Development and application of methods for pharmacokinetics (PK) analysis in PNG HIV/AIDS patients

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Discipline of Pharmacology

School of Biomedicine

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The University of Adelaide

October 2022

A thesis submitted for the degree of Doctor of Philosophy

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Citation list

1. **Andriguetti NB**, Barratt DT, Tucci J, Pumuye P, Somogyi AA. Instability of Efavirenz Metabolites Identified During Method Development and Validation. J Pharm Sci. 2021; 110(10):3362-3366. doi: 10.1016/j.xphs.2021.06.028.
2. **Bordin Andriguetti N**, Van Schalkwyk HK, Barratt DT, Tucci J, Pumuye P, Somogyi AA. Large variability in plasma efavirenz concentration in Papua New Guinea HIV/AIDS patients associated with high frequency of CYP2B6 516T allele. Clin Transl Sci. 2021; 14(6):2521-2531. doi: 10.1111/cts.13120.
3. **Andriguetti NB**, Barratt DT, Tucci J, Pumuye P, Somogyi AA. Assessment of lamivudine and tenofovir pharmacokinetic variability in a Papua New Guinea HIV population through a combined high throughput LCMS/MS assay. 2022. To be submitted.
4. **Andriguetti NB**, Barratt DT, Tucci J, Pumuye P, Somogyi AA. Determination of dolutegravir in dried blood spots and volumetric absorptive microsampling (VAMS) by LC-MS/MS. 2022. To be submitted.

Abstract

HIV is an infectious disease affecting millions of people around the world, mainly in low-income countries (e.g. Papua New Guinea-PNG). HIV has become a lifelong chronic disease, so treatment should be readily managed. Nevertheless, antiretrovirals have noted side effects, often with narrow therapeutic ranges and high variability between and within patients, which makes them good candidates for Therapeutic Drug Monitoring (TDM). Variability in drug concentrations can be due to non-genetic and genetic factors, therefore their study is required to ensure the patient is getting maximum benefits and minimum negative outcomes from the drug use. PNG has a high prevalence of HIV and unique pharmacogenetics profile, however nothing is known on pharmacokinetics or adverse effects. Therefore, studying this population is important to ensure maximum benefits of the treatment are achieved. TDM requires precise, accurate and selective methods for quantification of the drugs, so Chapter 2, describes the development and validation of a method for the quantification of efavirenz (EFV) and metabolites, showing for the first time the significant instability of two metabolites in plasma samples at high temperatures (samples are heated for virus inactivation). Chapter 3 investigated if *CYP2B6* c.516T allele and demographic factors affected plasma EFV and 8-hydroxy-EFV variability, and their association with adverse effects in 156 HIV/AIDS patients from PNG. The frequency of 516T allele was 53%. Genotype *per se* explained 38% of EFV variability and all patients with EFV plasma concentrations above the therapeutic range (> 4 μg mL^{-1}) carried the TT genotype. Even though c.516TT genotype is a strong predictor of high plasma EFV concentrations, it did not translate into increased incidence of side effects, most likely due to drug tolerance.

The current first-line treatment for HIV in PNG consists of a combination of dolutegravir (DTG), tenofovir (TNF) and lamivudine (3TC). Simultaneous drug analysis saves time and consumables, so Chapter 4 reports an attempt at the simultaneous quantification of TNF, 3TC and DTG. Due to differences in chemical properties of the drugs, two separate methods had to be developed. Besides the development and validation of a method to simultaneously quantify TNF and 3TC, Chapter 5 brings the application of the developed assay in PNG HIV patients and the association of TNF and 3TC plasma concentrations variability with demographic factors and patient's kidney function. The method was successfully applied to 121 patient samples and only a small proportion (TNF 8 %, 3TC 6 %) of the variability was explained by these parameters.

Chapter 6 describes the development of an assay to quantify DTG in microsampling devices, which are used as an alternative to conventional sampling (venous blood), particularly for their advantage regarding to transportation of samples.

In summary, methods for the quantification of HIV drugs in biological samples were developed and validated. Association of genetic and non-genetic factors with drug concentration variability was explored, however most of the variability was not explained by the parameters investigated. Therefore, future studies are needed to explore novel factors to predict HIV drug concentrations variability and its association with side effects.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Natalia Bordin Andriguetti

7th October 2022

Acknowledgments

This study was supported by the Australian Centre for HIV and Hepatitis Virology Research (ACH2) [Personalised medicine for HIV infection in PNG], Australia. I was personally granted a Research Training Program Scholarship (Australian Government). I also acknowledge the travel awards from the School of Biomedicine for my international conference and the travel grants received from the ASCEPT Board and IATDMCT Committee.

To the people who helped throughout my study, my sincerest gratitude. First, my acknowledgments to Professor Andrew Somogyi, for giving me the opportunity to be his student and further my research in method development and pharmacokinetics. Andrew, my sincerest thanks for all the help you have given me and for always being so prompt with your revisions and replies. Also, many thanks to my cosupervisor Dr. Daniel Barratt, for always helping me with basically everything in the lab, I am not sure if I could do it without your support Dan, so I really appreciate all the time you spent showing me how to read the manuals and especially how to do perform statistics. I was extremely privileged to work with such fantastic and supportive supervisors, thank you so very much Andrew and Dan.

I extend further thanks to: Dr. Paul Pumuye from the University of Papua New Guinea, for the patients' samples and the well done dried blood spots samplings; Professor Joseph Tucci from La Trobe University Bendigo Campus, for all the revisions and your support when I needed a bit of help in my trip to Melbourne; to the HIV patients from Papua New Guinea who participated in this study; Professor Joanne Bowen, for helping me organise my PhD; Dr. Stefan Musolino, for getting quotes and purchasing materials, also the support in the lab; and to my friend and colleague Helena van Schalkwyk, for all the shopping trips, chats and quality time spent together.

I would like to express my gratitude to my family – my Mum, Nadir, my Sis, Nadine and my Dad, Antoninho. Thank you for understanding that I had to move away to pursue my dream of becoming a Doctor of Philosophy, even when that place, which I now consider home, is on the other side of the World. Thank you so much for all the support and love you all have given me throughout this amazing challenge.

With all this support, I also wish to acknowledge myself, I am very proud of my achievement. I moved across the world leaving my home, my family and my friends, to further my studies and challenge myself to get my PhD degree in another language. I could not be happier than I am right now.

Finally, my favourite quote:

"The best things in life are on the other side of fear" – Will Smith -

List of Abbreviations

%CV	coefficient of variability
3TC	lamivudine
3TC-TP	lamivudine triphosphate
7-OH-EFV	7-hydroxy-efavirenz
8,14-diOH-EFV	8,14-dihydroxy-efavirenz
8-OH-EFV	8-hydroxy-efavirenz
ABCB1	ATP Binding Cassette Subfamily B Member 1
ABCC2	ATP Binding Cassette Subfamily C Member 2
ABCC4	ATP Binding Cassette Subfamily C Member 4
ABCG2	ATP Binding Cassette Subfamily G Member 2
ACN	acetonitrile
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral treatment
AUC	area under the curve
AZT	azidothymidine
BCRP	breast cancer resistance protein
BMI	body mass index
C_{24h}	concentrations 24h after dose
CAT	clot activator
CCR	cell co-receptor
CE	collision energy
CL	oral clearance
C_{max}	maximum concentration
CNS	central nervous system
CrCl	creatinine clearance
C_{trough}	trough plasma concentrations
CYP2B6	Cytochrome P450 2B6
CYP3A4	Cytochrome P450 3A4
DBS	dried blood spot
DNA	deoxyribonucleic acid
DPS	dried plasma spots

DTG	dolutegravir
EFV	efavirenz
eGFR	estimated glomerular filtration rate
FDA	US Food and Drug Administration
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HQC	high quality control
HT	haematocrit
INSTI	integrase strand transfer inhibitors
IS	internal standard
KTD	kidney tubular dysfunction
LC-MS/MS	liquid chromatography-mass spectrometry
LiHep	lithium heparin
LLE	liquid-liquid extraction
LLOQ	low limit of quantification
Ln	natural logarithm
LQC	low quality control
m/z	mass-to-charge ratio
MP	mobile phase
MQC	medium quality control
MR	metabolic ratio
MRM	multiple reaction monitoring
MRP2	multi-drug resistant protein 2
MRP4	multi-drug resistant protein 4
MS	mass spectrometer
MTBE	methyl tert-butyl ether
NNRTI	non-nucleoside reverse transcriptase inhibitor
NR1I2	nuclear receptor subfamily 1 group I member 2
NR1I3	nuclear receptor subfamily 1 group I member 3
NRTI	nucleoside reverse transcriptase inhibitor
OAT	organic anion transporters
OCT	organic cation transporters
PDA	photodiode array

PEEK	polyetheretherketone
P-gp	P-glycoprotein
PGx	pharmacogenetics
PI	protease inhibitor
PK	pharmacokinetics
PLHIV	people living with HIV
PNG	Papua New Guinea
PPT	protein precipitation
PRTD	proximal renal tubular dysfunction
QC	quality control
RTI	reverse-transcriptase inhibitor
S/N	signal-to-noise
SLC22A6	Solute Carrier Family 22 Member 6
SLC22A8	Solute Carrier Family 22 Member 8
SLE	Supported liquid extraction
SNP	single nucleotide polymorphism
SPE	Solid phase extraction
T_a	absorption time
TB	tuberculosis
TDF	tenofovir disoproxil fumarate
TDM	therapeutic drug monitoring
TNF	tenofovir
TNF-DP	tenofovir diphosphate
UGT	UDP-glucuronosyltransferase
UGT1A1	UDP-glucuronosyltransferase 1A1
UGT1A3	UDP-glucuronosyltransferase 1A3
UGT1A9	UDP-glucuronosyltransferase 1A9
UGT2B7	UDP-glucuronosyltransferase 2B7
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet
VAMS	volumetric absorptive microsampling
WHO	world health organization

Chapter 1: Introduction

1. Infectious Diseases: HIV

1.1 Overview

The Human Immunodeficiency Virus (HIV) causes an infection characterized by a loss of CD4+ T cells that leads to the impairment of the patient's immune system (1, 2). HIV continues to be a major public health problem, impacting on the life of over 38 million people worldwide. Since the first reported case in 1981, HIV infection has claimed more than 36 million lives (3). There are two main types of HIV, HIV-1 which is the most common (95% of all infections worldwide) and more aggressive type, and the HIV-2 type which is concentrated in West Africa, less infectious and progresses more slowly than HIV-1 (4). The treatment for HIV is well known and effectively reduces the progression of the disease by preventing replication of the virus (5). Combination therapy known as Highly Active Antiviral Therapy (HAART) involves two nucleoside reverse transcriptase inhibitors (NRTIs) with either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) (6), and is the common drug regimen. In 2019, 68% of people living with the virus worldwide were receiving antiretroviral treatment (3).

The HIV infection compromises the patient's immune system facilitating the development of other infectious diseases, such as tuberculosis (TB). The co-infection with these diseases accelerates the degradation of patient's immunological functions leading to a quick progress of both diseases (7, 8). Globally, TB is among the top 10 causes of death accounting for an estimated 1.4 million deaths in 2019, and is the number one cause of death in HIV positive patients (9). TB has an impact all over the world, but in 2019, the most affected areas were Southeast Asia with 44% of new cases, then African region accounting for 25% of cases and finally the Western Pacific region with 18% of the world cases (9). Effective treatment that

leads to cure has been introduced onto the market in late 1960's with a combination of antibiotics taken for 6 months (10).

Infectious diseases such as TB and HIV-1 impact significantly on the health of the 8.6 million inhabitants of Papua New Guinea (PNG), located in the Western Pacific region (11). In 2019, 52000 adults and children were living with HIV (30% increase since 2010), 3300 were newly infected, 62% were receiving antiretroviral treatment, and 18% were co-infected with TB (HIV/TB) (12). There is a high burden of TB in PNG, with incidence rates of 432/100,000 in 2019 (13), with levels of multidrug resistant TB on the rise and similar to other high TB burden countries of the region (14). Among the cases, 2700 (~7%) were co-infected with HIV-1 (9).

1.2 Western Pacific Region – Papua New Guinea

The Western Pacific Region is one of the six regions of the World Health Organization (WHO) and comprises 37 countries and areas (Figure 1) with nearly 1.9 billion inhabitants, including PNG accounting for 0.4% (8.9 million inhabitants) of the region's total population. The region is affected by a range of infectious disease, such as malaria, dengue, polio, rubella, HIV and TB (15).

Figure 1. Countries and areas of the WHO Western Pacific Region (sand-coloured countries), highlighted is Papua New Guinea (16).

The estimated number of people living with HIV (PLHIV) in the region is 1.9 million, with PNG the country with the highest prevalence (0.9%), followed by Cambodia (0.5%) (12, 15). Even with a jump from 20% in 2010 to 62% in 2019 of people receiving HAART treatment, the number of people living with HIV almost doubled in the same period (33000 in 2010 to 59000 in 2021) (12).

The number of TB infected people in the Western Pacific Region is similar to HIV, with 1.4 million notified cases with an additional estimate of about half a million more in 2019. On average 77% of the patients were receiving TB treatment in the same year (13).

2. HIV Infection and Treatment

The majority of HIV infections occur from unprotected sexual intercourse, particularly between homosexual men, and among drug addicts sharing contaminated needles (17, 18). Other ways of transmission occur through transfusion of contaminated blood, and between a mother and her infant during pregnancy, childbirth and breastfeeding if the mother is not receiving treatment (19). In PNG the majority of cases occur among sex workers (104 males to 100 females) (11).

Once the infection is established, HIV binds to the CD4+T cell receptor and co-receptors (CCR5), resulting in fusion with the host-cell membrane. Once inside the cell, the viral capsid uncoats and the viral genome and proteins are released into the cytoplasm. HIV converts its genetic material into HIV DNA, enters the nucleus of the host cell integrating to the host genetic material producing more HIV proteins to then be released into the bloodstream (Figure 2) (20). The infection caused by HIV leads to progressive deterioration of the immune system, by attacking and impairing its cells, resulting in chronic CD4+ T-cell count depletion if left untreated (21).

2.1 HIV Pathogenesis

For a few years after the first case, the only defence against the virus was prevention; then in the late 1980's the first drug (azidothymidine – AZT), a NRTI, for HIV treatment was approved (22). Even though therapy with AZT showed survival benefits for a short period (24 weeks), the US Food and Drug Administration (FDA) approved it for use in patients with advanced HIV (23). Two years following the approval, the first mechanism of HIV resistance to AZT was described (24).

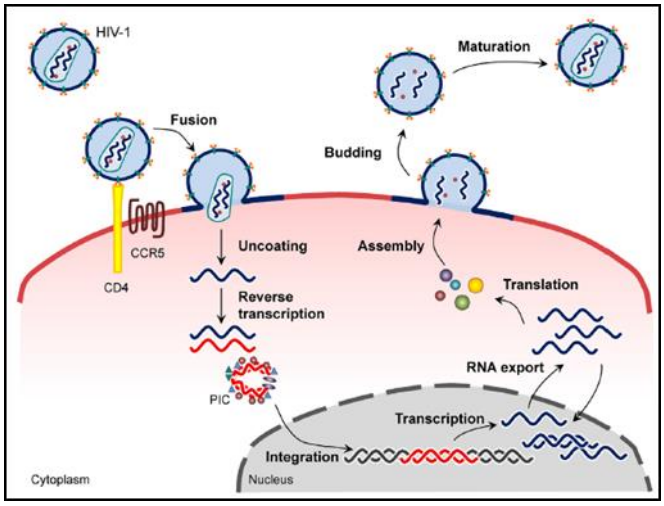

Figure 2. Pathogenesis of HIV (20).

Considering AZT failure for the treatment of HIV, a few other drugs were developed and approved throughout a decade until the US FDA in 1996 approved two new drug classes: PIs (saquinavir, ritonavir, indinavir, nelfinavir) and NNRTI nevirapine (25). Combination therapy capable of reducing viral replication changed the outcome of HIV infection from potentially lethal to a chronic manageable disease (17, 26). Antiretroviral therapy causes slowing of the illness progression by preventing the virus from replicating, usually involving a combination therapy of two NRTIs with either a PI or a NNRTI (i.e. HAART) (5). Efavirenz (EFV), a NNRTI, was approved by the FDA in 1998 (26), and remained the first line treatment for HIV/AIDS for two decades until newer and more expensive NNRTIs entered the market (6, 27, 28). Efavirenz is often prescribed in combination with two NRTIs, usually with tenofovir disoproxil fumarate (TDF) and lamivudine (3TC) (29).

Although NRTI and NNRTI are the most commonly used class of drugs for HIV therapy, other types are becoming more popular. The integrase strand transfer inhibitors (INSTI) are a class of antiviral drugs designed to block the action of the viral enzyme integrase. Since integration is a vital step in retroviral replication, blocking it can halt further spread of the virus (30). The most popular INSTI is dolutegravir (DTG), approved by the FDA in 2013. Based on numerous pieces of evidence for toxicities caused by EFV treatment (see below), and large clinical trials demonstrating the general benefits and safety of DTG, in 2019 the WHO recommended DTG as the preferred first-line treatment for HIV in all populations (31). Nevertheless, EFV remains as an alternative first-line treatment for adults and adolescents and as a first-line for HIV in China, India, Indonesia and Thailand (32).

2.2 Efavirenz

As a NNRTI, EFV binds to the enzyme HIV-1 reverse transcriptase preventing the virus from replicating, therefore reducing HIV RNA levels in patients (33, 34). The standard adult dosage recommended for EFV is 600 mg orally once daily, in combination with two NRTIs, preferably taken on an empty stomach. High-fat meals can increase the bioavailability of EFV, leading to adverse effects (29, 34, 35). No dose adjustment is necessary for patients with renal impairment, however EFV is not recommended for patients with moderate or severe hepatic impairment. Caution should be taken in administering in patients with mild hepatic impairment (29).

2.2.1 Physicochemical Properties

EFV is a hydrophobic molecule (octanol/pH 7.4 buffer ratio= 4.7; pKa= 10.2). Its empirical formula is $C_4H_9ClF_3NO_2$, and the structure is shown in figure 3 with a mass of 315.7 g/mol that reaches peak plasma concentrations of ~ 4 μg/mL 5 h after a standard 600 mg adult oral dose, attaining steady-state plasma concentrations in 6-10 days (29). EFV has a long

terminal half-life of 52-76 h after a single dose, and is highly plasma protein bound (> 99%), predominantly to albumin (34). After a 600 mg/day to steady state EFV, the oral clearance (CL) was 9.4 L/h and the volume of distribution was 252 L (36).

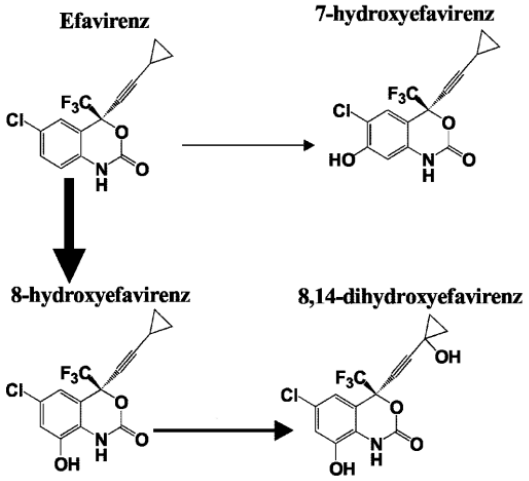

Figure 3. Metabolic pathway of EFV and chemical structure of EFV and main metabolites (37).

2.2.2 Metabolism

Efavirenz is metabolized in the liver primarily by CYP2B6 to the major inactive metabolite 8-hydroxy-efavirenz (8-OH-EFV), with further hydroxylation of 8-OH-EFV to 8,14-dihydroxy-efavirenz (8,14-diOH-EFV) (38). Furthermore, EFV can be hydroxylated to 7-hydroxy-efavirenz (7-OH-EFV) by CYP2A6 (Figure 4) (39). Chemical structures of the metabolites can be seen in Figure 3. The excretion of EFV occurs predominantly in urine following glucuronidation of the EFV hydroxylated metabolites by multiple hepatic UDP-glucuronosyltransferase (UGT) isoforms (33, 34). Mass balance of EFV oral was conducted in six volunteers who received EFV with radiolabelled material [^{14}C-EFV]. Mean urinary recovery of total radioactivity was 25% (86% was 8-OH-EFV and minor 7-OH-EFV, followed by glucuronide and sulfate conjugates) and 41% was recovery in faeces (2% of the

dose as 8-OH-EFV and over 90% as intact [^{14}C] EFV). Total recovery was 66% of the administered dose over 21 days (40).

EFV exhibits prolonged autoinduction of its own metabolism through the activation of the nuclear receptors NR1I3 and NR1I2 that regulate the expression of *CYP2B6* and *CYP3A4* genes (Figure 4) (33, 41). The extent of autoinduction can be influenced by the duration of therapy and the patient's genotype; for example, patients with the *CYP2B6*1* allele (see 2.2.3.2) exhibit greater autoinduction compared to *6*/6** (42-44).

