COMPARISON OF LOPINAVIR AND RITONAVIR DRUG CONCENTRATIONS IN PLASMA AND DRIED BLOOD SPOTS (DBS) IN HIV INFECTED PATIENTS IN THAILAND

BY

MISS YARDPIROON TAWON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (BIOCLINICAL SCIENCE)

GRADUATE STUDIES

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Thesis Title	COMPARISON OF LOPINAVIR AND RITONAVIR DRUG CONCENTRATIONS IN PLASMA AND DRIED BLOOD SPOTS (DBS) IN HIV-INFECTED ADULTS AND CHILDREN IN THAILAND
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ABSTRACT

Lopinavir/ritonavir (LPV/RTV) remains a commonly administered HIV protease inhibitor in HIV-infected patients in Thailand. Lopinavir is a HIV protease inhibitor administered with low dose ritonavir to enhance its bioavailability. Antiretroviral drug measurement can be useful for the clinical management of patients with drug toxicities, drug-drug interactions, as well as optimization of dosing for pregnant women and young children. Drug measurements are performed using plasma samples and they require storage and shipping under frozen conditions. Dried blood spots (DBS) is an alternative sample matrix for drug measurement as they can be stored at room temperature and shipped in the normal post. A new liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) assay was developed and validated to quantify lopinavir and ritonavir from DBS. Sample preparation involved a liquid-liquid extraction. Chromatographic separation was performed on a Gemini Polar Reversed Phase C18 column (150 x 2.0 mm ID, 5µm) using a stepwise gradient. The calibration curve was linear over the range 0.05 to 20 µg/mL. The lower limit of

quantification was 0.05 µg/mL. The assay average accuracy was 102-112% for lopinavir and 90-112% for ritonavir. The assay precision (inter- and intra assay) expressed as coefficient of variation (%CV) was <6% for lopinavir and <9.0% for ritonavir. The recoveries for lopinavir and ritonavir were 82.1% and 102.6%, respectively. Both drugs were stable in DBS stored at room temperature for at least 3 months. Sample hematocrit (30-60%) had no effect. Concentrations of lopinavir and ritonavir in paired plasma and DBS samples collected from 155 HIV-infected patients (median age 29 years, range 3 to 70), during 0.1-17 hours after the last dose and the hematocrit of samples ranged from 28.1 to 48.2%. Plasma and DBS concentrations for lopinavir and ritonavir were highly correlated (Pearson correlation r = 0.964 and r =0.990, respectively). The Bland-Altman plot indicated no proportional bias between the DBS and plasma assays (p>0.05). However, lopinavir and ritonavir concentrations were 29.3% and 24.6% lower in DBS than in plasma, respectively. In conclusion, the LC-MS/MS assay validated for the quantification of lopinavir and ritonavir in DBS is robust, accurate and precise. Lopinavir and ritonavir concentrations in DBS are lower than plasma. Current target antiretroviral drug concentrations are based on plasma concentration thresholds, therefore drug concentrations determined from DBS samples need to be adjusted to estimate the plasma concentrations before interpretation.

Keywords: lopinavir, ritonavir, dried blood spots, LC-MS/MS

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Miss Yardpiroon Tawon

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
3TC	Lamivudine
ABC	Abacavir
ATV	Atazanavir
ATV/r	Atazanavir boosted with Ritonavir
ART	Antiretroviral Treatment
ARV	Antiretroviral drug
AZT	Zidovudine
ºC	The Celsius temperature scale
CLSI	Clinical and Laboratory Standards Institute
%CV	Percent Coefficient of variation
CYP3A	Cytochrome P450, Family 3, Subfamily A
CYP3A4	Cytochrome P450, Family 3, Subfamily A member 4
DBS	Dried Blood Spots
EFV	Efavirenz
EMA	European Medicine Agency
ESI-LC-MS/MS	Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
Fbbp	Fraction of drug bound to proteins
FTC	Emtricitabine
Hct	Hematocrit
HIV	Human Immunodeficiency Virus
HPLC-MS/MS	High Performance Liquid Chromatography-Tandem Mass Spectrometry
HQC	High Concentration Validation Sample
IN	Integrase Inhibitors

LC-MS-MS	Liquid Chromatography-tandem Mass Spectrometry
LPV	Lopinavir
LPV/rtv	Combination of Lopinavir and Ritonavir
LQC	Low concentration Validation Sample
MALDI-QqQ-MS/MS	Matrix assisted laser desorption/ionization-triple quadrupole-tandem mass spectrometry
mL	milliliter
MQC	Medium Concentration Validation Sample
ng/mL	Nano gram per milliliter
NNRTI	Non-Nucleoside Reverse Transcriptase inhibitors
NRTI/NtRTI	Nucleoside/Nucleotide Reverse Transcriptase inhibitors
NVP	Nevirapine
PID	Patient Identification Code
PIs	Protease Inhibitors
PMTCT	The prevention of mother-to-child transmission
TDF	Tenofovir
TDM	Therapeutic Drug Monitoring
R^2	The coefficient of determination
RPV	Rilpivirine
RTV	Ritonavir
SD	Standard Deviation
SE	Standard Error
UHPLC-MS/MS	Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry
UNAIDS	The Joint United Nations Programme on HIV and AIDS
US FDA	Food and Drug Administration of the United States of America
µL	microliter
xg	Relative Centrifugal Force or G-Force

CHAPTER 1
INTRODUCTION

1.1 HIV Epidemic

HIV remains a major public health problem with an estimated 36.9 million people living with HIV globally at the end of 2014. There were also 2 million new infections and 1.2 million people died from AIDS related causes that year [(1)]. In Thailand there are approximately 450,00 people living with HIV [(2)]. The number of new infections reported in Thailand counties to decline with 8,535 people infected in 2014; however, this rate of decline is slower than expected. A major success has been the significant reduction in the rate of mother-to-child-transmission rate of HIV to 2.1%. Surprisingly, the number of AIDS-related deaths per year has remained relatively stable at 20,000 after a sharply decrease between 2000 and 2010 [(3, 4).]

1.2 Antiretroviral Treatment

Today, 15 million people are receiving antiretroviral therapy worldwide; however, this still only represents 41% of all adults and 32% of all children who need to access these life-saving treatments [(1)].

The US FDA has approved 26 individual antiretroviral drugs to treat HIV/AIDS [(5)]. Antiretroviral drugs act on different steps within in the HIV-life cycle and are classified into different classes based on their mechanisms of action: (1) nucleoside/nucleotide reverse transcriptase inhibitors (NRTI/NtRTI); (2) non-nucleoside reverse transcriptase inhibitors (NNRTI); (3) protease inhibitors (PIs); (4) integrase inhibitors (IN); (5) entry inhibitors; and (6) fusion inhibitors. A triple combination of antiretroviral drugs, from at least 2 drug classes, is recommended for antiretroviral therapy (ART).

In Thailand, the recommended first-line antiretroviral treatment regimens are composed of a dual NRTI backbone plus an NNRTI, e.g. TDF+3TC plus EFV (see Table 1). For patients who cannot take a NNRTI, the HIV protease lopinavir/ritonavir

is recommended to replace the NNRTI. This first line regimen is also recommendation for HIV-infected pregnant women [2].

Table 1.1 Recommended antiretroviral drug regimens for adults in Thailand [2]

NRTI backbone		NNRTIs		Others
Recommended		Recommended		Third drug/ Recommended
TDF/FTC		EFV	→	
TDF + 3TC				LPV/r
Alternative		or	For patient who cannot take NNRTIs	Or
ABC + 3TC AZT + 3TC	+	RPV NVP		ATV/r

Lopinavir/ritonavir-based ART is recommended as part of antiretroviral drug regimens for children. LPV/r is part of the preferred 1st line regimen for HIV-infected infants less than 3 years of age [2].

Table 1.2 Antiretroviral Treatment Guidelines for Children in Thailand 2014, [2]

	<1 year old	**1 - <3 years old**	**3– 12 years old**	**>12 years old**
Preferred regimens	AZT (or ABC) + 3TC + LPV/r	AZT (or ABC) + 3TC + LPV/r	AZT (or ABC) + 3TC + EFV	AZT (or ABC) + 3TC + EFV
Alternative regimens	• AZT (or ABC) + 3TC + NVP • d4T +3TC +LPV/r • d4T+3TC+NVP	• AZT (or ABC) + 3TC + NVP • d4T+3TC+LPV/r • d4T+3TC+NVP	• AZT (or ABC) + 3TC+ NVP • TDF+3TC +EFV (or NVP) • d4T+3TC +EFV (or NVP)	• AZT (or ABC) + 3TC + EFV (or NVP) • TDF+3TC +NVP or RPV • AZT (or ABC) + 3TC+ NVP

Lopinavir has a very low bioavailability when administered alone but is significantly increased when coadministered with low dose ritonavir (i.e. RTV acts a pharmacokinetic 'booster' for LPV). Lopinavir is a substrate for cytochrome CYP3A4 and RTV enhances the bioavailability of lopinavir through the potent inhibition of this enzyme. Lopinavir/ritonavir is co-formulated in a single tablet (200/50 mg or 100/25 mg) and is administered twice daily. LPV/r is approved by the U.S. FDA for the treatment of HIV infection in adults and children (starting from 14 days of age).

1.3 Therapeutic drug monitoring of antiretroviral drugs[4]

Therapeutic drug monitoring of antiretroviral plasma drug concentrations is not recommended as part of routine care but can be considered in special clinical circumstances as below:

- for patients with renal or hepatic impairment,
- pregnant women,
- infants/children,
- in cases of suspected drug-drug interactions.

1.4 Antiretroviral drugs measurement using Dried Blood Spots (DBS)

Standard drug measurement methods require plasma samples but these samples must be processed (i.e. centrifuge) and then stored at -20°C to -70°C. Moreover, if these plasma samples need to be shipped to a reference laboratory for analysis the samples must be shipped on dry-ice. Using dried blood spot (DBS) samples instead of plasma may help overcome these challenges. DBS samples are normally prepared using blood from a finger/heel stick or a small volume of venous blood (e.g. 50 μL). The blood is dropped onto a designated area of a filter paper card and air dried. DBS cards are then stored at room temperature in a zip-locked bag with a desiccant until analysis. There are several advantages of DBS sample collection. Firstly, it is relatively non-invasive and only a small volume of blood is needed, making it an ideal sampling method for babies and children. Secondly, DBS samples can be stored and shipped under ambient condition, which is particularly useful for resource limited

settings and remote areas. Thirdly, due to the dry matrix the sample is considered none pathogenic allowing shipment in the normal post. Drug measurements using DBS is increasingly being used in drug discovery, drug development, and therapeutic drug monitoring.

1.5 Development and Validation of Drugs Assays using Dried Blood Spots (DBS)

To ensure that a newly developed drug assay provides accurate, precise and reliable results it is necessary to validate the assay before testing clinical samples. Both the US FDA [6] and EMA [7] have guidelines for bioanalytical assay validation that included: (1) selectivity and specificity, (2) sensitivity, (3) linearity, (4) intra- and inter-day precision and accuracy, (5) stability (stock/spiking solution stability, stability in QC samples that undergo freeze–thaw condition, stability in blood), (6) dilution integrity and (7) carryover.

The US FDA does not currently accept standalone DBS data as a replacement for liquid matrices for registration studies. If a bioanalytical method for an analyte is already developed for a liquid matrix one can usually modify and apply it to DBS samples. DBS methods must be developed and validated to meet the same validation acceptance criteria for liquid matrices. Addition validation steps are also required for DBS assay, such as testing for a 'hematocrit effect', which is important when only a portion of the total blood spot is used for testing.

It may also be important to compare the drug concentrations in DBS and plasma as the concentration in DBS may not necessarily be equal to the concentration in plasma. For antiretroviral drugs the efficacy and toxic concentrations thresholds are based on plasma drug concentrations therefore it would be necessary to determine the agreement between DBS and plasma concentrations to facilitate interpretation of drug concentration results reported from DBS samples.

1.6 Objectives

The objectives of this research were to validate a new assay to quantify antiretroviral drugs in Dried Blood Spots (DBS) and to compare the drug concentrations obtained in paired plasma and DBS samples in HIV-infected patients receiving antiretroviral therapy. The specific objectives were:

1 To validate a method to quantify lopinavir and ritonavir concentrations in DBS using liquid chromatography-triple quadrupole mass spectrometry.

2 To assess the agreement between lopinavir and ritonavir concentrations in plasma and DBS using validated LC-MS/MS methods.

CHAPTER 2
REVIEW OF LITERATURE

The first publication reporting the quantification of antiretroviral drugs in DBS by LC-MS/MS was in 2005 by Koal *et al*. This assay simultaneously measured 9 antiretroviral drugs: 7 PIs (amprenavir, nelfinavir, indinavir, lopinavir, saquinavir, ritonavir, atazanavir) and 2 NNRTI (nevirapine and efavirenz). The retention times of all analytes were between 4.9 and 5.5 minutes. Drying the DBS for a minimum of 2 hours was necessary for optimal analyte detection. Validation results reported included the limits of detection (LOD 8 to 70 ng/mL), lower limits of quantification (LLOQ, 41 to 102 ng/mL), linearity (R^2, 0.9981 to 0.9999), linear concentration range (41 to 10,000 ng/mL), accuracies (92 to 113%) and recoveries (62 to 94%). No ion suppression effects of analytes were reported. All analytes were stable in whole blood at 56°C for 30 minutes before spotting on the DBS card [8].

In 2008, ter Heine et al developed and validated an LC/MS/MS assay to quantify 4 PIs (atazanavir, darunavir, lopinavir and ritonavir) and 2 NNRTIs (efavirenz and nevirapine) in DBS. This assay also included the newly approved PI darunavir. The retention times of all analytes were between 3.8 and 5.7 minutes. The influence of spot size was investigated and using 20 to 60 µL of blood per spot did not influence the amount of analyte presented in a 0.25 inch diameter punched-out disc. Validation data for intra- and inter- assay accuracy and precision for all analytes at all levels ranged from 96.2 to 113.9% and 3.1 to 13.3%, respectively. The recoveries were within the range of 94 to 109 % for all analytes. All analytes were stable for at least 7 days at 30°C in DBS and also stable in the final extract at 4°C for 3 days [9].

