DETERMINATION OF TOTAL, UNBOUND, AND INTRACELLULAR CONCENTRATIONS OF THE ANTIRETROVIRAL DRUGS EFAVIRENZ, LOPINAVIR, AND RITONAVIR

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PLAGIARISM DECLARATION

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ABSTRACT

Efavirenz, lopinavir, and ritonavir are antiretroviral drugs used for the treatment of HIV in South Africa. Plasma concentrations of these drugs are routinely monitored to ensure efficacy, minimise adverse effects, and adjust dosing. However, variability exists in patient treatment response and tolerability, which cannot always be explained by the therapeutic drug monitoring results. This may be due to variability in the amount of drug reaching the target site within the HIV-infected cells. Therefore, intracellular drug concentrations could provide a more accurate depiction of drug exposure. An alternative to intracellular drug concentrations could be the quantitation of drug not bound to plasma proteins as this is the portion able to diffuse into tissues and cells to exert a therapeutic effect.

A method is described for the quantification of intracellular efavirenz, lopinavir, and ritonavir from one million human peripheral blood mononuclear cells. In addition, the quantification of unbound efavirenz, lopinavir, and ritonavir from human plasma using ultracentrifugation is demonstrated, including a novel surrogate matrix. The two methods were validated according to the United States Food and Drug Administration and European Medicines Agency guidelines and proven to be accurate, precise, and reproducible. Both methods were submitted to the United States National Institute of Allergy and Infectious Diseases' Clinical Pharmacology Quality Assurance group for review and have been approved for use on clinical samples.

A proof-of-concept correlation study of intracellular, unbound, and total drug concentrations is described using blood samples from six HIV-positive patients. A further patient unresponsive to lopinavir treatment, despite total plasma concentrations within the normal therapeutic range, was also evaluated. Paired plasma and cell samples indicated that the drug reached the target site within the cells, eliminating a possible cause of treatment failure. These findings show the utility and validity of these methods in a clinical setting to provide an overall view of treatment response and support their novel application in individualised patient care in South Africa.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAG	Alpha-1-acid-glycoprotein
ABC	Abacavir
ACTG	AIDS Clinical Trial Group
AIDS	Acquired Immunodeficiency Syndrome
ALOQ	Above the upper limit of quantification
ARV	Antiretroviral
ATL	Adult T-cell Leukemia
AZT	Zidovudine
BCA	Bicinchoninic acid
BLOQ	Below the limit of quantitation
BSA	Bovine serum albumin
cART	Combination antiretroviral therapy
CNS	Central nervous system
CPQA	Clinical Pharmacology Quality Assurance
CPT	Cell preparation tube
CV(%)	Coefficient of variation
CYP3A4	Cytochrome P450-3A4
DNA	Deoxyribonucleic acid
EFV	Efavirenz
EMA	European Medicines Agency
ESI	Electro spray ionisation
FDA	Food and Drug Administration (United States of America)
FTC	Emtricitabine
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HPLC	High-performance liquid chromatography
HTLV	Human T-lymphotropic Virus
IMPAACT	International Maternal Paediatric Adolescent AIDS Clinical Trials Network
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LPV	Lopinavir
LPV/r	lopinavir/ritonavir
MCV	Mean corpuscular volume

MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
NAIAD/DAIDS	National Institute of Allergies and Infectious Diseases, Division of AIDS
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PK	Pharmacokinetic
QC	Quality control
RED	Rapid Equilibrium Dialysis
RNA	Ribonucleic acid
RTV	Ritonavir
S1	Highest calibration standard
S9	Lowest calibration standard
SPE	Solid-phase extraction
STDEV	Standard deviation
TB	Tuberculosis
TDF	Tenofovir
TDM	Therapeutic drug monitoring
UCT	University of Cape Town
ULOQ	Upper limit of quantification
UNMC	University of Nebraska Medical Center
UV	Ultraviolet
WS	Working solution
3TC	Lamivudine

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CHAPTER ONE: LITERATURE REVIEW

1. HUMAN IMMUNODEFICIENCY VIRUS

Sub-Saharan Africa, specifically South Africa, has the highest prevalence of Human Immunodeficiency Virus (HIV) infection worldwide.[1,2] In 2019, 19.1% of South African adults aged 15–49 years were HIV-positive.[3] There are many factors that contribute to the high prevalence of HIV in South Africa, including inadequate access to medical care, poverty, illiteracy, age-disparate sexual relationships, sexual violence, and other socio-economic issues.[4,5]

HIV is a retrovirus that infects human immune cells, namely the lymphocytes, monocytes, and dendritic cells. If left untreated, HIV can lead to Acquired Immunodeficiency Syndrome (AIDS) and ultimately immune system failure.[6,7] There are seven distinct phases of the HIV lifecycle, which are summarized as follows:

1. **Entry (also termed binding or attachment):** The HIV virus binds to the CD4 receptor on the surface of the host's immune cells. The virus also binds to either the CCR5 or CXCR4 co-receptors.
2. **Fusion:** The viral envelope fuses with the host cell membrane enabling release of the viral ribonucleic acid (RNA) and enzymes into the host cell.
3. **Reverse transcription:** The viral single-stranded RNA is converted into double-stranded HIV deoxyribonucleic acid (DNA) by the viral enzyme, reverse transcriptase.
4. **Integration:** Inside the host cell nucleus, the viral DNA is inserted into the host's DNA by the viral enzyme, integrase.
5. **Replication:** The host's cell machinery is used to transcribe and translate the HIV DNA into new HIV RNA and viral proteins.
6. **Assembly:** Viral protease enzymes activate the new HIV proteins, which, together with the newly transcribed HIV RNA, form new viruses.
7. **Budding:** The newly formed viruses exit the host cell using the host CD4 cell membrane to form the viral envelope. The CD4 receptors on the HIV envelope then allow for the new virus to bind to and infect other CD4 immune cells.[7–10]

Interestingly, however, it is not direct action by the virus itself that kills the patient; rather, HIV infection weakens the patient's immune response and increases their susceptibility to secondary infections such as tuberculosis (TB) which ultimately leads to patient death.[11]

2. HIV TREATMENT

Since the mid-1980s, when HIV was causatively linked to AIDS, there have been more anti-viral drugs approved by the United States Food and Drug Administration for the treatment of HIV than for all other viral infections combined.[12] Currently, six classes of antiretroviral (ARV) drugs are available to combat HIV infection and reduce viral load, defined according to the phase of the HIV lifecycle inhibited. The ARV classes and available drugs in each class, as of January 2022, are listed below:

- **Entry/attachment inhibitors:** *Fostemsavir, ibalizumab, and maraviroc.* These drugs bind to the host CCR5 receptors, acting as competitive inhibitors to HIV attachment and preventing viral infection.
- **Fusion inhibitors:** *Enfuvirtide.* By binging to the gp41 protein on the viral envelope, it prevents the virus from fusing with the host cell membrane.
- **Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs/ NtRTIs):** *Abacavir, emtricitabine, lamivudine, stavudine, tenofovir disoproxil fumerate, tenofovir alafenamide, and zidovudine.* These drugs prevent viral RNA from being transcribed into viral DNA by binding to the active site of the viral enzyme, reverse transcriptase.
- **Non-nucleoside reverse transcriptase inhibitors (NNRTIs):** *Doravirine, efavirenz, etravirine, nevirapine, and rilpivirine.* Similar to NRTIs/NtRTIs, NNRTIs also inhibit reverse transcription of viral RNA by binding to reverse transcriptase. They bind to sites on the enzyme other than the active site.
- **Integrase inhibitors:** *Bictegravir, cabotegravir, dolutegravir, elvitegravir, and raltegravir.* Inhibition of the viral enzyme integrase prevents the integration of viral DNA into the host DNA.
- **Protease inhibitors:** *Amprenavir (discontinued),*[13] *atazanavir, darunavir, fosamprenavir (prodrug of amprenavir), indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir.* These drugs act by inhibiting the viral protease enzymes, which are responsible for activating the new viral proteins and the assembly of the HIV capsule.[9,10,14,15]

Combination therapy, termed highly active antiretroviral therapy (HAART) or combination antiretroviral therapy (cART), consists of three ARV drugs from at least two different classes being administered simultaneously. HAART has dramatically reduced the morbidity and mortality associated with HIV/AIDS compared to monotherapy.[9,12] In 2019, 71% of HIV-positive adults (aged 15–49) and 47% of HIV-positive children (<15 years old) in South Africa were on antiretroviral therapy.[11] Therapeutic drug monitoring (TDM) is often required to routinely measure ARV concentrations in plasma and to adjust the dose accordingly to ensure efficacy and minimise adverse effects due to large inter-patient variability.[16–19]

The UCT Routine TDM Laboratory (an ISO 15189:2012 accredited laboratory) within Groote Schuur Hospital provides a clinical laboratory service to clinicians in Cape Town, South Africa. Even in a resource-strained setting such as South Africa, this laboratory offers routine drug monitoring of EFV, LPV, and RTV (as well as atazanavir, darunavir, dolutegravir, and nevirapine) at an international standard. The laboratory subscribes to the Clinical Pharmacology Quality Assurance proficiency testing program, whereby blinded external quality control plasma samples, at five different concentrations of the aforementioned ARVs are analysed in each batch. The laboratory's proficiency in measuring these ARVs is evaluated twice a year by comparing the closeness of the reported concentrations to the assigned concentrations, with an accepted 20% deviation.[20]

2.1. EFAVIRENZ

Efavirenz (EFV), an NNRTI, is one of the most prescribed ARVs in South Africa as it formed part of the first-line treatment until 2020. EFV is highly effective against the widespread HIV-1 type; however, it is not active against the less common, less infective HIV-2.[21,22] It is a lipophilic, weakly-acidic molecule with a molecular weight of 315.675 g/mol (Figure 1.1) and a very high affinity to albumin; with more than 99% of the circulating drug bound.[23,24]

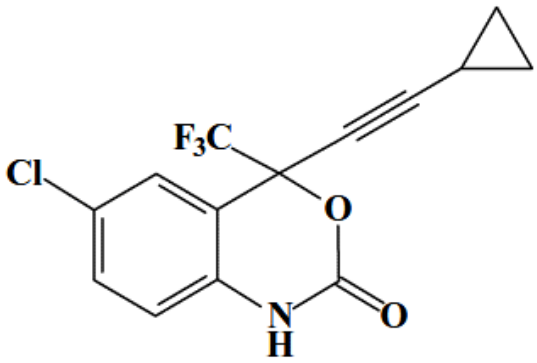

Figure 1.1. Chemical structure of EFV.[25]

Reported side effects include dizziness and headaches, while central nervous system toxicity is a risk when plasma EFV concentrations are above 4 µg/mL.[17,26] TDM is, therefore, essential to ensure that average EFV concentrations remain within the therapeutic range of 1–4 µg/mL, where viral load is suppressed and adverse effects are minimized.[27,28] Neurophysiological toxicity has been found to correlate with intracellular EFV concentrations and not with plasma EFV concentrations. It is hypothesized that the intracellular concentrations may reflect drug concentrations within other physiological compartments, such as the central nervous system.[29] Hepatotoxicity has also been associated with EFV use; however, it is more common in patients prescribed nevirapine, another NNRTI drug.[30]

While dolutegravir has since replaced EFV as the preferred treatment for newly-diagnosed patients, EFV is still widely used in South Africa.[19,31] Its use globally has steadily decreased with the introduction of dolutegravir-containing regimens in 2019, as these are cheaper and better tolerated.[31] Production of the EFV-emtricitabine-tenofovir disoproxil fumerate combination medication, *Atripla*, was terminated in 2021 due to lack of demand – although generic preparations are still available.[21] EFV-based regimens will likely persist for patients co-infected with TB, as EFV has little interaction with rifamycin.[32]

A major issue with EFV treatment, affecting both efficacy and severity of adverse effects, is the large inter-individual variability in plasma drug concentrations after dosing. The study by Marzolini *et al.* (2001) analysing 226 plasma samples from 130 HIV-infected patients on EFV found an inter-patient %CV of 118%, while the intra-patient %CV was 30%.[26] EFV concentrations were not affected by covariates such as sex, age, or height. The variability of EFV concentrations between patients may be explained by the fact that EFV is more than 99% bound to plasma proteins.[27]

Based on drug-drug interaction studies, EFV should not be administered in combination with lopinavir/ritonavir (LPV/r) as co-administration reduces LPV concentrations. Should co-administration be required, the dose of LPV/r should be increased and monitored closely.[33] EFV has also been shown to competitively bind to plasma proteins and displace other drugs, even other highly plasma-protein bound drugs such as Warfarin which is >99% bound.[34]

2.2. LOPINAVIR

LPV is a protease inhibitor commonly formulated with ritonavir (RTV) as a fixed combination, LPV/r. LPV undergoes hepatic metabolism and usually has a high extraction ratio, but RTV inhibits the liver enzyme cytochrome P450-3A4 (CYP3A4) that metabolizes protease inhibitors, including LPV; thereby increasing the bioavailability of LPV and lowering the hepatic extraction ratio.[35] Like most protease inhibitors, LPV is a large, lipophilic, weakly basic molecule. Its molecular weight is 628.814 g/mol[36] (Figure 1.2). It is highly bound to the plasma proteins, albumin, and alpha-1-acid-glycoprotein (AAG), with >98% of the drug bound. While LPV has a higher affinity for AAG, at physiological conditions, the binding to AAG is saturable while the binding to albumin is not, and because albumin is found in much higher concentrations, the binding capacity of LPV to albumin is higher than that of AAG.[37] Even in sick HIV-positive patients with elevated AAG concentrations, the effect of LPV-AAG binding is not clinically relevant.[38]

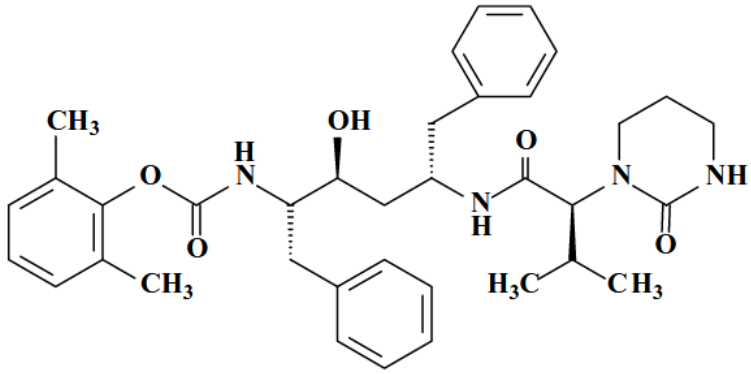

Figure 1.2. Chemical structure of LPV.[25]

The most common adverse effects associated with LPV/r are diarrhoea, asthenia (physical weakness and lack of energy), and elevated triglyceride and cholesterol levels. The increase in triglycerides results in an increased risk of developing pancreatitis in adult patients. Rare side effects include vomiting and rash, occurring in 1% and 2% of patients, respectively.[39]

LPV is considered safe, well-tolerated, and effective in reducing viral load in HIV-infected children aged 6 months to 12 years.[40] In 2008 and 2009, researchers showed that LPV may be used in HIV-positive infants aged between 6 weeks and 6 months, and between 14 days and 6 weeks, respectively, with similar exposures to those of older children despite the higher clearance in infants under 6 months old.[41,42] LPV/r is commonly used in HIV-positive pregnant women to prevent mother-to-child transmission of HIV. LPV is well-tolerated in pregnant women and does not require dose adjustments as the unbound concentrations of LPV do not change despite the low albumin levels associated with pregnancy.[43,44]

2.3. RITONAVIR

RTV is an HIV-protease inhibitor with a potent antiviral effect.[45] However, when co-formulated with a large concentration of LPV, the antiviral effect of RTV is negligible, and thus, the anti-HIV effect of LPV/r is due to LPV.[46] This is due to the fact that RTV has a high affinity for the CYP3A4 enzymes and is almost entirely metabolized before it can reach the site of HIV infection but, in doing so, greatly increases the bioavailability of LPV. Cobicistat is a derivative of RTV that also acts as a boosting agent, which is commonly co-formulated with atazanavir, darunavir, and elvitegravir to increase the efficacy of these ARVs.[32,47]

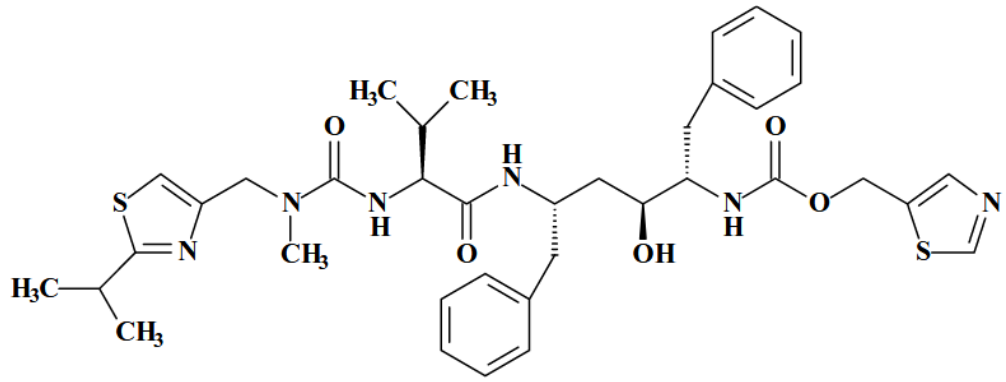

Figure 1.3. Chemical structure of RTV.[25]

RTV is a large molecule with a molecular weight of 720.948 g/mol (Figure 1.3).[48] Similar to LPV, RTV is more than 98% bound to albumin and AAG.[49] An *in vitro* study by Gulati *et al.* (2008) found that when RTV concentrations are above 50 µM, LPV protein binding is significantly displaced.[37] However, *in vivo*, this is unlikely as the low dose of RTV co-formulated with LPV does not reach therapeutic plasma concentrations due to its high metabolism by the CYP3A enzymes and, thus, will not reach the high,

saturating concentrations where competitive binding with LPV might occur.[17] The most common reported side effect of RTV is diarrhoea, followed by nausea, headaches, and weakness; however, these are generally considered to be minor.[45]

3. UNBOUND DRUG CONCENTRATIONS

Drugs are found in plasma in two forms: bound and unbound. The pharmacokinetic and pharmacodynamic effects of a drug are exerted by the unbound form.[50,51] This is because the unbound drug is available to diffuse into tissues and cells to have an effect, and to undergo metabolism and elimination, while the bound form is mostly bound to plasma proteins, typically albumin and AAG.[52] While the bound drug is not available to exert pharmacokinetic and pharmacodynamic effects, it can act as a reservoir, as drug-protein binding is a dynamic process and the bound drug will become unbound to maintain the equilibrium. This phenomenon is summarized as the "free-drug theory", which states that, in the absence of energy-dependent processes, bound and unbound drug concentrations reach equilibrium, and only the unbound drug is able to penetrate tissues and reach the target site.[53,54]

Different drugs exhibit different extents of binding and different affinities to albumin *versus* AAG. In general, lipophilic, weakly basic molecules bind to AAG, and weakly acidic molecules bind predominantly to albumin.[55] While some drugs may have a higher affinity for AAG, albumin is present in much higher concentrations (albumin accounts for 55% of all plasma proteins) and has a higher binding capacity and, therefore, is the primary plasma protein involved in drug-protein binding.[56]

It is important to understand the terminology used to describe drug binding. The unbound drug concentration is often referred to as the "free" drug concentration, which refers to the *concentration* of the drug not bound to plasma proteins. "Free fraction" is a term frequently used in literature that refers to the *ratio* of unbound drug to total drug, i.e. unbound drug: (bound + unbound drug) and is often expressed as a percentage. The two terms "free drug" and "free fraction" are not synonymous, and while free fraction data is more often reported and discussed, it is the free/unbound drug concentration that is the most clinically relevant.[57] For the sake of clarity, only the term "unbound concentrations" will be used from here onwards.

TDM of ARVs is currently based on the total plasma concentrations (bound and unbound). However, the percentage binding of a drug can influence the interpretation of total plasma concentrations as

variations in the unbound drug concentrations can lead to changes in drug efficacy and toxicity that cannot be explained by the total drug concentration data.[52] It has been suggested that unbound drug data is only clinically significant for drugs that are more than 80% plasma-protein bound. For drugs that are less than 80% bound, a change in the concentrations of plasma proteins to which the drug binds is considered to have no clinically significant effect on unbound concentrations.[58] Drugs with a higher percentage binding are susceptible to dramatic fluctuations in unbound drug concentrations caused by small changes in the concentrations of the proteins. Disease, malnourishment, infection, and pregnancy can markedly perturb the concentrations of AAG and/or albumin and, as a result, the protein binding.[43,59,60] This is especially important to consider when investigating highly protein-bound drugs where >98% of the drug is bound or drugs which exhibit non-linear protein binding (where the unbound fraction increases with concentration) and atypical non-linear protein binding (where the unbound fraction is markedly higher at very low and very high concentrations resulting in a U-shaped curve).[50,61] A small change in binding can have a significant effect on the apparent total drug concentration-effect relationship and thus, may skew the interpretation of the TDM results based on total drug concentration data alone.[43,62] A lack of unbound drug measurements may also lead to the misinterpretation of low total drug concentrations as reduced exposure and, consequently, increase the dose and the risk of toxicity.[52]

Interpretation of TDM results and the role that plasma protein binding has on drug exposure is also largely dependent on the major route of clearance of the drug, namely renal or hepatic clearance.[57] Drugs that undergo hepatic clearance can have a high, intermediate, or low hepatic extraction ratio depending on how much drug is cleared during a single pass through the liver.[63]

For renally-extracted drugs and drugs with a low hepatic extraction ratio, clearance is dependent on the unbound drug concentration – a decrease in binding results in increased clearance, and so, the total drug concentration will change, but the unbound drug concentration at equilibrium will not be affected (Figure 1.4). In this scenario, a change in protein binding could result in misinterpreted TDM results when only the total drug concentration is measured, i.e. a change in protein binding will not affect the concentration of the unbound drug, but the total plasma concentrations may appear to be too low, and the dose could be incorrectly increased which could lead to toxicity.[63]

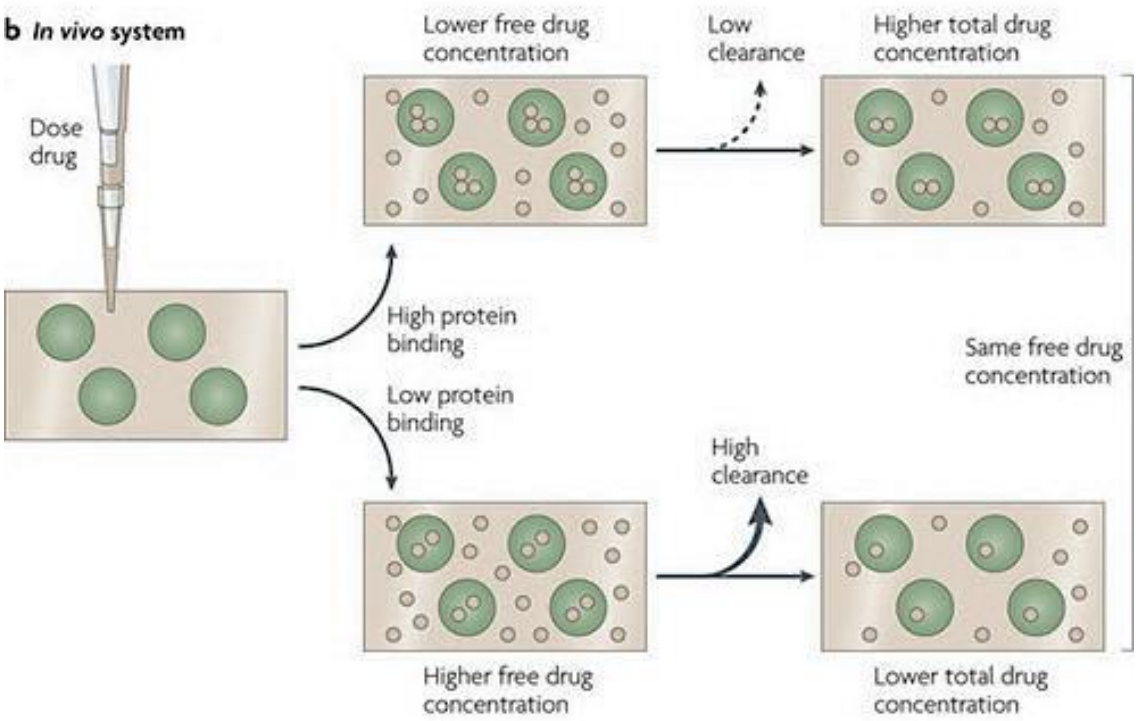

Figure 1.4. The effect of plasma protein binding on an *in vivo* system. (Adapted from Smith *et al.* (2010))

Hepatic clearance with a high extraction ratio is only dependent on blood flow, and changes in binding will not have any effect on the amount of drug cleared. Therefore, a change in binding will result in a change in unbound drug concentration (Figure 1.4).[57] In this scenario, a change in binding could lead to increased toxicity if the binding decreases or decreased efficacy if the binding increases and, therefore, requires dose adjustment. [51,63]

In healthy patients, the total drug concentration can be measured, and the data adjusted using the known percentage plasma-protein binding, clearance, and volume of distribution of the drug. However, during pregnancy, and in patients with hypoalbuminemia caused by malnourishment, age, or nephrotic syndrome, for example, this becomes much harder to model, and the total drug concentration may not be an accurate representation of pharmacologically active drug.[44,58] In this case, the most accurate way to monitor and adjust the patient dose is to quantify the unbound drug concentration.[57,58] It is especially important to measure the unbound concentrations of ARV drugs, as these drugs have narrow therapeutic indexes and the extent of protein binding is highly variable in HIV-positive patients.[54]

3.1. SEPARATION TECHNIQUES

There are several techniques available to separate the protein-bound and unbound drug, with ultracentrifugation, ultrafiltration, and equilibrium dialysis being the most common.

Equilibrium dialysis is often considered the "gold standard" to determine plasma-protein binding of drugs due to the fact that the drug-protein binding is determined at equilibrium, and the unbound and total plasma concentrations can be determined from a single sample.[56,64,65]

The principle behind equilibrium dialysis is the law of mass action. Two chambers are separated by a semi-permeable membrane, the plasma sample is added to the first chamber, and a dialysis buffer is added to the second. The molecular cut-off of the membrane is such that proteins (with the protein-bound concentrations of the drug of interest) are trapped, but the small molecular weight unbound drug is free to cross the membrane into the buffer. After sufficient equilibration at 37°C, the concentration of unbound drug is the same on both sides of the membrane. An aliquot from each chamber is then analysed to determine the total and unbound drug concentrations. Traditional equilibrium dialysis is very time- and labour-intensive (12–48 hours to reach equilibrium) and requires a large volume of plasma (~1 mL); however, commercially-available Rapid Equilibrium Dialysis (RED) devices have since reduced the time-to-equilibrium (4–6 hours) and the volume of plasma required.[65–67]

The major disadvantage of equilibrium dialysis (both traditional and RED) is the non-specific binding of analytes to the membrane, which can remove over 50% of the total drug concentration if the membrane is not properly pre-treated.[66] A second disadvantage associated with the membrane is the Gibbs-Donnan effect, where charged molecules sometimes fail to distribute across a semi-permeable membrane equally if ion strength and pH are not carefully controlled by the dialysis buffer in the second chamber.[53,61] The solubility of the analyte in the dialysis buffer must also be considered, as poor solubility of hydrophobic drugs in the buffer will underestimate the concentration of unbound drug in the sample. However, a pH change may alter the binding constant of certain drugs to albumin.[68] It is reported that the percentage binding of basic compounds increases with an increased pH, while no clear trend is apparent for acidic drugs.[69] The oncotic pressure exerted by the proteins on one side of the membrane can also cause a volume shift, whereby the dialysis buffer moves across the membrane into the plasma. This volume shift is reported to cause as much as a 10–30% increase in plasma volume, which would concentrate the amount of drug on the "unbound" side of the

membrane.[66] However, the concentration of unbound drug can be mathematically corrected if the initial and final volume of plasma and buffer is determined.

Ultrafiltration is considered the fastest and simplest method to determine the unbound drug concentration.[66,69] It is similar to equilibrium dialysis in that a semi-permeable membrane separates the unbound drug concentrations from that which is protein-bound. The plasma sample is added to the top chamber, and centrifugation or positive pressure moves the unbound drug and plasma water into the lower chamber. The liquid in the lower chamber is collected and analysed to determine the unbound drug concentration, while a second aliquot of unfiltered plasma is used to determine the total plasma concentration. The advantage of ultrafiltration over equilibrium dialysis is the lack of buffers. This avoids those issues discussed where buffers can disrupt drug-protein binding due to pH changes and eliminates the risk of volume shifts due to the oncotic pressure from the proteins. However, the non-specific binding of the analyte to the membrane and the Gibbs-Donnan effect are still considerations. The filter may be pre-treated to reduce non-specific binding, or the unbound concentration may be mathematically corrected if the non-specific binding is constant across different concentrations of analyte. The major disadvantage of ultrafiltration is the effect of molecular sieving, where the plasma water molecules cross the filter faster than the unbound analyte due to too much pressure. This is more common with larger molecular weight drugs and can result in an underestimation of the unbound drug concentration.[69]

Ultracentrifugation is a separation technique that does not make use of membranes or filters and, as a result, eliminates the issues around non-specific binding, dilution effects caused by volume shifts or molecular sieving, and the Gibbs-Donnan effect.[61,66,70] The lack of membranes also makes ultracentrifugation especially useful for lipophilic compounds, which can have limited permeation through dialysis membranes even when non-specific binding is negligible.[71] Ultracentrifugation at very high speeds separates the unbound drug from the protein-bound based on their sedimentation coefficients, resulting in three visually distinct layers: a yellow protein layer at the bottom, a middle aqueous layer, and a top thin fatty layer. The protein pellet will contain the plasma-protein bound drug, while the aqueous layer contains the unbound drug.[72] The middle aqueous layer is removed and analysed to determine the unbound drug concentration, while a second aliquot of plasma is required to determine the total plasma concentration. Low levels of proteins can be present within the aqueous layer. However, this protein contamination is said to have a negligible effect on the measured unbound drug concentration as the majority of the proteins detected in the aqueous layer are found to be small peptides and loose amino acids.[67,73] The study by Nakai *et al.* (2004) found that the unbound drug concentration determined by ultracentrifugation for ten drugs had a correlation factor

of 0.98 and a slope of 0.99 when compared to other methods such as equilibrium dialysis, despite the protein contamination observed. A later study by Kieltyka *et al.* (2016) also found a slight protein contamination of <1% of the starting protein concentration from human serum. However, again, this contamination did not impact the percentage protein binding determined by ultracentrifugation as their results correlated well with reported values (r^2 = 0.920) for 20 compounds covering a wide range of molecular weights from 236–780 g/mol.[71]

Ultracentrifugation can also be used for drug-lipoprotein binding testing due to the separation of lipoproteins into the top thin fatty layer.[70,71] The major disadvantages of ultracentrifugation are the low throughput and high cost of instrumentation compared to equilibrium dialysis and ultrafiltration.[61,74] Additionally, sedimentation of large molecular weight compounds can create an analyte gradient within the middle aqueous layer and result in variable unbound drug concentrations depending on where within this layer the sample is taken.[66,69]

Non-separative techniques also exist, such as spectroscopic methods (UV, fluorescence, infrared, *etc*.), which allow for the quantification of drug-protein binding at equilibrium in a solution, without separating the bound and unbound drug concentrations. These methods can provide a more accurate drug-protein binding constant since the sample is undisturbed.

Spectroscopic methods can also provide insight into the location of drug binding sites on the proteins and the mechanisms involved. However, such methods can only quantify changes in drug-protein binding and are not a direct measurement of the unbound drug concentrations. Another disadvantage is that these methods have poor sensitivity and require high drug concentrations. This may cause solubility issues and binding saturation when used *in vitro.* While *in vivo* sample concentrations may be too low to detect.[66]

Ultimately, each method has advantages and disadvantages and is highly drug-specific. Whichever method is chosen should be optimized for a given analyte to ensure accurate results.

4. INTRACELLULAR DRUG CONCENTRATIONS

The importance of measuring intracellular ARV concentrations is driven by the fact that only the drug inside the cells has a therapeutic effect against the virus.[75] Sub-therapeutic concentrations within the cell will also apply selective pressure on the virus, which could lead to the emergence of drug

resistance.[76] For many ARVs such as LPV, patient treatment response and tolerability have shown a large level of variability, with some patients even failing treatment. It has been suggested that this may be due to variability in the amount of drug reaching the target site within the HIV-infected cells, and, as a result, intracellular drug concentrations should be measured.[77,78] Quantifying the intracellular concentrations represents a more reliable and clinically-relevant measure of drug activity.[75]

Peripheral blood mononuclear cells (PBMCs) are a heterogeneous collection of the cells that HIV infects (lymphocytes, monocytes, and dendritic cells) and are relatively easy to isolate from whole blood samples and, as a result, are used to quantify intracellular drug concentrations.[75,79] PBMCs can be isolated from whole blood using a variety of techniques, namely BD Vacutainer CPT, Lymphoprep, SepMate, and Ficoll-Paque methods. These techniques all utilise the principle of density gradient centrifugation to separate the PBMCs from the red blood cells and plasma.[75,77,79,80] After isolation, the cells are washed with ice-cold phosphate-buffered saline (PBS) to remove any remaining plasma and drugs absorbed to the PBMC surface.[28] Regardless of the isolation procedure used, it is important to process the cells quickly and on ice to prevent drug efflux from the cells, which can lead to inaccurate results.[76,81–85]

A review by Bazzoli *et al.* (2010) summarizing studies focusing on intracellular ARV concentrations found that, while most of the studies also measured total plasma concentrations (and a few measured unbound plasma concentrations), only 8 out of 31 did any statistical analysis to determine if these concentrations were correlated.[86] Some ARVs have been reported as having a correlation between total steady-state plasma concentrations and intracellular concentrations such as stavudine, nelfinavir, and EFV, but the relationship is non-linear, and the correlation coefficients were reported as 0.459, 0.455, and 0.66, respectively, which suggests this correlation is weak.[80,87,88] The study by Tanaka *et al.* (2008) did, however, find that when the intracellular concentrations were correlated to the unbound plasma concentrations, the correlation coefficient increased to 0.76, which supports the fact that it is the unbound drug that can enter the cells. However, none of these studies focused on South African populations, and due to the genetic diversity observed in South Africa with respect to drug response, these findings might not reflect a South African population.[89,90]

To correlate plasma and intracellular concentrations, the intracellular drug concentrations must be converted from nanograms per million cells to nanograms per millilitre using the following formula:

$$\text{Intracellular concentration} = \frac{\text{lysate concentration x lysate volume}}{\text{cell number x mean cell volume}}$$

The cell number is typically measured by microscopy or a cell counter, and the cell volume is presumed to be 400 fL. The presumed 400 fL value was determined by Gao *et al.* (1993), who measured the mean corpuscular volume (MCV) of PBMCs[91] – where a corpuscle is any blood cell: red blood cell, white blood cell, or platelet. Gao *et al.* found the MCV to be 250 fL for inactivated cells and 380 fL for activated cells. Activation was initiated by a mitogenic agent assumed to make the cell more closely resemble an HIV-infected cell. The 380 fL value was later was rounded up to 400 fL. A study by Simiele *et al.* (2011) measured the MCV of 190 HIV-positive patients using a Coulter Counter and reported the mean volume to be 282.9 fL (range: 207.0–354.6 fL) which was more in agreement with Gao's "inactivated" cell volume. Due to the large range of MCV values, Simiele *et al.* recommend that one determines the MCV of PBMCs for every patient or to find an average for a target population instead of using the arbitrary value of 400 fL, which may underestimate the intracellular concentration in nanograms per millilitre.[79] However, in order to compare intracellular concentrations with published concentrations, 400 fL must be used as this is the most commonly used value in literature.[76–78,92–101]

5. BIOANALYTICAL METHODS

Almost all biological matrices require sample preparation for drug quantification. Sample preparation or extraction serves several purposes in bioanalytical methods. First, it acts as a sample "clean up" procedure by removing large endogenous matrix components such as proteins and other potential contaminants which may interfere with analyte detection. Secondly, it results in a sample that is more compatible with the chromatography system compared to the unextracted matrix. In some cases, sample extraction has the added benefit of removing other analytes that may interfere with quantification or concentrating the sample to assist with detection – however, this is dependent on the extraction technique used.[102]

5.1. EXTRACTION TECHNIQUES

The most widely used extraction techniques are protein precipitation, liquid-liquid extraction (LLE), and solid-phase extraction (SPE), each with unique advantages and disadvantages.

Protein precipitation involves adding a solvent of 2–5 times the volume of the sample to disrupt the solubility of proteins in the sample. Organic solvents such as acetone, acetonitrile, methanol, and isopropanol are the most used precipitation agents, whereby the organic nature of the solvent disrupts the hydrophobic interactions within the protein core. A high molarity salt solution can reduce the hydration of the proteins, while a change in pH due to the addition of an acid or base will change the ionization state of the proteins. Interfering with the intra-protein hydrophobic interactions, dehydrating the proteins, or changing the proteins' ionization state will cause the proteins to become insoluble and remove them from solution. The proteins can be separated from the supernatant by centrifugation. It is important to optimize the precipitation solution for the matrix and analyte of interest; since some solvents, acids/bases, and salt solutions can cause the analyte to be "trapped" within the precipitant and result in low analyte recovery.[103]

Protein precipitation may be used as a stand-alone technique or as a precursor to other sample preparations. Compared to LLE and SPE, protein precipitation is a cheap, easy, and quick method, although it has low specificity and may not remove smaller contaminants such as salts.[103,104]

LLE is reported to be the oldest extraction technique in analytical chemistry since Berthelot and Jungfleisch studied the partitioning of a solute between two liquids.[105,106] The process involves the use of two immiscible solvents (often an aqueous and non-polar organic solvent) and relies on the partition coefficient of the analytes. The two solvents are chosen based on the polarity and solubility of the analytes of interest to maximize the differential distribution of the analyte from the sample into the non-polar phase. Liquid samples such as urine and plasma can be directly extracted into a large volume of an organic solvent such as dichloromethane, ethyl acetate, hexane, or octane (5–20 times the volume of the sample). Liquid samples may also be diluted with an aqueous buffer to modify the pH or ionic strength of the sample. This can selectively enhance the analyte's affinity for the non-polar phase and improve analyte recovery.[103] As a bioanalytical extraction method, LLE is more labour-intensive than protein precipitation and is difficult to automate but has the benefit of increased specificity and a cleaner sample extract.

SPE uses a solid stationary phase and liquid mobile phase, much like chromatography. Initially, the packing material such as silica or cellulose beads were packed in the extraction column by hand to

form the stationary phase; however, pre-packed SPE cartridges became commercially available in the late 1970s.[103] These commercially-available cartridges have since been improved upon and offer a wide range of packing materials and modifications to allow for normal-phase, reversed-phase, mixed-phase, and ion-exchange extraction.

The SPE procedure involves five distinct steps: conditioning the solid-phase, equilibrating the solid-phase, loading the sample, washing contaminants from the sample, and eluting the analytes of interest from the solid-phase. Each of these steps needs to be optimized to develop a selective method for the chosen analytes and sample matrix. The combination of chosen stationary phase, wash solvents, and elution solvents results in a highly selective and very clean extraction method.[103] However, the lengthy method development process and cost of consumables are the main disadvantages of SPE compared to the simpler and cheaper protein precipitation and LLE.[104]

It is important to note that the extraction technique is highly analyte- and matrix-specific. Multiplex assays (where more than one analyte is analysed in the same method) are often difficult to develop as the analytes may have very different physicochemical properties and require different extraction solvents. Often a compromise must be found to ensure all analytes are extracted with accuracy and precision, sometimes at the expense of analyte recovery.

5.2. DETECTION BY HPLC-MS/MS

High-performance liquid chromatography (HPLC) is a separation technique that selectively retains and elutes analytes based on their physiochemical interactions with the stationary and mobile phases. HPLC is widely used in both quantitative and qualitative bioanalysis of involatile and thermally unstable compounds and is coupled to a detector. Common detectors include mass spectrometers, ultraviolet, and fluorescent detectors. Ultraviolet and fluorescent detectors are less sensitive than mass spectrometry due to potential background noise from the sample solvent. Mass spectrometry is also the most selective and robust method, especially when tandem mass spectrometry (MS/MS) is used. Therefore, HPLC-MS/MS is the gold standard for quantitative small molecule analysis.[107]

5.3. METHOD VALIDATION

Bioanalytical methods are rigorously tested and validated according to guidelines from regulatory institutions such as the United States' Food and Drug Administration (FDA) and European Medicines Agency (EMA) to ensure that methods are suitably accurate, precise, and robust for the analysis of clinical samples. The most current guidelines include the 2018 FDA Guidance for Industry and 2012 EMA Guidelines for Bioanalytical Method Validation.[108,109]

The Clinical Pharmacology Quality Assurance (CPQA) peer review program is managed by the University at Buffalo, State University of New York, under a contract by the US National Institute of Allergies and Infectious Diseases, Division of AIDS (NAIAD/DAIDS) to provide quality assurance for participating clinical pharmacology laboratories, including the Pharmacokinetic (PK) Laboratory at the University of Cape Town (UCT).[110,111] The program is intended to review assay methods according to the CPQA Guidelines for Bioanalytical Chromatographic Method Development, Validation, and Application to ensure assay quality prior to use on study samples.[112] The review of assay methods is blinded to eliminate potential bias and is performed by experts in pharmacology assay method development and validation.

The International Maternal Paediatric Adolescent AIDS Clinical Trials Network (IMPAACT) and AIDS Clinical Trial Group (ACTG) are two global clinical trial groups that require the laboratory assays involved in their trials to be CPQA-approved.

6. THE IMPORTANCE OF DEVELOPING THESE METHODS IN SOUTH AFRICA

The antiretroviral drugs EFV, LPV, and RTV are very commonly prescribed in South Africa. These drugs are very highly plasma-protein bound, and any variability in protein binding due to changes in protein concentrations or binding affinities may have a significant effect on the unbound drug concentration, which, in turn, affect how much drug is available to enter the immune cells and reach the site of action. There is very little known about the unbound and intracellular drug concentrations in South African populations. If the drug-protein binding decreases due to malnourishment or disease state, then the recommended dose may be too high and lead to an overdose and increased risk of side effects, which includes central nervous system toxicity.[113] It is also crucial to quantify the intracellular drug concentrations to know, with certainty, what drug concentrations are reaching the virus, and whether

these concentrations fall within the therapeutic window. By correlating the unbound concentrations in the plasma with the intracellular concentrations using PBMCs, the dose can be adjusted to below toxic concentrations, which is driven by the unbound drug concentrations in plasma, while maintaining the minimum required concentrations to lower viral load, driven by the intracellular drug concentrations. Investigating the relationship between the plasma and intracellular concentrations will add to the greater scientific knowledge on the pharmacokinetics of these drugs in a South African population.

While there are many published methods for the determination of unbound[24,28,52,76,80,114–116] or intracellular[24,28,76,78,80,95,98,100,101,117–122] EFV, LPV, and/or RTV currently published, only one[123] has been validated according to the updated FDA and EMA guidelines.[108,109] The method by De Nicolo *et al.* (2020) quantitated intracellular atazanavir, cobicistat, darunavir, dolutegravir, EFV, elvitegravir, etravirine, maraviroc, nevirapine, raltegravir, rilpivirine, and RTV in approximately 16 million PBMCs isolated from two 8 mL Vacutainer CPT tubes of whole blood. The method did not include LPV or unbound drug concentrations, and while the assay was successfully applied to 56 PBMC samples from 30 HIV-positive patients, no paired plasma and PBMC samples were analysed for EFV.[123]

A partially-validated method for the quantification of EFV, LPV, and RTV from approximately four million PBMCs was developed as a secondary objective in a doctoral thesis by Yuan Ren, the Division of Clinical Pharmacology, UCT, in 2009.[124] As is the case for the published intracellular methods,[24,28,76,78,80,95,98,100,101,117–122], this method was evaluated according to the 2001 FDA guidelines,[125], which have since been updated multiple times.[108,126] In addition, the EMA published its own set of guidelines in 2012.[109] The method used reserpine as the internal standard for all three analytes and did not include any sample preparation following cell lysis which could explain the poor precision and reproducibility of the method. Using deuterated internal standards for each analyte and optimizing the sample extraction could greatly improve the robustness of the method. Other validation experiments such as matrix effects, recovery, process efficiency, dilution integrity, and stability testing were not performed and are now requirements for a full validation. While the method served its purpose in the scope of the aforementioned thesis, it does not meet acceptance criteria for accuracy and precision and is not suitable for use in a clinical setting.

There is currently only one CPQA-approved method for the determination of intracellular EFV, LPV, and RTV.[127] This method was developed, and validated by, the Antiretroviral Pharmacology Lab at the University of Nebraska Medical Center (UNMC) and currently analyses all intracellular samples for NAIADS/DAIDS HIV clinical trial networks. By developing and validating a CPQA-approved method for

intracellular EFV, LPV, and RTV within the UCT PK Laboratory, samples from IMPAACT or ACTG clinical trials in Africa could be processed in South Africa instead of being sent to Nebraska.

The P1092 phase IV study by IMPAACT aims to evaluate the steady-state pharmacokinetics of lamivudine, zidovudine, and LPV/r in severely malnourished HIV-1 infected children. A secondary objective of the P1092 study requires that unbound LPV and RTV concentrations be determined using a CPQA-approved assay method. However, globally, there are no CPQA-approved methods for the quantification of unbound EFV, LPV, and RTV. By developing and validating such a method with CPQA-approval, this project can fulfil this secondary P1092 study objective and be used in future clinical trials.