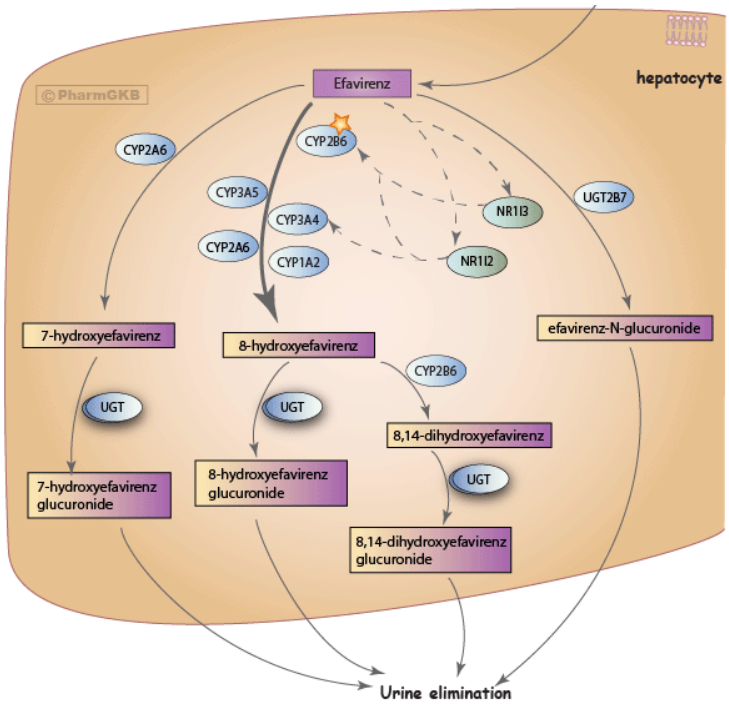

Figure 4. Schematic representation of EFV metabolism (33).

2.2.3 Variations in EFV disposition

Efavirenz concentrations can vary due to numerous reasons, including differences in metabolism, body weight, drug compliance, use of co-medications, presence of concomitant diseases (inter- or intra-individual variability), ethnicity, gender, as well as genetic factors (inter-individual variability) (45). The clearance of EFV is affected by body weight, ethnicity, co-medications, and genetics (46-49); although the effect of gender on EFV concentrations appears to be equivocal (48).

2.2.3.1 Non genetic factors

Csajka et al. (2003) characterized the population pharmacokinetics (PK) of EFV in HIV-positive patients (*n*=235), and the influence of covariates on EFV absorption and disposition (36). A one-compartment model with first-order absorption was used to describe the data. The interpatient variability of CL and volume of distribution was 55% (%CV). Of the tested demographic covariates (ethnicity, height, age, sex), only ethnicity showed a significant influence on EFV PK, where black patients had higher EFV concentrations, as shown by a significant reduction in CL of about 20%. Co-administration with other antiretroviral drugs was not significantly associated with EFV disposition (36). Robarge et al. (2017) describe developing a population PK model to estimate the contributors of genetic and non genetic factors to EFV disposition in 73 HIV-seronegative volunteers (48). A correlation between the volume of distribution and gender was identified, where females had on average a 20% greater volume. The correlation was adjusted for body mass index, however gender remained an independent predictor of volume of distribution (48).

Poeta et al. (2011) reported a significant inverse correlation between EFV plasma concentrations and body weight (P=0.013), where patients with lower weight had higher concentrations (46). In a study conducted by Stohr et al. (2008) (*n*=339), plasma EFV concentrations were significantly influenced by ethnicity (59% higher in black than in white patients, P<0.001), and a correlation between EFV concentrations and body weight was reported (P=0.002), where patients with higher weight had lower concentrations (47). Lee et al. (2014) and Stohr et al. (2008) reported a 26% and 40% decrease in EFV plasma concentrations, respectively, in patients receiving rifampicin when compared to patients not receiving rifampicin (47, 50). Rifampicin is part of the combination therapy against TB, and is known to induce the activity of the EFV main metabolic enzyme, CYP2B6, which results in enhanced efavirenz clearance and reduced plasma concentrations (51).

2.2.3.2 Genetic factors

The gene that encodes CYP2B6, the main enzyme involved in the phase I metabolism of EFV, is highly polymorphic, resulting in altered drug metabolism (52, 53). Single nucleotide polymorphism (SNP) is a variation at a single position in a DNA sequence, and SNPs are the most common genetic variation to alter drug concentrations in humans (54). More than 60 variant alleles have been identified in the promoter and coding regions, as well as in intronic sequences of the *CYP2B6* gene (55). However, the most frequently studied and clinically relevant for EFV metabolism is the *CYP2B6* c.516G>T (rs3745274) missense variant, which has been associated with decreased activity of the CYP2B6 enzyme, and increased plasma EFV concentrations in many different populations (49, 53, 56). This alteration in drug metabolism often leads to adverse events (57) and discontinuation of EFV treatment (58).

PNG has the highest frequency of the c.516G>T variant allele of all populations known to date. Mehlotra et al. (2006) and Tucci et al. (2018) reported a 65% and a 54% frequency of the c.516G>T variant allele, respectively (59, 60). In other populations, this T variant shows high variability, ranging from 16-46% in Caucasians, 16-40% in Asians and 35-45% in Africans (61). Besides 516G>T, a few other SNPs in the *CYP2B6* gene have been associated with alterations in EFV metabolism, but particularly for PNG population they are either very rare (60) or in general have no or modest effect on function (53).

In addition to CYP2B6, another phase I EFV metabolism enzyme (CYP2A6) has been studied (55). The distribution of CYP2A6 gene variants (48T>G and 479T>A) have been studied by our group (60), however the latter was not identified in 52 PNG HIV/AIDS patients, and the 48T>G presented a frequency of 7%, similar to that found in Europeans (61). Nonetheless, the clinical significance of CYP2A6 variants on EFV therapy is unclear

(33). As for the phase II metabolic enzymes, UGT2B7 c.802T>C has been significantly associated with variations in EFV concentrations, with patients carrying the variant C allele having lower plasma concentrations, in 94 HIV-infected Ghanaians (62). In a study by Mehlotra et al. (2007), the prevalence of UGT2B7 c.802T>C was found to be 28% among 153 PNG individuals, being similar to other populations (63, 64).

2.2.4 Efavirenz Adverse Effects

The use of EFV may lead to the development of adverse events such as Central Nervous System (CNS), psychiatric and hepatic toxicities that can lead to a decrease in patient compliance and further virological failure (65, 66). The CNS side effects associated with EFV include decreased concentration, dizziness, insomnia and vivid dreams; however, these symptoms seem to disappear gradually for most patients within 2-4 weeks after initiation of treatment, because either the patients tolerate or the severity declines and dissipates overtime (67-69). The development of these side effects has resulted in a narrow EFV therapeutic concentration range of 1-4 µg/mL, whereby patients with EFV concentrations above 4 µg/mL are at greater risk of developing toxicities, whilst patients below 1 µg/mL are more likely to experience treatment failure (70) [see 2.2.4 Therapeutic Monitoring]. An *in vitro* study showed that EFV, 8-OH-EFV and 7-OH-EFV induce neuronal damage in a dose-dependent manner and 8-OH-EFV was at least an order of magnitude more toxic than EFV and 7-OH-EFV (71).

The developing of side effects is associated with high plasma EFV concentrations, which is often related to genetic polymorphisms (see 2.2.3.2) (69, 72, 73). Mukonzo et al. (2013) showed that among the patients carrying *CYP2B6* c.516G>T variant allele, 93% experienced neuropsychiatric disorders and had higher EFV concentrations (> 4 µg/mL), however no follow-up was conduct to verify if side effects persisted (72). In a study conducted in South

Africa, patients who experienced severe side effects had median EFV concentrations of 14882 µg/mL and 33% of the patients who reported severe and moderate CNS EFV related side effects were homozygous for the c.516T variant. Patients carrying TT genotype (n=18) had an overall increase in EFV concentrations, but it was not statistically significant when compared between genotypes (p= 0.08) (69). In the same study, a follow-up was conducted at four weeks post initiation of therapy and all patients denied persistence of CNS symptoms. Limitations have been noted in this study, in that no other polymorphisms affecting EFV metabolism were studied; problems with DNA extraction and genotyping resulted in a decrease of sample size (n=80); and no drug quantification was performed in the follow-up (69). Haas et al. (2004) reported an association between the development of side effects, high EFV concentrations and presence of c.516T variant in the first week following treatment, however at week 24 no side effects were reported by the patients, even though EFV concentrations in those patients were still high compared to the patients that initially reported no symptoms (73). Therefore it was concluded that patients seem to develop tolerance to the side effects (73). However, some studies show an association between high EFV concentrations and the development of toxicities, whereas other studies show no relation (74, 75). In fact, in one of our studies we observed an inverse correlation between plasma EFV concentrations and reported side effects. Patients that reported side effects had lower EFV concentrations than those with no side effects (see chapter 3). Self-reported toxicities must be carefully interpreted because they often can be inaccurate and unreliable (69, 76). Another important point to consider when analysing side effects is the therapy length, as the patients can potentially develop resistance or tolerance (73). Also, the presence of other factors associated with the development of side effects, such as comorbidities and co-medications, needs to be investigated (76).

2.2.5 Therapeutic drug monitoring

In view of the high pharmacokinetic variability extensively reported in antiretroviral treated patients along with numerous side effects and narrow therapeutic range, therapeutic drug monitoring (TDM) of EFV is useful to minimize the adverse events whilst maintaining treatment efficacy (77). To perform TDM, drug measurements in biological samples are required, usually in plasma or serum because reference ranges for most of the drugs are established in these samples (78). Several methods have been developed and published for the measurement of EFV and metabolites in HIV/AIDS patients by liquid chromatography. The methods differ in relation to the sample preparation, sample volumes, detector used, biological sample, analysis of metabolites, column and mobile phase employed as well as the internal standard used (Table 1).

Most methods used a Mass Spectrometer (MS) for detection, which has more sensitivity and so requires smaller volume of samples for analysis as well as less volume for injection. Besides these advantages, MS generally allows shorter run times, which require less volume of solvents. All methods employed reverse-phase columns, which requires polar solvents as water, acetonitrile and methanol. In general, for plasma analysis using photodiode array (PDA) detector (measures entire wavelength in real time) a large volume of sample (> 100 µL) is required (79-81). Hoffman et al. (2013) developed a method to quantify EFV in a microsampling device (dried blood spot – DBS), allowing just a small volume of sample to be used (20 µL), however the amount of sample extract injected onto the system was high (100 µL), as a PDA (250 nm) was used for detection (82). Injecting high volumes of extracted sample into the system could cause interference in the analysis (by matrix components) or reduce the column life (82). The detection with MS has its advantages, however is not free of drawbacks; the cost of acquiring and maintaining is higher than the PDA system. Of all published methods mentioned in Table 1, just three of them used labelled

internal standard, which is known to improve the reproducibility of samples injected into the chromatographic system (83, 84) [see chapter 2]. As for sample extraction, different preparations can be performed in order to extract the drug from the matrix. Protein precipitation is the simplest and cheapest preparation, however it does not offer a clean extract, which could interfere in the analysis, and be detrimental for the column of the liquid chromatography system (83) and the MS interface. On the other hand, solid phase extraction offers a cleaner extract, however it is more laborious and costly (81, 85). Finally, liquid-liquid extraction exhibits good extraction efficiency, is more selective than protein precipitation and less expensive than solid phase extraction (86-89) [also see chapter 2].

To be clinically applied, the methods need to be validated according to the Bioanalytical Method Validation (e.g. FDA) guidelines (90), which requires the validation of numerous parameters, including linearity (calibration curve), precision and accuracy, recovery, selectivity and sensitivity, stability (under different conditions) and matrix effect, if MS is used. Most of the previous published methods did not measure EFV metabolites, however, in spite of them being not active in terms of antiretroviral activity, their accumulation could contribute to the development of adverse events (91).

Table 1. Methods for the quantification of EFV and metabolites by liquid chromatography.

Reference	Biological samples	Sample volume	Sample preparation	Injection volume	Analyte	Column	Mobile phase	Elution mode and flow rate	Detector
(79)	Plasma	300 µL	SPE, elution with MeOH	80 µL	Efavirenz	150×4.6 mm, 3 µm particle size Supelcosil LC8	Acetonitrile–50 m*M* potassium phosphate + triethylamine 1% pH 5.75 (55:45, v/v)	Isocratic, 1 mL/min	PDA
(86)	Plasma	50 µL	LLE with hexane and ethyl acetate	2.5 µL	Efavirenz	50×2.1 mm 1.7 µm particle size Acquity UPLC BEH C18	0.1% formic acid in water 0.1% formic acid in methanol	Gradient, 0.5 mL/min	MS
(87)	Plasma	100 µL	LLE with MTBE	5 µL	Efavirenz	50×4.6 mmmonolithic Onix C18	Acetonitrile/water (50:50, v/v + 5% of isopropyl alcohol)	Isocratic, 1.5 mL/min	MS
(88)	Plasma	200 µL	LLE with ethyl acetate	2 µL	Efavirenz, 8OH-EFV, 8,14diOH-EFV	100×2 mm, 3 µm particle size Luna C18	Acetonitrile/ 20 mM ammonium acetate + 0.1% formic acid (pH 3.8) (90:10, v/v).	Isocratic, 0.2 mL/min	MS
(80)	Plasma	500 µL	LLE with MTBE and Tris buffer	10 µL	Efavirenz + 9 antivirals	150×2.1 mm, 1.7 µm particle size UPLC BEH C18	Acetonitrile Triethylammonium phosphate 5 mmol/L	Gradient, 0.45 mL/min	PDA
(83)	Plasma	50 µL	PPE with acetonitrile	10 µL	Efavirenz	50 mm×2.1 mm, 3.5 µm particle size Xbridge C18	0.1% formic acid in water 0.1% formic acid in acetonitrile	Gradient, 0.3 mL/min	MS
(92)	Plasma	100 µL	PPE with acetonitrile	10 µL	Efavirenz	100×4.6 mm, 2.6 µm particle size Kinetex C18	Acetonitrile/ Water + 0.1% formic acid (75:25, v/v)	Isocratic, 0.3 mL/min	PDA

(81)	Plasma	500 µL	SPE, elution with methanol/acetonitrile	30 µL	Efavirenz + 5 antivirals	150×4.6 mm, 3.5 µm particle size XBridge C18	Acetonitrile 50 mM acetate buffer at pH 4.5	Gradient, 1mL/min	PDA
(89)	Plasma	250 µL	LLE with ethyl acetate	20 µL	Efavirenz	150×4.6mm, 5.0µm particle size Inertsil ODS	Phosphate buffer (pH 3.5)/acetonitrile (30:70, v/v)	Isocratic, 1.5 mL/min	MS
Chapter 2	Plasma	100 µL	SLE, elution with MTBE	1 µL	Efavirenz, 8OH-EFV, 7OH-EFV, 8,14diOH-EFV	100×2.1 mm, 1.7 µm particle size Kinetex C18	Methanol/water (65:35, v/v + 0.1% formic acid)	Isocratic, 0.4 mL/min	MS
(85)	Saliva	100 µL	SPE, elution with methanol	5 µL	Efavirenz	150mm×3mm, 2.6 µm particle size Kinetex C18	Methanol–water 10:90 (v/v) 5mM ammonium formate buffer in 97% MeOH, pH 5.5	Gradient, 0.4 mL/min	MS
(93)	DPS	100 µL	MTBE+ ammonium 15%	30 µL	Efavirenz + 8 antivirals	50mm×2.0mm, 3 µm particle size Atlantis T3 C18	0.05% formic acid in water 0.05% formic acid in acetonitrile	Gradient, 0.25 mL/min	MS
(84)	DBS	10 µL	Acetonitrile, methanol and 0.2 M zinc sulphate in water (1:1:2, v/v/v)	20 µL	Efavirenz + 5 antivirals	50mm×2.0mm Gemini C18	Methanol+ammonium acetate with acetic acid Methanol	Gradient, 0.25 mL/min	MS
(82)	DBS	20 µL	Elution buffer (10mM KH_2PO_4 w/ 75% ACN)	100 µL	Efavirenz	150mm×4.6mm MAC-MOD Ace 5 C18	10mM potassium phosphate buffer, pH 3.1-3.15/ACN (51:49 v/v)	Isocratic, 0.75 mL/min	PDA

(94)	Dried breast milk	20 µL	Elution with methanol	10 µL	Efavirenz	100 mm×2.1 mm, 3 µm particle size Fortis C18	1 mM ammonium acetate in water 1 mM ammonium acetate in acetonitrile	Gradient, 0.4 mL/min	MS

SPE, solid phase extraction; MeOH, methanol; ACN, acetonitrile; KH_2PO_4, monobasic potassium phosphate; LLE, liquid-liquid extraction; PPE, protein precipitation; MS, mass spectrometer; DBS, dried blood spots; SLE, supported liquid extraction; MTBE, methyl terc-butyl ether; PDA, photodiode array; DPS, dried plasma spots; 8OH-EFV, 8 hydroxy-efavirenz; 7OH-EFV, hydroxy-efavirenz; 8,14diOH-EFV, 8,14 dihydroxy-efavirenz.

2.2.6 Summary

Efavirenz has been used worldwide for the treatment of HIV/AIDS for more than two decades and continues to be the first-line regimen for adolescents and adults in many countries (AIDSMAP, 2019). Despite good efficacy, EFV has side effects in many populations (66, 91), and has high inter- and intra-patient variability in plasma concentrations (70). Toxicities experienced by patients under EFV treatment are usually related to high drug concentrations caused by interindividual differences, including demographic variates, co-administration of other drugs and especially genetic (*CYP2B6*) factors (45).

Papua New Guinea has the highest incidence of HIV in the Western Pacific Region as well as a very high frequency of the c.516G>T variant well known to cause increased EFV concentrations (12), however no PK study had been conducted so far to investigate the relationship between plasma drug concentrations and the presence of the c.516G>T variant in this population. In addition, no study had investigated the influence of demographic covariates on intra- or interpatient variability plasma concentrations in this population. A combined study of PK (along with TDM) and pharmacogenetics (PGx) in specific populations is important in order to assure efficacy and to avoid drug related toxicities and so discontinuation of treatment. Quantification of EFV concentrations is necessary for the application of TDM and so reliable methods are required. Several methods have been developed in order to quantify EFV, however few have analysed EFV metabolites, even though they had been associated with neurotoxicity *in vitro*. Moreover, none of those has studied the stability at high temperatures for all the main metabolites, which is important for the reliable measurements of the metabolites.

2.3 Tenofovir disoproxil fumarate (TDF) and lamivudine (3TC)

Tenofovir (TNF), a nucleotide reverse transcriptase inhibitors (NtRTI) and lamivudine (3TC), a nucleoside reverse transcriptase inhibitors (NRTI) are frequently prescribed worldwide as part of the HAART for the treatment of HIV (31). Recommended dosage for HIV-infected adults is 300 mg daily of each drug, whilst for paediatric patients the dose is reduced according to body weight. For patients with renal impairment, 3TC and TDF are adjusted in accordance with creatinine clearance (CrCl) (95, 96).

2.3.1 Tenofovir disoproxil fumarate

Tenofovir disoproxil fumarate is a hydrophilic molecule (octanol/phosphate buffer – pH 6.5, logP 1.3 at 25ºC) with a pKa of 3.75 and a total mass of 635.5 g/mol (97). TDF undergoes esterase hydrolysis (in gut and plasma) to TNF. Then, TNF is phosphorylated by cellular kinases to the monophosphate intermediate and then to the pharmacologically active metabolite tenofovir diphosphate (TNF-DP) (Figure 5) (97, 98). The oral bioavailability of TDF in fasted patients is low, approximately 25% (99). Following oral administration of a single dose (300 mg), peak plasma tenofovir concentrations of ~300 ng/mL are reached 1 to 2 hours post-dose, with a terminal half-life of approximately 17 hours (96). Lamorde et al. (2012) investigated the effect of food on the steady-state PK of TNF, showing that food intake increases the oral bioavailability, with a 4% and 19% increase in maximum concentration (C_{max}) and area under the curve (AUC), respectively (100). *In vitro*, TNF is less than 1% bound to plasma proteins (101). The apparent volume of distribution at steady-state is 1.3 ± 0.6 L/kg (96). Median trough plasma concentrations (C_{trough}) of TNF have been reported in different ethnicity groups, varying between 42 and 92 ng/mL (102-105).

