disconnection [Néron *et al.* 2007].

The leukoreduction chamber was rinsed with a 60 mL syringe fitted with 22-gauge needle by 50 mL of phosphate buffered saline (PBS) solution containing 2% of foetal calf serum (FCS, Highveld Biological PTY LTD, Lyndhurst, RSA). The leukocyte suspension was equally divided to 2 portions (approximately 30 mL of each portion). In two 50 mL-Falcon[®] tubes containing 15 mL of Histopaque solution (Sigma Chemical Co.), 30 mL of leukocyte suspension was carefully loaded without disturbing the interface of the two solution. The biphasic solution was centrifuged at 350 ×g for 30 minutes at room temperature, with slow acceleration and no break. The layer at interface was collected from the two tubes and pooled into one 50 mL tube. The interface layer was washed with 50 mL of PBS and centrifuged at 650 ×g for 10 minutes at room temperature, with break at low setting. The supernatant was aspirated off, and cell pellet was re-suspended in 50 mL of PBS, inverted gently for 10 times. The wash step was repeated twice [Colombo *et al.* 2005]. After the final wash step, the cell pellet was re-suspended in 25-30 mL of PBS solution, from which two 10 µL- aliquots of suspension were removed. One aliquot was diluted and mounted onto a Bright Microscope Line Counting Chamber (Hausser Scientific Company) under a coverslip for cell counting. Another aliquot was stained with same volume (10 µL) of Trypan blue solution for cell viability calculation. The formula for cell viability calculation is as follows:

$$\% \text{ of viability} = \frac{\text{Number of viable (unstained) PBMC}}{\text{Total number of PBMC}} \times 100$$

Depend on the cell count and viability, the suspension was centrifuged and re-suspended in RPMI-1640 growth media (Dutch Modification with NaHCO₃ at 1 g/L and 20 mM HEPES without L-glutamine; from Sigma Chemical Co.) to make a cell stock solution with a concentration of 8×10⁶ cells/mL.

2.3.2 *In vitro* ARV Drug Intracellular Accumulation Experiment

Efavirenz and lopinavir were spiked in 500 µL of RPMI growth media containing 10% of FCS in the absence and presence of drug efflux protein inhibitors (verapamil at 50 µM, furosemide at 50 µM, cyclosporine A at 20 µM and ritonavir at 5 mg/L) and inducer

(rifampicin at 4 mg/L). The same volume of cell stock solution was added (the final cell count= 4×10^6 cells/mL) and incubated at 37 °C for half an hour. The final concentration for efavirenz and lopinavir was 1.5 and 5 mg/L, respectively. The PBMC samples were then centrifuged at 350 ×g at 4 °C for 5 minutes. The supernatant was aspirated off. The bottom of the tube which contained cell pellet was clipped off into another 1.5 mL polypropylene Eppendorf tube and kept at -20 °C for concentration testing.

2.3.3 Intracellular ARV Concentration Testing

2.3.3.1 Preparation of Blank Control PBMCs

The blank control PBMCs were used for the preparation of LC/MS/MS calibration and quality control samples. PBMCs were isolated from leukoreduction chamber as described in Section 2.3.1. After cell counting and viability calculation, appropriate volume of PBS was added to the cell suspension to provide a concentration of 4×10^6 cells/mL. The diluted suspension was distributed as 1 mL-aliquots in 1.5 mL polypropylene Eppendorf tubes. These 1 mL-aliquots were centrifuged at 350 ×g for 5 minutes. The supernatant was aspirated off, leaving cell pellets at the bottom of the tubes. The blank PBMCs were stored at -20 °C.

2.3.3.2 Calibration Curve and Quality Control

Working solution contains the mixture of 50% methanol and 50% of HPLC-grade water with 0.05 µg/mL of reserpine as internal standard. The stock solution containing efavirenz, lopinavir and ritonavir at 1 mg/mL in methanol was diluted with working solution to obtain 9 concentrations for the calibration curve (0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 50.0, 75.0, 100.0 ng/mL) and 3 concentrations for quality control (0.75, 20.0, 80.0 ng/mL).

2.3.3.3 Extraction of ARV from PBMCs and LC/MS/MS Assay

A 300 µL-aliquot of calibration and quality control with reported concentrations (Section 2.3.3.2) was added to blank PBMC pellet. An aliquot of 500 µL of working solution was added to each polypropylene Eppendorf tube containing sample from *in vitro* ARV drug accumulation experiment (Section 2.3.2). The resulting suspension was vortexed at high

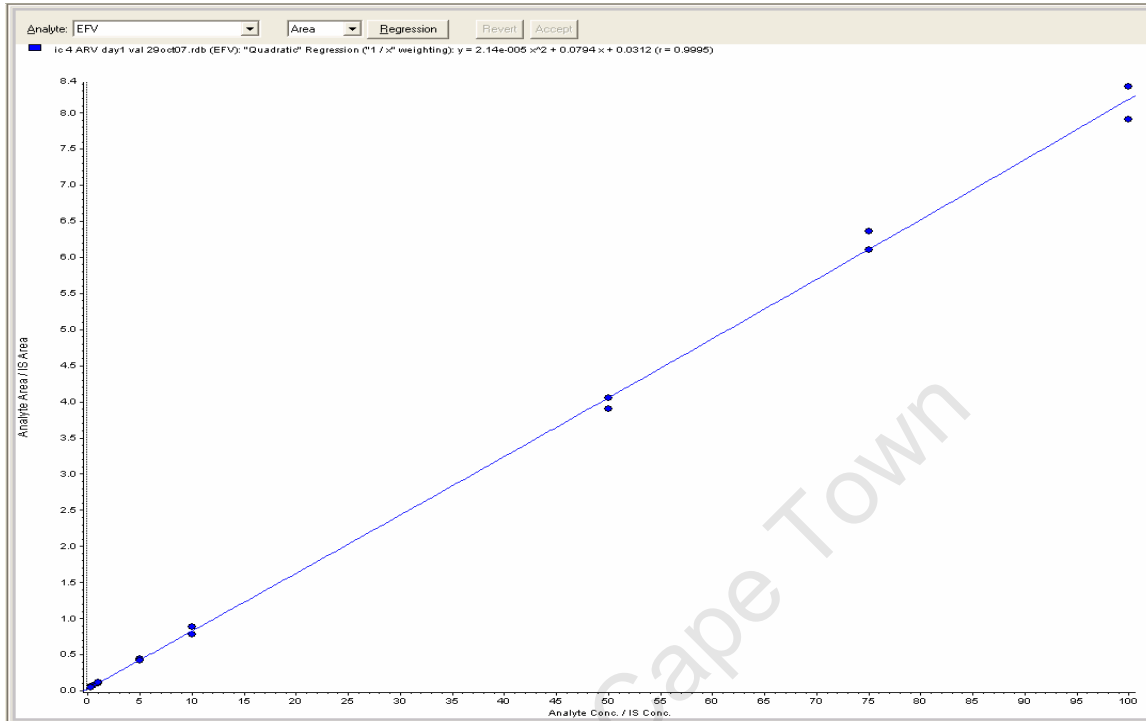
speed for 30 seconds and subsequently sonicated for 20 minutes. Samples were shaken for 3 hours by a vortex machine (at level 4) used as a shaker. Samples were then centrifuged at 12000 \times g for 5 minutes at room temperature. The clear extracts were transferred to vial inserts and placed in the autosampler tray for injection onto LC/MS/MS column.

Intracellular concentrations of efavirenz, lopinavir and ritonavir were quantified simultaneously by the same validated LC/MS/MS method used in plasma and DBS ARV drug concentration assays (Section 2.1.2). The efavirenz, lopinavir and ritonavir calibration curves for intracellular concentration testing were linear over the range of 0.1-100.0 ng/mL (Figure 2.5). The LLOQ was 0.1 ng/mL for these 3 drugs (Figure 2.6). Any sample with plasma concentration below the LLOQ was treated as 40% of LLOQ concentration in the data analysis. Any sample with the concentration above the upper limits of quantification was diluted with working solution and reanalyzed.

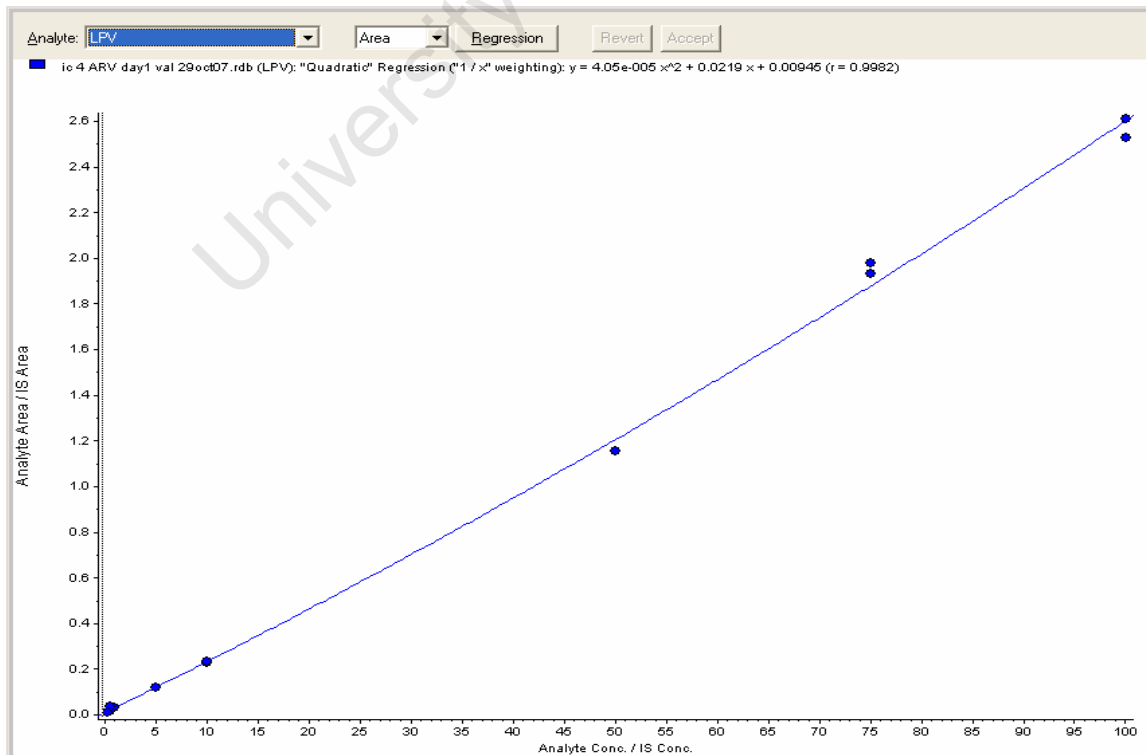
For efavirenz plasma assay: Accuracy ranged from 87.93% to 115.76%. The intra-day and inter-day precisions ranged from 2.56% to 14.62% and from 1.12% to 9.39%, respectively. For lopinavir assay: Accuracy ranged from 93.51% to 108.42%. The intra-day and inter-day precisions ranged from 0.01% to 24.32% and from 1.13% to 32.02%, respectively. For ritonavir assay: Accuracy ranged from 87.87% to 110.43%. The intra-day and inter-day precisions ranged from 0.88% to 27.84% and from 1.40% to 9.70%, respectively.

Figure 2.5 Intracellular calibration curves

a) Efavirenz intracellular calibration curve



b) Lopinavir intracellular calibration curve



c) Ritonavir intracellular calibration curve

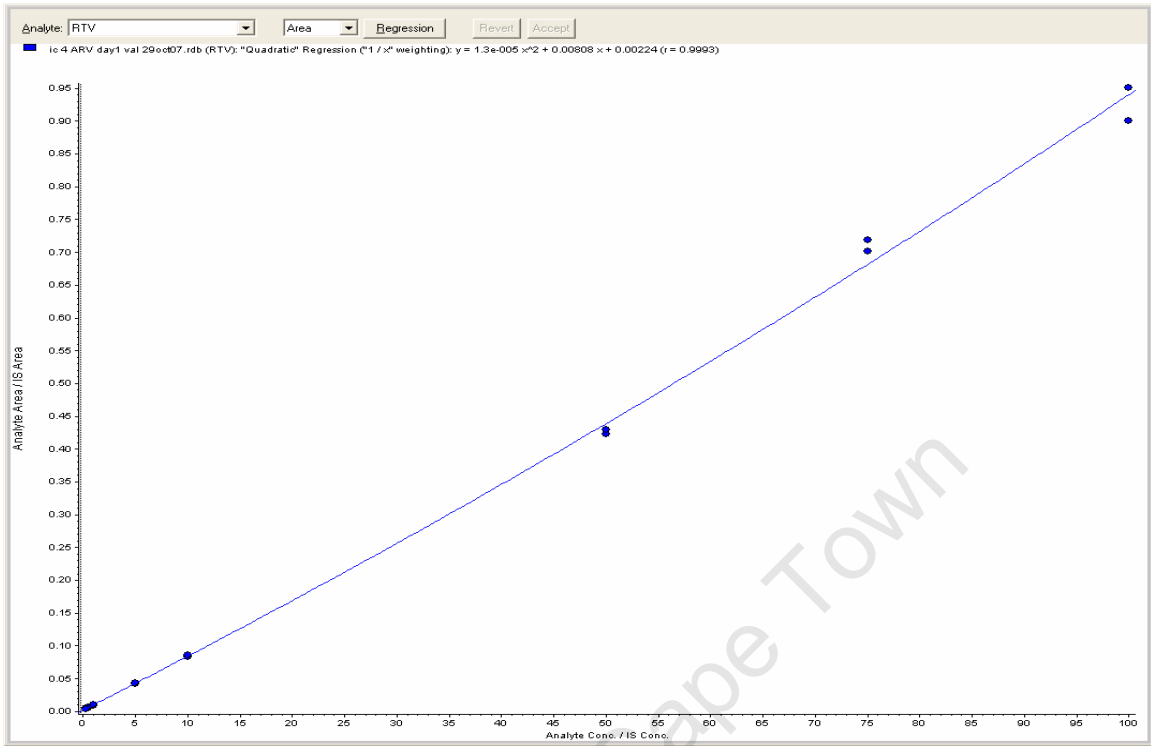
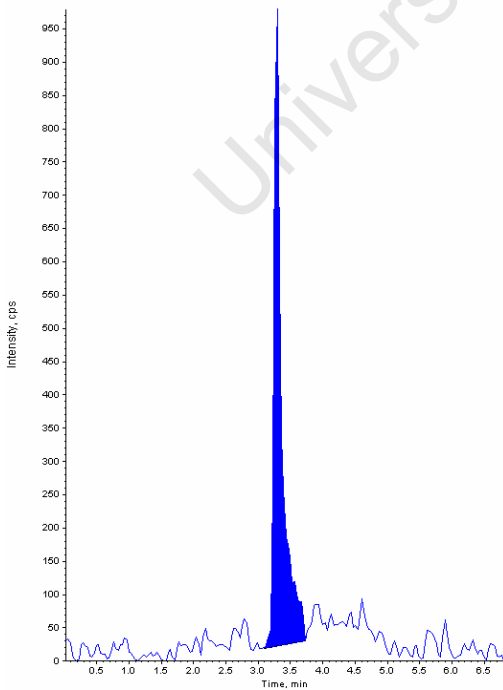


Figure 2.6 Chromatograms of intracellular LLOQ

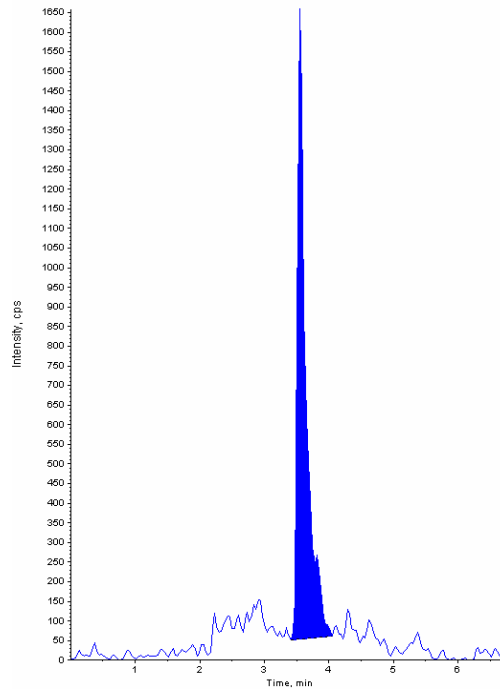
a) Efavirenz intracellular LLOQ

LLOQ - EFV (QC) 313.9/243.9 amu - sample 59 of 60 from ic Day1 valid 29oct07.wiff
Area: 8860, counts Height: 956, cps RT: 3.29 min



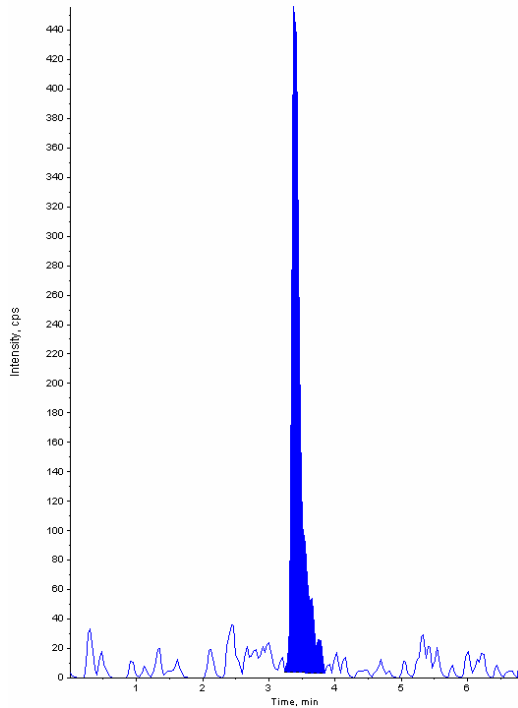
b) Lopinavir intracellular LLOQ

LLOQ - LPV (QC) 629.7/155.3 amu - sample 59 of 60 from ic Day1 valid 29oct07.wiff
Area: 14500, counts Height: 1610, cps RT: 3.55 min



c) Ritonavir intracellular LLOQ

■ LLOQ - RTV (QC) 721.1/140.0 amu - sample 59 of 60 from ic Day1 valid 29oct07.wiff
Area: 4110 counts Height: 451 cps RT: 3.38 min



2.3.4 Quantification of Drug Concentration in PBMCs

The relative drug concentrations were calculated from calibration curve. However, the number of cells needed to be taken into account. The concentrations must be converted to express the amount of drug in cell volume, assuming a cell volume of 0.4 pL for each PBMC [Furman *et al.* 1986]. The formula used for calculation of intracellular concentration was:

$$\text{Intracellular concentration (mg/L)} = \frac{\text{Total amount of drug}}{\text{PBMC volume} \times \text{Total viable cell number/mL}}$$

The total amount of drug was obtained by multiplying the relative drug concentration by 0.5 mL (the volume of working solution added to extract each sample). The viable cell number/mL in each pellet in this experiment was 4×10^6 cells/mL. Data were expressed as cellular accumulation ratios (the ratio of efavirenz and lopinavir concentrations associated with cell pellet to media).