An ultrafast and high-throughput method to determine lopinavir and ritonavir concentrations in plasma and DBS by matrix-associated laser desorption/ionization-triple quadrupole tandem mass spectrometry (MALDI-QqQ-MS/MS) has been reported by Meesters et al. The retention times for all analytes were <15 seconds because the MALDI-QQ technique does not need the liquid chromatographic separation of samples. The assay LOD for LPV and RTV were 24.5 and 41.8 ng/mL, respectively [approximately 30 times more sensitive than standard ESI-LC-MS/MS methods]. The intra- and inter-assay accuracy and precision for both

LPV and RTV ranged from 89.2 to 113.7% and 4.7 to 18.2%, respectively. LPV and RTV in DBS were stable at 4°C for 24 hours and in a desiccator at 20°C for 20 days [10].

Watanabe *et al* assessed a new device to try and simplify DBS collection. This new device was able to reduce the DBS drying time to 5 minutes using a microwave. The collection device was used as part of a new method to quantify 8 PIs (indinavir, ritonavir, lopinavir, saquinavir, amprenavir, nelfinavir, atazanavir and darunavir) in DBS. The LOD for all analytes was 100 ng/mL and all retention times were less than 3 minutes. The intra- and inter- assay accuracy and precision were within the standard acceptance criteria (<20% for LLOQ and <15% for the other concentrations). Extracted recoveries were more than 85% and matrix enhancement of about 10 to 15% was observed. All drugs were stable in the dark at room temperature for at least seven days, except nelfinavir [11].

Overall, there have been 4 publications for the quantification of lopinavir and ritonavir in DBS. None of these studies assessed the impact of different hematocrit values as part of the assay validation. A patient's hematocrit is an important factor to consider for DBS samples as it can impact the spread of blood on the card. This can be a problem when partial 'standard' size holes are punched for extraction as it may affect the volume of blood per punched spot. Another issue is that the drug concentrations in DBS may not necessary be equal to plasma concentrations because of drug-plasma proteins binding (fbpp). Lopinavir and ritonavir are highly protein bound (both >98%) therefore it is necessary to investigate the relative concentrations of each drug in paired plasma and DBS samples. Indeed, a major limitation of the published assays is that lack of data assessing the degree of agreement between lopinavir and ritonavir concentrations quantified in plasma and in DBS. Koal *et al* reported a good correlation (R^2=0.97) between antiretroviral drugs concentrations in paired plasma and DBS from 70 patients (containing either LPV, ATV, SQV, RTV and EFV [8]. In 2010, Meester *et al*. reported a good correlation between plasma and DBS concentrations in 19 HIV-infected children for both LPV (R^2=0.85) and RTV (R^2=0.77). Despite the strong correlation coefficients reported this does not demonstrate the agreement between the two methods to quantify the drug concentrations [10]. In order to compare a new measurement technique with an established one is necessary to determine whether the

results between the assays sufficiency agree so that the new assay can replace the previous one. A Bland-Altman analysis should be used to evaluate if a bias exists between two quantitative measurements i.e. between the plasma and DBS assays.

A summary of publications reporting LC-MS/MS assays that quantify lopinavir and ritonavir concentrations in DBS are show in Table 2.1.

Table 2.1 Summary of published methods to quantify lopinavir and ritonavir in DBS using LC-MS/MS

Ref#	Drug	Bioanalytical Method Summary	Hct Effect	DBS versus plasma conc.
Koal *et al.,* 2005[8]	APV, NFV, **LPV,** SQV, **RTV,** ATV, NVP, EFV	▪ **DBS Card:** PK DBS ▪ **Extraction Step:** Protein precipitation using 50:50 MeOH/0.2 M $ZnSO_4$ (v/v) ▪ **Instrument:** HPLC-MS/MS	Not Done	Linear regression of DBS versus plasma for 70 patient samples containing LPV, ATV, SQV, RTV and EFV shown an R^2 ≥0.9681 with a slope >1.0 for 2 hrs drying time DBS and R^2 ≥0.9772 with a slope >1.0 for 3 days drying time DBS
ter Heine *et al.,* 2008[9]	ATV, DRV, EFV, **LPV,** NVP, **RTV**	▪ **DBS Card:** Whatman 903 ▪ **Extraction Step:** Protein precipitation using Acetonitrile/Methanol/0.2M $ZnSO_4$ (1:1:2 v/v) ▪ **Instrument:** HPLC-MS/MS	Not Done	Not Done

Table 2.1 Summary of published methods to quantify lopinavir and ritonavir in DBS using LC-MS/MS (continue)

Ref#	Drug	Bioanalytical Method Summary	Hct Effect	DBS versus plasma conc.
Meesters *et al.,* 2010[10]	**LPV, RTV**	▪ **DBS Card:** FTA PK collecting cards, GE healthcare ▪ **Extraction Step:** Protein precipitation **Instrument:** MALDI-QqQ-MS/MS	Not Done	Linear regression of pair DBS versus plasma for 19 samples collected from patients who receiving Kaletra (LPV/rtv) in cohort study was determined and shown an R^2 = 0.8487 with slope < 1.0 for LPV and R^2=0.7679 with slope <1.0 for RTV.
Watanabe *et al.,* 2014[11]	IDV, RTV, **LPV, RTV,** SQV, APV, NFV, ATV, DRV	▪ **DBS Card:** Whatman 903 card cut and inserted in the lid of 1.5 mL micro-centrifuge tube. ▪ **Extraction Step:** Protein precipitation ▪ **Instrument:** Parallel UHPLC-MS/MS	Not Done	Not Done

CHAPTER 3
RESEARCH METHODOLOGY

3.1 DBS Assay Development and Validation

3.1.1 Antiretroviral Drug Powders: Controls and Standards

Drug*: Lopinavir*

Source: Toronto Research Chemicals Inc. (TRC), Catalog#L469480, Lot # 14-XJZ-78-1, Purity: 98%, Empirical Formula: $C_{37}H_{48}N_4O_5$, Molecular Weight: 628.80

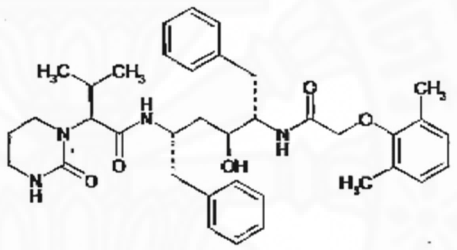


Figure 3.1 Structural formula of Lopinavir (LPV),

Drug*: Lopinavir-d8 (Internal Standard for Lopinavir, LPV-d8-IS)*

Source: Toronto Research Chemicals Inc. (TRC), Catalog#L469480, Lot # 14-XJZ-78-1, Purity: 98%, Empirical Formula: $C_{37}H_{40}D_8N_4O_5$, Molecular Weight: 636.85

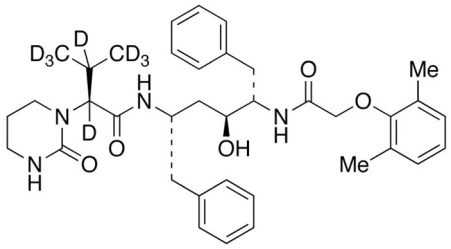


Figure 3.2 Structural formula of Lopinavir-d8 (LPV-d8)

Drug: *Ritonavir*

Source: Toronto Research Chemicals Inc. (TRC), Catalog#1-GBL-19-1, and Lot # 1-GBL-19-1, Purity: 98%, Empirical Formula: $C_{37}H_{48}N_6O_5S_2$, Molecular Weight: 720.94

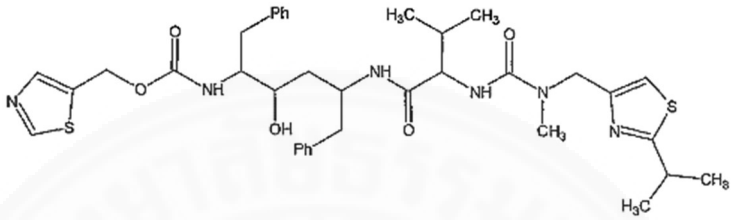


Figure 3.3 Structural formula of Ritonavir (RTV)

Drug: *Ritonavir-d6 (Internal Standard for Ritonavir, RTV-d6-IS)*

Source: Toronto Research Chemicals Inc. (TRC), Catalog#R535002, and Lot # 11-ELZ-106-1, Chemical Purity: 98%, Isotopic Purity: 99%, Empirical Formula: $C_{37}H_{42}D_6N_6O_5S_2$, Molecular Weight: 726.98

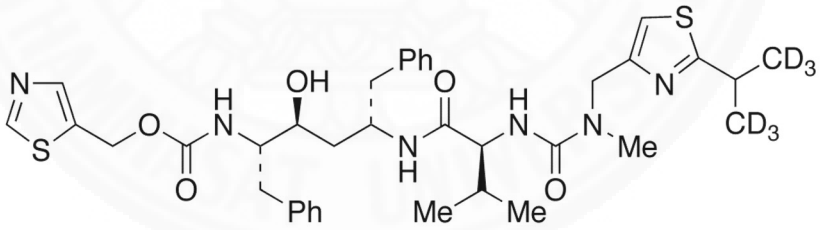


Figure 3.4 Structural formula of Ritonavir-d6 (RTV-d6)

3.1.2 LC-MS/MS Equipment

The quantification of lopinavir and ritonavir in plasma and DBS were performed using liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS). An Agilent HPLC 1100 series coupled with an Agilent Triple Quad MS 6430 system was used. A triple quadrupole mass spectrometer (TQMS) also known as tandem mass spectrometry (MS/MS or MS^2) consists of two quadrupole mass analyzers and a non-mass analyzer that acts as a cell for collision-induced dissociation. This configuration is often abbreviated QqQ or Q1q2Q3.

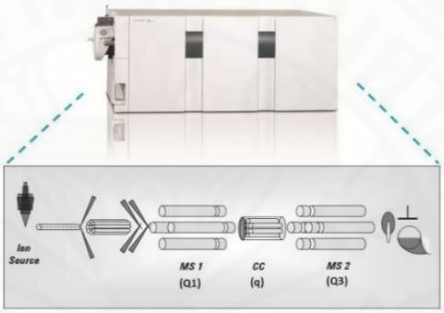


Figure 3.5 Diagram of the Triple Quadrupole Mass Spectrometry System

Each of the two mass filters (Q1 and Q3) contains four parallel, cylindrical metal rods. Both Q1 and Q3 are controlled by direct current (dc) and radio-frequency (rf) potentials, while the collision cell, q, is only subjected to RF potential. The RF potential associated with the collision cell (q) allows all ions that were selected for to pass through it.

3.1.3 Quantification of lopinavir and ritonavir concentrations in DBS

The DBS method was developed based on two published methods. The chromatographic conditions used were adapted from that reported by Rob ter Heine et al [9] and the drug extraction conditions was based that reported by A. Joubert et al [12].

3.1.3.1 Preparation of standard calibration samples, internal standards and quality controls stock solutions

Stock solutions of all analytes (LVP, LPV-d8, RTV and RTV-d6) were dissolving in methanol to make a final concentration of 2.0 mg/mL for LPV and RTV and 1 mg/mL for LPV-d8 and RTV-d6.

3.1.3.2 Preparation of Standard calibration samples and Quality Controls samples

The stock solution of LPV and RTV were diluted with human blank plasma to an intermediate working solution at a concentration of 100,000 ng/mL. This working solution was further diluted with whole blood (K2-EDTA) to prepare 9 calibration levels between 50 to 20,000 ng/mL. Internal Quality Control Samples were prepared similarly by diluting the intermediate working solution in plasma into whole blood (K2-EDTA) to yield three levels: QC low at 150 ng/mL, QC medium at 1,500 ng/mL and QC High at 16,000 ng/mL. Each calibrator and internal quality control samples were spotted (50 µL) on separate Whatman Protein Saver 903 Cards. DBS cards were dried at room temperature overnight and stored with a desiccant at -20ºC.

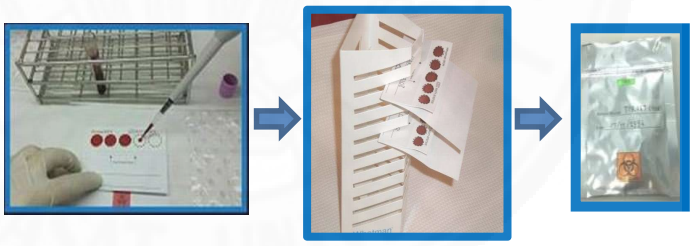


Figure 3.6 DBS Standards and QCs spotting technique

3.1.3.3 Preparation of Internal standards working solutions

A working solution of internal standards was prepared by mixing spiked stock solutions of LPV-d8 and RTV-d5 into DI water to obtain a final concentration of 500 ng/mL.

3.1.4 Drug Extraction Procedure from DBS

The entire DBS was cut out using a 1/2-inch hole punch and placed into an appropriately labelled 1.5 mL micro tube.

Figure 3.7 Example of punching out DBS

150 µL of the IS-mixture in water was added into the micro-tube and left to equilibrate at room temperature for 10 minutes. Firstly, methanol was added to precipitate the proteins and then the tubes sonicated for 15 minutes. Secondly, a liquid-liquid extraction was performed by adding 1 mL of ethyl acetate to the tubes. The tubes were vortexed for 1 minute followed by centrifugation at 12,000 rpm (16,060xg) for 5 minutes. The supernatant was transferred to a new tube and evaporated under nitrogen gas. Once the sample was dried, it was reconstitution in mobile phase B (10 mM ammonium acetate buffer: 10 mM acetic acid: methanol (43:22:35 %v/v). 10 µL of sample matrix was injected in to the HPLC-MS-QQQ by automatic injector.