In vitro studies show that tenofovir is not a substrate for the cytochrome P450 (CYP450) enzymes (96). Tenofovir is mostly eliminated unchanged in the urine by a combination of

active tubular secretion and glomerular filtration (99). The proteins involved in the transport into the kidneys tubular cells are the organic anion transporters (OAT) 1 and 3, encoded by *SLC22A6* and *SLC22A8* genes, respectively. Then, TNF is secreted to the tubular lumen by multi-drug resistant protein 2 (MRP2) and multi-drug resistant protein 4 (MRP4), encoded by *ABCC2* and *ABCC4* genes, respectively (106).

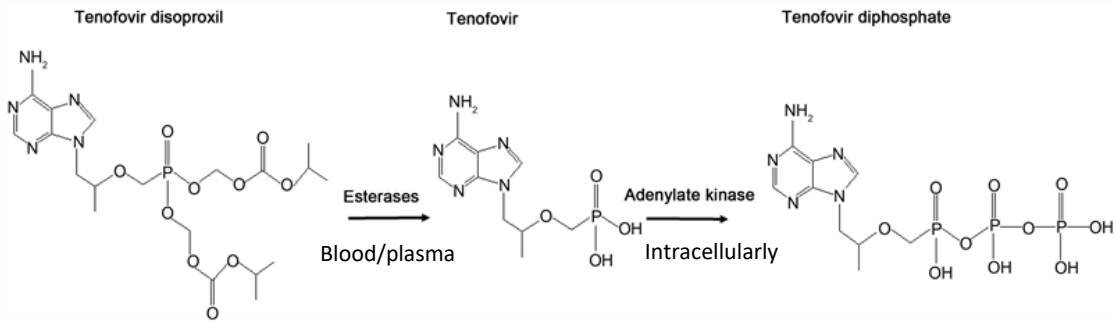

Figure 5. Metabolism of tenofovir disoproxil fumarate (107).

Gagnieu et al. (2008) developed a population pharmacokinetic model of TNF, describing the model as a 2-compartment model with first-order absorption rate (103). Inter- and intra-individual variability of TNF clearance was estimated in 175 patients including different covariates, as age, body weight, height, sex, serum creatinine, calcium and phosphate levels, blood urea nitrogen, and dose per interval or per day of all antiretrovirals comedications. They found a relatively high interpatient (20%) and interoccasion (30%) variation of TNF oral clearance (and AUC) after accounting for variability due to body weight and serum creatinine. In this model, other antiretroviral comedications had no influence on the variation of TNF clearance, however, Baxi et al. (2014) showed an association between TNF AUC and the concomitant use of ritonavir (1.33-fold increase in TNF AUC, p= 0.002) (108). Gervasoni et al. (2013) (n= 100), found body weight (p= 0.002) and serum creatinine (p= 0.003) to be independently associated with plasma TNF concentrations, in particular women with body weight < 50 kg had significantly higher concentrations compared to men and body weight > 50 kg (160±93 vs. 71±52 ng/mL, p< 0.001) (109).

Most common side effects following tenofovir use include nausea, vomiting, diarrhoea, flatulence, weakness, headache, dizziness, hypophosphataemia, and nephrotoxicity (96). Different degrees of renal toxicity have been reported, including proximal tubulopathy, nephritis, acute tubular necrosis, proximal renal tubular dysfunction (PRTD) and diabetes insipidus (98, 110-112).

High plasma TNF concentrations and covariates (age, estimated glomerular filtration rate (eGFR), CrCl, concomitant use of protease inhibitors, sex, body mass index (BMI)) were previously associated with the development of drug-related kidney and bone toxicities. Kidney toxicities included PRTD, proximal tubulopathy, nephritis, Fanconi Syndrome and kidney tubular dysfunction (KTD) (98, 108, 109, 113-115). Rodriguez-Novoa et al. (2010) showed that patients with plasma TNF concentrations >160 ng/mL were at a 5 times higher risk of experiencing KTD than patients with concentrations below this cut-off value (116). Poizot-Martin et al. (2013) suggested a threshold of > 90 ng/mL of TNF C_{trough} as a predictor of KTD (102).

Genetic polymorphisms in genes that encode the proteins involved in the transport of TNF have been studied and associated with higher TNF plasma concentrations. Kiser et al. (2008) were the first to identify a relation between *ABCC4* 3463 A>G genotype and TNF-DP, where the presence of the 3463G allele was associated with 35% increase TNF-DP concentrations (117). In a multivariate analysis, after controlling for body weight, eGFR, and concomitant use of ritonavir, Rungtivasuwan et al. (2015) showed that patients with *ABCC4* 4131TG or GG genotype had 30% higher mean TNF plasma concentrations than in patients with TT genotype (p= 0.007) (118). They also studied *ABCC4* 3463 A>G genotype, however no association was found in a Thai HIV-infected cohort (p= 0.177) (118).

2.3.2 Lamivudine

Lamivudine (3TC) has an octanol/saline partition coefficient of 0.12 and a molecular mass of 229.3 g/mol (pKa= 4.3), with the molecular formula of $C_8H_{11}N_3O_3S$ (119). The oral bioavailability is approximately 86%. Following administration of 150 mg twice daily, C_{max} is reached in 0.5 to 1.5 h post-dose (~1.4 µg/mL) and the $AUC_{(0\text{-}12\ h)}$ ~5 µ×h/mL, both increasing proportionally to oral dose over the range from 0.25 to 10 mg/kg (95, 120, 121). The absorption of 3TC was slower in the fed state compared to fasted state, with C_{max} 40% lower, however no different in systemic AUC was observed when 3TC is administered with or without food (95). The oral steady-state volume of distribution (1.3 L/kg) is independent of dose and does not correlate with weight (122) and the plasma protein binding is 36% (95, 120). The terminal half-life of 3TC is approximately 5 to 8 h (120, 123).

Lamivudine must be phosphorylated intracellularly to its active metabolite, lamivudine triphosphate (3TC-TP) (Figure 6) (124). Pharmacokinetic properties of 3TC were not altered by hepatic impairment, suggesting that it is not significantly metabolised by liver enzymes (125). The majority (~ 70%) of 3TC is eliminated unchanged in urine by active organic cation secretion and only around 5 to 10% is metabolised by the liver to trans-sulfoxide 3TC (125). Pharmacokinetics of 3TC are significantly altered in patients with renal impairment, therefore it is recommended to adjust the dosage according to their levels of kidney function (based on CrCl or eGFR) (126). The most common adverse effects are nausea, fatigue and malaise, headache, diarrhoea and cough (95); although rare, lactic acidosis and severe hepatomegaly with steatosis are life-threatening complications (127).

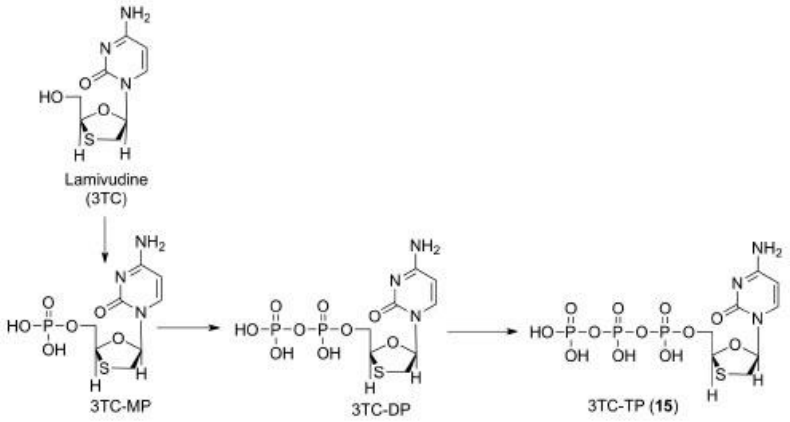

Figure 6. Intracellular metabolism of Lamivudine (124).

Different factors can alter 3TC PK, including drug-drug interaction, either co-administration with other antiretrovirals or non-retroviral drugs (126). Ceckova et al. (2018) observed a decrease in 3TC renal excretion when administered with EFV due to EFV inhibition of the organic cation transporters (OCT1, OCT2), consequently reducing 3TC recovery in urine and enhancing its retention in the renal tissue, potentially increasing drug toxicity (128). A decrease of 35% in 3TC clearance and an increase of 43% of the 3TC AUC was observed when co-administered with trimethoprim-sulfamethoxazole (122). In an adult healthy volunteer study, Adkison et al. (2018), found that sorbitol when administered during 3TC treatment cause a dose-dependent decrease in 3TC exposure mainly affecting its absorption. Sorbitol (13.4 g) caused a reduction of 3TC C_{max} and AUC by 55% and 44%, respectively (129).

Panhard et al. (2007) performed a population PK analysis whereby 3TC PK was best described using a one-compartment model with zero-order absorption and first-order elimination (130). They assessed the variability of PK parameters based on what co-administered protease inhibitor each patient was taking, their age and BMI. The mean absorption time (T_a) (how quickly the drug enters the bloodstream) for all 54 patients was 1.46 h, however for those receiving nelfinavir opposed to indinavir, 3TC T_a was 40% lower

(p< 0.0001). For an increase of 10 years of age, a 36% increase in 3TC T_a was found (p= 0.014). One BMI unit increase was associated with a 10% decrease in the 3TC T_a of 3TC (130).

2.3.3 Summary

Kidney toxicities reported among HIV patients taking TDF and 3TC have been associated with high drug concentrations of TNF (98, 109, 131). The variability of TNF concentrations has been studied in several populations, however as yet, not in PNG patients. As for 3TC, there is a lack of studies around the variability and the association of plasma concentrations with side effects. Therefore, as part of the present study, the variability and association between drug concentrations and the parameters (CrCl and eGFR) used to predict renal toxicities will be conducted in PNG patients, as well as the correlation between all HAART drug concentrations (EFV, 3TC and TNF).

2.4 Dolutegravir

Dolutegravir, an orally administered HIV INSTI, was approved by the FDA in 2013 (132, 133). Integrase strand transfer inhibitors block the action of the viral integrase enzyme, preventing virus integration into the host DNA (134). After assessing benefits and impediments to its use, the WHO changed its recommendation to this newer drug DTG as the preferred first-line treatment for all populations (31). In the most recent PNG National guidelines for HIV care and treatment (135), EFV was replaced by DTG as the first-line drug in combination therapy (DTG 50 mg + TDF 300 mg + 3TC 300 mg for adults). No dose adjustment is necessary for patients with mild or moderate hepatic impairment, however due to lack of investigation on DTG PK for severe hepatic impairment, for these patients DTG is not recommended (133, 136). In general, no dose adjustment is necessary for patients with renal impairment (133).

Dolutegravir is a slightly water soluble molecule (octanol/pH 7.4 buffer ratio= 1.62) with a mass of 419.4 g/mol and pKa of 8.2 (133). When administered once-daily (50 mg orally), it reaches peak plasma concentrations of ~3.5 µg/mL 2 to 3 hours post-dose, with a terminal half-life of approximately 12 to 15 hours, achieving steady state within 5 days (133, 137, 138). Dolutegravir is highly bound to human plasma proteins (>99%) mainly to albumin and, to a lesser extent, to alpha 1-acid glycoprotein; DTG has an apparent volume of distribution of 17.4 L, and an apparent clearance of 0.9 L/h (139, 140). The effect of food on DTG oral absorption was studied by Song et al. (2012), during phase III clinical trials on healthy subjects receiving DTG 50 mg (141). They showed that concomitant ingestion with food increased (33-66% for low- to high-fat meals) DTG exposure and reduced the rate of absorption, however this increase is not anticipated to affect clinical safety, hence the FDA suggests that DTG can be taken with or without food (133, 141).

The metabolic scheme of DTG is represented in Figure 7. Glucuronidation by UGT1A1 is the major pathway, forming the majority of an ether glucuronide of DTG (M2), which is also formed by UGT1A3 and UGT1A9 but at a much lower rate ~2% and 5%, respectively (142). An oxidative metabolite (M3) is formed by CYP3A4 (contributing 10-15% to DTG metabolism), and finally an *N*-dealkylated metabolite (M1) is formed through hydrolysis of M3 (143, 144). DTG is a substrate for the transporters P-glycoprotein (P-gp) (encoded by *ABCB1* gene) and breast cancer resistance protein (BCRP) (encoded by *ABCG2* gene), but displays no inhibition or induction of transporters (140).

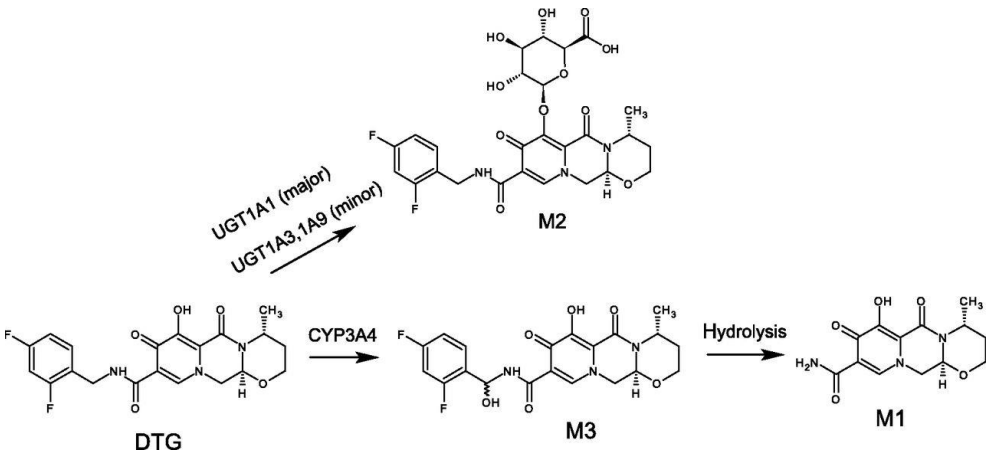

Figure 7. Metabolic scheme of DTG (143).

DTG mass balance was assessed by Castellino et al. (2013), following single oral dose of [^{14}C] DTG 20 mg in healthy human individuals (144). Sixty four percent of the total oral dose was excreted unchanged in faeces (3.1% excreted as oxidative metabolic products), while 31.6% was excreted in urine, represented by M2, M3 and M1 accounting for 18.9%, 3% and 3.6% of total dose, respectively. Absorption of DTG was rapid with at least 34% of the administered dose being absorbed, based on the fractions of the dose renally eliminated (31.6%) and excreted in the faeces as oxidative metabolic products (3.1%). The renal excretion of unchanged DTG was less than 1% of the total dose (144).

A population pharmacokinetic (PK) model of DTG in HIV treatment-naïve patients was developed by Zhang et al. (2015) (139). The PK analysis combined data from three clinical trials (total n= 563 subjects), exploring the influence of several covariates on the exposure of DTG. DTG PK was best described by a one compartment model with absorption lag time and first order absorption and elimination as the final base model. Gender was a predictor of oral bioavailability (21% higher in females), apparent clearance and volume of distribution increased with weight, clearance was 16% higher in smokers compared to non-smokers and a decrease in clearance was observed with increasing total bilirubin. However,

the magnitude of the effects of these covariates on steady-state plasma DTG exposure was relatively small (<32%) and was not considered clinically significant.

A few other covariates were studied, such as ethnicity, albumin, creatinine clearance, serum alanine and aspartate aminotransferase, but no influence on DTG PK was observed (139). In general, it is well tolerated and clinically effective in treatment-naïve patients (145, 146), treatment-experienced and INSTI-naïve patients (147), and INSTI-experienced patients (148). DTG PK variability is moderate, with Coefficients of Variation (CV%) of 25-50% for AUC, C_{trough} and the C_{max} (137, 139, 140, 149). DTG therapeutic range has not been established as yet, however mean plasma C_{trough} values were between 1000 – 4000 ng/mL (150-153). Development of neurological side effects have been reported at 6060 ng/mL (154).

Evidence from randomized controlled trials showed the total incidence of DTG adverse effects to be close to 90%, however these were predominantly mild reactions that ceased with time and might not be entirely drug related, and leading to a low discontinuation rate of therapy (~3%) (145, 147, 148). Common side effects are diarrhoea, fatigue, headache, and nausea but the proportion of severe reactions was 1-10% (133, 137, 155). Most recent studies have shown neuropsychiatric toxicities, including headache and peripheral neuropathy (156), and sleep disturbances, insomnia, depression, anxiety, irritability (157). Neurotoxicity was the cause of therapy discontinuation in 1.7% of patients (total n= 1950) (156), and 5% of the patients (total n= 517) (157). Hoffman et al. (2017) studied 985 INSTI treatment-experienced German patients, observing a similar proportion (5.6%) of the total patients having neuropsychiatric adverse effects leading to discontinuation of therapy with DTG (158). Particular concerns about the prescription of DTG to pregnant women have been discussed, as preliminary data suggest an increase in neural tube defects when using DTG at the time of conception (159).

The most frequently studied polymorphisms affecting DTG metabolism are those in the *UGT1A1* gene, especially those that result in decreased enzyme activity (*UGT1A1*28, *6* and **37*) (160-162). Chen et al. (2014) have reported an increase of 32% in DTG C_{max} and 46% in the AUC in poor metaboliser patients (**28/*28*; **28/*37*; **37*37*), compared to normal UGT1A1 function (**1/*1* and **1/*36*) (160). Elliot et al. (2020) observed a 27% increase in AUC_{0-24} in *UGT1A1*28* poor metabolisers (161).

Polymorphisms in other genes have also been studied, including *ABCG2* (c.421C>A; c.34G>A) and *NR1I2* (c.63396C>T), being associated with increased DTG plasma concentrations (161, 163). Elliot et al. (2020) reported a 28% increase in DTG C_{max} in patients homozygous for the *ABCG2* c.421A variant compared to homozygous for the c.421C allele; while for those homozygous for the *NR1I2* c.63396T variant allele, DTG C_{max} was 24% higher than for those homozygous for the c.63396C allele (161). Genetic variations in *ABCB1* gene are known to affect transporter function and thus substrate exposure. In particular, studies have indicated that a SNP in *ABCB1* 3435 C>T (rs1045642) alters P-gp expression and may affect the PK of some P-gp substrates (164, 165). However, no correlations between DTG plasma concentrations and any genotypes of *ABCB1* 1236 C>T, 2677 G>T/A, 3435 C>T, and 4036 A>G were found (163).

Dolutegravir replaced EFV as the first-line drug for the HIV treatment in PNG at the end of 2019 (135). So far, no studies have investigated the influence of demographic covariates on DTG intra- or interpatient variability in this population. Variability in DTG concentrations could contribute to the development of side effects, and as for EFV, TDM could be a useful tool in order to minimize the adverse events whilst maintaining the treatment efficacy. A few methods have been developed and validated to measure DTG in plasma (151, 152, 166-168); however, there are no published methods of the measurement of plasma DTG in microsampling devices, probably due to its relatively recent introduction to the market.

3. HIV drug monitoring

Drugs for HIV have narrow therapeutic indices with, in some cases, life-threatening toxicities, characteristics that qualify them as TDM targets (67, 77, 169). Therapeutic drug monitoring is used in order to reduce the occurrence of side effects and enhance efficacy, ultimately individualisation of the dose by maintaining drug concentrations within a target range (169). Drug quantification in the context of TDM requires precise, accurate and sensitive analytical technologies as liquid or gas chromatography (separation of the compounds), and a detector, usually ultraviolet (UV), PDA or the preferred MS (170).

The interpretation of drug concentration measurements in the context of TDM is usually based on reference ranges established in plasma or serum samples (171). The usual sampling strategy to estimate pharmacokinetics parameters of HIV drugs requires, after phlebotomy, plasma separation from venous blood. Several studies have reported method validations for the quantification of HIV (80, 81, 86, 89, 151, 152, 166-168, 172) drugs in human plasma. However, this conventional sampling approach requires special transport of samples (frozen samples), which is not ideal in the case of a remote site such as PNG. In fact, the transport of blood samples in one box with dry ice from PNG to Australia cost our research group almost AUD$7000. To overcome this sampling approach, the use of dried samples, such as dried blood spots (DBS) and volumetric absorptive microsampling (VAMS) could be an alternative for sample transportation from distant sites to reference laboratories, not requiring refrigeration (173-176).

Although microsampling devices have substantial advantages, they are not free from drawbacks. Clinical use requires extensive method validation and translation of the concentrations found in capillary blood to plasma (reference ranges) (173, 177-179). For complete microsampling device drug validation, a short plasma method validation needs to be performed to analyse the plasma of the same patients. Once the methods with the

microsampling devices are validated there is no need for plasma collection and analyses (174).

PNG is a low-income country with a high incidence of infectious diseases but insufficient infrastructure, financial, and health/medical resources to readily control and eradicate these diseases on its own. This is compounded by significant drug-resistance resulting from both drug-induced (i.e. non-optimized drug therapy) and organism-induced mechanisms (180). A clear need exists to better optimize the therapy of these diseases, and one of the most important steps to address the pharmacotherapy of these infectious diseases is through pharmacokinetic (monitoring the blood concentration) and drug efficacy monitoring.