2.4 *In vivo* Intracellular ARV Drug Concentration Testing

2.4.1 Isolation of Patient PBMCs

PBMCs were isolated from HIV-infected children receiving efavirenz or LPV/r, using the BD Vacutainer™ CPT™ Tubes (Becton Dickinson, Franklin Lakes, NJ, USA), which contain citrate as anticoagulant, a polyester gel layer, and a Ficoll Hypaque solution enabling the direct separation of mononuclear cells from other blood components. Approximately 4 mL of whole blood from each child was collected into a 4mL CPT™ tube and centrifuged immediately at 1800 ×g for 30 minutes at 4 °C, without brake. The whitish interface which contains mononuclear cells was transferred into a 15 mL polypropylene centrifuge tube. The collected cells were washed with 10 mL of cold PBS solution (4 °C) and centrifuged at 300 ×g for 15 min at 4 °C with brake at low setting. The supernatant was discarded. The washing procedure was repeated twice [Becton Dickinson Vacutainer™ system]. Supernatant wash solution was completely aspirated off after the third wash and an exact 1 mL of PBS was added to the cell pellet and carefully homogenized. One 10 µL-aliquot from the cell suspension was removed and diluted for cell counting. Another 10 µL-aliquot was taken out and stained with the same volume of Trypan blue solution for cell viability calculation. The remaining 980 µL cell suspension was centrifuged at 350 ×g for 5 minutes at 4 °C. The supernatant was aspirated off, leaving the PBMC pellet at the bottom of the vial which was immediately stored at –20 °C while waiting to be analyzed.

2.4.2 *In vivo* Intracellular Drug Extraction and LC/MS/MS Assay

The same procedures were followed as described in Section 2.3.3.3. The assay was performed in the laboratory of the Division of Clinical Pharmacology, University of Cape Town.

2.4.3 Quantification of *in vivo* Intracellular Drug Concentration

The formula used for calculation of intracellular concentration was stated in Section 2.3.4. The total amount of drug was obtained by multiplying the relative drug concentration by 0.5 mL (the volume of working solution added to each sample). The viable cell in

number/mL was obtained by multiplying cell count with cell viability in each sample. Cell volume is 0.4 pL for each PBMC [Furman *et al.* 1986].

University of Cape Town

Chapter 3

Effect of Rifampicin on Efavirenz Pharmacokinetics in HIV-infected Children with Tuberculosis

3.1 Introduction

The non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz, is recommended by the World Health Organization as a preferred first-line antiretroviral agent for children older than 3 years [WHO 2006]. As efavirenz is metabolized primarily by hepatic cytochrome P450 (CYP) 2B6, and rifampicin induces the expression of this enzyme, the potential pharmacokinetic interaction is a concern in HIV-infected children receiving concurrent treatment for TB and HIV. Adult studies show that concomitant rifampicin tends to reduce mean efavirenz plasma concentrations [Lopez-Cortes *et al.* 2002], and some authorities recommend increasing the efavirenz dose by 25% to compensate [Panel on Antiretroviral Guidelines for Adult and Adolescents, 2008]. No study has evaluated the effect of rifampicin-based antitubercular treatment on efavirenz plasma concentrations in children.

As low and high efavirenz concentrations in adults have been associated with virological failure and central nervous system side-effects, respectively [Marzolini *et al.* 2001; Csajka *et al.* 2003; Joshi 1999; van Leth *et al.* 2006; Stähle *et al.* 2004; González de Requena *et al.* 2004; Puthanakit *et al.* 2009; Cohen *et al.* 2009], it is important to maintain efavirenz trough concentrations within the recommended therapeutic range (1 to 4 mg/L) [la Porte *et al.* 2006; www.hivpharmacology.com]. The marked inter-patient and low intra-patient variability suggest that individual drug concentration monitoring may be a useful strategy for optimizing treatment [Marzolini *et al.* 2001]. However, in resource-limited settings where access to cART is rapidly expanding,

concentration-controlled dosing is not available outside of the research environment. There is a paucity of information about efavirenz plasma concentrations in children, especially African children.

In this chapter, a clinic-based, cross-sectional comparative study is described, in which the sparse sampling method was used to evaluate efavirenz plasma concentrations and determine the effect of rifampicin on efavirenz pharmacokinetics in African HIV-infected children with and without rifampicin-based antitubercular treatment.

3.2 Methods

3.2.1 Patients

A total of 30 children (aged 3-15 years old or weighing > 10kg) were enrolled: 15 TB/HIV co-infected children receiving standard doses of efavirenz as part of cART and rifampicin-based antitubercular treatment, and 15 HIV-infected children without tuberculosis receiving standard doses of efavirenz based cART. All children were dosed according to weight and the doses of efavirenz capsules were titrated to the nearest 50 mg in accordance with the Stocrin® product information leaflet [Merck Sharp & Dohme Limited, Modderfontein, South Africa, 1999]. Rifampicin-based antitubercular treatment regimens were administered in accordance with the South African National Tuberculosis Treatment Programme. Children were recruited at three sites: the HIV Clinic at Red Cross Children's Hospital, Cape Town; the Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto; and Brooklyn Chest Hospital, Cape Town. Institutional approval of the study was granted by the research ethics committees of the University of Cape Town, Stellenbosch University and the University of the Witwatersrand.

Additional group of 5 TB/HIV co-infected children was enrolled to evaluate the method used to estimate efavirenz 24-hour C_{\min} .

Exclusion criteria were: renal, hepatic, or intestinal disease (including malabsorption or diarrhoea); other active opportunistic infections; recent exposure to drugs described to have pharmacokinetic interactions with efavirenz; or reported missed doses of efavirenz

in the preceding 3 days. They were accompanied by a parent or legal guardian who provided written informed consent to participate in the study, and children older than 7 years gave their own assent.

3.2.2 Study Design

Co-infected children were on treatment for TB and HIV for at least 4 weeks before PK evaluation. Efavirenz plasma concentrations were evaluated a second time in this group of children at least 4 weeks after completing antitubercular treatment. The children without TB were established on cART for at least 4 weeks before efavirenz concentrations were measured. At least 12 hours after the dose of efavirenz, 3 blood samples were collected during a clinic visit with a time interval of no less than 2 hours between each sample. For the extra group of TB/HIV co-infected children, after collecting of 3 samples on 2 occasions described above, the additional 4th sample was collected at 24 hours after previous dose of efavirenz on both occasions. The exact times of blood sampling were recorded and the time of efavirenz ingestion the previous evening was reported by the accompanying adult.

Adults accompanying the participants were asked about treatment adherence to cART during the 3 days prior to PK sampling using a questionnaire. Viral load was tested by NASBA EasyQ (Biomerieux, Boxtel, the Netherlands). Viral load monitoring was performed at 6 month intervals as part of routine management.

3.2.3 Data Analysis

The plasma concentrations of efavirenz were determined by LC/MS/MS. The detailed methods were described in the Materials and Methods Chapter (Section 2.1). Estimation of the C_{\min} concentration of efavirenz in plasma at 24 hours after dose administration assumed first-order elimination of the drug during the sampling period [Kappelhoff *et al.* 2005]. The estimated C_{\min} was determined by extrapolation of the log-linear regression line of the 3 concentration vs. time points to 24 hours after the reported time of the dose given in the previous evening. The elimination rate constant (k) was defined as the absolute value of the slope of the log-linear regression line and the half-life was derived

from k (half-life = $0.693/k$). The MDI concentration of efavirenz was defined as the mean of the plasma concentrations taken between 16 and 20 hours after dose administration.

3.2.4 Statistical Analysis

Stata version 8.2 (Stata Corp., College Station, TX) was used to compute summary statistics, and to perform statistical analyses. Wilcoxon ranksum test (RS) and Wilcoxon matched-pairs signed-ranks test (SR) were used for independent groups and paired observations, respectively, with non-normally distributed data. The relative prediction error (bias) of estimation was the difference between the estimated and the true value relative to the true measurement. The relative imprecision was the standard deviation of the relative prediction error. The sensitivity and specificity were calculated using the following formulae:

$$\text{Sensitivity} = \frac{\text{number of true positive}}{\text{number of true positive} + \text{number of false negative}}$$

$$\text{Specificity} = \frac{\text{number of true negative}}{\text{number of true negative} + \text{number of false positive}}$$

3.3 Results

3.3.1 Efavirenz Study

The children's general characteristics are summarized in Table 3.1. The co-administered NRTIs were stavudine plus lamivudine in 28 (93%) children, lamivudine plus zidovudine in 1 (3%), and zidovudine plus didanosine in 1 (3%) child. The median durations of cART in TB/HIV co-infected children prior to the first and second pharmacokinetic assessments were 12 weeks (range 4, 55) and 28 weeks (range 20, 78), respectively. The median duration of cART in control group was 71 weeks (range 8, 173). Two children were under 3 years: 1 with TB/HIV co-infection (1.42 and 2.08 years, during and after antitubercular treatment, respectively) and the other was in the control group (2.25 years); both of them weighed more than 10 kg.

Three efavirenz concentrations were determined in all participants at each pharmacokinetic assessment. The efavirenz concentrations ranged from 0.36 to 10.60 mg/L and the sampling times ranged from 11.9 to 24.0 hours after dose administration. The regression lines used to estimate the C_{\min} values had a median R-squared value of 0.95 (IQR 0.85, 0.98). Median R-squared value was slightly reduced after antitubercular treatment when compared with during antitubercular treatment in the same group of children (median 0.88 IQR 0.38, 0.98 and 0.95 IQR 0.85, 0.98, respectively; SR $p= 0.094$). The estimated efavirenz plasma C_{\min} in TB/HIV co-infected children during and after rifampicin-based antitubercular treatment were similar (median 0.83 mg/L IQR 0.59, 6.57 and 0.86 mg/L IQR 0.61, 3.56, respectively; SR $p= 0.125$). Each child had at least one plasma efavirenz concentration measurement between 16 and 20 hours after dosing. The median MDI blood sampling time was 17.67 hours (IQR 17.45, 18.26). Median MDI concentrations were 1.24 mg/L (IQR 0.91, 7.38) and 1.23 mg/L (IQR 0.85, 4.18) during and after antitubercular treatment, respectively (SR $p= 0.078$). No significant differences were found in any of the PK parameters between TB/HIV co-infected group during antitubercular treatment with the control group (Table 3.1). Efavirenz half-life was correlated with higher efavirenz estimated C_{\min} and MDI concentrations (Spearman's $\rho= 0.685$, $p< 0.001$ and Spearman's $\rho= 0.521$, $p< 0.001$, respectively) and tended to be shorter during antitubercular treatment than after antitubercular treatment (Table 3.1). Nine (60%) and 8 (53%) of the children in the TB/HIV co-infected group had efavirenz estimated $C_{\min}< 1$ mg/L during and after rifampicin-based antitubercular treatment, respectively. Six children (40%) in the control group had estimated $C_{\min}< 1$ mg/L. Similarly, the MDI concentrations were < 1 mg/L in 6 children (40%) both during and after antitubercular treatment, whereas, 4 children (27%) in the control group had MDI concentrations < 1 mg/L.

Table 3.1 Characteristics and PK measures in 15 TB/HIV co-infected children during and after antitubercular treatment and 15 HIV-infected children without TB in control group (EFV= efavirenz). Categorical variables are expressed as n (%); continuous variables are summarized using median (interquartile range).

Characteristics	TB/HIV co-infected Group		<i>p</i> value*	Control Group	
	During TB treatment	After TB treatment		On cART only	<i>p</i> value**
n	15	15		15	
Male	9 (60%)	9 (60%)	1.000	9 (60%)	1.000
Median Age [years]	6.3 (4.3, 9.0)	7.1 (5.7, 9.2)	<0.001	6.8 (5.4, 9.1)	0.633
Median Weight [kg]	18.0 (15.7, 24.5)	20.5 (17.3, 24.8)	0.003	18.3 (17.3, 21.7)	0.756
Median Height [cm]	109.4 (101.5, 123.7)	113.4 (105.6, 124.3)	<0.001	110.0 (101.1, 115.0)	0.852
Median EFV dose [mg/kg]	13.9 (12.3, 15.2)	14.0 (12.8, 14.5)	0.050	13.9 (13.7, 14.5)	0.950
Median z-score Weight for age ¹	-1.10 (-1.63, -0.15)	-0.72 (-1.23, -0.19)	0.470	-1.33 (-2.03, -1.19)	0.663
PK Measures					
Median C _{min} [mg/L]	0.83 (0.59, 6.57)	0.86 (0.61, 3.56)	0.125	1.18 (0.46, 1.70)	0.756
Median MDI [mg/L]	1.24 (0.91, 7.38)	1.23 (0.85, 4.18)	0.078	1.58 (0.67, 2.20)	0.820
Median Half life [hours]	11.71 (9.18, 17.29)	24.66 (12.60, 44.14)	0.156	13.13 (8.15, 19.69)	0.852
R-squared value	0.95 (0.85, 0.98)	0.88 (0.38, 0.98)	0.094	0.95 (0.86, 0.97)	0.803

1. Z-score weight for age is an application of statistical theory to describe how far a child's weight is from the average weight of a child at the same age in the reference data (the National Centre for Health Statistics/WHO values).

* *p* values were obtained from comparison of TB/HIV co-infected children during and after antitubercular treatment (SR test).

***p* values were obtained from the comparison between TB/HIV co-infected children when receiving antitubercular treatment and children in control group only receiving efavirenz-based cART alone (RS test).

The distributions of efavirenz estimated C_{min} and MDI concentrations are shown in Figure 3.1. Wide inter-individual variation in efavirenz concentrations was apparent, particularly during antitubercular treatment, and marked bimodality of the efavirenz concentrations was observed. High C_{min} (> 4 mg/L) were estimated in 4 TB/HIV co-infected children during antitubercular treatment, and 2 children in the control group. Children with high estimated C_{min} had prolonged half-life values in comparison to the

remaining children (median 33.35 hours IQR 17.29, 66.65 vs. 10.87 hours IQR 8.10, 13.72; RS $p= 0.001$). Interestingly, amongst the 4 ‘slow metabolizers’ in TB/HIV co-infected group during antitubercular treatment, efavirenz concentrations were consistently decreased after antitubercular treatment with median changes of -2.64 mg/L (range $-3.01, -2.01$; SR $p= 0.068$) and -2.80 mg/L (range $-3.20, -2.41$; SR $p= 0.068$) in C_{\min} and MDI concentrations, respectively (Figure 3.1). In contrast, changes in median C_{\min} and MDI concentrations in the remaining patients were negligible (median change for C_{\min} 0.01 mg/L IQR $-0.21, 0.29$, SR $p= 1.00$; and for MDI concentration -0.06 mg/L IQR $-0.23, 0.22$; SR $p= 0.722$).

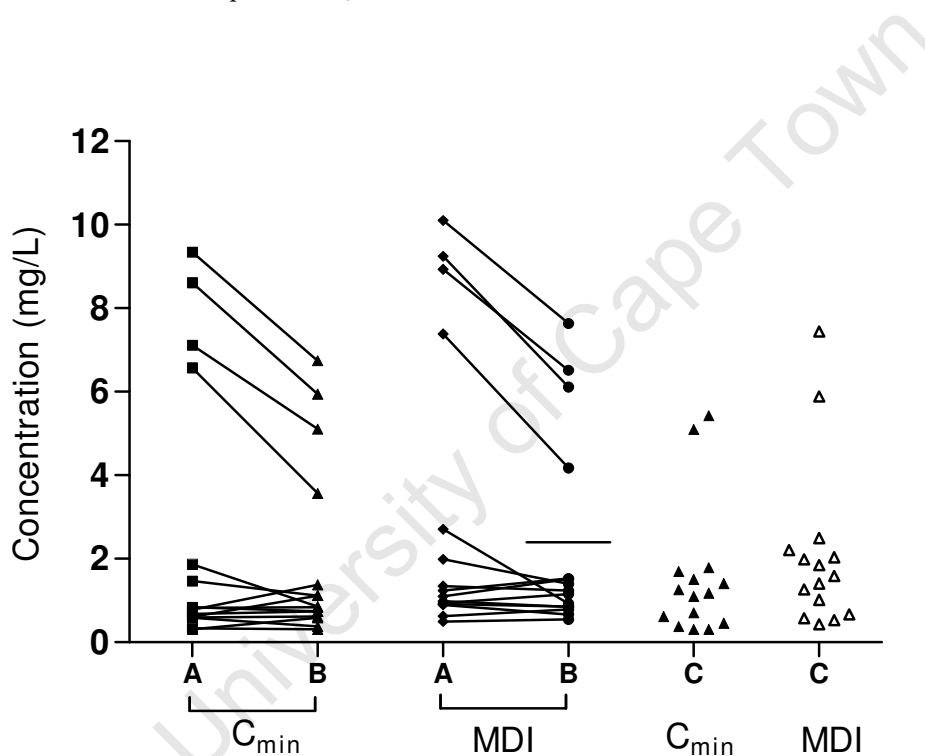


Figure 3.1 Distribution plot of efavirenz estimated C_{\min} concentrations and MDI concentrations. Solid lines indicate the respective C_{\min} and MDI during and after antitubercular treatment.

A: TB/HIV co-infected children receiving rifampicin-based antitubercular treatment and efavirenz-based cART.

B: The children included in “A” after completion of their antitubercular treatment and receiving efavirenz-based cART.

C: The control group of HIV infected children receiving efavirenz-based cART alone.

Efavirenz doses were adjusted in 5 children with low estimated C_{\min} and MDI concentrations (2 TB/HIV co-infected children and 3 in the control group). Efavirenz doses were increased by 100 mg in the 2 children during antitubercular treatment. Repeated estimated C_{\min} and MDI concentrations were above 1 mg/L after dose adjustment in both children. However, after changing back to standard doses on completion of antitubercular treatment, the estimated C_{\min} were lower than 1 mg/L in both children. Efavirenz doses were increased by 50 mg in the 3 children from the control group. The estimated C_{\min} and MDI concentrations were above 1 mg/L in 2 of the 3 children after dose adjustment.

Viral load results were available for 29 of the 30 children at least 6 months (6 had viral load results at 1 year) after initiating antiretroviral therapy. Twenty-two children (76%) with viral loads under the detectable limit of the assay (< 50 copies/mL) had significantly higher estimated C_{\min} than the remaining 7 children (median 1.22 mg/L IQR 0.67, 5.10 vs. 0.46 mg/L IQR 0.30, 1.40, respectively; RS $p=0.022$). These 7 children had viral load \log_{10} values ranging from 2.40 to 4.23. Five of 7 (71%) with detectable viral loads had estimated $C_{\min} < 1$ mg/L compared to 9 of 22 (41%) of those with viral suppression (Chi-squared test, $p=0.159$).