Not only could the methods developed promote South Africa's involvement in global HIV clinical trial networks and assist in growing the scientific knowledge about these ARVs, but the assay methods can also be used on an individual level to better manage patients' medications. Unbound and intracellular quantification of ARVs will likely never replace current TDM practices in South Africa; however, these tools can provide supplementary information to clinicians for patients whose treatment response cannot be explained by TDM results alone.

The aim of this study is to develop and validate laboratory assay methods for the accurate and precise quantification of unbound and intracellular concentrations of EFV, LPV, and RTV according to the highest international standards and, in doing so, increase the clinical trial research capacity of South Africa and provide tools to improve individualised treatment of South African HIV-positive patients. The development of these laboratory assay methods has been approved by the University of Cape Town Human Research Ethics Committee (HREC #447/2017, Appendix A).

7. AIM

To establish a methodology for the analysis of unbound and intracellular EFV, LPV, and RTV concentrations within a South African clinical setting.

8. OBJECTIVES

1. Develop and validate a bioanalytical method for the quantification of unbound EFV, LPV, and RTV from human plasma.
2. Develop and validate a bioanalytical method for the quantification of intracellular EFV, LPV, and RTV from human PBMCs.
3. Apply the methods to HIV-positive patient samples to evaluate the feasibility of the methods in a clinical setting in South Africa.

CHAPTER TWO: UNBOUND DRUG CONCENTRATIONS

1. CHAPTER SUMMARY

Therapeutic drug monitoring (TDM) of antiretrovirals currently considers the total drug concentration (bound and unbound to plasma proteins). However, the drug not bound to plasma proteins is the only form available to diffuse into tissues and cells and have a therapeutic effect.[53,54,57]

In healthy patients, the total drug concentration can be measured, and the data adjusted using the known percentage plasma-protein binding, clearance, and volume of distribution of the drug. However, in sick patients, this becomes much harder to model, and the total drug concentration may not be an accurate representation of pharmacologically-active drug. In this case, the most accurate way to monitor and adjust the patient dose is to quantify the unbound drug concentration.[57]

It is especially important to measure the unbound drug concentration when dealing with highly protein-bound drugs, where more than 98% of the drug is bound. A small change in binding can have a significant effect on the apparent total drug concentration-effect relationship and, thus, may skew the interpretation of the TDM results based on total drug concentration data alone.[51,57,62]

This chapter presents a method for the quantification of unbound EFV, LPV, and RTV in 250 µL human plasma. The method consists of ultracentrifugation followed by liquid-liquid extraction (LLE) of 50 µL of the aqueous layer using an ammonium acetate buffer and a mixture of hexane and ethyl acetate. With stable deuterated isotopes, EFV-d5, LPV-d8, and RTV-d6 as the internal standards.

The extraction procedure is followed by liquid chromatographic separation using an Agilent Poroshell 120 (EC-C18, 2.7 µm, 50 x 4.6 mm) analytical column. The method uses an isocratic mobile phase consisting of 0.1% formic acid in water and acetonitrile (25:75, v/v) at a flow rate of 500 µL/min over 4 minutes.

An AB Sciex API 5500 Q trap mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode was used to monitor the transition of the deprotonated precursor ion m/z 314.0 to product ion m/z 243.9 for EFV, and the protonated precursor ions m/z 629.4 and m/z 721.3 to product ions m/z m/z 447.1 and m/z 296.0 for LPV and RTV, respectively. MRM mode was used to monitor the

transition of the deprotonated precursor ion m/z 319.0 to product ion m/z 248.1 for EFV-d5 and the protonated precursor ions m/z 637.4 and m/z 727.4 to product ions m/z 447.1 and m/z 301.9 for LPV-d8 and RTV-d6, respectively. Electro Spray Ionisation was used for ion production.

The method was validated according to the FDA and EMA guidelines[108,109] and was shown to be accurate, precise, and reproducible. The ultracentrifuge component of the method was also validated for accuracy, precision, and reproducibility. The HPLC-MS/MS method was tested for sensitivity and specificity as well as crosstalk between each of the analytes and between the analytes and their respective internal standards. Further validations were conducted to confirm the absence of matrix effects, recovery, process efficiency, anticoagulant-cross, haemolysis, dilution integrity, and the stability of the analytes under various conditions. Finally, a novel surrogate matrix was investigated and validated to reduce the volume of plasma required for calibration standards and quality control samples.

The assay method and all validation data were submitted for CPQA review[110] and were subsequently approved for use on human plasma samples.

2. ASSAY DEVELOPMENT

2.1. CHEMICALS AND REAGENTS

Reference standards for EFV, LPV, RTV, and their respective stable deuterated internal standards, EFV-d5, LPV-d8, and RTV-d6, were purchased from Toronto Research Chemicals (Ontario, Canada). Their chemical structures are shown in Figure 2.1 below.

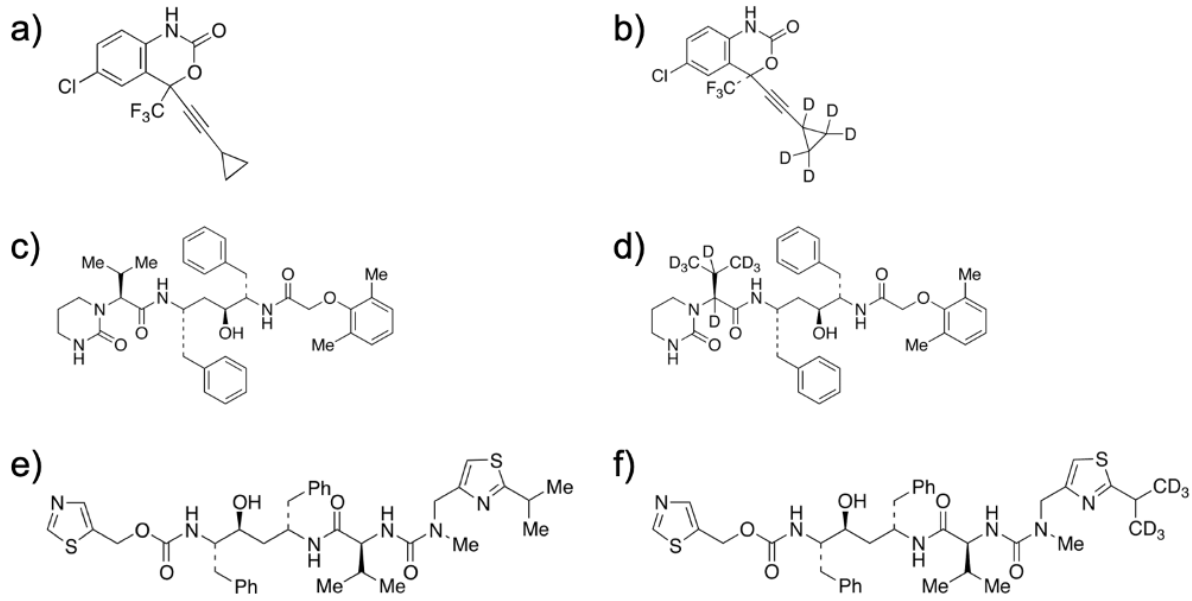

Figure 2.1. Chemical structure of a) EFV, b) EFV-d5, c) LPV, d) LPV-d5, e) RTV, and f) RTV-d6. (Images sourced from the respective certificates of analysis from Toronto Research Chemicals.)

Methanol, acetonitrile, and acetone were purchased from Honeywell (North Carolina, U.S) and formic acid from Fisher Chemical (Illinois, USA). All water used was filtered in-house (Millipore, 18.2 MΩ.cm at 25°C) using a Synergy Water Purification System from Merck Millipore (Darmstadt, Germany). Acetic acid, ammonium acetate, ammonium bicarbonate, ammonium formate, boric acid, ethyl acetate, hexane, isopropanol, phosphoric acid, and sodium hydroxide were purchased from Sigma-Aldrich (now a subsidiary of Merck, Germany).

All reference standards, internal standards, chemicals, and reagents were used within the expiry dates provided by the manufacturers.

2.2. ULTRACENTRIFUGE METHOD

Ultracentrifugation was chosen as the separation method for measuring unbound drug concentrations largely because an ultracentrifuge (Beckman Coulter Optima L-80 XP) was readily available, and the day-to-day costs involved are lower than other separation methods such as rapid equilibrium dialysis or ultrafiltration. Ultracentrifugation also avoids the use of buffers, membranes, or filters which can cause a loss of drug due to non-specific binding.[54,72] The lack of membranes also mitigates any errors caused by the Gibbs-Donnan effect, where charged molecules sometimes fail to distribute across a semi-permeable membrane equally if ion strength and pH are not carefully controlled.[53] Unfortunately, ultracentrifugation is more time-consuming and has a lower throughput compared to equilibrium dialysis and ultrafiltration as the number of samples analysed per day is limited to the rotor size[55], in this case, 72 samples per ultracentrifuge run.

Ultracentrifugation separates plasma into three distinct layers: a yellow protein pellet, a middle aqueous layer, and a top thin fatty layer (Figure 2.2). The protein pellet contains the plasma-protein bound drug concentration, while the aqueous layer contains the unbound drug concentrations.[72] The middle layer must be carefully removed without removing any of the top lipoprotein layer. Thereafter it is referred to as 'ultracentrifuged plasma".

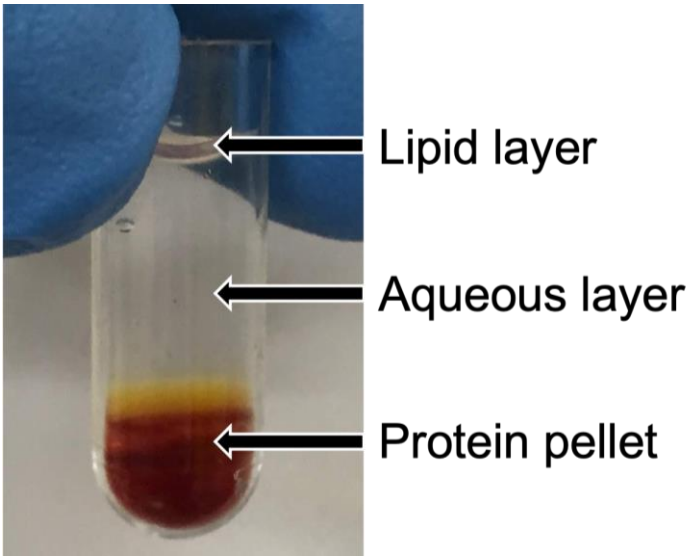

Figure 2.2. Photo of ultracentrifuged plasma showing the three distinct layers. *Note: the plasma sample presented here was slightly haemolysed, which caused the yellow protein pellet to be red.*

A micro-scale ultracentrifugation method described by Nakai *et al.* (2004) was shown to correlate with equilibrium dialysis and ultrafiltration methods for ten drugs with a slope of 0.99 and an r^2 value of 0.98. Their micro-scale method used a Beckman Coulter OptimaMAX ultracentrifuge with a TLA-100

(Beckman Coulter) rotor, which could sufficiently separate the unbound drug concentrations from 200 µL plasma when ultracentrifuged at 436 000 rcf at 4°C for only 140 minutes.[73] An 18-hour ultracentrifugation using a Beckman Coulter Optima TLX ultracentrifuge and TLA-120.1 (Beckman Coulter) rotor at 52 000 rcf was used by Kieltyka *et al.* (2016) for 20 compounds covering a wide range of molecular weights (236 – 780 g/mol). Determination of the percentage protein binding of these compounds from 500 µL human serum was shown to correlate with reported values in literature with an r^2 value of 0.920 despite the longer and slower ultracentrifugation.[71]

Samples were ultracentrifuged with a Ti 42.2, Beckman Coulter ultracentrifuge at 42 000 rpm (223 000 rcf). A small sample volume was important for future patient sample analysis. A sample volume of 200 µL plasma was tested, but it was difficult to accurately remove the middle, aqueous layer without disturbing the protein pellet, and, as a result, the sample volume was increased to 250 µL. While the sample volume is larger than that of Nakai *et al.* (2004), it is equal to or smaller than that required by other reported ultrafiltration or equilibrium dialysis methods measuring unbound EFV, LPV, or RTV, which use 250 µL,[52] 500 µL,[114] or 1.5 mL[76] per sample.

To determine the time required to separate the unbound drug concentrations from the plasma-protein bound drug concentrations, aliquots of 250 µL of blank human plasma were ultracentrifuged at 42 000 rpm for either 4, 6, or 18 hours at 4°C. The collected middle, aqueous layer of the ultracentrifuged plasma was analysed for protein levels using the Pierce™ Bicinchoninic Acid (BCA) protein assay kit microplate procedure from Thermo Fisher Scientific.[128] The BCA assay is a fast, simple, colourimetric assay where copper(II) sulphate is added to a sample, followed by bicinchoninic acid. Proteins in the sample reduce copper(II) to copper(I), which then chelate with the BCA. When no protein is present, the solution is blue-green in colour with a low UV absorbance – while the copper(I)-BCA complex is purple and exhibits a strong UV absorbance at 562 nm. The reduction of copper(II) to copper(I) and subsequent chelation to the BCA is proportional to the protein content in the sample. Therefore, the absorbance at 562 nm is proportional to the sample protein concentration. The Pierce™ BCA assay kit includes a 2 mg/mL bovine serum albumin (BSA) standard to prepare a nine-point calibration curve through zero with a protein concentration range of 25–2000 µg/mL. A blank sample containing no BCA was used to normalize the absorbance results.

The calculated protein content of the plasma samples ultracentrifuged for 4 hours was greater than 2000 µg/mL, which was the upper limit of quantification of the curve, while the samples ultracentrifuged for 6 hours had a calculated concentration of ~1000 µg/mL. The protein content of the plasma samples ultracentrifuged for 18 hours was found to be ~400 µg/mL, which is lower than

that reported in the literature.[73] A more in-depth analysis of the protein content of the ultracentrifuged plasma is discussed during the validation of the ultracentrifuged method in Section 3.13 of this chapter.

To determine if the protein contamination test results were an accurate indication of the unbound drug concentrations, human plasma was spiked with a 14.4 µg/mL of EFV (only EFV was used to test the centrifugation time to simplify the experiment and remove any drug-drug interaction effects). The plasma was incubated at 37°C for one hour for the drug-protein binding to reach equilibrium. Aliquots of 250 µL were ultracentrifuged at 42 000 rpm for either 4 or 18 hours at 8°C. (It was noted that the ultracentrifuge was often at 8°C at the end of the 18-hour run time instead of the set 4°C, despite the ultracentrifugation being within calibration, and when the temperature was kept at 4°C, some of the samples were frozen. Therefore, to reduce the risk of freezing and to achieve a more reliable temperature, the ultracentrifuge temperature was changed to 8°C.) The ultracentrifuged plasma and total plasma were extracted in triplicate and submitted for HPLC-MS/MS analysis to determine the percentage binding. The results are presented in Table 2.1.

Table 2.1. Calculated percentage binding from total and unbound EFV to determine effect of 4- *versus* 18-hour ultracentrifugation

	4 hours			18 hours		
	Total EFV (peak area ratio)	**Unbound EFV (peak area ratio)**	**%Bound**	**Total EFV (peak area ratio)**	**Unbound EFV (peak area ratio)**	**%Bound**
Sample 1	1.28	0.0435	96.6	2.59	0.00562	99.8
Sample 2	1.30	0.0214	98.4	2.51	0.00519	99.8
Sample 3	1.17	0.0708	93.9	2.29	0.00520	99.8
Average	1.25	0.0452	**96.3**	2.46	0.00534	**99.8**
STDEV	0.0700	0.0247	2.22	0.155	0.000245	0.0102
CV(%)	5.6	54.7	2.4	6.3	4.6	0.0

%Bound = (Total – Unbound)/Total x 100

The total EFV peak area ratios were different for the 4- and 18-hour experiments (1.25 and 2.46, respectively, even though they were prepared at the same concentration) as the 4- and 18-hour samples were extracted and analysed on two separate days, immediately after each ultracentrifuge run. The difference in total EFV peak area ratios is most likely due to a difference in internal standard

peak area between the two batches. The total and unbound samples for each experiment were extracted and analysed in the same batch.

The percentage plasma-protein binding of EFV is reported to be >99%.[24,55] The results of the 4- *versus* 18-hour ultracentrifugation experiment show that the 4-hour ultracentrifugation led to an overestimation in unbound drug concentration with a percentage binding of 96.3%. The 4-hour ultracentrifugation also led to very variable unbound EFV results with a CV(%) of 54.7%. The 18-hour results showed a percentage binding of >99% and an unbound EFV CV(%) of 4.6%, indicating that the method is accurate and precise for separating the unbound drug from that which is protein bound. These findings confirm that the protein contamination test results were an accurate indication of the efficiency of the ultracentrifuge to separate the bound and unbound drug concentrations. Therefore, 18-hour ultracentrifugation at 42 000 rpm at 8°C was used. An ultracentrifugation time greater than 18 hours was not tested as the results above, in conjunction with the ultracentrifugation validation (which will be discussed in Section 3.13 of this chapter), showed that 18 hours was accurate, precise, and reproducible.

2.3. EXTRACTION METHOD DEVELOPMENT

The method discussed in this chapter was the first extraction method for unbound drug analysis developed within the UCT Division of Clinical Pharmacology. Therefore, all three extraction techniques discussed in Chapter 1 (Section 5.1) were investigated, namely protein precipitation, liquid-liquid extraction (LLE), and solid-phase extraction (SPE).

Aliquots of 80 µL ultracentrifuged plasma were spiked with 125 ng/mL of EFV, LPV, and RTV and extracted in triplicate using one of the following precipitation solvents: 100% acetonitrile, 100% methanol, 0.1% formic acid in acetonitrile, 0.1% formic acid in methanol, and 100% ethyl acetate. Ethyl acetate is more commonly used as an LLE solvent; however, a method by Ramachandran *et al.* (2006) extracted EFV from plasma directly into ethyl acetate without the use of an aqueous buffer as a second solvent, and it was therefore included in this in the protein precipitation investigation.[129] In each case, 500 µL of internal standard-containing solvent was added to the ultracentrifuged plasma, whereafter the samples were vortexed for approximately 30 seconds, sonicated for 5 minutes, and centrifuged for 5 minutes at 16000 rcf. The supernatant (500 µL) was removed, dried under nitrogen at 40°C, and reconstituted in 200 µL mobile phase (a mixture of acetonitrile and 0.1% formic acid in water, 3:1, v/v). The extraction efficiency was analysed using the mean peak areas (Figures 2.3–2.5)

while the precision of each extraction solvent was evaluated using the mean peak area ratios (data not shown).

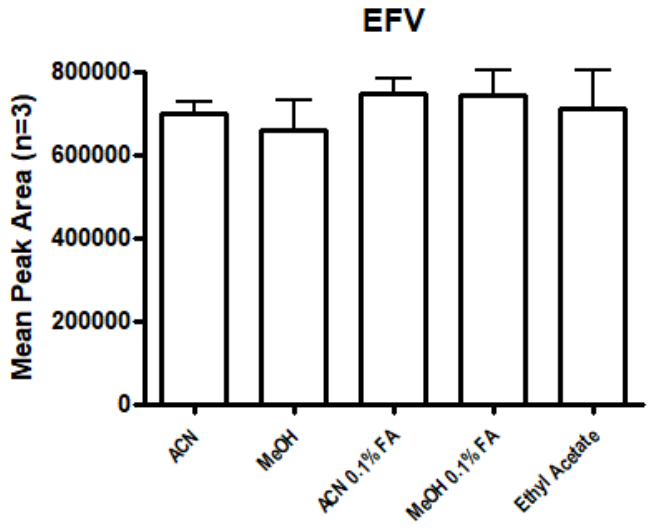

Figure 2.3. Mean peak areas of EFV when extracted using various solvents.

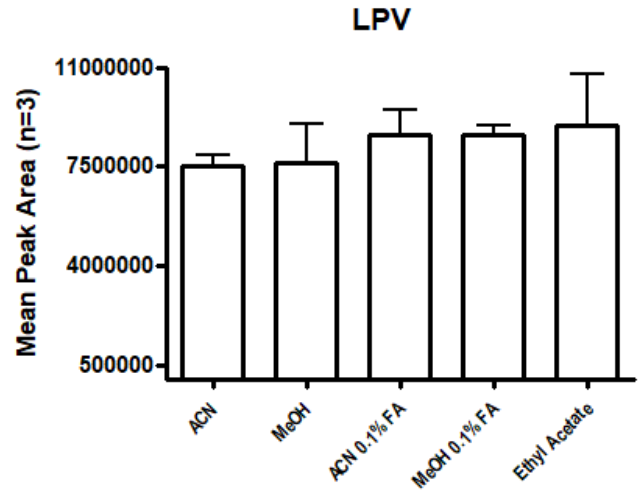

Figure 2.4. Mean peak areas of LPV when extracted using various solvents.

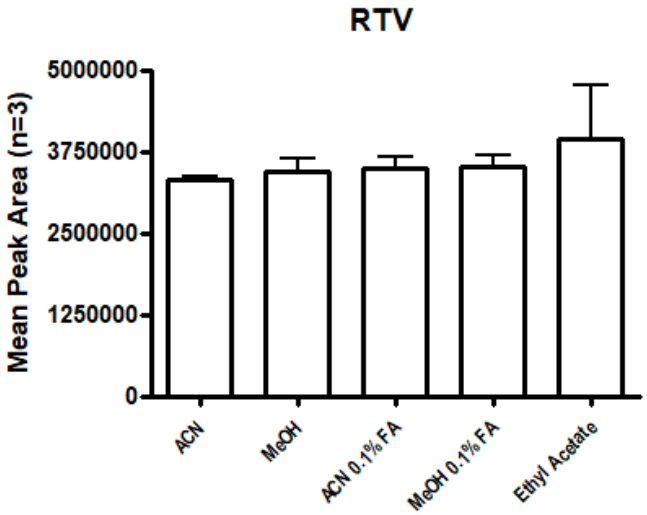

Figure 2.5. Mean peak areas of RTV when extracted using various solvents.

For all three analytes, there was no significant difference between the different solvents based on the results of a one-way ANOVA test at the $p<0.05$ level [EFV: $F(4,10) = 0.9977$, $p = 0.4526$. LPV: $F(4,10) = 1.009$, $p = 0.4476$. RTV: $F(4,10) = 1.059$, $p = 0.4253$]. Ethyl acetate had the greatest peak areas for LPV and RTV, and while the error bars in the figures show it had the highest variation in peak areas, the variability is comparable with the other solvents when normalized to the internal standard peak areas (the percentage coefficient of variation for all solvents was less than 10.7% for all three analytes). Therefore, ethyl acetate produced a high recovery (though not significantly higher than the other solvents) with a high degree of precision when normalized against the internal standard.

Next, an LLE method was investigated. The method required two optimization steps: the buffer type and pH of the aqueous layer and the non-polar organic solvent composition. Ethyl acetate is a common LLE solvent and was used during the buffer experiments. A Britton Robinson buffer was prepared by adding 5.724 mL acetic acid, 6.824 mL phosphoric acid, and 6.183 g boric acid to 1 L water. This was diluted with a 0.5 M sodium hydroxide solution to produce pH 3, 4, 5, 6, 7, 8, 9, and 10 buffers. Additionally, buffers were made using 100 mM ammonium acetate and adjusted with acetic acid to pH 6, 7, and 8. Aliquots of 80 µL ultracentrifuged plasma spiked with EFV, LPV, and RTV were diluted with 100 µL of each buffer in triplicate and vortexed for approximately 30 seconds. Ethyl acetate (500 µL) containing each internal standard was added, followed by a 1-minute vortex and 5-minute centrifugation at 16 000 rcf. The aqueous layers were frozen using a freezing plate at ~-30°C, and the organic layer was subsequently poured off and dried under nitrogen at 40°C. The samples were then reconstituted in 200 µL of a mixture of acetonitrile and 0.1% formic acid in water (3:1, v/v). As with the protein precipitation optimization results, extraction efficiency was analysed using the mean peak areas, while the precision of each buffer was evaluated using the mean peak area ratios.

EFV has a pKa of 10.2 and has poor solubility in water over a pH range of 1.2 – 8.0.[130] Likewise, LPV and RTV have pKa values of 13.4 and 13.7, respectively.[131,132] All three analytes are highly non-polar with logP values over 4.[131–133]. It is, therefore, unsurprising that the results of the buffer optimization experiment showed good recovery across all the low and neutral pH values. There was no significant difference in the recovery over a pH range from 3–8 as the analytes are already highly non-polar: so very little pH adjustment was needed to partition the analytes into the organic phase. Only the pH 9 and 10 buffers (where the analytes had improved solubility in the aqueous phase) had notably lower peak areas.

It was decided to proceed with 0.2% acetic acid in a 100 mM ammonium acetate buffer (~pH 5) for the non-polar organic phase experiments. This buffer was chosen as it was simpler to prepare than

the Britton Robinson buffer, and there was concern over the use of sodium hydroxide as this could contaminate the mass spectrometer if not sufficiently removed. As the buffer experiment showed no significant difference over a large pH range, the final buffer was prepared volumetrically to a concentration of 0.2% acetic acid, which was shown to give reproducible results when prepared on different days.

Ethyl acetate was again tested as the non-polar organic phase, as well as a mixture of ethyl acetate: hexane (1:1, v/v) as used by Avery *et al.* (2010) for the extraction of EFV from ultrafiltrate.[115] Because the ethyl acetate protein precipitation had yielded such good results, the non-polar solvents were tested with and without the addition of the ammonium acetate buffer. Aliquots of 80 µL ultracentrifuged plasma were either diluted with 100 µL of buffer in triplicate and vortexed for approximately 30 seconds or left undiluted (no buffer). Either 500 µL ethyl acetate or ethyl acetate: hexane (1:1, v/v) containing each internal standard was added, followed by a 1-minute vortex and 5-minute centrifugation at 16 000 rcf. The aqueous layers were frozen using a freezing plate at ~-30°C, and the organic layer was subsequently poured off and dried under nitrogen at 40°C. The samples were then reconstituted in 200 µL of a mixture of acetonitrile and 0.1% formic acid in water (3:1, v/v). Once again, the extraction efficiency was analysed using the mean peak areas as presented in Figures 2.6–2.8, while the precision of each test condition was evaluated using the mean peak area ratios (data not shown).

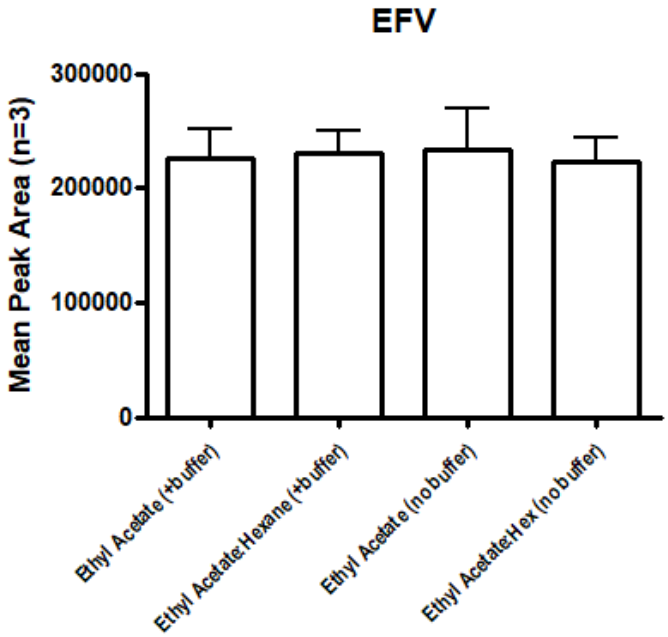

Figure 2.6. Mean peak areas of EFV when extracted using various LLE conditions.

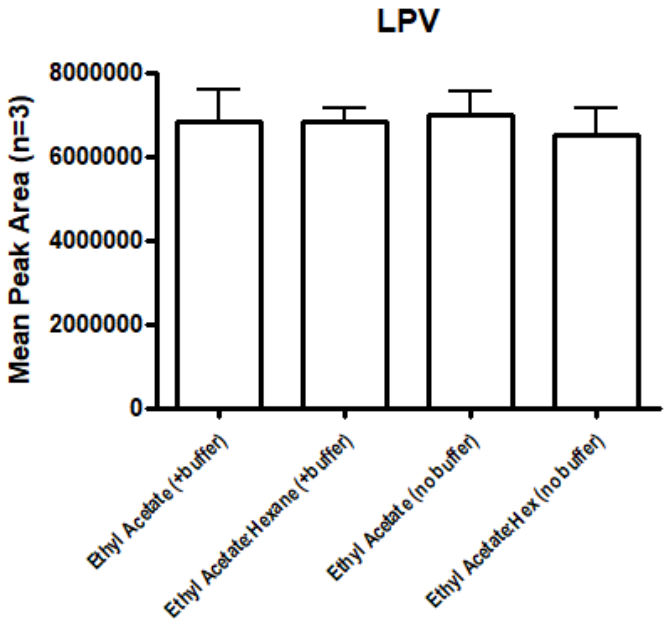

Figure 2.7. Mean peak areas of LPV when extracted using various LLE conditions.

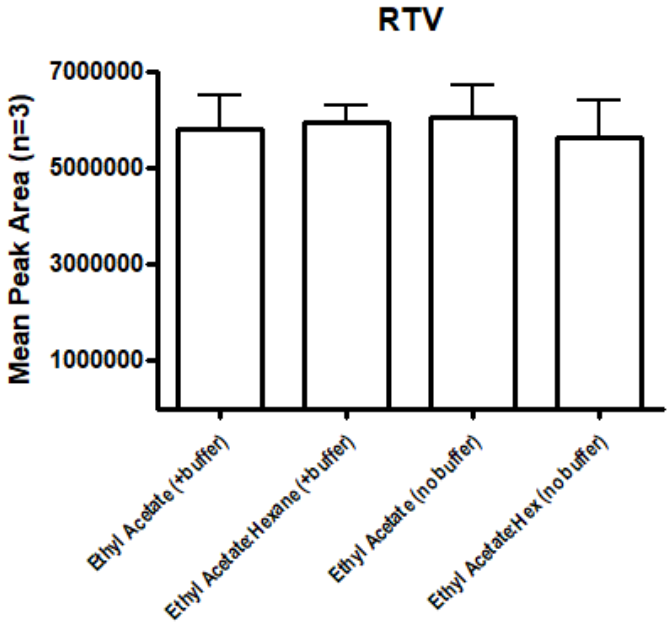

Figure 2.8. Mean peak areas of RTV when extracted using various LLE conditions.

Interestingly, the ethyl acetate (no buffer) once again resulted in the highest peak areas. However, these peak areas were not significantly different from the other test conditions according to a one-way ANOVA test at the $p<0.05$ level for EFV [$F(3,8) = 0.1051$, $p = 0.9548$], LPV [$F(3,8) = 0.3355$, $p = 0.8003$], or RTV [$F(3,8) = 0.2480$, $p = 0.8606$]. Since the plan was to compare the best LLE, SPE, and protein precipitation methods against each other using the same sample, the LLE conditions with the second-highest peak areas (though not significant) were chosen as the best LLE, namely, the ethyl

acetate and hexane mixture with the buffer – while the ethyl acetate (no buffer) was chosen as the best “protein precipitation” method.

SPE involves five distinct steps: conditioning the solid-phase, equilibrating the solid-phase, loading the sample, washing contaminants from the sample, and eluting the analytes of interest from the solid-phase. Each of these steps needs to be optimized to develop a selective method for the chosen analytes and sample matrix. The flow diagram in Figure 2.9 shows the various solvents tested in each step in the SPE process using Waters Sep-Pak® C18 (55–105 µm, 100 mg, 1 mL) cartridges. The equilibration, sample dilution, and wash tests were performed in triplicate on ultracentrifuged plasma containing a high (2000 ng/mL) and low (1.95 ng/mL) concentration of EFV, LPV, and RTV. The conditioning and elution tests were performed using a duplicate curve over a range of 1.95–2000 ng/mL prepared by 2-fold serial dilutions. In each case, 80 µL of ultracentrifuged plasma was spiked with 4 µL of working solution (prepared in a mixture of acetonitrile and water (4:1, v/v)) and, after elution, was dried and reconstituted in the mobile phase before being submitted for HPLC-MS/MS analysis.

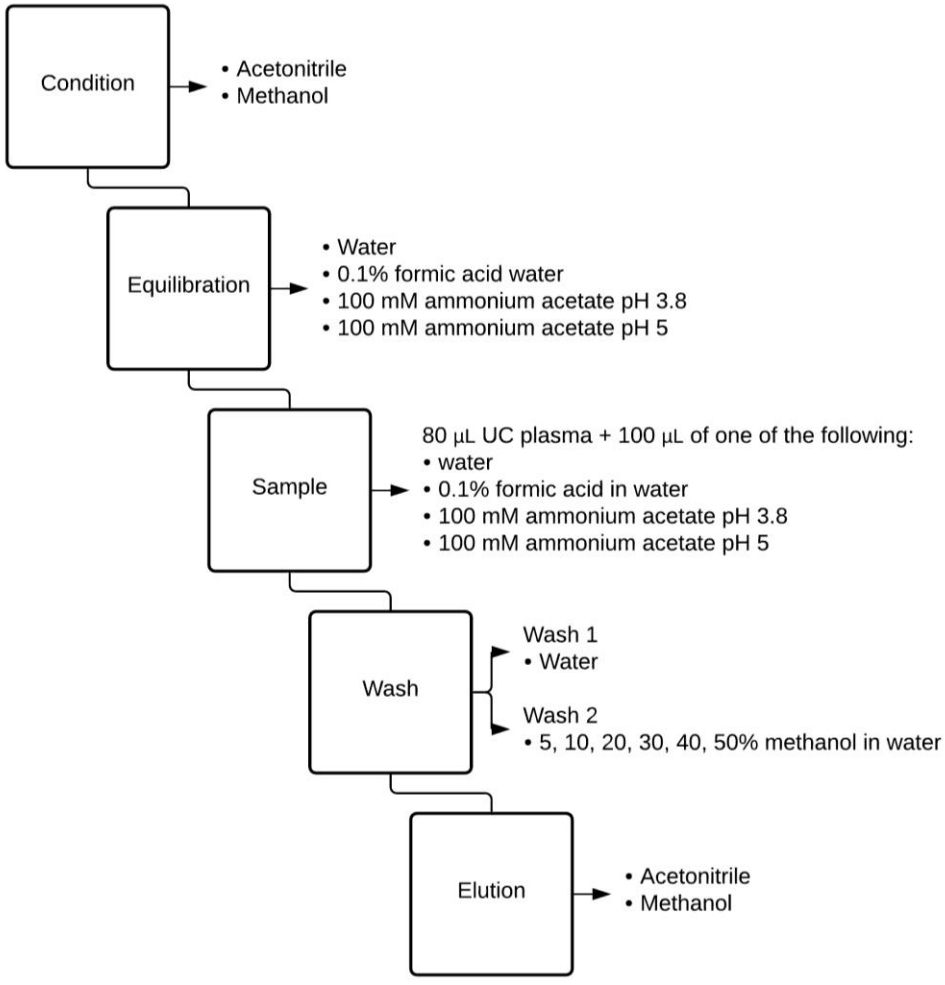

Figure 2.9. SPE conditions tested for each extraction step during method development using Waters Sep-Pak® C18 (55–105 µm, 100 mg, 1 mL) cartridges.

The use of methanol to condition the SPE cartridges and elute the analytes produced linear curves which were accurate (85–115% of the nominal concentration) at every concentration for all three analytes. The calibrations curves from samples conditioned and eluted using acetonitrile were also linear but failed for EFV at the low concentrations, even with a weighting of $1/x^2$. The peak areas of the EFV low concentrations eluted with acetonitrile had very low intensities, which could explain the poor accuracies. At the high and low concentrations, similar peak areas were obtained after using each of the different equilibration and sample dilution solvents for all three analytes, and, as a result, water was chosen.

These findings agree with those from the LLE buffer optimization experiments, where pH values under 8 had little effect on analyte recovery. The wash 2 results for the low concentration EFV, LPV, and RTV samples were blank for all the wash solutions tested. However, at the high concentration, breakthrough of EFV was observed at 50% methanol, breakthrough of RTV was seen at 40 and 50% methanol, while breakthrough of LPV occurred at concentrations over 20% methanol. As a result, 10% methanol in water was chosen as the wash solution as this was the highest percentage of methanol that could be used to remove contaminants without eluting the analytes of interest.

To ultimately decide which extraction method would proceed to method validation, the best of the three extraction methods were all compared on the same day and analysed in the same HPLC-MS/MS run. Eighteen aliquots of 80 µL ultracentrifuged plasma was spiked with 4 µL working solution to a concentration of 5.00 ng/mL. Six aliquots were extracted using 500 µL ethyl acetate containing 50 ng/mL internal standards as per the protein precipitation method. Six aliquots were extracted using 100 µL of a 0.2% acetic acid solution in a 100 mM ammonium acetate buffer and 500 µL of a hexane and ethyl acetate solution (1:1, v/v) containing 50.0 ng/mL internal standards. And finally, six aliquots were extracted using the optimized SPE method where the Waters Sep-Pak® C18 (55-105 µm, 100 mg, 1 mL) cartridges were conditioned with 1 mL methanol and equilibrated with 1 mL water. The samples were diluted with water containing 50 ng/mL internal standards and loaded onto the SPE cartridges, after which they were washed with 500 µL water and then 500 µL 10% methanol in water. Lastly, the samples were eluted using 1 mL methanol.

The protein precipitation supernatant, LLE organic phase, and SPE eluants were all dried under a gentle stream of nitrogen at 40°C and reconstituted in 200 µL mobile phase (acetonitrile: 0.1% formic acid in water, 3:1, v/v). The reconstituted samples were transferred to a 96-well plate and submitted for HPLC-MS/MS analysis. The resulting mean peak areas are presented in Figures 2.10–2.12.

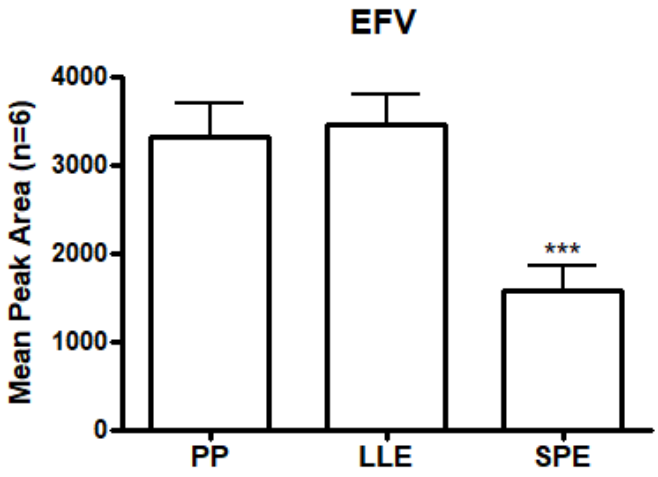

Figure 2.10. Peak areas of EFV when extracted using protein precipitation (PP), LLE, or SPE.

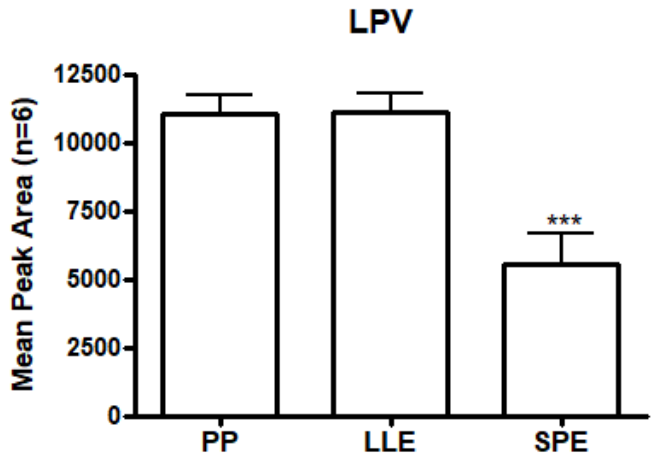

Figure 2.11. Peak areas of LPV when extracted using protein precipitation (PP), LLE, or SPE.

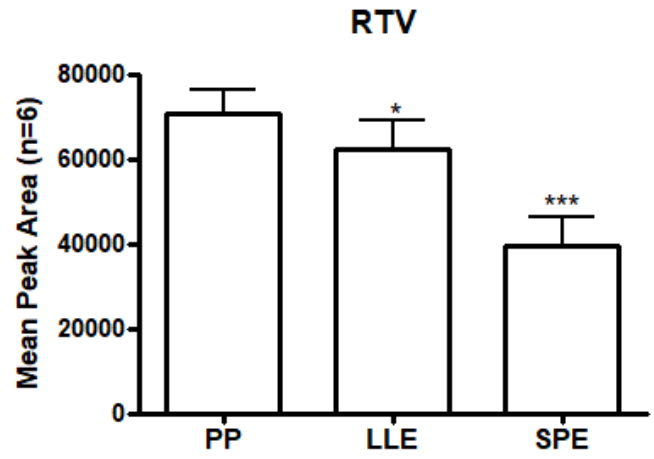

Figure 2.12. Peak areas of RTV when extracted using protein precipitation (PP), LLE, or SPE.

Separate two-tailed t-tests (95% confidence interval) were conducted to compare protein precipitation with LLE, protein precipitation with SPE, and LLE with SPE for each analyte. There was a significant difference between SPE and protein precipitation for EFV [$t(10) = 9.196$, $p < 0.0001$], LPV [$t(10) = 10.11$, $p < 0.0001$], and RTV [$t(10) = 8.600$, $p < 0.0001$]. There was also a significant difference between SPE and LLE for EFV [$t(10) = 10.48$, $p < 0.0001$], LPV [$t(10) = 10.18$, $p < 0.0001$], and RTV [$t(10) = 5.772$, $p = 0.0002$].

Therefore, for all three analytes, the mean peak area of analyte extracted using SPE was significantly less than that extracted by the other methods. During the SPE optimization experiments, it was found that the loading and wash steps did not cause breakthrough, and the 1 mL methanol elution was sufficient to remove all analytes and internal standards from the cartridge, and so, the loss of analyte must have occurred prior to sample loading. It was hypothesized that this difference was due to the poor solubility of the analytes in aqueous solutions. During the protein precipitation and LLE, the sample was extracted in the same tube in which the ultracentrifuged sample was stored. Therefore, any analyte that may have precipitated out of solution during storage was redissolved during extraction. However, during the SPE method, the sample is diluted with water prior to loading onto the SPE cartridges. Approximately half of the analyte concentration appears to be lost during this transfer step. A potential remedy for this issue would have been to add some organic solvent to the ultracentrifuged plasma before transferring to the SPE cartridges; however, this may have resulted in poor retention on the stationary phase. Ultimately, it was decided to not return to the method development stage and to proceed using one of the other optimized methods.

For EFV and LPV, there was no significant difference in analyte recovery using protein precipitation or LLE [EFV: $t(10) = 0.6426$, $p = 0.5350$. LPV: $t(10) = 0.1460$, $p = 0.8868$], while there was a significant difference between protein precipitation and LLE for RTV recovery [$t(10) = 2.270$, $p = 0.0466$]. Interestingly, these RTV results contradict the one-way ANOVA results from the LLE optimization experiment (Figure 2.8), where there was no significant difference found between the “ethyl acetate (no buffer)” and “hexane: ethyl acetate mixure” conditions. It is unclear what could have caused the different results as the volumes and procedures were the same in both cases (the ethyl acetate (no buffer) and hexane: ethyl acetate mixure conditions are simply refered to as “PP” and “LLE” in this experiment). The only difference in experimental design was the number of replicates – triplicate *versus* six-fold. Potentially, the smaller number of replicates used in the LLE optimization experiment lead to a Type II error (where the null hypothesis that there was no significant difference between the mean peak areas recovered using different extraction techniques was not rejected when it was

actually false) while the larger number of replicates in this experiment (Figure 2.12) reduced the chances of such an error occurring.

Even though the RTV results indicate that the highest RTV recovery was achieved using protein precipitation with 500 µL ethyl acetate – the LLE using 100 µL of 0.2% acetic acid in a 100 mM ammonium acetate buffer and a mixture of 500 µL hexane and ethyl acetate (1:1, v/v) was chosen as the final extraction method due to its higher specificity and cleaner sample extract.

Throughout the method development stage, 80 µL of ultracentrifuged plasma was used as the sample volume as this was the maximum volume of the middle aqueous layer that could be removed – it was decided to reduced the sample volume to 50 µL in the final method to reduce the risk of disturbing the protein layer which would contaminate the unbound drug concentration in patient samples.

2.4. SUMMARY OF ASSAY METHOD

After optimization, the final extraction method was performed as follows:

Plasma samples (250 µL) were ultracentrifuged at 42 000 rpm (230 000 rcf) for 18 hours at 8°C. Subsequently, 50 µL of the clear middle layer was removed without disturbing the protein pellet or removing any of the top lipoprotein layer and transferred to individual 1.5 mL polypropylene microcentrifuge tubes. These ultracentrifuged plasma samples were stored at ~-80°C until required.

On the day of extraction, 10 µL of blank acetonitrile and water solution (4:1, v/v) was added to each unknown and blank sample, while 10 µL of the corresponding working solution was added to the calibration standards and QC samples. Next, 100 µL of 0.2% acetic acid in a 100 mM ammonium acetate buffer was added to each sample and vortexed for 30 seconds. The samples were extracted using 500 µL of a mixture of hexane and ethyl acetate (1:1, v/v) containing internal standards (EFV-d5 and LPV-d8 at 33.3 ng/mL and RTV-d6 at 16.7 ng/mL). The double blank sample was extracted using 500 µL of blank hexane and ethyl acetate (1:1, v/v). The samples were vortexed for 1 minute and centrifuged for 5 minutes at 16 000 rcf. The entire extraction took place in the same 1.5 mL polypropylene tube that the sample was stored in to minimise any loss of analyte due to precipitation out of solution during storage.