3.1 Microsampling strategies – DBS and VAMS

DBS and VAMS are microsampling devices used as an alternative to standard blood sampling technique (Figure 8). One drop of capillary blood is required for sampling on these devices, usually collected from finger pricks. Following sampling, the devices must be left at room temperature to dry out for at least 3 hours. In high humidity countries such as PNG, it is essential that the DBS cards are stored with desiccants packets in order to reduce moisture and humidity. After this time, they can be packed, identified and sent to the reference laboratory by normal mail. Other advantages include minimally invasive sampling, often high analyte stability, minimal biological risk after drying (including HIV and TB patients) and the possibility of self-sampling by patients (177, 181). Method development and validation using these devices have been performed for many drugs, such as paclitaxel (177), docetaxel (179), topiramate (182), vancomycin (183), irinotecan (178), carbamazepine (184), efavirenz (82, 185), isoniazid (186) and rifampicin (187), lamivudine and tenofovir (188).

Figure 8. Illustration of blood microsampling devices, A. Whatman Protein Saver 903® for dried blood spots sampling, B. Volumetric Absorptive Microsampling – VAMS®, Neoteryx.

Despite the advantages of the DBS strategy towards plasma collection, this technique is not free of drawbacks. An extensive validation must be developed considering that varying haematocrits (HT) can affect the volume of blood in the spot, especially for DBS (189). Other disadvantages include contamination, poor quality of samples associated to self-sampling and also the need to translate the estimated concentrations in DBS to plasma (173).

To overcome the HT problem when validating with DBS, a novel microsampling approach has been used; VAMS shows advantages over DBS related to sampling volume accuracy, thus eliminating the HT effect on sample volume (175). VAMS is a device that includes a plastic handle and a globous tip, with a diameter of about 4 mm. After finger pricking, the tip is gently placed in the drop of blood formed in order to absorb a fixed volume of sample into its pores: 10 µL, 20 µL or 30 µL, accordingly to tip size. Then, the VAMS device is left to dry for about 3 hours and is ready to be used (175).

The clinical application of TDM using DBS and VAMS as the sampling strategy demands analytical methods with high sensitivity and specificity, such as liquid chromatography-mass spectrometry (LC-MS/MS), particularly considering the small amount of blood available for testing, usually in the range of 5 to 50 µl (173, 175). Additionally, before clinical application, these drug measurement assays require extensive validation, including

tests to evaluate the impact of blood haematocrit on accuracy in the case of DBS. Moreover, a clinical application study is mandatory to adequately translate DBS and VAMS concentrations to plasma levels, considering the therapeutic range that is established for plasma (171, 173, 174).

In the last few years, a few studies reported the development and validation of methods to quantify EFV in DBS, but not for its metabolites (82, 84, 185, 190). The latest approved drug against HIV, DTG, has not, to date, been quantified in any microsampling devices from HIV/AIDS patient samples. As a novel approach, VAMS was never used as a microsampling strategy for the quantification of EFV and metabolites, and neither to quantify DTG. The use of microsampling devices in the context of TDM could simplify the transportation logistics and allow the conduction of a PK study of these drugs in PNG population. Even though the population is predominantly poor metabolisers of EFV, no PK study has been conducted until now. The PK study is also important to address the question regarding interactions at the pharmacokinetic level between RIF and EFV already reported in the literature (191, 192).

4. Overall summary

HIV continues to be a major public health issue worldwide, but it is of particular concern in low-income countries. However, different from the past when HIV was a life-threatening illness, nowadays it has become a lifelong chronic disease and so treatment should be readily managed (193). Even though every year more and more people obtain access to treatments that control the progression of the disease, the rate of new infections is also increasing (3). The drugs used for HIV therapy have noted side effects, often a narrow therapeutic range and high inter- and intra-patient variability, which makes them good candidates for TDM (77, 194).

Therapeutic drug monitoring is applied in order to keep drug concentrations in range to ensure efficacy and avoid the occurrence of side effects. It also has been proposed to confirm short-term adherence (195); Calcagno et al. (2017) used TDM to demonstrated that poor adherence was an independent predictor of virological failure (196). In order to perform TDM, the quantification of the drugs has to be performed and for that, analytical methods have to be developed and validated (170). For the identification and quantification of plasma EFV, TNF, 3TC and DTG several methods have been previously published. The use of microsampling devices has been applied for the quantification of EFV, TNF and 3TC, however as DTG has been introduced onto the market later than the others, no methods have been published using DBS or VAMS to date. Microsampling devices do not require special transport, which is particularly advantageous for remote sites as PNG.

The variability in HIV drugs PK comes from non-genetic (demographics, comorbidities, drug-drug interactions) and genetic factors (polymorphisms in genes that encode enzymes responsible for the metabolism and/or transport of the drugs). These factors are often related to the occurrence of side effects in patients undertaking HIV drugs. The most studied genetic variant involved in EFV variability is *CYP2B6* c.516T and it has been associated with high EFV concentrations and consequently side effects. The highest frequency of this variant in the world was reported in PNG, however, no studies (PK *vs.* PGx) have been performed in this population. Papua New Guinea has also a very high incidence of HIV/AIDS which contributes to the need of studies to attain knowledge about this association (EFV PK *vs* PGx). Tenofovir and 3TC are not metabolised by the CYP450 enzymes, and the side effects associated with the use of these two NRTIs are kidney related because they are mainly renally eliminated. Previous studies have shown the association between high concentrations of TNF and variations on CrCl and eGFR. However, there is a lack of investigation on the variability of 3TC.

In the present thesis the variability in the pharmacokinetics of EFV, 3TC and TNF will be explored and analytical methods for the quantification of plasma EFV, 3TC and TNF will be developed, validated and clinically applied to PNG HIV/AIDS patients. Microsampling devices will be used to develop and validate a method to quantify DTG.

4.1 Research gaps and aims

The gaps to be addressed in this clinical pharmacology study in PNG HIV/AIDS patients are:

1. whether EFV and metabolites are stable under the high temperature conditions commonly used in preparation of samples;
2. whether and how much does the CYP2B6 c.516T allele affect plasma EFV and 8-hydroxyefavirenz concentrations;
3. whether the analysis of plasma 3TC, TNF and DTG can be simultaneously performed using the same extraction procedure and method detection;
4. whether demographic factors and renal function influence the variability in 3TC and TNF concentrations;
5. whether a method to quantify DTG in DBS and VAMS devices can be developed, validated and clinically applied in HIV/AIDS patients.

To bridge these research gaps, I aimed to assess:

1. the stability of EFV, 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV in plasma at 60ºC for one hour. Including stability under different conditions and other parameters of method validation following FDA bioanalytical method guidelines. Moreover, the clinical application of the method in PNG HIV/AIDS patients – addressed in Chapter 2.

2. the first investigation of the impact of CYP2B6 c.516T and patients demographics on plasma EFV and 8-OH-EFV concentrations, metabolic ratio (8-OH-EFV/EFV), and their association with adverse effects in PNG HIV/AIDS patients – addressed in Chapter 3.

3. the challenges of developing a LC-MS/MS method for the simultaneous quantification of TNF, 3TC and DTG in plasma samples – addressed in Chapter 4.

4. the first investigation of impact of demographic factors and renal function on the variability in 3TC and TNF plasma concentrations in a PNG HIV/AIDS population. Including the development and validation of a method for the simultaneous quantification of TNF and 3TC by LC-MS/MS and its clinical application in HIV patients – addressed in Chapter 5.

5. the development and validation of the first method for the quantification of DTG in DBS and VAMS by LC-MS/MS. This includes the application of the validated method to 15 PNG HIV/AIDS patients – addressed in Chapter 6.

Chapter 2: Method development and validation for the quantification of EFV and metabolites in human plasma by LC-MS/MS

Statement of Authorship

Title of Paper	Instability of Efavirenz Metabolites Identified During Method Development and Validation
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Journal of Pharmaceutical Sciences. 2021; 110(10):3362-3366. DOI: 10.1016/j.xphs.2021.06.028.

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Contribution to the Paper	Study design, performed all the experiments and statistical analysis, interpreted the results, wrote the first manuscript draft and acted as the corresponding author.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	30/08/2022

Journal of Pharmaceutical Sciences 110 (2021) 3362–3366

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Rapid Communication

Instability of Efavirenz Metabolites Identified During Method Development and Validation

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ARTICLE INFO

Article history:
Received 23 April 2021
Revised 20 June 2021
Accepted 21 June 2021
Available online 24 June 2021

Keywords:
HIV/AIDS
Efavirenz
Liquid Chromatography-Mass Spectrometry (LC-MS)
Chemical stability
Metabolites

ABSTRACT

Accurate quantification of efavirenz metabolites in patient samples is required to investigate their potential contribution to efavirenz adverse events. This study aimed to validate a LC-MS/MS method to quantify and investigate the stability of efavirenz and metabolites in human plasma. Compounds were extracted from plasma by supported liquid extraction and resolved on a C18 column. Validation was performed following FDA bioanalytical method validation guidelines. Stability under common conditions of sample pre-treatment and storage were assessed. Efavirenz and 8-hydroxyefavirenz were stable for all conditions tested. 7-Hydroxyefavirenz and 8,14-dihydroxyefavirenz were not stable in plasma at room temperature for 24 h (46%-69% loss), -20°C for 90 days (17%-50% loss), or 60°C for 1 h (90%-95% loss). Efavirenz and 8-hydroxyefavirenz concentrations in HIV/AIDS patient (n=5) plasma prepared from pre-treated (60°C for 1 h) whole blood varied from 517-8564 ng/mL and 131-813 ng/mL, respectively. 7-Hydroxyefavirenz and 8,14-dihydroxyefavirenz concentrations were below validated lower limits of quantification (0.25 and 0.5 ng/mL, respectively), most likely due to sample pre-treatment. This is the first report of 7-hydroxyefavirenz and 8,14-dihydroxyefavirenz instability under conditions commonly used in preparation of samples from HIV/AIDS patients. Alternative biosafety measures to heat pre-treatment must therefore be used for accurate quantification of plasma 7-hydroxyefavirenz and 8,14-dihydroxyefavirenz.

Introduction

The non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) has been used for the treatment of HIV-1 positive patients for more than 20 years.[1] EFV is recommended as an alternative first-line regimen (along with lamivudine and tenofovir) for adults and adolescents worldwide,[2] and a first-line treatment for HIV in China, India, Indonesia and Thailand.[3]

EFV is hepatically hydroxylated to 8-hydroxyefavirenz (8-OH-EFV) and 7-hydroxyefavirenz (7-OH-EFV). A dihydroxylated metabolite, 8,14-dihydroxyefavirenz (8,14-diOH-EFV), has also been detected in patients.[4,5] Efavirenz (Sustiva®) submission documents,[6] state that 25% of the ^{14}C dose could be recovered in urine (major 8-OH-EFV, minor 7-OH-EFV, primarily as glucuronide and sulphate conjugates) and 41% in faeces (primarily unchanged EFV), with 34% of the dose unaccounted.[6,7] 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV in unconjugated and conjugated forms have been identified in plasma and CSF with large interindividual variability, contributed by metabolizing enzyme polymorphism (CYP2B6).[1,6,8,9]

Adverse events, in particular central nervous system (e.g. headache, dizziness) and psychiatric (e.g. depression) toxicities, have been associated with plasma EFV concentrations above the therapeutic range of 1000 to 4000 ng mL^{-1}.[10,11] *In vitro*, 8-OH-EFV is an order of magnitude more neurotoxic than EFV and 7-OH-EFV, however, both EFV and 7-OH-EFV are neurotoxic but via a calcium-independent mechanism.[12]

Therefore, quantification of EFV and metabolites may be important because their accumulation could contribute to adverse events,[13] however clinical analyses of EFV metabolites are sparse. In addition, the stability of 7-OH-EFV and 8,14-diOH-EFV in plasma under different conditions, including heat pre-treatment of HIV/AIDS patient blood samples performed by most clinics as a biosafety measure, has not been reported.

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https://doi.org/10.1016/j.xphs.2021.06.028
0022-3549/

We describe a full LC-MS/MS method validation for the quantification of EFV, 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV in plasma, with application to patient samples, and the analysis of analyte stability under common conditions of sample pre-treatment and storage.

Materials and methods

Method application

Patient samples assessed for this study were obtained as part of a large pharmacokinetic, pharmacogenetic and safety study of EFV in Papua New Guinea approved by the University of Adelaide Human Research Ethics Committee (H-2017-167) and Medical Research Advisory Committee of the National Department of Health Government of Papua New Guinea (MRAC No. 16.32). Patients provided written informed consent. The methods that follow were applied to samples from five Papua New Guinean patients who were being treated with efavirenz (600 mg once daily). Whole blood was collected by venepuncture and heated at 60°C for one hour, then centrifuged to separate plasma. Plasma samples were frozen and transported to Adelaide on dry ice and maintained at -20°C until analysis.

Reagents and materials

EFV (lot number 7-ARD-114-5), 8-OH-EFV (1-JLI-23-3), 7-OH-EFV (1-MKM-114-1), 8,14-diOH-EFV (8-GBH-13-2) and labelled internal standards (efavirenz-d5 (8-MMH-114-6), 8-hydroxyefavirenz-d4 (8-GBH-55-3), 7-hydroxyefavirenz-d4 (2-QFY-70-2), 8,14-dihydroxyefavirenz-d4 (5-GBH-85-1) were purchased from Toronto Research Chemicals (North York, Canada). Methanol (MeOH), formic acid and water (all Fisher brand Optima LCMS-grade) were purchased from Thermo Fisher Scientific (Australia). HiPerSolv CHROMANORM® for HPLC (VWR) methyl tert-butyl ether (MTBE) was purchased from Bio-Strategy (Australia), and ammonium hydroxide from Merck (Darmstadt, Germany).

Preparation of drug solutions

Stock solutions of labelled and unlabelled EFV and metabolites were prepared by dissolution in 50% MeOH at 1 mg mL^{-1}. Standard solutions were obtained by combining analytes and diluting in 50% MeOH to obtain 7 different concentrations per analyte: from 250 to 100000 ng mL^{-1} for EFV; 125 to 50000 ng mL^{-1} for 8-OH-EFV; 2.5 to 1000 ng mL^{-1} for 7-OH-EFV; and 5 to 2000 ng mL^{-1} for 8,14-diOH-EFV (concentrations in the standard solution). Labelled internal standard solutions (IS) were prepared by combining and diluting stock solutions to obtain concentrations of 10000 ng mL^{-1} for EFV-d5, 5000 ng mL^{-1} for 8-OH-EFV-d4, 100 ng mL^{-1} for 7-OH-EFV-d4 and 2000 ng mL^{-1} for 8,14-di-OH-EFV-d4 in 50% MeOH.

Quality control (QC) solutions were prepared by combining analytes at three different concentrations (low (LQC), medium (MQC), high (HQC)) in 50% MeOH as follows: EFV 750, 7500, 75000 ng mL^{-1}; 8-OH-EFV 350, 3500, 35000 ng mL^{-1}; 7-OH-EFV 7.5, 75, 750 ng mL^{-1}; 8,14-di-OH-EFV 15, 150, 1500 ng mL^{-1}.

Chromatography and spectrometry conditions

EFV and metabolites were quantified using a Nexera UHPLC system coupled to a LCMS-8040 tandem mass spectrometer (Shimadzu, Tokyo, Japan). Compounds were separated using a Kinetex C18 column (100 × 2.1 mm, 1.7 μm) (Phenomenex, Torrance, CA, USA), at 40°C, and isocratic mobile phase of 0.1% formic acid (35%) and methanol (65%) at 0.4 mL min^{-1}. The autosampler was maintained at 15°C. Retention times for EFV, 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV were 3.3, 2.7, 2.1 and 1.2 min (void time = 0.6 min), respectively with a total run time of 4 min. Ionization by electrospray in negative mode: nebulizing gas flow 2.5 L min^{-1}; drying gas flow 20 L min^{-1}; heating block temperature 300°C; desolvation line temperature 250°C. The quantification mass transitions *(m/z)* for EFV, 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV were 314→69, 330→210, 330→258, 346→262, respectively. Transitions for EFV-d5, 8-OH-EFV-d4, 7-OH-EFV-d4 and 8,14-diOH-EFV-d4 were 319→69, 334→258, 334→258 and 350→229, respectively.

Sample preparation

To prepare calibrator and QC samples for extraction, 10 μL of standard or QC solution was added to 90 μL blank plasma (Transfusion Medicine Unit, Royal Adelaide Hospital, Adelaide, Australia). To 1.5 mL polypropylene tubes (Eppendorf) were added 100 μL of calibrator, QC or patient's plasma samples, 10 μL of IS and 100 μL of 0.5 M ammonium hydroxide. The tubes were vortex mixed and briefly centrifuged (12,000 x g, 1 min), then transferred to a Strata DE 200 μL 96-well plate (Phenomenex®). After 5 minutes, samples were eluted by gravity using 2 × 600 μL of MTBE into a 2 mL collection plate. Eluates were dried in a vacuum centrifugal evaporator (Genevac™ miVac – Thermo Fisher Scientific) at 50°C. Extracts were reconstituted in 100 μL of mobile phase and one microliter was injected.

General method validation

Method validation was performed following FDA bioanalytical method validation guidelines,[14] where the acceptance criteria are a maximum coefficient of variation (CV%) and inaccuracy of 15%, with the exception of the lower limit of quantification (LLOQ) (± 20% of the nominal concentrations). Validation parameters were linearity, precision, accuracy, sensitivity, selectivity, recovery, matrix effect, and stability. Linearity of analyte/IS peak area ratio against nominal concentration ($1/Y^2$ weighting) as well as of natural logarithm (Ln) of peak area ratios against Ln of nominal concentration (unweighted) were assessed by residual plots. Seven batches of blank human plasma were analysed to determine specificity. Within-assay and between-assay precision were calculated as CV%. Accuracy was evaluated by calculating the percentage difference between nominal concentrations and estimated concentrations from the calibration curve. Sensitivity was assessed at the lowest concentration of the calibration curve. Recovery efficiencies were evaluated by comparing peak area ratios of extracted QCs (high, medium, low) spiked with IS post-extraction with extracted blank samples spiked with corresponding QCs and IS post-extraction. For estimation of the matrix effect, blank plasma samples from five different human sources were extracted then spiked with QCs (high, medium, low) and IS, and compared to mobile phase spiked with corresponding QCs and IS.

Stability

Unless otherwise specified, analyte concentrations of all test and control condition samples described below were interpolated from freshly prepared calibration curves and duplicate QCs.

Freeze/thaw and storage stability

LQC and HQC samples were prepared in triplicate in plasma and analysed before and after 3 freeze/thaw cycles. For each cycle, the samples were frozen at -20°C, and kept at room temperature for one hour before re-freezing or analysis. LQCs and HQCs were prepared in triplicate and stored at -20°C for 90 days before analysis to evaluate long-term stability.

Processed sample/autosampler stability

Single LQC and HQC plasma extracts without IS were stored in the LC-MS/MS autosampler and one μL was injected at one hourly intervals for 12 h. A maximum deviation in analyte peak area of ± 15% from the beginning to the end of the series was deemed acceptable.[14]

Bench-top and pre-treatment stability

To avoid biological risk during transport and analysis, patients' samples were heated at 60°C for one hour immediately after blood collection in order to inactivate HIV. This same condition along with room temperature exposure for one, two and 24 h, and 4°C for 24 h was applied to evaluate bench-top stability of LQC and HQC in plasma in triplicate. Additional follow up tests were performed with individual analytes at HQC concentration in duplicate at room temperature for one and 24 h and at 60°C for one hour.

Specific stability tests for 7-hydroxyefavirenz and 8,14-diohydroxyefavirenz

Further experiments based on initial bench-top and pre-treatment stability results (see Results) were performed for 7-OH-EFV and 8,14-diOH-EFV. Phosphate buffer pH 7.4 (fresh plasma pH) and pH 8.2 (frozen plasma pH) were spiked with 7-OH-EFV and 8,14-diOH-EFV to HQC and LQC concentrations (in duplicate for each condition) and kept at room temperature for one and 24 h, and also for 1 h at 60°C, before analysis. Duplicates freshly prepared in buffer were used as controls.

Results and discussion

General method validation

General method validation results are presented in Table 1. Calibration curves of peak area ratio against nominal concentration were not linear for all analytes, whilst Ln (peak area ratio) against Ln (nominal concentration) were linear for all analytes, with coefficients of determination (R^2) ranging from 0.991 to 0.999. Accuracy (97-109%), imprecision (0.4-9.5% CV) and matrix effects (<12%) were acceptable for all analytes. Blank plasma samples showed no interfering peaks. Mean extraction efficiencies were 81% (79-83%), 89% (86-92%), 87% (83-91%) and 58% (56-60%) for EFV, 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV, respectively, having consistent results between the three different levels of QC.