3.3.2 Verification of Efavirenz C_{\min} Estimation

The true efavirenz plasma C_{\min} concentrations were measured at 24 hours after the dose given the previous evening in 5 TB/HIV co-infected children and repeated in 3 of them after completion of antitubercular treatment. Estimated efavirenz plasma C_{\min} concentrations determined by extrapolation from the log-linear regression line of the 3 concentration vs. time points at 24 hours after dosing and single MDI concentrations were compared with the true C_{\min} concentrations in these children (Table 3.2). The estimated C_{\min} for one child could not be calculated as he had an elevated efavirenz concentration at the third sampling point. The mean relative prediction error (bias) of estimated C_{\min} was -5.88% for the rest 7 samples, although a large relative imprecision (33.66%) was observed. Amongst the 7 true C_{\min} which had corresponding estimated C_{\min}

concentrations, 3 (43%) had efavirenz true C_{\min} and estimated C_{\min} below 1 mg/L. One child had efavirenz estimated $C_{\min} < 1$ mg/L, but had normal true C_{\min} . For this individual, the true C_{\min} was similar to the concentration at last sampling at 19.58 hours after dosing (1.06 and 1.05 mg/L, respectively), indicating a flat elimination curve in this child. However, both concentrations were on the borderline of the minimum recommended concentration. The sensitivity and specificity for detecting sub-therapeutic efavirenz trough concentration using the estimation method were 100% and 75%, respectively.

The concentrations at the second sampling point were used as the single MDI concentrations, with the sampling time ranging from 15.50 to 19.55 hours after efavirenz intake. The mean relative prediction error (bias) of using the single MDI concentrations was 23.78% with mean relative imprecision of 18.77%. The sensitivity and specificity of the single MDI concentrations were both 100%. The linear correlations between efavirenz estimated C_{\min} and single MDI concentrations with the true C_{\min} are shown in Figure 3.2. The goodness of fit (R^2) values for estimated C_{\min} and single MDI sample with the true C_{\min} were 0.84 and 0.97, respectively.

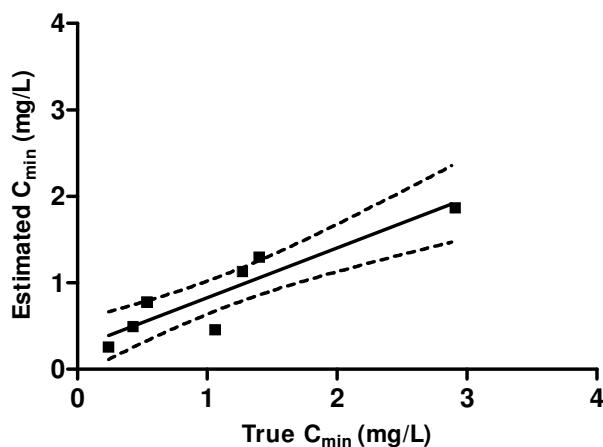
Table 3.2 Comparison between efavirenz estimated C_{\min} and single MDI concentrations with the true C_{\min} amongst 5 TB/HIV co-infected children (3 during and after antitubercular treatment and 2 during antitubercular treatment).

Subject	On/ off RIF	Estimated C_{\min} (mg/L)	True C_{\min} (mg/L)	Single MDI Sample (mg/L)
1	On	0.26	0.24	0.27
1	Off	- *	1.11	1.68
2	On	0.49	0.43	0.51
2	Off	0.78	0.54	0.84
3	On	1.30	1.40	1.58
3	Off	0.46	1.06	1.30
4	On	1.87	2.91	3.13
5	On	1.13	1.27	1.42

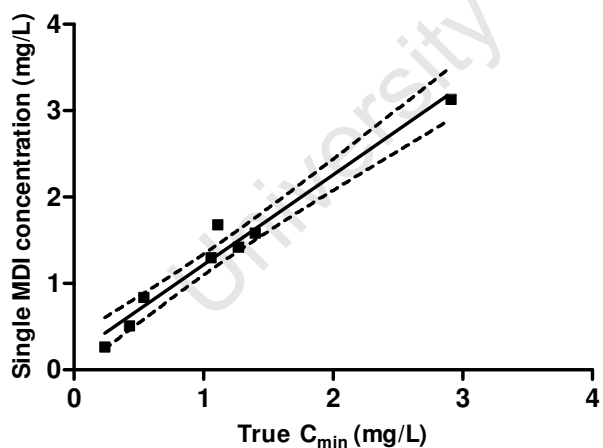
* C_{\min} could not be estimated due to poor correlation

Figure 3.2 Linear correlations between efavirenz estimated C_{\min} and single MDI concentrations with true C_{\min} (solid line). The 90% confidence interval (CI) was indicated by the dashed lines.

a) Correlation between estimated C_{\min} and true C_{\min}



b) Correlation between single MDI concentrations and true C_{\min}



3.4 Discussion

It was found that co-administration of rifampicin-based antitubercular treatment did not significantly reduce the key efavirenz PK parameter, the estimated C_{\min} concentration. Furthermore, the proportion of children with sub-therapeutic (< 1 mg/L) estimated C_{\min}

and MDI concentrations was not significantly affected by rifampicin-based antitubercular treatment. Similarly, studies amongst South African adults receiving standard doses of efavirenz have failed to demonstrate significant reductions in efavirenz concentrations during compared to after rifampicin-based antitubercular therapy [Friedland *et al.* 2006; Cohen *et al.* 2009]. Although a study in 8 Spanish patients reported decreases in median efavirenz C_{max} , C_{min} and AUC of 24%, 18% and 10% respectively after 7 days of concomitant rifampicin, none of these differences were statistically significant [Lopez-Cortes *et al.* 2002]. Conversely, a recently published analysis found a 35% reduction (95% CI -56.2%, -2.1%) in efavirenz TDM sample concentrations amongst 339 patients (17% on rifampicin) after adjustment for weight, ethnicity and concomitant zidovudine [Stöhr *et al.* 2008]. In this study, the half-life tended to be reduced during antitubercular treatment consistent with induction of efavirenz metabolism by rifampicin. However, this finding was not reflected in the closely similar median estimated C_{min} and MDI concentrations during and after antitubercular treatment. Physiological changes in response to treatment, improved nutritional status and maturation (e.g. growth, weight gain and altered serum protein concentrations) affecting volume of distribution and drug disposition may have resulted in higher peak concentrations of efavirenz without apparent differences in the estimated C_{min} and MDI concentrations. Furthermore, as no robust estimations of adherence to treatment were available, it cannot be excluded that there was improved adherence during antitubercular treatment given the wide variability in efavirenz concentrations. The time of the evening dose of efavirenz prior to PK sampling which could affect C_{min} estimations and MDI concentrations was not directly observed by study investigators. The study was underpowered to detect relatively small differences in efavirenz PK parameters between TB/HIV co-infected group during antitubercular treatment and the control group. However, using parametric assumptions for the within subject comparison, 33 TB/HIV co-infected children are required to detect the mean reduction of 0.7 mg/L (26%) in efavirenz concentrations during antitubercular treatment (significance level= 0.05; 90% power; 20% drop-out rate). A larger study is warranted to confidently define the effect of antitubercular treatment on efavirenz concentrations.

Wide inter-patient variation and bimodality were observed. Genetic polymorphisms could explain the high estimated C_{\min} in 6 children (20%). Exonic single nucleotide polymorphisms of CYP2B6 have been described and are associated with impaired metabolism and increased levels of efavirenz [Haas *et al.* 2004; Tsuchiya *et al.* 2004]. Some of these are more common amongst populations of African origin [Haas *et al.* 2004; Wang *et al.* 2006]. A weakness of this study is the lack of genetic information; however, genetic analysis was beyond the scope of this investigation.

Contrary to expectation, 4 'slow metabolizers' in the TB/HIV co-infected group had increased efavirenz concentrations during antitubercular treatment. A similar pattern was noted in 2 studies amongst South African adults [Friedland *et al.* 2006; Cohen *et al.* 2009]. It is interesting to speculate that the consistently higher efavirenz concentrations during antitubercular treatment amongst the 'slow metabolizers' might be due to inhibition of an accessory metabolic pathway by isoniazid. *In vitro* studies have shown that isoniazid inhibits CYP3A4 and CYP2A6 amongst other enzymes [Wen *et al.* 2002]. Furthermore, a recent study suggests that functional polymorphisms in CYP3A4 and CYP2A6 are important determinants of efavirenz exposure in patients with limited CYP2B6 function [di Lulio *et al.* 2008]. To our knowledge, the effect of the combination of isoniazid and rifampicin on the activity of different CYP isoenzymes is poorly characterized. Even so, the inhibitory action of isoniazid on accessory pathways of efavirenz metabolism may moderate the effect of rifampicin-induced CYP2B6 activity on efavirenz concentrations.

Although median body weight increased by 2.5 kg between the two sampling occasions, the doses of efavirenz were kept in line with the increased body weight. It was found a high proportion of children dosed correctly according to the manufacturer's instructions had sub-therapeutic efavirenz estimated C_{\min} and MDI concentrations. In the Pediatric AIDS Clinical Trials Group (PACTG) 382 study [Starr *et al.* 1999], 22 of the 50 children receiving efavirenz (44%) had their daily doses of efavirenz increased based on pharmacokinetic assessment of the 24-hour AUC at 2 weeks after starting the study regimen. Eleven (22%) of these 50 children had trough efavirenz concentrations of less

than 1 mg/L (personal communication, Professor Courtney V. Fletcher, University of Colorado Health Sciences Center, Denver, CO, 2006). In this study the efavirenz doses given corresponded to, or were slightly higher, than the starting doses used in the PACTG 382 study. The proportion of children with estimated minimum concentrations less than 1 mg/L was greater than in PACTG 382. Children in this study tended to be younger (7.15 ± 3.09 vs. 8.5 ± 3.3 years) than the children in the PACTG study.

In keeping with adult studies showing that low efavirenz concentrations are associated with virological failure [Marzolini *et al.* 2001; González de Requena *et al.* 2004; Cohen *et al.* 2009], children in this study with virological failure had significantly lower efavirenz estimated C_{\min} than those children who had undetectable viral loads. Virological failure was observed in 5 of the 14 children (36%) had low efavirenz estimated C_{\min} (<1 mg/L), compared to 13% of those who had estimated $C_{\min} > 1$ mg/L. This study raises concern that many children may be under-dosed using the current guidelines. A non-linear mixed effect PK model describing efavirenz concentrations in 33 children from the Netherlands suggested that an adult efavirenz dose should be given to children weighing ≥ 25 kg, and that dose should be allometrically scaled *a priori* for other weight levels [ter Heine *et al.* 2008]. Another study in 48 West African children observed that 19% children had efavirenz C_{\min} below the therapeutic concentration, and that children weighing less than 15 kg were more likely to have sub-therapeutic concentrations. They recommended a higher dose of efavirenz (25 mg/kg) for children under 6 years of age, 15 mg/kg dose for children between 6- 10 years, and 10 mg/kg efavirenz dose for children between 10- 15 years in order to optimize efavirenz C_{\min} concentrations [Hirt *et al.* 2009]. A study by Wintergerst *et al.* reported higher plasma efavirenz concentrations (median 2.8 mg/L; 8.8% < 1 mg/L) in 33 HIV-infected children (two thirds of whom were sampled 8 to 20 hours after the dose) than those observed in this study (median MDI concentration 1.4 mg/L; 33% < 1 mg/L) amongst slightly younger children (median 6.75 years range 1.42, 14.75 vs. 8.2 years range 2.1, 16.7) [Wintergerst *et al.* 2008]. A recent study in an older Thai paediatric population (median age 12.3 years range 3.1, 18.7) showed that efavirenz plasma concentrations were adequate in majority of Thai children (only 13% had efavirenz concentration < 1 mg/L) sampled at 14.8 ± 0.8 hours after the last dose

[Puthanakit *et al.* 2009]. Thus differences in the sampling strategies, study populations and age appear to contribute to the differences in the findings between studies.

It is important to conduct pharmacokinetic studies in the relevant paediatric populations. Biochemical and physiological changes induced by the disease states may alter the pharmacokinetics of efavirenz in patients with TB/HIV co-infection. Furthermore, there are important age-related factors affecting pharmacokinetics [King *et al.* 2002; Saitoh *et al.* 2007]. As the true C_{\min} occurs in the evening, a simple method was introduced to estimate efavirenz C_{\min} in the out-patient clinics. The elimination rate constant was derived from a relatively narrow sampling range, compared with the long elimination half-life of efavirenz. However, the last sampling time for all 30 children was very close to 24 hours (median 18.7 hour, range 16.3, 20.8).

The estimation method of efavirenz C_{\min} was more accuracy to predict 24-hour true C_{\min} than the single MDI concentrations, as 3 concentration points allowed to observe a decline in efavirenz concentrations during elimination phase. The current recommended minimum therapeutic concentration of efavirenz 24-hour C_{\min} was based on the results from studies using single MDI concentrations, which showed a better specificity for detecting sub-therapeutic efavirenz trough concentrations in this study. However, a larger samples are required to definitely validate the method.

In conclusion, trough and MDI concentrations of efavirenz in children appeared not to be substantially different during *vs.* after rifampicin-based antitubercular treatment or during *vs.* control group without antitubercular treatment. Larger studies are needed to confidently define the effect of antitubercular treatment on efavirenz concentrations. A substantial proportion of South African children with and without concomitant antitubercular treatment have sub-therapeutic efavirenz concentrations despite being correctly dosed according to the manufacturer. The recommended efavirenz doses should therefore be re-evaluated, and TDM should be considered in children who are receiving efavirenz-based cART.

Chapter 4

Effect of Rifampicin on Lopinavir and Ritonavir Pharmacokinetics in HIV-infected Children with Tuberculosis

4.1 Introduction

Rifampicin is a strong inducer of cytochrome P450 enzymes, notably CYP3A isoenzymes, and of P-glycoprotein. Lopinavir, a PI, is primarily metabolized by CYP3A isoenzymes and is also a substrate of P-glycoprotein [Lee *et al.* 1998; Vishnuvardhan *et al.* 2003]. Ritonavir is a potent inhibitor of CYP3A and P-glycoprotein [Koudriakova *et al.* 1998; Kumar *et al.* 1999; Eagling *et al.* 1997; Lee *et al.* 1998; Profit *et al.* 1999; Washington *et al.* 1998; Gutmann *et al.* 1999]. For this reason, ritonavir is co-formulated with lopinavir in a lopinavir: ritonavir ratio of 4:1 as Kaletra[®] (LPV/r) to maintain high plasma concentration of lopinavir throughout the dosing interval. Co-administration with rifampicin resulted in a 90 to 99% reduction in trough (C_{\min}) lopinavir concentrations in two studies in healthy adult volunteers [Bertz *et al.* 2000]. Therefore, co-administration of rifampicin and LPV/r is not recommended.

A study in healthy adult volunteers [la Porte *et al.* 2004] showed that adjusted dose regimens of LPV/r with concomitant rifampicin resulted in acceptable peak and trough lopinavir concentrations. Trough concentrations were best preserved by adding ritonavir to LPV/r to give a lopinavir: ritonavir ratio of 1:1.

LPV/r based cART is used in South African children who are aged between 6 months to 3 years, have failed NNRTI-based cART or been exposed to NNRTIs for the prevention

of MTCT. The objective of this study was to evaluate whether the approach of adjusted dose regimen of LPV/r (adding extra ritonavir to standard dose of LPV/r; lopinavir: ritonavir ratio 1:1) can be used to overcome the reduction effect of rifampicin-based antitubercular treatment on lopinavir concentrations in young children.

4.2 Methods

4.2.1 Patients

A total of 30 children (aged from 6 months to 15 years) were enrolled: 15 TB/HIV co-infected children receiving LPV/r with additional ritonavir (lopinavir: ritonavir ratio 1:1) as part of cART and concomitant rifampicin-based antitubercular treatment, and 15 HIV-infected children without tuberculosis receiving LPV/r based cART. Lopinavir 230 mg/m²/dose + ritonavir 57.5 mg/m²/dose (lopinavir: ritonavir ratio 4:1, Kaletra[®] liquid formulation) was given twice a day in combination with twice daily dual NRTIs to children in the control group, additional ritonavir 172.5 mg/m²/dose (lopinavir: ritonavir ratio 1:1) was given to children receiving antitubercular treatment. Doses calculated according to body surface area were rounded up to the nearest 0.1 mL. Children were recruited at three sites: the HIV Clinic at Red Cross Children's Hospital, Cape Town; the Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto; and Brooklyn Chest Hospital, Cape Town. Institutional approval of the study was granted by the research ethics committees of the University of Cape Town, Stellenbosch University and the University of the Witwatersrand.

Exclusion criteria were: renal, hepatic, or intestinal disease (including malabsorption or diarrhoea); active opportunistic infections; recent exposure to drugs described to have PK interactions with lopinavir; or reported missed doses of LPV/r in the preceding 3 days. They were accompanied by a parent or legal guardian who provided written informed consent to participate in the study. Rifampicin-based antitubercular treatment regimens were administered in accordance with the National Tuberculosis Treatment Programme.

4.2.2 Study Design

Co-infected children were on treatment for TB and HIV for at least 4 weeks before PK evaluation. Lopinavir and ritonavir concentrations were evaluated a second time in this group of children at least 4 weeks after completing antitubercular treatment and receiving standard doses of LPV/r. The children without TB were established on cART for at least 4 weeks before lopinavir and ritonavir concentrations were measured. The exact time of the morning dose of lopinavir-ritonavir was recorded, and 8 blood samples were collected at 0 (pre-dose sample), 2, 3, 4, 5, 6, 8 and 12 hours after drug intake.

4.2.3 Data Analysis

Plasma concentrations of lopinavir and ritonavir were determined by LC/MS/MS. The detailed methods were described in the Materials and Methods Chapter (Section 2.1). WinNonlin version 4.1 (Pharsight Corp., Mountain View, CA) was used to characterize the PK parameters of lopinavir from concentration-time curves by non-compartmental analysis. The maximum concentration (C_{max}) and the time to C_{max} (T_{max}) were determined directly from the concentration-time data. Lopinavir C_{min} was the concentration measured at the 12-hour time point. Area under the curve (AUC_{0-12}) was calculated at the steady state with a dosing interval of 12 hours ($Tau = 12$ hours). The terminal half-life was determined by linear regression of the elimination phase from the plasma concentration-time curve.

4.2.4 Statistical Analysis

Wilcoxon RS test and Wilcoxon matched-pairs SR test were used for independent groups and paired observations, respectively. A probability of ≤ 0.05 was considered statistically significant for all tests. Stata version 8.2 (Stata Corp., College Station, TX) was used for the analyses.

4.3 Results

4.3.1 The Effect of Rifampicin on Lopinavir PK Measures

The children's general characteristics are summarized in Table 4.1. The co-administered NRTIs were stavudine plus lamivudine in 22 (73%) and lamivudine plus zidovudine in 8 (27%) children. The median volume of LPV/r administered to these children was 1.8 mL,

ranging from 1.0 to 2.1 mL. The maximum total volume of LPV/r with additional ritonavir administered was 3.5 mL when receiving concomitant antitubercular treatment. Drug administration was observed by study nurses after pre-dose sampling, and no vomiting incident has been recorded shortly after drug intake.