3.1.5 Chromatographic and Mass Spectrometric Conditions for DBS Assay

The chromatographic and MS conditions were based on a published method to quantify LPV and RTV in human plasma [4]. Chromatographic separations were performed on a Gemini Polar Reversed Phase C18 column (150 mm x 2.0 mm ID, particle size 5µm) connected with Security Guard Cartridges AQ C18: 4.0 mm x 2.0 mm ID. A stepwise gradient was used at a flow rate of 0.25 mL/min. At time zero the flow consist of 15% mobile phase A (100% Methanol) and 85% mobile phase B (10 mM ammonium acetate buffer: 10 mM acetic acid: methanol (43:22:35 %v/v). The percentage of mobile phase A was increased to 85% from time 0 to 0.1 minutes. The

85% mobile phase A and 15% mobile phase B was maintained from 0.1 to 10 minutes. At 10.1 minutes the percentage of mobile phase A was decreased from 85% to 15% and continues to recondition the system with 15% of mobile phase A and 85% of mobile phase B until 15 minutes. Total run time was 15 minutes. The triple quadrupole mass spectrometer was operated in positive mode. The source temperature was set at 350°C. The nebulizer was set at 50 psi (air). The drying gas (N_2) was flow at 10 L/min of gas flow. The multiple reaction monitoring (MRM) mode used and the MRM transitions for each analyte are given in Table 3.1

Table 3.1 MS QQQ Mass Spectrometer Parameters

Analyte Compound	**Precursor Ion**	**Product Ion**	**Dwell**	**Frag (V)**	**CE (V)**
LPV	629.80	447.2	50	128	8
LPV-d8	637.86	191.2	50	128	18
Internal Standard	**Precursor Ion**	**Product Ion**	**Dwell**	**Frag (V)**	**CE (V)**
RTV	721.90	296.1	50	128	16
RTV-d6	727.99	302.2	50	168	14

3.2 Assay Validation for the quantification of lopinavir and ritonavir in dried blood spots

The DBS method was validated following the FDA [6] and EMA guidelines [7] for bioanalytical assay validation. The validation parameters included: (1) matrix effect, percentage recovery and process efficiency; (2) selectivity/concomitant medications; (3) intra- and inter-day precision and accuracy; (4) carry-over; and (5) stability. The hematocrit effect and the variation of DBS spotting were also determined.

3.2.1 Matrix effect, Percentage recovery and Process Efficiency

The matrix effect, percentage recovery and process efficiency of components in DBS were determined by comparing the signal response at three levels: Low QC Sample (LQC), Medium QC Sample (MQC) and High QC Sample (HQC).

3.2.1.1 Matrix Effect/Ion Suppression

The potential matrix effect of components in DBS were determined by comparing the signal response from the following sets of samples:

Set 1: **No matrix**: LQC, MQC and HQC were prepared in the diluent; 5 replicates of LQC, MQC and HQC were analyzed.

Set 2: **Post-extraction spike**: blank DBS samples were extracted. The extracted matrix is spiked with analytes to generate LQC, MQC and HQC; 5 replicates of LQC, MQC and HQC were analyzed, each replicate is from a different source of blank DBS.

Calculation: the matrix effects were determined by calculating the ratio of the signal of the Post-extraction spike / No matrix. This ratio is determined for both analyte and internal standard at each level of validation sample:

$$\text{Matrix Effect} = \frac{\text{Signal Response in } \textbf{Post extraction}}{\text{Signal response in } \textbf{No matrix}} \times 100$$

Acceptance criteria: if the difference between the mean Post-extraction spike/No matrix ratio is <5% it can be considered that there is no major matrix effect between the analyte and internal standard.

3.2.1.2 Percentage Recovery

The recovery of analytes during the extraction process were determined by comparing the signal response from the following sets of samples:

Set 1: **Pre-extraction spike**: blank DBS samples spiked with analytes to LQC, MQC and HQC; 5 replicates of LQC, MQC and HQC were extracted and analyzed, each replicate is from a different source of blank DBS.

Set 2: **Post-extraction spike**: blank DBS samples were extracted. The extracted matrix was spiked with analytes to LQC, MQC and HQC; 5

replicates of LQC, MQC and HQC were analyzed, each replicate was from a different source of blank DBS.

Calculation: the percentage recovery was determined by calculating the ratio of the signal of the Pre-extraction spike/Post-extraction spike. This ratio is determined for the analyte and internal standard at each level of validation sample.

$$\text{Percentage recovery} = \frac{\text{Signal Response in } \mathbf{Pre\ extraction} \times 100}{\text{Signal response in } \mathbf{Post\ extraction}}$$

Acceptance criteria: the coefficient of variation (%CV) of the percentage recoveries for all of QC levels should be ≤ 15%. The percent recoveries of the analytes not need to be 100%, but the extent of recovery of an analytes and of the internal standard should be consistent, precise, and reproducible.

3.2.1.3 Process efficiency

The Process efficiency of the analytes was determined by comparing the signal response from the following Sets of samples:

Set 1: **No matrix**: LQC, MQC and HQC were prepared in the diluent: 5 replicates of LQC, MQC and HQC were analyzed.

Set 2: **Pre-extraction spike**: Blank DBS samples spiked with analytes to generate LQC, MQC and HQC; 5 replicates of LQC, MQC and HQC should be extracted and analyzed, each replicate is from a different source of blank DBS.

Calculation: the percentage recovery is determined by calculating the ratio of the signal of the Pre-extraction spike/No matrix. This ratio is determined for the analyte and internal standard at each level of validation sample.

$$\text{Process Efficiency} = \frac{\text{Signal Response in } \mathbf{Pre\ extraction} \times 100}{\text{Signal response in } \mathbf{No\ matrix}}$$

Acceptance criteria: process efficiency of the analytes need not be 100%, but the extent of recovery of an analytes and of the internal standard should be consistent, precise, and reproducible.

3.2.2 Selectivity/Concomitant Medications

Possible interference of endogenous compounds in the matrix and co-administered drugs with each analyte were assessed by:

- Analysis of DBS samples prepared from 6 individual sources of blank whole blood (matrix sample processed without analyte and internal standard),
- Analysis of DBS samples prepared from the whole blood spiked with the other antiretroviral drugs.

3.2.3 Sensitivity/Lower limit of quantification (LLOQ)

A LLOQ of 50 ng/mL were assessed, which is the same concentration as the LLOQ for the plasma assay. This concentration point was considered from the carry over result and the response need to be at least five times the response compared to blank response.

3.2.4 Calibration Standard (CS) for inter-method performance

A calibration curve was prepared by spiking whole blood with 9 points of known concentrations over the range 50 to 20,000 ng/mL then spotted on Whatman 903 filter paper. The back calculated values and reproducibility (inter-day) for each calibration standard method for all standard curves was calculated and reported.

Calculation:

Accuracy: $\%\text{Deviation} = \frac{(\text{Observed conc.} - \text{Theoretical conc.})}{(\text{Theoretical concentration})} \times 100$

Precision: $\%\text{CV} = \frac{\text{SD}}{\text{Mean}} \times 100$

Acceptance criteria: at least 75% of standards, with a minimum of six standard levels, must fulfill the following criterion; ± 20% deviation of the LLOQ from nominal concentration; ± 15% deviation of standards other than LLOQ from nominal concentration.

3.2.5 Accuracy and Precision (intra/inter-method performance)

The accuracy and precision of the method was determined by analysis of samples spiked with known amounts of drugs at 4 levels of the calibration curve:

(1) Lower Limit of Quantification (LLOQ) at 50 ng/mL

(2) Low concentration Validation Sample (LQC) at 150 ng/mL (within 3x LLOQ)

(3) Mid concentration Validation Sample (MQC) at 1,500 ng/mL (mid-range of curve)

(4) High concentration Validation Sample (HQC) at 16,000 ng/mL (≥ 80% of highest calibration level)

Six replicate DBS samples of LLOQ, LQC, MQC and HQC in 3 separate analytical runs will be analyzed.

Calculation:

Accuracy: $\%\text{Deviation} = \frac{(\text{Observed conc.} - \text{Theoretical conc.})}{(\text{Theoretical concentration})} \times 100$

Precision: $\%\text{CV} = \frac{\text{SD}}{\text{Mean}} \times 100$

Acceptance criteria for accuracy: means of the LQC, MQC and HQC validation samples must be within 15% of the theoretical value and the mean of the LLOQ validation samples must be within 20% of the theoretical value.

Acceptance criteria for precision: means of the LQC, MQC and HQC validation samples must have %CVs of less than 15% and the mean of the LLOQ validation samples %CV must be less than 20%.

3.2.6 Carry-over

Carry-over were assessed by injecting extracted blank samples after a high concentration sample or calibration standard at the upper limit of quantification. Inject blank samples at least three samples.

Calculation:

$$\%\text{Different} = \frac{(\text{Response in observed blank} - \text{Response in LLOQ})}{(\text{Response in LLOQ})} \times 100$$

Acceptance criteria:

- At the analyte retention time, the mean response in blank sample must be ≤20% of the mean analyte response in the acceptable LLOQ standards.
- IS response ≤5% of the mean IS response in the acceptable LLOQ standards.

3.2.7 Stability

At least five spots of the LQC and HQC were determined and evaluated for the stability of the analyses during samples collection and handling as following;

- Freeze-thaw cycles, five freeze-thaw cycles of DBS samples at -20°C and thawed at room temperature,

- Short Term Stability in DBS at any variation of temperature (room temperature (20-25°C), refrigerated condition (4-8°C)) for one week,

- Stability in Injection Matrix (re-inject triplicate low and high validation samples from one validation day, values are read off the same standard curve, and compared to each other).

- Long-Term Stability (samples were analyzed after one week, one month and three months and 1 year).

The stability of processed samples was determined as follows.

Calculations:

Accuracy from control:

$$\%\text{Different} = \frac{(\text{Treated conc.} - \text{Control conc.})}{(\text{Control concentration})} \times 100$$

Accuracy from theoretical value:

$$\%\text{Different} = \frac{(\text{Treated conc.} - \text{Theoretical conc.})}{(\text{Theoretical concentration})} \times 100$$

Acceptance criteria: means of validation samples must be within 15% of original value.

3.2.8 Hematocrit effect

Hematocrit is currently identified as the single most important parameter influencing the spread of blood on DBS cards, which could impact the validity of the results generated by DBS methods, affecting the spot formation, spot size, drying time, homogeneity, and ultimately, the robustness and reproducibility of the methods. DBS samples were prepared at low level (QC Low) and high level (QC High) from whole blood with 30% hematocrit and 60% hematocrit then analyzed and compared with the control DBS sets prepared from whole blood with 45% hematocrit.

Calculations:

Accuracy from control:

$$\%\text{Different} = \frac{(\text{Treated conc.} - \text{Control conc.})}{(\text{Control concentration})} \times 100$$

Acceptance criteria: difference between means of the treated (low or high % hematocrit) and untreated (normal % hematocrit) validation samples must be less than 15%.

3.2.9 DBS spotting technique

The spotting technique can affect the validity of the results even though the exactly volume of blood is used for DBS spotting. The validation of DBS spotting effect was performed by spotting the DBS with the blood at LQC and HQC on Whatman 903 filter paper using a calibrated capillary (50µL) and then compared with the control sets prepared by using a micropipette (50µL).

Calculations:

Accuracy from control:

$$\%\text{Different} = \frac{(\text{Treated conc.} - \text{Control conc.})}{(\text{Control concentration})} \times 100$$

Acceptance criteria: difference between means of the treated (spot by using calibrated capillary) and untreated (using a micropipette) validation samples must be less than 15%.

3.3 Study Design for Comparison of Methods

To assess the agreement between lopinavir and ritonavir concentrations in plasma and DBS a retrospective analysis of stored plasma and DBS samples collected within an observational cohort study of HIV-infected patients was performed. These samples were sent to the PHPT-AMS pharmacology laboratory at the Faculty of Associated Medical Science, Chiang Mai University for therapeutic drug monitoring (TDM) of antiretroviral drugs.

Plasma samples were measured using a previously internally validated method within the Program for HIV Prevention and Treatment laboratory at the Faculty of Associated Medical Sciences, Chiang Mai University. This method was originally developed by Rob ter Heine et al[9]. Lopinavir and ritonavir concentrations DBS samples were quantified using the new validated method. The LC-MS/MS assay to quantify lopinavir and ritonavir in plasma was served as the 'standard' method and the DBS LC-MS/MS assay was the 'candidate' method.

To ensure the reliable results duplicate internal QC samples (low, medium and high level) were included in every assay run. The assay run was accepted if the QC acceptance criteria were met (e.g. at least 67% of the QC samples were <15% from nominal values).

3.3.1 Sample size for method comparison

The Clinical and Laboratory Standards Institute (CLSI) Guidelines for 'Measurement Procedure Comparison and Bias Estimation Using Patient Samples' state that for method comparison and bias estimation a minimum of 40 patient specimens are required. If the methods being compared use either a different chemical reaction, difference principle of measurement or different matrix then large numbers of patient specimens need to be assessed, i.e. up to 100 to 200 samples are recommended [13]. To assess whether lopinavir and ritonavir concentrations in plasma and DBS are similar, 180 paired plasma and DBS samples from patients receiving LPV/RTV were tested.

3.3.2 Paired Plasma and DBS Sample Collection from HIV-infected Patients

As part of the routine follow up in the PHPT cohort study a whole blood samples was collected in spray dried-EDTA tubes. These blood tubes were transferred to the local hospital laboratory within 1 hour of collection to prepare the DBS and plasma samples (Figure 3.6).