Stability

General stability

Results of stability experiments are provided in Table 2. All analytes were stable after at least 3 freeze/thaw cycles, storage at room temperature for up to 2 h, storage at 4°C for at least 24 h, and in the autosampler for at least 12 hours. Stability of EFV and 8-OH-EFV was acceptable after storage at room temperature for 24 h, -20°C for 90 days, and 60°C for 1h, however it was not acceptable for 7-OH-EFV and 8,14-diOH-EFV, with a maximum loss of 95% in concentration (see Table 2).

Stability tests of each analyte separately in plasma at 60°C for 1 h showed similar results, with no detectable conversion of one analyte to another. This further supports that EFV and 8-OH-EFV are stable, and that 7-OH-EFV and 8,14-diOH-EFV are not converted to EFV or 8-OH-EFV, under these conditions.

Our findings align with those of previous studies demonstrating EFV stability in human plasma tested under different conditions,[15-17] including stability after the blood heating process.[18,19] The stability of 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV has been less studied. Kim et al.[4] reported that after 5 h at room temperature 8-OH-EFV and EFV were stable (<15% deviation), but even though 8,14-diOH-EFV was quantified, no stability data were presented. Here we demonstrate stability of EFV and 8-OH-EFV, and instability of 7-OH-EFV and 8,14-diOH-EFV, beyond conditions previously tested.

Specific stability tests for 7-hydroxyefavirenz and 8,14-dihydroxyefavirenz

Contrary to spiked plasma, the bench-top stabilities of 7-OH-EFV and 8,14-diOH-EFV in pH 7.4 and pH 8.2 phosphate buffer were acceptable for up to 24 h at room temperature (Table 2). When heated to 60°C for 1 h in buffer, decreases in 7-OH-EFV and 8,14-diOH-EFV concentrations were less than in plasma, but still unacceptable for both analytes at pH 8.2, and 7-OH-EFV at pH 7.4 (20-52% loss, Table 2).

Clinical application

EFV concentrations varied between 517 and 8564 ng mL^{-1} and 8-OH-EFV concentrations from 131 to 813 ng mL^{-1}. Three patients had EFV concentrations within, one below and one above, the EFV therapeutic range (1000 - 4000 ng mL^{-1}).[10] Our method had sufficient sensitivity to quantify 7-OH-EFV and 8,14-diOH-EFV

Table 1
EFV and metabolites accuracy and precision, sensitivity and matrix effect.

QC sample	Analyte	Nominal concentration ng mL^{-1}	Precision (mean CV %)		Accuracy (mean %, n=15)	Matrix effect (mean %, n=5)
			Inter-day (n = 3)	Intra-day (n = 5)		
LLOQ	EFV	25	5.6	2.7	98	-
	8OH-EFV	12.5	2.0	2.8	97	-
	7OH-EFV	0.25	9.5	2.2	104	-
	8,14diOH-EFV	0.5	4.3	6.5	101	-
LQC	EFV	75	4.2	3.9	102	-6.1
	8OH-EFV	35	2.9	2.4	103	-6.8
	7OH-EFV	0.75	3.3	3.0	102	-5.1
	8,14diOH-EFV	1.5	5.3	2.5	105	-11.2
MQC	EFV	750	1.2	1.6	106	-10.1
	8OH-EFV	350	1.1	1.8	109	-8.3
	7OH-EFV	7.5	3.3	1.7	103	-11.4
	8,14diOH-EFV	15	2.0	1.9	103	-9.1
HQC	EFV	7500	2.0	1.9	104	-6.0
	8OH-EFV	3500	0.5	1.2	103	-5.6
	7OH-EFV	75	1.4	3.8	107	-10.4
	8,14diOH-EFV	150	2.4	0.4	100	-6.5

QC, quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; EFV, efavirenz; 8OH-EFV, 8-hydroxyefavirenz; 7OH-EFV, 7-hydroxyefavirenz; 8,14diOH-EFV, 8,14-dihydroxyefavirenz; CV%, coefficient of variation.

Table 2
Plasma and buffer stability tested at different conditions for EFV and metabolites.

QC sample	Analyte	Nominal concentration ng mL^{-1}	Auto-sampler stability 12 h (% change vs 0 h, n=1)	Freeze/thaw stability (after 3rd cycle) (triplicate mean % change)	Stability room temperature (triplicate mean % change vs 0 h)		Stability 4°C 24 h (triplicate mean % change vs 0 h)	Stability 60°C 1 h (triplicate mean % change vs 0 h)	Long-term stability (90 days) (triplicate mean % change vs 0 h)	Buffer pH	Stability in buffer room temperature (duplicate mean % change vs 0 h)		Stability in buffer 60°C 1 h (duplicate mean % change vs 0 h)
					1 h	2 h	24 h				1 h	24 h	
LQC	EFV	75	-4.35	-3.28	+0.9	+11.9	+0.8	+2.4	+4.2	+7.3	-	-	-
	8OH-EFV	35	-2.69	-3.44	+12.8	+1.0	-2.64	+8.6	-9.6	-3.4	-	-	-
	7OH-EFV	0.75	-5.46	-5.8	+9.5	+11.2	-54.2	+9.3	-90.5	-17.5	-	-	-
	8,14diOH-EFV	1.5	-10.27	+4.69	+4.8	+9.0	-67.7	+2.0	-94.2	-50	-	-	-
HQC	EFV	7500	-5.37	+0.67	+9.9	+5.6	+7.0	+5.8	+7.0	+0.9	-	-	-
	8OH-EFV	3500	+1.09	-1.44	+7.0	+8.4	-0.6	+3.0	-14.3	-3.8	-	-	-
	7OH-EFV	75	-6.67	-9.49	+8.4	+2.9	-46.1	+8.6	-94.4	-16.5	7.4	-1.0	+3.7	-20.3
											8.2	-8.0	-7.7	-29.3
	8,14diOH-EFV	150	-3.71	-7.14	+7.2	-0.7	-69.2	-4.4	-94.6	-47.7	7.4	+0.2	+0.2	-11.7
											8.2	-7.9	-7.9	-51.8

QC, quality control; LQC, low quality control; HQC, high quality control; EFV, efavirenz; 8OH-EFV, 8-hydroxyefavirenz; 7OH-EFV, 7-hydroxyefavirenz; 8,14diOH-EFV, 8,14-dihydroxyefavirenz.

concentrations previously reported in studies,[4,5] however it is unclear as to whether those studies were able to confirm stability of the metabolites under the conditions of their assays. In the samples we analysed, 7-OH-EFV and 8,14-diOH-EFV concentrations were below the validated LLOQ, being very likely related to the lack of stability under the pre-treatment conditions (see *General stability*) applied to patient samples in this study.

Conclusion

This is the first report of stability failure for the EFV metabolites, 7-OH-EFV and 8,14-diOH-EFV. Based on our study we conclude that these two metabolites are not stable under high temperature conditions commonly used in preparation of samples from HIV/AIDS patients. Therefore, for the purpose of pharmacokinetic studies, we recommend substituting heating pre-treatment with alternative biosafety measures (e.g. PPE and physical containment controls) in order to ensure the reliability of the results for 7-OH-EFV and 8,14-diOH-EFV concentrations. The mechanism of this instability remains unknown, but sample matrix factors (e.g. plasma, pH) appear to be important.

Funding

This work was supported by the Australian Centre for HIV and Hepatitis Virology Research (ACH2) [Personalised medicine for HIV infection in PNG]. Daniel Barratt receives salary support from an Australian Research Council Future Fellowship (FT180100565).

Declaration of Competing Interest

The authors declare no conflict of interest.

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Chapter 3: Impact of CYP2B6 c.516T and patients demographics on plasma EFV and 8-OH-EFV concentrations in PNG HIV/AIDS patients

Statement of Authorship

Title of Paper	Large variability in plasma efavirenz concentration in Papua New Guinea HIV/AIDS patients associated with high frequency of CYP2B6 516T allele
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Clinical and Translation Science. 2021; 14(6):2521-2531. DOI: 10.1111/cts.

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Contribution to the Paper	Study design, performed experiments and statistical analysis, data interpretation, wrote the first manuscript draft and acted as the corresponding author.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	30/08/2022

Received: 20 May 2021 | Revised: 20 June 2021 | Accepted: 2 July 2021

DOI: 10.1111/cts.13120

ARTICLE

Large variability in plasma efavirenz concentration in Papua New Guinea HIV/AIDS patients associated with high frequency of CYP2B6 516T allele

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Funding information
This work was funded by the Australian Centre for HIV and Hepatitis Virology Research (ACH2). Daniel Thomas Barratt receives salary support from an Australian Research Council Future Fellowship (FT180100565).

Abstract

Papua New Guinea (PNG) has a high HIV/AIDS prevalence and very high frequency of the CYP2B6 c.516G>T (rs3745274) variant. We have conducted the first investigation of the impact of c.516G>T and patient demographics on plasma efavirenz (EFV) and 8-hydroxyefavirenz (8OH-EFV) concentrations, metabolic ratio (8OH-EFV/EFV) (MR), and their association with adverse effects, in PNG patients with HIV/AIDS. For 156 PNG patients with HIV/AIDS taking EFV 600 mg/day (for 3–156 months), plasma EFV and 8OH-EFV concentrations were quantified, *CYP2B6* c.516G>T genotyped, and demographic and self-reported adverse effects data recorded. Genotype differences in EFV and 8OH-EFV concentrations, MR, and percent within therapeutic range (1000–4000 ng/ml) were examined, in addition to EFV and 8OH-EFV concentration differences between patients experiencing adverse effects. *CYP2B6* c.516T allele frequency was 53%. Plasma EFV ($p < 0.0001$), 8OH-EFV ($p < 0.01$), and MR ($p < 0.0001$) differed significantly between genotypes, with genotype explaining 38%, 10%, and 50% of variability, respectively. Plasma EFV concentrations were significantly higher in T/T (median = 5168 ng/ml) than G/G (1036 ng/ml, post hoc $p < 0.0001$) and G/T (1502 ng/ml, $p < 0.0001$) genotypes, with all patients above therapeutic range ($n = 23$) being T/T genotype ($p < 0.0001$). EFV and 8OH-EFV concentrations were not significantly higher in patients experiencing adverse effects. In PNG HIV/AIDS population where the 516T frequency is very high, it explains a substantial portion of variability (38%) in EFV disposition; however, at least for the patients receiving EFV long term, this does not translate into significant side effects.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

High efavirenz (EFV) concentrations are strongly associated with the development of adverse effects, particularly central nervous system (CNS) and psychiatric

toxicities. Papua New Guinea (PNG) has a high HIV/AIDS prevalence and the highest frequency of the *CYP2B6* c.516G>T decreased function variant allele of any population assessed to date.

WHAT QUESTION DID THIS STUDY ADDRESS?

Whether there is a gene-dose association among the c.516G>T genotype, EFV and metabolite concentrations, and adverse events.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

In PNG, the *CYP2B6* c.516T/T genotype was strongly associated with substantially higher plasma EFV concentrations and lower metabolic ratio (8-hydroxy-efvairenz/efavirenz) in a gene-dose manner. In PNG, plasma efavirenz and 8-hydroxy-efavirenz concentrations were not significantly higher in patients experiencing adverse effects after the first 3 months of treatment.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

For PNG people taking EFV, early (<3 months) pharmacokinetic, and nonpharmacokinetic, mechanisms of CNS toxicity need investigation.

INTRODUCTION

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor, frequently prescribed as a component of highly active antiretroviral therapy used for the treatment of patients infected with HIV.[1] EFV was introduced onto the market in 1998[2] and has been recommended by the World Health Organization as the first-line drug for HIV treatment for more than 20 years.[3] Although EFV was recently replaced by dolutegravir as the recommended first-line drug,[4] it remains as an alternative first-line regimen for adolescents and adults, and as a first-line therapy in a few countries in Asia and the Western Pacific regions.[5]

Despite its efficacy in the treatment of HIV,[2,6] EFV often leads to the development of adverse effects, in particular those of the central nervous system (CNS) and psychiatric toxicities.[7,8] The likelihood of developing these adverse effects and for achieving therapeutic efficacy has been associated with total plasma EFV concentrations within its narrow therapeutic plasma concentration range of 1000–4000 ng/ml.[9-11] Patients with EFV concentrations above 4000 ng/ml are at greater risk of developing toxicities, whereas patients below 1000 ng/ml are more likely to experience treatment failure.[9] Although the occurrence of adverse effects has been associated with drug concentrations in some studies, other studies did not find an increased risk of CNS and psychiatric toxicities in patients with high EFV plasma concentrations.[12-15]

EFV shows large variability between patients in terms of plasma concentrations, drug-related toxicity, and drug response.[16] This variability is multifactorial, and includes differences in metabolism, ethnicity, gender, body weight, drug compliance, use of comedications, presence of concomitant diseases, as well as genetic factors.[17] Variations in body weight, ethnicity, and comedication affect the clearance of EFV[18,19] yet the association between gender and plasma EFV concentrations appears to be equivocal.[20] Genetic polymorphisms, especially those associated with drug metabolism, have been studied in many different populations, and the effect on plasma EFV concentrations is unequivocal.[11,20,21]

Cytochrome P450 CYP2B6 is the main isoenzyme involved in hepatic EFV metabolism. About 70% of EFV is metabolized to 8-hydroxy-efavirenz (8OH-EFV), predominantly by CYP2B6.[22] CYP2B6 is also involved in the formation of the EFV secondary metabolite, 8,14-dihydroxyefavirenz.[23] The gene that encodes CYP2B6 is highly polymorphic, resulting in altered drug metabolism.[21,24] The single nucleotide polymorphism (SNP) most frequently studied is the c.516G>T (rs3745274) missense variant, which has been associated with decreased activity of the CYP2B6 isoenzyme, and increased plasma EFV concentrations.[11,21,23,25,26,27] This SNP has also been associated with the occurrence of adverse events and EFV discontinuation.[28,29] The c.516T allele frequency varies significantly between different ethnic populations ranging from 16% in Finnish and Southern Han Chinese populations to up to 65% in Papua New Guinea (PNG) populations who have the highest frequency of the variant allele known to date.[30,31]

Even though PNG shows the highest prevalence of the c.516T variant, there are no studies evaluating the relationship among plasma EFV concentrations, adverse effects and *CYP2B6* polymorphisms.[32,33] In the present study, we investigated (a) the impact of *CYP2B6* c.516G>T genotypes and patient demographics such as age, gender

and body weight on plasma EFV and 8OH-EFV concentrations and the metabolic ratio (MR), and (b) the association between plasma EFV and 8OH-EFV concentrations, MR as well as 516G>T genotypes and reported side effects.

METHODS

Patients and sample collection

One hundred fifty-six patients receiving EFV combination antiretroviral therapy at the HIV Heduru Clinic (Port Moresby General Hospital – PNG) from October 2017 to June 2018 were enrolled in the study, after giving written informed consent. This study was approved by the Medical Research Advisory Committee of the National Department of Health of the Government of Papua New Guinea (MRAC No. 16.32) and the University of Adelaide Human Research Ethics Committee (H-2017–167). All patients were receiving EFV 600 mg once daily with tenofovir disoproxil fumarate 300 mg and lamivudine 300 mg as a single tablet (duration of therapy ranged between 3 months and 13 years). Demographic data, such as age, body weight, gender, region, comedication, and comorbidities, were recorded, in addition to the time of dosing over the previous 3 days. Self-reported CNS (tiredness, dizziness, drowsiness, insomnia, and impaired concentration) and psychiatric (agitation, depression, aggression, and euphoria) side effects were also captured with yes/no symptom questionnaires.

Venous blood samples (5 ml) were collected between 8.5 and 22 h post last dose into clot activator (CAT) and lithium heparin (LiHep) blood collection tubes (BD vacutainers, Melbourne, Australia). CAT tubes were allowed to clot at room temperature then heat treated at 60°C for 1 h and stored at −20°C. LiHep tubes were also heat treated at 60°C for 1 h in a water bath, then centrifuged (2500 rpm for 20 min) to separate the plasma, which was stored at −20°C until transportation. The samples (CAT tubes, LiHep plasma, and LiHep buffy coat/red blood cell fraction) were transported on dry ice from PNG to Adelaide (Australia) and stored at −20°C until analysis.

EFV and 8OH-EFV plasma concentrations

Steady-state EFV and 8OH-EFV plasma concentrations were determined using a Nexera ultra-high-performance liquid chromatography (UHPLC) system coupled to a liquid chromatography mass spectrometry (LCMS)-8040 triple quadrupole mass spectrometer (Shimadzu, Tokyo, Japan).[34]

Plasma (100 µl) was added into a tube containing 10 µl of labeled internal standard (1 µg/ml for EFV-d5, 0.5 µg/ml for 8OH-EFV-d4) and 100 µl of 0.5 M ammonium hydroxide. This was vortexed and pipetted into the wells of a Strata DE 200 µl 96-well plate placed on a 2 ml collection plate (Phenomenex, Lane Cove, NSW, Australia). Samples were then eluted using MTBE. Eluates were then dried in a vacuum centrifugal evaporator and reconstituted in mobile phase before injecting 1 µl onto the UHPLC system.

Compounds were separated on a Kinetex C18 PS column (100 × 2.1 mm, 1.7 µm) (Phenomenex) maintained at 40°C. The mobile phase comprised of 35% 0.1% formic acid in LCMS-grade water and 65% LCMS-grade methanol, with a flow of 0.4 ml/min. The chromatogram was run for 4 min with retention times for EFV and 8OH-EFV of 3.3 and 2.7 min, respectively. Mass spectra were acquired in negative ionization mode using electrospray ionization. Mass transitions used to quantify EFV and 8OH-EFV were m/z 314 → 69 and m/z 330 → 210, respectively.

The validated calibration ranges for EFV and 8OH-EFV were 50–10000 ng/ml and 12.5–5000 ng/ml, respectively. Intra- and inter-day imprecision and inaccuracy for EFV and 8OH-EFV were all within 10%. Both analytes were shown to be stable at room temperature for 24 h, −20°C for 90 days, 60°C for 1 h, after at least three freeze/thaw cycles, and in the autosampler for at least 12 h.[34]

DNA extraction and genotyping

DNA was extracted from defrosted and vortexed CAT tube and LiHep buffy coat/red blood cell samples using Maxwell 16 Blood DNA Purification kits (Promega, Sydney, Australia). Purity of the extracted DNA was tested by absorbance (260 nm:280 nm) using Synergy Mx plate reader with Take3 plate (Biotek Instruments Inc., USA). DNA was quantified by Quanti-IT dsDNA broad-range assay kit (Thermo Fisher, Adelaide, Australia) according to the manufacturer's instructions. For samples with low DNA yield (<100 ng), multiple extractions were performed, combined, and concentrated using ReliaPrep DNA Clean-up and Concentration System kits according to the manufacturer's instructions (Promega). *CYP2B6* c.516G>T (rs3745274) genotype was determined as part of a custom Agena MassArray Panel through the Australian Genome Research Facility (AGRF, Brisbane, Australia).

Statistical analysis

Patient demographics (age, gender, and body weight) were summarized as median and range. The following statistical analyses were performed using GraphPad Prism 8.0.0 unless otherwise specified. Chi-squared analysis was used to test for genotype deviation from Hardy–Weinberg equilibrium. Median and range was applied to describe plasma

EFV and 8OH-EFV concentrations and MR, calculated as plasma 8OH-EFV concentration/EFV concentration.

Differences between c.516G>T genotypes in plasma EFV and 8OH-EFV concentrations and MR were determined using the nonparametric Kruskal-Wallis test where a p value of less than 0.05 was regarded as statistically significant. Dunn's multiple comparison test was performed as a post hoc test to compare differences between genotype groups. To test for "gene-dose" effects, Jonckheere-Terpstra exact (permutation) tests for ordered differences were performed in R version 1.2.5033 (RStudio, PBC) using the jonckheere.test function (two-sided with 200,000 permutations) of the clinfun package.[35] Chi-square test was applied to test for genotype differences in the likelihood of being below (<1000 ng/ml), within (1000–4000 ng/ml) or above (>4000 ng/ml) the EFV therapeutic range, with p less than 0.05 considered statistically significant.

To examine the multiple contributions of genetic and demographic variables to EFV concentrations, multiple linear regression analyses were conducted in R. Distributions of continuous variables (weight and age) were initially checked using histograms, quantile–quantile plots, and Box-Cox power transformations (R packages::-functions used were: graphics[36]::hist, stats[36]::qqnorm and qqline, and MASS[37]::boxcox, respectively). Linear regression (stats::lm) analyses were performed separately for EFV, 8OH-EFV, and MR testing for main effects of genotype, age, gender, and weight (weight was not tested for MR). Models were checked for linearity, homoscedasticity, normality, and potential high leverage outliers (stats::plot). Significant explanatory variables were identified by F-test (car::Anova). Relative contributions (R^2) of significant explanatory variables were assessed using the averaging over orderings method (relaimpo[38]::calc.relimp).