Full adherence to their antiretroviral therapy during the 3 days prior to PK evaluation was reported for all 30 participants. The median duration of antiretroviral treatment was 20 weeks, ranging from 4-173 weeks. Twenty-eight children had viral load information up to 6 months after initiation of antiretroviral treatment. Twenty of 28 children (71%) had viral loads under the detectable limit of the assay (<50 copies/mL) after at least 6 months of antiretroviral therapy. Of the remaining 8 children, 4 from each group had viral load \log_{10} values ranging from 2.54 to 4.72. Ten of 15 TB/HIV co-infected children had repeated PK sampling after antitubercular treatment. Two children during antitubercular treatment, two children after antitubercular treatment and 1 child without tuberculosis had slightly higher than normal (5 to 30 units/L) ALT concentrations (35 and 40, 35 and 43, and 42 units/L in the 2 groups respectively). However, they were all below 1.5 times of upper normal limit (UNL) of ALT normal range. The ALT concentrations after antitubercular treatment were not available for the 2 children with elevated ALT concentrations during antitubercular treatment (technique problems occurred on 1 sample, and the other child was lost to follow-up). The 2 children, whose ALT concentrations were abnormal after antitubercular treatment, had ALT concentrations within the normal range during antitubercular treatment. Five of the TB/HIV co-infected children (33%) were established on cART before they were treated for TB. None of the five developed elevated ALT concentrations during or after antitubercular treatment.

The lopinavir PK measures are shown in Table 4.1, and the concentration-time curves are shown in Figure 4.1. Large inter-patient variability was observed. No significant differences were found between any of the PK measures during vs. after rifampicin-based antitubercular treatment in the 10 children who were sampled during and after antitubercular treatment, although C_{\max} tended to lower during antitubercular treatment (median 10.50 mg/L IQR 7.06, 14.30 vs. 14.44 mg/L IQR 9.82, 16.76; SR $p=0.075$). The

actual time at the 12-hour-sampling point was significantly earlier in TB/HIV co-infected children after antitubercular treatment than in those during treatment (median 11.50 hour IQR 10.38, 12.00 vs. 11.58 hour IQR 11.17, 12.00; SR $p=0.048$), however, no significant difference was found between lopinavir C_{\min} during and after antitubercular treatment. Significant reductions in lopinavir C_{\max} (median 10.50 mg/L IQR 7.06, 14.30 vs. 14.20 mg/L IQR 11.90, 23.50; RS $p=0.018$) and in AUC_{0-12} (median 80.86 mg·h/L IQR 50.87, 121.74 vs. 117.83 mg·h/L IQR 80.41, 176.08; RS $p=0.036$), but not in lopinavir C_{\min} (median 3.94 mg/L IQR 2.26, 7.66 vs. 4.64 mg/L IQR 2.32, 10.40; RS $p=0.468$), were observed in children during antitubercular treatment compared with children in the control group receiving standard dose of LPV/r without concomitant rifampicin. Statistical significant differences were not found in any of the lopinavir PK parameters between TB/HIV co-infected children after antitubercular treatment and children in the control group without TB.

The median BSA was increased significantly after antitubercular treatment ($p=0.005$), but lopinavir was dosed accordingly, resulting in no difference in lopinavir dose/BSA ($p=0.508$). Both groups had lopinavir doses slightly higher than the recommended dosage (230 mg/m²). All children but two had lopinavir C_{\min} above 1 mg/L. The two children with low lopinavir C_{\min} were receiving additional ritonavir with rifampicin-based antitubercular treatment, while their pre-dose sample concentrations were 2.17 and 4.29 mg/L, the half-life of lopinavir in these children were substantially shorter (0.97 and 1.75 hours) than that observed in any of the other children. One of these 2 children had a low dose of additional ritonavir due to a dosing error (49.5 vs. 172.5 mg/m²) when receiving concomitant rifampicin. Two children from the control group not receiving rifampicin had lopinavir pre-dose concentration below 1 mg/L, but normal C_{\min} concentrations. They were suspected to have missing doses prior to PK sampling. The results from Wilcoxon RS and SR tests remained unaffected after these 3 children were excluded from the analysis. All children after rifampicin-based antitubercular treatment had lopinavir C_{\min} and pre-dose concentration above 1 mg/L, including the two children who had lopinavir $C_{\min} < 1$ mg/L during antitubercular treatment. The half-life of lopinavir in these children was increased after antitubercular treatment (4.08 vs. 0.97 and 3.53 vs. 1.75 hours).

Table 4.1 General characteristics and lopinavir PK measures of children in TB/HIV co-infected group during and after antitubercular treatment and in control group. Categorical variables are expressed as n (%); continuous variables are summarized using median (IQR). (RIF=rifampicin; LPV= lopinavir)

Characteristics	During TB treatment (RIF and LPV/r = 1:1)	After TB treatment (LPV/r= 4:1)	<i>p</i> * value	Control (LPV/r = 4:1)	<i>p</i> ** value
n	15	10	-	15	-
Female	8 (53%)	7 (70%)	0.405	10 (67%)	0.456
Median Age [months]	16 (14, 24)	25 (19, 31)	0.005	29 (22, 34)	0.004
Median Weight [kg]	8.6 (7.8, 9.9)	11.0 (10.0, 12.6)	0.006	11.6 (10.5, 13.4)	0.002
Median Height [cm]	77.0 (71.4, 79.0)	80.3 (79.0, 83.2)	0.006	82.5 (79, 87.9)	0.002
Median BSA ¹ [m ²]	0.42 (0.41, 0.46)	0.49 (0.47, 0.53)	0.005	0.51 (0.48, 0.55)	0.001
Median LPV dose/BSA [mg/ m ²]	291.9 (274.3, 308.6)	289.1 (285.8, 302.7)	0.508	265.2 (248.8, 289.3)	0.034
Median z-score Weight for age	-1.97 (-2.93, -0.98)	-0.97 (-1.51, -0.4)	0.139	-1.00 (-1.85, -0.48)	0.085
Lopinavir PK measures					
Median T _{max} ² (hour)	3.00 (2.00, 4.07)	3.54 (2.97, 5.00)	0.221	3.92 (2.78, 4.00)	0.467
Median C _{max} ³ (mg/L)	10.50 (7.06, 14.30)	14.44 (9.82, 16.76)	0.075	14.20 (11.90, 23.50)	0.018
Median C _{min} ⁴ (mg/L)	3.94 (2.26, 7.66)	8.52 (2.94, 10.78)	0.139	4.64 (2.32, 10.40)	0.468
Median AUC ₀₋₁₂ ⁵ (mg-h/L)	80.86 (50.87, 121.74)	114.95 (76.60, 160.54)	0.114	117.83 (80.41, 176.08)	0.036
Median C _{pre-dose} ⁶ (mg/L)	5.20 (4.18, 9.30)	7.86 (5.54, 14.54)	0.386	8.12 (6.34, 13.00)	0.310
Median Half life (hour)	7.58 (4.01, 16.61)	10.54 (4.08, 15.62)	0.575	4.86 (3.82, 8.29)	0.254

1. BSA- body surface area
2. T_{max}- time to maximum concentration
3. C_{max}- maximum concentration

4. C_{\min} - concentration at 12-hour point (the evening trough)

5. AUC_{0-12} - area under the curve to 12 hours

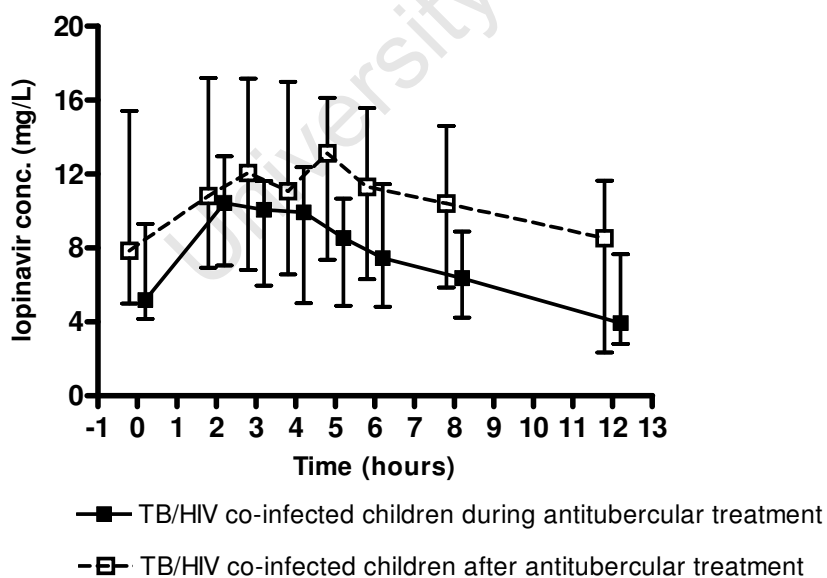
6. $C_{\text{pre-dose}}$ - Pre-dose concentration (the morning trough)

* p values were obtained from comparison of 10 TB/HIV co-infected children during and after antitubercular treatment (SR test).

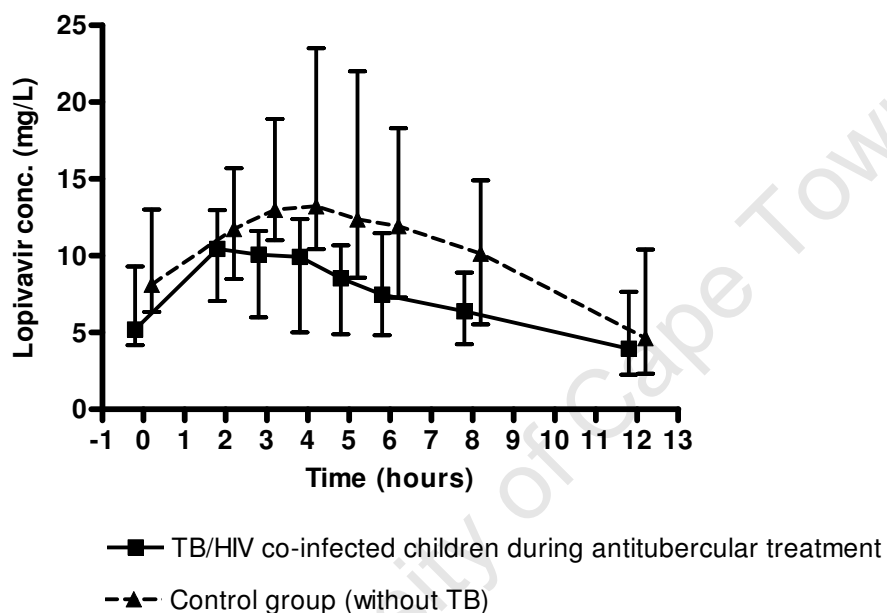
** p values were obtained from comparison made between TB/HIV co-infected children during antitubercular treatment and the control group (RS test).

Figure 4.1 Lopinavir concentration vs. time curves

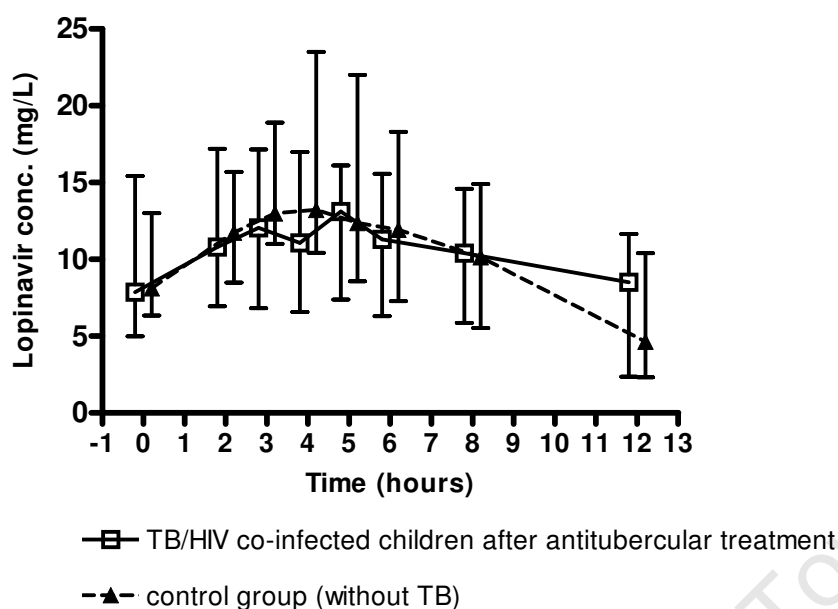
a) Lopinavir concentration vs. time curves during and after antitubercular treatment. The concentration-time curve for TB/HIV co-infected children receiving LPV/r with additional ritonavir (lopinavir: ritonavir ratio 1:1) and concomitant rifampicin is shown by the solid line; the curve for children in the same group after rifampicin-based antitubercular treatment, receiving standard doses of LPV/r (lopinavir: ritonavir ratio 4:1) is shown by the dashed line. The time points of the two occasions have been separated for clarity. Median concentrations and interquartile ranges are indicated at each time point.



b) Lopinavir concentration vs. time curves during antitubercular treatment and for the control group. The concentration-time curve for TB/HIV co-infected children receiving LPV/r with additional ritonavir (lopinavir: ritonavir ratio 1:1) and concomitant rifampicin is shown by the solid line; the curve for children in control group without tuberculosis is shown by the dashed line. The time points of the two groups have been separated for clarity. Median concentrations and interquartile ranges are indicated at each time point.



c) Lopinavir concentration vs. time curves after antitubercular treatment and for the control group. The concentration-time curve for TB/HIV co-infected children after antitubercular treatment is shown by the solid line; the curve for children in control group without tuberculosis is shown by the dashed line. Children in both groups were receiving standard doses of LPV/r (lopinavir: ritonavir ratio 4:1). The time points of the two groups have been separated for clarity. Median concentrations and interquartile ranges are indicated at each time point.



4.3.2 Evaluation of Ritonavir Plasma Concentrations and PK Measures

Ritonavir plasma concentrations were analyzed simultaneously with lopinavir concentrations. The median ritonavir plasma concentration was 0.42 mg/L (IQR 0.20, 0.88). As a result of the increased doses of ritonavir (lopinavir: ritonavir ratio 1:1) in TB/HIV co-infected children during antitubercular treatment, all ritonavir PK parameters except the half-life and T_{max} were significantly higher when compared to those after antitubercular treatment and to those in control group (Table 4.2; Figure 4.2). The median ratio of adjusted lopinavir: ritonavir during antitubercular treatment was 1.00 (IQR 0.95, 1.00). One child had a low dose of additional ritonavir due to a dosing error (49.5 vs. 172.5 mg/m²) (Section 4.3.1), resulting in lower ritonavir plasma concentrations of this child (median 0.40 mg/L IQR 0.18, 0.77 and 1.11 mg/L IQR 0.42, 2.39, respectively; $p=0.015$). The statistical differences between groups remained unaffected after this child together with 2 children from the control group, who were suspected to have missing doses prior to PK sampling (Section 4.3.1) were excluded from analysis. After resuming standard doses of LPV/r, no statistically significant differences were found in any of the ritonavir PK parameters between TB/HIV co-infected children after antitubercular treatment and children in the control group without TB.

Ritonavir C_{\min} , AUC_{0-12} and half-life were correlated with lopinavir C_{\min} , AUC_{0-12} and half-life (spearman's $\rho = 0.679$, $p < 0.001$; spearman's $\rho = 0.461$, $p = 0.003$; and spearman's $\rho = 0.539$, $p < 0.001$ for C_{\min} , AUC_{0-12} and half-life, respectively).

Table 4.2 Ritonavir PK measures for children in the TB/HIV co-infected group during and after antitubercular treatment and in the control group. Continuous variables are summarized using median (IQR). (RIF= rifampicin; RTV= ritonavir)

Ritonavir PK measures	During TB treatment (RIF and LPV/r = 1:1)	After TB treatment (LPV/r= 4:1)	<i>p</i> value*	Control (LPV/r = 4:1)	<i>p</i> value**
n	15	10	-	15	-
Median RTV dose/BSA [mg/ m ²]	301.3 (285.6, 308.6)	72.3 (71.5, 75.7)	0.005	66.3 (62.2, 72.3)	<0.001
Median T_{\max}^1 (hour)	2.05 (2.00, 3.00)	3.54 (2.02, 5.00)	0.059	3.92 (2.75, 4.00)	0.026
Median C_{\max}^2 (mg/L)	2.32 (1.56, 4.99)	0.54 (0.39, 0.87)	0.009	0.61 (0.33, 0.93)	0.001
Median C_{\min}^3 (mg/L)	0.22 (0.11, 0.85)	0.15 (0.06, 0.22)	0.037	0.11 (0.05, 0.17)	0.027
Median AUC_{0-12}^4 (mg·h/L)	9.45 (5.17, 27.03)	4.04 (2.53, 5.18)	0.009	3.97 (1.84, 5.36)	0.003
Median $C_{\text{pre-dose}}^5$ (mg/L)	0.68 (0.19, 2.33)	0.26 (0.15, 0.40)	0.005	0.19 (0.09, 0.28)	0.011
Median Half life (hour)	3.13 (2.31, 4.50)	3.53 (3.27, 4.26)	0.441	3.72 (2.85, 4.54)	0.178

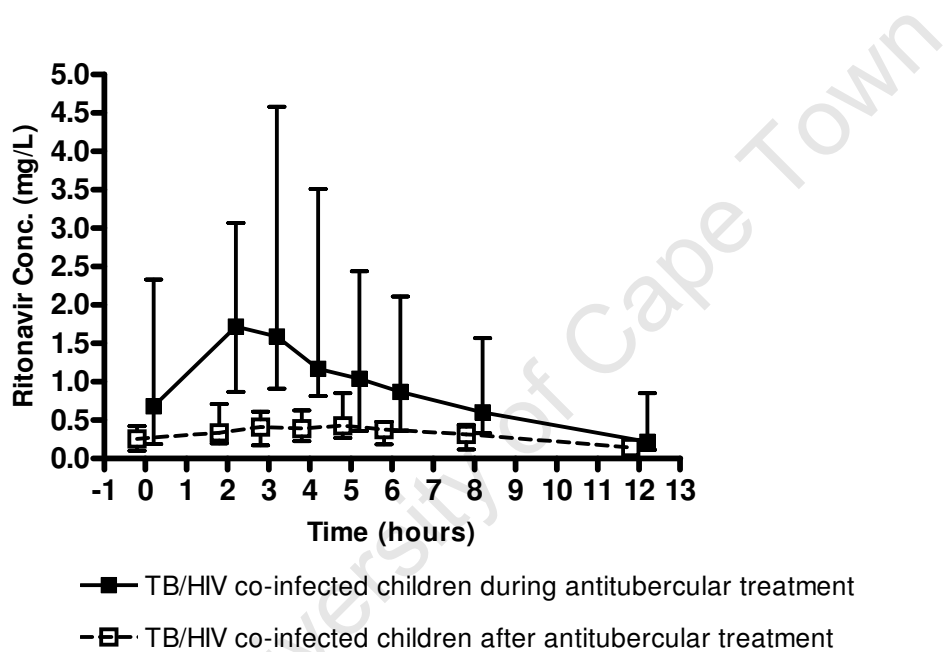
1. T_{\max} - time to maximum concentration
2. C_{\max} - maximum concentration
3. C_{\min} - concentration at 12-hour point (the evening trough)
4. AUC_{0-12} - area under the curve to 12 hours
5. $C_{\text{pre-dose}}$ - Pre-dose concentration (the morning trough)

* *p* values were obtained from comparison of 10 TB/HIV co-infected children during and after antitubercular treatment (SR test).