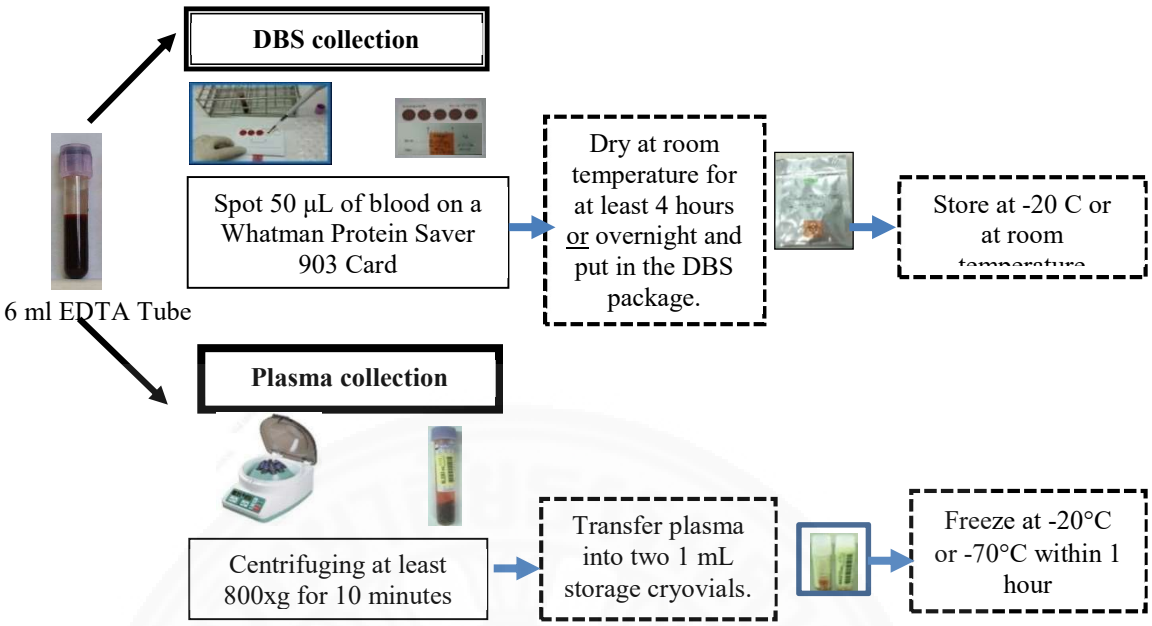


Figure 3.8 Summary of the process to prepare the paired plasma and DBS samples

3.3.2.1 DBS Sample Preparation

Dried Blood Spots samples were prepared by applying 50 µL of whole blood to a single spot on a Whatman Protein Saver 903 Card using a pipette or calibrated capillary. The process was repeated four times to fill all five spots on the card. The blood spot was allowed to dry at room temperature for at least 4 hours. Once dried, the DBS card was put in a gas-impermeable zip-lock bag containing a desiccant pack and stored at -20°C.

Figure 3.9 DBS sample (50 uL per spot), storage bag and desiccants

Invalid DBS samples were excluded from this analysis. Blood spots considered invalid were those that did not fill the entire spot, over-sized spot, light colour (suspected low hematocrit), dark red colour (suspected high hematocrit). Examples of invalid DBS cards are show below:

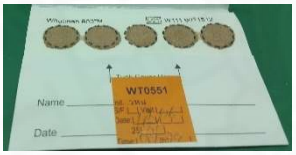
	
Small DBS Spots	**Over size spots and blood fused between spots**
	
Layered spots (did not mix well before spotting)	**Sample clot and not allowed to dry before putting in the storage bag.**
	
DBS samples with suspected lower Hct (light colour)	**DBS samples with suspected high Hct (Dark Red Colour)**

Figure 3.10: Examples of invalid DBS sample

3.3.2.2 Preparation of Plasma Samples

The remaining whole blood from DBS preparation was centrifuged (800xg for 10 minutes) at room temperature. The separated plasma was transferred into 1 mL cryovial tube with a screw cap (at least 0.15 mL/tube) and stored at -20°C or -70°C until analysis.

3.3.3 Statistical Analysis[14), (15), (16)]

The comparison of lopinavir and ritonavir concentrations in plasma and dried blood spots were performed using linear regresssion analysis and Bland-Altman plots [14-16]. Statistic analyses was performed using SPSS Statistic Software (version 22). The percentage error was also calculated and reported. The method to determine lopinavir and ritonavir in plasma served as the 'standard' method and the method to determine lopinavir and ritonavir in DBS was the 'candidate' method.

3.3.3.1 Comparison plots (Correlation Analysis)

The drug concentrations of lopinavir and ritonavir were plotted and displayed on a X-Y comparison plot, with the results from the standard method on the x-axis (independent variable) and the data from the candidate method plotted on an y-axis. The line of equality on which all points would lie if the two method give exactly the same reading was also plotted. Frequency distributions and scatter diagrams were plotted to inspect the distribution of data and explore the relationshsip between lopinavir and ritonavir contrations measured in plasma and DBS.

3.3.3.2 Bland-Altman analysis

The agreement between the drug concentrations of lopinavir and ritonavir measured from plasma and DBS was analyzed using a Bland-Altman Plot. The Bland-Altman Plot is a XY scatter plotthe Y axis shows the percent deviation between the lopinavir and ritonavir drug concentrations measured from DBS and Plasma [(Measurement from DBS – Measurement from plasma)/ Measurement from plasma)]; and the X-axis represents the average of lopinavir and ritonavir concentrations from DBS and plasma [(Concentrations from DBS + Concentrations from Plasma)/2]. To assess if the data were normally distributed a Shapiro-Wilk's test was performed. The drug measurement in DBS (DBS assay) was calssified as the independent variable and the data from the candidate method. The overall mean perccent deviration between drug concentrations measured in plasma and DBS represents the 'bias' and quantifies how much higher or lower values are with the new method compared with the standard method. Ideally, the bias should be less than 20% in the context of this study. The standard deviation (SD) of all the individual differences were calculated as a measure of variability. The limits of agreement represents the range of values in which agreement between methods lie for

approximately 95% of samples. The 95% confidence limits of the Normal distribution was used to define the limites of agreement (mean percent deviation +/-1.96 SD).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Validation of an LC-MS/MS assay to measure lopinavir/ritonavir in DBS

4.1.1 LC-MS/MS chromatograms

A typical chromatogram showing the MRM chromatographic peaks of all analytes are shown in Figures 4.1 to 4.6. The retention times of RTV and LPV were 7.8 and 8.5 minutes, respectively. The stable-isotope-labeled internal standards (RTV-d6 and LPV-d8) had the same retention time as the analytes.

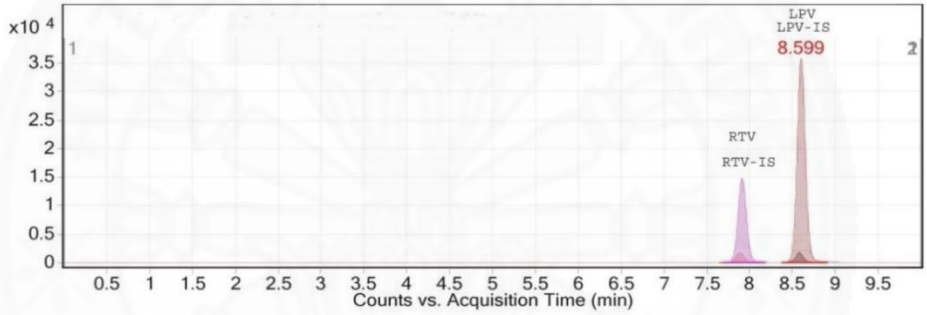


Figure 4.1: MRM chromatogram for lopinavir and ritonavir. RTV and RTV-d6-IS peaks were at 7.8 minutes; LPV and LPV-d8-IS peaks were at 8.5 minutes.

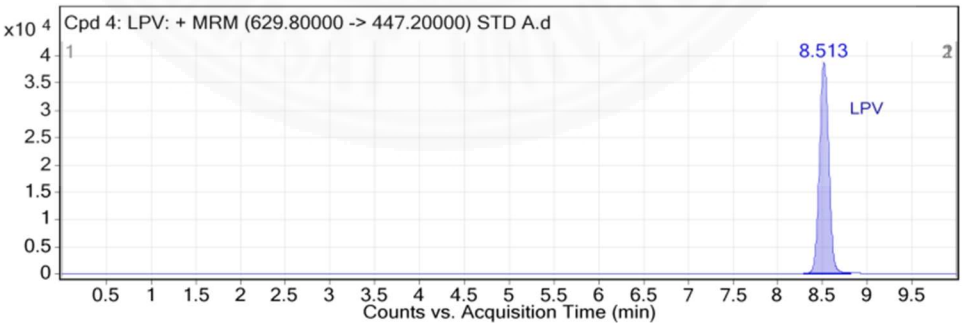


Figure 4.2: MRM Chromatogram of LPV STD A: 20,000 ng/mL (m/z 629.80->447.20)

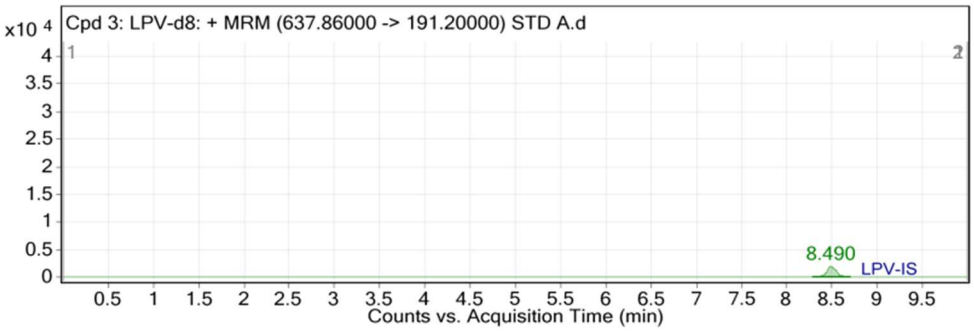


Figure 4.3: MRM Chromatogram of LPV-d8-IS: 500 ng/mL (m/z 637.86->101.20)

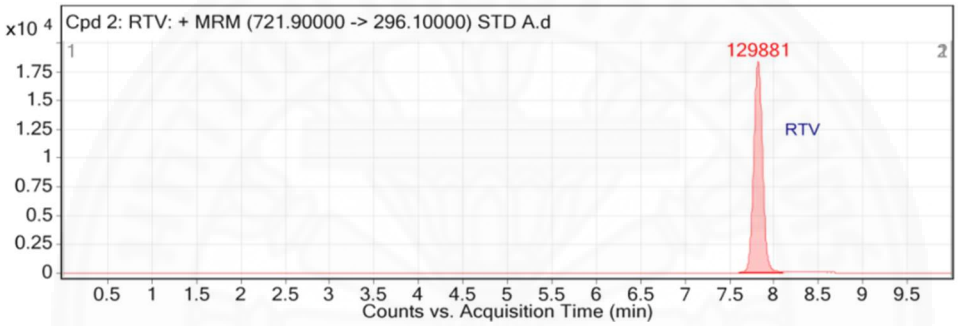


Figure 4.4: MRM Chromatogram of RTV STD A: 20,000 ng/mL (m/z 721.9->296.1)

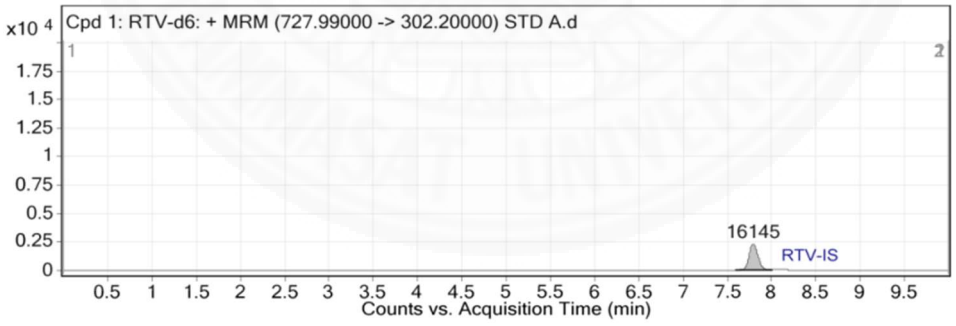


Figure 4.5: MRM Chromatogram of RTV-d6-IS: 500 ng/mL, (m/z 727.99->302.20)

4.1.2 DBS Assay Validation results

4.1.2.1 Matrix Effect, Percentage Recovery and Process efficiency

The % matrix effect for RTV and LPV were 93.5% and 120.7%, respectively. The % matrix effects for internal standards were 93.3% for RTV-d6-IS and 107.5% for LPV-d8-IS. There was an enhancement matrix effect for LPV and minor suppression for RTV. All analytes (LPV and RTV) were quantified using the area ratio of analytes and internal standard. The peak area of the isotopic internal standards had an enhancement for LPV-d8-IS and minor suppression for RTV-d6-IS, similar to their corresponding analytes. The matrix effect was considered acceptable: % matrix effect of area ratio for LPV/LPV-d8-IS and RTV/LPV-d6-IS peak area were 102.1% and 95.7%, respectively. The % recovery and % process effect were also acceptable for all analytes (Table 4.1).

Table 4.1 Matrix Effect (ME), Recovery (RE), and Process Efficiency (PE) for LPV and RTV in DBS Summary Table

Analyte	Validation parameters		Analyte	IS	Area Ratio (Analyte/IS)
LPV	**Matrix Effect (ME)**	Range	96.7 - 139.3	96.2 - 117.9	92.0 - 118.2
		Mean	120.7	107.5	102.1
	Recovery (RE)	Range	74.7 - 92.7	86.6 - 101.6	82.5 - 107.6
		Mean	82.1	92.9	100.4
	Process efficiency (PE)	Range	89.7 - 109.9	97.7 - 102.1	91.0 - 96.0
		Mean	97.9	99.3	97.6
RTV	**Matrix Effect (ME)**	Range	85.4 - 96.0	86.4 - 105.7	90.9 - 113.4
		Mean	93.5	93.3	95.7
	Recovery (RE)	Range	97.7 - 108.7	93.1 - 107.0	98.6 - 105.2
		Mean	102.6	100.9	101.8
	Process efficiency (PE)	Range	92.9 - 100.4	90.0 - 98.5	95.6 - 111.8
		Mean	95.7	93.7	102.7

The % matrix effect, % recovery and % process effect for each analyte and the internal standard in DBS are shown in **Tables A1-A2** (see Appendix A).