For the evaluation of side effects, patients were divided into four groups as follows: no side effects, CNS side effects but no psychiatric side effects, psychiatric side effects but no CNS side effects, and both CNS and psychiatric side effects. Plasma EFV and 8OH-EFV concentrations and MR were summarized as medians and range for each of the four groups. Kruskal-Wallis and Dunn's multiple comparison test were applied to evaluate the relationship between drug concentrations and the occurrence of side effects. For the association between the occurrence of side effects and genotype, the patients were divided into two groups, none and CNS and/or psychiatric side effects, and group proportions compared between genotypes by χ^2 test. Missing data were handled by pairwise exclusion within each analysis. All reported p values are unadjusted for multiple comparisons.

The power of the study to detect a significant difference in EFV plasma concentrations between side effect groups was estimated by performing 10,000 iterations of Kruskal-Wallis tests following random samplings from simulated population (side effect group) distributions of plasma concentrations. Power to detect a significant difference in side effect incidence between *CYP2B6* genotype groups was estimated by performing 10,000 iterations of χ^2 tests following random samplings from simulated population (genotype group) distributions of side effects.

RESULTS

Participant characteristics, drug concentrations, and genotypes

Participant characteristics and genotype frequencies are summarized in Table 1. Demographic data were not available for all participants, and sufficient DNA for genotyping was obtained for 112 patients, resulting in 102 subjects with complete genotype, plasma EFV and 8OH-EFV concentration, and demographic data. *CYP2B6* c.516 G>T

TABLE 1 Demographic data for 156 PNG HIV/AIDS patients receiving 600 mg per day EFV

		N
Age (years), median (range)	37 (14–65)	150[a]
Weight (kg), median (range)	61 (36–120)	148[a]
Female, n (%)	99 (66%)	151[a]
Region		155
Highland, *n* (%)	65 (42)	
Coastal, *n* (%)	90 (58)	
Genotype *n* (%) (*CYP2B6* c.516 G>T)		
G/G	28 (25%)	
G/T	52 (45%)	
T/T	35 (30%)	
No genotype results	41	
Comedications (*n*)		156
None	146	
Isoniazid	5	
Cotrimoxazole	4	
HREZ[b]	1	
Carbimazole	1	
Comorbidities (*n*)		156
None	148	
Tuberculosis	6	
Cancer	1	
Thyroid disorder	1	

Abbreviations: EFV, efavirenz; PNG, Papua New Guinea.

[a]Data not recorded in all case record forms.

[b]HREZ, fixed dose combination of isoniazid, rifampicin, ethambutol and pyrazinamide.

genotype distribution did not deviate significantly from Hardy-Weinberg equilibrium ($p = 0.36$).

The median (range) plasma EFV concentrations of 153 patients was 1575 (42 to 13,212) ng/ml, with three patients below the EFV lower limit of quantification. Ninety-two (61%) patients had plasma EFV concentrations within, 33 (22%) below, and 28 (17%) above the proposed therapeutic window of 1000–4000 ng/ml. The median (range) of plasma 8OH-EFV concentration was 202 (25 to 935) ng/ml. Median (range) MR was 0.13 (0.01 to 5.0). Full de-identified data are available in Supplementary Table S1.

CYP2B6 c.516G>T genotype differences in EFV and 8OH-EFV concentrations and MR

Univariate analysis showed that plasma EFV (Kruskal-Wallis $p < 0.0001$) and 8OH-EFV concentrations ($p < 0.01$), and the MR ($p < 0.0001$), differed significantly between 516G>T genotypes (Figure 1). Plasma EFV concentrations in T/T genotype patients (median = 5168 ng/ml) were 58% higher than G/G (1036 ng/ml, post hoc $p < 0.0001$), and 36% higher than G/T (1502 ng/ml, post hoc $p < 0.0001$). The difference in plasma EFV concentrations between G/G and G/T genotypes was also statistically significant (post hoc $p < 0.01$). The Jonckheere-Terpstra test confirmed a significant ($p < 1 \times 10^{-5}$) "gene-dose" effect for plasma EFV. MR in T/T genotype patients (median 0.04) was 79% lower than G/G (0.19, post hoc $p < 0.0001$), and 78% lower than G/T (0.18, post hoc $p < 0.0001$), with no significant difference between G/G and G/T genotypes. The Jonckheere-Terpstra test confirmed a significant ($p < 1 \times 10^{-5}$) "gene-dose" effect for MR. Plasma 8OH-EFV concentrations were significantly higher in G/T patients (293 ng/ml) compared to G/G (155 ng/ml) and T/T (137 ng/ml) patients (both post hoc $p < 0.05$), with no significant difference between G/G and T/T genotypes. The Jonckheere-Terpstra test indicated no significant gene-dose effect ($p = 0.7$) for plasma 8OH-EFV.

There were significant ($p < 0.001$) genotype differences in the proportions of patients below, within, and above the therapeutic plasma EFV concentration range (Table 2). G/G genotype patients were the most likely to be below the therapeutic range (42% vs. 12% and 15%), and G/T patients the most likely (85%) to be within the therapeutic range, whereas all patients with EFV plasma concentrations greater than 4000 ng/ml (above EFV therapeutic range) were T/T.

Contributions of genetic and demographic variables to EFV disposition

Initial distribution and linear regression analyses indicated a need for log transformation of plasma EFV and 8OH-EFV concentrations, MR, and weight (but not for age) to meet linear regression model assumptions. Genotype was the only significant explanatory variable for (natural log [ln]-transformed) plasma EFV concentrations (see Table 3), explaining 38% of variability. For (ln) plasma 8OH-EFV concentrations, genotype and (ln) weight variables were significant, explaining 10% and 7% of the variability, respectively (Table 4). Genotype was the most significant explanatory variable for (ln) MR followed by gender (Table 5), explaining 50% and 3% of the variability, respectively.

Drug concentrations, genotype, and side effects

A total of 149 patients were included in the analysis of the association between drug concentrations and the

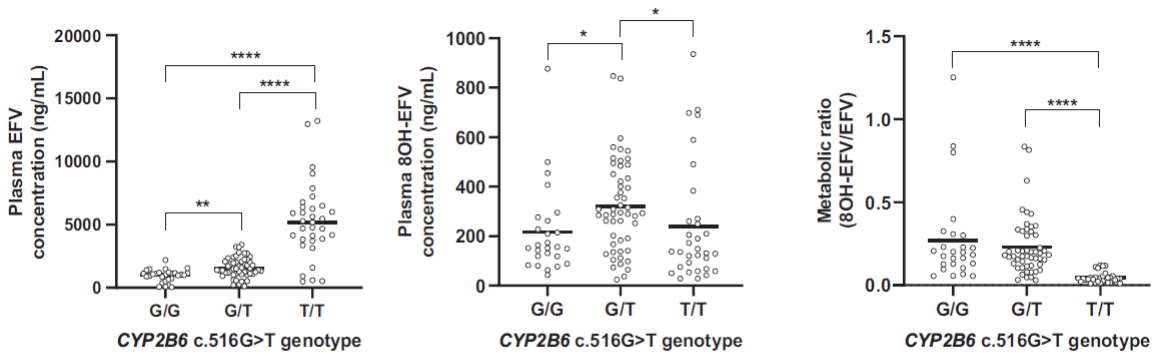

FIGURE 1 *CYP2B6* c.516G>T genotype differences in plasma EFV and 8OH-EFV concentrations and Metabolic Ratio (8OH-EFV/EFV) in 115 Papua New Guinea (PNG) patients with HIV/AIDS receiving EFV 600 mg/day. Dunn's post hoc *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

Genotype	EFV therapeutic concentration range		
	Below (<1000 ng/ml)	Within (1000–4000 ng/ml)	Above (>4000 ng/ml)
G/G, *n* (%)	11[a] (42)	15 (58)	0 (0)
G/T, *n* (%)	8 (15)	45 (85)	0 (0)
T/T, *n* (%)	4 (12)	6 (18)	23 (70)
Chi-square $p < 0.0001$			

TABLE 2 *CYP2B6* c.516G>T genotype differences in the proportion of patients within the EFV therapeutic plasma concentration range among 112 PNG patients with HIV/AIDS receiving EFV 600 mg/day

Abbreviations: EFV, efavirenz; PNG, Papua New Guinea.

[a]*n*: percentage within genotype group in brackets.

TABLE 3 Predictors of ln-transformed plasma EFV concentrations in 102 PNG patients with HIV/AIDS

Variable	Co-efficient estimate (95% CI)	*p*-value	Contribution[a] to model (R^2)
Age (years)	0.0066 (−0.0089 to 0.022)	0.4	0.004
Male gender	0.19 (−0.16 to 0.55)	0.056	0.004
ln(Weight) (kg)	−0.75 (−1.5 to 0.021)	0.3	0.036
CYP2B6 c.516G>T genotype		3×10^{-11}	0.38
G/G	(reference genotype)		
G/T	0.61 (0.2 to 1.0)**		
T/T	1.7 (1.25 to 2.14)***,#		
(Intercept)	9.4 (6.1 to 12.6)		

Note: Co-efficient estimates are for ln-transformed plasma EFV concentrations.

Abbreviations: CI, confidence interval; EFV, efavirenz; PNG, Papua New Guinea.

[a]Averaging over orderings method. Post hoc **$p < 0.01$ and ***$p < 0.001$ versus G/G genotype. #$p < 0.001$ versus G/T genotype.

Variable	Co-efficient estimate (95% CI)	*p*-value	Contribution[a] to model (R^2)
Age (years)	−0.002 (−0.017 to 0.012)	0.72	0.003
Male gender	−0.26 (−0.6 to 0.071)	0.12	0.034
ln(Weight) (kg)	−0.97 (−1.7 to −0.25)	0.009	0.069
CYP2B6 c.516G>T genotype		0.002	0.10
G/G	(reference genotype)		
G/T	0.44 (0.056 to 0.83)		
T/T	−0.17 (−0.6 to 0.26)**		
(Intercept)	9.3 (6.3 to 12.3)		

TABLE 4 Predictors of ln-transformed plasma 8OH-EFV concentrations in 102 PNG patients with HIV/AIDS

Note: Co-efficient estimates are for ln-transformed plasma 8OH-EFV concentrations.

Abbreviations: CI, confidence interval; EFV, efavirenz; PNG, Papua New Guinea.

[a]Averaging over orderings method. Post hoc **$p < 0.01$ versus G/T genotype.

occurrence of side effects; seven patients had concentrations below the lower limit of quantification for one or both analytes and were excluded in this analysis to prevent bias caused by possible medication nonadherence due to side effects. Fifty-one percent of patients reported one or more side effects. Overall, no significant ($p > 0.05$) association between side effects and plasma 8OH-EFV concentrations or MR was found (Table 6). When comparing plasma EFV concentrations between groups, a significant ($p = 0.02$) difference was found between those with CNS and psychiatric side effects and those without (none). Median plasma EFV concentrations were higher in those patients that did not report any side effects (2011 ng/ml vs. 1140 ng/ml). No significant ($p > 0.05$) association between side effects and treatment time was found.

TABLE 5 Predictors of ln-transformed metabolic ratio (plasma 8OH-EFV/EFV) in 102 PNG patients with HIV/AIDS

Variable	Co-efficient estimate (95% CI)	*p*-value	Contribution[a] to model (R^2)
Age (years)	−0.008 (−0.02 to 0.005)	0.2	0.01
Male gender	−0.4 (−0.7 to −0.1)	0.008	0.03
CYP2B6 c.516G>T genotype		2.2×10^{-16}	0.5
G/G	(reference genotype)		
G/T	−0.02 (−0.38 to 0.32)		
T/T	−1.63 (−2.02 to −1.24)***,#		
(Intercept)	−1.2 (−1.75 to −0.65)		

Note: Co-efficient estimates are for ln-transformed metabolic ratio.

Abbreviations: CI, confidence interval; EFV, efavirenz; PNG, Papua New Guinea.

[a]Averaging over orderings method. Post hoc ***$p < 0.001$ versus G/G genotype. #$p < 0.001$ versus G/T genotype.

TABLE 6 Comparison of plasma EFV and 8OH-EFV concentrations and MRs between those without (none) and those with side effects in 149 PNG patients with HIV/AIDS receiving EFV 600 mg/day

Side effects	*N* (%)	EFV ng/ml, median (range)	8OH-EFV ng/ml, median (range)	MR, median (range)
None	73 (49)	2011 (95–13,212)	199 (42–935)	0.12 (0.01–1.25)
CNS only	53 (36)	1497 (508–7874)	214 (29–876)	0.16 (0.02–0.84)
Psychiatric only	8 (6)	1492 (1026–5293)	348 (58–837)	0.18 (0.04–0.82)
CNS and Psychiatric	15 (9)	1140 (272–12,952)[a]	136 (25–711)	0.1 (0.03–0.4)
Kruskal-Wallis *P*		0.02	0.06	0.3

Abbreviations: 8OH-EFV: 8-hydroxyefavirenz; CNS, central nervous system (tiredness, dizziness, drowsiness, insomnia, and impaired concentration); EFV, efavirenz; MR, metabolic ratio (8OH-EFV/EFV); PNG, Papua New Guinea.

[a]None versus CNS and Psychiatric post hoc $p = 0.02$ (Dunn's multiple comparison test).

The incidence of side effects in patients with G/G, G/T, and T/T genotypes was 65% (17/26), 57% (29/51), and 41% (14/34), respectively. No statistically significant difference between genotypes per se and the occurrence of side effects was found ($p = 0.15$). Kruskal-Wallis test simulations indicated that, for the expected higher concentrations in patients with side effects, the study had greater than 90% power (alpha = 0.05) to identify median plasma EFV concentrations 50% higher in CNS-only or psychiatric-only groups, and 100% higher in CNS and psychiatric combined group, compared to a median of 1500 ng/ml in the no side effect group. However, the study had only ~ 53% power to detect the observed side effect group differences in plasma EFV concentrations (Table 6), whereas χ^2 test simulations indicated 40% power to identify the observed genotype differences in side effect incidence as significant.

DISCUSSION

The influence of genetic polymorphisms on EFV exposure, especially those genes encoding the enzymes involved in EFV metabolism, has been extensively studied and documented in many different populations.[8,11,21,25,26,39,40] PNG has the highest incidence and prevalence of HIV infections in the Western Pacific Region.[32] Probably due to logistical and clinical reasons, the present study is the first to investigate the relationship between EFV pharmacogenetics and drug concentrations, as well as the influence of patient demographics and the occurrence of adverse events to drug concentrations, in a PNG HIV/AIDS population. In the present study, the frequency of the 516T variant allele was 53%, being consistent with previous findings for PNG.[30,32] The T allele frequency shows high variability between populations, ranging from 16 to 46% in Europeans, 16 to 33% in East Asians, and 35 to 45% in Sub-Saharan Africans.[31,39]

A statistically significant increase in EFV concentrations was found in homozygous carriers of the *CYP2B6* c.516G>T variant allele, with median concentrations being fivefold and approximately threefold higher than G/G and G/T, respectively. Previous studies have replicated the association between *CYP2B6* c.516G>T and plasma EFV concentrations, including studies of patients from South Africa,[11] Zimbabwe,[40] Ghana,[39] China,[21] Switzerland,[13]

Taiwan,[41] and Chile.[42] In our study, 23 patients had concentrations above 4000 ng/ml and all were homozygous for the variant allele, demonstrating the potential clinical relevance of the effect size of *CYP2B6* c.516G>T genotypes in PNG patients with HIV/AIDS.

Multiple linear regression analyses were conducted to examine contributions of genetic and demographic variables, such as age, gender, and body weight, to EFV concentrations. Previous studies showed contradictory results relating to the contribution of age, gender, and body weight to EFV disposition,[18-20] and, in our study, we could not establish a contribution of patient demographics to EFV disposition. *CYP2B6* c.516G>T genotype was the only significant explanatory variable for plasma EFV concentrations, explaining 38% of variability. The variability in EFV concentrations caused by genotypes was also reported in an AIDS Clinical Trials Group (ACTG) study, including White, Black, and Hispanic populations ($n = 240$),[43] where the *CYP2B6* c.516G>T SNP explained 33% of variance. Part of the remaining variation in EFV concentrations in the PNG population might further be explained by other SNPs in *CYP2B6* as well as polymorphisms in other genes involved in EFV metabolism (discussed below).[32,42,44] Genotype was also the most significant contributor to variability in MR (50%).

Although *CYP2B6* is the main enzyme involved in the formation of 8OH-EFV, CYP3A5, CYP1A2, and CYP2A6 also contribute to EFV metabolism, but to a lesser extent.[1] CYP2B6 is also responsible for the further hydroxylation of 8OH-EFV to 8,14diOH-EFV,[22] possibly with other SNPs contributing to the variation in 8OH-EFV concentrations. EFV and all its three hydroxylated metabolites are subject to glucuronidation by UGT enzymes, which have SNPs associated with variation in plasma EFV concentrations.[44] These alternative metabolic pathways could potentially explain why the patients with T/T genotype in the present study had significantly lower MR but not lower plasma 8OH-EFV concentrations.

An important aspect to be considered in the present study is that patients were sampled at least 3 months, and up to 13 years, following initiation of treatment. EFV enhances its own metabolism by inducing the expression of *CYP2B6* through activation of the nuclear receptors NR1I3 and NR1I2.[2,45] Habtwold et al.[46] reported the long-term effect of EFV autoinduction, in which a 32% increase in median plasma 8OH-EFV concentration and a 20% decrease in MR (EFV divided by 8OH-EFV), equivalent of 20% increase in MR in our study (MR = 8OH-EFV/EFV), was seen by week 16 of treatment compared with week 4. The same authors observed that the change in 8OH-EFV and MR over time was significant in women and in *CYP2B6*1* and *UGT2B7*1* carriers.[46] The extent of such autoinduction can also be influenced by different sampling time-points (different sampling days) and the patient's genotype; for instance, greater EFV autoinduction in patients with *CYP2B6*1* allele compared to *6/*6.[47]

Contrary to expectations, high plasma EFV concentrations were not associated with a higher incidence of side effects. We hypothesize that treatment duration (>3 months) could be a factor in the development of tolerance to the side effects. Two previous studies reported an association between high plasma EFV concentrations and toxicities at the beginning of treatment, but CNS symptoms did not persist beyond a few weeks.[11,48] Moreover, not all studies have confirmed the plasma EFV concentration and side effects association.[12-15] However, this does not explain why patients who reported toxicities had lower EFV concentrations, compared to the non side effects group (Table 6). This requires further investigation in particular as to whether nonadherence due to toxicities might be playing a role as adherence, including the number of doses missed in the last month, was not formally assessed in this study apart from the times of dosing for 3 days prior to blood sampling.

The relationship between *CYP2B6* c.516G>T genotypes and the occurrence of side effects has been investigated in many studies. Gallien et al.[29] reported the probability of the occurrence of CNS toxicity to be 16% higher in patients with c.516T/T genotype compared to G/G ($p = 0.02$), although no association between EFV concentrations and side effects was found. However, in other studies, no association between *CYP2B6* c.516G>T genotype and increased risk of toxicities was established.[8,40,49,50] Vujkovic et al.[28] found an association between 516G>T genotypes and side effects, however, in their study the patients with G/G genotype reported adverse events more often than G/T or T/T ($p = 0.041$). In the present study, although no statistical significance was found, the same pattern was observed. These discrepancies suggest that the relationship between EFV metabolism and side effects has more external factors than just known genetic metabolism factors.

There are several limitations to the present study. There was sufficient sample size to clearly demonstrate genotype effects on plasma EFV concentrations/MR, although the sample size for the reported psychiatric and CNS and psychiatric side effects groups was relatively small (complete information was not available for all patients, as well as insufficient sample for DNA extraction in all patients), resulting in limited power to detect significant associations between side effects and plasma concentrations or genotype.

Some of the participants (~5%) had comorbidities, including tuberculosis. In consideration of whether concomitant medications (Table 1) may interfere in data interpretation, one patient was receiving rifampicin

(HREZ combination; see Table 1), which has previously been reported to affect EFV concentrations.[41] However, the drug concentrations for this patient did not differ from the other patients' concentrations, and the plasma EFV concentration was within the therapeutic range. The other comedications (isoniazid, cotrimoxazole, and carbimazole) are not expected to influence EFV pharmacokinetics. For the assessment of adverse effects, a key consideration is the self-reporting of side effects, which has the potential for bias in the interpretation of these data. In addition, we only tested the 516G>T polymorphism based on it being the major SNP affecting EFV pharmacokinetics as all others studied in the PNG population[32] are either very rare or have no or modest effect on function.[26]

In conclusion, the relationship between *CYP2B6* c.516G>T genotype, drug concentrations, and adverse effects, in PNG HIV/AIDS patients was assessed for the first time. *CYP2B6* c.516G>T genotypes are a strong predictor of plasma EFV concentrations and MR, with the 516T/T genotype strongly associated with higher plasma efavirenz concentrations and lower MR. However, *CYP2B6* c.516T/T genotype effects on EFV disposition do not translate into increased incidence of side effects after the first 3 months of treatment in PNG patients with HIV/AIDS. Whether the high proportion of PNG *CYP2B6* c.516T/T genotype patients above the therapeutic range impacts adverse events in the early stages of treatment requires specific investigation.