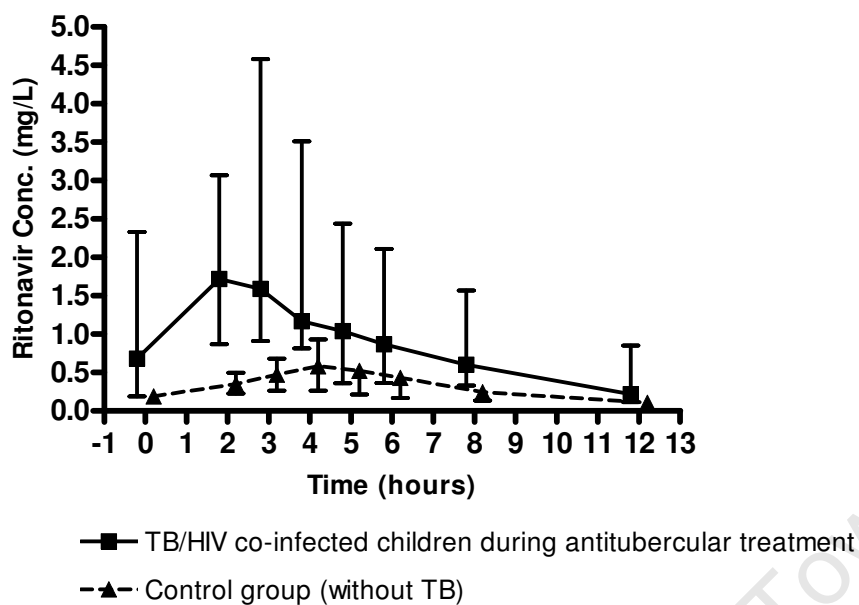
** *p* values were obtained from comparison made between TB/HIV co-infected children during antitubercular treatment and the control group (RS test).

Figure 4.2 Ritonavir concentration vs. time curves

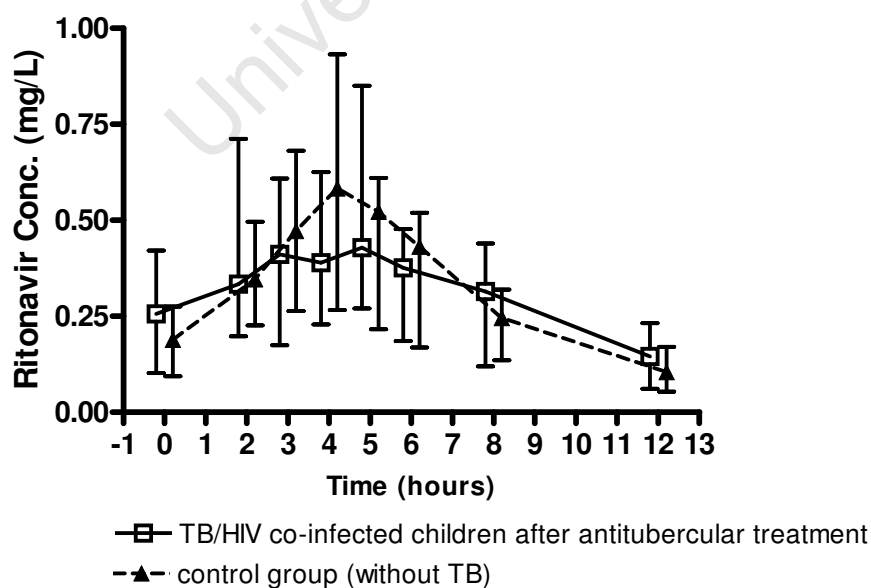
a) Ritonavir concentration vs. time curves during and after antitubercular treatment. The concentration-time curve for TB/HIV co-infected children receiving LPV/r with additional ritonavir (lopinavir: ritonavir ratio 1:1) and concomitant rifampicin is shown by the solid line; the curve for children in the same group after rifampicin-based antitubercular treatment, receiving standard doses of LPV/r (lopinavir: ritonavir ratio 4:1) is shown by the dashed line. The time points of the two occasions have been separated for clarity. Median concentrations and interquartile ranges are indicated at each time point.



b) Ritonavir concentration vs. time curves during antitubercular treatment and for the control group. The concentration-time curve for TB/HIV co-infected children receiving LPV/r with additional ritonavir (lopinavir: ritonavir ratio 1:1) and concomitant rifampicin is shown by the solid line; the curve for children in control group without tuberculosis is shown by the dashed line. The time points of the two groups have been separated for clarity. Median concentrations and interquartile ranges are indicated at each time point.



c) Ritonavir concentration vs. time curve after antitubercular treatment and for the control group. The concentration-time curve for TB/HIV co-infected children after antitubercular treatment is shown by the solid line; the curve for children in control group without tuberculosis is shown by the dashed line. Children in both groups were receiving standard doses of LPV/r (lopinavir: ritonavir ratio 4:1). The time points of the two groups have been separated for clarity. Median concentrations and interquartile ranges are indicated at each time point.



4.4 Discussion

To our knowledge, this is the first paediatric study to evaluate lopinavir and ritonavir pharmacokinetics and the drug-drug interaction between an adjusted dose regimen of LPV/r and rifampicin in a TB/HIV co-infected population. Although the median lopinavir C_{\max} , C_{\min} and AUC_{0-12} were reduced by 27%, 54% and 30%, respectively, in children receiving additional ritonavir during antitubercular treatment compared to those in the same children on standard doses of LPV/r after antitubercular treatment, the differences failed to reach statistical significance. The within group comparison allowed us to compare paired observations which would have a lower variability in PI concentrations than the inter-individual variability between groups [Guiard-Schmid *et al.* 2003]. Unfortunately, the power of the within group comparison was restricted by the high drop-out rate (33%). Significant reductions in the median lopinavir C_{\max} and AUC_{0-12} were found in children receiving additional ritonavir with rifampicin when compared to the control group, despite higher lopinavir doses in the children receiving antitubercular treatment. Previous studies have shown that the reduced lopinavir exposure was associated with the younger age [Verweel *et al.* 2007; Chadwick *et al.* 2008; Chadwick *et al.* 2009]. In this study, the younger age of children in TB/HIV co-infected group than those in the control group and other differences between the 2 groups (for example, co-infection with TB and disease states) may have impact on the statistical differences. However, the key parameter of lopinavir PK, C_{\min} , was not significantly different in the between group comparison.

Lopinavir presented kinetics that are consistent with circadian rhythm. Although lopinavir C_{\min} (the evening trough) was 34% lower than the pre-dose concentration (the morning trough), 38 of total 40 (95%) lopinavir C_{\min} concentrations were above the recommended minimum therapeutic concentration (1 mg/L) [la Porte *et al.* 2006; www.hivpharmacology.com]. In contrast with a previous report [Verweel *et al.* 2007], a correlation was not found between lopinavir plasma concentrations and age (Spearman's $\rho = -0.194$, $p = 0.353$; $n = 25$, children after antitubercular treatment plus control group). However, children in this setting received higher doses of LPV/r than children in their

study (median 286.4 mg/m² range 238.8, 339.0 vs. median 227 mg/m² range 189, 254), and children in this study was younger (median age 2.3 years range 0.8, 3.9 vs. median 5.6 years range 0.4, 13.2).

A safety and efficacy paediatric study [Sáez-Llorens *et al.* 2003] demonstrated that LPV/r (lopinavir: ritonavir ratio 4:1) at a higher dose of 300/75 mg/m² twice daily is well-tolerated in children. Only 1 in 100 children were discontinued due to study drug-related adverse events. In this study, slightly elevated ALT levels were observed in the same number of children during and after antitubercular treatment. None of the children had their treatment interrupted due to elevations of ALT. In contrast, 38% of adult healthy normal volunteers were withdrawn as a result of adverse events, and 28% had raised liver enzyme concentrations during treatment with adjusted doses of lopinavir and ritonavir together with rifampicin [la Porte *et al.* 2004]. It was found that the combination of additional ritonavir (lopinavir: ritonavir ratio 1:1) with rifampicin was generally well tolerated in children. However, further investigation regarding toxicity and efficacy of LPV/r with extra ritonavir and rifampicin-based antitubercular treatment needs to be carried out.

Rifabutin can be used in antitubercular treatment instead of rifampicin in patients on LPV/r-based HAART, as rifabutin does not affect lopinavir concentrations [Centers for Disease Control, 2004]. This approach is not a feasible option for most tuberculosis control programs in developing countries as rifabutin is currently prohibitively expensive. There is no suitable rifabutin formulation for pediatric practice, which precludes the use of rifabutin in very young children. Furthermore, in high burden countries tuberculosis control programs rely on standard treatment regimens in fixed dose combinations administered by nurses.

For ritonavir, a less than proportional increase in ritonavir plasma concentrations were achieved during antitubercular treatment with adjusted doses of LPV/r (lopinavir: ritonavir ratio 1:1) compared to those achieved with standard doses of LPV/r (lopinavir: ritonavir ratio 4:1) after antitubercular treatment. Whereas, la Porte *et al.* reported

considerably higher ritonavir concentrations during antitubercular treatment. There are important age-related factors affecting pharmacokinetics, of particular relevance to lopinavir and ritonavir is the activity of cytochrome P450 enzyme system [King *et al.* 2002], may explain the inconsistency in the findings between the 2 studies. Disease states could also alter the PK of ARV drugs in patients with TB/HIV co-infection, for example by altering the concentrations of the drug-transporting proteins. Therefore, it is important to conduct PK studies in the relevant paediatric diseased population rather than to extrapolate from adults, particularly from healthy volunteers.

A positive relationship was found between the half-life of ritonavir and lopinavir, indicating similar systematic elimination for ritonavir and lopinavir. This study also revealed the positive associations of C_{\min} and AUC_{0-12} between ritonavir and lopinavir. This is in keeping with the findings from previous adult study [Guiard-Schmid *et al.* 2003], suggesting high plasma concentration of ritonavir accentuates its enhancement effect on lopinavir.

The main limitations of this study are small sample size and the high drop-out rate in children after antitubercular treatment due to family relocating. Plasma concentrations of PIs display large inter-patient variability, as illustrated by the coefficient of variation. The inter-individual variability for lopinavir C_{\min} were 86.2% and 68.2% during and after antitubercular treatment in TB/HIV co-infected group and 111.4% for the control group. For ritonavir, the inter-individual variability of C_{\min} concentrations was 159.8% and 87.0% during and after antitubercular treatment and 108.3% for the control group. This is another factor which limits the power of this study. However it is difficult to recruit large numbers of paediatric patients for PK studies.

In conclusion, this study in children confirmed the findings of a previous adult healthy volunteer study [la Porte *et al.* 2004] that the reduction of lopinavir trough concentrations caused by rifampicin can be attenuated by adding extra ritonavir to LPV/r (lopinavir: ritonavir ratio 1:1). This adjusted dose regimen of LPV/r achieved adequate trough lopinavir concentrations in the majority of HIV-infected children when co-administered

with rifampicin-based antitubercular treatment. However, due to the large inter-patient variability observed, TDM should be considered for children receiving concomitant rifampicin for optimal dosing. Hepatic ALT levels should also be monitored regularly as hepatotoxicity is a concern when rifampicin-based antitubercular treatment is used with adjusted doses of LPV/r.

University of Cape Town

Chapter 5

The Use of Dried Blood Spots as an Alternative Method to Plasma for ARV Concentration Assays

5.1 Introduction

As the accessibility to cART in developing countries increases, TDM is recommended to optimize ARV efficacy and to reduce toxicity of the treatment for HIV-infection [Back *et al.* 2002; Gerber *et al.* 2003; Boffito *et al.* 2005; Rendón *et al.* 2005]. Serum and plasma are the common matrices for routine TDM of NNRTI and PI in patient samples. Conventional methods using plasma or serum samples require expensive sample collection, processing techniques, storage and transportation conditions which are difficult or unavailable in resource-limited settings. Furthermore, the volume of blood required should be minimized for studies in children.

DBS method has been used as an alternative to plasma for diagnosis of HIV-1 infection [Cassol *et al.* 1991; Cassol *et al.* 1992; Jacob *et al.* 2008], HIV-1 antibody testing [Solomon *et al.* 2002], HIV-1 drug resistance genotyping [Youngpairoj *et al.* 2008], CD4+ lymphocyte counts [Mwaba *et al.* 2003], and viral load measurement [Mwaba *et al.* 2003; Brambilla *et al.* 2003]. Koal *et al.* [Koal *et al.* 2005] described a robust LC/MS/MS method for multicomponent analysis of PI and NNRTI drugs in DBS and showed concentrations correlated well between DBS and plasma. However, there is no data to support the use of a DBS method in paediatric PK study and in TDM practice. In this chapter, the DBS method was applied in a paediatric patient population and correlations

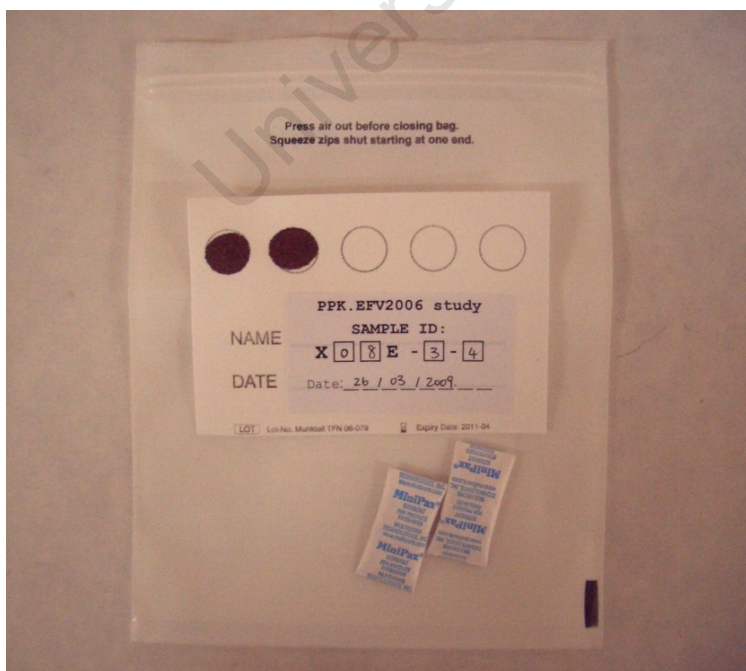
between DBS and plasma concentrations were evaluated for efavirenz, lopinavir and ritonavir. The relatively large number of samples evaluated allowed good definition of variability in the findings between samples. Storage conditions, patient characteristics and treatment factors which may affect the stability and DBS/plasma correlations of efavirenz, lopinavir and ritonavir (key components of cART in South African children) were also evaluated.

5.2 Methods

5.2.1 DBS Sample Preparation and Assay

DBS samples were obtained from efavirenz and lopinavir-ritonavir studies during plasma sampling. Exactly 50 μL aliquots from heparinized whole blood samples were spotted within 30 minutes after sampling onto 903 TFN filter paper cards with pre-marked circles (Munktell, Niederschlag, Germany) (Figure 5.1). The cards were dried at room temperature for 2 hours and stored in sealed plastic bags with desiccant at 4 °C. The concentrations of efavirenz, lopinavir and ritonavir in DBS samples were determined simultaneously by validated LC/MS/MS methods. The detailed procedures were described in the Materials and Methods Chapter (Section 2.2).

Figure 5.1 DBS samples dried on filter paper with plastic bag and desiccant.



5.2.2 Statistical Analysis

Stata version 10.0 (Stata Corp., College Station, TX) was used to compute summary statistics. Spearman rank correlation test was used to determine the correlation between two variables in nonparametric data. Wilcoxon matched-pairs SR test and Wilcoxon RS test were used to compare paired and unpaired observations for skewed data, respectively. One-way Analysis of Variance (ANOVA) followed by Bonferroni's correction was used to compare the mean DBS/plasma ratios at each time point for lopinavir and ritonavir.

Variables of interest were individually tested in the first instance for associations with DBS/plasma ratios by univariate analyses. Variables with p values < 0.2 were included in the multivariate analysis to determine the overall effects. The appropriate model of multivariate analysis was chosen based on the Wald Chi squared values.

A probability of ≤ 0.05 was considered statistically significant for all tests.

5.3 Results

5.3.1 Patient Samples

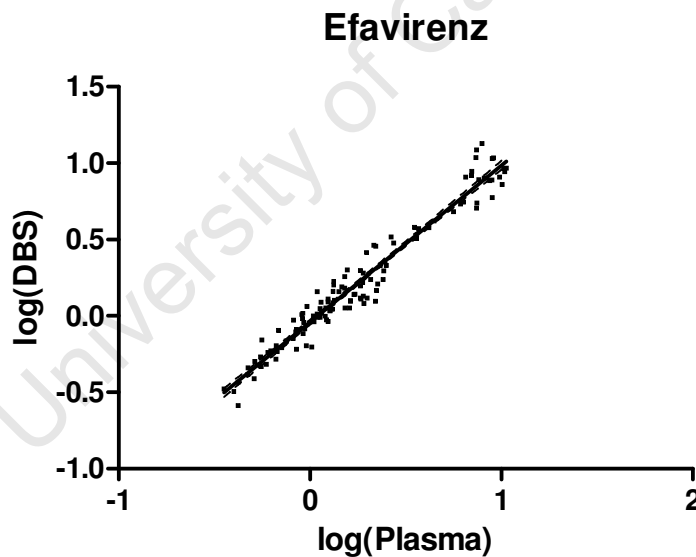
A total of 135 patient samples containing efavirenz as part of cART were analyzed, in which 45 samples contained rifampicin; 45 samples were from the same children after completion of rifampicin-based antitubercular treatment and 45 were from children without TB.