The aqueous phase was frozen at ~-30°C using a freezing plate for 10–15 minutes. The organic phase was poured off into borosilicate tubes and dried at 40°C under a gentle stream of nitrogen until

complete solvent evaporation was achieved, after which it was reconstituted in 200 µL of acetonitrile and 0.1% formic acid in water (3:1, v/v). The reconstituted samples were transferred to a 96-well plate and submitted for HPLC-MS/MS analysis.

2.5. CHROMATOGRAPHY

The routine TDM laboratory within the UCT Division of Clinical Pharmacology analyses plasma samples from Groote Schuur Hospital every two weeks for the standard TDM purposes of monitoring of EFV, LPV, and RTV. The laboratory uses a validated multiplex HPLC-MS/MS method for the determination of total EFV, LPV, and RTV from 10 µL of human plasma using an Agilent 1200 HPLC system coupled to an AB Sciex API 4000 Triple Quad mass spectrometer. The TDM method is validated for concentrations of greater than 195 ng/mL for EFV and LPV, and 48.8 ng/mL for RTV; however, the unbound drug method requires a concentration range in the low nanogram per millilitre range. Therefore, the sensitivity of the TDM method needed to be greatly improved and a more sensitive HPLC-MS/MS system was used – namely, an Agilent 1260 HPLC system coupled to an AB Sciex API 5500 QTrap mass spectrometer.

Originally, this project only planned on developing a method for unbound EFV and LPV, and therefore, RTV is not included in some of the method development experiments. Prior to validation, RTV was added to the method as it is co-formulated with LPV.[134]

The TDM method analytical column and mobile phases were used as the starting point for the chromatographic method development. The method consisted of separation using a Phenomenex Luna PFP (110A, 5 µm, 50 x 2 mm) analytical column kept at 20°C with an isocratic mobile phase consisting of 0.1% formic acid in water and 100% acetonitrile (1:1, v/v) at a flow rate of 350 µL/min over 4 minutes.

A second analytical column, an Agilent Poroshell 120 (EC-C18, 2.7 µm, 50 x 4.6 mm), was also investigated as the routine laboratory found the Luna PFP column to be prone to modification over time and the front of the EFV peak overlapped with the tail of the LPV peak (as shown in Figure 2.13). While complete baseline separation is not necessary on modern triple-quadrupole mass spectrometers, it can make a multiplex method more robust and mitigate potential issues with crosstalk between analytes. Due to the very low LLOQ concentrations required for unbound drug analysis, it was decided that even a marginal improvement in analyte separation would be preferred.

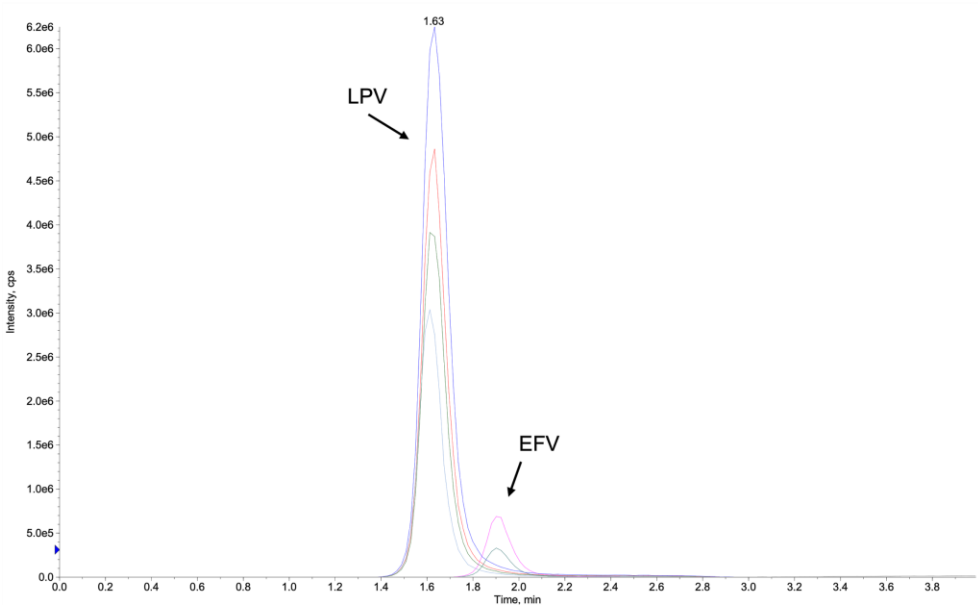

Figure 2.13. Chromatography using the TDM HPLC method and Luna PFP analytical column.

Various ratios of mobile phase A (0.1% formic acid in water) and B (acetonitrile) were investigated using the Poroshell column, as well as various flow rates and column temperatures to try to achieve better separation of the analytes. A gradient method seemed to only move the EFV peak earlier and resulted in more overlap with the LPV peak, so an isocratic elution was chosen. The following combinations of 0.1% formic acid in water: acetonitrile (v/v) were tested: 50:50, 40:60, 30:70, and 25:75. All the ratios produced symmetrical peaks; however, the methods with the lower percentages of organic required a run time of 5 minutes, while 75% organic resulted in consistent retention times with a 4 minute run time, as shown in Figure 2.14. The addition of 0.1% formic acid to the acetonitrile in mobile phase B was found to have no effect on analyte retention or peak height, and the use of methanol as the organic mobile phase (or a ratio of methanol: acetonitrile) resulted in less symmetrical peak shapes.

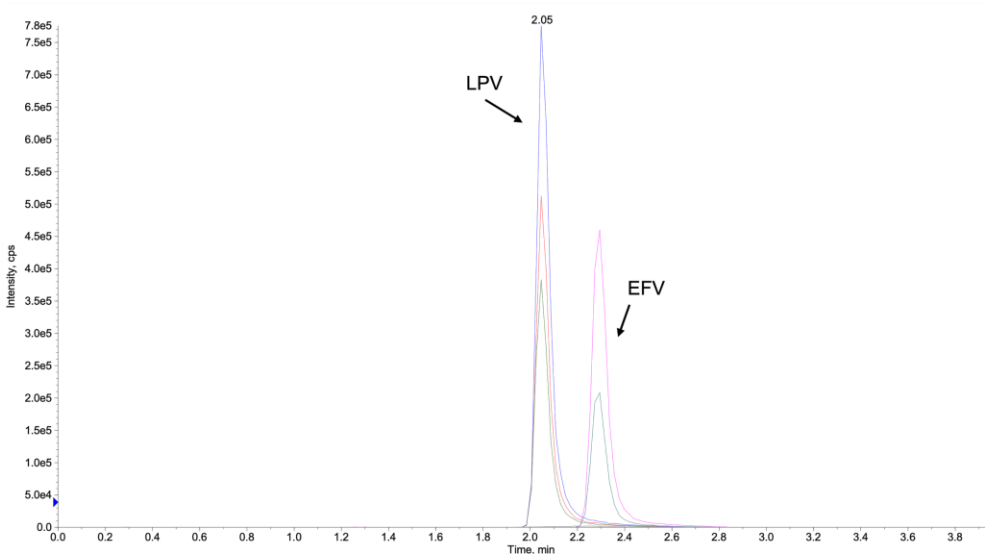

Figure 2.14. Chromatography using the Poroshell analytical column, 25:75 (v/v) mobile phase A:B, 40°C column temperature, and 400 µL/min flow rate.

During the development of the extraction method, RTV was added to the method. Fortunately, it showed good peak shapes and reproducible retention times when the optimised Poroshell method was used. However, the RTV peak co-eluted with the LPV peak, and so the chromatographic parameters were altered slightly. The mobile phase composition, run time, and column temperature remained 0.1% formic acid in water and 100% acetonitrile (25:75, v/v), 4 minutes, and 40°C, respectively. Better separation of the RTV and LPV peaks was achieved by simply increasing the flow rate to 500 µL/min; however, some peak overlap still occurred. The increase in flow rate is also why the final retention times of the analytes shown in Figure 2.15 are earlier than that in Figure 2.14.

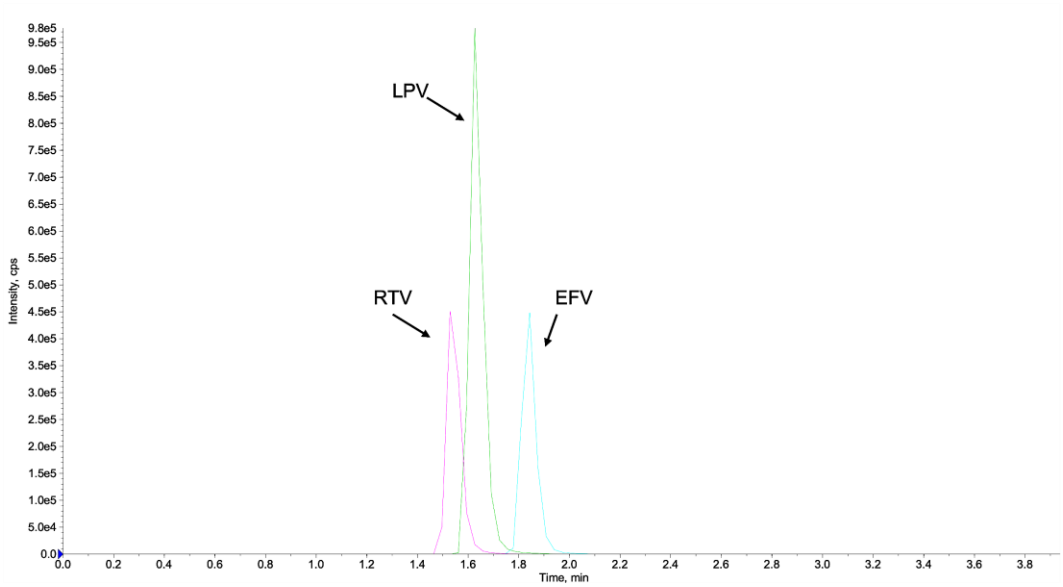

Figure 2.15. Final chromatography using a Poroshell analytical column, 25:75 v/v mobile phase A:B, 40°C column temperature, and 500 µL/min flow rate.

While complete baseline separation of the analytes was not fully achieved, the selectivity of the mass spectrometer meant that this was not a requirement. Had the crosstalk experiments failed to meet acceptance criteria during validation, the chromatography would have had to be reoptimized until the analytes were completely baseline separated. Fortunately, no interference between the analytes was present, as shown during the crosstalk validation experiments in Section 3.6 of this chapter.

Ultimately, separation of EFV, LPV, and RTV was achieved using reverse-phase chromatography with a Poroshell 120 column (EC-C18, 2.7 µm, 50 x 4.6 mm) kept at 40°C. An isocratic mobile phase system consisting of 25% mobile phase A (0.1% formic acid in LC-MS grade water) to 75% mobile phase B (acetonitrile) was used at a flow rate of 500 µL/min over a total run time of 4 minutes. The sample injection volume was 10 µL, and the autosampler temperature was kept at 8°C.

2.6. MASS SPECTROMETRY

Initially, all three analytes were ionized in the positive ionization mode as per the existing TDM method. However, EFV was later changed to the negative ionization mode as this greatly improved the sensitivity. The Electro Spray Ionisation (ESI) mode, MRM transitions, and main instrument parameters of each analyte and internal standard after infusion are summarized in Table 2.2.

Table 2.2. Main instrument parameters and MRM transitions

	ESI mode	Precursor ion (m/z)	Product ions (m/z)	Declustering potential (V)	Collision energy (V)	Entrance potential (V)	Exit potential (V)
EFV	Negative	314.0	243.9 229.9	-125	-22	-10	-21
EFV-d5	Negative	319.0	248.1	-65	-24	-10	-13
LPV	Positive	629.4	447.1 155.0	131	19	10	36
LPV-d8	Positive	637.4	447.1	171	19	10	38
RTV	Positive	721.3	296.0 139.9	146	23	10	24
RTV-d6	Positive	727.4	301.9	116	23	10	12

The first product ion of the analytes listed in Table 2.2 was used as the quantifier ion and was used for the quantification of calibration standards, QCs, and unknown samples. The second product ion listed was used as the qualifier ion and was used as a confirmatory result that the peaks seen in unknown samples were the analyte of interest.

Interestingly, it was found that the use of a split (where ~60% of the flow was diverted to waste) improved the ionization and signal-to-noise ratio of EFV. A decrease in sensitivity was consequently observed for LPV and RTV; however, the signal-to-noise of the lowest limit of quantitation samples (LLOQs) was still acceptable and therefore, improving the sensitivity of EFV was prioritized above the other analytes.

Flow injection analysis was performed, in triplicate to optimize the following source parameters: collision gas, curtain gas, nebulizer gas (gas 1), and turbo gas (gas 2). The mean peak area was used to determine the best setting for each source parameter, as well as the percentage variation between the triplicate results. While a greater peak area means better ionization and improved sensitivity, it was also important to consider how consistent the ionization was between injections. For each of the parameters tested, it was decided to use the best setting for EFV; due to the general poorer sensitivity of the analyte, even if this was not the optimal setting for LPV.

The source temperature and ion spray voltage were optimized manually by injecting the same sample six times with various source temperatures and ion spray voltages. The first injection of each setting was not included in the average and standard deviation to allow the system to properly equilibrate during that first injection. The results of the source temperature optimization are shown in Figure 2.16.

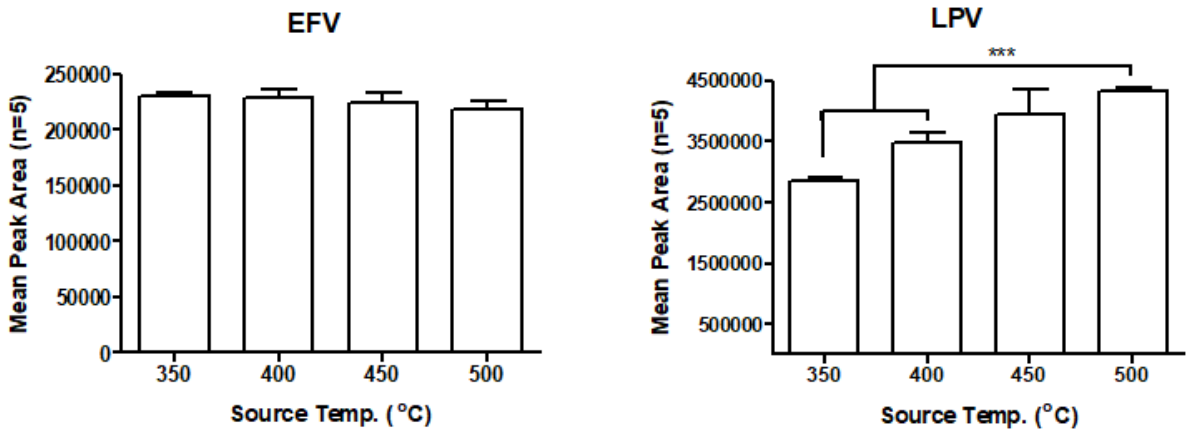

Figure 2.16. Effect of source temperature on mean analyte peak area.

There was no significant difference in EFV peak area for the different temperatures based on a one-way ANOVA test at the $p<0.05$ level [$F(3,16) = 3.228$, $p = 0.0505$], and so, the LPV results were used to decide which source temperature was optimal. The source temperature was chosen to be 500°C as this gave significantly better peak areas for LPV compared to 350°C [$t(8) = 55.26$, $p <0.0001$] and 400°C [$t(8) = 11.38$, $p <0.0001$]. While there was no significant difference between 450°C and 500°C for LPV [$t(8) = 2.053$, $p = 0.0741$], 500°C resulted in less variable peak areas. RTV was not yet included in the

method at the time of this experiment; however, it ionized well under the chosen conditions, and therefore, the source optimization was not repeated to include RTV. Had RTV shown poor reproducibility or poor sensitivity, the source optimization would have been repeated.

Unlike the source temperature, which had to be the same setting for all analytes, the ion spray voltage settings could be different for the positive and negative ionization modes. The allowed range of voltages is 0–5500 V and -45000–0 V for the positive and negative ionization modes, respectively. Ion spray voltages of 4000, 4500, 5000, and 5500 V were tested in the positive ionization mode for LPV and -3500, -4000, -4500 V were tested in the negative ionization mode for EFV. The optimal ion spray voltage for EFV and LPV, respectively, was found to be -4500 V and 5000 V. Once again, had RTV not ionized well under the final positive mode conditions, the experiment would have been repeated.

To summarize, the final parameter settings for collision gas, curtain gas, nebulizer gas (gas 1), and turbo gas (gas 2) were set to medium, 30 psi, 60 psi, and 60 psi, respectively, for all analytes. The ion spray voltage in the positive mode was set to 5000 V for LPV, LPV-d8, RTV, and RTV-d6, while the ion spray voltage in the negative mode was set to -4500 V for EFV and EFV-d5. The dwell time was set to 150 ms for EFV and EFV-d5 and 200 ms for LPV, LPV-d8, RTV, and RTV-d6. The source temperature was kept at 500°C, and a split (red Peek tubing inner diameter 0.0127 cm x 49.5 cm) diverting ~60% to waste was used to improve the ionization of EFV.

3. ASSAY VALIDATION

The method was validated according to the FDA and EMA guidelines for bioanalytical methods.[108,109] The validation included the evaluation of the method's accuracy, precision, reproducibility, sensitivity and specificity, crosstalk, matrix effects, recovery, process efficiency, anticoagulant effects, haemolysis, dilution integrity, and the stability of the analytes under various conditions. The accuracy, precision, and reproducibility of the ultracentrifuge method were also evaluated during the validation. In addition, a novel surrogate matrix was investigated and validated to reduce the volume of plasma required for calibration standards and quality control samples.

3.1. PREPARATION OF STOCK SOLUTIONS

Stock solutions were prepared volumetrically for each analyte and internal standard to a final concentration of 1 mg/mL. The weighed mass of each analyte was adjusted for purity before the solvent was added; however, no mass adjustments were made for the internal standards. Stock solutions for EFV and EFV-d5 were prepared in acetonitrile, while LPV, LPV-d8, RTV, and RTV-d6 were prepared in methanol. All stock solutions were stored at ~-80°C.

3.2. PREPARATION OF WORKING SOLUTIONS

The 1 mg/mL stock solutions were used to spike two sets of working solutions. The first set was prepared volumetrically in a solution of acetonitrile and water (4:1, v/v) according to the dilution scheme presented in Table 2.3. These working solutions (WS 1–9) were used on the day of each extraction to spike the calibration standards. WS 0 was not used to spike calibration standard samples but was included in the dilution scheme to dilute the 1 mg/mL stock solution to a 3000 ng/mL working solution for EFV and LPV and 750 ng/mL for RTV. The second set of working solutions was prepared independently by a second analyst, as shown in Table 2.4, and was used to spike the quality control samples.

Table 2.3. Preparation of calibration standard working solutions

Working solution	Blank solvent volume (µL)	Stock solution volume (µL)			Dilution source	Dilution source volume (µL)	WS Conc. (ng/mL)	
		EFV	LPV	RTV			EFV, LPV	RTV
WS 0	810	40.0	40.0	10.0			44 444	11 111
WS 1	3730				WS 0	270	3000	750
WS 2	900				WS 1	2100	2100	525
WS 3	900				WS 2	1200	1200	300
WS 4	1800				WS 3	600	300	75.0
WS 5	1200				WS 4	800	120	30.0
WS 6	1800				WS 5	600	30.0	7.50
WS 7	1000				WS 6	1000	12.0	3.00
WS 8	1200				WS 7	800	6.00	1.50
WS 9	750				WS 8	750	3.00	0.750

Table 2.4. Preparation of quality control working solutions

Working solution	Blank solvent volume (µL)	Stock solution volume (µL)			Dilution source	Dilution source volume (µL)	WS Conc. (ng/mL)	
		EFV	LPV	RTV			EFV, LPV	RTV
WS 0	810	40.0	40.0	10.0			44 444	11 111
WS DIL	3460				WS 0	540	6000	1500
WS H	2400				WS DIL	1600	2400	600
WS M	2500				WS H	1500	900	225
WS SYS	1500				WS M	1500	450	113
WS X	600				WS SYS	200	113	28.1
WS L	3470				WS X	280	8.40	2.10
WS LLOQ	1800				WS L	1000	3.00	0.750

The working solution referred to as “WS X” in Table 2.4. was not used to spike quality control samples but was included in the dilution scheme to dilute 450 ng/mL to 8.40 ng/mL. All working solutions were stored as aliquots of 150 µL in 1.5 mL polypropylene microcentrifuge tubes at ~-80°C until required. While only 20 µL of each working solution was needed per sample, the aliquot volume was 150 µL to allow for six-fold analysis per tube and to prevent working solution concentration due to evaporation. The calibration standard working solutions were previously stored as 50 µL aliquots; however, over time, the concentrations were observed to increase when compared to the quality control working solutions which were stored as aliquots of 150 µL. It was concluded that the smaller volume led to a higher rate of evaporation and consequent sample concentration, and therefore, a larger aliquot volume was used. Sample evaporation was shown to be mitigated when an aliquot volume of 150 µL was used – when compared to freshly prepared working solutions.

3.3. PREPARATION OF STANDARDS AND QUALITY CONTROLS

The calibration ranges were chosen based on the theoretical percentage binding of the analytes and the calibration range of the method for the quantification of total EFV, LPV, and RTV plasma concentrations used routinely for TDM within the UCT Division of Clinical Pharmacology. The total plasma concentration range is 0.0195–20.0 µg/mL for EFV and LPV, and 0.00488–5.00 µg/mL for RTV. The range for RTV is a quarter of the other analytes as RTV is co-formulated with LPV as 100 mg RTV and 400 mg LPV.[134] The percentage plasma-protein bindings of EFV, LPV, and RTV are all greater than 98%,[24,55,116,135], which results in their theoretical unbound concentrations lying in the low nanogram per millilitre range. The final calibration ranges were, therefore, chosen as 0.500–500 ng/mL for EFV and LPV and 0.125–125 ng/mL for RTV, which are in agreement with published methods.[52,76,115]

Aliquots of 250 µL blank human plasma were ultracentrifuged as per the ultracentrifuge method, and 50 µL of the middle, aqueous layer was removed and stored in individual 1.5 mL polypropylene microcentrifuge tubes. Calibration standards were prepared by adding 10 µL of each working solution to the 50 µL aliquots of blank ultracentrifuged plasma on the day of extraction to obtain the desired calibration standard concentrations, as shown in Table 2.5. The quality control samples were used to validate the calibration curve and were prepared using the same methodology as the calibration standards (Table 2.6).

Table 2.5. Preparation of calibration standards from working solutions

Calibration standard	Working solution used	Volume of WS (µL)	Volume of blank matrix (µL)	Concentration (ng/mL)	
				EFV, LPV	RTV
S1	WS 1	10.0	50.0	500	125
S2	WS 2	10.0	50.0	350	87.5
S3	WS 3	10.0	50.0	200	50.0
S4	WS 4	10.0	50.0	50.0	12.5
S5	WS 5	10.0	50.0	20.0	5.00
S6	WS 6	10.0	50.0	5.00	1.25
S7	WS 7	10.0	50.0	2.50	0.625
S8	WS 8	10.0	50.0	1.00	0.250
S9	WS 9	10.0	50.0	0.500	0.125

Table 2.6. Preparation of quality control samples from working solutions

Quality control	Working solution used	Volume of WS (µL)	Volume of blank matrix (µL)	Concentration (ng/mL)	
				EFV, LPV	RTV
QC DIL	WS DIL	10.0	50.0	1000	250
QC H	WS H	10.0	50.0	400	100
QC M	WS M	10.0	50.0	150	37.5
SYS	WS SYS	10.0	50.0	75.0	18.8
QC L	WS L	10.0	50.0	1.40	0.350
QC LLOQ	WS LLOQ	10.0	50.0	0.500	0.125

The dilution quality control (QC DIL) was used during a validation batch to validate the dilution process should the calculated concentration of an unknown sample be reported as above the upper limit of quantitation (ULOQ), i.e. greater than 500 ng/mL for EFV and LPV or greater than 125 ng/mL for RTV. The high, medium, and low quality controls (QC H, QC M, and QC L respectively), and QC LLOQ (lower limit of quantification) were used in six-fold during all validations, while only the QC H, QC M, and QC L were used in duplicate during sample analysis. A system check (SYS) sample was included in every batch to serve as a reference sample for instrument sensitivity and analyte retention time, and to evaluate system equilibration prior to injecting a batch.

3.4. INTER- AND INTRA-DAY ACCURACY AND PRECISION

In order to show that the method was accurate, precise, and reproducible, duplicate standards and six-fold QC samples were prepared and extracted in three separate batches over three different days. The intra-day accuracy and precision were based on the three individual batches, and the inter-day accuracy, precision, and reproducibility were assessed using the combined results of the three batches, resulting in six replicates of each standard and 18 replicates of each quality control. According to the FDA and EMA guidelines, accuracy is measured as the closeness of the observed concentration of the analyte to the nominal concentration and is expressed as percentage accuracy. While precision is measured as the closeness of the replicates at a given concentration to each other, expressed as the coefficient of variation (CV(%)).[108,109]

Using Analyst software version 1.6.3, the calibration standard peak area ratios (analyte peak area/ internal standard peak area) were plotted against the nominal concentrations to generate the calibration curves (Figures 2.17–2.19). The curves each fit a quadratic regression with a weighting of 1/concentration for EFV and 1/concentration^2 for LPV and RTV.

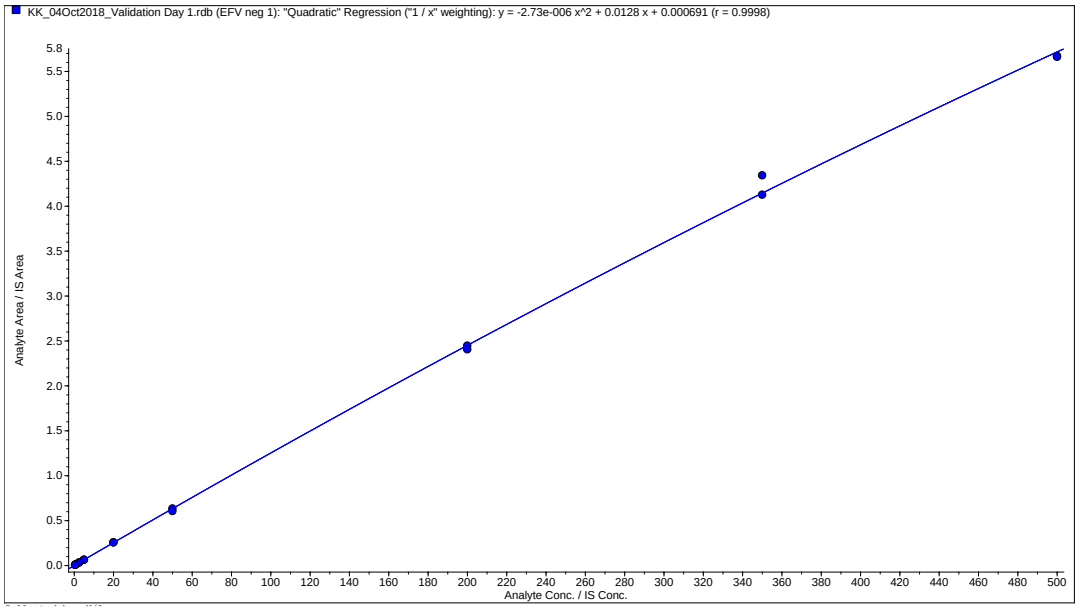

Figure 2.17. Calibration curve from validation day 1: EFV.

The EFV curves showed good fit as the r values for day 1, day 2, and day 3 were 0.9998, 0.9998, and 0.9999, respectively.

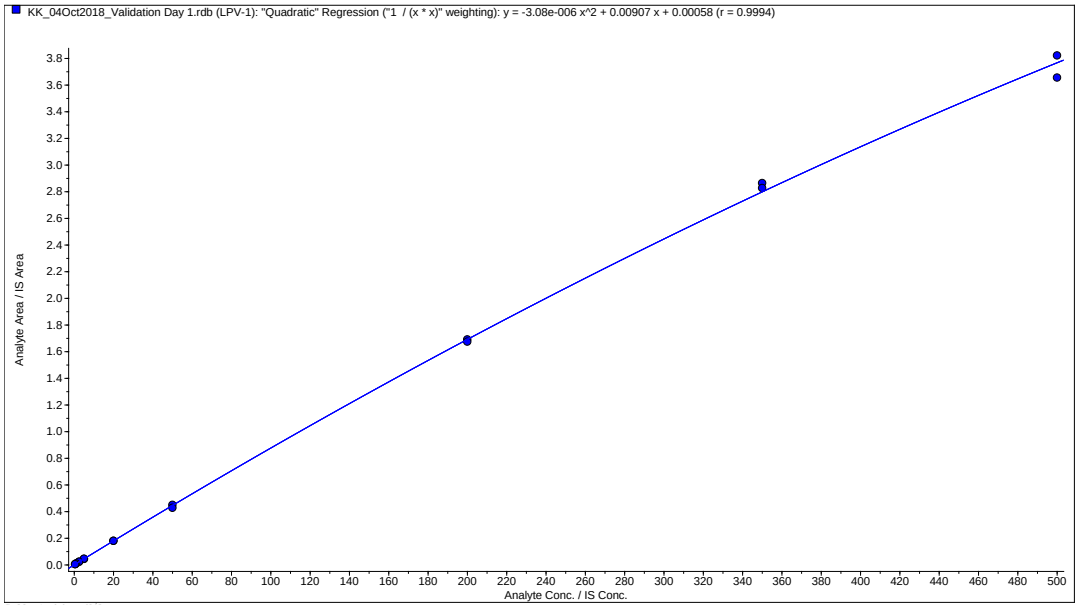

Figure 2.18. Calibration curve from validation day 1: LPV.

The LPV curves showed good fit with r values for day 1, day 2, and day 3 of 0.9994, 0.9992, and 0.9998, respectively.

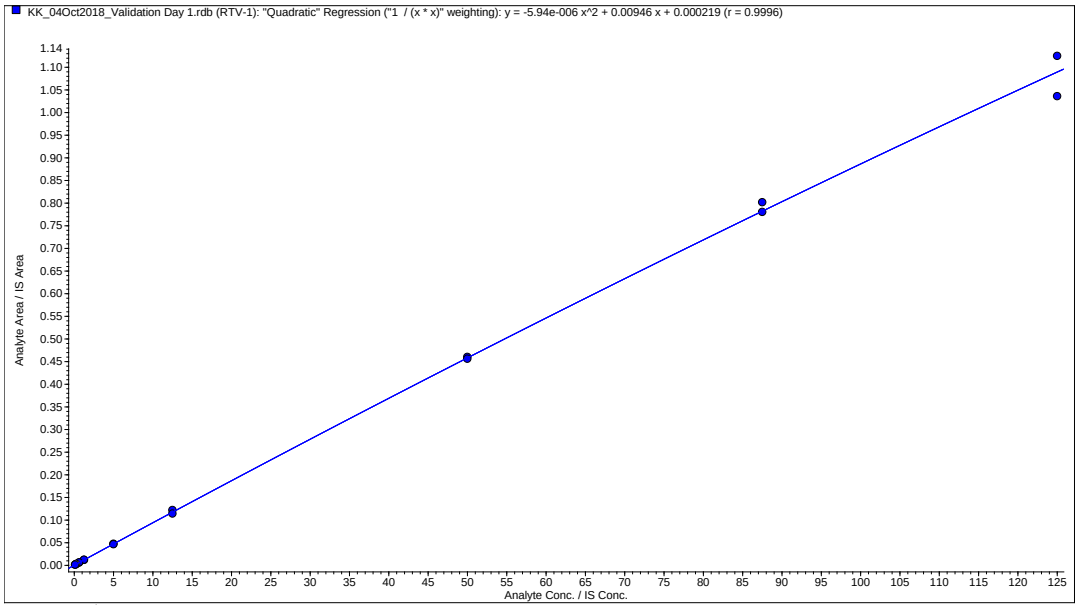

Figure 2.19. Calibration curve from validation day 1: RTV.

The RTV curves also showed good fit with r values for day 1, day 2, and day 3 of 0.9996, 0.9992, and 0.9984, respectively.

The FDA and EMA guidelines stipulate that for a bioanalytical method to be considered accurate and precise, the percentage accuracies must be within 85–115%, and the coefficients of variation must be less than 15% for all standards and quality controls, with the exception of the lowest standard (S9) and the QC LLOQ where the percentage accuracies must be within 80–120%, and the coefficients of variation must be less than 20%. Calibration standards may be excluded should they fail to meet acceptance criteria. However, quality control samples that do not meet acceptance criteria may not be excluded. Should a quality control sample be a statistical outlier, calculations excluding the outlier may be reported, provided that the calculations including this outlier are also reported.[108,109]

The intra-day results for EFV, LPV, and RTV met acceptance criteria with percentage accuracies between 89.8–105.6% for standards and 88.3–113.6% for quality controls and coefficients of variation below 6.8% for standards, below 9.7% for high-, medium-, and low-concentration quality controls, and below 15.5% for the LLOQs.

The same acceptance criteria apply for the inter-day accuracy and precision, namely that the percentage accuracies and coefficients of variation must be within 15% for all standards and quality control samples, with the exception of the lowest standard (S9) and the LLOQ, which must be within 20%. A summary of the inter-day results for each analyte is presented in Tables 2.7–2.12.

Table 2.7. Inter-day accuracy and precision results for calibration standards: EFV

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal conc. (ng/mL)	500	350	200	50.0	20.0	5.00	2.50	1.00	0.500
Average calculated conc. (ng/mL)	499	351	199	49.8	20.3	5.02	2.56	1.01	0.476
n	6	6	6	6	6	5*	6	6	6
STDEV	11.1	9.14	3.64	1.49	0.384	0.167	0.0918	0.0349	0.0219
CV(%)	2.2	2.6	1.8	3.0	1.9	3.3	3.6	3.5	4.6
%Accuracy	99.9	100.3	99.6	99.6	101.7	100.3	102.2	101.1	95.2

** Failed calibration standard excluded*

Table 2.8. Inter-day accuracy and precision results for quality controls: EFV

	QC H	QC M	QC L	QC LLOQ
Nominal conc. (ng/mL)	400	150	1.50	0.500
Average calculated conc. (ng/mL)	388	142	1.33	0.467
n	18	18	17*	18
STDEV	14.2	6.24	0.145	0.0399
CV(%)	3.7	4.4	10.9	8.5
%Accuracy	96.9	94.6	94.7	93.5

** Statistical outlier excluded due to spiking error where the working solution was added twice*

As shown in Tables 2.7 and 2.8, the inter-day percentage accuracies for EFV were 95.2–102.2% for the standards and 93.5–96.9% for the quality controls and were within acceptable limits. The coefficients of variation for the standards and quality controls were all less than 15% and were also within acceptable limits.

Table 2.9. Inter-day accuracy and precision results for calibration standards: LPV

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal conc. (ng/mL)	500	350	200	50.0	20.0	5.00	2.50	1.00	0.500
Average calculated conc. (ng/mL)	502	350	199	49.1	20.0	5.03	2.52	1.01	0.498
n	6	6	6	6	6	5*	6	6	6
STDEV	17.1	10.8	3.72	0.870	0.261	0.171	0.0594	0.0522	0.0121
CV(%)	3.4	3.1	1.9	1.8	1.3	3.4	2.4	5.2	2.4
%Accuracy	100.3	100.1	99.7	98.3	100.2	100.6	100.9	100.6	99.5

** Failed calibration standard excluded*

Table 2.10. Inter-day accuracy and precision results for quality controls: LPV

	QC H	QC M	QC L	QC LLOQ
Nominal conc. (ng/mL)	400	150	1.50	0.500
Average calculated conc. (ng/mL)	410	150	1.35	0.491
n	18	18	17*	18
STDEV	17.2	5.97	0.141	0.0367
CV(%)	4.2	4.0	10.4	7.5
%Accuracy	102.4	100.1	96.5	98.2

** Statistical outlier excluded due to spiking error where the working solution was added twice*

The inter-day percentage accuracies for LPV ranged from 98.3–100.9% for the standards and 96.5–102.4% for the quality controls and were within acceptable limits (Tables 2.9 and 2.10). The coefficients of variation were all less than 15% and were also within acceptable limits.

Table 2.11. Inter-day accuracy and precision results for calibration standards: RTV

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal conc. (ng/mL)	125	87.5	50.0	12.5	5.00	1.25	0.625	0.250	0.125
Average calculated conc. (ng/mL)	126	87.7	49.3	12.4	5.00	1.26	0.641	0.254	0.125
n	6	6	6	6	6	5*	6	6	6
STDEV	4.13	1.77	1.24	0.320	0.146	0.0279	0.0187	0.0228	0.00908
CV(%)	3.3	2.0	2.5	2.6	2.9	2.2	2.9	9.0	7.2
%Accuracy	100.6	100.2	98.5	98.9	99.9	100.7	102.5	101.4	100.2

** Failed calibration standard excluded*

Table 2.12. Inter-day accuracy and precision results for quality controls: RTV

	QC H	QC M	QC L	QC LLOQ
Nominal conc. (ng/mL)	100	37.5	0.350	0.125
Average calculated conc. (ng/mL)	110	39.8	0.372	0.132
n	18	18	17*	18
STDEV	4.73	1.61	0.0442	0.00983
CV(%)	4.3	4.1	11.9	7.5
%Accuracy	110.2	106.2	106.2	105.4

** Statistical outlier excluded due to spiking error where the working solution was added twice*

The inter-day percentage accuracies for RTV were 98.5–102.5% for the standards and 105.4–110.2% for the quality controls (Tables 2.11 and 2.12) and were within acceptable limits. The coefficients of variation were all less than 15% and were also within acceptable limits.

In summary, the intra- and inter-day accuracy and precision met the acceptance criteria for each analyte at all standard and quality control concentrations. This indicates that the method developed was accurate, precise, and reproducible for the analysis of unbound EFV, LPV, and RTV from ultracentrifuged plasma over a range of 0.500–500 ng/mL for EFV and LPV and 0.125–125 ng/mL for RTV. This is the same range validated by Avery *et al.* (2010) for unbound EFV in ultrafiltrate.[115] These ranges are also similar to the ranges validated by Ehrhardt *et al.* (2007), where the unbound LPV and RTV calibration ranges in ultrafiltrate were 0.200 – 500 ng/mL, and by Fayet *et al.* (2008), where the range of unbound EFV, LPV, and RTV in ultrafiltrate was 1.00–1000 ng/mL.[52,76] However, each of these methods were validated according to the 2001 FDA guidelines,[125] which have since been updated. The method discussed in this chapter has been validated according to the current guidelines from the FDA and EMA, which require a more thorough validation.[108,109,136]

3.5. SENSITIVITY AND SPECIFICITY

In order to assess the sensitivity of the method, six different sources of ultracentrifuged plasma (from six different lots of human plasma) were spiked at the lowest limit of quantitation (LLOQ) and extracted. The LLOQ of EFV and LPV was 0.500 ng/mL, while the LLOQ of RTV was 0.125 ng/mL. The signal-to-noise ratio of each peak was calculated using the S/N script (Analyst software version 1.6.3), where the noise was selected from the 15 seconds preceding the analyte peak. In addition, the six lots of blank ultracentrifuged plasma were extracted without internal standards (double blanks) and injected after the highest standard (S1) to evaluate any carry-over effects.

Raw chromatograms of the LLOQ samples for EFV, LPV, and RTV are shown in Figures 2.20–2.22, respectively. The signal-to-noise ratios at the LLOQ are required to be greater than or equal to five to ensure that the analytes can be quantified with accuracy and precision.[108,109] The signal-to-noise ratios at LLOQ extracted from six different lots of matrix were all greater than 76.6 for EFV, 210 for LPV, and 89.1 for RTV, indicating that the mass spectrometry method was sensitive for all three analytes. Any samples with a calculated concentration of greater than or equal to 0.500 ng/mL for EFV and LPV and 0.125 ng/mL for RTV can be reported, while any samples with a calculated concentration below these concentrations must be reported as “below the limit of quantitation” or “BLOQ.”

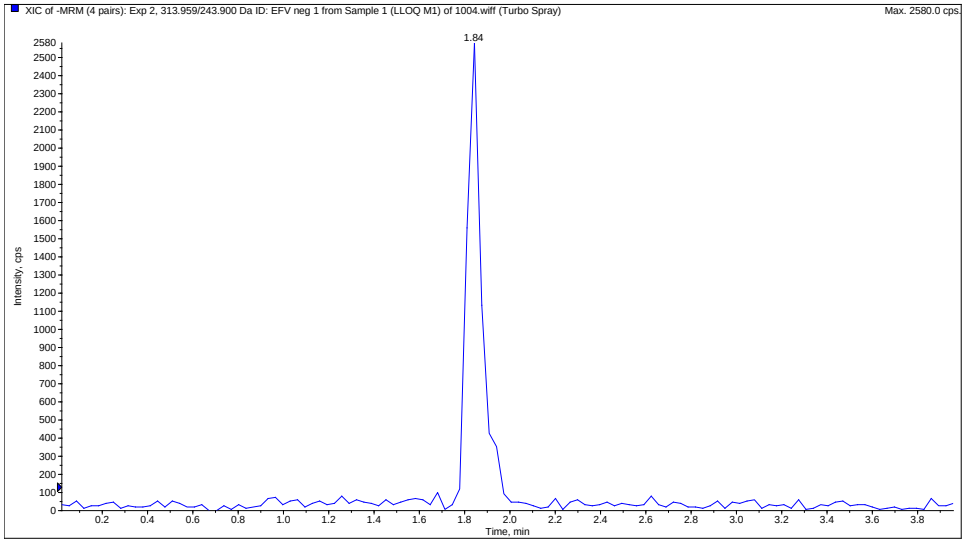

Figure 2.20. Representative chromatogram of EFV at LLOQ (0.500 ng/mL).

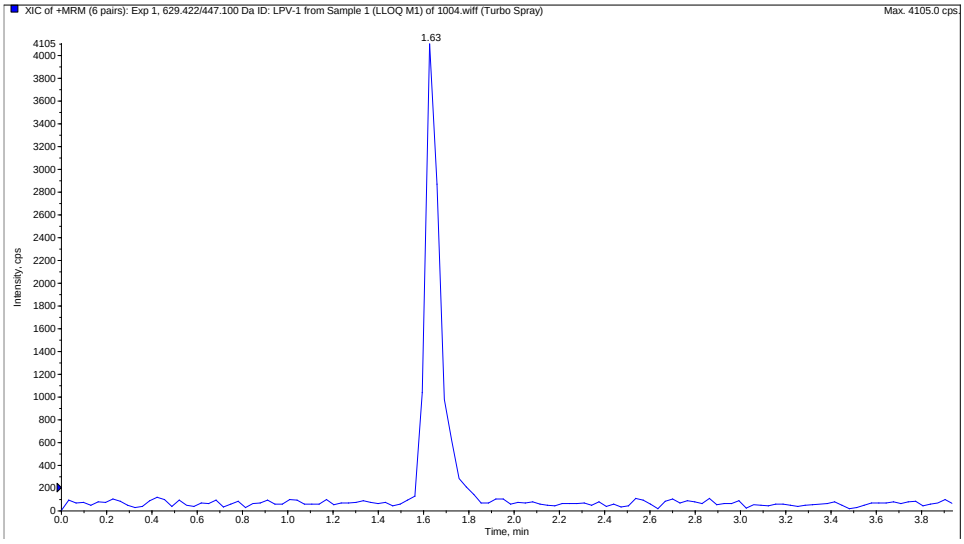

Figure 2.21. Representative chromatogram of LPV at LLOQ (0.500 ng/mL).

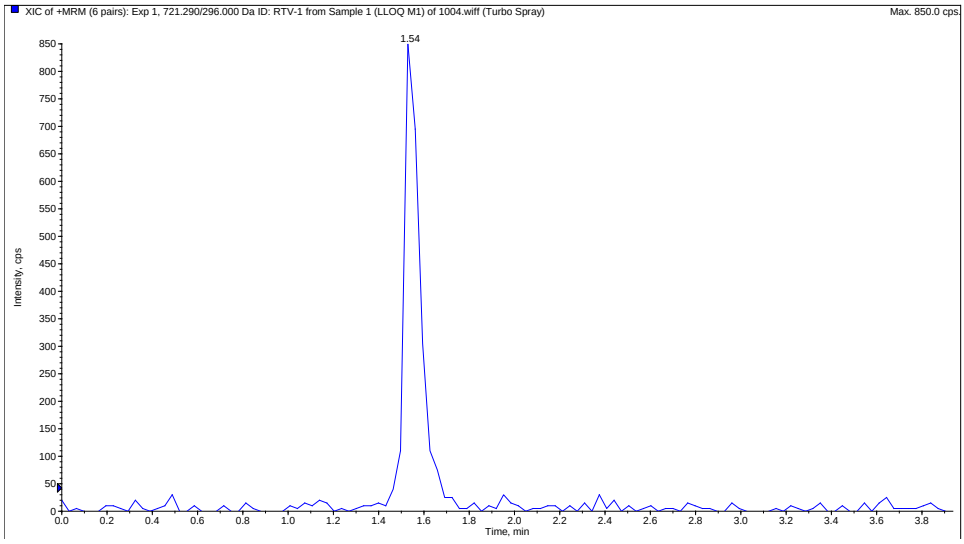

Figure 2.22. Representative chromatogram of RTV at LLOQ (0.125 ng/mL).