ACKNOWLEDGEMENTS

The authors acknowledge use of the services and facilities of the Australian Genome Research Facility (AGRF), as well as the extensive assistance of the HIV Heduru Clinic (Port Moresby General Hospital) staff.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

N.B.A., H.K.V.S., D.B., A.S., P.P., and J.T wrote manuscript. N.B.A., A.S., H.K.V.S., P.P., J.T., and D.B. designed the research. N.B.A., J.T., P.P., and H.K.V.S. performed the research. N.B.A. and D.B. analyzed the data. J.T., P.P., and A.S. contributed new reagents/analytical tools.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Bordin Andriguetti N, Van Schalkwyk HK, Barratt DT, Tucci J, Pumuye P, Somogyi AA. Large variability in plasma efavirenz concentration in Papua New Guinea HIV/AIDS patients associated with high frequency of CYP2B6 516T allele. *Clin Transl Sci*. 2021;00:1–11. https://doi.org/10.1111/cts.13120

Chapter 4: Experiments and challenges to develop a method for the simultaneous analysis of dolutegravir, lamivudine and tenofovir by LC-MS/MS

1. Introduction

The thesis aimed to develop and validate analytical methods for the quantification of HIV drugs in different biological matrices. A method for the quantification of efavirenz (EFV) and metabolites in human plasma was developed, validated and clinically applied to HIV/AIDS Papua New Guinea (PNG) patients (refer to publication 1 – chapter 2 and publication 2 – chapter 3). The next step was to develop a method to quantify EFV in microsampling devices; however at the end of 2019 PNG replaced efavirenz with dolutegravir (DTG) as the first-line drug against HIV, following the World Health Organization recommendations (31). For PNG adults, DTG is prescribed once daily in a combination therapy (DTG 50 mg + tenofovir disoproxil fumarate (TDF) 300 mg + lamivudine (3TC) 300 mg) (135).

Dolutegravir is a relatively new drug that has been used worldwide by HIV/AIDS patients for just a few years, and as such, its side effects have yet not been entirely elucidated. Aside from clinical trials, there is a lack of pharmacokinetic (PK) studies in different populations (197). A variety of factors can affect the PK of a drug, including genetic factors, demographic characteristics (such as age, gender, ethnicity, body weight), concomitant use of other drugs or the presence of other diseases. To perform a PK study, a validated method for the quantification of the drug is necessary, and for the majority of the drugs, the reference ranges are established in plasma samples (173). However, this conventional sampling method has many disadvantages, including special storage and transportation (-20ºC on dry ice) from the site of collection to the analysis facility. For remote sites such as in PNG, this can often be difficult, and a sampling approach that does not require special transportation or storage would help to overcome this problem.

Microsampling devices such as dried blood spots (DBS) and volumetric absorptive microsampling (VAMS) are the most commonly used alternatives, not requiring

refrigeration or special hazard signs or boxes for transportation (174, 175). However, to validate a method using these devices, it is necessary to translate the concentrations found in capillary blood to plasma (reference ranges), which requires a prior partial validation in plasma samples (173). DTG, 3TC and tenofovir (TNF, the active component of TDF in plasma) differ in key physicochemical properties that impact on drug extraction and chromatography (Table 1). This chapter details the associated challenges involved in developing a bioanalytical method to simultaneously quantify DTG, 3TC and tenofovir (TNF) by liquid chromatography coupled with mass spectrometry (LC-MS/MS), and the troubleshooting involved to meet validation requirements.

Table 1. DTG, 3TC and TDF physicochemical properties.

	Polar/nonpolar	Acid/Base	pKa	LogP	Aqueous/organic solubility
DTG	nonpolar	weak acid	8.2	2.2	Soluble in DMSO, slightly soluble in MeOH and water
3TC	polar	weak base	4.3	-1.4	Slightly soluble in MeOH and water
TDF	polar	weak base	3.75	1.25	Highly soluble in water

DTG, dolutegravir. 3TC, lamivudine, TDF, tenofovir disoproxil fumarate. DMSO, disoproxil sulfoxide. MeOH, methanol.

2. Experiments

2.1 Experiment 1 – Preparation of stock solutions and MS optimisation

Stock solutions of DTG, 3TC, TNF, all from Toronto Research Chemicals® (TRC®, North York, Canada) and labelled internal standards (IS) – 3TC-d3 (TRC®), DTG-d5 (BDG synthesis®) and TNF-d6 (Cayman Chemical®), were prepared from powder using an analytical balance and dissolved in either HPLC-grade methanol (MeOH) or HPLC-grade water. DTG and its labelled internal standard, DTG-d5 were prepared in MeOH at 0.7 mg/mL and 1 mg/mL, respectively. 3TC and 3TC-d3 were each prepared in MeOH at 1 mg/mL. TNF and TNF-d6 were each prepared in water at 1 mg/mL. Intermediate solutions of all analytes were prepared in 50% MeOH at 10 µg/mL in order to perform mass

spectrometry (MS) optimization. The MS instrument used was a triple quadrupole, consisting of two mass filters (Q1 and Q3) and a collision cell (q2) with ionization by electrospray.

One µL of each intermediate solution was directly injected into the MS in order to select the mass-to-charge ratio (*m/z*) transitions and collision energies (CE) for quantification and qualification of the analytes. The optimization of the CE is necessary to increase fragment ion intensity (198). The mobile phase (MP) used for MS optimization comprised 50% 0.1% formic acid in LCMS-grade water and 50% 0.1% formic acid LCMS-grade methanol, at a flow rate of 0.2 mL/min.

The precursor ion is the ion selected at Q1 that dissociates (in q2) to smaller fragment ions (product ions) selected at Q3. The precursor ion is often the total molar mass (M) of the compound plus or minus an adduct (e.g. $[M+H]^+$). For the qualitative identification of the precursor ion, a full scan in Q1 was performed between 100 and 1000 *m/z*, followed by a narrower scan (span 400-450 m/z for DTG, 200-250 m/z for 3TC and 250-300 m/z for TNF) for more precise precursor ion m/z identification. Candidate product ions are then identified using a Q3 scan (precursor ion m/z down to 100 m/z) over different collision energies. The precursor ion collides with Argon gas in q2 to create fragments or product ions, which are selected in Q3. Full scan in Q3 is performed to select the product ions by applying collision energy from 5V to 40V, giving qualitative and semi-quantitative information (fragment m/z and relative abundance) (Figure 1). Once the product ions are selected, based on their abundance, this Multiple Reaction Monitoring (MRM) approach is applied to provide best selectivity and signal-to-noise (S/N) ratio for quantification. The final step is an automated iterative process performed by the system software, where it optimizes the cone voltage, Q1 and Q3 bias for each *m/z* transition selected for quantification.

The process detailed above is universally applied to all study compounds, in the present study the process was performed for DTG, 3TC, TNF and for each of the respective labelled internal standards. Results are presented in Table 2. Following the selection of the transitions for each analyte, injection of a known concentration was performed into the LC-MS/MS for each analyte in order to assess the S/N ratio of each transition. The transitions selected for quantification are based on the best sensitivity (highest S/N ratio). Quantification ions selected for DTG, DTG-d5, 3TC, 3TC-d3, TNF and TNF-d6 were 277.05, 277.05, 112.1, 115.1, 136.1 and 136.1, respectively.

Table 2. Selected qualification and quantification ions for DTG, 3TC, TNF and their labelled internal standards (IS).

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*), CE (V)	
		Quantification	Qualification
DTG	420.1	277.05, 26	127.05, 33
DTG-d5	425.1	277.05, 27	132.1, 31
3TC	230.1	112.1, 12	-[a]
3TC-d3	233.1	115.1, 11	-[a]
TNF	288.1	136.1, 25	176.1, 25
TNF-d6	294.1	136.1, 25	182.1, 25

VCE (V): collision energy (voltage). [a]No qualification ion transition because 3TC parent ion only breaks into one fragment.

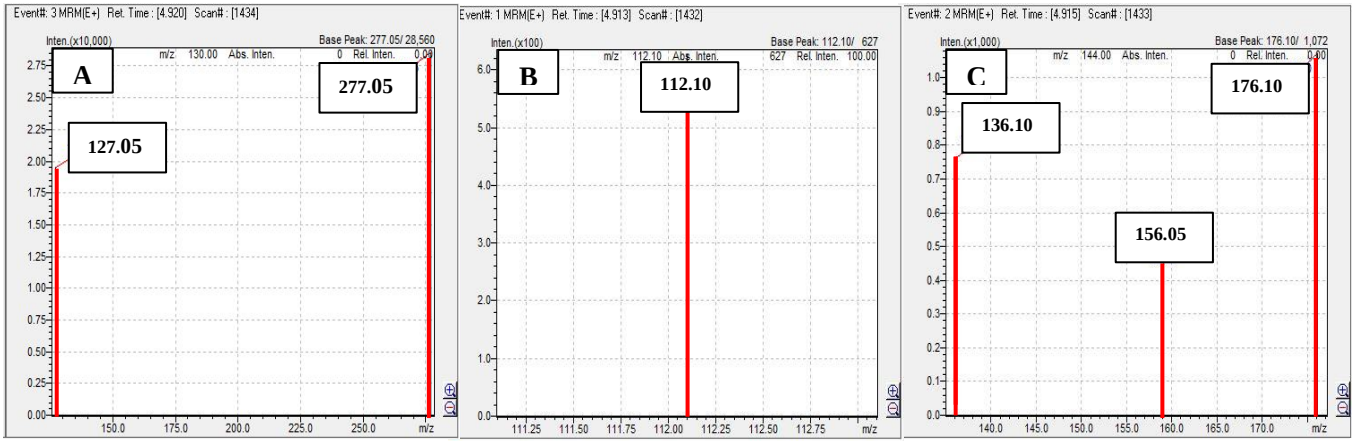

Figure 1. Product ion scans for DTG (A), 3TC (B) and TNF (C).

2.2 Experiment 2 – Optimising the mobile phase

Each unlabelled analyte was prepared at 1 µg/mL in MP (Table 2 Injection solution) and injected into the system using a Kinetex® C18 (100 x 2.1 mm, 1.7 µm) Phenomenex® column (Torrance, Canada), at a flow rate of 0.4 mL/min in gradient mode (see details below) for 8.5 min.

The fundamental MP solvents for LC-MS are water, MeOH or ACN, often with the addition of volatile buffer (e.g. ammonium acetate and ammonium formate), acid or base to adjust the pH in order to achieve separation of target compounds. The most common additive in the MP for LC-MS is formic acid, which can also help to improve ionisation in positive ionization mode. In the present study, both MeOH and ACN were tested with water and formic acid. Different conditions tested are summarized in Table 3. The MP scouting gradient was as follows: 1% to 90% organic mobile phase (%B) over 4 min, hold at 90% for 1 min and back to l% initial conditions up to 8.5 min. Using a scouting gradient provides information about what percentage of organic solvent analytes elute at, thus one can potentially reduce the MP gradient time or select an isocratic MP. TNF and 3TC are polar compounds so they elute quickly at a low percentage of organic solvent; on the other hand, DTG due to its hydrophobicity needs a higher percentage of organic solvent to elute.

Table 3. Chromatography assessment under different injection solution and mobile phase conditions using Kinetex C18 column and gradient program*.

Condition	Injection solution (mix of 3TC, DTG, TNF)	Mobile phase	Formic acid (%)	Retention time
1	50% ACN/ 50% H2O	H2O/ACN	0.1	3TC – 0.5 min DTG and TNF not visible
2	10% ACN/ 90% H2O	H2O/ACN	0.1	3TC – 1.25 min TNF – 2.25 min (peak tail) DTG not visible
3	10% MeOH/ 90% H2O	H2O/MeOH	0.1	3TC – 0.5 min TNF – 1 min (split peak) DTG – 8 min

4	100% H2O	H2O/MeOH	0.2	3TC – 0.5 min TNF – 0.8 min (extra peak at 1.7 min) DTG – 8 min (tail)
5	100% H2O	H2O/ACN	0.2	3TC – 0.5 min TNF – 0.8 min (double peak and extra peak at 1.7 min) DTG – 8 min (tail)

*One to 90% organic mobile phase (B) over 4 min, hold at 90% B for 1 min and back to l% B for 3.5 min.

The use of either organic solvent showed similar results and for logistic reasons, MeOH was selected. TNF is highly soluble in water and for this reason, the starting drug standard injection solution chosen was 100% H_2O. Moreover, to try to overcome the TNF split peak problem (see below), 0.2% of formic acid was trialled, however, TNF still produced double peaks and even an extra peak (Figure 2). Split or broad peaks in LC are often due to mechanical (all peaks are affected) or chemical problems (one peak is affected). Mechanical issues include blocked frit and column overload, column degradation, void at the column inlet, wrong column type, and bubbles in the system causing pump problems (fluctuation in pressure). Chemical problems are generally associated with the mobile phase compatibility, particularly the pH, with the analytes' chemical properties.

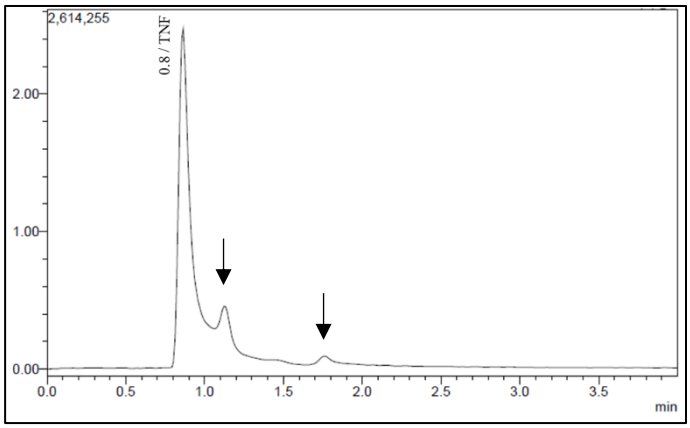

Figure 2. Chromatogram of TNF 1 µg/mL using injection and mobile phase condition 6 (Table 2). Single chromatogram illustration of TNF (total ion chromatogram – TIC) analysed with condition 6. Auto-scale to intensity signal. The arrows refer to the double peak and the extra peak.

The use of 0.2% formic acid in the MP makes the pH 2-2.5, and for this reason, the column was replaced by a Kinetex PS C18 that supports lower pH (1.5 to 10) and can support 100% aqueous. The gradient was modified as follows: 0%B (MeOH with 0.2% formic acid) hold for 1 min; ramp up to 90%B from 1 to 4 min; hold at 90%B for 1 min; and back to initial conditions from 5 to 7 min, to match with the injection solution (100% H_2O). This resulted in retention times of 1.4, 1.6 and 4.6 min for 3TC, TNF and DTG, respectively. This condition resulted in a tail in the TNF peak, and carry-over of TNF in subsequent blank mobile phase injections.

To remove the carry-over effect, first it is important to understand why it is occurring. Several reasons are related to the occurrence of carry-over effects, including analyte retention on injection needle surfaces; precipitation in the injection needle, injection loop, pre-column or column due to low solubility in the injection solution or mobile phase; and insufficient elution time before the next injection. Injection solution and mobile phase were matching, and the needle rinse solution (R0 – needle dipping) was 50% MeOH. External rinse (only) of the needle (R0) is standard in the default method, so the multi-rinse option with 0.2% formic acid isopropanol:methanol (20:80, v/v) was added for further internal (R2) and external (R1) rinse of the needle, however TNF and DTG were still eluting in the blank sample. I tested different rinse volumes, speed and mode (before and/or after aspiration), and needle dip time. Again, the carry-over effect was not resolved with needle washing.

Therefore, I decided to work around the mobile phase. Reverse phase columns are the most common used in HPLC methods, where the stationary phase is a nonpolar hydrocarbon and the mobile phase is a polar liquid. In order to retain highly polar compounds, including 3TC and TNF, I reduced the amount of organic solvent in the MP, making the MP weaker, e.g., 100% aqueous. However, DTG is a nonpolar analyte which requires a high percentage of organic (~80%B) to be eluted from the column; this being the reason for a gradient MP.

Initiating the gradient with 0%B was giving sharp peaks for 3TC and TNF, nonetheless carry-over of TNF and DTG in the blank samples was still present. An experiment was performed to check whether changing the %B in the start of the gradient would dismiss the carry-over effect. Drug standard solution used for injection in gradient mode usually needs to match or have lower organic phase %B than the beginning of the gradient, and at this point I had 0%B which means the standards were in 100% aqueous with 0.1% formic acid.

Several different gradients were tested based on the chromatographic conditions of a previously published method for the simultaneous quantification of 3TC and TNF in plasma (Table 4) (199). This required a new column to be purchased (ACQUITY® UPLC HSS T3 Column, 1.8 µm, 2.1 mm x 150 mm, Waters®) and used for further testing as it was chosen based on previous publications that developed and validated methods for the quantification of DTG, 3TC and TNF (167, 199).

Table 4. Chromatographic gradient of mobile phases A and B (199).

Time (min)	Flow(mL/min)	Mobile phase A%	Mobile phase B%
0.00	0.4	99	1
0.10	0.4	99	1
0.50	0.4	96	4
1.50	0.4	94	6
2.70	0.4	90	10
2.80	0.4	1	99
3.50	0.4	1	99
3.60	0.4	99	1
5.00	0.4	99	1

Mobile phase A – HPLC grade water + formic acid 0.05%; mobile phase B – acetonitrile + formic acid 0.05%.

Even though DTG was not included in this method, it was worth trying the conditions to check the reproducibility of the TNF peak. The following MP conditions were tested (MP A 0.05% formic acid in water and MP B 0.05% formic acid in ACN at a flow rate of 0.5 mL/min):

- Condition 1 – Chromatographic gradient described in table 3.

- Outcome: Good resolution overall; carry-overs for TNF and DTG (Figure 3).

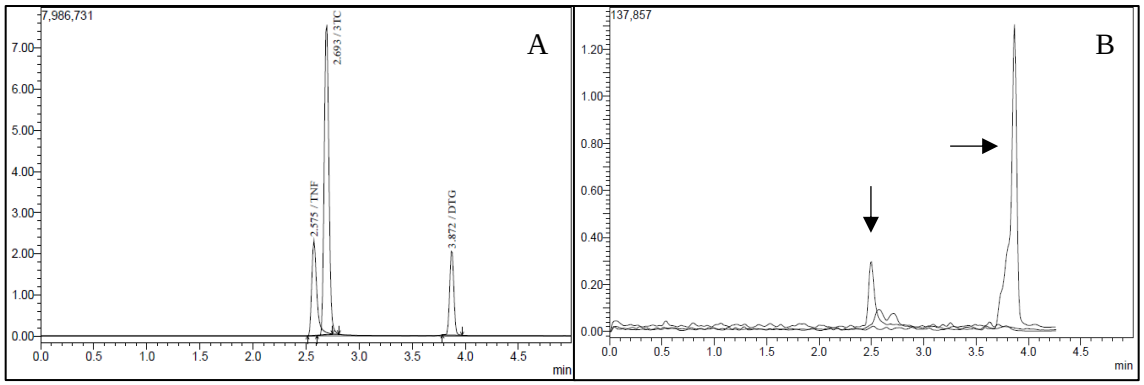

Figure 3. Condition 1: Mobile phase gradient from table 4. A, chromatogram illustration after a single injection of a mixture of DTG, 3TC and TNF at 1 µg/mL each in 100% aqueous with 0.1% formic acid with MP condition 1 (Table 3); B, chromatogram illustration of a single injection of a blank mobile phase showing the carry-over effect at 2.5 min TNF and 3.8 min DTG arrows). Auto-scale to intensity signal.

- Condition 2: 0-2.7 min 50%B; 2.8-3.5 min 90%B; 3.6-5 min 50%B.

- Outcome: Poor chromatogram resolution between 3TC and TNF and poor peak shape for TNF; no carry-over (Figure 4).