4.1.2.2 Selectivity/Concomitant Medications

No interfering signals were detected for RTV and RTV-IS (7.8 minutes) and LPV and LPV-IS (8.5 minutes) in blank samples from five different sources (Figures 4.7 to 4.11). No interference with the concomitant antiretroviral medications: lamivudine, abacavir and zidovudine were observed (Figure 4.12).

Figure 4.6: MRM Chromatogram of blank DBS Source 1 at the mass transition for LPV (m/z 629.80->447.20), LPV-IS (m/z 637.86->191.20), RTV (m/z 721.90->296.10) and RTV-IS (m/z 727.99->302.20).

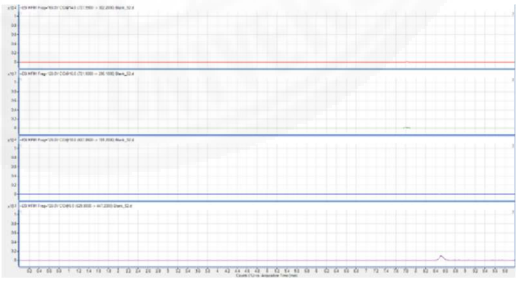


Figure 4.7: MRM Chromatogram of blank DBS Source 2 at the mass transition for LPV (m/z 629.80->447.20), LPV-IS (m/z 637.86->191.20), RTV (m/z 721.90->296.10) and RTV-IS (m/z 727.99->302.20).

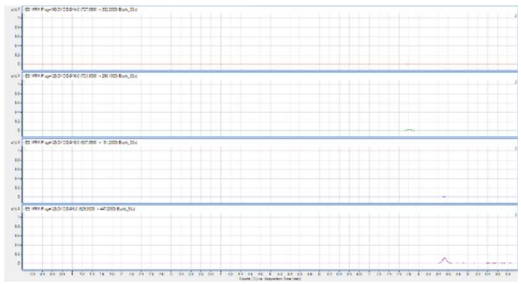


Figure 4.8: MRM Chromatogram of blank DBS Source 3 at the mass transition for LPV (m/z 629.80->447.20), LPV-IS (m/z 637.86->191.20), RTV (m/z 721.90->296.10) and RTV-IS (m/z 727.99->302.20).

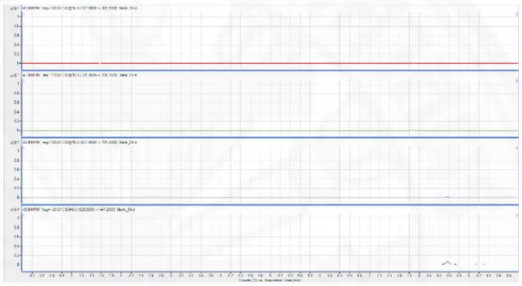


Figure 4.9: MRM Chromatogram of blank DBS Source 4 at the mass transition for LPV (m/z 629.80->447.20), LPV-IS (m/z 637.86->191.20), RTV (m/z 721.90->296.10) and RTV-IS (m/z 727.99->302.20).

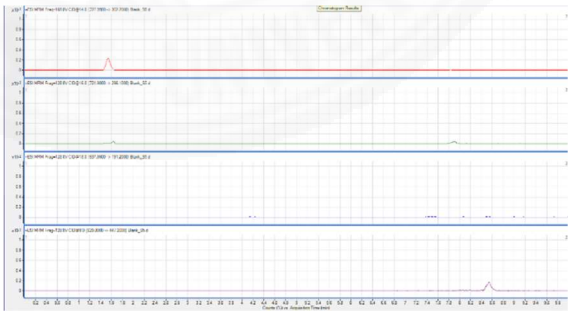


Figure 4.10: MRM Chromatogram of blank DBS Source 5 at the mass transition for LPV (m/z 629.80->447.20), LPV-IS (m/z 637.86->191.20), RTV (m/z 721.90->296.10) and RTV-IS (m/z 727.99->302.20).

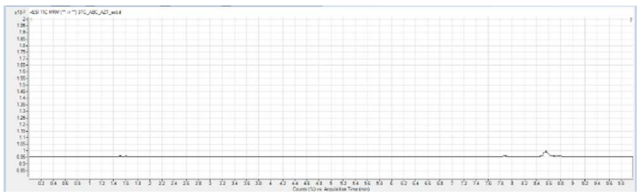


Figure 4.11: Chromatogram of DBS sample prepared from whole blood spiked with lamivudine, abacavir and zidovudine.

4.1.2.3 Sensitivity/Lower limit of quantification (LLOQ)

A small peak was detected at the retention time of LPV (8.5 minutes) but the response was not significant as it was <5 times response of the LLOQ. [Figures 4.13 to 4.14].

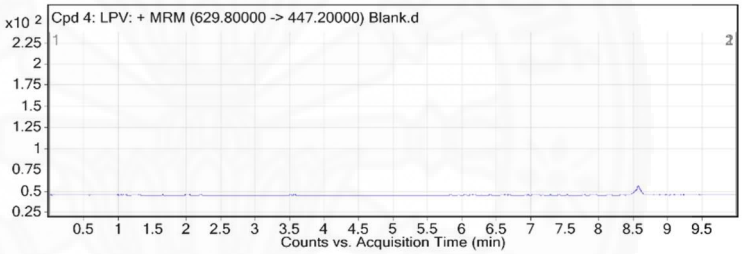


Figure 4.12: MRM Chromatogram of blank DBS at the mass transition for LPV (m/z 629.80->447.20).

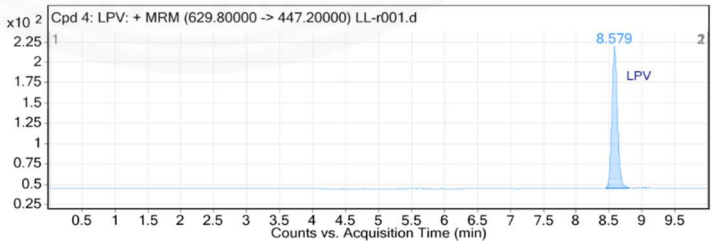


Figure 4.13: MRM Chromatogram of blank DBS at the mass transition for LPV (m/z 629.80->447.20).

No interfering signals were detected at the retention time of RTV (7.8 minutes) [Figures 4.14 to 4.15].

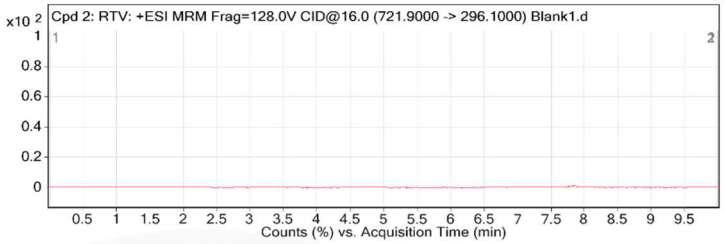


Figure 4.14: MRM Chromatogram of blank DBS at the mass transition for RTV (m/z 721.90->296.10).

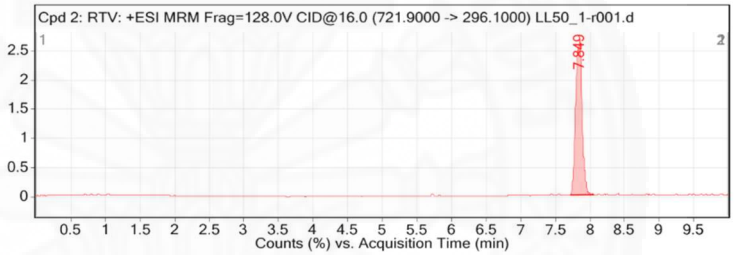


Figure 4.15: MRM Chromatogram of RTV LLOQ: 50 ng/mL (m/z 721.90->296.10).

4.1.2.4 Calibration Curves

The calibration curves over the range 50-20,000 ng/mL for LPV and RTV were plotted and fitted using $1/x^2$ weighted linear regression of the peak area ratios (drug peak area/I.S. peak area) versus concentrations. The calibration curves were run in singlet in 3 separate analytical runs. For each curve the slope, intercept, and correlation coefficient (linearity), as well as the accuracy and precision of the back calculated concentration for each calibration level (i.e. SD, %CV and % deviation from target) was determined and the results are shown in Tables B1-B2 (see Appendix B). Typical calibration curves for each analytes are also shown below Tables B1 – B2. (Note: For the Day#3 calibration curve the 100 ng/ml calibration standard (#2) was excluded)

All the calibration curves showed good linear correlation. The coefficient of determination (R^2) for the calibration curve for lopinavir and

ritonavir were >0.990 and >0.994, respectively. The accuracy and precision of the back calculated concentrations at each calibration level were within the acceptable limits. The lower LLOQ was 50 ng/mL for all both LPV and RTV.

4.1.2.5 Inter and Intra Assay Accuracy and precision

For both lopinavir and ritonavir, the accuracy and precision at the LLOQ, LQC, MQC and HQC were determined and the results are shown in Tables 3a-3b (see Appendix).

4.1.2.6 Intra-assay accuracy results (%dev)

For the LQC, MQC and HQC ranged from +3.0% to +11.5% for lopinavir and +3.9% to +11.5% for ritonavir. At the LLOQ, the %dev ranged from +1.9% to +4.0% for lopinavir and -9.8% to +5.6% for ritonavir.

4.1.2.7 Intra-assay precision results (%CV)

For the LQC, MQC and HQC ranged from 0.8% to 4.9% for lopinavir and 1.4% to 6.4% for ritonavir. At the LLOQ, the %CV ranged from 3.1% to 5.6% for lopinavir and 2.7% to 5.6% for ritonavir.

4.1.2.8 Inter-assay accuracy results (%dev)

For the LQC, MQC and HQC ranged from +6.7% to +10.8% for lopinavir and +7.1% to +10.1% for ritonavir. At the LLOQ, the inter-assay %dev was +2.9% for lopinavir and -1.6% for ritonavir.

4.1.2.9 Inter-assay precision results (%CV)

For the LVS, MVS and HVS ranged from 2.6% to 4.1% for lopinavir and 2.9% to 4.3% for ritonavir. At the LLOQ, the inter-assay %CV was 4.4% for lopinavir and 8.0% for ritonavir.

Overall, the intra- and inter-day accuracy and precision for lopinavir and ritonavir were within the acceptable limits. Means of the LQC (150 ng/mL), MQC (1,500 ng/mL) and HQC (16,000 ng/mL) validation samples were within 15% of the theoretical value and the mean of the LLOQ validation samples (50 ng/mL) were within 20% of the theoretical value. A summary of the assay precision and accuracy results are shown in Table C1-C2 (Appendix C). The assay average accuracy was 102-112% for lopinavir and 90-112% for ritonavir. The assay precision (inter- and intra assay) expressed as coefficient of variation (%CV) was <5% for lopinavir and <8.0% for ritonavir (Table 4.2).

Table 4.2 Inter- and intra assay accuracy and precision for LPV and RTV

Analyte	Level	Conc. (ng/mL)	Intra Assay (n = 6)			Inter Assay (n=18)		
			% Accuracy	% Variation	SD	% Accuracy	% Variation	SD
LPV	LLOQ	50	101.93 - 104.04	3.11 - 5.57	1.63-2.84	102.90	4.38	2.25
	Low	150	103.03 - 109.46	0.81 - 4.82	1.33-7.45	106.67	4.09	6.54
	Med	1500	108.40 - 109.33	1.54 - 4.04	25.04 - 66.01	108.92	2.57	42.07
	High	16000	109.60 - 111.50	1.88 - 4.94	334.30 - 886.95	110.75	3.17	562.49
RTV	LLOQ	50	90.18 - 105.59	2.65 - 5.61	1.20 – 2.81	98.43	8.04	3.96
	Low	150	103.92 - 110.90	2.65 - 5.25	2.87 – 8.18	107.07	4.23	6.80
	Med	1500	109.00 - 110.75	1.37 - 4.68	22.81 – 76.48	110.13	2.94	48.55
	High	16000	106.74 - 111.54	1.72 - 6.38	307.25 – 1101.95	108.72	4.31	750.41

4.1.2.10 Carry-over

The % carry-over from the injection of the highest calibration standard LPV and RTV were less than 20% of the peak area at the LLOQ level and less than 5% for the internal standards; Tables D1 – D2 (see Appendix D).

4.1.2.11 Stability

Both LPV and RTV were shown to be stable in whole blood at 4°C for 24 hours before spotting and in DBS after 5 freeze thaw cycles, 2 to 8°C for 1 week and at -20ºC and at room temperature for 3 months. A summary of the stability results are shown in Table 4.3. Overall, the stability results of LPV and RTV in DBS were within the acceptable limits (±15% of original value); Tables E1 – E7 (Appendix E).

4.1.2.12 The effect of Hematocrit and DBS preparation technique for LPV and RTV in DBS

There was no effect from hematocrit on the measurement of LPV and RTV in DBS for hematocrit values between 30-60%. DBS spotting using a calibrated capillary was not different from a using micropipette. A summary of the results for the effect of hematocrit and method of DBS spotting are shown in Table 4.4. Overall, the effect of hematocrit and DBS preparation results of LPV and RTV in DBS were within the acceptable limits (±15% of original value); Tables F1 – F3 (see Appendix F).