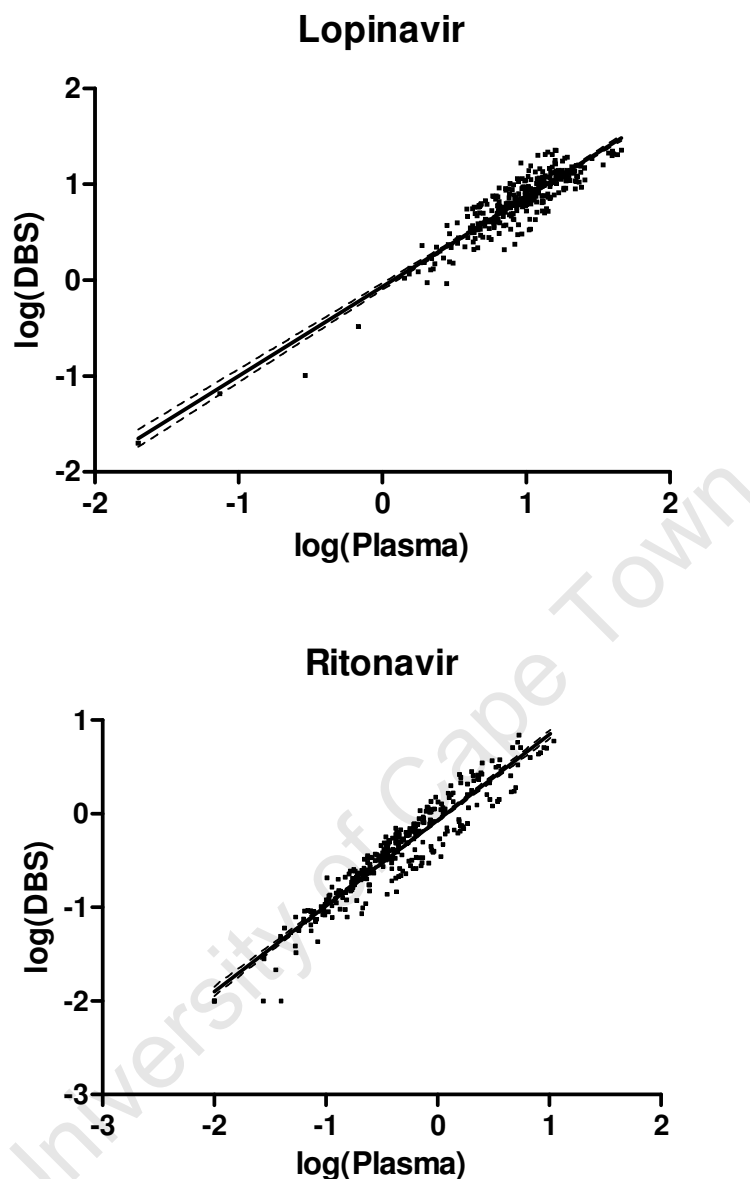
Of the 320 samples containing lopinavir and ritonavir that were analyzed, 120 samples were obtained from children receiving adjusted doses of LPV/r in a ratio of lopinavir:ritonavir= 1:1 with concomitant rifampicin, 80 samples were from the same children after completion of antitubercular treatment, receiving standard doses of LPV/r and 120 samples were from children without TB, receiving standard doses of LPV/r. Two samples had both plasma and DBS lopinavir and ritonavir concentrations below LLOQ. Another 3 samples had ritonavir DBS concentrations below LLOQ. Samples with concentrations below the LLOQ were treated as 40% of LLOQ concentrations.

5.3.2 Log/log Linear Correlation between DBS and Plasma

Figure 5.2 shows the log/log linear correlation (90% CI) between DBS and plasma concentrations. There were very strong linear correlations for all 3 drugs: efavirenz ($p < 0.001$, spearman's rho= 0.963; slope= 1.027); lopinavir ($p < 0.001$, spearman's rho= 0.860; slope= 0.931) and ritonavir ($p < 0.001$, spearman's rho= 0.932; slope= 0.914). Although the correlation was reduced during antitubercular treatment for lopinavir (spearman's rho= 0.778 vs. 0.863, n= 120 vs. 200), concomitant antitubercular treatment had little effect on the linear correlation of efavirenz and ritonavir (spearman's rho= 0.944 vs. 0.962, n= 45 vs. 90; spearman's rho= 0.918 vs. 0.908, n= 120 vs. 200).

Figure 5.2 The 10-based logarithms of DBS vs. plasma concentrations with linear correlation line (solid line) and 90% confidence interval (dashed line).





5.3.3 Distribution of ARV Drugs between Plasma and Whole Blood

The DBS/plasma drug concentration ratios are summarized in Table 5.1. The DBS/plasma ratios with and without concomitant antitubercular treatment were compared within the same group of children (during *vs.* after antitubercular treatment; SR test), and between children with and without TB (after antitubercular treatment *vs.* controls without TB; RS test). The DBS/Plasma ratios during and after antitubercular treatment were not significantly different except for ritonavir ($p=0.318$, $p=0.051$ and $p<0.001$ for efavirenz,

lopinavir and ritonavir, respectively). However, significant differences in DBS/plasma ratios were found when TB/HIV co-infected children after antitubercular treatment were compared with children in control group without TB ($p= 0.004$, $p< 0.001$ and $p< 0.001$, for efavirenz, lopinavir and ritonavir, respectively).

Univariate and multivariate analyses were used to identify factors, including ALB, HB, patient group differences (TB/HIV co-infected group and the control group), and the presence of concomitant rifampicin-based antitubercular treatment, which might affect the DBS/plasma ratios. In univariate analyses, efavirenz and lopinavir DBS/plasma ratios were not affected by ALB levels, whereas HB had no effect on ritonavir ratios ($p> 0.2$). Multivariate analyses showed that HB was negatively correlated with efavirenz DBS/plasma ratios (coefficient= -0.101 ; 95% CI $-0.138, -0.065$; $p< 0.0001$). Patient group differences rather than presence of rifampicin-containing antitubercular treatment also had significant effect on efavirenz ratios: co-infection with TB was associated with a 14.3% increase in the efavirenz ratio (coefficient= 0.143 ; 95% CI $0.046, 0.240$; $p= 0.004$). Whereas, presence of rifampicin was associated with 12.0% and 32.3% reductions in lopinavir and ritonavir DBS/plasma ratios, respectively (for lopinavir coefficient= -0.120 ; 95% CI $-0.181, -0.059$; $p< 0.0001$ and for ritonavir coefficient= -0.323 ; 95% CI $-0.410, -0.237$; $p< 0.0001$). HB and ALB levels did not show significant associations with DBS/plasma concentration ratios in multivariate analyses for lopinavir and ritonavir, respectively. Effects of each co-variant on efavirenz, lopinavir and ritonavir DBS/plasma concentration ratios evaluated by multivariate analyses were summarized in Table 5.2.

Table 5.1 Median (IQR) DBS/plasma ratios for efavirenz, lopinavir and ritonavir.

		Efavirenz	Lopinavir	Ritonavir
TB/HIV During TB treatment	N	45	120	120
	Median ratio (IQR)	0.98 (0.87, 1.17)	0.79 (0.61, 1.03)	1.07 (0.55, 1.28)
TB/HIV After TB treatment	N	45	80	80
	Median ratio (IQR)	0.97 (0.86, 1.13)	0.79 (0.70, 1.07)	1.16 (1.03, 1.31)
HIV control Without TB treatment	N	45	120	120
	Median ratio (IQR)	0.85 (0.73, 0.93)	0.67 (0.53, 0.82)	0.94 (0.61, 1.08)
All Subjects	N	135	320	320
	Median ratio (IQR)	0.93 (0.83, 1.08)	0.73 (0.61, 0.90)	1.05 (0.74, 1.21)

Table 5.2 The effects of each co-variant on DBS/plasma concentration ratios of efavirenz, lopinavir and ritonavir are summarized using coefficients (95% CI). Wald Chi squared (Wald Chi²) values of the final multivariate models are also indicated.

Factors	Efavirenz	Lopinavir	Ritonavir
HB	-0.101 (-0.138, -0.065) <i>p</i> < 0.0001	-0.046 (-0.098, 0.006) <i>p</i> = 0.086	-*
ALB	-*	-*	-0.007 (-0.026, 0.012) <i>p</i> = 0.443
Rifampicin	-0.048 (-0.110, 0.015) <i>p</i> = 0.137	-0.120 (-0.181, -0.059) <i>p</i> < 0.0001	-0.323 (-0.410, -0.237) <i>p</i> < 0.0001
Group (TB co-infection)	0.143 (0.046, 0.240) <i>p</i> = 0.004	0.133 (-0.011, 0.277) <i>p</i> = 0.071	0.159 (-0.104, 0.422) <i>p</i> = 0.236
Wald Chi² value	42.53	25.85	67.81

* indicates co-variant which had *p* > 0.2 in univariate analyses and excluded from multivariate analysis models

5.3.4 Comparison of PK Measures Between Plasma and DBS

Efavirenz DBS and plasma MDI concentrations were very similar (median 1.27 IQR 0.82, 2.61 vs. 1.34 IQR 0.90, 2.49; *p* = 0.708, respectively). Efavirenz estimated C_{\min} concentrations were also closely similar between DBS and plasma samples (median 1.01 IQR 0.59, 2.31 vs. 0.86 IQR 0.60, 1.78; *p* = 0.997, respectively). Elimination rate constant

(k) values of the log-linear regression lines were comparable between plasma and DBS methods ($k = -0.11$ and $k = -0.12$, respectively) (Figure 5.3 a I). PK parameters of lopinavir and ritonavir were compared between plasma and DBS samples in Table 5.3. In keeping with the finding that the median lopinavir concentration was 27% lower in DBS than in plasma samples (Table 5.1), lopinavir C_{\max} and AUC_{0-12} were reduced by 29.84% ($p = 0.008$) and 24.88% ($p = 0.048$) in DBS, respectively. Lopinavir C_{\min} and pre-dose concentrations were 16.60% and 16.10% lower in DBS samples, however, due to wide variability, statistically significant differences were not described. Ritonavir PK measures were comparable between plasma and DBS (Table 5.3; Figure 5.3 b I and c I).

DBS/plasma ratios for these 3 drugs were not affected by the time interval after dose. The plot of DBS/plasma ratio against sampling time after drug intake is shown in Figure 5.3 II. Best fit linear regression line was used to illustrate the trend of DBS/plasma ratio changes over elimination phase for efavirenz. The slope of the best fit line was -0.012 with insignificant deviation from zero ($p = 0.081$). Although there were no significant differences in median DBS/plasma ratio at each time point for lopinavir and ritonavir ($p = 0.989$ and $p = 0.992$, respectively; one-way ANOVA), median lopinavir DBS/plasma concentration ratios tended to be lower at 3- 5 hours than those at pre-dose and 12 hours after dose intake.

Efavirenz had the lowest inter- and intra-individual variability in DBS/plasma concentration ratios amongst these 3 drugs. The inter- and intra-individual variability for efavirenz, lopinavir and ritonavir were 21% and 8.6%, 34% and 10.8%, and 33% and 14.2%, respectively. The inter-individual variability of TB/HIV co-infected children during antitubercular treatment were 21.5, 36.3% and 41.0%, whereas, the variability were reduced in the same group of children after antitubercular treatment (17.8%, 31.8 and 16.7% for efavirenz, lopinavir and ritonavir, respectively). The variability of efavirenz, lopinavir and ritonavir for children in control group without TB were 20.0%, 24.5% and 31.2%, respectively.

Table 5.3 Comparison of lopinavir and ritonavir PK measures between plasma and DBS. Continuous variables are summarized using median (IQR). The differences of PK measures between DBS and plasma are expressed in absolute percentage values.

PK Measures (All Points)	Lopinavir				Ritonavir			
	Plasma	DBS	Difference (%)	P value	Plasma	DBS	Difference (%)	P Value
Median T_{max}^1 (hour)	3.31 (2.14, 4.08)	3.03 (2.15, 4.00)	8.46%	0.643	3.00 (2.04, 4.02)	3.00 (2.00, 4.00)	0.00%	0.732
Median C_{max}^2 (mg/L)	13.07 (9.29, 16.79)	9.17 (6.74, 13.25)	29.84%	0.008	0.87 (0.44, 1.61)	0.75 (0.40, 1.64)	13.79%	0.620
Median C_{min}^3 (mg/L)	4.70 (2.57, 9.53)	3.92 (1.96, 7.79)	16.60%	0.281	0.16 (0.07, 0.36)	0.17 (0.07, 0.30)	6.25%	0.904
Median AUC_{0-12}^4 (mg.hour/L)	99.85 (67.34, 155.42)	75.01 (51.75, 117.31)	24.88%	0.048	4.67 (2.65, 9.26)	4.84 (2.39, 9.14)	3.64%	0.817
Median $C_{pre-dose}^5$ (mg/L)	7.08 (4.43, 12.95)	5.94 (2.90, 9.19)	16.10%	0.120	0.26 (0.14, 0.57)	0.25 (0.12, 0.48)	3.85%	0.704
Median Half-life (hour)	7.46 (3.97, 11.75)	7.13 (4.36, 12.51)	4.42%	0.969	3.50 (2.85, 4.46)	3.30 (2.49, 4.61)	5.71%	0.901

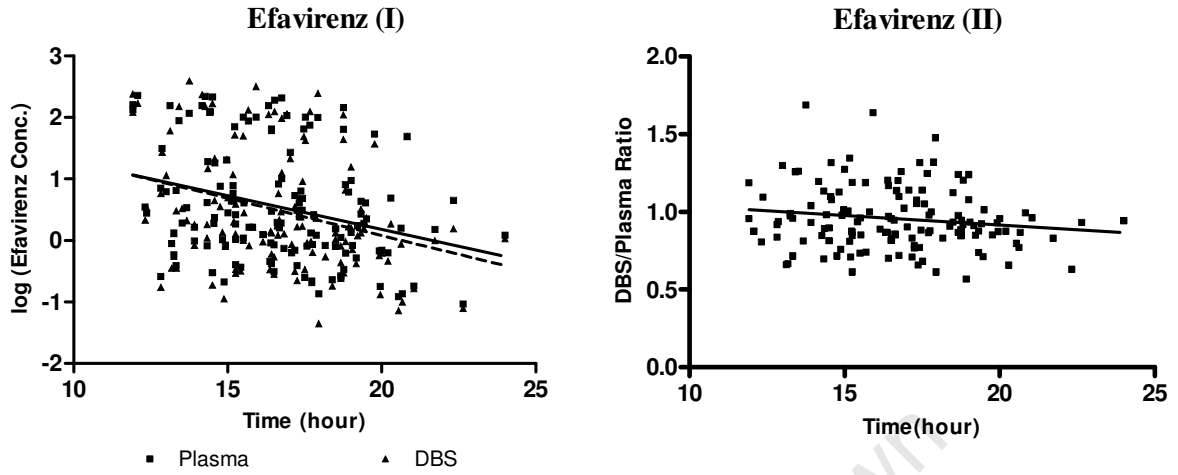
1. T_{max} - time to maximum concentration
2. C_{max} - maximum concentration
3. C_{min} - concentration at 12-hour point
4. AUC_{0-12} - area under the curve to 12 hours
5. $C_{pre-dose}$ - Pre-dose concentration

Figure 5.3 Plasma and DBS concentration vs. time curves, and plots of DBS/plasma ratio against sampling time after drug intake.

a) Efavirenz

(I) The scatter plot of efavirenz plasma (■) and DBS (▲) concentrations. The plasma log-linear regression line is indicated by solid line, and the DBS log-linear regression line is indicated by dash line.

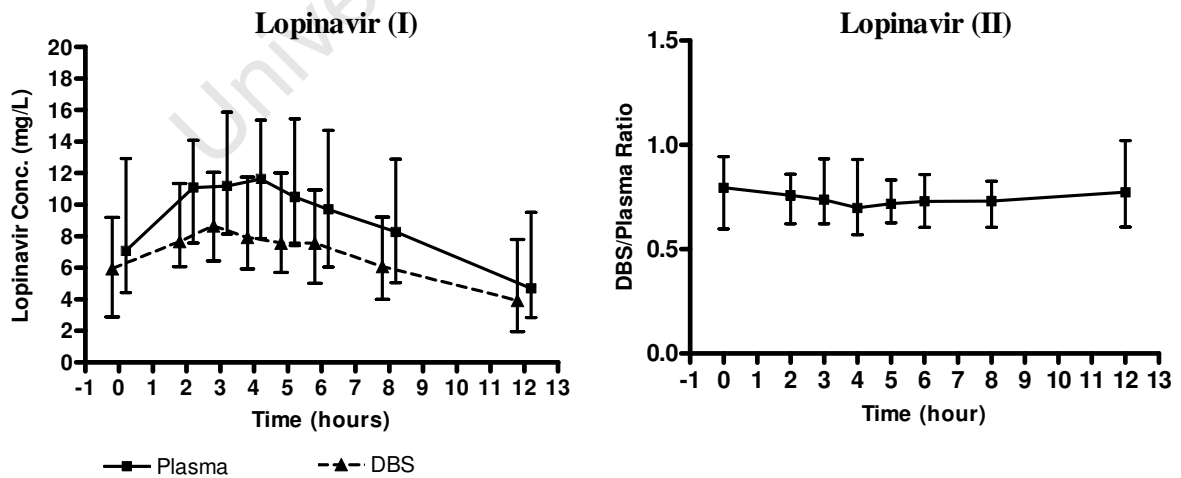
(II) Efavirenz DBS/plasma ratios vs. time interval after drug intake. The best fit linear regression line illustrates the trend of DBS/plasma ratio change over time (solid line).



b) Lopinavir

(I) Lopinavir DBS and plasma concentration vs. time curves. The concentration-time curve for plasma is shown by the solid line; the DBS concentration-time curve is shown by the dashed line. The time points of the two methods have been separated for clarity. Median (IQR) concentrations are indicated at each time point.

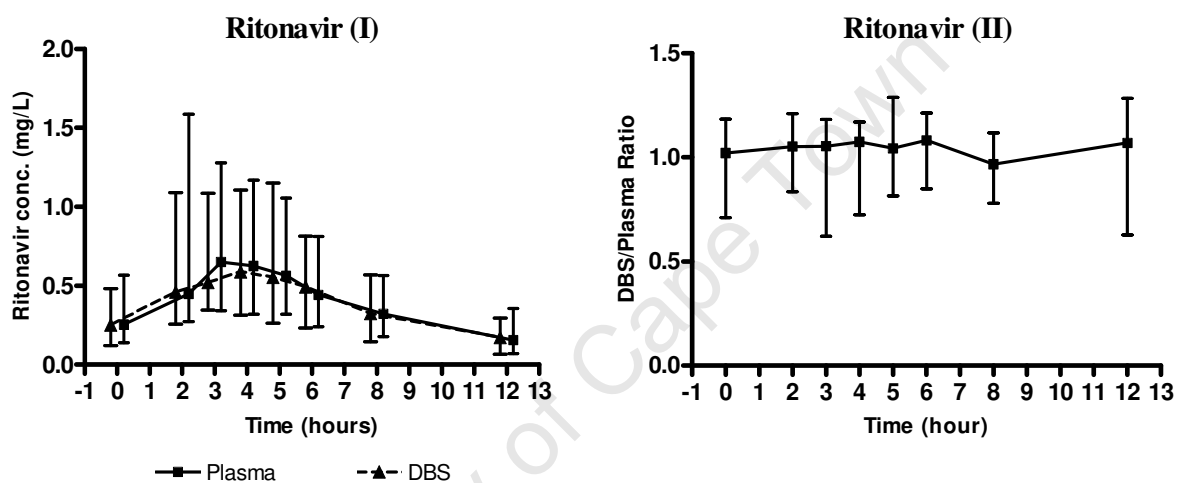
(II) Lopinavir DBS/plasma ratios vs. time interval after drug intake. The median (IQR) of DBS/plasma ratios at each time point is indicated and connected by solid line.



c) Ritonavir

(I) Ritonavir DBS and plasma concentration *vs.* time curves. The plasma concentration-time curve is shown by the solid line; the DBS concentration-time curve is shown by the dashed line. The time points of the two methods have been separated for clarity. Median (IQR) concentrations are indicated at each time point.