The double blank samples showed no carry-over for the analytes or internal standards when injected after the highest standard (Figure 2.23 a, b, and c). The double blank chromatograms showed that there was very low background noise throughout the run time and no peaks at the retention times of the analytes, indicating that the method was highly specific for the analytes and further explaining the very high signal-to-noise ratios calculated for the LLOQ samples.

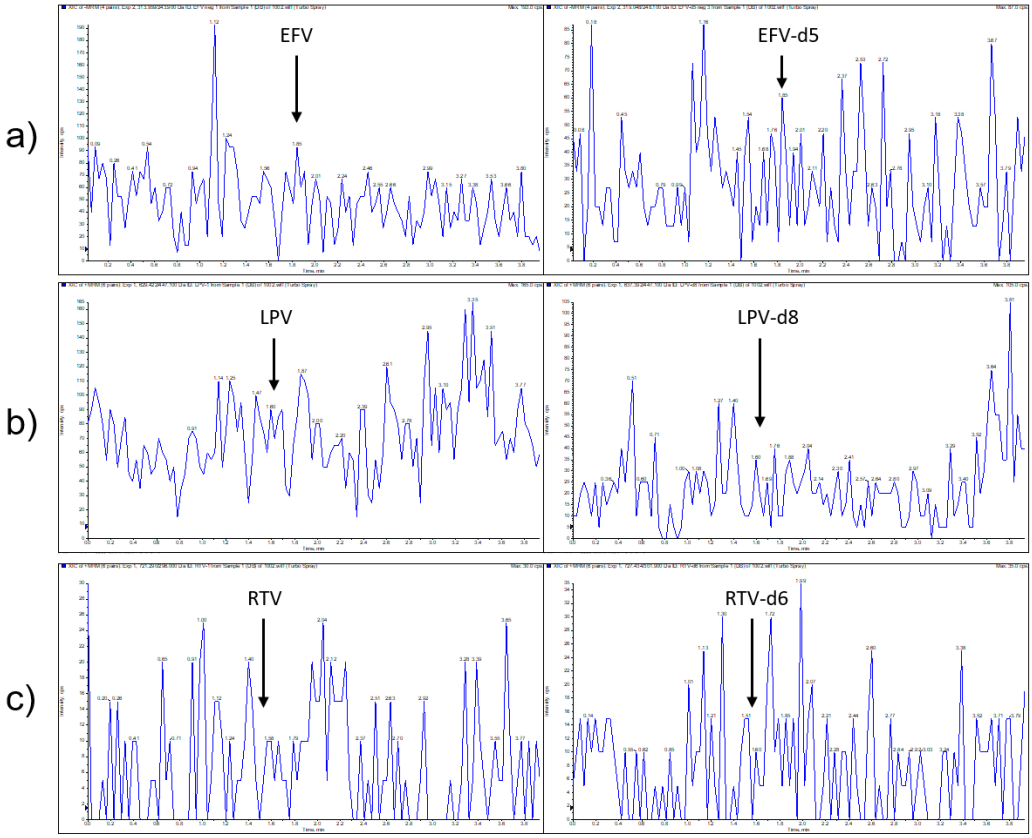

Figure 2.23. Representative chromatograms of double blank samples for **a)** EFV, **b)** LPV, **c)** RTV and their respective internal standards.

Compared to a published method by Fayet *et al.* (2008), this method is slightly more sensitive for EFV and LPV with a lower limit of 0.500 ng/mL compared to 1.00 ng/mL and four times more sensitive for RTV with an LLOQ of 0.125 ng/mL compared to 1.00 ng/mL.[52] The method is also ten times more sensitive for unbound EFV compared to the method by Almond *et al.* (2008), where the LLOQ of EFV from 200 µL ultrafiltrate was 5.00 ng/mL.[24]

3.6. CROSSTALK

Crosstalk refers to any contribution of one analyte to a different analyte's MRM channel, resulting in a false-positive result. Crosstalk can also occur between an analyte and its corresponding internal standard's MRM channel.

Six blank ultracentrifuged samples (without analyte but containing internal standards) were extracted to determine the possible contribution of the internal standard to the analyte MRM channel. Chromatograms of blank samples are presented in Figure 2.24 a, b, and c. Crosstalk between the three analytes was evaluated by extracting a sample containing each analyte separately at the highest standard concentration. For example, a sample would contain EFV at the highest concentration – but no LPV or RTV – and any subsequent peaks in the LPV or RTV channels would, therefore, be due to crosstalk from the EFV channel.

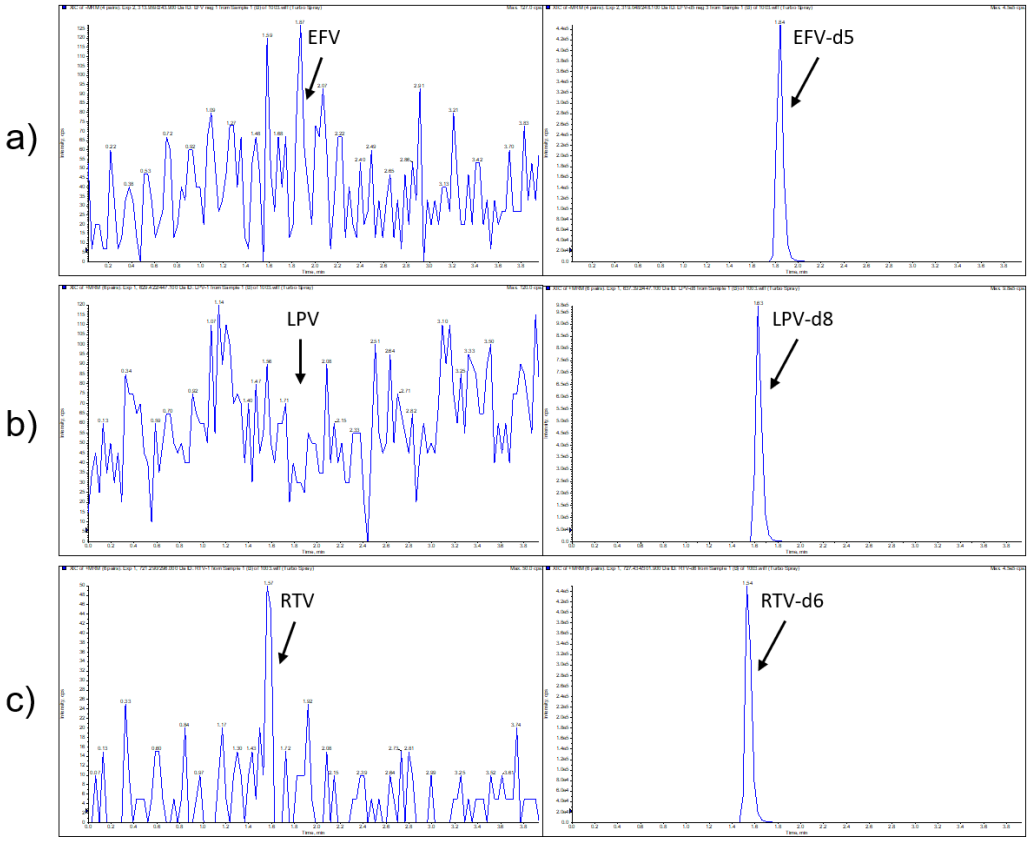

Figure 2.24. Representative chromatograms of blank samples for **a)** EFV, **b)** LPV, **c)** RTV and their respective internal standards.

The blank samples (without analytes but with internal standards) showed that there was no contribution of the internal standards to the analyte MRM channels as there were no peaks in the analyte channels. The sample containing EFV showed no peaks in the LPV and RTV channels, the LPV

sample showed no peaks in the EFV and RTV channels, and the RTV sample showed no peaks in the EFV and LPV channels, indicating that there is no crosstalk between any of the three analytes (chromatograms not shown).

3.7. MATRIX EFFECTS

In a bioanalytical method using HPLC-MS/MS, matrix effects refer to any substances present in a sample other than the analytes of interest that may impact ionization in the source. It is important to mitigate possible matrix effects by removing them during extraction and having an appropriate internal standard that will compensate for any ion enhancement or suppression.

To evaluate matrix effects, the Matuszewski method was used.[137] Six different blank sources of ultracentrifuged plasma (from six different lots of human plasma) were extracted without internal standards. After extraction, the samples were spiked at the theorectical post-extraction low, medium, and high concentrations (taking into account the dilution steps included in the method) and at one concentration of the internal standards.

The peak area ratios of each of the quality control levels *versus* the respective concentrations were used to calculate the regression of each of the ultracentrifuged plasma sources (Tables 2.13–2.15).

Table 2.13. Regression results to determine matrix effects: EFV

	High Conc. 400 ng/mL Peak Area Ratio	**Medium Conc. 150 ng/mL Peak Area Ratio**	**Low Conc. 1.40 ng/mL Peak Area Ratio**	**Peak Area Ratio *versus* Conc. Regression Slope**
Matrix 1	2.99	1.01	0.00876	0.00752
Matrix 2	3.05	1.04	0.00922	0.00767
Matrix 3	3.06	1.05	0.00933	0.00769
Matrix 4	3.09	1.10	0.00865	0.00775
Matrix 5	2.98	1.08	0.00875	0.00746
Matrix 6	2.84	1.04	0.00945	0.00711
Average	**3.00**	**1.05**	**0.00903**	**0.00753**
STDEV	**0.0902**	**0.0315**	**0.000346**	**0.000236**
CV(%)	**3.0**	**3.0**	**3.8**	**3.1**

Table 2.14. Regression results to determine matrix effects: LPV

	High Conc. 400 ng/mL Peak Area Ratio	Medium Conc. 150 ng/mL Peak Area Ratio	Low Conc. 1.40 ng/mL Peak Area Ratio	Peak Area Ratio *versus* Conc. Regression Slope
Matrix 1	1.79	0.699	0.00692	0.00445
Matrix 2	1.73	0.704	0.00722	0.00431
Matrix 3	1.79	0.732	0.00686	0.00445
Matrix 4	1.75	0.721	0.00683	0.00435
Matrix 5	1.75	0.714	0.00648	0.00435
Matrix 6	1.67	0.682	0.00677	0.00416
Average	**1.75**	**0.709**	**0.00684**	**0.00434**
STDEV	**0.0429**	**0.0175**	**0.000240**	**0.000109**
CV(%)	**2.5**	**2.5**	**3.5**	**2.5**

Table 2.15. Regression results to determine matrix effects: RTV

	High Conc. 100 ng/mL Peak Area Ratio	Medium Conc. 37.5 ng/mL Peak Area Ratio	Low Conc. 0.350 ng/mL Peak Area Ratio	Peak Area Ratio *versus* Conc. Regression Slope
Matrix 1	1.29	0.492	0.00448	0.0129
Matrix 2	1.24	0.477	0.00473	0.0124
Matrix 3	1.29	0.498	0.00479	0.0129
Matrix 4	1.31	0.502	0.00463	0.0130
Matrix 5	1.24	0.494	0.00439	0.0123
Matrix 6	1.18	0.475	0.00421	0.0118
Average	**1.26**	**0.489**	**0.00454**	**0.0125**
STDEV	**0.0449**	**0.0114**	**0.000220**	**0.000462**
CV(%)	**3.6**	**2.3**	**4.9**	**3.7**

The mean variability of the individual slopes was determined to be 3.1% for EFV, 2.5% for LPV, and 3.7% for RTV, which all meet the acceptance criteria of less than 5% as stipulated in the FDA and EMA guidelines.[108,109] This means that there were no background components co-extracted with the analytes that would adversely affect the ionization of the analytes, such as causing ion suppression or enhancement. The low level of matrix effects may be due to the extraction method removing interfering components, but also due to the ultracentrifugation process, which separates the proteins and lipids and effectively serves as a clean-up step.

3.8. RECOVERY

It is important that the analyte is not removed from the sample or lost during the extraction procedure. The percentage of analyte recovered from the sample need not be 100% but must be consistent across different concentrations and different matrix sources.

Six lots of ultracentrifuged plasma were spiked at high, medium, and low concentrations and extracted as per the methodology described in Section 2.4 of this chapter. These were referred to as the "test" samples. The same six lots of ultracentrifuged plasma were extracted blank and spiked at high, medium, and low levels after extraction, accounting for the dilution steps included in the method. These samples were used as the reference samples, which represent 100% recovery. The test sample peak areas were compared to the reference sample peak areas to calculate the percentage recovery during extraction. The percentage recovery for each of the analytes is summarized in the tables below (Tables 2.16–2.17).

Table 2.16. Recovery results for EFV

	High Concentration (400 ng/mL)		**Medium Concentration (150 ng/mL)**		**Low Concentration (1.40 ng/mL)**	
	Reference: Peak Area	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**
Sample 1	5990000	4660000	2230000	1860000	23800	19800
Sample 2	5570000	4720000	2260000	1940000	22400	20600
Sample 3	4820000	4880000	2110000	1980000	18900	19700
Sample 4	5670000	5130000	2200000	1970000	18400	19200
Sample 5	5650000	5060000	2310000	1970000	18900	[45400]
Sample 6	5400000	5450000	2050000	2000000	19100	20400
Average	**5516667**	**4983333**	**2193333**	**1953333**	**20250**	**19940**
STDEV	**391697**	**293167**	**96885**	**49666**	**2263**	**564**
CV(%)	**7.1**	**5.9**	**4.4**	**2.5**	**11.2**	**2.8**
%Recovery		**90.3**		**89.1**		**98.5**
					Average %Recovery	**92.6**
					Average CV(%)	**5.5**

[ ] outlier excluded due to spiking error where the working solution was added twice

Table 2.17. Recovery results for LPV

	High Concentration (400 ng/mL)		Medium Concentration (150 ng/mL)		Low Concentration (1.40 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Sample 1	17500000	14400000	6800000	6650000	71200	51200
Sample 2	16900000	13600000	7310000	6170000	71200	62800
Sample 3	15200000	15000000	7000000	6120000	66500	65500
Sample 4	17400000	15100000	7440000	6270000	66700	54500
Sample 5	18700000	15100000	7370000	5600000	71800	[103000]
Sample 6	18900000	11500000	7450000	5460000	71800	48300
Average	**17433333**	**14116667**	**7228333**	**6045000**	**69867**	**56460**
STDEV	**1344123**	**1407717**	**266939**	**442301**	**2545**	**7416**
CV(%)	**7.7**	**10.0**	**3.7**	**7.3**	**3.6**	**13.1**
%Recovery		**81.0**		**83.6**		**80.8**
					Average %Recovery	**81.8**
					Average CV(%)	**1.9**

[ ] outlier excluded due to spiking error where the working solution was added twice

Table 2.18. Recovery results for RTV

	High Concentration (100 ng/mL)		Medium Concentration (37.5 ng/mL)		Low Concentration (0.350 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Sample 1	3350000	3190000	1290000	1410000	13800	11700
Sample 2	3560000	2880000	1490000	1270000	16500	13400
Sample 3	3180000	3080000	1410000	1190000	17000	14200
Sample 4	4020000	3160000	1470000	1260000	18400	11600
Sample 5	4250000	2820000	1470000	940000	16600	[24200]
Sample 6	4570000	2220000	1750000	1060000	16100	9990
Average	**3821667**	**2891667**	**1480000**	**1188333**	**16400**	**12178**
STDEV	**545066**	**361354**	**151129**	**166783**	**1501**	**1653**
CV(%)	**14.3**	**12.5**	**10.2**	**14.0**	**9.2**	**13.6**
%Recovery		**75.7**		**80.3**		**74.3**
					Average %Recovery	**76.7**
					Average CV(%)	**4.1**

[ ] outlier excluded due to spiking error where the working solution was added twice

The mean recovery for EFV, LPV, and RTV across concentration levels was determined to be 92.6%, 81.8%, and 76.7%, respectively. While the recoveries were all greater than 75%, more importantly, recovery was consistent and precise across the different concentrations, with a required precision of less than 15%. The coefficient of variation for each quality control level was less than 15% for all three analytes, and the precision across the high, medium, and low concentrations were 5.5%, 1.9%, and 4.1% for EFV, LPV, and RTV, respectively.

3.9. PROCESS EFFICIENCY

Process efficiency assesses the effect of both recovery and matrix effects on an analyte's response. It is tested using neat solvent (mobile phase) spiked in triplicate at the theoretical final concentrations of each quality control level as the reference samples, representing 100% recovery and zero matrix effects. Test samples were spiked into six different lots of ultracentrifuged plasma at high, medium, and low concentrations and were extracted as per the methodology described in Section 2.4 of this chapter. These peak areas were compared to the reference peak areas to calculate the percentage process efficiency. As with recovery, process efficiency need not be 100% but must be consistent across different concentrations and plasma sources.

The analyte peak areas observed after extraction are compared to the peak areas of the neat samples and expressed as percentage process efficiency (Tables 2.19–2.21).

Table 2.19. Process efficiency results for EFV

	High Concentration (400 ng/mL)		**Medium Concentration (150 ng/mL)**		**Low Concentration (1.40 ng/mL)**	
	Reference: Peak Area	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**
Matrix 1	6020000	4660000	2490000	1860000	24300	19800
Matrix 2	5770000	4720000	2550000	1940000	25100	20600
Matrix 3	6070000	4880000	2340000	1980000	24600	19700
Matrix 4		5130000		1970000		19200
Matrix 5		5060000		1970000		[45400]
Matrix 6		5450000		2000000		20400
Average	**5953333**	**4983333**	**2460000**	**1953333**	**24667**	**19940**
STDEV	**49666**	**293167**	**108167**	**45338**	**330**	**564**
CV(%)	**0.8**	**5.9**	**4.4**	**2.3**	**1.3**	**2.8**
%Recovery		**83.7**		**79.4**		**80.8**
					Average %Process Efficiency	**81.3**
					Average CV(%)	**2.7**

[ ] outlier excluded due to spiking error where the working solution was added twice

Table 2.20. Process efficiency results for LPV

	High Concentration (400 ng/mL)		Medium Concentration (150 ng/mL)		Low Concentration (1.40 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Matrix 1	18500000	14400000	7630000	6650000	76300	51200
Matrix 2	17400000	13600000	7550000	6170000	76900	62800
Matrix 3	17700000	15000000	6690000	6120000	80000	65500
Matrix 4		15100000		6270000		54500
Matrix 5		15100000		5600000		[103000]
Matrix 6		11500000		5460000		48300
Average	**17866667**	**14116667**	**7290000**	**6045000**	**77733**	**56460**
STDEV	**442301**	**1407717**	**521153**	**403764**	**1621**	**7416**
CV(%)	**2.5**	**10.0**	**7.1**	**6.7**	**2.1**	**13.1**
%Recovery		**79.0**		**82.9**		**72.6**
					Average %Process Efficiency	**78.2**
					Average CV(%)	**6.6**

[ ] outlier excluded due to spiking error where the working solution was added twice

Table 2.21. Process efficiency results for RTV

	High Concentration (100 ng/mL)		Medium Concentration (37.5 ng/mL)		Low Concentration (0.350 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Matrix 1	3220000	3190000	1130000	1410000	13300	11700
Matrix 2	3130000	2880000	1220000	1270000	12500	13400
Matrix 3	2990000	3080000	1090000	1190000	12900	14200
Matrix 4		3160000		1260000		11600
Matrix 5		2820000		940000		[24200]
Matrix 6		2220000		1060000		9990
Average	**3113333**	**2891667**	**1146667**	**1188333**	**12900**	**12178**
STDEV	**115902**	**361354**	**66583**	**166783**	**400**	**1653**
CV(%)	**3.7**	**12.5**	**5.8**	**14.0**	**3.1**	**13.6**
%Recovery		**92.9**		**103.6**		**94.4**
					Average %Process Efficiency	**97.0**
					Average CV(%)	**6.0**

[ ] outlier excluded due to spiking error where the working solution was added twice

The mean process efficiency of EFV, LPV, and RTV is 81.3%, 78.2%, and 97.0%, respectively. The mean process efficiency of EFV was 11.3% lower than its mean recovery, which suggests that there is some ion suppression occurring in the source of the mass spectrometer. Conversely, the mean process efficiency of RTV was 20.3% higher than its mean recovery, which suggests that there is some ion enhancement occurring. However, as the matrix effects experiment for these analytes showed, these effects were consistent across different lots of plasma and were compensated for by the internal standards and, therefore, do not negatively impact the method. The mean process efficiency and

recovery of LPV only differed by 3.6%, indicating that the ionization of LPV was not influenced by any background components present in the sample.

While it is interesting to note the differences between the recovery and process efficiency results, they do not determine whether a method does or does not meet acceptance criteria. The precision of the measured process efficiency should not exceed 15% for any concentration, and the reproducibility between concentration levels should also not be greater than 15%.[108,109] The precision at each concentration was below 15%, and the reproducibility between the concentrations was 2.7%, 6.6%, and 6.0% for EFV, LPV, and RTV, respectively.

3.10. Dilution Integrity

During analysis of unknown patient samples, a calculated concentration may be reported as greater than the ULOQ. As this concentration lies outside of the validated range and is calculated by extrapolating the calibration curve, this result may not be reported with confidence. To evaluate if these samples may be diluted to within the validated range and re-extracted with accuracy and precision, six QC DIL samples were prepared at twice the ULOQ (1000 ng/mL for EFV and LPV and 250 ng/mL for RTV). These samples were diluted 1:4 pre-extraction with blank ultracentrifuged plasma, extracted as normal, and compared with the nominal concentration to calculate the percentage accuracy. Due to the highly aqueous nature of the ultracentrifuged plasma and the solubility issues noted during method development, a post-extraction dilution was also evaluated. Undiluted QC DIL samples were extracted in six-fold and were then diluted 1:4 with extracted blank samples (which contained internal standards) post-extraction. The resulting precision and accuracy data are shown (Tables 2.22–2.24).

Table 2.22. Dilution integrity results for EFV following a 5-fold pre- and post-extraction dilution

	Nominal Conc. (ng/mL)	Pre-extraction dilution calculated conc. (ng/mL)	Post-extraction dilution calculated conc. (ng/mL)
Sample 1	**1000**	536	844
Sample 2		474	832
Sample 3		560	898
Sample 4		467	778
Sample 5		576	874
Sample 6		520	923
	Average	**522**	**858**
	STDEV	**44.6**	**51.7**
	CV(%)	**8.5**	**6.0**
	%Accuracy	**52.2**	**85.8**

Table 2.23. Dilution integrity results for LPV following a 5-fold pre- and post-extraction dilution

	Nominal Conc. (ng/mL)	Pre-extraction dilution calculated conc. (ng/mL)	Post-extraction dilution calculated conc. (ng/mL)
Sample 1	**1000**	507	1010
Sample 2		443	1050
Sample 3		527	1090
Sample 4		443	934
Sample 5		579	1070
Sample 6		545	1100
	Average	**507**	**1042**
	STDEV	**55.3**	**62.0**
	CV(%)	**10.9**	**5.9**
	%Accuracy	**50.7**	**104.2**

Table 2.24. Dilution integrity results for RTV following a 5-fold pre- and post-extraction dilution

	Nominal Conc. (ng/mL)	Pre-extraction dilution calculated conc. (ng/mL)	Post-extraction dilution calculated conc. (ng/mL)
Sample 1	**250**	153	264
Sample 2		131	279
Sample 3		153	281
Sample 4		139	242
Sample 5		172	299
Sample 6		156	295
	Average	**150**	**277**
	STDEV	**13.9**	**21.1**
	CV(%)	**9.3**	**7.6**
	%Accuracy	**60.2**	**110.8**

The same acceptance criteria apply as for the high-, medium-, and low-concentration quality control samples, namely that the percentage accuracy and precision must be within 15%. The pre-extraction diluted samples did not meet these acceptance criteria for accuracy, with an average percentage accuracy of 52.5%, 50.7%, and 60.2% for EFV, LPV, and RTV, respectively. This indicates that reported concentrations above the upper limit of the validated calibration curve may not be diluted before they are extracted. It is hypothesized that this low percentage accuracy is due to the poor solubility of the analytes in aqueous solutions. EFV, LPV, and RTV have respective logP values of 4.457, 4.688, and 5.222[131–133], which could result in approximately half the analyte molecules precipitating out of solution when diluted with the highly aqueous ultracentrifuged plasma prior to extraction.

This effect was not noticeable when diluted post-extraction with blank extracted matrix, likely due to the organic nature of the sample and diluent. The calculated precision and accuracy for the post-extraction dilutions of EFV, LPV, and RTV fall within the accepted limits. Therefore, patient samples that have a reported concentration above the upper limit of quantification or “ALOQ” (up to a

maximum concentration of 1000 ng/mL for EFV and LPV and 250 ng/mL for RTV) may be diluted 1:4 with the "blank" from that batch (which was extracted using the same internal standard-containing extraction solution) and may be reanalysed reliably. It is, therefore, advisable to include additional extracted blank samples in any patient sample batch in the event that dilutions may be required.

The use of a post-extraction dilution for ALOQ samples is particularly useful for this assay as it mitigates the need for the second aliquot of 250 µL plasma to be ultracentrifuged, especially as the plasma may not be thawed and refrozen prior to ultracentrifugation (see Section 3.16.3 for freeze-thaw stability).

3.11. ANTICOAGULANT CROSS-VALIDATION

The method validated uses ultracentrifuged plasma originating from plasma containing K3EDTA as the anticoagulant for all the calibration standards and quality control samples. However, at times, clinical samples are collected in tubes containing other anticoagulants, and it is important to determine if such samples may still be analysed accurately and precisely. To evaluate the effect that a different anticoagulant may have on the ionization of the analytes and internal standards, quality control samples were prepared in six-fold in ultracentrifuged plasma originating from plasma containing either K2EDTA or lithium heparin as the anticoagulant. These test samples were analysed against a calibration curve prepared in the validated K3EDTA-containing matrix. The results are presented in Tables 2.25–2.27 below. To evaluate whether the use of a different anticoagulant had any effect on the selectivity of the method, a double blank sample was extracted from ultracentrifuged plasma originating from plasma containing K3EDTA, K2EDTA, or lithium heparin as the anticoagulant.

Table 2.25. Anticoagulant cross-validation results: EFV

	K2EDTA			**Lithium Heparin**		
	QC High	**QC Med**	**QC Low**	**QC High**	**QC Med**	**QC Low**
Nominal conc. (ng/mL)	400	150	1.40	400	150	1.40
Average calculated conc. (ng/mL)	390	146	1.31	392	147	1.31
n	6	6	6	6	6	6
STDEV	7.14	3.50	0.0653	3.87	1.75	0.0387
CV(%)	1.8	2.4	5.0	1.0	1.2	2.9
%Accuracy	97.5	97.6	93.3	98.0	98.2	93.7

Table 2.26. Anticoagulant cross-validation results: LPV

	K2EDTA			Lithium Heparin		
	QC High	**QC Med**	**QC Low**	**QC High**	**QC Med**	**QC Low**
Nominal conc. (ng/mL)	400	150	1.40	400	150	1.40
Average calculated conc. (ng/mL)	413	158	1.40	419	159	1.39
n	6	6	6	6	6	6
STDEV	12.2	3.44	0.0592	11.3	3.06	0.0314
CV(%)	2.9	2.2	4.2	2.7	1.9	2.3
%Accuracy	103.3	105.6	99.8	104.7	105.9	99.5

Table 2.27. Anticoagulant cross-validation results: RTV

	K2EDTA			Lithium Heparin		
	QC High	**QC Med**	**QC Low**	**QC High**	**QC Med**	**QC Low**
Nominal conc. (ng/mL)	400	37.5	0.350	100	37.5	0.350
Average calculated conc. (ng/mL)	110	41.3	0.376	112	41.9	0.375
n	6	6	6	6	6	6
STDEV	3.14	0.973	0.0142	2.64	0.532	0.0156
CV(%)	2.8	2.4	3.8	2.4	1.3	4.2
%Accuracy	110.3	110.0	107.5	111.8	111.8	107.0

The same acceptance criteria apply as for the inter- and intra-day quality control samples; namely, the percentage accuracy and precision must be within 15%. The results (Tables 2.25–2.27) show that the accuracy and precision for both anticoagulants at each concentration were within the acceptable limits for all three analytes. Likewise, the same acceptance criteria apply to the double blank samples, where any peak in the analyte channel should not be greater than 20% of the LLOQ peak, and any peak in the internal standard channel should not be greater than 5% of the internal standard peak at working concentrations. No interfering peaks were observed in either the analyte or internal standard channels for any of the three analytes when K2EDTA or lithium heparin were used instead of K3EDTA (chromatograms not shown). Overall, these results indicate that the use of K2EDTA or lithium heparin does not influence the accuracy, precision, or selectivity of the method, and clinical samples containing these anticoagulants may be analysed with confidence.

3.12. EFFECT OF HAEMOLYSIS

During sample collection, whole blood is centrifuged to separate the red blood cells from the plasma. Sometimes the red blood cells burst – known as haemolysis – and their contents partition into the plasma. Haemolysis may occur during phlebotomy due to an incorrect needle size or tube used, excessive tourniquet, or during sample handling due to prolonged time before centrifugation or excessive centrifuge speed.[138] Haemolyzed plasma contains charged species such as haemoglobin and increased potassium levels which may affect the ionization of analytes and internal standards compared to non-haemolyzed samples.[139] Sample extraction and chromatography may remove any interfering molecules, or the internal standard may compensate for any change in analyte response; however, it is important to test for this during method validation.

Haemolysis was tested at 2% haemolysed blood in plasma which was ultracentrifuged as normal. Six-fold high- and low-quality controls were prepared in haemolyzed ultracentrifuged plasma and in non-haemolyzed ultracentrifuged plasma and extracted. The resulting peak area ratios are presented in Tables 2.28–2.30.

Table 2.28. Effect of 2% haemolysis on EFV peak area ratios

	High conc. (400 ng/mL)		Low conc. (1.40 ng/mL)	
	No haemolysis	**2% haemolysis**	**No haemolysis**	**2% haemolysis**
Average peak area ratio	3.68	3.67	0.0152	0.0135
n	6	6	6	6
STDEV	0.162	0.146	0.00193	0.000531
CV(%)	4.4	4.0	12.7	3.9
%Difference		-0.4		-10.7

Table 2.29. Effect of 2% haemolysis on LPV peak area ratios

	High conc. (400 ng/mL)		Low conc. (1.40 ng/mL)	
	No haemolysis	**2% haemolysis**	**No haemolysis**	**2% haemolysis**
Average peak area ratio	2.18	2.18	0.00899	0.00847
n	6	6	5*	6
STDEV	0.0795	0.0717	0.00123	0.000255
CV(%)	3.6	3.3	13.7	3.0
%Difference		0.2		-5.8

** Statistical outlier excluded*

Table 2.30. Effect of 2% haemolysis on RTV peak area ratios

	High conc. (100 ng/mL)		Low conc. (0.350 ng/mL)	
	No haemolysis	**2% haemolysis**	**No haemolysis**	**2% haemolysis**
Average peak area ratio	1.02	1.02	0.00374	0.00374
n	6	6	5*	6
STDEV	0.0364	0.0359	0.000347	0.000141
CV(%)	3.6	3.5	9.3	3.8
%Difference		-0.3		-0.1

** Statistical outlier excluded*

A percentage difference of greater than 15% between the test and reference samples indicates that 2% haemolysis negatively impacts the ionization of the analytes and that the internal standard failed to compensate. In such a case, haemolyzed plasma samples would have to be rejected. As presented in Tables 2.28–2.30, the peak area ratios of the haemolyzed quality control samples were all within 10.7% of the reference non-haemolyzed samples, which shows that 2% haemolysis has no significant effect on the quantification of EFV, LPV, and RTV. The coefficients of variation for the haemolyzed samples were less than 4.0% for all three analytes, indicating that 2% haemolysis also had no significant effect on the precision of the assay. A double blank sample originating from haemolysed plasma that was ultracentrifuged and extracted as normal showed no interfering peaks in any of the analyte or internal standard channels, indicating that the presence of 2% haemolysis also has no effect on the selectivity of the method (chromatograms not shown).

It is interesting to note that after ultracentrifugation, there was visually no difference in the aqueous layer observed between the haemolyzed and non-haemolyzed samples. The protein pellet at the bottom of the tube was yellow-orange for the reference samples and red for the haemolyzed samples, indicating that the haemoglobin and red blood cell fragments were removed during ultracentrifugation. Any additional small molecules from the haemolyzed red blood cells that could not be seen with the naked eye, such as potassium, could have contaminated the aqueous middle layer; however, such contaminants were either removed during sample clean up, compensated for by the internal standards, or they had no significant effect on analyte ionization.

3.13. ULTRACENTRIFUGE METHOD VALIDATION

To the best of the authors' knowledge, validated methods for the quantification of unbound drug concentrations using HPLC-MS/MS have only assessed the accuracy, precision, and reproducibility of the method after the unbound drug has been separated from that to which it is bound. The three-day

validation is performed in ultracentrifuged plasma, filtrate, or dialysate, depending on the separation method used (ultracentrifugation, ultrafiltration, or equilibrium dialysis, respectively), where the analytes of interest are added after the separation procedure. The acceptance criteria stipulated by the FDA and EMA guidelines for method validations[108,109] are, therefore, only evaluating the extraction and HPLC-MS/MS methods. Typically, equilibrium dialysis is used as a reference method to which other separation methods are compared. While the separation method may be optimized or standardized, its reproducibility is not held to the same strict acceptance criteria.

To evaluate if the ultracentrifuge method was performing reliably, plasma samples from three different sources were ultracentrifuged in six-fold over three independent runs. The collected ultracentrifuged plasma was analysed for protein levels using the Pierce™ BCA protein assay kit microplate procedure from Thermo Fisher Scientific, as described in Section 2.2. The assay kit includes a 2 mg/mL BSA standard which was used to prepare a nine-point calibration curve through zero with a protein concentration range of 25.0–2000 µg/mL. The mean absorbance value of duplicate blank samples, containing no BCA, was subtracted from the absorbance values for each duplicate standard and unknown sample to normalize the results; and to draw the curve through zero as advised by the assay kit user guide. The mean absorbance values were plotted against their respective protein concentration to generate the standard curve presented in Figure 2.25. While one would expect absorbance values to fit a linear regression, the assay kit user guide states that a "quadratic of best-fit curve provides more accurate results than a purely linear fit."[128]

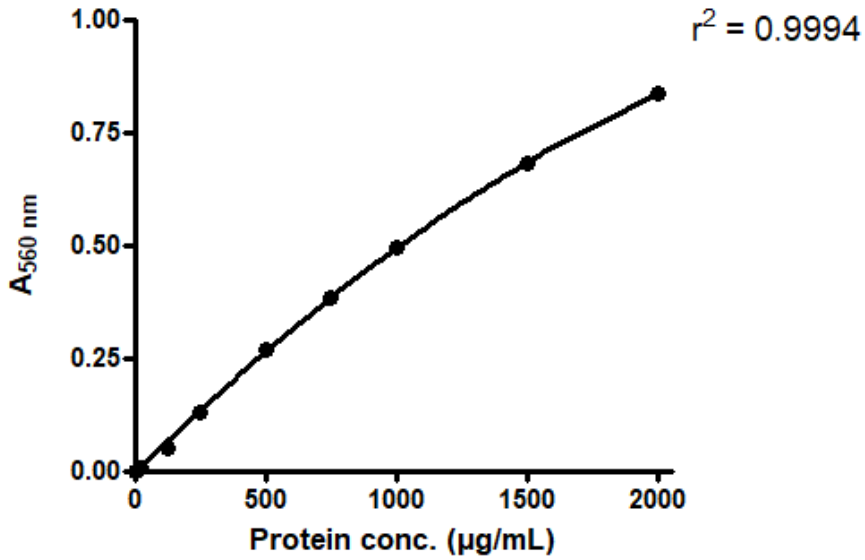

Figure 2.25. Calibration curve of mean absorbance *versus* protein concentration (n=2) to determine the protein concentration of ultracentrifuged plasma samples.

The calculated protein concentration of each aliquot of ultracentrifuged plasma collected over the three independent runs was determined. The intra- and inter-day precision and reproducibility results are presented in Table 2.31.

Table 2.31. Ultracentrifuge intra- and inter-day validation: Calculated protein concentrations (µg/mL)

	Day 1	Day 2	Day 3
Sample 1	452	398	388
Sample 2	366	408	369
Sample 3	360	463	422
Sample 4	387	420	480
Sample 5	354	526	404
Sample 6	469	363	392
Average protein conc. (µg/mL)	398	430	409
STDEV	50.0	57.3	38.8
Intra-day CV(%)	12.6	13.3	9.5
%Difference from Day 1		**8.0**	**2.8**
Overall average protein conc. in µg/mL (n = 18)			412
STDEV (n = 18)			48.3
Inter-day CV(%)			**11.7**

There are no specific acceptance criteria for an ultracentrifugation method; however, the same acceptance criteria were applied as for a standard three-day bioanalytical method validation. Namely, the intra- and inter-day precision must be less than 15% to show that the method is precise every day and over separate runs. The ultracentrifuge method developed and validated in this chapter had an intra-day precision of less than 13.3% and an overall precision of 11.7%, which shows that each sample will be separated into its three layers to the same degree, even when different lots of plasma were used. The percentage difference between each validation day was less than 8.0%, indicating that the ultracentrifuge method was reproducible. This is the first report of an ultracentrifuge method being validated to the same strict acceptance criteria as an extraction or the HPLC-MS/MS method.

It is difficult to assess the accuracy of the ultracentrifuge method as there is no nominal concentration with which to compare. A study by Nakai *et al.* (2004) investigated the protein contamination of the middle, aqueous layer of ultracentrifuged plasma and its effect on unbound drug concentrations. They found a mean protein concentration of 702 ± 100 µg/mL but concluded that this protein contamination had a negligible effect on the measured unbound drug level as the majority of the protein detected was found to be small peptides and tyrosine and tryptophan amino acids, whose presence form the basis of the BCA test. This is supported by the fact that albumin contributed only 50 µg/mL of the total 702 µg/mL protein contamination, and no AAG was detected.[73] In practice, Nakai *et al.* (2004) found that the unbound drug concentration determined by ultracentrifugation had a

correlation factor of 0.99 when compared to other methods such as equilibrium dialysis, despite the protein contamination observed. This validation found an even lower level of total protein contamination of 412 ± 48.3 µg/mL in the aqueous layer, suggesting that the drug levels found in the aqueous layer do accurately represent the unbound drug concentration.

3.14. SURROGATE MATRIX

To collect enough blank ultracentrifuged plasma for the calibration curve and quality control samples, a large volume of plasma is required (~5 mL of blank human plasma per duplicate curve). Ultracentrifuging the blank plasma is a timely process that reduces the number of unknown samples that can be processed per day as the ultracentrifuge rotor only holds 72 samples at a time. Therefore, to reduce the volume of plasma required per batch and to increase the number of unknown samples ultracentrifuged per batch, the use of a surrogate matrix was investigated. Based on the BCA test results used in the ultracentrifuge 3-day validation in Section 3.13 of this chapter, as well as the published results from Nakai *et al.* (2004), a 1:200 dilution of plasma in water was used to mimic the protein content of ultracentrifuged plasma. To evaluate the accuracy of the surrogate matrix, a set of high-, medium-, and low-concentration quality control samples were prepared in 1:200 diluted plasma. These samples were extracted as per the methodology described in Section 2.4 and analysed against a calibration curve prepared in ultracentrifuged plasma. The measured concentrations and calculated differences are presented (Tables 2.32–2.34). A double blank 1:200 diluted plasma sample was also extracted to investigate the effect of using the surrogate matrix on the method's selectivity.

Table 2.32. Surrogate matrix accuracy and precision results: EFV

	1:200 diluted plasma		
	QC High	**QC Med**	**QC Low**
Nominal conc. (ng/mL)	400	150	1.40
Average calculated conc. (ng/mL)	370	133	1.38
n	6	6	6
STDEV	10.9	2.79	0.0564
CV(%)	3.0	2.1	4.2
%Accuracy	92.4	88.6	95.1

Table 2.33. Surrogate matrix accuracy and precision results: LPV

	1:200 diluted plasma		
	QC High	**QC Med**	**QC Low**
Nominal conc. (ng/mL)	400	150	1.40
Average calculated conc. (ng/mL)	395	143	1.26
n	6	6	6
STDEV	10.9	4.03	0.0519
CV(%)	2.8	2.8	4.1
%Accuracy	98.7	95.6	89.9

Table 2.34. Surrogate matrix accuracy and precision results: RTV

	1:200 diluted plasma		
	QC High	**QC Med**	**QC Low**
Nominal conc. (ng/mL)	100	37.5	0.350
Average calculated conc. (ng/mL)	105	38.1	0.355
n	6	6	6
STDEV	2.59	0.828	0.0263
CV(%)	2.5	2.2	7.4
%Accuracy	104.5	101.6	101.4

The resulting precision and accuracy fall within the accepted values, which indicate that the use of 1:200 diluted plasma in water is an acceptable surrogate matrix for ultracentrifuged plasma to be used for EFV, LPV, and RTV calibration curve and quality control samples preparation. No interfering peaks were observed in the analyte channel or internal standard channel for all three analytes when 1:200 diluted plasma was used instead of ultracentrifuged plasma, indicating that the use of this surrogate matrix has no effect on the selectivity of the method (chromatograms not shown).

The validation of this surrogate matrix means that during patient sample analysis, only 5 µL of blank human plasma is required to prepare 1 mL of 1:200 diluted plasma for the calibration standards, quality control samples, double blank, blank, and system check samples per batch. This is far less than the 5 mL of blank human plasma required to collect 1 mL of ultracentrifuged plasma. Additionally, the use of a surrogate matrix reduces the number of overnight ultracentrifuge runs required to complete a study, as the ultracentrifuge rotor can be filled with only unknown samples.

3.15. ANALYTE STABILITY ASSESSMENTS

Various stability assessments were performed to evaluate the stability of EFV, LPV, and RTV in different conditions. The stability tests in solvents were performed to determine if there was any analyte degradation in the stock or working solutions during storage or on-bench. The stability tests in matrix were planned to try and mimic the conditions that a patient sample might go through to determine if the concentration of analyte would be affected under certain conditions.

3.15.1. STOCK AND WORKING SOLUTION STABILITY

Stability of the 1 mg/mL stock solutions at ~-80°C has already been proven within the Division of Clinical Pharmacology, UCT for 601 days for EFV in acetonitrile, 266 days for LPV in methanol, and 304 days for RTV in methanol, and was not retested during this validation. See Appendix B, C, and D, respectively, for the data.

Stability of the three analytes at ~-80°C in the working solution solvent (acetonitrile: water (4:1, v/v)) was tested by comparison with freshly prepared working solutions. After the given storage period, both the test and fresh working solutions were diluted with internal standard-containing reconstitution solution and submitted for HPLC-MS/MS analysis. Tables 2.35–2.37 show the resulting analyte/internal standard peak area ratios for EFV for 38 days at ~-80°C, and LPV and RTV for 16 days at ~-80°C at the highest and lowest working solution concentrations (WS 1 and WS 9, respectively). By testing stability at the highest and lowest concentrations, one can infer stability under the test conditions for all concentrations within this range. Short term stability of WS 0 was tested at room temperature for 7 hours and met the acceptance criteria for all three analytes. However, the long term stability of WS 0 was not tested as this working solution is never stored and is only included in the dilution scheme to dilute the 1 mg/mL stock solution to a 3000 ng/mL working solution for EFV and LPV and 750 ng/mL for RTV.

Table 2.35. Working solution stability of EFV when stored at ~-80°C for 38 days

	WS 1 (3000 ng/mL)		WS 9 (3.00 ng/mL)	
	Fresh	**~-80°C**	**Fresh**	**~-80°C**
Average peak area ratio	5.30	5.70	0.0110	0.0119
n	6	6	6	6
STDEV	0.0666	0.0943	0.000602	0.000735
CV(%)	1.3	1.7	5.5	6.2
%Difference		7.4		8.6

Table 2.36. Working solution stability of LPV when stored at ~-80°C for 16 days

	WS 1 (3000 ng/mL)		WS 9 (3.00 ng/mL)	
	Fresh	**~-80°C**	**Fresh**	**~-80°C**
Average peak area ratio	0.449	0.438	0.00598	0.00558
n	6	6	6	6
STDEV	0.0118	0.00416	0.000315	0.000247
CV(%)	2.6	1.0	5.3	4.4
%Difference		-2.6		-6.6

Table 2.37. Working solution stability of RTV when stored at ~-80°C for 16 days

	WS 1 (750 ng/mL)		WS 9 (0.750 ng/mL)	
	Fresh	**~-80°C**	**Fresh**	**~-80°C**
Average peak area ratio	0.290	0.312	0.00322	0.00333
n	6	6	6	6
STDEV	0.00461	0.00607	0.000287	0.000108
CV(%)	1.6	1.9	8.9	3.2
%Difference		7.8		3.5

The acceptance criteria for working solution stability as stipulated by the FDA and EMA guidelines is that the percentage difference must be less than 10% for an analyte to be considered stable in the tested solvent and storage conditions. The results presented in Tables 2.35–2.37 indicate that EFV is stable for a minimum of 38 days when stored at ~-80°C, while LPV and RTV are stable for a minimum of 16 days. The difference in storage time for the three analytes is not because LPV and RTV failed after 38 days, but rather that a preparation issue occurred with EFV during the 16-day fresh working solutions and was repeated after 38 days where LPV and RTV were not included. A potential oversight of this validation was not including LPV and RTV in the 38-day working solution stability test. There is little reason to believe that these analytes would be unstable after a longer storage period as stock solution stability at ~-80°C has been shown for over eight months when stored in pure solvent (Appendix B, C, and D). However, testing would be required to confirm that the addition of 20% water would not affect the analytes' stability. Nevertheless, working solutions older than 16 days were not used during validation or sample analysis.