Table 4.3 Stability Data for LPV and RTV in DBS under various conditions and storage

Stability Condition/ Variation parameters	LPV				RTV			
	Low		High		Low		High	
	% mean diff	%CV	% mean diff	%CV	% mean diff	%CV	% mean diff	%CV
In whole blood at 4°C for 24 hours	105.79	3.05	95.11	7.48	107.48	3.54	92.85	7.35
Freeze Thaw 5 cycles	105.44	7.35	97.41	1.75	103.78	8.36	93.01	1.76
In injection matrix at 2-8°C for 1 week	107.67	0.75	100.14	0.43	110.24	1.31	98.57	0.37
In DBS at room temperature for 1 week	109.96	2.05	108.74	4.29	110.52	3.61	103.65	5.55
In DBS at 2-8°C for 1 week	112.46	0.66	105.96	5.28	111.22	1.14	102.82	8.40
In DBS at room temperature for 3 months	92.19	4.55	97.57	5.52	92.99	7.07	92.73	5.62
In DBS at -20°C for 3 months	95.46	7.68	98.44	0.72	93.67	8.05	98.26	0.21

Table 4.4 Effect of Hematocrit and DBS spotting methods for LPV and RTV in DBS

Variation parameters	LPV				RTV			
	Low		High		Low		High	
	% mean diff	%CV	% mean diff	%CV	% mean diff	%CV	% mean diff	%CV
Hct (30%)	98.09	5.60	98.05	2.26	102.40	4.56	99.57	2.72
Hct (60%)	98.44	4.41	100.23	3.74	104.30	4.82	99.83	4.69
Prepare DBS by using 50 uL calibrated capillary tube	92.09	2.26	101.93	0.79	102.15	7.28	105.44	0.43

4.2 Comparison of DBS and plasma concentrations in HIV-infected patients

4.2.1 Paired patient plasma and DBS samples

Concentrations of lopinavir and ritonavir were testes from paired plasma and DBS samples collected from 180 HIV-infected patients. Twenty-five samples had both lopinavir and ritonavir concentration below the LLOQ (50 ng/mL) in both plasma and DBS samples and these samples were excluded from the analysis. Three samples had lopinavir above the LLOQ but ritonavir below the LLOQ but were retained in the analysis. The characteristics of the 155 patients included are summarized in Table 4.5. The median age was 29 years (range 3 to 70), samples collected between 0.1 to17 hours after the last doses, hematocrit range from 28.1-48.2% (n=116 samples).

Table 4.5 Patient characteristics

Characteristic	Median (range)
Age (years)	29 (3-70)
%Hematocrit (available from 116 patients)	34 (28.1 – 48.2)
Duration time after last drug intake (hours)	2.4 (0.1 – 17)

4.2.2 Correlation between lopinavir (LPV) drug concentrations in DBS and plasma

Plasma and DBS concentrations for lopinavir (LPV) was highly correlated (Pearson correlation r=0.964). The DBS concentrations fall under the equality line demonstrating that lopinavir (LPV) concentrations in DBS are lower than in plasma. The relationship between lopinavir (LPV) concentrations in DBS and plasma is shown in Figure 4.16.

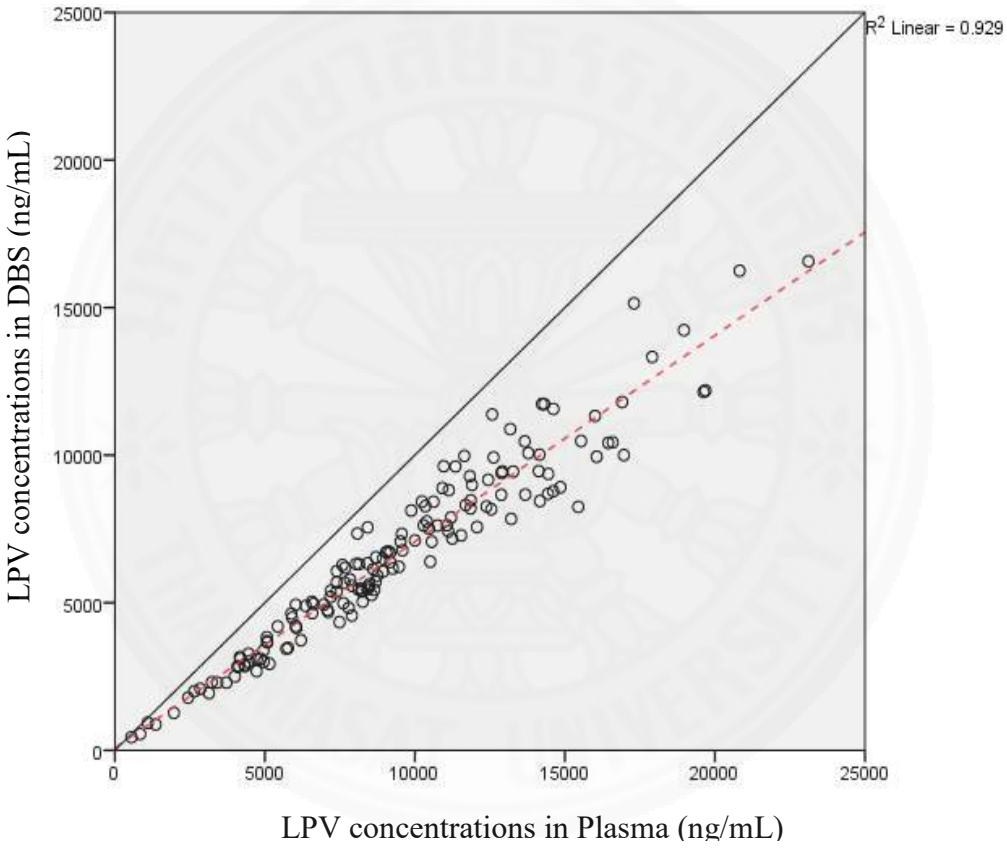


Figure 4.16: Comparison plot of LPV concentrations measured in DBS vs. plasma

4.2.3 Bland-Altman plot of percent deviation between lopinavir (LPV) concentrations in DBS (DBS assay) versus plasma (plasma assay)

A Shapiro-Wilk's test ($p>0.05$) showed that the percent deviation between lopinavir (LPV) drug concentrations measured from DBS and plasma were approximately normally distributed, with skewness of 0.32 (SE = 0.195) and a kurtosis of -0.292 (SE = 0.387).

The Bland-Alman plot for lopinavir (LPV) is shown in Figure 4.17. The percent deviation of DBS concentration from plasma [(DBS conc.-Plasma conc.)/Plasma conc.] was 29.3% lower (95% confidence interval (CI): -86.5% to 28.1%; p=0.189).

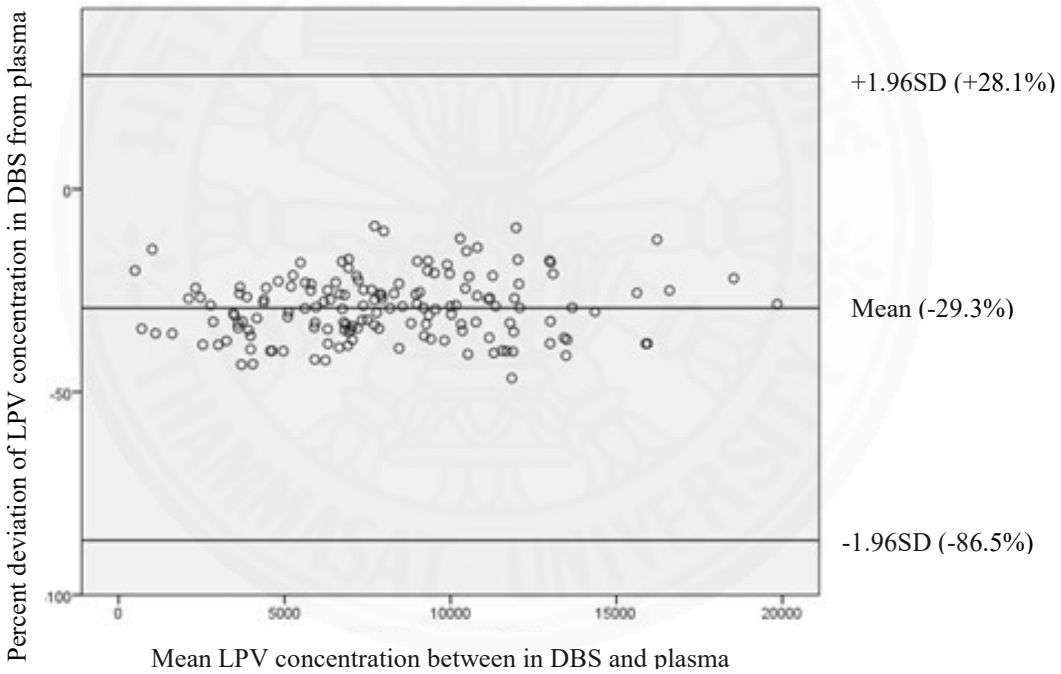


Figure 4.17 Bland-Altman plot of percent deviation between LPV concentrations in DBS (DBS assay) versus with plasma (plasma assay)

4.2.4 Correlation between ritonavir (RTV) drug concentrations in DBS and plasma

Plasma and DBS concentrations for ritonavir (RTV) was highly correlated (Pearson correlation r=0.990). The DBS concentrations fall under the equality line demonstrating that ritonavir (RTV) concentrations in DBS are lower than in plasma. The relationship between ritonavir (RTV) concentrations in DBS and plasma is shown in Figure 4.18.

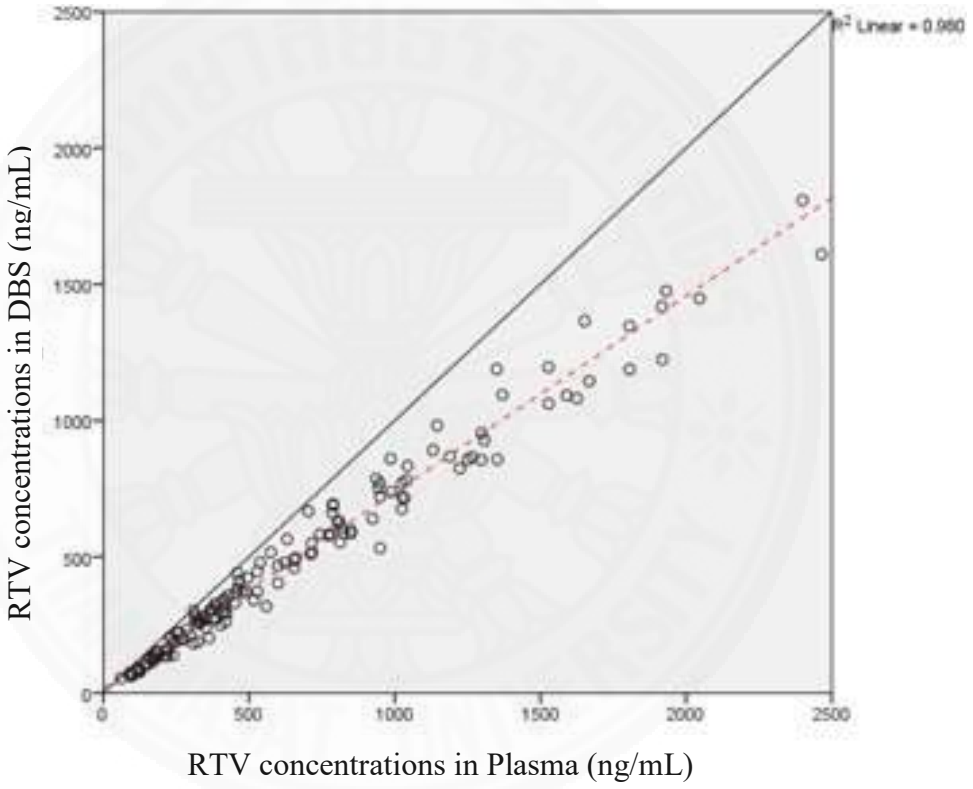


Figure 4.18 Comparison plot of RTV concentrations measured in DBS vs. plasma

4.2.5 Bland-Altman plot of percent deviation between ritonavir (RTV) concentrations in DBS (DBS assay) versus plasma (plasma assay)

Shapiro-Wilk's test ($p>0.05$) showed that the percent deviation between ritonavir (RTV) drug concentrations measured from DBS and plasma were approximately normally distributed, with skewness of 0.058 (SE = 0.196) and a kurtosis of -0.125 (SE = 0.390).

The Bland-Alman plot for ritonavir (RTV) is shown in Figure 4.19. The percent deviation of DBS concentration from plasma [(DBS conc.-Plasma conc.)/Plasma conc.] was 24.6% lower (95% CI: -72.8% to +23.6%; p=0.304).

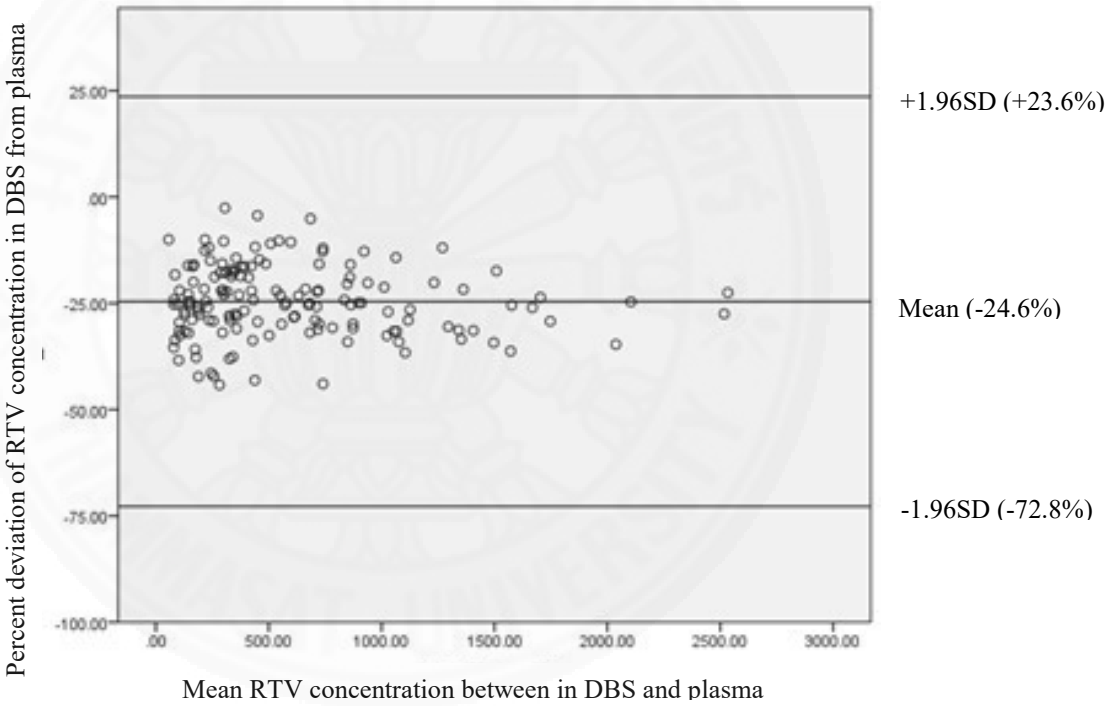


Figure 4.19 Bland-Altman plot of percent deviation between LPV concentrations in DBS (DBS assay) versus with plasma (plasma assay)

4.3 Discussion

We report a new validated method for the quantification of lopinavir and ritonavir in DBS. The ion suppression and matrix effect were reduced by using a 2-step extraction process (i.e. precipitation following by liquid-liquid extraction). The average assay accuracy (inter- and intra assay) was 102-112% for lopinavir and 90-112% for ritonavir. The assay precision (inter- and intra assay) expressed as coefficient of variation (%CV) was <6.0% for lopinavir and <9.0% for ritonavir. The recoveries for lopinavir and ritonavir were 82.1% and 102.6%, respectively. Both drugs were stable in DBS stored at room temperature for at 3 months; however, it will be important to demonstrate the long-term stability of lopinavir and ritonavir in DBS, e.g. for up to one year, to demonstrate the applicability of DBS samples to resource-limited settings.