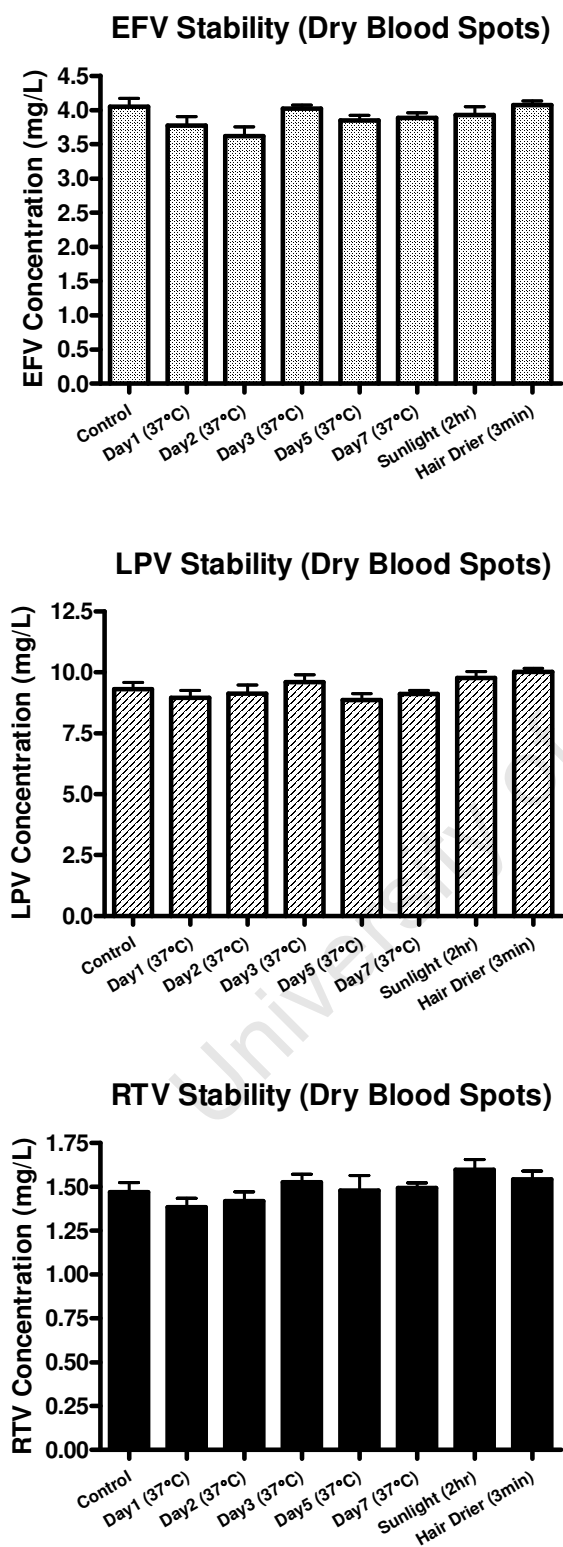
(II) Ritonavir DBS/plasma ratios *vs.* time interval after drug intake. The median (IQR) of DBS/plasma ratios at each time point is indicated and connected by solid line.



5.3.5 Stabilities of ARV Drugs in DBS Samples

The stabilities of efavirenz, lopinavir and ritonavir in DBS samples under various conditions are shown in Figure 5.4. All 3 drugs showed good stabilities at 37°C for 7 days, under the high heat setting of a hair drier for 3 minutes, as well as exposure to sunlight for 2 hours.

Figure 5.4 The stabilities of efavirenz, lopinavir and ritonavir in DBS samples under various conditions.



5.4 Discussion

As ARV plasma concentrations are associated with virological response and development of toxicity, it is critical to maintain drug concentrations within a therapeutic range in order to achieve long-term efficacy from cART. TDM is recommended to identify toxic levels of NNRTIs and subtherapeutic concentrations of PIs to reach optimal individualized dosing [Rendón *et al.* 2005]. Despite the fact that HIV and AIDS pandemic occurs primarily in less developed countries of the globe, the TDM service is seldom available in these regions as it requires expensive processing techniques, storage and transportation conditions. A previous study [Koal *et al.* 2005] evaluated 70 samples containing total of 5 ARV drugs using plasma and DBS methods, and reported that concentrations correlated well between plasma and DBS in adults. In this study, the correlations between DBS and plasma concentrations were defined for efavirenz, lopinavir and ritonavir in children and the factors that affecting the correlations were also identified.

In this study, efavirenz and ritonavir showed strong log/log linear correlations between DBS and plasma concentrations. The distribution between whole blood and plasma was close to a 1:1 ratio for efavirenz and ritonavir. And their PK measures calculated from DBS concentrations were also comparable with those calculated from plasma concentrations. However, the median lopinavir concentration was 27% (IQR 39%, 10%) lower in DBS in comparison to plasma samples, which is in keeping with the findings from the previous study [Koal *et al.* 2005]. It was also found that differences between DBS and plasma concentrations tended to be larger at lopinavir C_{max} than those at trough concentrations. The differences in extraction efficacy is unlikely to be the cause of unequal drug distribution between plasma and red blood cells, as the extraction method was standardised and carried out at all times. Protein binding may play an important role in the drug distribution, however further investigation is needed. Moreover, building compartmental population models could be a useful tool to elucidate the mechanism of lopinavir distribution between plasma and red blood cells over time interval after drug intake.

Multivariate analyses indicated a significant negative correlation between efavirenz DBS/plasma concentration ratios with HB concentrations. This could be because HB and ALB concentrations were correlated (spearman's $\rho = 0.364$, $p = 0.0001$), and higher ALB concentrations are expected to be associated with increased efavirenz plasma concentrations and consequently with decreased DBS/plasma ratios, as 99% of efavirenz in plasma is bound to ALB. However, a correlation was not found between DBS/plasma ratios and ALB concentrations. This may be due to missing ALB concentrations in some instances: there were 129 vs. 114 observations for HB and ALB, respectively. Rifampicin-based antitubercular treatment did not have a significant impact on efavirenz DBS/plasma concentration ratios. However, the presence of rifampicin-based antitubercular treatment was significantly associated with reduced lopinavir and ritonavir DBS/plasma concentration ratios. One explanation is that as an inducer of energy-dependent drug efflux proteins, the presence of rifampicin could cause pumping free drugs out of cells, resulting in reduced intracellular concentrations. However as red blood cells, which make up the majority of cells in the DBS samples, are not expected to have active transmembrane efflux pumps, a more likely explanation is that lopinavir and ritonavir are bound predominantly to AAG [Denissen *et al.* 1997], which is an acute phase reactant and is elevated in many acute and chronic infections. It is likely that the TB/HIV co-infected children had higher AAG concentrations (and consequently lower DBS/plasma concentration ratios) at the first PK assessment during antitubercular treatment than they had after antitubercular treatment. However, AAG concentrations were not determined, which is one of the limitations in this study.

Antiretroviral drugs showed good stabilities in DBS samples at high temperature for up to 7 days and under exposure to sunlight for up to 2 hours which make the requirements for storage and transportation less stringent. DBS samples can be dried using a hair drier on a high heat setting for 3 minutes which significantly shortens the drying period. The DBS method is also cost-effective as it simplifies sample collection/ preparation procedure and storage/transportation conditions. A small amount of blood is required by using the DBS

method, which could be sampled from a finger prick and may make TDM and PK studies more convenient for study participants especially for children.

In summary, strong log/log linear correlations between DBS and plasma, low intra-individual variability in DBS/plasma concentration ratios as well as less stringent sample collection and storage support the use of DBS as a field-friendly method in research and TDM practice for efavirenz, lopinavir and ritonavir. However, the pre-dose concentrations of lopinavir in DBS samples need to be increased by 16% when used to predict plasma concentrations in routine TDM samples. Furthermore, the frequently co-administered rifampicin may contribute to even larger variation between DBS and plasma concentrations of lopinavir.

University of Cape Town

Chapter 6

***In vitro* and *in vivo* Intracellular Accumulation of Antiretroviral Agents in Human Peripheral Blood Mononuclear Cells**

6.1 Introduction

Plasma concentrations of efavirenz and lopinavir have been associated with treatment efficacy [Marzolini *et al.* 2001; Csajka *et al.* 2003; Cohen *et al.* 2009; Masquelier *et al.* 2002]. TDM may be applied to ensure plasma concentrations of these ARV drugs fall into their therapeutic ranges. However, as the target of efavirenz and lopinavir is within HIV-infected cells, measurement of intracellular concentrations of efavirenz and lopinavir could provide understanding of drug exposure at the action site. Furthermore, P-gp and MRP are transmembrane efflux proteins, which share overlapping substrate specificity with CYP3A4. Co-administration of the inhibitor/inducer of these drug efflux proteins may alter the intracellular accumulation of efavirenz and lopinavir.

Studies have shown that efavirenz is a substrate for P-gp [Chandler *et al.* 2003], and PIs are not only substrates for P-gp [Profit *et al.* 1999; Washington *et al.* 1998; Lee *et al.* 1998; Gutmann *et al.* 1999] but also for MRP, including both MRP1 and MRP2 [Gutmann *et al.* 1999; Srinivas *et al.* 1998]. Previous *in vitro* study [Janneh *et al.* 2005] has shown that specific and non-specific inhibitors of P-gp, MRP1 and MRP2 significantly increased the intracellular accumulation of saquinavir in human PBMCs. Furthermore, ritonavir (in combination with lopinavir) and rifampicin, which are frequently co-administered with efavirenz and lopinavir, have also been identified as the inhibitor and inducer of these energy-dependent drug efflux proteins, respectively. However, modulation effects of low-dose ritonavir (as a pharmacoenhancer) and rifampicin at the concentrations reflecting the average drug concentrations in

HIV-infected patients on intracellular accumulation of efavirenz and lopinavir are unknown. In this chapter, *in vitro* effects of several drug efflux protein inhibitors as well as ritonavir and rifampicin on the intracellular accumulation of efavirenz and lopinavir were demonstrated using healthy human PBMCs.

The *in vivo* intracellular concentrations of various ARV drugs have been determined in adult Caucasian population [Chaillou *et al.* 2002; Colombo *et al.* 2006; Ford *et al.* 2004 a; Ford *et al.* 2004 b; Khoo *et al.* 2002; Ford *et al.* 2004 c; Almond *et al.* 2005 a; Almond *et al.* 2005 b; Crommentuyn *et al.* 2004; Colombo *et al.* 2004; Hoggard *et al.* 2002]. There is no information on intracellular concentrations in diseased paediatric population. In this chapter, efavirenz and lopinavir/ritonavir intracellular trough concentrations were evaluated in South African HIV-infected children.

6.2 Methods

6.2.1 *In vitro* ARV accumulation assay

PBMCs (4×10^6 cells/mL) were incubated in RPMI growth medium at 37 °C for half an hour in the absence and presence of drug efflux protein inhibitors: verapamil (non-specific inhibitor of drug efflux proteins; at 50 µM), furosemide (inhibitor of MRP1 and MRP2; at 50 µM), cyclosporine A (inhibitor of P-gp; at 20 µM) and ritonavir (inhibitor of P-gp, MRP1 and MRP2; at 5 mg/L) and an inducer: rifampicin (inducer of P-gp; at 4 mg/L). Efavirenz and lopinavir in PBMCs were extracted and concentrations were determined by validated LC/MS/MS method. Data were expressed as cellular accumulation ratios (the ratio of efavirenz and lopinavir concentrations associated with cell pellet to medium, assuming a cell volume of 0.4 pL for each PBMC). More detailed procedures were described in Section 2.3.

6.2.2 *In vivo* Intracellular ARV Drug Concentration Testing

6.2.2.1 Efavirenz Children

The extra group of TB/HIV co-infected children receiving standard doses of efavirenz as part of cART and rifampicin-based antitubercular treatment was recruited. The inclusion and exclusion criteria were stated in Section 3.2.1. Children (n= 5) who were receiving

dual treatment for at least 4 weeks had intracellular efavirenz concentrations evaluated at 24 hours after the dose of efavirenz. Three of them had their efavirenz intracellular concentrations evaluated a second time at least 4 weeks after completing antitubercular treatment.

6.2.2.2 Lopinavir-Ritonavir Children

TB/HIV co-infected children receiving double dose Kaletra[®] (460/115 mg/m²) twice daily as part of cART and rifampicin-based antitubercular treatment were enrolled. Lopinavir and ritonavir intracellular concentrations were determined after children established on double dose Kaletra[®] for at least 4 weeks. Lopinavir and ritonavir intracellular concentrations were evaluated again in the same group of children at least 4 weeks after completing antitubercular treatment receiving twice daily standard dose of LPV/r (230/57.5 mg/m²; Kaletra[®]). Intracellular samples were collected before morning dose or 12 hours after morning drug intake.

All intracellular samples were collected from children recruited at the HIV Clinic, Red Cross Children's Hospital in Cape Town. Institutional approvals of these two studies were granted by the research ethics committees of the University of Cape Town. Intracellular samples were stored at -20 °C while awaiting quantification of concentrations. Efavirenz and lopinavir/ritonavir intracellular concentrations were extracted and determined by validated LC/MS/MS method (Section 2.4).

6.2.3 Statistic Analysis

Two-tailed t-test and One-way ANOVA followed by Bonferroni's correction were used to compare two and more than two groups of observations, respectively, for normally distributed data.

6.3 Results

6.3.1 *In vitro* Effects of Drug Efflux Protein Inhibitors, Ritonavir and Rifampicin on Intracellular Accumulation of Efavirenz and Lopinavir in PBMC Samples

Wide inter-individual variability in the baseline accumulation of efavirenz and lopinavir (the control group, in the absence of P-gp modulators) were observed (Table 6.1). Verapamil (at 50 μM) significantly increased the efavirenz intracellular accumulation in 1 of 3 PBMC samples compared to the efavirenz control (62.48 ± 11.04 vs. 41.89 ± 5.38 ; $p=0.041$). Whereas, significant effects were not found in the accumulation of lopinavir in any of these samples. The intracellular accumulation of efavirenz and lopinavir were not altered by the presence of cyclosporin A (at 20 μM) in 2 PBMC samples. In contrast, furosemide (at 50 μM) significantly increased efavirenz and lopinavir intracellular accumulation in PBMCs ($p=0.018$ and $p=0.016$ for efavirenz and $p=0.034$ for lopinavir). Ritonavir at the concentration of 5 mg/L had no significant effect on intracellular accumulation of efavirenz or lopinavir in 2 PBMC samples. The presence of rifampicin (at 4 mg/L) significantly increased efavirenz accumulation in 1 of 3 samples ($p=0.003$) and a borderline increase was found on lopinavir accumulation in the same PBMC sample ($p=0.053$).

Table 6.1 Effects of drug efflux protein inhibitors/inducer on the intracellular accumulation of efavirenz and lopinavir.

PBMC Samples	1	2	3	4	5	6	7
Efavirenz Control	41.89 \pm 5.38	41.92 \pm 8.90	96.68 \pm 10.57	83.78 \pm 8.24	75.60 \pm 18.27	57.42 \pm 16.50	72.09 \pm 11.07
Verapamil	62.48 \pm 11.04*	51.95 \pm 11.45	92.85 \pm 9.51	-	-	-	-
Furosemide	-	-	-	-	-	87.00 \pm 10.05*	90.00 \pm 6.99*
Cyclosporin A	-	-	-	69.75 \pm 4.00	79.88 \pm 12.10	-	-
Ritonavir	-	-	-	77.18 \pm 15.45	86.18 \pm 4.15	-	-
Rifampicin	70.88 \pm 9.62*	48.45 \pm 4.32	103.05 \pm 10.37	-	-	-	-
PBMC Samples	1	2	3	4	5	6	
Lopinavir Control	190.65 \pm 24.12	89.93 \pm 22.42	86.63 \pm 16.34	85.13 \pm 4.92	89.34 \pm 21.17	60.50 \pm 3.66	
Verapamil	219.47 \pm 21.25	85.13 \pm 11.40	87.08 \pm 14.52	-	-	-	
Furosemide	-	-	-	-	-	76.77 \pm 11.31*	
Cyclosporin A	-	-	-	102.60 \pm 25.86	71.81 \pm 22.35	-	
Ritonavir	-	-	-	72.53 \pm 8.15	68.18 \pm 16.01	-	
Rifampicin	237.23 \pm 21.64	105.38 \pm 16.84	88.05 \pm 4.89	-	-	-	

* indicates statistically significant difference in intracellular accumulation of efavirenz and lopinavir compared to control.

6.3.2 *In vivo* Intracellular Concentrations of Efavirenz, Lopinavir and Ritonavir

Efavirenz intracellular concentrations were determined in 5 TB/HIV co-infected children during rifampicin-based antitubercular treatment. The isolation of PBMCs failed for patient 1 during antitubercular treatment due to haemolysis of blood sample. The median efavirenz intracellular/plasma concentration ratio for rest of the samples was 0.91 (IQR 0.54, 1.19). Three children had efavirenz intracellular concentrations evaluated again after antitubercular treatment: Patient 2 had a higher efavirenz intracellular/plasma concentration ratio during antitubercular treatment compared to that after treatment (1.00 and 0.61 for during and after antitubercular treatment, respectively), whereas efavirenz intracellular/plasma concentration ratio was increased after antitubercular treatment in patient 3 (0.79 vs. 0.27). The efavirenz intracellular concentration of patient 1 was not available during antitubercular treatment. The median efavirenz intracellular concentration for all 7 samples was 0.84 mg/L (IQR 0.38, 2.36). The median efavirenz intracellular/plasma concentration ratio was 0.81 (IQR 0.61, 1.38) (Table 6.2 and Figure 6.1).

Lopinavir and ritonavir intracellular concentrations were determined in 6 TB/HIV co-infected children receiving double dose LPV/r during antitubercular treatment. Patients 6 had both plasma and intracellular concentrations of lopinavir below LLOQ concentrations. Patient 7 had lopinavir intracellular concentration lower than LLOQ. Patients 6, 7 and 10 had ritonavir plasma concentrations below LLOQ, however, their ritonavir intracellular concentrations were within the quantification range. The 40% of LLOQ concentrations was adopted for samples with < LLOQ concentrations as described in Material and Method chapter. The median lopinavir and ritonavir intracellular concentrations were 0.20 mg/L (IQR 0.004, 0.30) and 0.11 mg/L (IQR 0.02, 0.22), respectively. The median lopinavir and ritonavir intracellular/plasma concentration ratios of all 6 samples were 0.22 (IQR 0.09, 0.31) and 4.17 (IQR 1.30, 7.33), respectively (Table 6.2 and Figure 6.1). This study was prematurely terminated due to sub-therapeutic

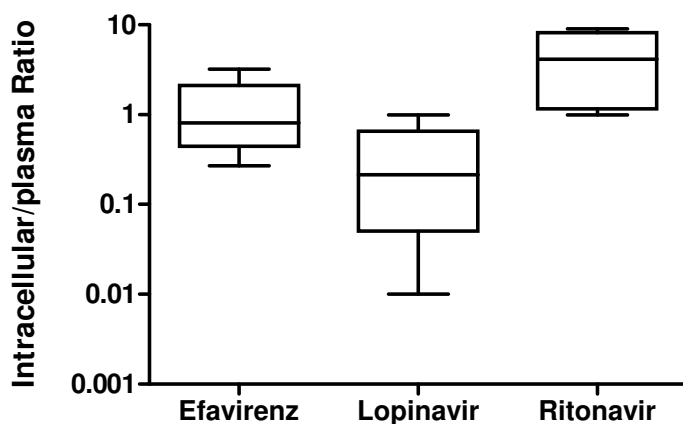
lopinavir plasma concentrations observed in the majority of participants (data not shown). Lopinavir/ritonavir intracellular concentrations in children after antitubercular treatment were not available for evaluation of the effects of rifampicin on intracellular concentrations of PIs.