To evaluate the stability of the analytes in the working solutions while on bench prior to extraction, aliquots of WS 1 and WS 9 were stored at room temperature for 24 hours. Thereafter, these aliquots were diluted with internal standard-containing reconstitution solution and compared to reference aliquots that were kept at ~-80°C (Tables 2.38–2.40).

Table 2.38. Working solution stability of EFV when stored at room temperature for 24 hours

	WS 1 (3000 ng/mL)		WS 9 (3.00 ng/mL)	
	~-80°C	Room temp.	~-80°C	Room temp.
Average peak area ratio	1.05	1.04	0.0108	0.0111
n	6	6	6	6
STDEV	0.0126	0.0134	0.000435	0.000501
CV(%)	1.2	1.3	4.0	4.5
%Difference		-1.1		2.7

Table 2.39. Working solution stability of LPV when stored at room temperature for 24 hours

	WS 1 (3000 ng/mL)		WS 9 (3.00 ng/mL)	
	~-80°C	Room temp.	~-80°C	Room temp.
Average peak area ratio	0.438	0.434	0.00461	0.00454
n	6	6	6	6
STDEV	0.00416	0.00599	0.000193	0.000156
CV(%)	1.0	1.4	4.2	3.4
%Difference		-0.9		-1.4

Table 2.40. Working solution stability of RTV when stored at room temperature for 24 hours

	WS 1 (750 ng/mL)		WS 9 (0.750 ng/mL)	
	~-80°C	Room temp.	~-80°C	Room temp.
Average peak area ratio	0.312	0.312	0.00333	0.00329
n	6	6	6	6
STDEV	0.00607	0.00585	0.000108	0.000201
CV(%)	1.9	1.9	3.2	6.1
%Difference		-0.3		-1.2

Again, the percentage difference between the test and reference samples must be less than 10% for an analyte to be considered stable under those conditions. The difference between the working solutions left at room temperature compared to those stored at ~-80°C was less than 3% for all three analytes; therefore, all three analytes are stable at room temperature for 24 hours.

Lastly, the stability of the deuterated internal standard stock solutions was tested for 7 hours at room temperature. This was done to ensure that the internal standards were stable for the duration of the extraction procedure. Table 2.41 summarizes the three internal standards' stabilities on-bench compared to aliquots kept at ~-80°C for the same period of time.

Table 2.41. Stability of each internal standard when at room temperature for 7 hours

	EFV-d5		LPV-d8		RTV-d6	
	~-80°C	**RT**	**~-80°C**	**RT**	**~-80°C**	**RT**
Average peak area	2900000	2981667	8390000	8135000	2350000	2388333
n	6	6	6	6	6	6
STDEV	137986	165459	147105	309758	52154	78081
CV(%)	4.8	5.5	1.8	3.8	2.2	3.3
%Difference		2.8		-3.0		1.6

EFV-d5, LPV-d8, and RTV-d6 stock solutions were shown to be stable on-bench for 7 hours at room temperature with percentage differences of less than 10%. This indicates that there is no breakdown of the internal standards for the duration of the extraction procedure.

3.15.2. BENCHTOP STABILITY

Benchtop stability refers to the time that samples will be left at room temperature prior to being extracted. Should benchtop stability fail at room temperature, it should be tested for a shorter period of time or on ice. For this assay, benchtop stability needs to be considered in two parts: stability of the analytes in plasma prior to being ultracentrifuged, and stability of the analytes in ultracentrifuged plasma prior to being extracted. Stability of EFV, LPV, and RTV in plasma has already been shown for four hours at room temperature as part of the routine TDM assay validation (see Appendix E for data). During the validation of the unbound drug method, high- and low-quality control samples were prepared in ultracentrifuged plasma and left on-bench for approximately six hours at room temperature. After six hours, the stability samples were extracted with a freshly prepared valid calibration curve. The measured concentrations of the benchtop quality control samples and the calculated percentage differences are presented in Table 2.42.

Table 2.42. Analyte stability in ultracentrifuged plasma for ~6 hours on-bench

	EFV		LPV		RTV	
Nominal conc. (ng/mL)	400	1.40	400	1.40	100	0.350
Average calculated conc. (ng/mL)	378	1.22	403	1.27	106	0.347
n	6	6	6	6	6	6
STDEV	10.0	0.0569	11.7	0.0690	2.05	0.0220
CV(%)	2.6	4.7	2.9	5.4	1.9	6.3
%Difference from nominal	-5.6	-13.1	0.7	-9.2	6.4	-0.8

According to the FDA and EMA guidelines for bioanalytical method validations, analytes are considered stable on-bench if the percentage difference from the nominal and percentage coefficient of variation is below 15%.[108,109] The results in Table 2.42 show that all three analytes meet these acceptance criteria at the high- and low-quality control concentrations, indicating that EFV, LPV, and RTV are stable in ultracentrifuged plasma for at least six hours at room temperature.

3.15.3. FREEZE-THAW STABILITY

Freeze-thaw stability testing during validation should attempt to mimic the sample handling conditions as closely as possible. As with the benchtop stability experiment, one needs to evaluate the freeze-thaw stability of patient samples in two parts: freeze-thaw stability in plasma prior to ultracentrifugation and freeze-thaw stability in ultracentrifuged plasma prior to extraction.

3.15.3.1. FREEZE-THAW STABILITY AFTER ULTRACENTRIFUGATION

First, the freeze-thaw stability of EFV, LPV, and RTV was investigated in ultracentrifuged plasma. High and low quality control samples were prepared in six-fold and frozen at ~-80°C. These samples were then subjected to three thawing and refreezing cycles, where the samples were thawed at room temperature for approximately four hours and frozen for at least 24 hours per cycle. The freeze-thaw samples were then extracted with a fresh calibration curve and assessed against the nominal concentration for accuracy and precision (Table 2.43).

Table 2.43. Stability of EFV, LPV, and RTV after 3 freeze-thaw cycles in ultracentrifuged plasma

	EFV		**LPV**		**RTV**	
Nominal conc. (ng/mL)	400	1.40	400	1.40	100	0.350
Average calculated conc. (ng/mL)	404	1.37	434	1.47	107	0.384
n	6	6	6	5*	6	5*
STDEV	9.37	0.105	13.0	0.0743	2.73	0.0353
CV(%)	2.3	7.7	3.0	5.1	2.5	9.2
%Difference from nominal	1.1	-1.9	8.5	4.9	7.3	9.8

** Statistical outlier excluded*

The results presented in Table 2.43 show that the percentage differences from the nominal concentrations and percentage coefficients of variation are within the required 15%, which indicates

that, after ultracentrifugation, all three analytes are stable in ultracentrifuged plasma for three freeze-thaw cycles.

3.15.3.2. FREEZE-THAW STABILITY IN PLASMA PRIOR TO ULTRACENTRIFUGATION

In order to ascertain whether plasma samples may be thawed and refrozen prior to ultracentrifugation, blank human plasma was spiked at a high and a low concentration for each analyte separately, followed by a one-hour incubation at ~37°C to mimic *in vivo* conditions and allow adequate equilibration time for plasma-protein binding. These plasma samples were then frozen at ~-80°C and put through three or five consecutive freeze and thaw cycles prior to ultracentrifugation and extraction. Each cycle consisted of sufficient thawing time at room temperature, a 30-second vortex and 5-minute sonication to mimic patient sample handling, followed by at least 24 hours of freezing time. These samples were analysed using samples only thawed on the day of ultracentrifugation as the reference. It was not possible to compare to the nominal concentration (as the freeze-thaw stability in ultracentrifuged plasma experiment did) as the analytes were spiked prior to ultracentrifugation, and, therefore, the spiked concentration refers to the total concentration and not the unbound concentration, which is what the standard curve assesses.

The results showed that RTV concentrations were within 15% of the reference samples and, therefore, were stable over three and five freeze-thaw cycles. However, the EFV and LPV samples failed to meet acceptance criteria with percentage differences greater than 15% (see Appendix F for data). While the EFV and LPV results did not meet acceptance criteria, the percentage differences were consistent across the high and low concentrations. This implied that a factor of 1.56 or 2.80 could be applied to the EFV concentrations of samples that have undergone three or five freeze-thaw cycles, respectively, to calculate the concentration of EFV after a single freeze-thaw cycle. Likewise, a factor of 1.26 and 1.33 could be applied to LPV concentrations for samples that went through three and five freeze-thaw cycles, respectively.

To confirm the spiked plasma sample results, freeze-thaw stability was also assessed using authentic patient samples. Plasma samples from patients currently on EFV (n=10) or LPV/r (n=12) therapy were sent to the Division of Clinical Pharmacology, UCT, for routine monitoring of these analytes. Prior to storing the plasma at ~-80°C, four aliquots of >250 µL were made from each sample. One aliquot was analysed for routine TDM purposes, and the remaining three were used to evaluate the effect of freezing and thawing the plasma on the accuracy and precision of unbound EFV, LPV, and RTV determination. The use of the remnant plasma for this purpose was approved by the University of

Cape Town Human Research Ethics Committee (HREC #272/2018, Appendix G). On day 1 of the experiment, one aliquot of each TDM sample was thawed and refrozen. As with the spiked sample validation experiment, each freeze-thaw cycle consisted of sufficient thawing time at room temperature, a 30-second vortex and 5-minute sonication to mimic patient sample handling, followed by at least 24 hours of freezing time. On day 2, the first aliquots of each sample were thawed for a second time, and the second aliquot of each sample was also thawed. On day 3, all aliquots were thawed and ultracentrifuged together in the same run resulting in an aliquot of each sample having undergone one, two, or three freeze-thaw cycles. The samples thawed for the first time on the day of ultracentrifugation were used as the reference (Tables 2.44–2.46) for EFV, LPV, and RTV, respectively. A limitation of this experiment was the lack of a fresh reference sample to determine the effect of a single freezing event. However, clinical study plasma samples will have to be frozen after collection for shipping and storage, and therefore, a single freezing event was chosen as the reference.

Table 2.44. Stability of EFV after 2 and 3 freeze-thaw cycles in plasma (TDM samples)

	Ref	2 x freeze-thaw cycles			3 x freeze-thaw cycles		
Sample ID	**Conc. (ng/mL)**	**Conc. (ng/mL)**	**%Diff. from Ref**	**Pass/Fail**	**Conc. (ng/mL)**	**%Diff. from Ref**	**Pass/Fail**
TDM 01	109	156	43.1	Fail	153	40.4	Fail
TDM 02	13.3	13.1	-1.5	**Pass**	15.1	13.5	**Pass**
TDM 03	9.69	9.9	2.2	**Pass**	10.5	8.4	**Pass**
TDM 04	20.2	20.8	3.0	**Pass**	50.7	151.0	Fail
TDM 05	21.7	23.6	8.8	**Pass**	25.6	18.0	Fail
TDM 06	59.7	59.9	0.3	**Pass**	42.3	-29.1	Fail
TDM 07	0.937	1.30	38.7	Fail	1.19	27.0	Fail
TDM 08	375	455	21.3	Fail	392	4.5	**Pass**
TDM 09	6.88	9.12	32.6	Fail	*	N/A	N/A
TDM 10	19.5	5.11	-73.8	Fail	5.55	-71.5	Fail
Pass = %Diff of <15% from Ref Fail = %Diff of >15% from Ref				**50.0% Pass**			**33.3% Pass**

* Sample contaminated with protein pellet during aqueous layer removal

Table 2.45. Stability of LPV after 2 and 3 freeze-thaw cycles in plasma (TDM samples)

	Ref	2 x freeze-thaw cycles			3 x freeze-thaw cycles		
Sample ID	**Conc. (ng/mL)**	**Conc. (ng/mL)**	**%Diff. from Ref**	**Pass/Fail**	**Conc. (ng/mL)**	**%Diff. from Ref**	**Pass/Fail**
TDM 11	94.1	105	11.6	**Pass**	99.9	6.2	**Pass**
TDM 12	53.1	46.0	-13.4	**Pass**	45.6	-14.1	**Pass**
TDM 13	28.1	67.6	140.6	Fail	71.8	155.5	Fail
TDM 14	123	101	-17.9	Fail	104	-15.4	Fail
TDM 15	19.1	0.47	-97.5	Fail	0.509	-97.3	Fail
TDM 16	78.1	388	396.8	Fail	615	687.5	Fail
TDM 17	332	582	75.3	Fail	665	100.3	Fail
TDM 18	0.886	1.12	26.4	Fail	0.898	1.4	**Pass**
TDM 19	9.31*	8.40*	-9.8	**Pass**	7.79*	-16.3	Fail
TDM 20	400	10.1	-97.5	Fail	16.2	-96.0	Fail
TDM 21	71.9	35.4	-50.8	Fail	1.86	-97.4	Fail
TDM 22	50.6	44.4	-12.3	**Pass**	62.7	23.9	Fail
Pass = %Diff of <15% from Ref Fail = %Diff of >15% from Ref				**33.3% Pass**			**25.0% Pass**

* Concentrations were ALOQ; peak area ratios reported instead

Table 2.46. Stability of RTV after 2 and 3 freeze-thaw cycles in plasma (TDM samples)

	Ref	2 x freeze-thaw cycles			3 x freeze-thaw cycles		
Sample ID	**Conc. (ng/mL)**	**Conc. (ng/mL)**	**%Diff. from Ref**	**Pass/Fail**	**Conc. (ng/mL)**	**%Diff. from Ref**	**Pass/Fail**
TDM 11	3.63	3.57	-1.7	**Pass**	3.33	-8.3	**Pass**
TDM 12	3.55	3.41	-3.9	**Pass**	3.30	-7.0	**Pass**
TDM 13	2.17	2.91	34.1	Fail	3.27	50.7	Fail
TDM 14	3.7	3.53	-4.6	**Pass**	3.32	-10.3	**Pass**
TDM 15	0.691	BLOQ	N/A	N/A	BLOQ	N/A	N/A
TDM 16	3.27	11.4	248.6	Fail	15.3	367.9	Fail
TDM 17	9.89	15.3	54.7	Fail	17.0	71.9	Fail
TDM 18	BLOQ	BLOQ	N/A	N/A	BLOQ	N/A	N/A
TDM 19	94.8*	75.2*	-20.7	Fail	56.9*	-40.0	Fail
TDM 20	2.47	0.256	-89.6	Fail	0.263	-89.4	Fail
TDM 21	1.11	0.517	-53.4	Fail	BLOQ	N/A	N/A
TDM 22	5.64	5.59	-0.9	**Pass**	6.20	9.9	**Pass**
Pass = %Diff of <15% from Ref Fail = %Diff of >15% from Ref				**40.0% Pass**			**44.4% Pass**

* Concentrations were ALOQ; peak area ratios reported instead

The EFV TDM sample data (Table 2.44) indicate that half the samples meet the acceptance criteria after two freeze-thaws, and only a third of samples met the acceptance criteria after three freeze-thaw cycles. Those samples with a percentage difference of greater than 15% showed no clear direction or order of change with a range of -73.8–43.1% change in EFV levels after two freeze-thaw

cycles and -71.5–151.0% after three freeze-thaw cycles. This wide range in EFV concentration changes indicates that freezing and thawing plasma samples multiple times will give inaccurate unbound EFV results and that a factor cannot be applied to back-calculate the original concentration of EFV.

Similarly, the LPV TDM results in Table 2.45 showed that only a third of the samples met acceptance criteria after two freeze-thaws, and a quarter of samples met acceptance criteria after three freeze-thaw cycles. As with the EFV results, there was no clear direction or order of change, with a range of -97.5–396.8% change in LPV levels after two freeze-thaw cycles and -97.4–687.5% after three freeze-thaw cycles. Once again, these results contradict the spiked sample results and show that the use of a factor to adjust results after multiple freeze-thaw cycles is not possible.

The RTV TDM results (Table 2.46) also contradict the spiked RTV results. When RTV was spiked into plasma, the data showed that samples could be frozen and thawed up to five times without impacting the quantification of unbound RTV. However, when TDM samples were used, more than half the samples had percentage differences of greater than 15% after only two freeze-thaw cycles.

The indiscriminate percentage changes observed for all three analytes in the TDM samples are likely due to disruptions in plasma-protein binding and not due to analyte stability, as all other validation experiments have shown these analytes to be stable under various conditions. Regardless, the results indicate that plasma samples may not be thawed and refrozen prior to ultracentrifugation. Therefore, during patient sample analysis, either multiple aliquots of plasma must be made or, should only one aliquot be available, analysis of other analytes or determination of total EFV, LPV, and RTV must be scheduled for after unbound drug concentration quantification. Alternatively, when the sample is thawed for the first time, 250 µL must be removed and ultracentrifuged before refreezing.

Although the spiked plasma results should be disregarded, and conclusions should only be made using the TDM patient data, both experiments are discussed in this chapter as it is interesting to note the different conclusions that can be drawn based on the two experiment designs.

3.15.4. REINJECTION REPRODUCIBILITY

After extraction, samples are added to a 96-well plate and submitted for HPLC-MS/MS analysis. However, it is not always possible to analyse the samples on the day of extraction due to instrument availability. Additionally, a batch may need to be reinjected in the case of instrument failure. It is,

therefore, important to determine whether or not a batch may be reinjected and for how long extracted samples may be stored without affecting the accuracy and precision of the method.

Following the initial injection of the second accuracy and precision batch (validation day 2), the extracted samples were left in the autosampler (at ~8°C) for ten days, after which the batch was reinjected. Reinjection reproducibility was assessed by evaluating whether the batch passed or failed as a whole. The reinjection reproducibility results from reinjecting the standards and quality controls from validation day 2 after ten days are summarised in Tables 2.47–2.49.

Table 2.47. EFV reinjection reproducibility: validation 2 reinjected after ten days

Sample ID	Nominal Conc. (ng/mL)	Mean Observed Conc. (ng/mL)	STDEV	CV(%)	%Accuracy	n
S1	500	500	18.2	3.6	100.0	2 of 2
S2	350	350	13.3	3.8	100.0	2 of 2
S3	200	200	5.38	2.7	100.2	2 of 2
S4	50.0	49.3	0.713	1.4	98.6	2 of 2
S5	20.0	20.3	0.791	3.9	101.3	2 of 2
S6	5.00	5.10	0.273	5.4	102.0	2 of 2
S7	2.50	2.55	0.126	4.9	102.1	2 of 2
S8	1.00	0.966	0.0568	5.9	96.6	2 of 2
S9	0.500	0.496	0.0530	10.7	99.1	2 of 2
QC H	400	386	3.62	0.9	96.6	6 of 6
QC M	150	147	5.51	3.8	97.7	6 of 6
QC L	1.40	1.38	0.0931	6.7	98.8	6 of 6
QC LLOQ	0.500	0.536	0.0270	5.0	107.2	6 of 6

Table 2.48. LPV reinjection reproducibility: validation 2 reinjected after ten days

Sample ID	Nominal Conc. (ng/mL)	Mean Observed Conc. (ng/mL)	STDEV	CV(%)	%Accuracy	n
S1	500	502	2.06	0.4	100.3	2 of 2
S2	350	347	3.74	1.1	99.1	2 of 2
S3	200	202	1.10	0.5	100.9	2 of 2
S4	50.0	49.1	0.808	1.6	98.1	2 of 2
S5	20.0	20.4	1.01	5.0	101.8	2 of 2
S6	5.00	5.27	0.345	6.5	105.4	2 of 2
S7	2.50	2.56	0.196	7.7	102.5	2 of 2
S8	1.00	0.947	0.0450	4.8	94.7	2 of 2
S9	0.500	0.486	0.0000180	0.0	97.1	2 of 2
QC H	400	421	7.0	1.7	105.2	6 of 6
QC M	150	155	3.6	2.3	103.6	6 of 6
QC L	1.40	1.42	0.132	9.3	101.4	6 of 6
QC LLOQ	0.500	0.539	0.0400	7.4	107.9	6 of 6

Table 2.49. RTV reinjection reproducibility: validation 2 reinjected after ten days

Sample ID	Nominal Conc. (ng/mL)	Mean Observed Conc. (ng/mL)	STDEV	CV(%)	%Accuracy	n
S1	125	125	4.18	3.3	100.2	2 of 2
S2	87.5	86.9	1.59	1.8	99.3	2 of 2
S3	50.0	50.4	0.681	1.4	100.8	2 of 2
S4	12.5	12.4	0.0910	0.7	99.0	2 of 2
S5	5.00	5.03	0.351	7.0	100.6	2 of 2
S6	1.25	1.27	0.0766	6.0	101.8	2 of 2
S7	0.625	0.631	0.0287	4.5	101.0	2 of 2
S8	0.250	0.238	0.0142	6.0	95.0	2 of 2
S9	0.125	0.128	0.00736	5.8	102.3	2 of 2
QC H	100	112	3.74	3.3	112.2	6 of 6
QC M	37.5	41.3	1.72	4.2	110.2	6 of 6
QC L	0.350	0.376	0.0132	3.5	107.4	6 of 6
QC LLOQ	0.125	0.133	0.0123	9.2	106.1	6 of 6

The accuracies of each standard and quality control from the reinjected batch fell within a range of 94.7–112.2%. A batch is required to have at least 75% of its standards and 66.7% of its quality controls pass (with 50% at each level) to be considered valid.[108,109] All of the calibration standards and quality control samples met these requirements for EFV, LPV, and RTV. The coefficients of variation for each quality control concentration were all equal to or less than 9.3%, which shows that the precision of the method is still acceptable. Therefore, should it not be possible to inject the batch on the day of extraction or should instrument interruptions occur, the extracted samples may be injected and analysed accurately and precisely for up to ten days after extraction, when stored in the autosampler at ~8°C.

3.15.5. AUTOSAMPLER STABILITY

Autosampler stability is assessed using the same results as for reinjection reproducibility, whereby the extracted samples from validation day 2 were left in the autosampler (at ~8°C) and reinjected after ten days. The difference between the two experiments is that reinjection reproducibility evaluates whether a batch passes or fails as a whole, while autosampler stability evaluates the stability of the analytes and how well the internal standards compensate, thereby determining if a batch may be reinjected in part.

The peak area ratios of the high- and low-quality control samples were compared to the results from the original injection to determine absolute autosampler stability. The percentage differences between the mean peak area ratios of the initial injection and the reinjection are shown in Tables 2.50–2.52 below.

Table 2.50. Ten-day autosampler stability of EFV in extracted samples

	High Concentration		Low Concentration	
	Initial injection	**Reinjection**	**Initial injection**	**Reinjection**
Average Peak Area Ratio	3.42	3.30	0.0127	0.0128
STDEV	0.0419	0.0304	0.000288	0.000829
CV(%)	1.2	0.9	2.3	6.5
n	6	6	6	6
%Difference		-3.6		1.2

Table 2.51. Ten-day autosampler stability of LPV in extracted samples

	High Concentration		Low Concentration	
	Initial injection	**Reinjection**	**Initial injection**	**Reinjection**
Average Peak Area Ratio	2.89	2.92	0.0115	0.0123
STDEV	0.0485	0.0408	0.000229	0.00104
CV(%)	1.7	1.4	2.0	8.5
n	6	6	6	6
%Difference		1.1		6.3

Table 2.52. Ten-day autosampler stability of RTV in extracted samples

	High Concentration		Low Concentration	
	Initial injection	**Reinjection**	**Initial injection**	**Reinjection**
Average Peak Area Ratio	0.855	0.871	0.00320	0.00333
STDEV	0.0152	0.0271	0.000229	0.000108
CV(%)	1.8	3.1	7.2	3.3
n	6	6	6	6
%Difference		1.9		4.1

The percentage differences after ten days for the high- and low-quality controls were respectively -3.6% and 1.2% for EFV, 1.1% and 6.3% for LPV, and 1.9% and 4.1% for RTV. These meet the acceptance criteria such that the reinjection results are within 15% of the initial peak area ratio. This data indicates that if a run stops midway, the batch may be reinjected in part, starting from the last accepted standard or quality control sample that was injected prior to the instrument interruption, after careful evaluation of instrument response.

4. CHAPTER CONCLUSIONS

This chapter describes the development and validation of a method for the separation and quantification of unbound EFV, LPV, and RTV from human plasma. The method uses ultracentrifugation to separate the unbound drug from the plasma-protein bound drug, followed by LLE of the ultracentrifuged plasma and quantitation of the analytes using HPLC-MS/MS analysis.

During the bioanalytical method validation, the method was tested as per the most recent FDA and EMA guidelines[108,109] and was shown to be robust, accurate, and precise over a range of 0.500–500 ng/mL for EFV and LPV and 0.125–125 ng/mL for RTV even when other anti-coagulants or 2% haemolyzed plasma samples were used. A novel surrogate matrix was investigated and validated to reduce the volume of plasma required for calibration standards and quality control samples and to increase the number of unknown samples that can be ultracentrifuged per run. In future, the surrogate matrix, a 1:200 dilution of plasma in water, may also be used for method development of other unbound drug methods, which will reduce the volume of plasma and time required.

The ultracentrifuge method itself was also shown to be reproducible across different sources of plasma and different days, which indicates that the unbound drug concentrations of patient samples determined using this method will be reliable. To the best of the authors' knowledge, this is the first assay to validate the precision and reproducibility of an ultracentrifuge method by quantifying the amount of protein contamination in the protein-free layer of ultracentrifuged plasma.

Endogenous matrix components were found to have no adverse effects on the reproducibility of the method when ultracentrifuged human plasma originating from six different sources was analysed. Recovery was greater than 75% and reproducible at each level of the dynamic range for all three analytes. Five-fold (1:4) dilutions were valid for the analytes when diluted post-extraction with an extracted blank sample; however, pre-extraction dilutions were shown to be inaccurate and may not be used. No significant carry-over of EFV, LPV, or RTV were observed with the autosampler, and no crosstalk was observed between the analytes or their internal standards.

The stock and working solutions were stable at room temperature, 4°C and ~-20°C for up to 24 hours. This 24-hour stability was anticipated as stock solution stability at ~-80°C has already been shown for 601 days for EFV in acetonitrile, 266 days for LPV in methanol, and 304 days for RTV in methanol. All three analytes were stable in ultracentrifuged plasma at room temperature for up to ~6 hours and when subjected to three freeze-thaw cycles. Importantly, plasma samples may not be thawed and refrozen prior to ultracentrifugation without disrupting the plasma-protein binding and yielding

inaccurate results. Therefore, an additional plasma aliquot must be kept for any unbound EFV, LPV, or RTV analysis. EFV, LPV, and RTV were stable in whole blood at room temperature and on ice for up to 2 hours. All analytes were shown to be stable on-instrument over a period of 10 days. Stability in plasma has been shown at ~-80°C for up to 6 (EFV), 5 (LPV), and 6 (RTV) years, while stability in ultracentrifuged plasma was shown for 11 days when stored at ~-80°C.

The assay method and all validation data presented in this chapter have been submitted to the CPQA group (NAIADS/DAIDS)[112] for peer review and have been approved for use on human samples in clinical applications. As of February 2022, this assay method is the only CPQA-approved assay for the quantification of unbound EFV, LPV, and RTV across all participating laboratories worldwide.[127] The approval documentation can be found in Appendix H. A clinical application of this method is discussed in Chapter Four, in conjunction with the intracellular concentration assay method described in Chapter Three.

CHAPTER THREE: INTRACELLULAR DRUG CONCENTRATIONS

1. CHAPTER SUMMARY

For many antiretrovirals, patient treatment response and tolerability have shown a large level of variability, with some patients even failing treatment. It has been suggested that this may be due to variability in the amount of drug reaching the target site within the HIV-infected cells, and as a result, intracellular drug levels are being investigated.[77] Subtherapeutic concentrations within the cell will apply selective pressure on the virus and could lead to the emergence of drug resistance, while high concentrations may lead to increased toxicity and decreased patient compliance.[76] Peripheral blood mononuclear cells (PBMCs) are a heterogeneous collection of cells that HIV infects and are relatively easy to isolate from whole blood. As a result, these cells are used to measure intracellular drug concentrations.[79]

A method is presented for the quantification of EFV, LPV, and RTV in one million PBMCs collected from whole blood using sodium citrate cell preparation tubes (CPT). The method uses methanol to lyse the cells, followed by solid-phase extraction (SPE) to remove any salts and contaminants remaining from the cell isolation procedure and to reconstitute the sample in a solvent compatible with the chromatographic method. Stable isotopically labelled EFV-d5, LPV-d8, and RTV-d6 are used as the internal standards.

The extraction procedure is followed by liquid chromatographic separation using an Agilent Poroshell 120 (EC-C18, 2.7 µm, 50 x 4.6 mm) analytical column. An isocratic mobile phase consisting of 0.1% formic acid in water and acetonitrile (25:75, v/v) at a flow rate of 500 µL/minute over 4 minutes was used. The retention times for EFV, LPV, RTV, EFV-d5, LPV-d8, and RTV-d6 were ~1.8, ~1.6, ~1.5, ~1.8, ~1.6, and ~1.5 minutes, respectively.

An AB Sciex API 5500 Q trap mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode was used to monitor the transition of the deprotonated precursor ion m/z 314.0 to product ion m/z 243.9 for EFV and the protonated precursor ions m/z 629.4 and m/z 721.3 to product

ions m/z m/z 447.1 and m/z 296.0 for LPV and RTV, respectively. MRM mode was also used to monitor the transition of the deprotonated precursor ion m/z 319.0 to product ion m/z 248.1 for EFV-d5 and the protonated precursor ions m/z 637.4 and m/z 727.4 to product ions m/z 447.1 and m/z 301.9 for LPV-d8 and RTV-d6, respectively. Electro Spray Ionisation was used for ion production.

The method was validated according to the FDA and EMA guidelines[108,109] and was shown to be accurate, precise, and reproducible. Additional validation experiments included sensitivity, crosstalk, matrix effects, recovery, process efficiency, dilution integrity, and the stability of the analytes under various conditions.

The assay method and all validation data were submitted for CPQA review.[110] Subsequently, the method has been approved for use on human samples in NAIAD/DAIDS HIV Clinical Trial Networks and other clinical applications.

2. ASSAY DEVELOPMENT

2.1. CHEMICALS AND REAGENTS

Reference standards for EFV, LPV, RTV and their respective stable deuterated internal standards, EFV-d5, LPV-d8, and RTV-d6, were all purchased from Toronto Research Chemicals (Ontario, Canada); for their chemical structures, see Figure 2.1 in Chapter Two.

Methanol, acetonitrile, and acetone were purchased from Honeywell (North Carolina, USA), isopropanol from Merck (Darmstadt, Germany), and formic acid from Fisher Chemical (Illinois, USA). The phosphate-buffered saline (PBS) and trypan blue used for the cell isolation and counting were purchased from Sigma (Missouri, USA). All water used was filtered in-house (Millipore, 18.2 mΩ.cm at 25°C) using a Synergy Water Purification System from Merck Millipore (Darmstadt, Germany). PBMC isolation was achieved using BD Vacutainer cell preparation tubes from Becton, Dickinson & Company (New Jersey, USA).

All reference standards, internal standards, chemicals, and reagents were used within the expiry dates provided by the manufacturers.

2.2. PBMC ISOLATION

While there are multiple reported isolation methods to separate PBMCs from the plasma and red blood cells, such as the Ficoll method, these methods have multiple transfer steps and are more time- and labour-intensive compared to the BD Vacutainer cell preparation tube (CPT) method.[140] It is important to isolate the PBMCs within two hours of sampling to maintain cell viability[94] and, therefore, the BD Vacutainer CPT method was chosen as it is a rapid and simple isolation method that makes it easier to process multiple samples at the same time – without impacting the yield of PBMCs.[141]

PBMCs were collected with informed consent from eight healthy in-house volunteers for use in method development and validation (HREC #773/2015, Appendix I). Approximately 16 mL of whole blood was collected per healthy volunteer in sodium heparin BD Vacutainer CPT tubes and gently inverted 5–10 times at room temperature to mix the anticoagulant. The tubes were centrifuged at 1800 rcf with no brake for 30 minutes at 21°C, which resulted in four distinct layers, as shown in Figure 3.1.

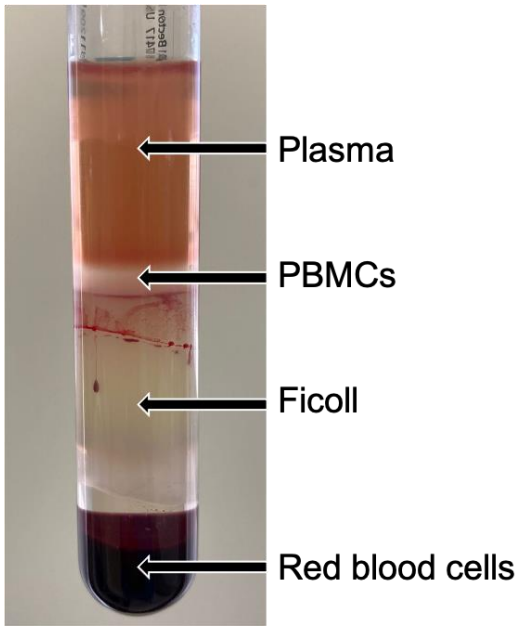

Figure 3.1. Photo of BD Vacutainer cell preparation tube after centrifugation.

The plasma was removed and discarded. The PBMC layers were transferred to 15 mL conical tubes, and ice-cold Dulbecco's PBS was added to bring the volume to 15 mL to wash off any plasma that may have been transferred with the cells. It is important to wash off the plasma as any analyte in the plasma will contaminate the cell sample and lead to inaccurate intracellular results. It is also important that the PBMCs be kept cold (on ice or at 4°C) during the wash steps to prevent drug efflux from the cells, which would also lead to inaccurate results.[76,81–85] The tubes were inverted five times to mix the cells and the PBS, followed by centrifugation at 300 rcf for 15 minutes at 4°C. The supernatant was removed and replaced with 10 mL ice-cold PBS for a second wash. The cells were re-suspended by inverting the tubes five times and centrifuged at 300 rcf for 10 minutes at 4°C. The supernatant was again removed and replaced with 1 mL ice-cold PBS for cell counting.

A 20 µL aliquot of each cell suspension was removed and added to 20 µL of Trypan Blue, resulting in a 1:1 dilution (v/v). A 20 µL aliquot of the 1:1 dilution was added to 80 µL Trypan Blue resulting in a final cell dilution of 1:5 (v/v). From this final cell dilution, 10 µL was added into the chamber of a cell counter plate and counted using a Bio-Rad TC20 automated cell counter (Bio-Rad Laboratories Inc., California, USA) with the lower and upper limits set to 6 and 8 µm, respectively. The gate limits were determined by the automated cell counter during the first PBMC isolation and were confirmed during a second isolation. All subsequent isolations used these gate limits for cell counting. The calculated cells/mL value was multiplied by five to determine the number of cells in the 1 mL cell suspension. To determine the aliquot volume required to make aliquots of one million cells, 980 µL (the volume of the cell suspension remaining after counting) was divided by the calculated number of cells. Aliquots of one million cells were transferred to individual polypropylene microcentrifuge tubes and

centrifuged at 850 rcf for 5 minutes at 4°C. The supernatant was removed, and the cell pellets were stored at approximately -80°C until required.

2.3. EXTRACTION METHOD DEVELOPMENT

There is no standard procedure for PBMC lysis as some laboratories use a mixture of methanol and water in the ratio (50:50, v/v), while others use the ratio (60:40, v/v),[82,142,143], or (70:30, v/v). Most PBMC work uses a lysing solution of methanol and water (70:30, v/v).[85,122,144–147] Other lysing solutions are used, such as combinations of methanol and a TRIS buffer instead of water,[120,148,149] a 2 mM tripotassium phosphate solution and tert-butylmethylether,[76] or 100% methanol.[77,83,84] There is often no clear explanation of the rationale for the chosen lysing solution, but it appears that compatibility with the extraction and detection method is the justification for many research groups. It was decided to lyse the cells with 100% methanol and then dilute with water to a ratio compatible with extraction (the ratios tested are explained in further detail below).

The use of methanol to lyse the cells is technically a protein precipitation extraction method and could have been the only sample preparation required; however, additional sample clean-up was investigated to remove any intracellular salts or PBS remaining from the cell washing procedure.

Liquid-liquid extraction (LLE) was found to be the optimal extraction method for the unbound EFV, LPV, and RTV from ultracentrifuged plasma, as discussed in Chapter Two, as this was more selective than protein precipitation and had a higher recovery than SPE. The poor recovery of the SPE method was hypothesized to be due to the lack of analyte solubility in the aqueous matrix. However, in this assay, the three analytes are all dissolved in the cell lysate, and as a result, very little analyte was lost when loading the samples on the solid-phase cartridges. SPE is also a much more selective method and thus, was chosen as the optimal extraction technique.

SPE involves five distinct steps: conditioning the solid-phase, equilibrating the solid-phase, loading the sample, washing contaminants from the sample, and eluting the analytes of interest from the solid-phase. Each of these steps needs to be optimized to develop a selective method for the chosen analytes and sample matrix. The equilibration step and aqueous wash step were not optimized for this extraction method as they were already explored during the SPE optimization for unbound EFV, LPV, and RTV (see Chapter Two, Section 2.3), where it was shown that 500 µL water was sufficient to

equilibrate the cartridges and to wash the polar compounds off the cartridges without analyte breakthrough.

Reversed-phase SPE was used due to the analytes' affinity for non-polar environments and poor solubilities in aqueous solutions (logP values all greater than 4).[23,36,150] Two SPE cartridges were tested: Phenomenex Strata-X (33 µm, 200 mg, 3 mL) and Waters Sep-Pak® C18 (55-105 µm, 100 mg, 1 mL) cartridges. The Phenomenex Strata-X cartridges contain a modified polymer-based sorbent made from styrene and divinylbenzene, which allows for analyte retention through three different interactions, namely hydrophobic interactions, pi-pi bonds, and hydrogen bonds. The Strata-X cartridges are, therefore, referred to as a "universal SPE material" as they are able to retain polar, non-polar, acidic, basic, and neutral compounds.[151] The Waters Sep-Pak® C18 cartridges are a more traditional reversed-phase SPE that utilise a silica-based sorbent with a polar surface and C18 functional groups to retain polar and non-polar analytes through hydrophobic interactions.

The ratio of methanol to water in the loading step was tested to evaluate any analyte breakthrough and to decide on the cell lysing solution. Solutions of 25%, 50%, and 75% methanol in water (v/v), as well as 100% methanol, were prepared and spiked with 40 ng/mL EFV and LPV, and 10 ng/mL RTV. A 500 µL aliquot of each solution was loaded onto both cartridge types in triplicate, and the eluent collected and analysed using HPLC-MS/MS. Each of the spiked solvents (without running through the cartridges) were also analysed as a reference to calculate the percentage breakthrough.

Figures 3.2–3.4 show the breakthrough results from the SPE loading step optimization experiment. Only the Waters Sep-Pak® cartridge results are shown; however, the Phenomenex Strata-X cartridges showed similar results with the exception of RTV, which showed no breakthrough even when loaded with 100% methanol.

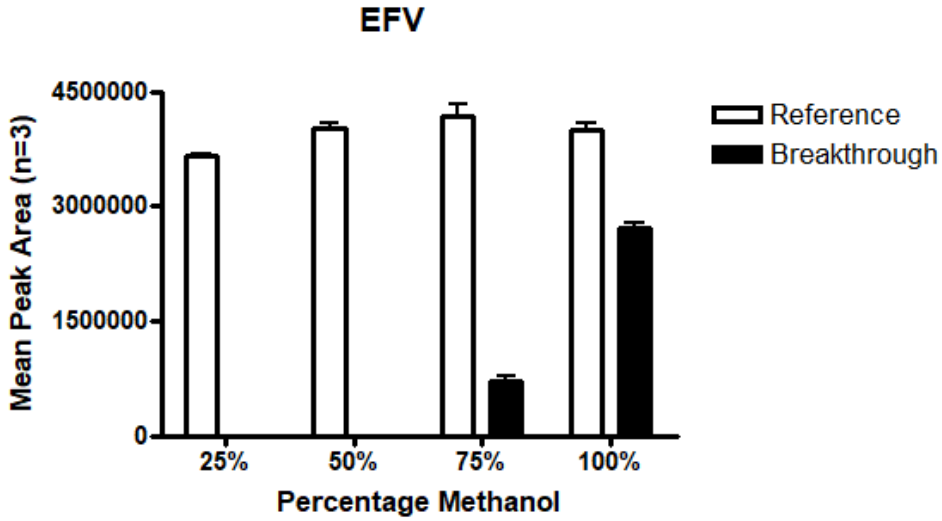

Figure 3.2. Results showing breakthrough of EFV from the Waters Sep-Pak® C18 cartridges.

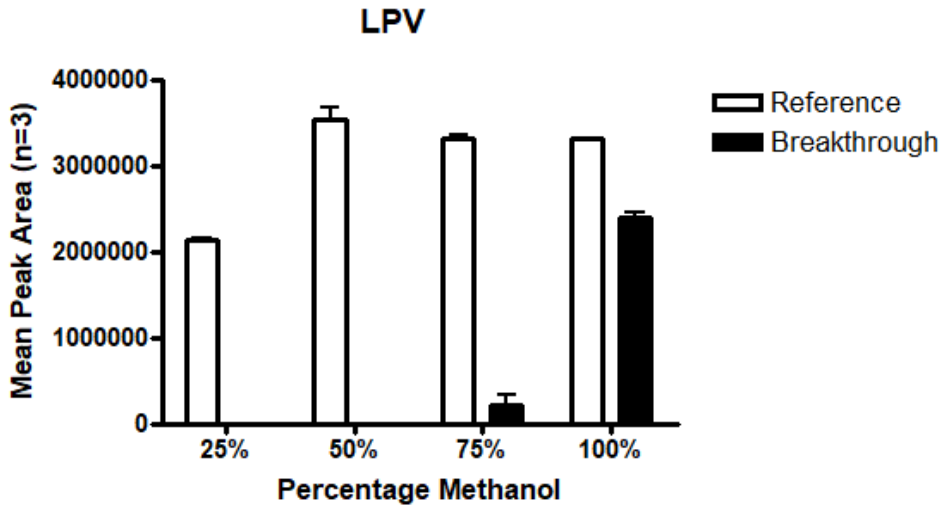

Figure 3.3. Results showing breakthrough of LPV from the Waters Sep-Pak® C18 cartridges.

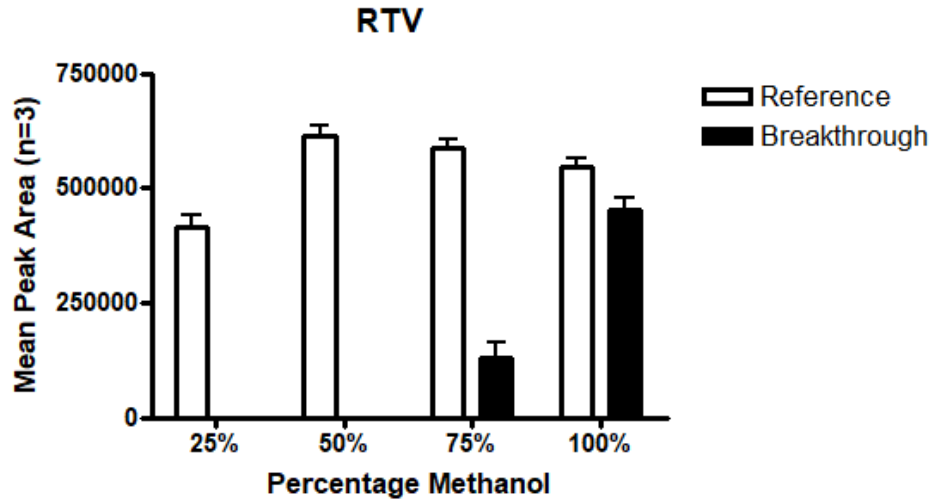

Figure 3.4. Results showing breakthrough of RTV from the Waters Sep-Pak® C18 cartridges.

No breakthrough was observed for all three analytes for the 25% and 50% methanol samples. Breakthrough did occur for the 75% and 100% methanol samples for EFV, LPV, and RTV and, as a result, were not suitable as loading solvents, as the percentage organic was too high to allow for adequate analyte interactions with the stationary phase of the cartridges. The reference samples prepared in 25% methanol showed peak areas that were approximately 9.6%, 36.9%, and 28.9% lower than the mean peak areas seen in the other reference samples for EFV, LPV, and RTV, respectively. It was suspected this was due to the analytes' poor solubility in a 75% aqueous solution, which resulted in a portion of analyte precipitating out of solution and not being transferred to the cartridge; therefore, 25% methanol in water was not a suitable loading solvent.[23,36,150] This experiment was later repeated at a higher concentration (500 ng/mL for EFV and LPV, and 125 ng/mL for RTV compared to 40 ng/mL and 10 ng/mL, respectively) and again, no breakthrough was detected using 50% methanol in water as the loading solvent. Consequently, a 1:1 ratio of methanol and water was chosen as the

loading solvent as it showed good solubility of the analytes and no breakthrough from the cartridges. These findings were in agreement with the literature.[98]

Next, the elution solvent was investigated. The Strata-X and Sep-Pak® C18 cartridges were conditioned with 500 µL acetonitrile or methanol and equilibrated with 500 µL water. Triplicate samples at high (500 ng/mL for EFV and LPV and 125 ng/mL for RTV) and low (2.00 ng/mL for EFV and LPV and 0.500 ng/mL for RTV) concentrations were loaded onto each cartridge type, followed by elution with either 500 µL acetonitrile or 500 µL methanol (cartridges preconditioned with acetonitrile were eluted with acetonitrile and the cartridges preconditioned with methanol were eluted with methanol). A second elution of 500 µL acetonitrile or methanol was performed and collected in separate tubes to evaluate if a single elution step would be enough to remove all the retained analytes from the cartridges. All eluents were dried at ~40°C under a gentle stream of nitrogen and reconstituted in 200 µL mobile phase (a mixture of acetonitrile and 0.1% formic acid in water (3:1, v/v)) and submitted for HPLC-MS/MS analysis.