Several LC-MS/MS assays for the quantification of lopinavir and ritonavir in DBS have been reported[9,10,11,12] but the impact of different hematocrit values was not included as part of the assay validation. A patient's hematocrit is an important factor for DBS samples as it can affect the spread of blood on the card and thus the volume of blood per punched spot. This is particularly important when only a 'partial punch' hole is used for testing. We observed no effect of sample hematocrit in samples with low hematocrit (30%), at normal hematocrit (45%) and at high hematocrit (60%) during the assay validation.

Limited data assessing the degree of agreement between lopinavir and ritonavir concentrations quantified in plasma and in DBS samples are available. Koal *et al*[9] reported a good correlation (r=0.984) between antiretroviral drugs concentrations in paired plasma and DBS from 70 patients (containing either atazanavir, saquinavir, LPV, RTV or efavirenz). Roland *et al.*[10] reported a strong correlation between plasma and DBS concentrations in 19 HIV-infected children for both LPV (r=0.921) and RTV (r=0.877). Despite the strong correlation no information on the level of agreement between the two assays was reported. We also found a strong correlation between LPV and RTV concentrations between DBS and plasma concentrations. Although the Bland-Almond plots showed that there was no proportional bias (p>0.05) between the DBS and plasma methods the lopinavir concentrations were 29.3% lower and ritonavir 24.6% lower in DBS than in plasma. The different drug concentrations in DBS and plasma could be due to the diffusion of the drug between blood cells and fraction of

drug bound to plasma proteins (fbpp). This observed difference between the sample matrixes could lead to a different interpretation of the drug level results. Also, given that standard efficacy and toxic antiretroviral concentration thresholds for clinical management are based on plasma drug concentrations using LPV or RTV blood concentrations without correction would not be acceptable. A relationship to predict plasma concentrations from DBS concentrations for lopinavir and ritonavir should be investigated (i.e. using linear or polynomial regression methods). Once the equations describing these relationships are derived they would need to be validated using a separate dataset to that used to generate the equations.

Even though no hematocrit effect was observed during the assay validation it may be important to assess samples with a hematocrit less than 30% and above 60%. For example, in the patient samples tested there was a trend that patients with a low hematocrit had a smaller deviation between drug concentrations in DBS and plasma. Preparing standard calibration samples from blood with different levels of hematocrit may help to solve this problem (in our study the standard calibration samples were prepared from blood with 45% hematocrit).

Finally, a limitation of our study was that DBS samples were prepared from a venous blood sample. This was decided as the DBS needed to be prepared using an exact volume of blood and mixed well before spotting. Often DBS are prepared from a finger or heel prick and it will be important to determine if the LPV and RTV concentrations in a DBS prepared from a venous or finger/heel prick (i.e. capillary) are similar.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions and Recommendations

The validated LC-MS/MS assay for the quantification of lopinavir and ritonavir in Dried Blood Spots (DBS) was robust, accurate and precise. DBS sample collection is ideal for neonates and small children as it is less invasive and only requires a small blood volume. In addition, DBS samples are considered non-infectious so can be shipped by standard post mail without temperature control. DBS samples are also preferable in remote areas with no equipment for plasma collection and facilities for sample storage and shipment under frozen condition.

In paired DBS-plasma samples from HIV-infected patients both lopinavir and ritonavir concentrations were lower in DBS than in plasma samples. Current efficacy and toxic antiretroviral concentration thresholds for clinical management are based on plasma drug concentrations. Thus, DBS results need to be adjusted to estimate the corresponding plasma concentration before interpretation. The DBS assay developed can be useful for the clinical management by providing qualitative results or semi-quantitative results to monitor drug adherence and drug toxicity of the patients.

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APPENDICES

APPENDIX A

MATRIX EFFECTS (ME), RECOVERY (RE), AND PROCESS EFFICIENCY (PE) RESULTS

TABLE A1: Matrix Effect (ME), Recovery (RE), and Process Efficiency (PE) for LPV in DBS

Level	Matrix Effect Post-E/Non-E			Recovery Effect Pre-E/Post-E			Process Effect Pre-E/Non-E		
	Analyte	IS	Area ratio (Analyte/IS)	Analyte	IS	Area ratio (Analyte/IS)	Analyte	IS	Area ratio (Analyte/IS)
Low	139	118	118	79	87	108	110	102	91
Med	126	109	96	75	91	83	94	98	96
High	97	97	92	93	102	91	90	98	92
Mean	121	108	102	82	93	100	98	99	98

Note: Non-E=No matrix set, Pre-Ex=Pre-extraction spike set, Post-Ex=Post-extraction spike set

TABLE A2: Matrix Effect (ME), Recovery (RE), and Process Efficiency (PE) for RTV in DBS

Matrix Effect Post-E/Non-E			**Recovery Effect Pre-E/Post-E**			**Process Effect Pre-E/Non-E**		
Analyte	**IS**	**Area ratio (Analyte/IS)**	**Analyte**	**IS**	**Area ratio (Analyte/IS)**	**Analyte**	**IS**	**Area ratio (Analyte/IS)**
99	88	113	101	102	99	100	90	112
85	86	99	109	107	102	93	93	101
96	106	91	98	93	105	94	99	96
9ก	93	101	103	101	102	96	94	103

Note: Non-E=No matrix set, Pre-Ex=Pre-extraction spike set, Post-Ex=Post-extraction spike set

APPENDIX B

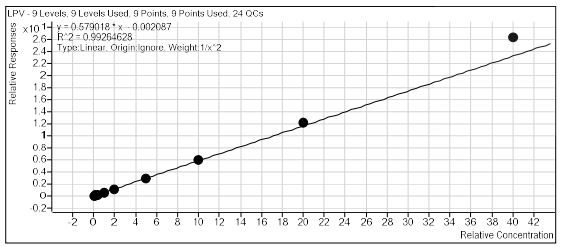
BACK CALCULATED VALUES FOR THE CALIBRATION STANDARDS

Table B1: Back calculated values for the Calibration Standards for **lopinavir (LPV) in DBS**

LPV	Run ID	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8	Cal 9	Slope	R^2	Y-Intercept	Weighting
21/Jan/15	1	54	90	186	486	940	2448	5115	10495	22672	0.57902	0.99265	-0.002087	1/x2
25/Jan/15	2	48	94	183	495	933	2507	5018	11066	21041	0.50814	0.99489	0.000820	1/x2
28/Jan/15	3	54	85	187	514	930	2505	5090	10486	21918	0.49128	0.99175	-0.000877	1/x2
Theoretical Conc.		**50**	**100**	**200**	**500**	**1,000**	**2,500**	**5,000**	**10,000**	**20,000**				
mean		52	90	185	498	934	2486	5075	10682	21877	0.52614	0.99310		
SD		4	4	2	15	5	34	50	332	816	0.04656	0.00161		
%CV		6.89	4.95	0.98	2.91	0.56	1.35	0.99	3.11	3.73	8.84923	0.16259		
%dev		3.99	-10.07	-7.32	-0.36	-6.58	-0.54	1.49	6.82	9.38				
n		3	3	3	3	3	3	3	3	3	3	3		

Note: For the Day#3 calibration curve, the 100 ng/ml calibration standard (#2) was excluded.

Calibration Curve Run Day #1



Calibration Curve Run Day #2

Calibration Curve Run Day #3

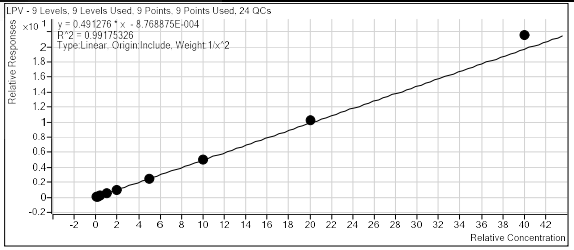
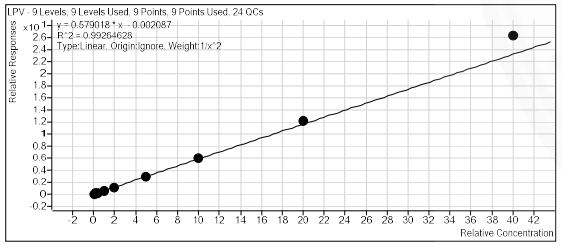
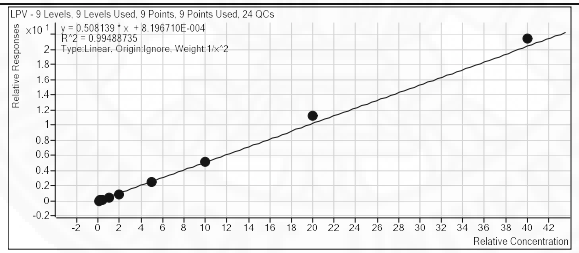
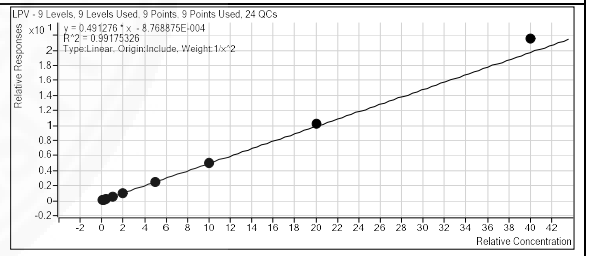


Table B2: Back calculated values for the Calibration Standards for **ritonavir (RTV) in DBS**

RTV	Run ID	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8	Cal 9	Slope	R^2	Y-Intercept	Weighting
21/Jan/15	1	53	92	189	497	931	2497	5137	10509	21538	0.22100	0.99563	-0.000084	1/x2
25/Jan/15	2	51	96	185	502	970	2469	4998	11092	20082	0.20008	0.99651	-0.000237	1/x2
28/Jan/15	3	51		184	512	906	2532	4932	10364	21844	0.18718	0.99566	0.003016	1/x2
Theoretical Conc.		**50**	**100**	**200**	**500**	**1,000**	**2,500**	**5,000**	**10,000**	**20,000**				
mean		52	94	186	504	936	2499	5022	10655	21154	0.20	0.995936		
SD		1	3	3	8	32	32	105	385	941	0.02	0.000498		
%CV		2.05	3.39	1.49	1.57	3.40	1.28	2.08	3.62	4.45	8.42	0.049983		
%dev		3.36	-5.94	-7.02	0.74	-6.42	-0.03	0.45	6.55	5.77				
n		3	2	3	3	3	3	3	3	3	3	3		

Calibration Curve Run Day #1	**Calibration Curve Run Day #2**	**Calibration Curve Run Day #3**
		

APPENDIX C

PRECISION AND ACCURACY RESULTS

Table C1 Precision and Accuracy for **lopinavir (LPV) in DBS**

Sample	LLOQ	LQC	MQC	HQC		Intra-assay Statistics			
#	50	150	1,500	16,000		LLOQ	LQC	MQC	HQC
1	46	162	1634	16027	**Mean**	51	161	1626	17537
2	53	160	1667	18184	**SD**	3	5	25	867
3	50	157	1596	17988	**%dev**	1.93	7.51	8.40	9.60
4	53	154	1614	18064	**%CV**	5.57	3.36	1.54	4.94
5	52	168	1609	18013	**n**	6	6	6	6
6	52	167	1636	16944		Intra Day 1			
1	53	162	1545	17163	**Mean**	52	164	1635	17840
2	54	164	1558	18151	**SD**	2	1	66	387
3	53	165	1676	18196	**%dev**	4.04	9.46	9.02	11.50
4	51	165	1670	18051	**%CV**	3.11	0.81	4.04	2.17
5	50	165	1665	17771	**n**	6	6	6	6
6	51	164	1698	17705		Intra Day 2			
1	52	161	1669	17676	**Mean**	51	155	1640	17785
2	49	148	1666	18158	**SD**	2	7	30	334
3	54	146	1609	18087	**%dev**	2.74	3.03	9.33	11.16
4	52	164	1621	17247	**%CV**	4.74	4.82	1.84	1.88
5	54	150	1667	17888	**n**	6	6	6	6
6	48	158	1608	17654		Intra Day 3			

Inter-assay Statistics	Theoretical Conc.	50	150	1,500	16,000
	mean	51.5	160.0	1634	17720
	SD	2	7	42	562
	%CV	4.38	4.09	2.57	3.17
	%dev	2.90	6.67	8.92	10.75
	Accuracy (%)	102.90	102.90	106.67	108.92
	n	18	18	18	18