Table 6.2 Intracellular efavirenz and lopinavir/ritonavir concentrations and intracellular/plasma concentration ratios in children with or without rifampicin-based antitubercular treatment.

Patient	On/off RIF	Time after Dose (hours)	Plasma (mg/L)	Intracellular (mg/L)	Ratio*
Efavirenz					
1	On	24.00	0.24	No yield	-
1	Off	24.08	1.11	3.58	3.23
2	On	24.00	0.43	0.43	1.00
2	Off	24.01	0.54	0.33	0.61
3	On	24.00	1.40	0.38	0.27
3	Off	24.00	1.06	0.84	0.79
4	On	24.00	2.91	2.36	0.81
5	On	24.00	1.27	1.75	1.38
Lopinavir					
6	On	12.00	0.02	0.004	0.20
7	On	11.95	0.07	0.001	0.01
8	On	Pre-dose	3.22	0.30	0.09
9	On	Pre-dose	0.97	0.22	0.23
10	On	12.00	0.18	0.18	1.00
11	On	Pre-dose	2.32	0.72	0.31
Ritonavir					
6	On	12.00	0.01	0.02	2.00
7	On	11.95	0.01	0.01	1.00
8	On	Pre-dose	0.10	0.13	1.30
9	On	Pre-dose	0.03	0.22	7.33
10	On	12.00	0.01	0.09	9.00
11	On	Pre-dose	0.06	0.38	6.33

*ratio of intracellular concentration to plasma concentration

Figure 6.1 The intracellular/plasma concentration ratios for efavirenz, lopinavir and ritonavir. The box and whisker plots represent the median, IQR and range of the ratios.



6.4 Discussion

6.4.1 *In vitro* Intracellular Accumulation of Efavirenz and Lopinavir in PBMC Samples

Furosemide, a MRP1 and MRP2 inhibitor, significantly increased efavirenz and lopinavir accumulation in healthy human PBMC samples by 1.2- 1.5 fold, which was comparable with the average 1.9-fold increase on saquinavir accumulation in a previous study [Janneh *et al.* 2005]. Whereas, neither verapamil nor cyclosporin A had significant effects on efavirenz or lopinavir intracellular accumulation. Although ritonavir has been reported to be at least as good a P-gp inhibitor as cyclosporin A in renal proximal tubules isolated from teleost fish [Gutmann *et al.* 1999], it was found that the inhibitory effect of ritonavir on drug efflux proteins at the concentration reflecting its average plasma concentration in HIV-infected patients did not alter efavirenz or lopinavir accumulation in PBMC samples. Similar observation was reported by an *in vivo* study which showed that co-administration of ritonavir did not boost the median intracellular/plasma concentration ratios of saquinavir or indinavir [Khoo *et al.* 2002]. The reason for the failure of ritonavir to increase intracellular accumulation of PIs could be that the concentrations required for ritonavir inhibiting P-gp and MRP are much higher than those required for inhibition of HIV replication and than those used for boosting [Srinivas *et al.* 1998]. Therefore, the intracellular accumulation of efavirenz and lopinavir are not likely to differ with the

presence of low-dose ritonavir. A study using P-gp-deficient mice concluded that ritonavir was a weak inhibitor of P-gp [Huisman *et al.* 2001], thus the improved bioavailability of PIs in combination with ritonavir may primarily result from CYP3A4 inhibition. Despite being an inducer of P-gp, rifampicin increased the accumulation for both efavirenz and lopinavir to different extents in all 3 PBMC samples, suggesting the presence of rifampicin may compete with ARV drugs for protein binding, resulting in the increased concentrations of unbound ARV drugs and consequently more free drug is available for distribution into the PBMCs.

6.4.2 *In vivo* Intracellular Concentrations of Efavirenz, Lopinavir and Ritonavir

Although *in vitro* experiment could provide conceptual insights into intracellular localization properties of ARV drugs and the influences of concomitant drugs on cellular accumulation, factors such as age and disease states could alter the plasma protein content and therefore drug accumulation. In fact, an *in vitro* study has shown that HIV infection appeared to reduce intracellular accumulation of PIs in cells expressing efflux transporters [Jones *et al.* 2001]. It is important to determine the *in vivo* intracellular concentrations of ARV drugs in diseased population.

This study in children showed the comparable median intracellular efavirenz plasma/intracellular concentration ratio with those observed by Rotger *et al.* and Colombo *et al.* (geometric mean reported) (0.81 vs. 0.9 and 0.71, respectively) [Rotger *et al.* 2005; Colombo *et al.* 2006], however, less than reported by Almond *et al.* (median 0.81 range 0.27, 3.23 vs. median 1.3 range 0.7, 3.3) [Almond *et al.* 2005 a], suggesting that the factors such as age, population differences and disease states may contribute to the differences. Due to smaller samples size, the correlation between intracellular and plasma concentrations of efavirenz in this study was not as good as reported by Colombo *et al.* (slope= 0.90, $R^2= 0.38$, $p= 0.272$ vs. slope= 0.69, $R^2= 0.58$, $p< 0.0001$) [Colombo *et al.* 2006]. In chapter 3, we reported that sub-therapeutic efavirenz plasma concentrations were found in large proportion of African paediatric population, moreover, the cellular efavirenz exposure in children was similar or less than that in adults. For this reason, further investigation regarding determination of the relationship between intracellular

efavirenz exposure and long-term treatment efficacy needs to be carried out. Larger sample size is needed to draw definite conclusion about the effect of concomitant rifampicin on efavirenz intracellular/plasma ratio.

The intracellular accumulation of ritonavir was much higher than that of lopinavir when double dose LPV/r was given twice daily with concomitant rifampicin-based antitubercular treatment. In this study, lopinavir intracellular/plasma concentration ratios were lower than previously reported values (median 0.22 IQR 0.09, 0.31 vs. median 1.18 IQR 0.74, 2.06 [Crommentuyn *et al.* 2004]; 1.55 IQR 0.67, 3.80 [Hoggard *et al.* 2002] and mean 0.65 standard error 0.12 [Colombo *et al.* 2004]). Furthermore, intracellular/plasma concentration ratios of lopinavir trough concentrations were much lower than those reported amongst adults with virological suppression after receiving LPV/r (400/100 mg twice daily) for 6 months (median 0.22 IQR 0.09, 0.31 vs. median 2.4 IQR 1.6, 3.1), and all lopinavir trough concentrations were below the reported intracellular lopinavir C_{\min} efficacy threshold (8 mg/L) [Breilh *et al.* 2004]. However, the virological outcomes were not available as the study was terminated due to sub-therapeutic lopinavir plasma concentrations observed in the majority of children receiving double dose of LPV/r with concomitant antitubercular treatment.

Ritonavir intracellular/plasma concentration ratios (median 4.17 IQR 1.30, 7.33) was comparable with values reported by Crommentuyn *et al.* and Hoggard *et al.* (median 4.59 IQR 3.20, 7.70 and mean 5.28, respectively), and greater than reported by Colombo *et al.* (mean 0.94 standard error 0.18) [Colombo *et al.* 2004]. Factors such as age, population differences, disease status, dosing strategies and concomitant medications could contribute to the differences of the findings. Poor correlations were observed between intracellular and plasma concentrations for lopinavir and ritonavir (slope= 0.14, $R^2= 0.51$, $p= 0.112$ and slope= 1.85, $R^2= 0.23$, $p= 0.330$, respectively).

In summary, intracellular accumulation is a dynamic process influenced by oral bioavailability, plasma protein binding, physiochemical properties, intracellular trapping and influx/efflux active transporters. The power of this study was limited by the small

sample size; however it is difficult to recruit large numbers of paediatric patients for pharmacokinetic studies. Further investigations regarding P-gp expression on PBMCs and evaluations of free drug concentrations are required.

University of Cape Town

Chapter 7

Conclusion

Conclusion

In this PhD project, the plasma, whole blood and intracellular concentrations of efavirenz, lopinavir and ritonavir were evaluated in HIV-infected African children with and without concomitant rifampicin-based antitubercular treatment.

The main objectives of the project and their outcomes are summarized below.

Objective 1: *To develop and validate a simple and rapid efavirenz, lopinavir and ritonavir concentration testing methods requiring small volumes of plasma using LC/MS/MS.*

The LC/MS/MS methods were successfully developed and validated to simultaneously determine efavirenz, lopinavir and ritonavir concentrations in plasma.

Objective 2: *In African children receiving cART with and without tuberculosis, to evaluate the effect of concomitant rifampicin-based antitubercular treatment on efavirenz concentrations.*

It was found that efavirenz concentrations were not significantly different during and after antitubercular treatment, indicating rifampicin is not an important determinant of efavirenz concentrations in children. However, the study may be underpowered to detect a moderate reduction in efavirenz concentrations due to small sample size and large inter-individual variabilities. A larger study is warranted to confidently define the effect of

antitubercular treatment on efavirenz concentrations. It is not possible to draw conclusions about the association between efavirenz concentrations and treatment outcomes in the study population as sample size was small and viral load information was not available for all participants at a standardized time point. However, as efavirenz concentrations were below the recommended therapeutic range in large proportion of children with or without concomitant rifampicin, findings raise concerns about the adequacy of current efavirenz dosing guidelines in children, and TDM is recommended to optimize individual dosing.

Objective 3: *In African children receiving cART with and without tuberculosis, to evaluate the lopinavir and ritonavir concentrations during rifampicin-based antitubercular treatment with an adjusted dose of lopinavir/ritonavir (lopinavir: ritonavir ratio of 1:1) in comparison to those after antitubercular treatment and in a group of controls.*

Large inter-individual variability was observed in lopinavir and ritonavir concentrations. This study showed that the addition of extra ritonavir to LPV/r (lopinavir: ritonavir ratio 1:1) during rifampicin-based antitubercular treatment prevented significant reductions in the lopinavir key PK parameter, C_{min} . The recommended minimum therapeutic concentration was achieved in the majority of children with and without antitubercular treatment. Therefore, in the context of the limited options available for young children with prior exposure to nevirapine, or maternal NNRTIs, LPV/r with additional ritonavir (lopinavir: ritonavir ratio 1:1) is an acceptable approach to treat children receiving concomitant rifampicin-based antitubercular treatment. However, due to the large inter-patient variability observed, TDM is recommended for this group of children. Although the adjusted dose of LPV/r was generally well tolerated in children, further investigation regarding the toxicity and efficacy of LPV/r with extra ritonavir and rifampicin-based antitubercular treatment is needed.

Objective 4: *To develop and validate the use of a DBS method to facilitate the measurement of ARV drug concentrations in research and TDM practice in resource-limited settings.*

The LC/MS/MS methods were successfully developed and validated to simultaneously determine efavirenz, lopinavir and ritonavir concentrations in DBS samples.

There were strong log/log linear correlations between plasma and DBS concentrations for efavirenz, lopinavir and ritonavir. However, co-variants such as HB concentrations and co-administration of rifampicin influenced DBS/plasma drug concentration ratios, indicating that these factors affect the drug distributions between the cellular blood component and plasma. All 3 drugs showed good stabilities in DBS samples. Overall, DBS is a simple and field-friendly method which can facilitate TDM and research studies in children. Further investigation regarding the effects of disease states and concomitant treatment on drug protein binding may provide explanations to the uneven drug distribution between blood cells and plasma.

Objective 5: *To determine intracellular efavirenz, lopinavir and ritonavir trough concentrations in HIV-infected children with and without concomitant rifampicin-based antitubercular treatment (in vivo).*

The LC/MS/MS methods for simultaneously determining intracellular concentrations of efavirenz, lopinavir and ritonavir were successfully developed and validated.

This small pilot study is the first to evaluate the intracellular concentrations of ARV drugs in HIV-infected children. The intracellular/plasma concentration ratios observed (median 0.81, 0.22, 4.17 for efavirenz, lopinavir and ritonavir, respectively) were different to those reported in adults. Factors such as age, population differences, disease states, dosing strategies and concomitant medications might partly explain the differences. Future investigations regarding free drug concentrations and the expression levels of drug efflux proteins on lymphocytes could establish the relationship between total and free drug concentrations with intracellular drug exposure, plasma protein binding and expression of efflux transporters.

Objective 6: To evaluate the effects of rifampicin and ritonavir on efavirenz and lopinavir intracellular accumulation in human PBMCs (*in vitro*)

It was found that ritonavir, an inhibitor of transmembrane drug efflux proteins, had no effect on lopinavir intracellular accumulation in the PBMCs. Rifampicin increased the accumulation for both efavirenz and lopinavir, suggesting that competition for protein binding may play an important role. A limitation of this study is that the expression level of each different type of drug efflux proteins on PBMCs, which could help to identify the specificity of ARV drugs to different types of drug efflux proteins, was not measured.

In summary, this study added important value on optimizing ARV treatment in HIV infected children with concomitant antitubercular treatment, especially for whom living in high-burden countries with limited options of ARV and antitubercular drugs available. Future investigation regarding identification of the factors which could affect on intracellular ARV drug accumulation in order to improve the drug accumulation within the target cells might maximize the potency of existing cART.

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Appendix

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Table A.1 The previously reported efavirenz PK parameters (in children).

Efavirenz PK						
Study	Dose (mg/kg)	C _{min} (mg/L)	C _{max} (mg/L)	AUC (mg·h/L)	CL/F (L/h/kg)	% below Target*
Starr <i>et al.</i> 1999 (n=50) (2 weeks) Median 8 years (range 3.8, 16.8)	Mean 11.7	-	-	Mean 68.8	-	22% (based on C _{min}) 44% (based on AUC)
von Hentig <i>et al.</i> 2006 (n=11) 4- 10 years	10-15	Mean 1.29 (95% CI 0.89, 1.70)	Mean 5.55 (95% CI 3.95, 7.15)	Mean 63.6 (95% CI 44.2, 83.0)	-	63.6%
King <i>et al.</i> 2009 (n= 15) Median 13 years (range 10, 16)	Median 10.7 (range 3.4, 13.7)	Median 0.77 (range 0.2, 2.5)	Median 3.57 mg/L (range 1.4, 7.6)	Median 40.87 (range 13.7, 86.2)	Median 8.5 (range 2.3, 22.9)	53% (C _{min}) 14% (AUC)
Hirt <i>et al.</i> 2009 (n= 48) Median 6.35 years (range 2.77, 14.7)	Mean 14.4	Mean 1.64	Mean 3.71	Mean 65.2	Mean 0.21	19% (C _{min})
Wintergerst <i>et al.</i> 2008 (n= 33) Median 8.2 years (range 2.1- 16.7)	Median 13.3 (range 9.7, 22.5)	MDI (at first sampling) 2/3 sampled between 8- 12 hours Median 2.8 (range 0.13, 11.6) mg/L		-	Median 0.30 (range 0.07, 1.0)	8.8% (MDI)
Puthanakit <i>et al.</i> 2009 (n= 63) Median 12.3 years (range 3.1- 18.7)	Mean± SD 12.0± 1.6	Sample time mean(± SD) 14.8± 0.8 hours MDI Mean± SD 3.14± 3.31 mg/L		-	-	13% (MDI)

SD= standard deviation; C_{min}- trough concentration; AUC- area under the curve; MDI- mid-dosing interval; CL/F- Apparent Elimination Clearance

* Minimum target concentration= 1 mg/L; minimum target AUC= 60 mg·h/L

Table A.2 The previously reported lopinavir PK parameters (in children).

Study	Lopinavir PK Measures						
	Dose (mg/m ²)	C _{min} (mg/L)	C _{max} (mg/L)	T _{max} (hours)	AUC (mg·h/L)	CL/F (L/h/kg)	Half-life (hours)
Sáez-Llorens <i>et al.</i> 2003 (n= 12) 6months-12 years	230/57.5	mean± SD 3.35± 2.14	mean± SD 8.16± 2.94	mean± SD 3.8± 1.6	mean± SD 72.6± 31.1	-	mean± SD 5.8± 3.0
Verweel <i>et al.</i> 2007 (n=23) Median 5.6 years (range 0.4, 13.2)	230/57.5	3.68± 2.48 Median 4.23 (IQR 0.67, 5.48)	9.33± 3.27 Median 10.6 (IQR 6.63, 11.87)	2.61± 2.09	75.3± 33.7 Median 83.9 (IQR 46.6, 106.4)	Median 0.11 (IQR 0.08, 0.24)	6.59± 4.19 Median 5.73 (IQR 3.04, 8.96)
Chadwick <i>et al.</i> 2008 (n= 18) Median 3.4 months (range 1.6, 5.9)	300/75	Mean 2.0	Mean 9.4	-	Mean 74.5 Median 67.52 (range 23.66, 164.04)	Median 0.187 (range 0.073, 0.610)	Median 3.68 (range 1.2, 13.11)
Chadwick <i>et al.</i> 2009 (n= 9) Median 5.7 weeks (range 3.6, 5.9)	300/75	Mean 1.4	Mean 5.17 Median 4.76 (range 2.84, 7.28)	-	Mean 43.4 Median 36.6 (range 27.9, 62.6)	Mean 0.43 Median 0.37 (range 0.15, 0.75)	Mean 3.67 Median 3.51 (range 2.06, 5.80)

SD= standard deviation; C_{min}- trough concentration; AUC- area under the curve; T_{max}- Time to Maximum Concentration; CL/F- Apparent Elimination Clearance

Table A.3 The previously reported intracellular/plasma concentration ratios for efavirenz, lopinavir and ritonavir.

	Intracellular/plasma Concentration Ratios	
Study	Efavirenz	
Rotger <i>et al.</i> 2005	Median 0.9	
Colombo <i>et al.</i> 2006 (n= 62)	Geometric mean 0.69 (geometric CV 101)	
Almond <i>et al.</i> 2005 a (n= 10)	Median 1.3 (range 0.7- 3.3)	
	Lopinavir	Ritonavir
Crommentuyn <i>et al.</i> 2004 (n= 11) LPV/RTV combination	Median 1.18 (IQR 0.74, 2.06)	Median 4.59 (IQR 3.20, 7.70)
Hoggard <i>et al.</i> 2002 (n= 12) LPV/RTV combination	Median 1.55 (range 0.67, 3.80)	Median 5.28
Colombo <i>et al.</i> 2004 (n= 16) LPV/RTV combination	Mean 0.65 (standard error 0.12)	Mean 0.94 (standard error 0.18)
Breilh <i>et al.</i> 2004 (n= 38) LPV/RTV combination	virological success vs. failure (1 month) Median 3.2 (IQR 2.6, 3.8) vs. 2.3 (IQR 1.9, 2.8)	-
	virological success vs. failure (6 month) Median 2.4 (IQR 1.6, 3.1) vs. 1.4 (IQR 1.3, 1.7)	
Khoo <i>et al.</i> 2002 (n= 6) RTV single agent	-	Median 1.00 (range 0.60, 2.28)

CV- Coefficient of variation; IQR- Interquartile range; LPV- Lopinavir; RTV- Ritonavir