The first elution of 500 µL acetonitrile or 500 µL methanol had large peak areas for all three analytes, with a small peak area observed in the second elution. To compare the two elution solvents, the peak areas of the first and second elution were summed for each sample. The results (Tables 3.1–3.3) showed that acetonitrile eluted EFV and RTV better than methanol, while methanol eluted LPV better.

Table 3.1. Peak area results from eluting EFV using acetonitrile or methanol from different cartridges

	Sep-Pak C18				Strata-X			
	Low Conc.		High Conc.		Low Conc.		High Conc.	
	ACN	MeOH	ACN	MeOH	ACN	MeOH	ACN	MeOH
Mean peak area (n=3)	185333	171000	41900000	40866667	185333	167667	40966667	35866667
STDEV	7234	13229	608276	230940	7024	10786	776745	4565450
CV(%)	3.9	7.7	1.5	0.6	3.8	6.4	1.9	12.7
%Difference	ref	-7.7	ref	-2.5	ref	-9.5	ref	-12.4

Table 3.2. Peak area results from eluting LPV using acetonitrile or methanol from different cartridges

	Sep-Pak C18				Strata-X			
	Low Conc.		High Conc.		Low Conc.		High Conc.	
	ACN	MeOH	ACN	MeOH	ACN	MeOH	ACN	MeOH
Mean peak area (n=3)	125333	134000	31933333	32433333	106667	107667	29100000	29466667
STDEV	11240	5292	896289	503322	5774	3215	692820	1365040
CV(%)	9.0	3.9	2.8	1.6	5.4	3.0	2.4	4.6
%Difference	ref	6.9	ref	1.6	ref	0.9	ref	1.3

Table 3.3. Peak area results from eluting RTV using acetonitrile or methanol from different cartridges

	Sep-Pak C18				Strata-X			
	Low Conc.		High Conc.		Low Conc.		High Conc.	
	ACN	MeOH	ACN	MeOH	ACN	MeOH	ACN	MeOH
Mean peak area (n=3)	29000	26100	7763333	7316667	21867	20967	6546667	6175000
STDEV	800	1562	172143	110151	58	1242	174738	106066
CV(%)	2.8	6.0	2.2	1.5	0.3	5.9	2.7	1.7
%Difference	ref	-10.0	ref	-5.8	ref	-4.1	ref	-5.7

The percentage difference between eluting with acetonitrile and methanol were all equal to or less than 12.4%, which shows that there was a minimal difference between the two elution solvents and the two cartridge types. It was decided to focus on the EFV results to choose which elution solvent and cartridge to use for the final method as EFV had the poorest sensitivity of the three analytes, and therefore, any marginal improvement in EFV recovery was prioritised over LPV and RTV.

As a result, acetonitrile was chosen as the preconditioning and elution solvents as these results had higher peak areas and greater precision (lower coefficients of variation) compared to methanol (Table 3.1). There was no statistically significant difference for EFV between the Sep-Pak® C18 and Strata-X stationary phases when conditioned and eluted with acetonitrile at the high [$t(4) = 1.639$, $p = 0.1766$] or low concentration [$t(4) = 0.000$, $p = 1.000$], and so, it was decided to continue using the Strata-X cartridges, as these were more readily available.

2.4. SUMMARY OF ASSAY EXTRACTION METHOD

After optimization, the final extraction method was performed as follows:

An aliquot containing one million PBMCs was lysed using 250 µL of methanol containing internal standards (EFV-d5 and LPV-d8 at 40 ng/mL and RTV-d6 at 20 ng/mL). The double blank sample was lysed with 250 µL of blank methanol. All samples were vortexed for 30 seconds and sonicated for 5 minutes to ensure complete cell lysis. Unknown samples and the double blank sample were diluted with 250 µL of water, while the calibration standards and quality control samples were diluted with 230 µL of water and 20 µL of the corresponding working solution, resulting in a final sample volume of 500 µL. The difference in solvent composition between the unknowns and standards is 4% and is considered negligible.

Phenomenex Strata-X (33 µm, 200 mg, 3 mL) cartridges were conditioned with 500 µL of acetonitrile followed by equilibration with 500 µL water. The samples were vortexed briefly and loaded onto the cartridges. The cartridges were rinsed with 1 mL water, followed by 1 mL 40% methanol in water. Finally, the cartridges were eluted three times using 500 µL of acetonitrile (the third elution of 500 µL acetonitrile was included in the final method to ensure that all the analytes were removed from the cartridges, as a small peak was still observable in the second elution step.)

The eluted samples were dried at 40°C under a gentle stream of nitrogen until complete solvent evaporation was achieved, after which it was reconstituted in 200 µL of a mixture of acetonitrile and 0.1% formic acid in water (3:1, v/v). The reconstituted samples were transferred to a 96-well plate and submitted for HPLC-MS/MS analysis.

2.5. CHROMATOGRAPHY

The chromatography method described in Chapter Two for the unbound drug assay was also used for the intracellular drug analysis. To summarize, an Agilent 1260 HPLC system coupled to an AB Sciex API 5500 QTrap mass spectrometer was used. Separation of EFV, LPV, and RTV was achieved using reversed-phase chromatography with an Agilent Poroshell 120 column (EC-C18, 2.7 µm, 50 x 4.6 mm) kept at 40°C. An isocratic mobile phase system consisting of 25% mobile phase A (0.1% formic acid in LC-MS grade water) to 75% mobile phase B (acetonitrile) was used at a flow rate of 500 µL/minute. The total run time was 4 minutes.

The only difference compared to the unbound drug assay was the injection volume used. The injection volume was reduced from 10 µL (as used in the unbound drug assay) to 5 µL as the larger injection volume resulted in very quadratic calibration curves for LPV, which made it difficult to differentiate between the highest two standards and resulted in poor accuracy at the low concentrations.

2.6. MASS SPECTROMETRY

The same mass spectrometry settings from the unbound drug assay described in Chapter Two were used in the intracellular drug assay. The ESI mode, MRM transitions, and main instrument parameters of each analyte and internal standard after infusion are summarized in Table 3.4 below. The ion spray

voltage in the positive mode was set to 5000 V for LPV, LPV-d8, RTV, and RTV-d6 while the ion spray voltage in the negative mode was set to -4500 V for EFV and EFV-d5. The source temperature, collision gas, curtain gas, nebulizer gas (gas 1), and turbo gas (gas 2) were set to 500°C, medium, 30 psi, 60 psi, and 60 psi, respectively, for all analytes. The dwell time was set to 150 ms for EFV and EFV-d5 and 200 ms for LPV, LPV-d8, RTV, and RTV-d6. A split (red Peek tubing inner diameter 0.0127 cm x 49.5 cm) diverting ~60% to waste was used to improve the ionization of EFV.

Table 3.4. Main instrument parameters and MRM transitions

	ESI mode	Precursor ion (m/z)	Product ions (m/z)	Declustering potential (V)	Collision energy (V)	Entrance potential (V)	Exit potential (V)
EFV	Negative	314.0	243.9 229.9	-125	-22	-10	-21
EFV-d5	Negative	319.0	248.1	-65	-24	-10	-13
LPV	Positive	629.4	447.1 155.0	131	19	10	36
LPV-d8	Positive	637.4	447.1	171	19	10	38
RTV	Positive	721.3	296.0 139.9	146	23	10	24
RTV-d6	Positive	727.4	301.9	116	23	10	12

The first product ion of the analytes listed in Table 3.4. was used as the quantifier ion and was used for the quantification of calibration standards, quality controls, and unknown samples. The second product ion listed was used as the qualifier ion and was used as a confirmatory result that the peaks seen in unknown samples were the analyte of interest.

3. ASSAY VALIDATION

The method was validated according to the FDA and EMA guidelines for bioanalytical methods.[108,109] The validation included the evaluation of the method's accuracy, precision, reproducibility, sensitivity, specificity, crosstalk, matrix effects, recovery, process efficiency, dilution integrity, and the stability of the analytes under various conditions.

3.1. PREPARATION OF STOCK SOLUTIONS

Stock solutions were prepared volumetrically for each analyte and internal standard to a final concentration of 1 mg/mL. The weighed mass of each analyte was adjusted for purity before the solvent was added; however, no mass adjustments were made for the internal standards. Stock solutions for EFV and EFV-d5 were prepared in acetonitrile, while LPV, LPV-d8, RTV, and RTV-d6 were prepared in methanol. All stock solutions were stored at ~-80°C.

3.2. PREPARATION OF WORKING SOLUTIONS

The 1 mg/mL stock solutions were used to spike two sets of working solutions. The first set was prepared volumetrically in acetonitrile: water (4:1, v/v) according to the dilution scheme presented in Table 3.5. These working solutions were later used to spike the calibration standards. The second set of working solutions was prepared independently by a second analyst, as shown in Table 3.6, and was later used to spike the quality control samples.

Table 3.5. Dilution scheme for calibration standard working solutions

Working solution	Blank solvent volume (µL)	Volume stock solution (µL)			Dilution source	Dilution source volume (µL)	WS Conc. (ng/mL)	
		EFV	LPV	RTV			EFV, LPV	RTV
WS 1	9330	120	120	30.0			12500	3125
WS 2	1200				WS 1	2800	8750	2188
WS 3	1350				WS 2	1800	5000	1250
WS 4	2700				WS 3	900	1250	313
WS 5	2100				WS 4	1400	500	125
WS 6	3600				WS 5	1200	125	31.3
WS 7	2000				WS 6	2000	62.5	15.6
WS 8	2550				WS 7	1700	25.0	6.25
WS 9	1400				WS 8	1400	12.5	3.13

Table 3.6. Dilution scheme for quality control working solutions

Working solution	Blank solvent volume (µL)	Volume stock solution (µL)			Dilution source	Dilution source volume (µL)	WS Conc. (ng/mL)	
		EFV	LPV	RTV			EFV, LPV	RTV
WS DIL	4530	120	120	30.0			25000	6250
WS H	3300				WS DIL	2200	10000	2500
WS M	2500				WS H	1500	3750	938
WS X	2500				WS M	500	625	156
WS SYS	4160				WS X	840	105	26.3
WS L	3000				WS SYS	1500	35.0	8.75
WS LLOQ	2520				WS L	1400	12.5	3.13

The working solution referred to as "WS X" in Table 3.6. was not used to spike quality control samples but was included in the dilution scheme to dilute 3750 ng/mL to 105 ng/mL. All working solutions were stored as aliquots of 150 µL in 1.5 mL polypropylene microcentrifuge tubes at ~-80°C until required. While only 20 µL of each working solution was needed per sample, the aliquot volume was 150 µL to allow for six-fold analysis per tube and to prevent working solution concentration due to evaporation (which was seen when the calibration standard working solutions were previously stored as 50 µL aliquots).

3.3. Preparation of standards and quality controls

It was decided to report the calibration range in nanograms per millilitre of lysate instead of nanograms per million cells, as this eliminates the need for a mean cell volume in the calculation. This also means that only the patient samples' mean cell volumes need to be determined in order to directly compare the intracellular concentrations in nanograms per millilitre, and these results are not

skewed by presuming the mean cell volume of the calibration standards. Using referenced volumes has been shown to provide an overestimation of patient cell volumes resulting in an underestimated intracellular drug concentration.[79,101] Calibration standards and quality controls were prepared by spiking an aliquot of one million blank PBMCs at room temperature using the stored working solutions presented in Tables 3.5. and 3.6. A 20 µL aliquot of each working solution was spiked into 250 µL methanol (containing internal standards) in a 1.5 ml polypropylene microcentrifuge tube containing one million PBMCs at the time of sample analysis to lyse the cells. This was then diluted with 230 µL water, resulting in a final lysate volume of 500 µL to obtain the desired calibration standard concentrations over a range from 0.500–500 ng/mL for EFV and LPV and 0.125–125 ng/mL for RTV, as shown in Table 3.7. The volume of the one million cell pellet is considered negligible (<1 µL) and was not included in the calculations. The quality control samples were used to validate the calibration curve and were prepared using the same methodology as the calibration standards (Table 3.8).

Table 3.7. Spiking scheme for calibration standards from working solutions

Calibration standard	Working solution used	Volume WS spiked into lysate (µL)	Volume blank lysate (µL)	Conc. in lysate (ng/mL)	
				EFV, LPV	RTV
S1	WS 1	20.0	480	500	125
S2	WS 2	20.0	480	350	87.5
S3	WS 3	20.0	480	200	50.0
S4	WS 4	20.0	480	50.0	12.5
S5	WS 5	20.0	480	20.0	5.00
S6	WS 6	20.0	480	5.00	1.25
S7	WS 7	20.0	480	2.50	0.625
S8	WS 8	20.0	480	1.00	0.250
S9	WS 9	20.0	480	0.500	0.125

Table 3.8. Spiking scheme for quality control samples from working solutions

Quality control	Working solution used	Volume WS spiked into lysate (µL)	Volume blank lysate (µL)	Conc. in lysate (ng/mL)	
				EFV, LPV	RTV
QC DIL	WS DIL	20.0	480	1000	250
QC H	WS H	20.0	480	400	100
QC M	WS M	20.0	480	150	37.5
SYS	WS SYS	20.0	480	4.20	1.05
QC L	WS L	20.0	480	1.40	0.350
QC LLOQ	WS LLOQ	20.0	480	0.500	0.125

The QC DIL was used during a validation batch to validate the dilution process should the calculated concentration of an unknown sample be reported as above the upper limit of quantification (ULOQ), i.e. greater than 500 ng/mL for EFV and LPV or greater than 125 ng/mL for RTV. The QC H, QC M, QC L, and QC LLOQ were used in six-fold during all validations, while only the QC H, QC M, and QC L were used in duplicate during sample analysis. A system check sample was included in every batch to serve

as a reference sample for instrument sensitivity and analyte retention time, and to evaluate system equilibration prior to injecting a batch.

3.4. INTER- AND INTRA-DAY ACCURACY AND PRECISION

In order to show that the method was accurate, precise, and reproducible, duplicate standards and six-fold quality control samples were prepared and extracted in three separate batches over three different days. The calibration range was 0.500 – 500 ng/mL for EFV and LPV and 0.125 – 125 ng/mL for RTV. The intra-day accuracy and precision were based on the three individual batches, and the inter-day accuracy, precision, and reproducibility were assessed using the combined results of the three batches, resulting in six replicates of each standard and 18 replicates of each quality control.

Accuracy is measured as the closeness of the observed concentration of the analyte to the nominal concentration and is expressed as percentage accuracy. While precision is measured as the closeness of the replicates at a given concentration to each other, expressed as the coefficient of variation (CV(%)).

The calibration standard peak area ratios (analyte peak area/ internal standard peak area) were plotted against the nominal concentrations to generate the calibration curves (Figures 3.5–3.7). The curves each fit a quadratic regression with a weighting of 1/concentration for EFV and LPV, and 1/concentration^2 for RTV.

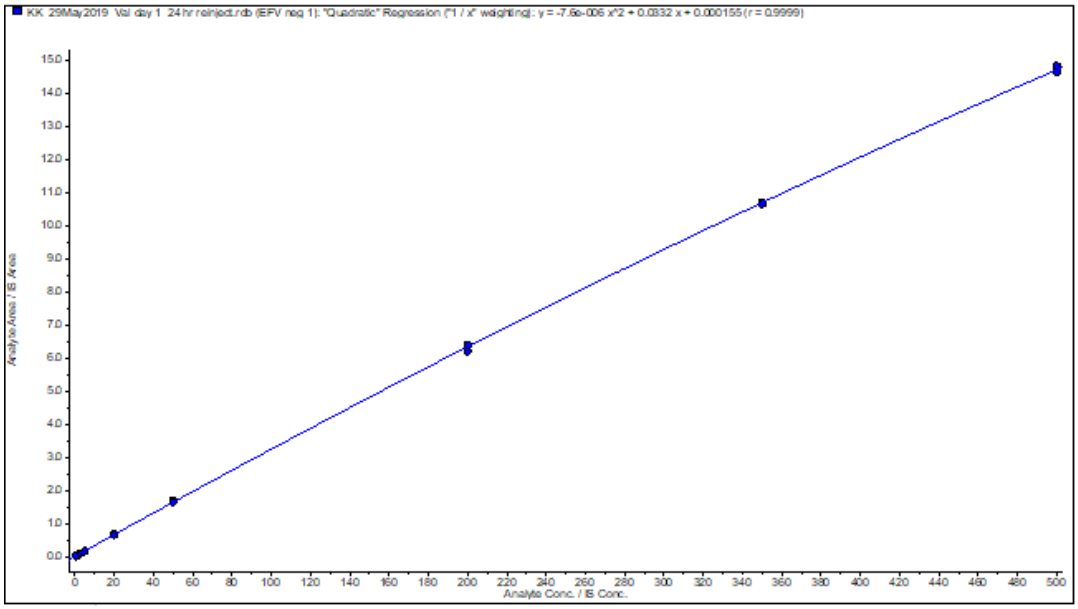

Figure 3.5. Calibration curve from validation day 1: EFV.

The EFV curves showed good fit as the r values for day 1, day 2, and day 3 were 0.9999, 0.9998, and 0.9999, respectively.

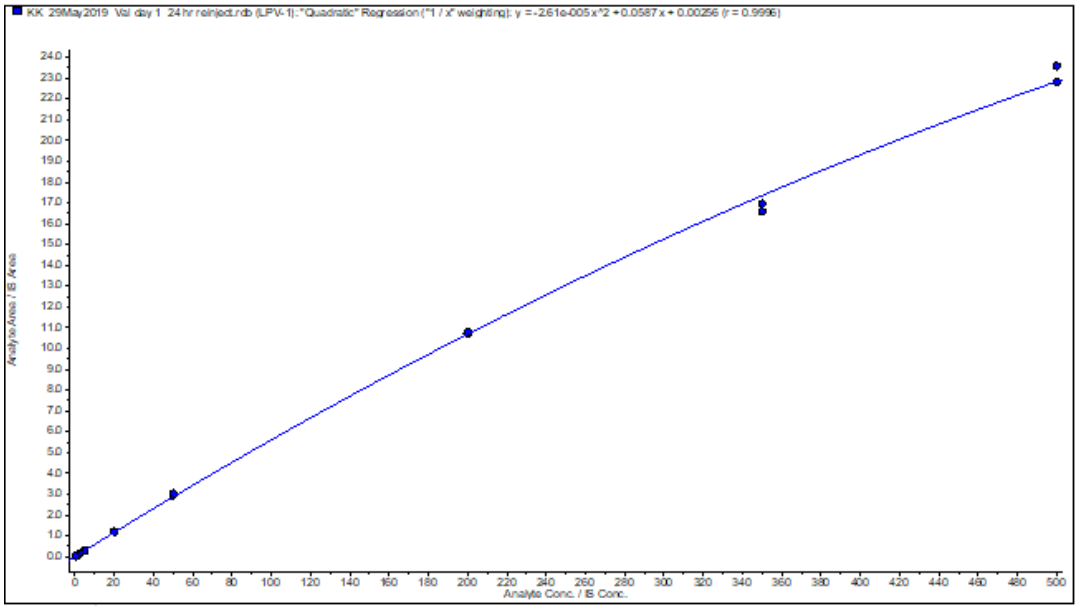

Figure 3.6. Calibration curve from validation day 1: LPV.

The LPV curves were the most quadratic of the three analytes but still showed a good fit. The r values for LPV day 1, day 2, and day 3 were 0.9996, 0.9999, and 0.9995, respectively.

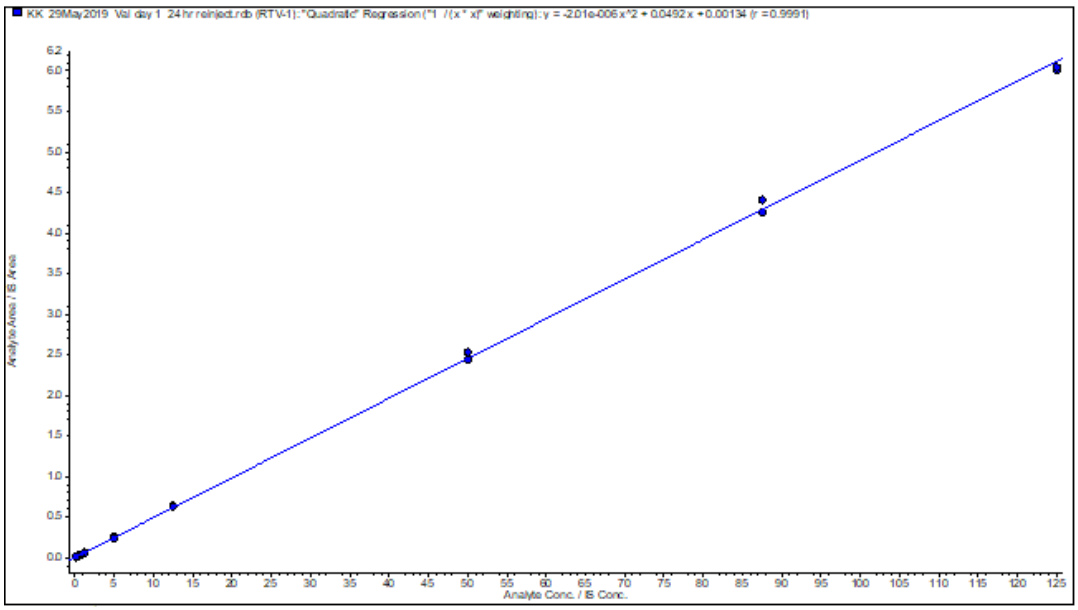

Figure 3.7. Calibration curve from validation day 1: RTV.

The RTV curves showed good fit with r values for day 1, day 2, and day 3 of 0.9996, 0.9980, and 0.9998, respectively.

The FDA and EMA stipulate that for a bioanalytical method to be considered accurate and precise, the percentage accuracies must be within 85 – 115%, and the coefficients of variation must be less than 15% for all standards and quality controls, with the exception of the lowest standard (S9) and the QC LLOQ where the percentage accuracies must be within 80–120%, and the coefficients of variation must be less than 20%. Calibration standards may be excluded should they fail to meet acceptance criteria, provided 75% of standards pass; however, quality control samples that do not meet acceptance criteria may not be excluded. Should a quality control sample be a statistical outlier, calculations excluding the outlier may be reported, provided that the calculations including this outlier are also reported.[108]

The intra-day results for EFV, LPV, and RTV met acceptance criteria with percentage accuracies between 93.6–104.4% for standards and 92.6–113.3% for quality controls and coefficients of variation below 6.4% for standards, below 6.3% for high-, medium-, and low-concentration quality controls, and below 19.1% for the LLOQs.

The same acceptance criteria apply for the inter-day accuracy and precision, namely that the percentage accuracies and coefficients of variation must be within 15% for all standards and quality control samples, with the exception of the lowest standard (S9) and the LLOQ, which must be within 20%. A summary of the inter-day results for each analyte is presented in Tables 3.9–3.14.

Table 3.9. Inter-day accuracy and precision results for calibration standards: EFV

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal conc. (ng/mL)	500	350	200	50.0	20.0	5.00	2.50	1.00	0.500
Average calculated conc. (ng/mL)	501	349	200	50.8	20.1	4.95	2.53	0.987	0.497
n	6	6	5*	5*	6	6	6	5*	6
STDEV	9.33	5.96	2.88	1.08	0.256	0.206	0.0344	0.0289	0.0251
CV(%)	1.9	1.7	1.4	2.1	1.3	4.2	1.4	2.9	5.1
%Accuracy	100.2	99.6	99.8	101.6	100.6	98.9	101.3	98.7	99.3

** Failed calibration standard excluded*

Table 3.10. Inter-day accuracy and precision results for quality controls: EFV

	QC H	QC M	QC L	QC LLOQ
Nominal conc. (ng/mL)	400	150	1.50	0.500
Average calculated conc. (ng/mL)	424	156	1.46	0.543
n	18	18	18	18
STDEV	10.3	4.44	0.0433	0.0709
CV(%)	2.4	2.8	3.0	13.0
%Accuracy	105.9	104.1	104.3	108.6

The inter-day percentage accuracies for EFV were 98.7–101.6% for the standards and 104.1–108.6% for the quality controls and were within acceptable limits (Tables 3.9–3.10). The coefficients of variation for the standards and quality controls were all less than 15% and were also within acceptable limits.

Table 3.11. Inter-day accuracy and precision results for calibration standards: LPV

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal conc. (ng/mL)	500	350	200	50.0	20.0	5.00	2.50	1.00	0.500
Average calculated conc. (ng/mL)	516	339	201	51.5	20.2	5.07	2.51	0.975	0.488
n	6	6	5*	5*	6	6	6	5*	6
STDEV	21.1	10.4	2.95	2.40	0.596	0.194	0.0668	0.0354	0.0189
CV(%)	4.1	3.1	1.5	4.7	3.0	3.8	2.7	3.6	3.9
%Accuracy	103.1	96.9	100.6	103.0	100.8	101.5	100.5	97.5	97.6

** Failed calibration standard excluded*

Table 3.12. Inter-day accuracy and precision results for quality controls: LPV

	QC H	QC M	QC L	QC LLOQ
Nominal conc. (ng/mL)	400	150	1.50	0.500
Average calculated conc. (ng/mL)	423	160	1.53	0.539
n	18	18	18	18
STDEV	19.9	6.24	0.0629	0.0710
CV(%)	4.7	3.9	4.1	13.2
%Accuracy	105.8	106.4	109.3	107.8

The inter-day percentage accuracies for LPV ranged from 96.9–103.1% for the standards and 105.8–109.3% for the quality controls and were within acceptable limits (Tables 3.11–3.12). The coefficients of variation were all less than 15% and were also within acceptable limits.

Table 3.13. Inter-day accuracy and precision results for calibration standards: RTV

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal conc. (ng/mL)	125	87.5	50.0	12.5	5.00	1.25	0.625	0.250	0.125
Average calculated conc. (ng/mL)	125	87.4	50.0	12.6	5.00	1.25	0.621	0.248	0.126
n	6	6	5*	5*	6	6	6	5*	6
STDEV	2.40	2.32	1.41	0.227	0.118	0.0469	0.0169	0.00559	0.0125
CV(%)	1.9	2.7	2.8	2.2	2.4	3.8	2.7	2.3	9.9
%Accuracy	99.9	99.9	100.0	101.0	99.9	100.0	99.4	99.4	100.5

** Failed calibration standard excluded*

Table 3.14. Inter-day accuracy and precision results for quality controls: RTV

	QC H	QC M	QC L	QC LLOQ
Nominal conc. (ng/mL)	100	37.5	0.350	0.125
Average calculated conc. (ng/mL)	101	37.5	0.355	0.130
n	18	18	18	18
STDEV	4.34	1.41	0.0181	0.0219
CV(%)	4.3	3.8	5.1	16.8
%Accuracy	100.6	100.0	101.3	104.1

The inter-day percentage accuracies for RTV were 99.4–101.0% for the standards and 100.0–104.1% for the quality controls (Tables 3.13 and 3.14) and were within acceptable limits. The coefficients of variation were less than or equal to 9.9% for the standards and 5.1% for the quality controls (other than the LLOQ) and were within the acceptable limits of less than 15%. The QC LLOQ had a coefficient of variation of 16.8%, which meets the acceptance criteria of less than 20% for the lowest concentration.

In summary, the intra- and inter-day accuracy and precision met the acceptance criteria for each analyte at all standard and quality control concentrations. This indicates that the method developed was accurate, precise, and reproducible for the analysis of intracellular EFV, LPV, and RTV from human PBMCs over a range of 0.500–500 ng/mL for EFV and LPV and 0.125–125 ng/mL for RTV which is the same range as the unbound drug method discussed in Chapter Two and is similar to the ranges validated by Ter Heine *et al.* (2009) where the PBMC lysate calibration ranges were 1.00–500 ng/mL for EFV, LPV, and RTV and by Colombo *et al.* (2005) where the range for quantifying EFV, LPV and RTV in PBMC lysate was 0.400–100 ng/mL.[98,101] However, these methods required four million PBMCs or more per sample and were validated prior to the 2012 EMA and 2013/2018 FDA guidelines.

3.5. SENSITIVITY AND SPECIFICITY

In order to assess the sensitivity of the method, six different lots of matrix (PBMCs originating from six different lots of whole blood donated by healthy volunteers) were spiked at LLOQ and extracted. The LLOQ of EFV and LPV was 0.500 ng/mL, while the LLOQ of RTV was 0.125 ng/mL. The signal-to-noise ratio of each peak was calculated using the S/N script (Analyst software version 1.6.3), where the noise was selected from the 15 seconds preceding the analyte peak.

Six lots of blank matrix were extracted without internal standards (double blanks) and injected after the highest standard (S1) to evaluate the assay's specificity for each analyte and any carry-over effects.

Raw chromatograms of the LLOQs for EFV, LPV, and RTV are shown in Figures 3.8–3.10, respectively. The signal-to-noise ratios at the LLOQ are required to be greater than or equal to five to ensure that the analytes can be quantified with accuracy and precision.[108,109] The signal-to-noise ratios at the LLOQ level extracted from six different lots of matrix were all greater than 76.6 for EFV, 210 for LPV, and 89.1 for RTV, indicating that the mass spectrometry method was sensitive for all three analytes. Any samples with a calculated concentration of greater than or equal to 0.500 ng/mL for EFV and LPV, and 0.125 ng/mL for RTV can be reported; while any samples with a calculated concentration below these concentrations must be reported as "below the limit of quantitation" or "BLOQ."

It is difficult to compare this sensitivity with other published methods due to the variability in concentration units without first converting the reported LLOQs to nanograms per millilitre of lysate. Some methods quantifying intracellular EFV, LPV, and RTV have validated LLOQs of 1 ng/mL,[101] 0.5 ng/mL,[98] or 0.125 ng/mL[122], which are comparable to the LLOQs of this method. Other methods report LLOQs of 0.1 ng/3 million cells for LPV and RTV[76], which corresponds to a lysate concentration of 4 ng/mL (25 µL lysate volume and 400 fL cell volume) or 2 ng/3 million cells for EFV and LPV and 1 ng/3 million cells for RTV[119] which corresponds to a lysate concentration of 10 ng/mL for EFV and LPV and 5 ng/mL for RTV (200 µL lysate volume and 400 fL cell volume) which are far less sensitive and require a greater cell number than the method validated in this chapter. A method published by De Nicolò *et al.* (2020) validated an LLOQ of 0.390 ng/sample for EFV and 0.0390 ng/sample for RTV, where each sample consisted of 16 million cells, 500 µL lysate, and 100 µL working solutions.[123] This results in a respective LLOQ in lysate of 0.650 ng/mL for EFV and 0.0650 ng/mL for RTV. While the De Nicolò *et al*. (2020) method is more sensitive for RTV, it requires 16 million cells per sample from 16 mL of whole blood (two 8 mL CPT tubes) whereas the method validated in this chapter only requires one million cells per sample.

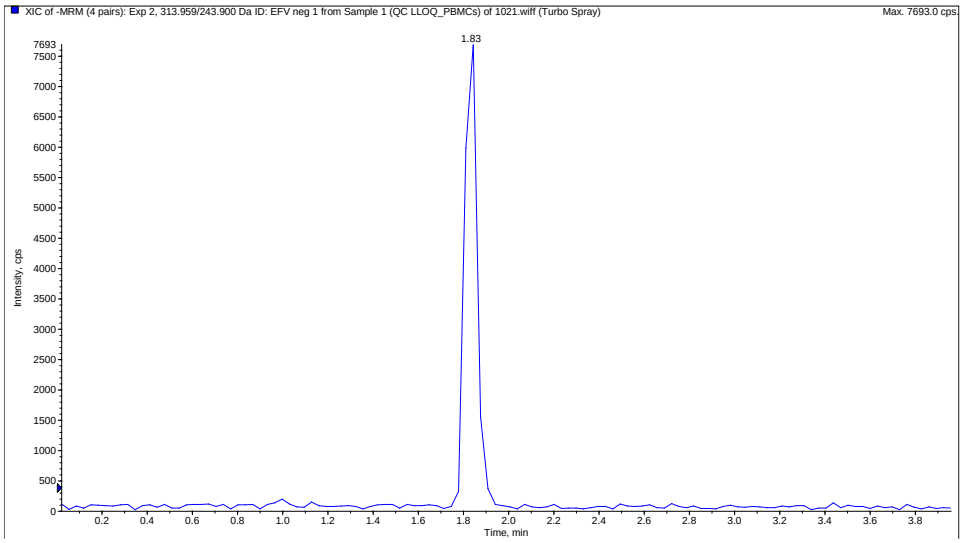

Figure 3.8. Representative chromatogram of EFV at LLOQ (0.500 ng/mL).

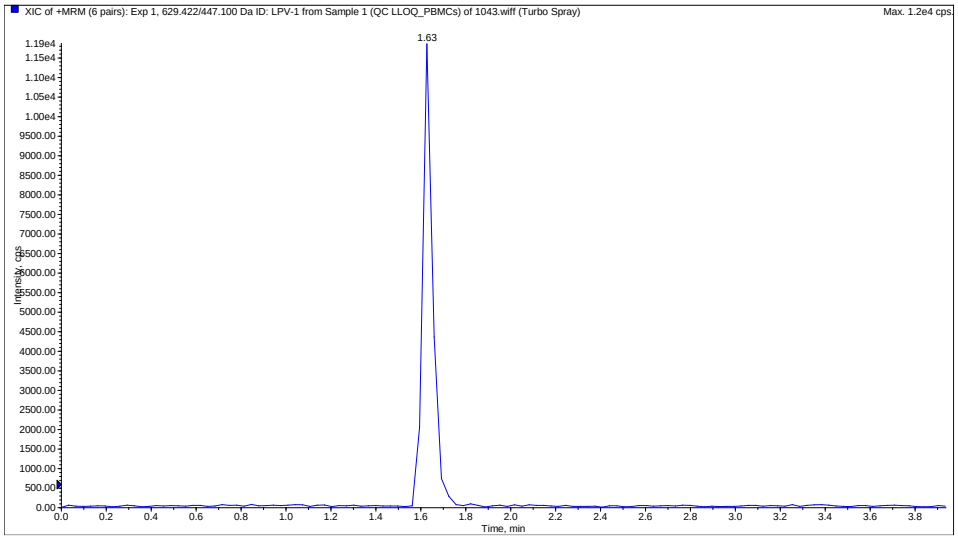

Figure 3.9. Representative chromatogram of LPV at LLOQ (0.500 ng/mL).

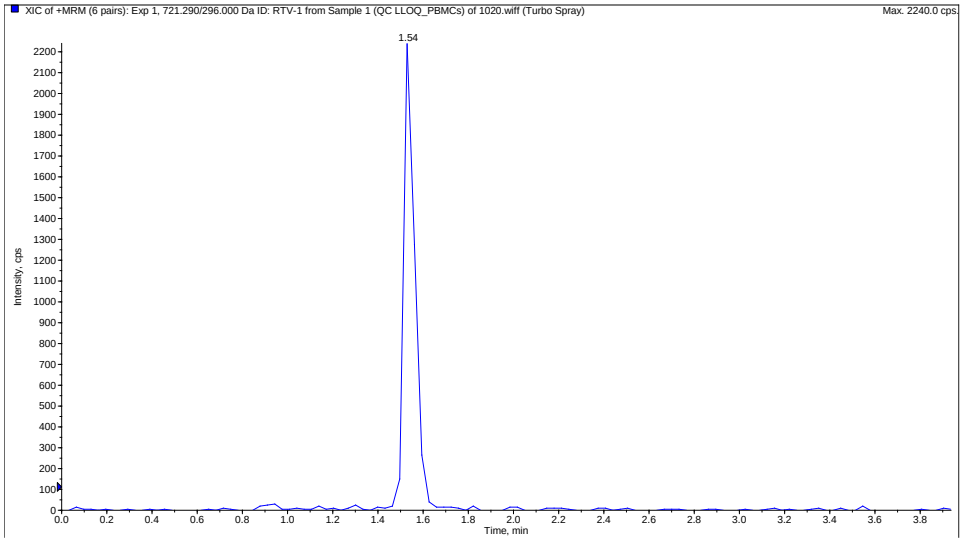

Figure 3.10. Representative chromatogram of RTV at LLOQ (0.125 ng/mL).

The double blank samples showed no carry-over for the analytes or internal standards when injected after the highest standard (Figure 3.11 a–c). The double blank chromatograms showed that there was very low background noise throughout the run time and no peaks at the retention times of the analytes, indicating that the method was highly specific for the analytes and further explained the very high signal-to-noise ratios calculated for the LLOQs.

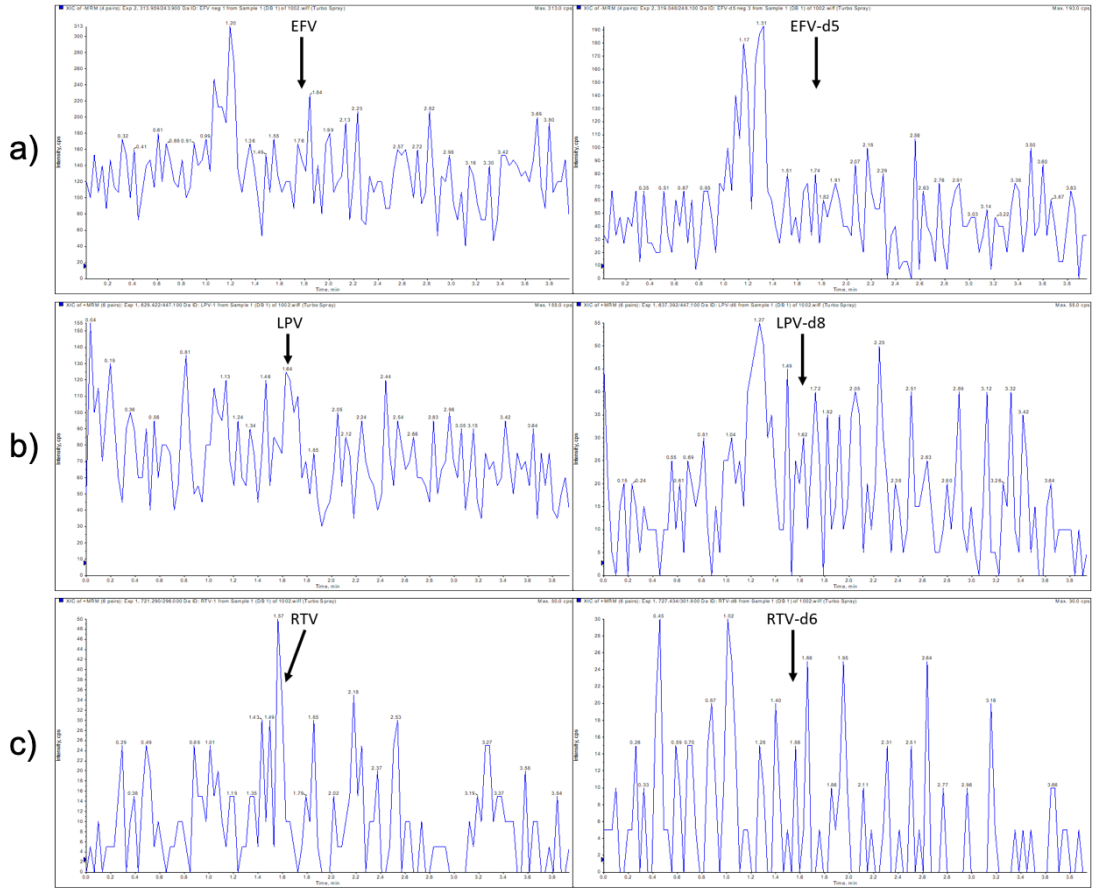

Figure 3.11. Representative chromatograms of double blank samples for **a)** EFV, **b)** LPV, **c)** RTV, and their respective internal standards.

3.6. CROSSTALK

Crosstalk refers to any contribution of an analyte to a different analyte's MRM channel, resulting in false-positive results. Crosstalk can also occur between an analyte and its corresponding internal standard's MRM channels.

Crosstalk between EFV, LPV, and RTV was assessed during the validation for unbound EFV, LPV, and RTV (see Chapter Two, Section 3.6). Crosstalk between the internal standards and respective analytes

was also evaluated in Chapter Two, however, the concentrations of internal standards in the intracellular method were different from that previously tested. Therefore, the contribution of the internal standards on the analyte MRM channel was re-evaluated.

Six blank samples (without analyte, containing internal standards) originating from six different lots of whole blood were extracted to determine the possible contribution of the internal standard to the analyte.

The blank samples (without analytes but with internal standards) showed that there was no contribution of the internal standards to the analyte MRM channels as there were no peaks in the analyte channels (Figure 3.12 a–c).

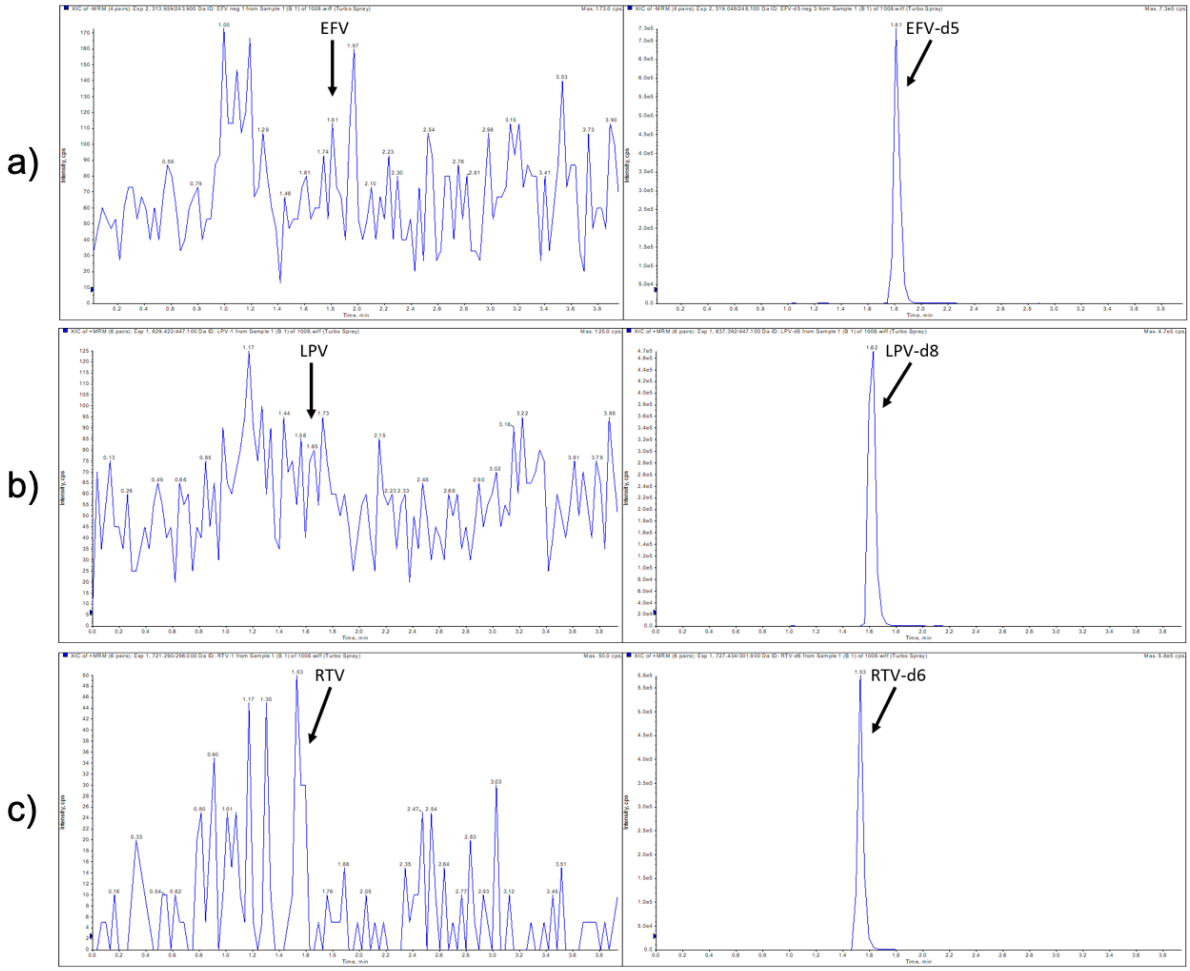

Figure 3.12. Representative chromatograms of blank samples for **a)** EFV, **b)** LPV, **c)** RTV, and their respective internal standards.

3.7. MATRIX EFFECTS

In a bioanalytical method using HPLC-MS/MS, matrix effects refer to any substances present in a sample other than the analytes of interest that may impact ionization in the source. It is important to mitigate possible matrix effects by removing any endogenous matrix components that cause the interference during extraction and having an appropriate internal standard that will compensate for any ion enhancement or suppression.

To evaluate matrix effects, the Matuszewski method was used.[137] Six different sources of PBMCs (isolated from whole blood donated by six different healthy volunteers) were extracted without analytes and without internal standards. After extraction, the samples were spiked at low, medium, and high concentrations, taking into account the concentration steps included in the method, and at one concentration of the internal standards.

The peak area ratios of each of the high-, medium-, and low-concentration quality control levels *versus* their respective concentrations was used to calculate the regression slope of each of the PBMC sources (Tables 3.15–3.17).