Table C2 Precision and Accuracy for **ritonavir (RTV) in DBS**

Sample	**LLOQ**	**LQC**	**MQC**	**HQC**		**Intra-assay Statistics**			
#	**50**	**150**	**1,500**	**16,000**		**LLOQ**	**LQC**	**MQC**	**HQC**
1	45	164	1688	15471	**Mean**	50	160	1660	17261
2	49	163	1641	18242	**SD**	3	4	23	1102
3	52	162	1636	17745	**%dev**	-0.47	6.39	10.63	7.88
4	50	154	1641	17841	**%CV**	5.61	2.38	1.37	6.38
5	51	157	1674	17943	**n**	6	6	6	6
6	52	158	1677	16327		Intra Day 1			
1	52	167	1530	16293	**Mean**	53	166	1635	17078
2	57	167	1548	16879	**SD**	3	3	76	468
3	55	162	1691	17274	**%dev**	5.59	10.90	9.00	6.74
4	50	165	1651	17208	**%CV**	5.31	1.73	4.68	2.74
5	51	170	1698	17695	**n**	6	6	6	6
6	51	168	1693	17120		Intra Day 2			
1	44	163	1630	17808	**Mean**	45	156	1661	17847
2	44	164	1669	17808	**SD**	1	8	34	307
3	47	146	1652	17421	**%dev**	-9.82	3.92	10.75	11.54
4	45	163	1669	17754	**%CV**	2.65	5.25	2.02	1.72
5	46	150	1719	18370	**n**	6	6	6	6
6	44	150	1628	17921		Intra Day 3			

Inter-assay Statistics	**Theoretical Conc.**	**50**	**150**	**1,500**	**16,000**
	mean	49	161	1652	17396
	SD	4	7	49	750
	%CV	8.04	4.23	2.94	4.31
	%dev	-1.57	7.07	10.13	8.72
	Accuracy (%)	98.43	98.43	107.07	110.13
	n	18	18	18	18

APPENDIX D

CALCULATION TABLE FOR CARRY OVER CHECKING

Table D1: **Carry-over for lopinavir (LPV), in DBS**

LPV	Blank	Response	
		Analyte	IS
Run ID	Sample #		
1	1	0.00	0.00
	2	0.00	0.00
	3	0.00	0.00
Analyte response (LLOQ)		6661	43881
mean response in blank		0.00	0.00
%different from LLOQ		-100.00	-100.00

Table D2: **Carry-over for ritonavir (RTV), in DBS**

RTV	Blank	Response	
		Analyte	IS
Run ID	Sample #		
1	1	0.00	0.00
	2	0.00	0.00
	3	0.00	0.00
Analyte response (LLOQ)		2,365	39,390
mean response in blank		0.00	0.00
%different from LLOQ		-100.00	-100.00

APPENDIX E

STABILITY TESTING RESULTS

Table E1 Stability in DBS for 1 week at room temperature for LPV and RTV

RT (20-25°C) TREATED	ID	Low QC	High QC	theoretical conc. (ng/mL)	Low QC 150	High QC 16,000
LPV	1	162	18152	**mean**	165	17,399
Time: 1 week				**SD**	3	747
in DBS	2	164	16659	**%CV**	2.05	4.29
				%dev (theoretical)	9.96	8.74
	3	169	17386	**%dev (Controls)**	3.40	-0.01
				n	3	3
CONTROLS	1	162	16027	**mean**	160	17400
LPV				**SD**	2	1193
in DBS	2	160	18184	**%CV**	1.46	6.86
				%dev	6.35	8.75
	3	157	17988	**n**	3	3
RTV	1	160	17547	**mean**	166	16,583
Time: 1 week				**SD**	6	920
in DBS	2	171	15714	**%CV**	3.61	5.55
				%dev (theoretical)	10.52	3.65
	3	166	16490	**%dev (Controls)**	1.80	-3.32
				n	3	3
CONTROLS	1	164	15471	**mean**	163	17153
RTV				**SD**	1	1477
in DBS	2	163	18242	**%CV**	0.49	8.61
				%dev	8.57	7.21
	3	162	17745	**n**	3	3

Table E2 Stability in DBS for 1 week at 2-8°C for LPV and RTV

2-8°C TREATED	**ID**	**Low QC**	**High QC**	**theoretical conc. (ng/mL)**	**Low QC** 150	**High QC** 16,000
LPV	1	168	15923	**mean**	169	16,953
Time: 1 week				**SD**	1	895
DBS	2	168	17405	**%CV**	0.66	5.28
				%dev (theoretical)	12.46	5.96
	3	170	17531	**%dev (Controls)**	5.74	-2.57
				n	3	3
CONTROLS	1	162	16027	**mean**	160	17400
LPV				**SD**	2	1193
in DBS	2	160	18184	**%CV**	1.46	6.86
				%dev	6.35	8.75
	3	157	17988	**n**	3	3
RTV	1	168	14857	**mean**	167	16,451
Time: 1 week				**SD**	2	1382
in DBS	2	168	17306	**%CV**	1.14	8.40
				%dev (theoretical)	11.22	2.82
	3	165	17190	**%dev (Controls)**	2.44	-4.09
				n	3	3
CONTROLS	1	164	15471	**mean**	163	17153
RTV				**SD**	1	1477
in DBS	2	163	18242	**%CV**	0.49	8.61
				%dev	8.57	7.21
	3	162	17745	**n**	3	3

Table E3 Stability injection matrix for 1 week at 4°C for LPV and RTV

Temp (4°C) TREATED	ID	Low QC	High QC	theoretical conc. (ng/mL)	Low QC 150	High QC 16000
LPV	1	163	16102	**mean**	162	16023
				SD	1	70
Injection Matrix	2	161	15971	**%CV**	0.75	0.43
				%dev (theoretical)	7.67	0.14
Time: 1 week	3	161	15996	**%dev (Controls)**	5.57	-2.60
				n	3	3
CONTROLS	1	155	16318	**mean**	153	16451
				SD	2	138
LPV Freshly analysis	2	153	16441	**%CV**	1.49	0.84
				%dev	1.99	2.82
	3	151	16593	**n**	3	3
RTV	1	167	15705	**mean**	165	15771
				SD	2	58
Injection Matrix	2	166	15813	**%CV**	1.31	0.37
				%dev (theoretical)	10.24	-1.43
Time: 1 week	3	163	15795	**%dev (Controls)**	12.84	-2.71
				n	3	3
CONTROLS	1	149	16161	**mean**	147	16210
				SD	7	48
RTV Freshly analysis	2	151	16256	**%CV**	3.84	0.29
				%dev	-2.30	1.31
	3	140	16214	**n**	3	3

Table E4 Stability in DBS after freeze-thaw five cycles for LPV and RTV

	ID	Low QC	High QC		Low QC	High QC
TREATED				**theoretical conc**	150	16000
LPV	1	171	15889	**mean**	158	15585
FreezeThaw				**SD**	11	272
5 cycles	2	157	15362	**%CV**	7.35	1.75
				%dev (theoretical)	5.44	-2.59
	3	147	15505	**%dev (Controls)**	0.71	-10.80
				n	3	3
LPV	1	157	17624	**mean**	157	17473
CONTROLS				**SD**	9	524
Un-	2	148	17905	**%CV**	5.93	3.00
FreezeThaw				**%dev**	4.69	9.21
	3	166	16890	**n**	3	3
RTV	1	169	15122	**mean**	156	14881
FreezeThaw				**SD**	13	258
5 cycles	2	155	14913	**%CV**	8.36	1.73
				%dev (theoretical)	3.78	-6.99
	3	143	14609	**%dev (Controls)**	-1.27	-10.45
				n	3	3
RTV	1	155	16675	**mean**	158	16619
CONTROLS				**SD**	10	475
Un-	2	149	17063	**%CV**	6.51	2.86
FreezeThaw				**%dev**	5.11	3.87
	3	169	16118	**n**	3	3

Table E5 Stability in whole blood storage at 4 °C for 24 hours before spotting

	ID	Low QC	High QC		Low QC	High QC
TREATED				**theoretical conc**	150	16000
LPV	1	153	14080	**mean**	159	15201
24 Hrs in				**SD**	5	1137
Whole blood	2	161	16354	**%CV**	3.05	7.48
				%dev (theoretical)	5.79	-4.99
	3	162	15169	**%dev (Controls)**	0.30	-13.53
				n	3	3
LPV	1	158	17732	**mean**	158	17580
CONTROLS				**SD**	9	527
Spot	2	149	18015	**%CV**	5.93	3.00
immediately				**%dev**	5.47	9.88
	3	168	16994	**n**	3	3
RTV	1	155	13661	**mean**	161	14856
24 Hrs in				**SD**	8	1093
Whole blood	2	164	15804	**%CV**	3.54	7.35
				%dev (theoretical)	7.48	-7.15
	3	165	15103	**%dev (Controls)**	1.19	-11.63
				n	3	3
RTV	1	157	16869	**mean**	159	16811
CONTROLS				**SD**	11	480
Spot	2	150	17261	**%CV**	6.63	2.86
immediately				**%dev**	6.22	5.07
	3	171	16305	**n**	3	3

Table E6 Stability in DBS at -20 °C for 3 months for LPV and RTV

Stored at -20°	ID	Low QC	High QC		Low QC	High QC
				theoretical conc.	150	16000
LPV	1	145	15837	**mean**	143	15751
Time:				**SD**	11	113
3 months	2	153	15792	**%CV**	7.68	0.72
				%dev (theoretical)	-4.54	-1.56
	3	132	15622			
				n	3	3
RTV	1	143	15684	**mean**	141	15722
Time: 3				**SD**	11	34
months	2	151	15733	**%CV**	8.05	0.21
				%dev (theoretical)	-6.33	-1.74
	3	128	15748	**%dev (Controls)**	-3.95	-1.22
				n	3	3

Table E7 Stability in DBS at Room temperature for 3 months for LPV

at RT (20-25°C)	ID	Low QC	High QC		Low QC	High QC
				theoretical conc.	150	16000
LPV	1	141	14645	**mean**	138	15612
				SD	6	861
Time: 3	2	143	15893	**%CV**	4.55	5.52
months in				**%dev (theoretical)**	-7.81	-2.43
DBS	3	131	16297	**%dev (Controls)**	-4.59	-5.71
				n	3	3
RTV	1	138	13936	**mean**	139	14837
				SD	10	833
Time: 3	2	150	14998	**%CV**	7.07	5.62
months in				**%dev (theoretical)**	-7.01	-7.27
DBS	3	130	15579	**%dev (Controls)**	-4.64	-6.77
				n	3	3

APPENDIX F

HEMATOCRIT EFFECT AND DBS PREPARATION TECHNIQUE

Table F1 in DBS prepared from 30% Hematocrit of blood for LPV and RTV

	ID	Low QC	High QC		Low QC	High QC
				theoretical conc.	150	16000
LPV	1	140	15433	**mean**	147	15687
Hct30				**SD**	8	354
	2	156	16092	**%CV**	5.60	2.26
				%dev (theoretical)	-1.91	-1.95
	3	145	15537	**%dev (Controls)**	-3.83	-4.64
				n	3	3
LPV	1	155	16318	**mean**	153	16451
CONTROLS				**SD**	2	138
Hct45	2	153	16441	**%CV**	1.49	0.84
				%dev	1.99	2.82
	3	151	16593	**n**	3	3
RTV	1	146	15742	**mean**	154	15930
Hct30				**SD**	7	433
	2	160	16426	**%CV**	4.56	2.72
				%dev (theoretical)	2.40	-0.43
	3	154	15623	**%dev (Controls)**	4.82	-1.73
				n	3	3
				theoretical conc.	150	16000
RTV	1	149	16161	**mean**	147	16210
CONTROLS				**SD**	6	48
Hct45	2	151	16256	**%CV**	3.84	0.29
				%dev	-2.30	1.31
	3	140	16214	**n**	3	3

Table F2 in DBS prepared from 60% Hematocrit of blood for LPV and RTV

		Low QC	High QC		Low QC	High QC
	ID			**theoretical conc.**	150	16,000
LPV	1	148	15976	**mean**	148	16,036
Hct60				**SD**	7	600
	2	154	16664	**%CV**	4.41	3.74
				%dev (theoretical)	-1.56	0.23
	3	141	15468	**%dev (Controls)**	-3.48	-2.52
				n	3	3
LPV	1	155	16318	**mean**	153	16451
CONTROLS				**SD**	2	138
Hct45	2	153	16441	**%CV**	1.49	0.84
				%dev	1.99	2.82
	3	151	16593	**n**	3	3
RTV	1	159	16347	**mean**	156	15,973
Hct60				**SD**	8	749
	2	162	16461	**%CV**	4.82	4.69
				%dev (theoretical)	4.30	-0.17
	3	148	15111	**%dev (Controls)**	6.76	-1.46
				n	3	3
RTV	1	149	16161	**mean**	147	16210
CONTROLS				**SD**	6	48
Hct45	2	151	16256	**%CV**	3.84	0.29
				%dev	-2.30	1.31
	3	140	16214	**n**	3	3

Table F3 Variation from prepare DBS by using 50 uL calibrated capillary tube

	ID	Low QC	High QC		Low QC	High QC
LPV				**theoretical conc.**	150	16,000
	1	136	16195	**mean**	138	16,308
Calibrated				**SD**	3	128
50uL	2	142	16282	**%CV**	2.26	0.79
Capillary				**%dev (theoretical)**	-7.91	1.93
	3	137	16448	**%dev (Controls)**	-6.67	1.05
				n	3	3
CONTROLS	1	155	16287	**mean**	148	16139
LPV				**SD**	6	175
using pipette	2	145	15946	**%CV**	3.99	1.09
				%dev	-1.32	0.87
	3	145	16184	**n**	3	3
RTV	1	159	16876	**mean**	153	16,871
				SD	11	72
Calibrated	2	160	16796	**%CV**	7.28	0.43
50uL				**%dev (theoretical)**	2.15	5.44
Capillary	3	140	16941	**%dev (Controls)**	-1.15	-0.52
				n	3	3
CONTROLS	1	164	17913	**mean**	155	16959
RTV				**SD**	8	826
using pipette	2	152	16519	**%CV**	5.01	4.87
				%dev	3.34	6.00
	3	149	16447	**n**	3	3

BIOGRAPHY

Name	Miss Yardpiroon Tawon
Date of Birth	May 5, 1982
Educational Attainment	2000-2003: Medical Technology, Bachelor of Science, B.Sc.
Work Position	Medical Technologist IRD URI174/PHPT laboratory
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