Table 3.15. Regression results to determine matrix effects: EFV

	High Conc. 400 ng/mL Peak Area Ratio	**Medium Conc. 150 ng/mL Peak Area Ratio**	**Low Conc. 1.40 ng/mL Peak Area Ratio**	**Peak Area Ratio *versus* Conc. Regression Slope**
Matrix 1	55.4	22.0	0.235	0.138
Matrix 2	57.6	22.8	0.243	0.143
Matrix 3	56.9	23.8	0.249	0.141
Matrix 4	57.3	22.5	0.248	0.143
Matrix 5	57.2	22.9	0.230	0.142
Matrix 6	57.6	23.0	0.223	0.143
Average	**57.0**	**22.8**	**0.238**	**0.142**
STDEV	**0.817**	**0.618**	**0.0106**	**0.00206**
CV(%)	**1.4**	**2.7**	**4.5**	**1.5**

Table 3.16. Regression results to determine matrix effects: LPV

	High Conc. 400 ng/mL Peak Area Ratio	Medium Conc. 150 ng/mL Peak Area Ratio	Low Conc. 1.40 ng/mL Peak Area Ratio	Peak Area Ratio *versus* Conc. Regression Slope
Matrix 1	18.6	7.70	0.0910	0.0460
Matrix 2	19.3	7.91	0.0851	0.0478
Matrix 3	18.8	8.56	0.0964	0.0463
Matrix 4	18.3	8.44	0.0951	0.0451
Matrix 5	18.9	8.37	0.0897	0.0467
Matrix 6	18.8	9.08	0.0864	0.0460
Average	**18.8**	**8.35**	**0.0906**	**0.0463**
STDEV	**0.313**	**0.490**	**0.00453**	**0.000897**
CV(%)	**1.7**	**5.9**	**5.0**	**1.9**

Table 3.17. Regression results to determine matrix effects: RTV

	High Conc. 100 ng/mL Peak Area Ratio	Medium Conc. 37.5 ng/mL Peak Area Ratio	Low Conc. 0.350 ng/mL Peak Area Ratio	Peak Area Ratio *versus* Conc. Regression Slope
Matrix 1	5.15	1.85	0.0198	0.0516
Matrix 2	5.25	1.92	0.0210	0.0525
Matrix 3	5.29	2.04	0.0216	0.0527
Matrix 4	5.15	1.90	0.0211	0.0515
Matrix 5	5.20	2.00	0.0192	0.0519
Matrix 6	5.29	2.02	0.0191	0.0528
Average	**5.22**	**1.95**	**0.0203**	**0.0522**
STDEV	**0.0635**	**0.0749**	**0.00106**	**0.000578**
CV(%)	**1.2**	**3.8**	**5.2**	**1.1**

The mean variability of the individual regression slopes was determined to be 1.5% for EFV, 1.9% for LPV, and 1.1% for RTV, which all meet the acceptance criteria of less than 5% as stipulated in the FDA and EMA guidelines.[108,109] This means that there were no significant endogenous matrix components co-eluting with the analytes that would adversely affect the ionization of the analytes, such as causing ion suppression or enhancement.[137]

3.8. RECOVERY

During sample extraction, some analyte may not be removed from the sample or may be lost during the extraction procedure. The percentage of analyte recovered from the sample need not be 100% but must be consistent across different concentrations and different matrix sources.

Six lots of matrix were spiked at high, medium, and low concentrations and extracted as per the methodology described in Section 2.4 of this chapter. These were referred to as the "test" samples. The same six lots of matrix were extracted blank and spiked at high, medium, and low levels after extraction, accounting for the concentration steps included in the method. These samples were used as the reference samples, which represent 100% recovery. The test sample peak areas were compared to the reference sample peak areas to calculate the percentage recovery during extraction.

The mean recovery for EFV, LPV, and RTV across concentrations was determined to be 92.9%, 91.7%, and 91.0%, respectively (Tables 3.18–3.20). Recoveries were all greater than 90% and, more importantly, were consistent and precise across the different concentrations, with a required precision of less than 15%.

Table 3.18. Recovery results for EFV

	High Concentration (400 ng/mL)		**Medium Concentration (150 ng/mL)**		**Low Concentration (1.40 ng/mL)**	
	Reference: Peak Area	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**
Sample 1	23000000	23000000	9930000	9720000	117000	95900
Sample 2	23600000	22000000	9820000	9530000	122000	101000
Sample 3	22300000	21900000	10100000	9940000	120000	104000
Sample 4	22800000	21400000	9590000	9140000	108000	93500
Sample 5	22600000	21100000	9500000	8970000	103000	93000
Sample 6	22300000	21700000	10100000	9170000	99300	95300
Average	**22766667**	**21850000**	**9840000**	**9411667**	**111550**	**97117**
STDEV	**492612**	**653452**	**253693**	**378281**	**9445**	**4411**
CV(%)	**2.2**	**3.0**	**2.6**	**4.0**	**8.5**	**4.5**
%Recovery		**96.0**		**95.6**		**87.1**
					Average %Recovery	**92.9**
					Average CV(%)	**5.4**

Table 3.19. Recovery results for LPV

	High Concentration (400 ng/mL)		Medium Concentration (150 ng/mL)		Low Concentration (1.40 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Sample 1	23200000	22700000	10400000	10600000	131000	108000
Sample 2	23700000	21500000	11000000	10600000	126000	108000
Sample 3	23100000	21600000	10700000	10700000	135000	116000
Sample 4	22200000	20900000	10300000	9340000	116000	94500
Sample 5	22500000	20600000	9790000	9740000	113000	95500
Sample 6	22700000	21500000	10900000	9660000	108000	99700
Average	**22900000**	**21466667**	**10515000**	**10106667**	**121500**	**103617**
STDEV	**540370**	**722957**	**447828**	**593386**	**10747**	**8439**
CV(%)	**2.4**	**3.4**	**4.3**	**5.9**	**8.8**	**8.1**
%Recovery		**93.7**		**96.1**		**85.3**
					Average %Recovery	**91.7**
					Average CV(%)	**6.2**

Table 3.20. Recovery results for RTV

	High Concentration (100 ng/mL)		Medium Concentration (37.5 ng/mL)		Low Concentration (0.350 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Sample 1	5250000	5300000	2090000	2030000	22800	20100
Sample 2	5350000	4880000	2170000	1980000	23500	19300
Sample 3	5340000	4860000	2080000	2040000	24800	22300
Sample 4	5130000	4680000	1940000	1790000	21100	17000
Sample 5	5200000	4570000	1920000	1870000	20300	18900
Sample 6	5340000	4560000	2010000	1810000	19900	18100
Average	**5268333**	**4808333**	**2035000**	**1920000**	**22067**	**19283**
STDEV	**90646**	**277302**	**96073**	**110995**	**1940**	**1818**
CV(%)	**1.7**	**5.8**	**4.7**	**5.8**	**8.8**	**9.4**
%Recovery		**91.3**		**94.3**		**87.4**
					Average %Recovery	**91.0**
					Average CV(%)	**3.8**

The coefficient of variation for each quality control concentration was less than 10% for all three analytes, and the reproducibility across the high, medium, and low concentrations was 5.4%, 6.2%, and 3.8% for EFV, LPV, and RTV, respectively which shows that the recovery of analyte is consistent across the calibration range.

3.9. PROCESS EFFICIENCY

Process efficiency assesses the effect of both recovery and matrix effects on an analyte's response. It is tested using neat solvent (mobile phase) spiked in triplicate at the theoretical final concentrations of each quality control level as the reference samples, representing 100% recovery and zero matrix effects. Test samples were spiked into six different lots of matrix at high, medium, and low concentrations and were extracted as per the methodology described in Section 2.4. These peak areas were compared to the reference peak areas to calculate the percentage process efficiency. As with recovery, process efficiency need not be 100% but must be consistent across different concentration levels and matrix sources.

The mean process efficiency of EFV, LPV, and RTV is 80.5%, 86.2%, and 90.1%, respectively (Tables 3.21–3.23). The mean process efficiencies for EFV and LPV are 12.4% and 5.5%: lower than their respective mean recoveries. This suggests that there is some ion suppression occurring; however, as the matrix effects experiment showed, these effects were consistent across different lots of matrix. The mean process efficiency and recovery of RTV only differed by 0.9%, indicating that the ionization of RTV was not influenced by any background components present in the sample.

Table 3.21. Process efficiency results for EFV

	High Concentration (400 ng/mL)		**Medium Concentration (150 ng/mL)**		**Low Concentration (1.40 ng/mL)**	
	Reference: Peak Area	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**	**Reference: Peak Area**	**Test: Peak Area**
Matrix 1	25600000	23000000	11700000	9720000	129000	95900
Matrix 2	25600000	22000000	11700000	9530000	126000	101000
Matrix 3	25300000	21900000	11700000	9940000	132000	104000
Matrix 4		21400000		9140000		93500
Matrix 5		21100000		8970000		93000
Matrix 6		21700000		9170000		95300
Average	**25500000**	**21850000**	**11700000**	**9411667**	**129000**	**97117**
STDEV	**378281**	**653452**	**0.00**	**345322**	**2449**	**4411**
CV(%)	**1.5**	**3.0**	**0.0**	**3.7**	**1.9**	**4.5**
%Recovery		**85.7**		**80.4**		**75.3**
					Average %Process Efficiency	**80.5**
					Average CV(%)	**6.5**

Table 3.22. Process efficiency results for LPV

	High Concentration (400 ng/mL)		Medium Concentration (150 ng/mL)		Low Concentration (1.40 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Matrix 1	24400000	22700000	11700000	10600000	132000	108000
Matrix 2	23300000	21500000	11300000	10600000	124000	108000
Matrix 3	23700000	21600000	11300000	10700000	132000	116000
Matrix 4		20900000		9340000		94500
Matrix 5		20600000		9740000		95500
Matrix 6		21500000		9660000		99700
Average	**23800000**	**21466667**	**11433333**	**10106667**	**129333**	**103617**
STDEV	**593386**	**722957**	**230940**	**541685**	**3771**	**8439**
CV(%)	**2.5**	**3.4**	**2.0**	**5.4**	**2.9**	**8.1**
%Recovery		**90.2**		**88.4**		**80.1**
					Average %Process Efficiency	**86.2**
					Average CV(%)	**6.2**

Table 3.23. Process efficiency results for RTV

	High Concentration (100 ng/mL)		Medium Concentration (37.5 ng/mL)		Low Concentration (0.350 ng/mL)	
	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area	Reference: Peak Area	Test: Peak Area
Matrix 1	5370000	5300000	2100000	2030000	21000	20100
Matrix 2	5330000	4880000	2080000	1980000	22100	19300
Matrix 3	5380000	4860000	2030000	2040000	22800	22300
Matrix 4		4680000		1790000		17000
Matrix 5		4570000		1870000		18900
Matrix 6		4560000		1810000		18100
Average	**5360000**	**4808333**	**2070000**	**1920000**	**21967**	**19283**
STDEV	**26458**	**277302**	**36056**	**110995**	**907**	**1818**
CV(%)	**0.5**	**5.8**	**1.7**	**5.8**	**4.1**	**9.4**
%Recovery		**89.7**		**92.8**		**87.8**
					Average %Process Efficiency	**90.1**
					Average CV(%)	**2.8**

The acceptance criteria were met for all three analytes. The precision of the measured process efficiency should not exceed 15% for any concentration, and the reproducibility between concentration levels should also not be greater than 15%.[108,109] The precision at each concentration was below 10%, and the reproducibility between the concentrations was 6.5%, 6.2%, and 2.8% for EFV, LPV, and RTV, respectively.

3.10. DILUTION INTEGRITY

During sample analysis of unknown patient samples, a calculated concentration may be reported as greater than the ULOQ. As this concentration lies outside of the validated range, this result may not be reported with confidence. To evaluate if these samples may be diluted to within the validated range and re-extracted with accuracy and precision, six QC DIL samples were prepared at twice the ULOQ (1000 ng/mL for EFV and LPV and 250 ng/mL for RTV). These samples were diluted 1:4 (v/v) with blank lysate, extracted as normal, and compared with the nominal concentration to calculate the percentage accuracy.

Published work has validated a 1:2 and 1:3 dilution of PBMC lysate for EFV, LPV, and RTV with precision and accuracy.[98] In this study, a 1:4 dilution was investigated. The concentrations of QC DIL samples that were diluted 1:4 with blank lysate were determined (incorporating the dilution factor of five) and compared to the nominal concentrations (Table 3.24).

Table 3.24. Dilution integrity results following a 5-fold dilution with blank cell lysate

	EFV	LPV	RTV
Nominal conc. (ng/mL)	1000	1000	250
Average calculated conc. (ng/mL)	1026	1086	240
n	6	6	6
STDEV	18.9	31.6	3.62
CV(%)	1.8	2.9	1.5
%Accuracy	102.6	108.6	95.8

The same acceptance criteria apply as for the high-, medium-, and low-quality controls; namely, the percentage accuracy and precision must be within 15%. The dilution results met all acceptance criteria meaning that concentrations calculated as above the upper limit may be reanalysed reliably when the extracted sample is diluted into the calibration range (1:4) with blank lysate.

3.11. ANALYTE STABILITY ASSESSMENTS

Various stability assessments were performed to evaluate the stability of EFV, LPV, and RTV in different conditions. The stability tests in solvents were performed to determine if there was any analyte degradation in the stock or working solutions during storage or on-bench. The stability tests

in matrix were planned to try and mimic the conditions that a patient sample might go through to determine if the concentration of analyte would be affected under such conditions.

3.11.1. STOCK SOLUTION STABILITY

Long term storage stability at ~80°C was shown for the stock solutions, as shown in Appendix B, C, and D. A 1 mg/mL stock of EFV in acetonitrile is stable for 601 days and 1 mg/mL stock of LPV or RTV in methanol is stable for 266 and 304 days, respectively. Based on the stock solution stability, working solutions may be prepared approximately eight months after the stock solutions are made. This is important as working solutions are often not prepared on the same day that the stock solutions were prepared.

Each of the internal standards used, EFV-d5, LPV-d8, and RTV-d6 were shown to be stable at room temperature for up to 7 hours (see Chapter Two, Section 3.15.1), which shows that the internal standards will not degrade over the course of the extraction.

3.11.2. WORKING SOLUTION STABILITY

During the stability testing for the unbound drug method described in Chapter Two, Section 3, working solution stability was shown for 38 days at ~-80°C and 24 hours at room temperature, ~4°C and ~-20°C. The highest concentration of analytes in these working solutions was 44.4 µg/mL for EFV and LPV and 11.1 µg/mL for RTV, and the lowest concentration was 3.00 ng/mL for EFV and LPV and 0.750 ng/mL for RTV. The working solution concentrations used in the PBMC method fall within the range tested in Chapter Two and were prepared in the same solvent composition (acetonitrile: water, 4:1, v/v) and therefore infer stability of EFV, LPV, and RTV in the working solutions presented in Tables 3.5 and 3.6 of Section 3.2 of this chapter.

3.11.3. BENCHTOP STABILITY

Benchtop stability could only be tested in lysate as it was not feasible to spike known concentrations into the cells prior to lysis. In order to ascertain the benchtop stability in lysate, low and high quality

controls were spiked in cell lysate and left on the bench for approximately five hours at room temperature. Five hours was anticipated to be the maximum time that future study samples might be left thawed until extracted.

The measured concentrations and calculated percentage differences of the high- and low-concentration quality control samples that had been left on-bench for five hours prior to extraction are summarized in Table 3.25.

Table 3.25. Analyte stability in lysate after ~5 hours on-bench

	EFV		LPV		RTV	
Nominal conc. (ng/mL)	400	1.40	400	1.40	100	0.350
Average calculated conc. (ng/mL)	430	1.45	436	1.51	98.4	0.347
n	6	6	6	6	6	6
STDEV	51.5	0.0440	24.8	0.0412	3.68	0.00965
CV(%)	12.0	3.0	5.7	2.7	3.7	2.8
%Difference from nominal	7.5	3.7	9.1	7.7	-1.7	-1.0

The percentage differences from the nominal concentrations and the coefficients of variation are required to be less than 15%. All three analytes met these acceptance criteria. Therefore, samples may be left on-bench for up to five hours prior to extraction without compromising the results.

3.11.4. FREEZE-THAW STABILITY

The freeze-thaw stability evaluation of intracellular analytes is difficult without incurred samples or PBMCs containing certified concentrations of analytes which are not currently commercially available.[78] The experiment was consequently performed in lysate, and the results analysed as peak areas compared to freshly spiked quality control peak areas to infer the absolute analyte stability during freezing and thawing.[98]

Therefore, to determine if samples may be frozen and thawed multiple times, high and low quality controls were spiked in cell lysate and subjected to three freezing and thawing cycles. Each freeze-thaw cycle consisted of approximately 24 hours at ~-80°C and 2 hours at room temperature, which included a 30-second vortex and a 5-minute sonication to mimic patient sample handling. The freeze-thaw quality control test samples were then extracted with freshly prepared valid quality control reference samples.

Typically, the calculated concentrations of the freeze-thaw samples are compared to their respective nominal concentrations; however, in this experiment, the calculated concentrations or peak area ratios could not be used because the test and reference samples were lysed with lysing solution containing internal standards prepared on different days (day of cell lysis for the test samples prior to freezing and thawing and the day of extraction for the fresh samples), resulting in varied internal standard peak areas. Therefore, the analyte peak areas were used to calculate the percentage differences to assess absolute analyte stability over three freeze-thaw cycles (Tables 3.26–3.28).

The peak area ratios are included in the EFV low concentration table (Table 3.26) to show that the high coefficient of variation seen for the EFV QC L sample peak areas (CV(%) = 19.1%) was due to instrument variability, as when normalised to the internal standard peak areas, the coefficient of variation of peak area ratios was 6.5%.

Table 3.26. Stability of EFV in lysate after 3 freeze-thaw cycles

	High Concentration		Low Concentration			
	Ref QC H	Test QC H	Ref QC L			Test QC L
	Analyte Peak Area	Analyte Peak Area	Analyte Peak Area	ISTD Peak Area	Peak Area Ratio	Analyte Peak Area
Average	25016667	22916667	114067	2156667	0.0525	125333
n	6	6	6	6	6	6
STDEV	793515	3019547	21753	273691	0.00340	5645
CV(%)	3.2	13.2	19.1	12.7	6.5	4.5
%Difference		-8.4				9.9*

** The percentage difference was still calculated using the Ref QC L analyte peak area. The ISTD peak area and peak area ratio data are only included to show that the ISTD compensated for the instrument variability.*

Table 3.27. Stability of LPV in lysate after 3 freeze-thaw cycles

	High Concentration		Low Concentration	
	Ref QC H	Test QC H	Ref QC L	Test QC L
Average Peak Areas	33633333	32566667	228500	215833
n	6	6	6	6
STDEV	524087	571548	14734	21995
CV(%)	1.6	1.8	6.4	10.2
%Difference		-3.2		-5.5

Table 3.28. Stability of RTV in lysate after 3 freeze-thaw cycles

	High Concentration		Low Concentration	
	Ref QC H	**Test QC H**	**Ref QC L**	**Test QC L**
Average Peak Areas	6140000	6008333	27917	26833
n	6	6	6	6
STDEV	901288	687413	3103	2745
CV(%)	14.7	11.4	11.1	10.2
%Difference		-2.1		-3.9

All quality control samples that were put through the three freeze-thaw cycles were within the acceptable limits for accuracy and precision, as the percentage differences ranged from -5.5–9.9%, and the coefficients of variation were all less than 15%. These results show that all three analytes will be stable during three freeze-thaw cycles if isolated PBMCs are frozen and thawed prior to cell lysis, which confirms the findings of Colombo *et al.* (2005), who showed that EFV, LPV, and RTV were stable in PBMC lysate, inferring stability of the analytes prior to lysis, after three freeze-thaw cycles.[98] Many research groups validating intracellular drug methods either reference freeze-thaw stability of the analytes in human plasma to infer stability in PBMCs[24,120,122] or do not mention freeze-thaw stability in PBMCs at all.[76,101,119,142,152]

3.11.5. REINJECTION REPRODUCIBILITY

It is important to determine the stability of the analytes after extraction as it is not always possible to analyse the batch on the day of extraction, and sometimes a batch needs to be reinjected due to instrument interruptions that can occur during HPLC-MS/MS analysis. To evaluate how long a batch may be left in the autosampler, the extracted samples from validation day 1 were left in the autosampler (at ~8°C) for five days, after which the batch was reinjected. Reinjection reproducibility was assessed by evaluating whether the batch passed or failed as a whole.

The reinjection reproducibility results from reinjecting the standards and quality controls from validation day 1 after five days are summarised in Tables 3.29–3.31.

Table 3.29. EFV reinjection reproducibility: validation 1 reinjected after five days

Sample ID	Nominal Conc. (ng/mL)	Mean Observed Conc. (ng/mL)	STDEV	CV(%)	%Accuracy	n
S1	500	501	10.4	2.1	100.2	2 of 2
S2	350	350	5.93	1.7	99.9	2 of 2
S3	200	199	1.95	1.0	99.4	2 of 2
S4	50.0	50.9	0.871	1.7	101.7	2 of 2
S5	20.0	20.0	0.211	1.1	99.8	2 of 2
S6	5.00	5.01	0.0874	1.7	100.2	2 of 2
S7	2.50	2.55	0.0324	1.3	101.8	2 of 2
S8	1.00	0.972	0.0168	1.7	97.2	2 of 2
S9	0.500	0.499	0.0195	3.9	99.8	2 of 2
QC H	400	429	6.95	1.6	107.2	6 of 6
QC M	150	155	3.59	2.3	103.6	6 of 6
QC L	1.40	1.43	0.0287	2.0	102.2	6 of 6
QC LLOQ	0.500	0.512	0.0108	2.1	102.4	6 of 6

Table 3.30. LPV reinjection reproducibility: validation 1 reinjected after five days

Sample ID	Nominal Conc. (ng/mL)	Mean Observed Conc. (ng/mL)	STDEV	CV(%)	%Accuracy	n
S1	500	499	2.34	0.5	99.8	2 of 2
S2	350	356	14.4	4.1	101.6	2 of 2
S3	200	192	5.02	2.6	96.2	2 of 2
S4	50.0	52.5	2.04	3.9	105.0	2 of 2
S5	20.0	20.5	0.0579	0.3	102.4	2 of 2
S6	5.00	5.06	0.114	2.2	101.2	2 of 2
S7	2.50	2.60	0.00523	0.2	104.0	2 of 2
S8	1.00	0.982	0.0650	6.6	98.2	2 of 2
S9	0.500	0.458	0.0209	4.6	91.7	2 of 2
QC H	400	435	10.7	2.5	108.7	6 of 6
QC M	150	162	7.76	4.8	107.9	6 of 6
QC L	1.40	1.49	0.0341	2.3	106.6	6 of 6
QC LLOQ	0.500	0.524	0.0244	4.7	104.8	6 of 6

Table 3.31. RTV reinjection reproducibility: validation 1 reinjected after five days

Sample ID	Nominal Conc. (ng/mL)	Mean Observed Conc. (ng/mL)	STDEV	CV(%)	%Accuracy	n
S1	125	126	1.63	1.3	100.7	2 of 2
S2	87.5	86.5	1.91	2.2	98.8	2 of 2
S3	50.0	50.0	0.359	0.7	100.0	2 of 2
S4	12.5	12.8	0.562	4.4	102.4	2 of 2
S5	5.00	4.94	0.160	3.2	98.9	2 of 2
S6	1.25	1.24	0.00691	0.6	99.0	2 of 2
S7	0.625	0.610	0.0301	4.9	97.7	2 of 2
S8	0.250	0.260	0.00382	1.5	104.2	2 of 2
S9	0.125	0.123	0.00517	4.2	98.5	2 of 2
QC H	100	101	4.16	4.1	101.1	6 of 6
QC M	37.5	36.5	0.741	2.0	97.4	6 of 6
QC L	0.350	0.352	0.00710	2.0	100.5	6 of 6
QC LLOQ	0.125	0.124	0.00661	5.3	99.4	6 of 6

The accuracies of each standard and quality control from the reinjected batch fell within a range of 91.7–108.7%. All the calibration standards and quality control samples passed for EFV, LPV, and RTV. A batch is required to have at least 75% of its standards and 66.7% of its quality controls pass (with 50% at each level) to be considered valid.[108,109] The coefficients of variation for each concentration level were all equal to or less than 6.6%, which shows that the precision of the batch is still acceptable. Therefore, should it not be possible to inject the batch on the day of extraction or should instrument interruptions occur, the samples may be injected and analysed with confidence and accuracy up to five days after extraction, when stored in the autosampler at ~8°C.

3.11.6. AUTOSAMPLER STABILITY

Autosampler stability is assessed using the same results as for reinjection reproducibility, whereby the extracted samples from validation day 1 were left in the autosampler (at ~8°C) and reinjected after five days. However, instead of evaluating if the batch passes or fails as a whole, autosampler stability determines if a batch may be reinjected in part. The peak area ratios of the high and low quality control samples were compared to the results from the original injection to determine absolute autosampler stability.

The percentage differences after five days for the high and low quality controls were respectively -4.8% and -1.0% for EFV, 6.2% and -8.1% for LPV, and 9.8% and 14.9% for RTV (Tables 3.32–3.34). These meet the acceptance criteria such that the percentage differences are less than ±15%.

Table 3.32. Five days autosampler stability of EFV in extracted samples

	High Concentration		Low Concentration	
	Initial injection	**Reinjection**	**Initial injection**	**Reinjection**
Average Peak Area Ratio	13.0	12.4	0.0480	0.0475
n	6	6	6	6
STDEV	0.134	0.195	0.000896	0.000876
CV(%)	1.0	1.6	1.9	1.8
%Difference		-4.8		-1.0

Table 3.33. Five days autosampler stability of LPV in extracted samples

	High Concentration		Low Concentration	
	Initial injection	**Reinjection**	**Initial injection**	**Reinjection**
Average Peak Area Ratio	19.8	21.0	0.0910	0.0836
n	6	6	6	6
STDEV	0.802	0.459	0.00229	0.00203
CV(%)	4.1	2.2	2.5	2.4
%Difference		6.2		-8.1

Table 3.34. Five days autosampler stability of RTV in extracted samples

	High Concentration		Low Concentration	
	Initial injection	**Reinjection**	**Initial injection**	**Reinjection**
Average Peak Area Ratio	5.08	5.58	0.0182	0.0209
n	6	6	6	6
STDEV	0.184	0.200	0.000589	0.000414
CV(%)	3.6	3.6	3.2	2.0
%Difference		9.8		14.9

The autosampler stability for RTV at the low concentration just met acceptance criteria with reinjection peak area ratios 14.9% higher than the initial injection results. The observed increase is likely due to evaporation of the injection solution in the 96-well plate; however, the internal standard adequately compensates, and the FDA and EMA acceptance criteria are still met.[108,109] This indicates that batches may be reinjected in part after five days when stored in the autosampler at ~8°C. These findings are in agreement with those found by Ter Heine *et al.* (2009), who showed autosampler stability of EFV, as well as LPV and RTV, in extracted human PBMCs over a range of 1.00–500 ng/mL for four days.[101] Colombo *et al.* (2005) has shown autosampler stability of extracted PBMC samples for EFV, LPV, and RTV for 24 hours[98] while other PBMC research groups have either referenced

autosampler stability of EFV, LPV, and RTV determined from plasma methods[122] or have not tested or referenced autosampler stability at all.[24,76,119]

4. CHAPTER CONCLUSIONS

This chapter describes the development and validation of a method for the quantification of intracellular EFV, LPV, and RTV from human PBMCs. The method uses BD Vacutainer cell preparations tubes to quickly and easily isolate the cells from whole blood, followed by SPE of the cell lysate and HPLC-MS/MS analysis.

During the bioanalytical method validation, the method was rigorously tested as per the FDA and EMA guidelines and was shown to be robust, accurate, and precise over a range of 0.500–500 ng/mL of lysate for EFV and LPV and 0.125–125 ng/mL of lysate for RTV. The ranges were reported in nanograms per millilitre of lysate as opposed to nanograms per million cells, as this would simplify future calculations for patient intracellular concentrations. Conversion between the concentration in lysate and intracellular concentration requires the mean cell volume of the sample, which can differ depending on the literature cited. Using referenced volumes has been shown to be an overestimation of patient cell volumes resulting in an underestimated intracellular drug concentration. By validating the method using the concentration in lysate, it allows for the principal investigator of a study to choose their preferred cited cell volume or for the cell volume of each study sample to be individually measured and used in the calculation for more accurate results.

Endogenous matrix components were found to have no adverse effects on the reproducibility of the method when PBMCs originating from six different sources of whole blood were analysed. Recovery was greater than 85% and reproducible for all three analytes. Five-fold (1:4) dilutions were valid for the analytes when diluted with blank lysate. No significant carry-over of EFV, LPV, or RTV were observed with the autosampler, and no crosstalk was observed between the analytes and their respective internal standards.

Various stability experiments were performed in cell lysate, but unfortunately, the stability of the analytes within the cells prior to cell lysis could not be evaluated without the use of incurred patient samples and could only be inferred from the stability of the analytes in solvent. All three analytes were stable in cell lysate at room temperature for up to ~5 hours and when subjected to three freeze-thaw cycles. Autosampler stability and reinjection reproducibility were shown for five days for EFV, LPV, and RTV when stored in the autosampler at approximately 8°C.

The assay method and all validation data presented in this chapter have been submitted to the CPQA group (NAIADS/DAIDS)[112] for peer review and have been approved for use on human samples in clinical applications. As of February 2022, this assay method is the only CPQA-approved assay in Africa

for the quantification of intracellular EFV, LPV, and RTV.[127] The approval documentation can be found in Appendix J. A clinical application of this method is discussed in Chapter Four, in conjunction with the method that was described in Chapter Two.

CHAPTER FOUR: CLINICAL APPLICATION OF ASSAY METHODS

1. CHAPTER SUMMARY

Sub-Saharan Africa has the highest prevalence of HIV infection worldwide.[11] In South Africa, 19.1% of adults aged 15–49 years are HIV-positive.[3] Of these, 71% of HIV-positive adults and 47% of HIV-positive children in South Africa are on antiretroviral therapy.[11] Since the mid-1980s, when HIV was causatively linked to AIDS, there have been more drugs approved by the FDA for the treatment of HIV than for all other viral infections combined.[12] This thesis has focused on three of these ARVs: EFV, LPV, and RTV.

EFV is an NNRTI that formed part of the first-line treatment for newly diagnosed patients until 2020. While dolutegravir has replaced EFV as the preferred first-line treatment, EFV is still widely used in South Africa.[19,31] LPV is a protease inhibitor that is co-formulated with RTV as a fixed combination (LPV/r) used in the second-line treatment of HIV. *In vivo,* LPV undergoes hepatic metabolism and usually has a high extraction ratio, but RTV inhibits the CYP3A4 enzyme that metabolizes LPV, thereby increasing the bioavailability of LPV and lowering its hepatic extraction ratio.[35] RTV also inhibits the drug efflux pump P-glycoprotein, of which LPV is a substrate, further increasing the bioavailability of LPV.[153]

Chapter Two describes the development and validation of a method to determine the unbound drug concentrations in plasma of EFV, LPV, and RTV. For drugs that are less than 80% bound to plasma proteins, a change in the protein concentration to which the drug binds is considered to have no clinically significant effect on unbound concentrations.[58] However, drugs with a higher percentage binding are susceptible to dramatic fluctuations in unbound drug concentrations caused by small changes in the concentrations of the proteins. Disease, infections, and pregnancy can markedly perturb the concentrations of AAG and/or albumin and, as a result, the protein binding capacity of blood plasma.[43,59,60] EFV, LPV, and RTV are highly plasma-protein bound (>98%) to albumin and AAG and have large inter-patient variability. Sub-therapeutic concentrations can lead to treatment failure and drug resistance, while too high levels can cause adverse effects.[26,77]

In healthy patients, the total drug concentration can be measured, and the data adjusted using the known percentage plasma-protein binding, clearance, and volume of distribution of the drug. However, during pregnancy and in patients with hypoalbuminemia caused by malnourishment or nephrotic syndrome, for example, this becomes much harder to model, and the total drug concentration may not be an accurate representation of the pharmacologically-active drug.[44,58] In this case, the most accurate way to monitor and adjust the patient's dose is to quantify the unbound drug concentration.[57]

Alternatively, it has been suggested that the variability in treatment response to ART may be due to the variability in the amount of drug reaching the target site within the HIV-infected cells, and as a result, intracellular drug concentrations should be measured in patients failing treatment.[77] A review by Bazzoli *et al.* (2010) summarising studies focusing on intracellular ARV concentrations found that, while most of the studies also measured plasma concentrations, only 8 out of 31 did any statistical analysis to determine if these concentrations were correlated.[86] However, none of these studies focused on South African populations. Genetic diversity is highest in people of African ancestry with respect to drug response, and therefore, these findings might not reflect a South African population.[89,90] Chapter Three describes the development and validation of a method to determine the intracellular drug levels of EFV, LPV, and RTV in PBMCs.

This chapter aims to act as a proof-of-concept study for the assays described in Chapters Two and Three by comparing the plasma total, plasma unbound, and intracellular concentrations of EFV and LPV in HIV-positive patients. Plasma and PBMC samples were collected from remnant whole blood submitted for therapeutic drug monitoring of EFV from six patients, while plasma and PBMCs from a single patient currently receiving LPV-based therapy were collected at various time points.

The unbound and intracellular assays, when used in conjunction with the routine TDM assay for total plasma analysis, provided a tool for physicians to evaluate the relationship between total, unbound, and intracellular concentrations in a complicated patient. The clinical application supports the use of the assays as a form of individualized medicine in future patients who are not responding to treatment or are presenting with side effects that the TDM results alone cannot explain.

2. PROOF-OF-CONCEPT STUDY

To evaluate the validity and feasibility of the methods developed in Chapters Two and Three, a proof-of-concept study was implemented. The study made use of remnant whole blood from samples submitted to the TDM laboratory within the Division of Clinical Pharmacology for monitoring of EFV or LPV/RTV. The use of these remnant samples did not impact the TDM service nor deliver additional results to the requesting clinician. Ethical approval was granted by the UCT Human Research Ethics Committee (HREC #259/2021, Appendix K).

2.1. METHODOLOGY

2.1.1. SAMPLE COLLECTION

Samples were collected over a period of two months with the following inclusion and exclusion criteria:

Inclusion criteria:

- Samples from adults (over the age of 18 years old)
- Samples from patients currently receiving EFV or LPV/r therapy
- Samples from patients providing whole blood to Groote Schuur Hospital for routine TDM purposes

Exclusion criteria:

- Samples from children under 18 years of age
- Samples sent to the TDM laboratory as fresh or frozen plasma

The chosen maximum sample size was small, as the purpose of the study was to evaluate the assay applicability in HIV-positive clinical samples. It was not possible to perform this proof-of-concept study using laboratory spiked samples as the spiked drug concentrations will not distribute into the cells biologically after spiking *in vitro* and, therefore, the use of samples from patients on ART was required. The study was not designed to answer specific questions about the effects or impact of the chosen drugs, nor was extensive statistical analysis performed. The relationship between the total, unbound, and intracellular concentrations were evaluated using correlation coefficients.

A detailed data safety and monitoring plan was not required, as the study was not designed to answer specific questions about the chosen drugs, nor did it include any behavioural or psychosocial interventions. Likewise, as there was no recruitment or enrolment of participants, no informed consent or reimbursement was required.

2.1.2. SAMPLE PROCESSING

Whole blood samples collected into EDTA-lined tubes were received by the TDM laboratory and processed according to the flow diagram shown in Figure 4.1.

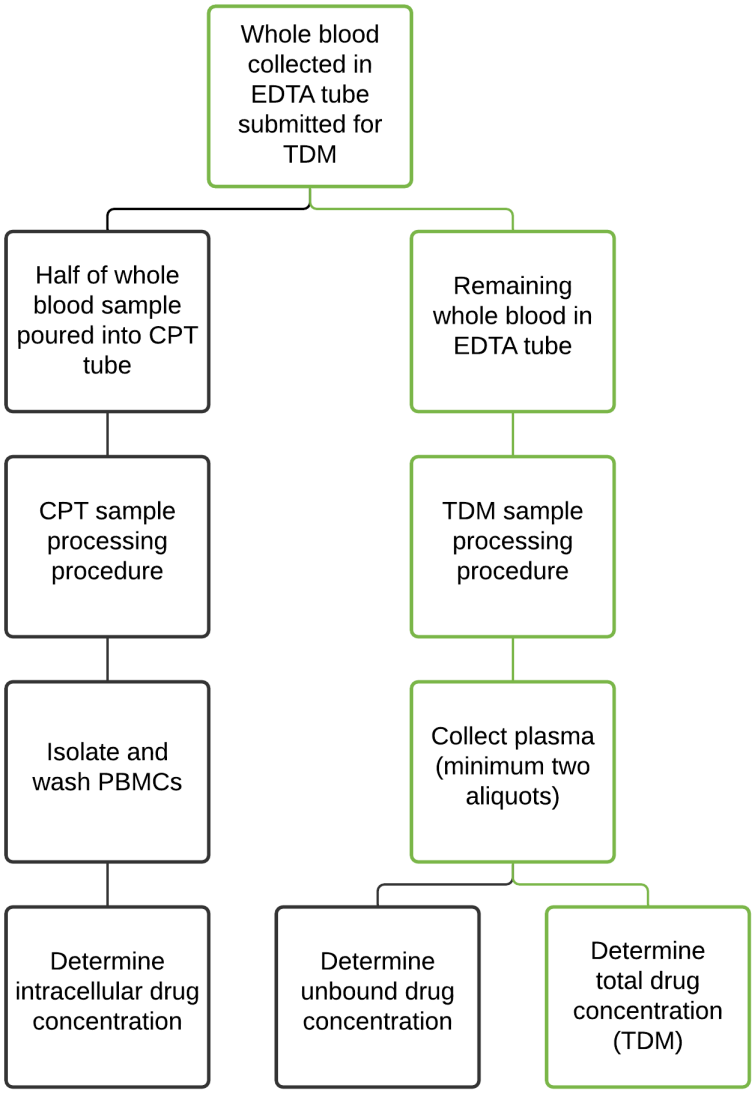

Figure 4.1. Whole blood sample processing procedure. The pathway in green shows the uninterrupted flow of samples analysed for TDM purposes.

Upon receipt, half of the whole blood was immediately poured off into a BD Vacutainer CPT tube. The remaining whole blood was left in the EDTA-lined tube and processed as per the TDM procedure (centrifuged at 1500–2000 rcf for 10 minutes) to collect the plasma. This plasma was stored at ~-80°C as at least two aliquots (for total and unbound drug analysis) as the unbound drug assay required plasma that had not been thawed and refrozen. The CPT tube was processed in parallel as per the PBMC isolation procedure described in Chapter Three, Section 2.2. Cell pellets of approximately one million PBMCs were isolated, washed, and stored at ~-80°C within two hours of sample receipt.

During sample processing, a laboratory ID was assigned to each sample and subsequent aliquots. Only the laboratory ID was recorded on the aliquots to pair the EDTA tube and BD Vacutainer CPT tube for each sample. The patient's name or any other identifiable information was not made available.

Over a period of two months, ten samples met the inclusion criteria for EFV analysis. One sample could not be processed for analysis in PBMCs due to insufficient volume transferred into the CPT tube. Prior to sample analysis, nine paired plasma and PBMC samples were collected for EFV analysis. Of these, three were found to be BLOQ for plasma total EFV (<0.0195 µg/mL) when analysed for TDM purposes and were subsequently not analysed for unbound or intracellular EFV concentrations, resulting in a final sample size of six paired plasma and PBMC samples. For the purposes of providing proof-of-concept for the assay methods described in Chapters Two and Three of this thesis, a sample size of six was deemed sufficient.

No whole blood samples were received for monitoring of LPV/r during this period. The case study discussed in Section 3 of this chapter centred around LPV/r analysis and, therefore, it was decided not to extend the collection period of TDM samples in the hope that LPV/r samples would arrive but rather proceed with only the EFV samples.

2.1.3. SAMPLE ANALYSIS

Plasma sample analysis for the total EFV concentrations was performed within the routine TDM laboratory, whereby 10 µL of plasma was extracted by protein precipitation with 120 µL of acetonitrile containing 200 ng/mL EFV-d5. The supernatant is dried under nitrogen gas at ~40°C and reconstituted in 100 µL of a mixture of acetonitrile and 0.1% formic acid in water (1:1, v/v), followed by HPLC-MS/MS analysis. Concentrations of EFV were reportable within a validated range of 0.0195–20.0 µg/mL.

Samples with total concentrations less than 0.0195 µg/mL were not analysed for unbound or intracellular EFV.

The second aliquot of plasma was ultracentrifuged and extracted according to the method described in Chapter Two, Section 2.4, to determine the unbound EFV concentrations. The PBMC pellets containing approximately one million cells were lysed and extracted according to the method described in Chapter Three, Section 2.4. The resulting concentration of EFV in lysate was converted to the intracellular concentration using the following formula:

$$\text{Intracellular concentration} = \frac{\text{lysate concentration x lysate volume}}{\text{cell number x mean cell volume}}$$

This conversion was performed using two reported values for the mean cell volume (MCV) to investigate the effect of assumed MCV on the interpretation of the results. The first MCV value being 400 fL which is the most commonly used value in literature.[76–78,92–101] The second MCV used in the conversion of these data was 282 fL, which is closer to Gao's "inactivated" cell volume. Studies by D'Avolio *et al.* (2011) measuring the MCV from 20 and 86 HIV-positive participants reported mean values of 281.2 fL and 282.5 fL, respectively.[122] Similarly, Simiele *et al.* (2011) reported a mean volume of 282.9 fL from 190 HIV-positive participants.[79]

2.2. RESULTS AND DISCUSSION

The resulting intracellular, plasma total, and unbound EFV concentrations of each TDM sample are presented in Table 4.1.

Table 4.1. Plasma total, unbound, and intracellular concentrations of EFV and subsequent percentage protein binding and intracellular accumulation ratios

Patient	Total plasma conc. (µg/mL)	Unbound plasma conc. (µg/mL)	Intracellular conc. (µg/mL) 400 fL MCV	Intracellular conc. (µg/mL) 282 fL MCV	Plasma-protein binding (%)	Intracellular: total ratio (400 fL MCV)
TDM 1	ALOQ (>20.0)	0.203	3.24	4.59	99.0*	0.162*
TDM 2	9.07	0.379	3.84	5.44	95.8	0.423
TDM 3	1.74	0.0167	0.574	0.814	99.0	0.330
TDM 4	6.14	0.146	5.14	7.29	97.6	0.837
TDM 5	3.22	0.411	8.60	12.2	87.2	2.67
TDM 6	0.452	0.0797	1.81	2.57	82.4	4.01

* 20.0 µg/mL was used as the total plasma concentration in these calculations.

TDM 1 had a total plasma concentration above the upper limit of quantification (ALOQ) of 20.0 µg/mL and was excluded from the correlation calculations (Figure 4.2 a and b) as it is TDM practice to report such samples as “ALOQ" and not dilute and reanalyse. The wide range of total EFV concentrations (0.452 to >20.0 µg/mL) agrees with the large inter-patient CV(%) of 118% reported in literature.[26] This variability could also be due to different dosages or different times of sampling after dose; however, no information about dose or sampling times was available.

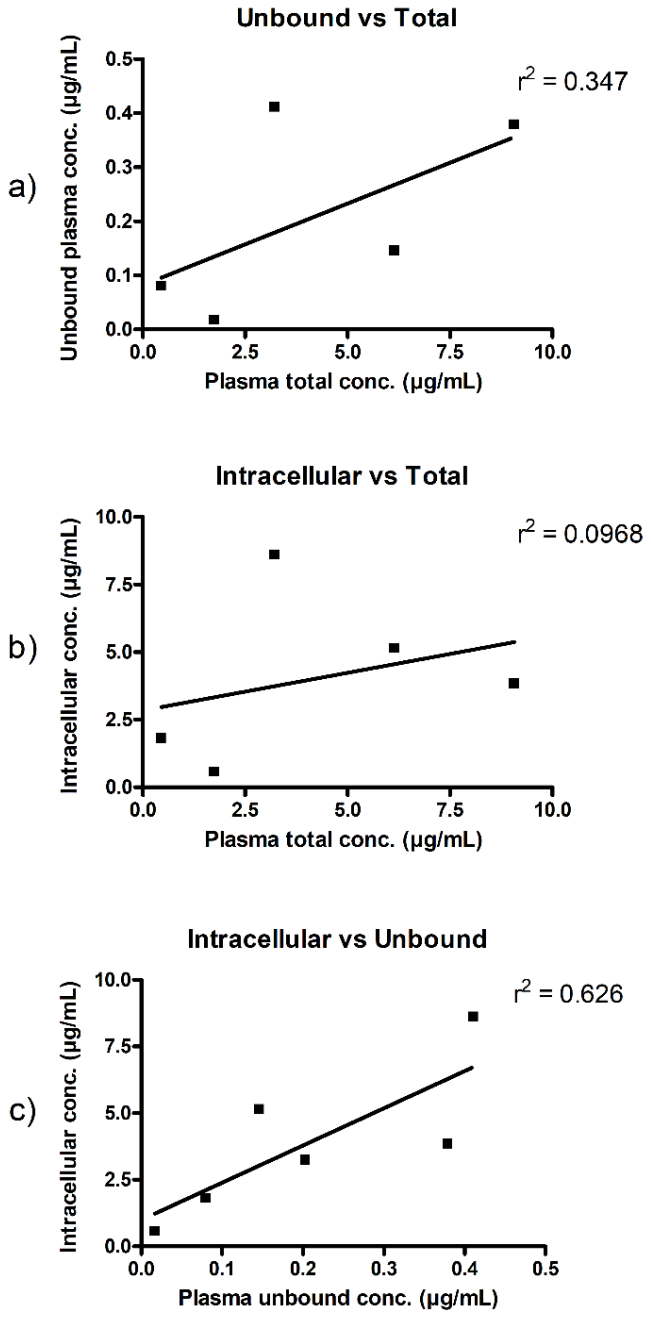

Figure 4.2. Graphs showing the correlation between a) unbound plasma and total plasma concentrations, b) intracellular, and total plasma concentrations, and c) intracellular and unbound plasma concentrations of EFV from remnant whole blood samples collected for TDM purposes from adults currently receiving EFV. *Note: an MCV of 400 fL was used for the intracellular concentrations.*

The unbound EFV concentrations (range 16.7–411 ng/mL) had a weak correlation with the total plasma levels with a correlation factor (r^2) of 0.347, as shown in Figure 4.2a. Four of the six patients had calculated percentage protein bindings of 98% (CV(%) = 1.5%) while TDM 5 and 6 had 87% and 82% protein-bound EFV, respectively. The lower percentage of protein binding observed with TDM 5 and 6 could be due to the patients' disease state causing different protein profiles as EFV is reported to be 86% bound to albumin in the absence of other plasma proteins,[154] however no information on the patients' protein profile was available. A study by Fayet *et al.* (2008) found that EFV protein-binding was consistent over a dosing interval, and so, any differences in time of sampling after dose is unlikely to be the cause of these low protein-binding results.[52]

As shown in Figure 4.2b, the intracellular EFV concentrations (range 0.574–8.60 µg/mL, assuming an MCV of 400 fL) did not correlate with the total plasma concentrations at all, with an r^2 value of 0.0968. Patients TDM 1–4 had intracellular concentrations lower than the total plasma concentrations (mean intracellular: plasma accumulation ratio of 0.438 ± 0.287), while patients TDM 5 and 6 had intracellular concentrations greater than the respective plasma concentrations (mean intracellular: plasma accumulation ratio of 3.34 ± 0.947) which suggests that the total plasma concentrations do not accurately represent the EFV levels at the target site within the cells. The high variability of EFV intracellular accumulation ratios is not surprising as a large inter-patient accumulation ratio CV(%) of 78% has been reported in literature.[78]

Using the 400 fL MCV value in the calculation can underestimate the intracellular concentration by a factor of 0.7 compared to the 282 fL value. For five of the six TDM samples, the use of either MCV value did not change the pattern of intracellular EFV compared to total plasma EFV (Table 4.1). For TDM 4, however, using an MCV value of 282 fL instead of 400 fL changed the intracellular: plasma total accumulation ratio from 0.837 to 1.19. This highlights a potential issue. Should an intracellular concentration be calculated using the 400 fL value and be reported as below the desired concentration, a physician may decide to increase the dose when it is not necessary. While the 252 fL value may also not be the true MCV value, the discrepancy supports the need for measuring the MCV in specific patients or populations and warrants further investigation.

The unbound plasma concentrations and intracellular concentrations had the strongest correlation (Figure 4.1c, $r^2 = 0.626$), which supports the theory that only the unbound drug is able to enter the cells. This agrees with a study by Tanaka *et al.* (2008) in which a strong correlation factor ($r^2 = 0.76$) was reported to exist between unbound and intracellular EFV concentrations. They reported on EFV concentrations from samples of 13 HIV-positive Japanese males and could also indicate a relatively

high correlation (r^2 = 0.66) between total EFV plasma concentrations with both unbound and intracellular concentrations. Another study correlating the total, unbound, and intracellular EFV concentrations in 10 British HIV-positive adults (6 males and 4 females; 7 Caucasian and 3 Black) reported conflicting findings. They found no correlation between the unbound and intracellular EFV concentrations (r^2 = 0.13) while the total and intracellular concentrations had the strongest correlation (r^2 = 0.59).[24] The relatively poor correlations described in this chapter (total plasma concentration to unbound and intracellular concentrations) may be due to the smaller sample size or other factors such as disease state, dose, time of last dose etc.

Perhaps most interestingly, there appears to be a strong inverse relationship (r^2 = 0.968) between the percentage protein binding and the intracellular accumulation ratio, as shown in Figure 4.3.

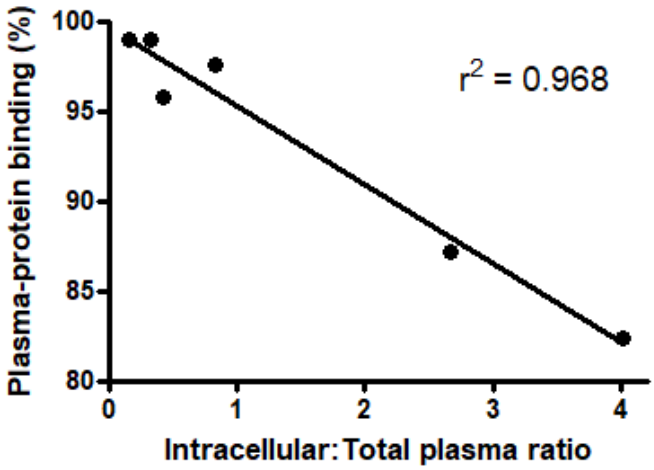

Figure 4.3. Graph showing the inverse linear relationship between the percentage plasma-protein binding and the intracellular accumulation ratio of EFV.

This indicates that the lower the percentage of protein binding, the more unbound drug is available to enter and accumulate inside the cells, which highly supports the free-drug theory. While the unbound *versus* intracellular concentration results were well correlated (r^2 = 0.626) and, when the total plasma concentrations are also taken into account when comparing the unbound and intracellular drug concentrations, the correlation becomes much stronger (r^2 = 0.968). This shows how closely the three compartments (total, unbound, and intracellular) are linked despite the small sample size and lack of controlled dosing and sampling conditions.

The strong correlation also implies that it may be possible to develop a mathematical model to predict intracellular EFV concentrations given the plasma total and unbound concentrations. If feasible, such a model could be useful for clinicians as the intracellular concentrations are the most clinically relevant, and yet plasma is much easier to collect and process compared to PBMCs. However, a study

with a much larger sample size and more controlled conditions would be required to investigate if such a model is possible.

3. LPV/R CASE STUDY

In 2020, the Department of Immunology of the University of Pretoria approached the UCT Division of Clinical Pharmacology about a complicated case. A 50-year-old female (hereafter referred to as the Pretoria Patient) tested positive for HIV in 2005 and, despite various ART regimens, has never been virally suppressed. According to her physicians, she is an adherent patient who understands her illness, never misses a clinic visit, and is not taking any other medications or herbs that are known to interfere with the absorption and metabolism of the drugs – with routine TDM levels to support this. Furthermore, drug resistance tests performed in 2012, 2016, and 2020 all showed no resistance. Table 4.2 shows her history of regimens, viraemias, and CD4 cell counts.

Table 4.2. ART regimens, viraemias, and CD4 cell counts of the Pretoria Patient

Date	ART Regimen*	Viraemia (copies/mL)	CD4 Cell Count (cells/µL)	CD4%
2010	-	-	1 565	-
2011	TDF, FTC, EFV	-	-	-
2015	AZT, 3TC, LPV/r	-	-	-
May 2016		67 852	10 897	87.5
Nov 2016		59 856	16 303	83.7
May 2017	3TC "holding regimen"	85 200	12 000	79.0
Aug 2017		346 000	11 644	79.5
Jan 2018		627 000	16 203	86.3
July 2018		279 749	-	-
Mar 2019	ABC, 3TC, LPV/r	270 145	-	-
Dec 2019		819 637	14 362	75.7
Apr 2020	AZT, 3TC, LPV/r	1 323 020	21 116	78.2

***** Tenofovir (TDF), emtricitabine (FTC), zidovudine (AZT), lamivudine (3TC), abacavir (ABC)

A viraemia of <50 copies/mL is considered virally suppressed, while >1000 copies/mL is indicative of virologic failure (due to either poor adherence or drug resistance).[155,156] A CD4 count of approximately 500–1500 cells/µL is expected for healthy and HIV-controlled adults, whereas HIV-uncontrolled adults tend to have CD4 counts of <500 cells/µL.[155,157] The CD4% refers to the percentage of lymphocytes that are CD4 cells. Typically, HIV-negative adults will have a CD4 percentage of around 40% compared to 25% or less for uncontrolled HIV-positive individuals.[158] The CD4% value helps contextualize the CD4 count absolute values. For instance, the CD4 count could increase over time, but if the percentage remains the same, this means that the patient's total immune cell count is increasing and not the CD4 count specifically. As shown in Table 4.2, the patient's CD4 cell count and HIV viraemia have increased

over the course of treatment with levels outside of the normal range. The CD4 percentage has remained ~80% over the years, which is double the normal ratio of healthy adults. As of 2020, the patient had a CD4 count approximately 200 times the norm and a viraemia of greater than a million copies/mL.

The Pretoria Patient is also positive for Human T-lymphotropic Virus (HTLV), which, when co-infected with HIV, results in higher CD4 counts[159] and increased HIV transcription[160], which could potentially explain the unusually high CD4 count and viraemia. A similar case was reported by Nasreddine *et al.* (2019), where an HIV-1/HLTV-1 co-infected male failed to respond to ART.[161] The paper reported a CD4 count of 21 000 cells/µL and a viraemia of 13 400 copies/mL, which did not improve even when Direct Observed Therapy was initiated and genotyping indicated no drug resistance. Only once the male patient was treated for HTLV-associated Adult T-cell Leukemia (ATL) could the HIV viral load be controlled, which suggested the HTLV-associated ATL was linked to the HIV treatment failure. The authors proposed that the HTLV could be causing an increased expression of the p-glycoprotein efflux transporters, which could be removing the ARVs from the cells, thereby decreasing the accumulation of the drugs at their target sites.

The Pretoria Patient has not been diagnosed with ATL – with skin biopsies, lumbar punctures, and bone marrow aspirates all being non-conclusive. However, ART failure due to an HTLV-induced overexpression of the drug efflux transporters was still a valid hypothesis. While RTV is known to inhibit the drug efflux pump p-glycoprotein[153], it is possible that the levels of RTV reaching the cells are too low to inhibit all the efflux transporters if they are highly over-expressed.

To test this hypothesis, plasma and PBMC samples were collected to determine the intracellular LPV concentrations compared to the respective plasma concentrations.

3.1. METHODOLOGY

3.1.1. SAMPLE COLLECTION AND PROCESSING

Collection and processing of the whole blood samples were performed by the Department of Immunology at the University of Pretoria. Informed consent was given for the intracellular, plasma total, and plasma unbound analysis of LPV to be used in this study (see Appendix L).

Whole blood was drawn into CPT and EDTA-lined tubes at the time points listed in Table 4.3. At each time point, the EDTA-lined tube was centrifuged at 1500–2000 rcf for 10 minutes to collect the plasma, which was separated into multiple aliquots of 250 µL and stored at ~-80°C. At the same time, each CPT tube was processed for PBMC isolation as described in Chapter Three, Section 2.2. Cell pellets of approximately one million PBMCs were isolated, washed, and stored at ~-80°C within two hours of each blood sampling.

Table 4.3. Timing of blood sampling and sample collection

Time after dose (hours)	Samples collected
0 (15 min pre-dose)	Plasma and PBMCs
1	Plasma and PBMCs
2	Plasma and PBMCs
4	Plasma only (CPT broke during processing)
6	Plasma and PBMCs
7	Plasma and PBMCs

The plasma and PBMC samples were transported on dry ice at ~-80°C to the UCT Division of Clinical Pharmacology for analysis.

3.1.2. SAMPLE ANALYSIS

Analysis of the Pretoria Patient's samples for LPV and RTV was performed together with the EFV samples collected during the proof-of-concept study described in Section 2.1.3. Briefly, the total plasma concentrations were determined within the TDM laboratory using the routine TDM assay, while the plasma unbound and intracellular concentrations were determined using the methods developed in Chapters Two and Three, respectively.

The resulting concentrations of LPV and RTV in lysate were converted to the intracellular concentrations using the same formula indicated in Section 2.1.3. Unlike the EFV proof-of-concept study, this conversion was only performed using the 400 fL MCV value as this allowed for direct comparison with intracellular LPV/r concentrations reported in the literature.[76,77]

3.2. RESULTS AND DISCUSSION

The resulting intracellular, plasma total and unbound LPV concentrations of each time point are presented in Figure 4.4.

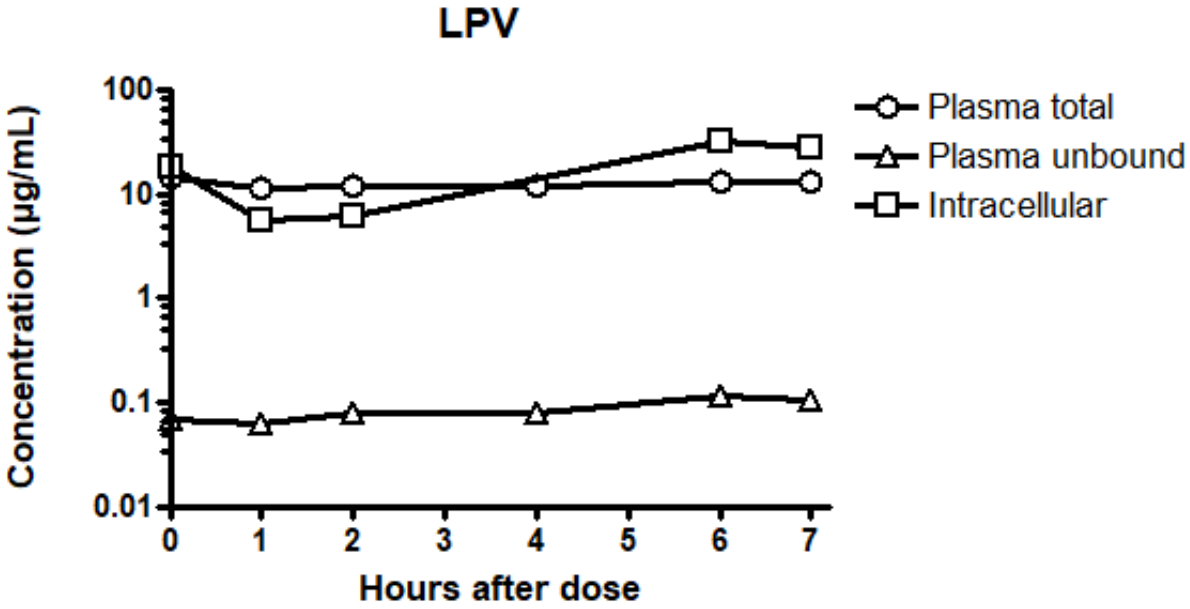

Figure 4.4. LPV total plasma concentration, unbound plasma concentration, and intracellular concentration vs time profile in the Pretoria Patient after a single oral dose of Kaletra® 400 mg LPV/100 mg RTV. Time 0 hours represents 15 minutes pre-dose. (Note: 4 hours after dose does not have an intracellular concentration as the CPT tube broke during sample collection).

The intracellular LPV concentrations (range 5.73–33.0 µg/mL) are comparable with the total plasma concentrations (range 11.2–14.5 µg/mL) with a correlation factor (r^2) of 0.808, although the intracellular concentrations were more variable over time. The unbound LPV plasma concentrations (range 63.5–117 ng/mL) correlated with the total plasma concentrations with a factor of 0.891 and equated to a mean plasma protein binding of 99.3% (CV(%) = 0.2), which is in agreement with literature.[36,76,114] The unbound plasma and intracellular concentrations had a correlation factor of 0.792. It is interesting that the unbound and intracellular results had the lowest correlation factor when it is thought that the unbound drug concentrations would correlate better than the total plasma concentrations.[55] However, all three correlations were strong considering the small number of data points.

Figure 4.5 shows two figures appended from studies in healthy volunteers showing LPV intracellular and plasma profiles.[76,77] In both cases, the intracellular concentrations are within a similar range as the total plasma concentrations, similar to the data in Figure 4.4. However, the Pretoria Patient's

results differ in two significant aspects. Firstly, the concentrations in the Pretoria Patient are much higher than those in Figure 4.5 despite the same given dose of LPV and secondly, the concentration-time profile is a flat line which does not show the expected decline between two and seven hours post-dose. Slow or low clearance of the drug could potentially explain the high concentrations observed (even pre-dose when one would expect low concentrations), which could also explain the flat profile; however, further investigations would be required, which are not within the scope of this study.

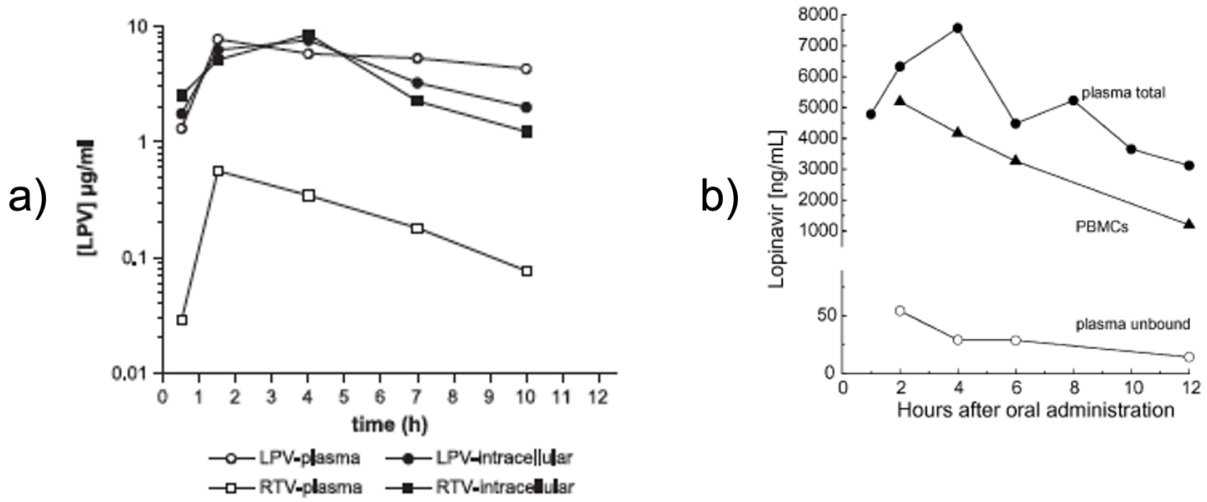

Figure 4.5. LPV plasma and intracellular concentration-time profiles after a single oral dose (Kaletra® 400 mg LPV/100 mg RTV) each in a healthy volunteer amended from a) Azoulay et al. (2004) and b) Ehrhardt et al. (2007).

The data in Figures 4.4 and 4.5 confirm that the intracellular concentration of LPV in the Pretoria Patient reaches the therapeutic levels within the cells. Therefore, the high viraemia values are likely not due to the lack of LPV at the target site. While these findings do not explain the cause of the virological failure in this patient, they do eliminate a suspected possible cause. It would have been potentially useful to know the plasma and intracellular concentrations of the other ARVs in the treatment regimen – zidovudine and lamivudine – to determine if all the drugs were reaching the target site; however, such methods are not available in our laboratory at present. Unfortunately, any further investigation into this complicated case is not within the scope of this thesis and will be left to the patient's primary physicians.

4. CHAPTER CONCLUSIONS

The methods developed in Chapters Two and Three were applied to analyse paired plasma and PBMC samples from six patients receiving EFV therapy and one patient receiving LPV/r therapy. The proof-of-concept study and the Pretoria Patient case report described in this chapter show the feasibility and usefulness of these methods in clinical settings.

The correlations between total, unbound, and intracellular LPV concentrations were strong ($r^2 \geq 0.792$) within a single individual over time, as shown by the Pretoria patient. However, the Pretoria patient was an unusual case, and these results may not be generalisable for other patients receiving LPV therapy. The EFV data show weaker correlations between the three physiological compartments (total, unbound, and intracellular), although this could be because the data were from six separate individuals where a high inter-patient variability is expected. However, when the EFV unbound, and intracellular concentrations are normalised to the total plasma concentrations and are compared as percentage protein binding and intracellular accumulation ratios, the correlation is very close to one with an inverse linear relationship ($r^2 = 0.968$). These results support the free-drug theory whereby a lower percentage binding to plasma proteins results in a higher degree of unbound drug available to diffuse into the cells, resulting in a higher intracellular accumulation ratio. This warrants investigation into the possibility of a mathematical model to predict intracellular EFV concentrations from plasma total and unbound concentrations.

A limitation of intracellular and unbound drug work that has been noted in literature is the large volume of whole blood required, which makes sampling over multiple time points difficult.[10] The EFV proof-of-concept study shows how total, unbound, and intracellular concentrations can be determined from a single 8 mL blood sample, which would make PK sampling over multiple time points feasible.

Overall, these results show the validity and feasibility of the methods developed during this thesis for the quantification of unbound and intracellular concentrations of EFV and LPV, which could be used in individualised medicine when patients are not responding to treatment or having side effects that cannot be explained by the routine drug monitoring results. The assays may also be used in future studies and clinical trials which require CQPA-approved methods.

CHAPTER FIVE: CONCLUSIONS

1. THESIS SUMMARY

While there are many published methods for the determination of unbound or intracellular concentrations of EFV, LPV, and RTV, there are very few studies that analyse both fractions in the same patient, and no published studies have quantified paired total, unbound, and intracellular concentrations in South African HIV-positive patients using assays validated according to the latest FDA, EMA, and CPQA guidelines.[108,109,112]

The method described in Chapter Two appears to be the first fully validated method according to FDA and EMA guidelines for the quantification of unbound EFV, LPV, and RTV using ultracentrifugation. This assay has been peer-reviewed and approved by CPQA. This assay has also added to the field of unbound drug analysis an alternative surrogate matrix which was shown to be an accurate replacement of ultracentrifuged plasma to be used for method development, calibration standards, and quality control samples.

An earlier method for intracellular EFV, LPV, and RTV has been used by the UCT Division of Clinical Pharmacology laboratory; however, this method was not fully validated and failed to meet current FDA, EMA, and CPQA guidelines. The intracellular assay described in Chapter Three has been shown to be more accurate, precise, and robust than the previous method. The new method is also the first and only validated method in South Africa for the quantification of intracellular EFV, LPV, and RTV that has been peer-reviewed and approved by CPQA.

The proof-of-concept study described in Chapter Four shows the utility of the assays in a clinical setting. The unbound and intracellular assays may be used in conjunction with the routine TDM assay for total plasma analysis as a tool to evaluate the relationship between total, unbound, and intracellular concentrations in individual patients. The total, unbound, and intracellular relationship tool may be applied to patients who are not responding to treatment or are presenting side effects that the TDM results alone cannot explain as a form of individualized medicine. The fact that PBMC and plasma samples could be collected from a single blood collection tube taken for the purposes of

TDM, without disrupting the routine TDM procedure is especially promising – as clinicians may request all three assays without additional blood draws.

Ultimately, the research aims and objectives were met. Bioanalytical methods were developed and validated according to the latest FDA and EMA guidelines for the quantitative determination of unbound EFV, LPV, and RTV from human plasma and for the quantitative determination of intracellular EFV, LPV, and RTV from human PBMCs. The methods were successfully used for the analysis of paired plasma and PBMC samples from HIV-positive patients. This demonstrated the ease with which a clinician could have unbound and intracellular results, in addition to the standard TDM results, to help manage patient treatment and support their application in individualized medicine for complicated patients.

2. LIMITATIONS

One of the limitations of the intracellular method validation was the lack of incurred samples. Without incurred samples, where multiple aliquots exist of the same sample, it was impossible to do stability testing of intracellular concentrations prior to cell lysis. All long-term or freeze-thaw stability testing, therefore, had to be performed in cell lysate, which cannot give the absolute stability of the analytes in matrix. The proof-of-concept study discussed in Chapter Four used a small volume of whole blood to collect the PBMCs samples, as half the blood volume was needed to collect plasma. Of the final six EFV-containing TDM samples, only two had sufficient cell volume for more than one aliquot of one million cells and therefore could not be used to perform long-term or freeze-thaw testing as both tests require analysis in six-fold.

A second limitation of the intracellular method was the inability to measure the MCV of each patient sample. This meant that the MCV value had to be assumed as 400 fL or 282 fL as reported in the literature, which may not have accurately reflected the true cell volume of the study population as neither reported values are from South African HIV-positive patients.

The focus of this doctorate was on developing and validating bioanalytical methods to be used in a clinical laboratory. The proof-of-concept study showed the feasibility of the methods in a clinical setting. However, the small sample size did not allow for extensive statistical analysis on the relationship between the plasma total, unbound, and intracellular concentrations of the three ARVs and, therefore, did not add statistically significant information to the larger scientific knowledge base

about EFV, LPV, or RTV. In addition, the Pretoria patient was an unusual case and the resulting correlations between total, unbound, and intracellular LPV may not be generalisable for other patients receiving LPV therapy.

Since 2017, dolutegravir has been replacing EFV in South Africa's first- and second-line ARV combination therapies as it is cheaper, has fewer side effects, and is less likely to lead to drug resistance, according to the WHO.[31] While EFV is still widely used in South Africa, the usefulness of including EFV in the methods is likely to reduce over time with the decreased use of the drug. For the methods to be used to their full potential in South Africa, it may be prudent to include dolutegravir and other commonly prescribed ARVs such as emtricitabine and tenofovir in the methods in future.

3. CONTRIBUTIONS

The methods described in this thesis have been used to analyse samples from two clinical trials. The method to determine unbound LPV concentrations in human plasma was used to analyse plasma samples as part of a secondary objective of the P1092 phase IV study by the International Maternal Paediatric Adolescent AIDS Clinical Trials Network (IMPAACT) intended to evaluate the steady-state pharmacokinetics of zidovudine, lamivudine, and LPV/r in severely malnourished HIV-1 infected children.

The method to determine intracellular LPV concentrations was used to analyse PBMC samples as a secondary objective in the DATiC study, a pharmacokinetic study investigating the dosing of ARVs and first-line TB drugs in children.

3.1. CONFERENCE PROCEEDINGS

The validation data of the method described in Chapter Two was presented at the 2018 South African Society of Basic and Clinical Pharmacology (SASBCP) conference held in Pretoria, South Africa. The presentation entitled "*Determination of unbound concentrations of efavirenz, lopinavir, and ritonavir in human plasma using ultracentrifugation and LC-MS/MS*", was awarded first place for basic pharmacology podium presentation in the Young Scientist Award competition.

4. FUTURE PROSPECTS

The PK Laboratory at the University of Cape Town analyses samples for two global clinical trial groups: IMPAACT and AIDS Clinical Trial Group (ACTG). Both networks require the laboratory assays involved in their trials to be approved by the Clinical Pharmacology Quality Assurance (CPQA) group (NAIADS/DAIDS).[112] As of February 2022, the intracellular method described in this thesis is the only CPQA-approved method for the quantification of intracellular EFV, LPV, and RTV in South Africa, while the unbound EFV, LPV, RTV assay is the only such method across all participating laboratories worldwide.[127] Both assay methods may be used in future studies and clinical trials which require CQPA-approved methods.

The Pretoria patient case discussed in Chapter Four shows the applicability of the methods in individualized patient care. While the results did not indicate why the patient was failing treatment, they did refute the hypothesis that the intracellular lopinavir concentrations were at subtherapeutic concentrations and added to the physicians' knowledge base for future potential treatment decisions regarding this patient. The methods developed may be used in future such cases where standard therapeutic drug monitoring is found to be insufficient.

The strong correlation (r^2 = 0.968) between the percentage protein binding and the intracellular accumulation ratio of EFV across six different HIV-positive individuals suggests that the intracellular concentrations could be calculated from the unbound and total plasma concentrations. Plasma is much easier to collect and process compared to PBMCs, and yet the intracellular concentrations are the most clinically relevant.[75,78] Despite the small sample size, these findings suggest that a model could be possible to determine the intracellular concentrations of EFV from paired plasma total and unbound concentrations and warrants further investigation; however, a much larger, statistically significant sample size would be required.

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APPENDIX A

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS017: Annual Progress Report / Renewal

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report	Approved until/next renewal date	30/11/21
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee		Date Signed	25/10/2020

Note: Please note that incomplete submissions will not be reviewed.
Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.

Please clarify your plan for research-related activities during COVID-19 lockdown

Principal Investigator to complete the following:

1. Protocol Information

Date (when submitting this form)	19Oct2020		
HREC REF Number	447/2017	Current Ethics Approval was granted until	30Nov2020
Protocol title	Determination of free, total, and intracellular concentrations of antiretroviral drugs efavirenz, lopinavir, and ritonavir		
Principal Investigator	Laboratory Director, A/Prof Lubbe Wiesner		
Department / Office Internal Mail Address	K50, Division of Clinical Pharmacology, Old Main Building, Groote Schuur Hospital		
1.1 Does this protocol receive US Federal funding?		☐ Yes	☒ No

2. Protocol status (tick ✓)

☒	Research-related activities are ongoing
☐	Data collection is complete, data analysis only
Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository	
180/2011: DATIC. 885/2014: P[illegible]/2020. 272/2018. Ongoing PhD student research	

ETHICS COMMITTEE
23 OCT 2020
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval		39
Total number of records or specimens collected, reviewed or stored since last progress report		9
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes, please list and attach with this report	☒ Yes	☐ No
272/2018 data presented as a podium presentation at the 2019 South African Society of Basic and Clinical Pharmacology conference		

4. Signature

Signature of PI		Date	19 Oct 2020

APPENDIX B

Table 9.2. Longer term stock solution stability of EFV at ~-80°C for 601 days

SS3-EFV#250 CS_21Jul2016 EFV#416 SC_28Nov2014	Reference: SS3		Test: EFV #416 SC_28Nov2014	
	analyte peak area	ISTD peak area	analyte peak area	ISTD peak area
Sample 1	606000	573000	583000	566000
Sample 2	601000	567000	596000	587000
Sample 3	581000	552000	595000	590000
Sample 4	609000	584000	595000	585000
Sample 5	610000	560000	613000	580000
Sample 6	625000	578000	600000	577000
Average	605333	569000	597000	580833
STDEV	14376	11798	9695	8658
CV(%)	2.4	2.1	1.6	1.5
% Difference			-1.4	

	Reference: SS3	Test: EFV #416 SC_28Nov2014
	peak area ratio	peak area ratio
Peak area 1	1.06	1.03
Peak area 2	1.06	1.02
Peak Area 3	1.05	1.01
Peak Area 4	1.04	1.02
Peak Area 5	1.09	1.06
Peak Area 6	1.08	1.04
Average	1.06	1.03
STDEV	0.0178	0.0181
CV(%)	1.7	1.8
% Difference		-3.4

APPENDIX C

Addendum

A method was first validated for the qualitative assessment of atazanavir, darunavir, efavirenz, lopinavir and nevirapine in human CPD plasma in October 2016 (VL2016/176). Additional assessments were carried out to support this validation. Longer term stock solution stability of ATZ and LPV in methanol was determined at ~-80°C. Long term stability of all five analytes in CPD plasma was evaluated at the intended storage temperature (~-80°C) to cover the duration of storage of clinical study samples.

1. Stock Solution Accuracy and Stability for ATZ and LPV

Long term stock solution stability (266 days at ~-80°C) for ATZ and LPV stock solutions (1mg/ml) in MeOH was tested on 07 March 2018 by comparing stock solutions prepared on 14 June 2017 (WvD_14Jun2017_LPV/ ATZ) to reference stock solutions prepared on the day of stability testing (CS_07Mar2018_LPV_SS1/ TKh_07Mar2018_ATZ_SS2). The reference stock solutions were tested for accuracy by comparing it to stock solutions prepared by different analysts (TKh_07Mar2018_LPV_SS2/ SC_07Mar2018_SS3) on the same day using ultraviolet-visible spectrophotometry. The absorption of each stock solution, prepared in triplicate in methanol at 10.0 µg/ml, were measured at λ = 249 nm for ATZ and λ = 202 nm for LPV. The results are presented in tables 1 and 2 for ATZ and LPV respectively.

Table 1. Longer term stock solution stability of atazanavir at ~-80 °C for 266 days.

	λ = 249 nm		
	TKh_07Mar2018_ATZ_SS2	SC_07Mar2018_ATZ_SS3	WvD_14Jun2017_ATZ
Absorbance A	0.223	0.228	0.233
Absorbance B	0.237	0.240	0.224
Absorbance C	0.245	0.231	0.235
Average	0.235	0.233	0.231
STDEV	0.0112	0.00599	0.00588
CV(%)	4.8	2.6	2.5
% Difference		-0.9	-1.8

Table 2. Longer term stock solution stability of lopinavir at ~-80 °C for 266 days.

	λ = 202 nm		
	CS_07Mar2018_LPV_SS1	TKh_07Mar2018_LPV_SS2	WvD_14Jun2017_LPV
Absorbance A	0.623	0.636	0.589
Absorbance B	0.651	0.642	0.620
Absorbance C	0.622	0.642	0.628
Average	0.632	0.640	0.613
STDEV	0.0161	0.00348	0.0206
CV(%)	2.5	0.5	3.4
% Difference		1.2	-3.1

APPENDIX D

Addendum

A method was first validated for the quantification of Lopinavir (LPV), Ritonavir (RTV), Efavirenz (EFV) and Nevirapine (NVP) in human plasma in 2013 (VL2013/150). Additional assessments were carried out for LPV, RTV, EFV and NVP to support these validations and to cover the treatment and condition of clinical samples analysed for various clinical studies. RTV stock solution stability was assessed for up to 304 days. Extended bench top stability for all four analytes in plasma was established for 24 hours at room temperature and for 48 hours at ~4°C. In addition, stability of NVP in plasma was established for up to 72 hours at room temperature. Long-term stability of the analytes in plasma was evaluated at the intended storage temperature to cover the duration of storage of clinical study samples. Whole blood stability of the analytes was also evaluated in samples at room temperature and on ice at various time points, up to a maximum of 96 hours. In addition, the inclusion of a sonication step in the extraction procedure, after samples have been thawed and prior to aliquoting the samples, was validated. This was included to resolve the issue of failing CPQAs, as it was found that samples stored in larger volumes gave inaccurate results if not sonicated prior to aliquoting.

1. Stock Solution Stability (RTV)

Stock solution stability for RTV for up to 304 days in methanol at ~-80°C was tested on the 12Feb2015 by comparing a stock solution prepared on 20Feb2014 (AvW_20Feb2014_RTV) to a reference stock solution prepared after 304 days (AF_12Nov2014_RTV). The reference stock solution was tested for accuracy by comparing it to a stock solution prepared by a different analyst (VM_12Nov2014_RTV) on the same day using mass spectrometry. The results are presented in table 1.

Table 1. Longer term stock solution stability of RTV at ~-80 °C for 304 days.

	AF_12Nov2014_RTV			AvW_20Feb2014_RTV		
	analyte peak area	**ISTD peak area**	**peak area ratio**	**analyte peak area**	**ISTD peak area**	**peak area ratio**
Sample 1	10800000	494000	21.9	10900000	477000	22.9
Sample 2	10700000	510000	21.0	10700000	484000	22.1
Sample 3	11000000	489000	22.5	10700000	494000	21.7
Sample 4	11200000	490000	22.9	10800000	501000	21.6
Sample 5	10800000	498000	21.7	11100000	493000	22.5
Sample 6	11000000	506000	21.7	11100000	507000	21.9
		Average	21.9		**Average**	22.1
		STDEV	0.660		**STDEV**	0.504
		CV(%)	3.0		**CV(%)**	2.3
					%Difference	0.7

Acceptance Criteria: For stability, a high CV(%) (higher than 15%) of the measured values and a difference of more than 10% from the reference solution could indicate instability in the stock/working solution.

Discussion: The two stock solutions were shown to be accurate, with a %Difference of 0.7%, concluding that RTV is stable in methanol for up to 304 days. Stock solution stability for EFV and NVP in acetonitrile stored at

APPENDIX E

Table 66 On bench stability of LPV

	High Concentration (LPV)		Low Concentration (LPV)	
	Observed QC Mean (µg/ml)	Observed B/T (µg/ml)	Observed QC Mean (µg/ml)	Observed B/T (µg/ml)
Sample 1	13.9	14.1	0.0559	0.0543
Sample 2		14.2		0.0561
Sample 3		14.0		0.0537
Sample 4		13.8		0.0557
Sample 5		13.7		0.0559
Sample 6		14.0		0.0534
Average		14.0	Average	0.0549
STDEV		0.186	STDEV	0.00119
CV(%)		1.3	% CV	2.2
% Difference		0.8	% Difference	-1.9

Table 67 On bench stability of RTV

	High Concentration (RTV)		Low Concentration (RTV)	
	Observed QC Mean (µg/ml)	Observed B/T (µg/ml)	Observed QC Mean (µg/ml)	Observed B/T (µg/ml)
Sample 1	3.65	3.70	0.0149	0.0144
Sample 2		3.56		0.0144
Sample 3		3.60		0.0148
Sample 4		3.53		0.0142
Sample 5		3.61		0.0144
Sample 6		3.62		0.0139
Average		3.60	Average	0.0144
STDEV		0.0582	STDEV	0.000295
CV(%)		1.6	% CV	2.1
% Difference		-1.4	% Difference	-4.0

Table 68 On bench stability of EFV

	High Concentration (EFV)		Low Concentration (EFV)	
	Observed QC Mean (µg/ml)	Observed B/T (µg/ml)	Observed QC Mean (µg/ml)	Observed B/T (µg/ml)
Sample 1	14.2	14.0	0.0593	0.0552
Sample 2		14.3		0.0528
Sample 3		15.1		0.0574
Sample 4		13.9		0.0554
Sample 5		14.4		0.0563
Sample 6		13.5		0.0577
Average		14.2	Average	0.0558
STDEV		0.544	STDEV	0.00179
CV(%)		3.8	% CV	3.2
% Difference		0.3	% Difference	-5.9

APPENDIX F

Table 4.28. Freeze and thaw stability of EFV: Spiked plasma

	High Conc.			Low Conc.		
	1 F/T (ref) Peak Area Ratio	3 F/T Peak Area Ratio	5 F/T Peak Area Ratio	1 F/T (ref) Peak Area Ratio	3 F/T Peak Area Ratio	5 F/T Peak Area Ratio
Sample 1	12.5	5.67	1.64	0.0683	0.0353	0.0438
Sample 2	10.9	5.19	1.87	[0.103]	0.0332	0.0381
Sample 3	5.9	8.22	1.84	0.0777	0.0385	0.0263
Sample 4	6.43	7.62	[5.90]	0.0563	[0.124]	0.0333
Sample 5	12.5	5.34	1.71	0.0763	0.036	0.0384
Sample 6	6.49	9.25	1.94	0.0623	0.0359	0.0317
Average	**9.13**	**6.88**	**1.8**	**0.0682**	**0.0358**	**0.0353**
STDEV	**3.19**	**1.71**	**0.121**	**0.00911**	**0.00192**	**0.00611**
CV(%)	**34.9**	**24.9**	**6.7**	**13.4**	**5.4**	**17.3**
%Difference	**Ref**	**-24.6**	**-80.3**	**Ref**	**-47.5**	**-48.2**
					Average %Difference after 3 F/T	**-36.1**
					Average %Difference after 5 F/T	**-64.3**

[]Significant outlier. P < 0.05

Table 4.29. Freeze and thaw stability of LPV: Spiked plasma

	High Conc.			Low Conc.		
	1 F/T (ref) Peak Area Ratio	3 F/T Peak Area Ratio	5 F/T Peak Area Ratio	1 F/T (ref) Peak Area Ratio	3 F/T Peak Area Ratio	5 F/T Peak Area Ratio
Sample 1	4.28	4.92	3.98	0.0246	0.0197	0.0173
Sample 2	5.29	4.26	4.07	0.024	0.0209	0.0183
Sample 3	5.73	4.23	4.02	0.0246	0.0200	0.0167
Sample 4	4.83	3.63	4.43	0.0271	0.0189	0.0182
Sample 5	[9.20]	3.71	4.06	0.0236	0.0193	0.0178
Sample 6	6.20	3.94	4.27	0.0209	0.0182	0.0163
Average	**5.26**	**4.12**	**4.14**	**0.0241**	**0.0195**	**0.0174**
STDEV	**0.751**	**0.471**	**0.174**	**0.00200**	**0.000930**	**0.000818**
CV(%)	**14.3**	**11.4**	**4.2**	**8.3**	**4.8**	**4.7**
%Difference	**Ref**	**-21.8**	**-21.4**	**Ref**	**-19.2**	**-27.7**
					Average %Difference after 3 F/T	**-20.5**
					Average %Difference after 5 F/T	**-24.6**

[]Significant outlier. P < 0.05

Table 4.30. Freeze and thaw stability of RTV: Spiked plasma

	High Conc.			Low Conc.		
	1 F/T (ref) Peak Area Ratio	3 F/T Peak Area Ratio	5 F/T Peak Area Ratio	1 F/T (ref) Peak Area Ratio	3 F/T Peak Area Ratio	5 F/T Peak Area Ratio
Sample 1	0.338	0.365	0.367	0.0102	0.00935	0.00839
Sample 2	0.342	0.324	0.332	0.00981	0.00946	0.0102
Sample 3	0.340	0.363	0.326	0.00969	0.0102	0.00867
Sample 4	0.394	0.327	0.339	0.00974	0.0103	0.00878
Sample 5	[1.58]	0.342	[0.656]	0.0114	0.0100	0.00898
Sample 6	0.410	0.399	0.307	0.0113	0.00961	0.00912
Average	**0.365**	**0.353**	**0.334**	**0.0104**	**0.00981**	**0.00903**
STDEV	**0.03433**	**0.0281**	**0.0216**	**0.000798**	**0.000392**	**0.000638**
CV(%)	**9.4**	**7.9**	**6.5**	**7.7**	**4.0**	**7.1**
%Difference	**Ref**	**-3.1**	**-8.4**	**Ref**	**-5.3**	**-12.9**
					Average %Difference after 3 F/T	**-4.2**
					Average %Difference after 5 F/T	**-10.7**

[]Significant outlier. P < 0.05

APPENDIX G

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

30 April 2018

HREC REF: 272/2018

Dr L Wiesner
Division of Pharmacology
K-45
OMB

Dear Dr Wiesner

PROJECT TITLE: REQUEST TO USE REMNANT TDM SPECIMENS FOR THE PURPOSES OF ASSAY VALIDATION OF FREE DRUG CONCENTRATION DETERMINATION

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 May 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical

APPENDIX H

AVR SOP Submission Utility FSTRF

AVR SOP Review Comments for Submission ID: 2019123cpqa635

Other AVR

IS acceptance criteria should be discussed with CPQA

Other SOP

Changes made

Final Summary

Nice work good job.

Score

Your AVR and/or SOP has been assigned a score of 1.
Your assay support documents are final; no further submissions are necessary.
Please update your stability information as it becomes available.

APPENDIX I

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS017: Annual Progress Report / Renewal

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☐ Approved	Annual progress report	Approved until/next renewal date	30.4.23
☐ Not approved	See attached comments		
Signature Chairperson of the HREC		Date Signed	9/3/2022

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	09 March 2022		
HREC REF Number	773/2015	Current Ethics Approval was granted until	30 April 2022
Protocol title	Blood donations for PK Calibration purposes, Clinical Pharmacology		
Principal Investigator	Laboratory Director: Assoc. Prof. Lubbe Wiesner		
Department / Office Internal Mail Address	Division of Clinical Pharmacology, K45, OMB, GSH		
1.1 Does this protocol receive US Federal funding?		☐ Yes	X No

2. Protocol status (tick ✓)

✓	Research-related activities are ongoing
☐	Data collection is complete, data analysis only
Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository	
Whole blood continues to be donated in house for the purposes of assay testing and validation experiments.	

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval		126
Total number of records or specimens collected, reviewed or stored since last progress report		18
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes, please list and attach with this report.	☐ Yes	X No

4. Signature

Signature of PI	Lubbe Wiesner Digitally signed by Lubbe Wiesner Date: 2022.03.09 10:58:55 +02'00'	Date	

APPENDIX J

AVR SOP Submission Utility FSTRF

AVR SOP Review Comments for Submission ID: 20191113cpqa99

Other AVR

ok

Other SOP

ok

Final Summary

Thank you for addressing all my comments. No further corrections need to be made to this AVR and SOP except to update it with appropriate stability assessments in harvested PBMC samples obtained from incurred samples.

I look forward to reviewing this data.

Thank you.

Score

Your AVR and/or SOP has been assigned a score of 1.
Your assay support documents are final; no further submissions are necessary.
Please update your stability information as it becomes available.

APPENDIX K

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room G50- Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: hrec-submissions@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

28 April 2021

HREC REF: 259/2021

A/Prof L Wiesner
Division of Cardiology
K-45 OMB
Email: lubbe.wiesner@uct.ac.za
Student: krgkat004@myuct.ac.za

Dear A/Prof Wiesner

PROJECT TITLE: DETERMINATION OF FREE, TOTAL, AND INTRACELLULAR CONCENTRATIONS OF ANTIRETROVIRAL DRUGS EFAVIRENZ, LOPINAVIR, AND RITONAVIR FROM REMNANT TDM WHOLE BLOOD SAMPLES: A PROOF-OF-CONCEPT STUDY. SUB-STUDY LINKED TO: 447/2017 & 386/2010. PHD CANDIDATE: MS K KRIEGLER

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020 & 06 July 2020.

Approval is granted for one year until the 30 April 2022.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: - Ms Katherine Kriegler will also be involved in this study.

Please quote the HREC REF 259/2021 in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

APPENDIX L

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
Department of Immunology

14 March 2022

To Whom It May Concern:

RE: PERMISSION TO USE PATIENT DATA

With this letter I give Katie Kriegler, PhD student at the University of Cape Town, permission to use the data generated from a patient sample (reference number 19292) I had sent to their laboratory for further investigation of possible drug resistance. Ms Kriegler may use the patient data as a clinical example of the laboratory methods she had developed.

Kind regards

Professor Theresa Rossouw
MBChB, MPH, DPhil, PhD

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