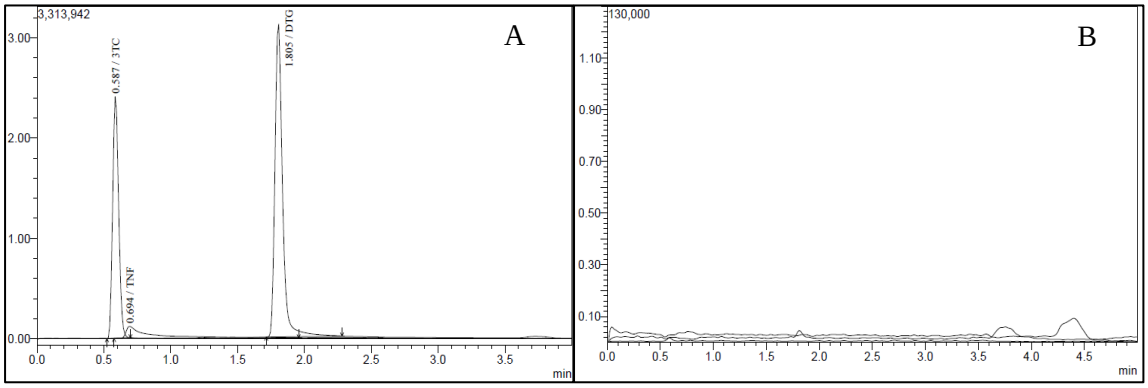

Figure 4. Condition 2 – Starting with 50% MP B. A, chromatogram illustration after a single injection of a mixture of DTG, 3TC and TNF at 0.5 µg/mL each in 50% aqueous with 0.1% formic acid MP with condition 2; B, chromatogram illustration of a single injection of a blank mobile phase.

- Condition 3: 0-2.7 min 25%B; 2.8-3.5 min 90%B; 3.6-5 min 25%B.

- Outcome: 3TC and TNF elute at the same retention time; DTG carry-over (Figure 5).

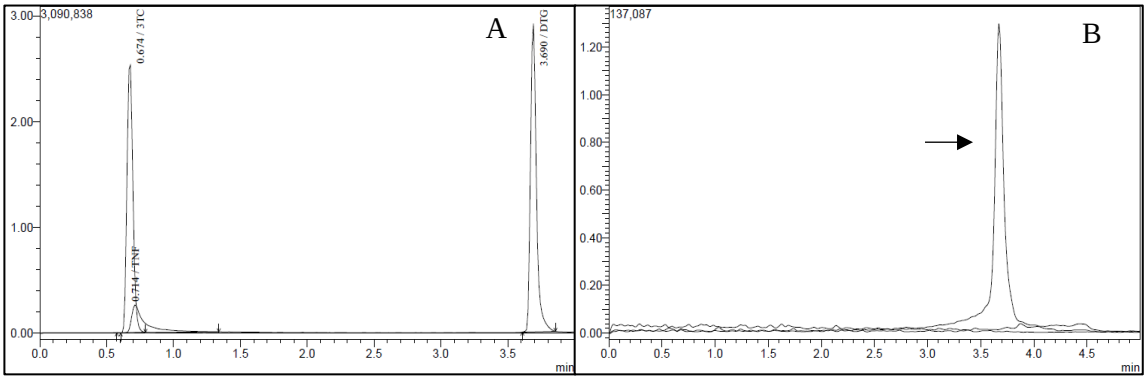

Figure 5. Condition 3 – Starting with 25% MP B.A, chromatogram illustration after a single injection of a mixture of DTG, 3TC and TNF at 0.5 µg/mL each in 25% aqueous with 0.1% formic acid MP with condition 3; B, chromatogram illustration of a single injection of a blank mobile phase showing the carry-over effect at 3.8 min DTG (arrow). Auto-scale to intensity signal.

The following tests were performed with MP A 0.1% formic acid in water and MP B 0.1% formic acid in ACN at a flow rate of 0.5 mL/min, in order to help and improve resolution and ionization, thus more sensitivity.

- Condition 4: Gradient 2%B hold for 0.1 min; ramp up to 6%B from 0.2 to 1.5 min; ramp up to 10%B from 1.6 to 2.7 min; up to 98%B at 2.8 min and hold for 0.7 min; back to initial conditions from 3.6 to 5 min.

- Outcome: Good peak shapes and resolution; slight carry-over for DTG (30% of the expected LLOQ) (Figure 6).

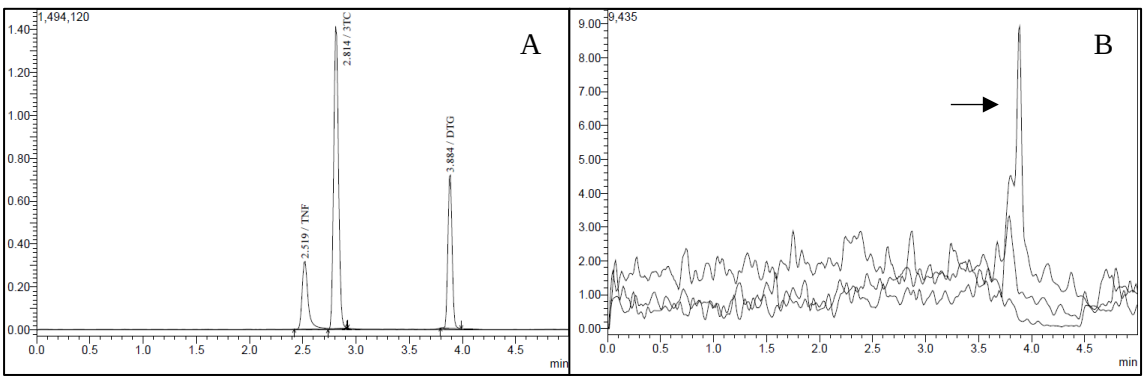

Figure 6. Condition 4 – Gradient starting with 2% MP B.A, chromatogram illustration after a single injection of a mixture of DTG, 3TC and TNF at 0.5 µg/mL each in 98% aqueous

with 0.1% formic acid each with MP condition 4; B, chromatogram illustration of a single injection of a blank plasma showing the carry-over effect at 3.8 min DTG (arrow). Auto-scale to intensity signal.

- Condition 5: Gradient 3%B hold for 0.1 min; ramp up to 6%B from 0.2 to 1.5 min; ramp up to 10%B from 1.6 to 2.7 min; up to 97%B at 2.8 min and hold for 0.7 min; back to initial conditions from 3.6 to 5 min

- Outcome: Good peak shapes and resolution; carry-over for DTG (Figure 7).

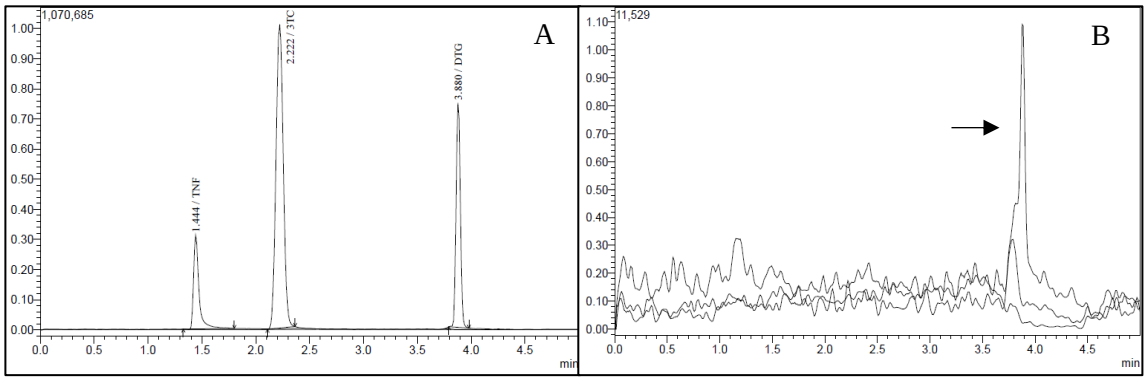

Figure 7. Condition 5 – Gradient starting with 3% MP B. A, chromatogram illustration after a single injection of a mixture of DTG, 3TC and TNF at 0.5 µg/mL each in 97% aqueous with 0.1% formic acid with MP condition 5; B, chromatogram illustration of a single injection of a blank mobile phase showing the carry-over effect at 3.8 min DTG (arrow). Auto-scale to intensity signal.

Additional experimentation identified that the only way to eliminate the DTG carry-over effect entirely was to begin the gradient with 50%B; however this condition was causing severe peak tailing for TNF and with very low sensitivity.

One last test was performed to try to improve the TNF peak shape. The Shimadzu technician suggested testing a different connection between the injector and the column. Stainless steel tubing is commonly used in UHPLC system as it holds high pressures, however it can, sometimes, cause peak tailing due to the interaction between phosphate residues in the analytes and stainless steel. TNF has a phosphate group in its chemical structure, which could be chelating with the stainless steel tubing and causing the tailing in the TNF peak. The test was performed using polyetheretherketone (PEEK) polymer tubing, which is

biocompatible, chemically inert to most solvents and almost non-reactive to sensitive components. Unfortunately, the replacing of the connection did not help to improve the TNF peak shape. Whilst still having problems with peak shape for TNF, I wanted to try and analyse it using the same method as for 3TC and DTG, without having to develop a new single TNF assay. The DTG and TNF carry-over effects were occurring when the concentration of organic solvent was < 50%B and < 2%B in the beginning of the run, respectively. Using 50%B in the beginning of the run eliminates the carry-over for both compounds, however it reduces the chromatography resolution (between 3TC and TNF), and for TNF it causes a poor peak shape and reduces the peak area (see Figure 4A above).

2.3 Experiment 3 – Optimising the interface parameters

Optimization of the MS interface parameters helps to improve signal-to-noise by maximising analyte ionisation. The first step for the optimization of the interface parameters is to learn what the organic concentration is at the retention time of each analyte (i.e. when each compound reaches the interface). An equation is used for gradient elution:

%B at retention time = (initial B% + (retention time x Δ%B/min)) – ((dwell volume/ flow rate) x Δ%B/min), where Δ%B is %B final - %B initial

The injection of a mixture in 50% ACN with 0.1% formic acid, with initial %B = 50%, final B = 90% (so gradient change = 40), gradient time = 3 min, flow rate of 0.5 mL/min and dwell volume = 0.12 mL, resulted in the following retention times: 0.6, 0.67 and 1.6 min for 3TC, TNF and DTG, respectively. Therefore, the concentrations of organic at their retention times were 55% for 3TC and TNF and 68% for DTG. The interface parameters are then optimized by performing repeat direct injections of each compound (1 µg/mL) separately at their calculated %B. The following parameters are iteratively changed, one by one, to check

the improvement in signal-noise: position of the probe; nebulizing gas flow rate; drying gas flow rate; heating block temperature, and desolvation line temperature.

Tenofovir did not shown any improvement in signal-noise following the optimization of the interface parameters. For DTG and 3TC, the matched parameters that resulted in best signal-noise for both were probe in neutral position, nebulizing gas of 1.5 L/min; drying gas of 15 L/min; heating block 450 ºC; desolvation line 300 ºC.

2.4 Experiment 4 – Preparation of calibration curve

Even though TNF peak shape did not improve after applying different approaches (see above), I still decided to prepare a calibration curve of standard solutions of a mix of DTG, 3TC and TNF. The calibration curve prepared had 7 concentrations, from 35 to 8400 ng/mL for DTG and 10 to 2400 ng/mL for 3TC and TNF. The calibration curve range was determined based on previous publications (166, 167, 200, 201) that reported healthy and HIV-positive subjects' trough concentrations (C_{min}), ranging between 50-180 ng/mL for TNF, 200-1500 ng/mL for 3TC, and 200-5000 ng/mL for DTG.

Four injections of each calibrator were performed resulting in good peak shape and enough sensitivity considering the LLOQ for DTG and 3TC; however TNF was detected only from the fifth calibrator (600 ng/mL) with poor peak shape as expected. This calibration curve test was the last attempt to validate a method to quantify these three compounds simultaneously. I then decided to pursue method development with only DTG and 3TC as they both showed good resolution and sensitivity. The next step was to test different sample preparations to find the most efficient and reproducible extraction of these two drugs from plasma samples.

2.5 Experiment 5 – Sample extraction of DTG and 3TC

Due to its simplicity, protein precipitation (PPT) was chosen to be the first sample preparation method tested to extract 3TC and DTG from plasma samples. For PPT, ACN was used as the precipitating reagent (200 µL + 100 µL of spiked plasma + 10 µL of labelled internal standards). The samples were vortexed for 1 min and centrifuged for 10 min at 18000 x g. The supernatant (180 µL) was transferred to another polypropylene tube and dried in a sample concentrator (50ºC for 35 min). The samples were resuspended with 100 µL of mobile phase (water and ACN with 0.1 % formic acid, 50:50, v/v), filtered in 0.1 µm membrane and 1 µL was injected in the LC system. The recovery for DTG was approximately 90% however for 3TC it was less than 25% of the nominal concentration. Disadvantages of using PPT include poor sample clean-up, where some other plasma components, such as phospholipids, can interfere in the ionization by either suppressing or enhancing ion production (202).

Before considering PPT as being not effective for 3TC, I tested different protocols' varying conditions that can have an impact on recovery, such as the drying step, filter plates and extraction solvents. Extraction solvents for PPT are strategically chosen according to their miscibility with the sample solution, ability to precipitate plasma proteins, and compatibility (e.g. polarity or hydrophobicity) with the target analyte, however even though compatible, the use of different solvents can alter the analyte recovery rate (203). The drying process and consequent concentration of the sample, which is often applied in sample preparation, contributes to greater sensitivity; however, some analytes are not stable at certain temperatures necessary for this process, or are otherwise lost (e.g. analyte irreversibly binds to the plastic or glassware, or simply deteriorates) in the drying process. Filtering samples after resuspension helps to clean the injection solution especially when samples are extracted by PPT, however some analytes can be retained in the membrane of the filter. The peak areas obtained from all the tests below were compared to the peak area of the nominal

concentration of standard drug in MP. The drying process was tested with water instead of plasma to avoid equipment/column blockage. Water was spiked with 3TC drug standard and the same process as mentioned above for extraction was performed in duplicate for each condition. For the first condition, the samples were dried as mentioned above and for the second the samples were injected without the drying process. Both conditions were compared and resulted in the same concentrations, concluding that the drying step was not affecting the recovery of 3TC.

The use of different extraction reagents was also tested to check if any could improve recovery. I tested the frequently employed solvents for PPT: 100% ACN; 50% ACN/ 50% MeOH, and 100% MTBE. In addition, I performed a liquid-liquid extraction with 100% ethyl acetate (3:1 to plasma). The extractions with ACN and MeOH had similar results, recovery of 30%. No 3TC was recovered from the plasma using MTBE as a precipitation reagent and only 4% of 3TC was extracted when using ethyl acetate. The filtration process of the resuspended sample was assessed by injecting extracted samples without filtering and comparing the results to filtered samples. This resulted in similar peak areas for both samples, revealing that the sample filtration process does not affect the recovery of 3TC. From these tests, I could conclude that PPT and liquid-liquid sample extraction techniques do not produce acceptable recovery for 3TC, possibly due to matrix interferences not eliminated with this poor specific method.

After testing different conditions to improve the 3TC recovery rate, I also tested the matrix effect on ionisation when using PPT. Spiked mobile phase with 3TC was compared to extracted blank plasma spiked with 3TC in the end of the extraction, during resuspension in MP. The matrix effect was approximately -70% revealing that ion suppression was probably being caused by phospholipids, particularly because of the early retention time of 3TC. To test this potential interference, ISOLUTE® PLD+ Protein and Phospholipid Removal Plates

(Biotage®) were used for sample preparation, a supported PPT that retains phospholipids. Blank plasma samples were extracted using PLD+ plates and spiked with 3TC in the end. The peak areas obtained were compared to spiked MP, resulting in a reduction of 65% of the peak area, still showing unacceptable ion suppression.

After the unsuccessful attempts with PPT and PLD+ plates, the next sample preparation tested was Supported Liquid Extraction (SLE). Spiked and blank samples (spiked at the end) were prepared using four dilution reagents (H_2O, ammonia, ammonium acetate and 0.1% formic acid in water) and two elution solvents (ethyl acetate 100% and dichloromethane:isopropanol 95:5 v/v) in different combinations. The matrix effect was approximately 5% for both 3TC and DTG, which is acceptable, however the highest recovery of 3TC obtained was 10% when using ethyl acetate as elution solvent.

The last available option to test was Solid Phase Extraction (SPE). SPE is a highly selective technique used to prepare complex samples for chromatographic analysis, however, it is laborious and more expensive than other methods. The available SPE plates were Oasis® MCX (cation exchange) micro-elution 96-well plates (Waters®). Following the standard manufacturer protocol for sample preparation, the recovery for DTG and 3TC was 1-4% and 90-101%, respectively; and the matrix effect 8-14% and 1-9%, respectively. Even though the matrix effect showed acceptable result for both compounds, DTG is practically not extracted with this protocol. Looking at all the results from different sample preparation tests, I concluded that DTG and 3TC need to be analysed using separate methods.

3. Conclusion

Several attempts were performed in order to develop a method to quantify DTG, 3TC and TNF simultaneously in plasma. However, after months of experiments trying to improve TNF peak resolution and sensitivity, I reached the conclusion that the only way to have an

acceptable TNF signal with no carry-over effect is to perform a gradient elution starting with a low percentage of organic solvent. This condition does not suit DTG analyses, which needs a higher percentage of organic at the beginning of the run to flush the compound out of the column in a single run, resulting in no carry-over effect. For 3TC, starting the gradient with low or high organic results in the same good resolution. There are no published methods in the literature with the simultaneous quantification of these three compounds in human plasma, only in tablet dosage form (204).

Although DTG and 3TC had acceptable resolution and sensitivity using the same instrument parameters, different sample preparations need to be applied in order to get good recovery and reproducibility. For this reason, I decided to develop and validate two separate methods with different extraction methods and MP, one to analyse DTG using PPT as sample preparation and one analysing both 3TC and TNF performing SPE to extract the compounds from the plasma samples of HIV/AIDS patients (refer to chapter 5). A bioanalytical method to quantify DTG in DBS and VAMS will also be developed, validated and clinically applied on HIV/AIDS patients from Papua New Guinea (refer to chapter 6).

Chapter 5: Influence of demographic factors and renal function on 3TC and TNF variability

Statement of Authorship

Title of Paper	Assessment of lamivudine and tenofovir pharmacokinetic variability in a Papua New Guinea HIV population through a combined high throughput LCMS/MS assay
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	To be submitted

Principal Author

Name of Principal Author (Candidate)	Natalia Bordin Andriguetti		
Contribution to the Paper	Study design, performed experiments and statistical analysis, data interpretation, wrote the first manuscript draft and will act as the corresponding author.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/09/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Daniel Barratt		
Contribution to the Paper	Study design, assisted with statistical analysis and manuscript critical review.		
Signature		Date	02/09/22

Name of Co-Author	Joseph Tucci		
Contribution to the Paper	Data analysis, manuscript critical review.		
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Contribution to the Paper	Recruited patients and provided extra patient data.		
Signature		Date	8/09/2022

Name of Co-Author	Andrew Somogyi		
Contribution to the Paper	Study design, data analysis and manuscript critical review.		
Signature		Date	30/08/2022

Assessment of lamivudine and tenofovir pharmacokinetic variability in a Papua New Guinea HIV population through a combined high throughput LC-MS/MS assay

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

FUNDING

This work was funded by the Australian Centre for HIV and Hepatitis Virology Research (ACH2). Daniel Barratt receives salary support from an Australian Research Council Future Fellowship (FT180100565).

Abstract

Background Demographic covariates and kidney function contribute to variability in plasma lamivudine (3TC) and tenofovir (TNF) concentrations that often leads to the development of side effects. Although these drugs are prescribed for the treatment of HIV in Papua New Guinea (PNG) patients, their pharmacokinetic (PK) variabilities in this population have not been studied. **Aim** To assess the PK variability of 3TC and TNF in PNG HIV/AIDS patients. **Methods** Development and validation of an LC-MS/MS assay for the simultaneous quantification of 3TC and TNF in plasma was performed following FDA bioanalytical method validation guidelines. For 121 PNG HIV/AIDS patients, age, body weight, gender and serum creatinine levels data were recorded, and plasma 3TC and TNF concentrations quantified. Creatinine clearance (CrCl) and estimated glomerular filtration rate (eGFR) were calculated using Cockcroft-Gault equation and MDRD equations, respectively. Univariate and multivariate analysis were performed to assess the association of demographic covariates and kidney function with plasma 3TC and TNF concentrations. **Results** The LC-MS/MS method showed adequate performance for the quantification of 3TC and TNF. Median (range) plasma 3TC and TNF concentrations were 188 (15.5 to 1099) ng mL^{-1} and 64.4 (15 to 251) ng mL^{-1}, respectively. Lower eGFR (p= 0.022) and CrCl (p= 0.011) were associated with higher TNF concentrations. For 3TC only low CrCl was associated with significantly higher drug concentrations (p= 0.043), (eGFR p=0.11). Only a very small percentage (<8%) of the variation in drug concentrations was explained by the parameters tested. **Conclusions** A specific, precise and accurate method was developed and validated for simultaneous quantification of plasma TNF and 3TC, and was successfully applied to reveal, for the first time, large interpatient variability in TNF and 3TC exposure in PNG HIV/AIDS patients. Only a small proportion of this variability could be explained by the demographic and kidney function parameters tested.

1. Introduction

Papua New Guinea (PNG) has the highest prevalence and incidence of HIV in the Western Pacific Region (1). Tenofovir (TNF), a nucleotide reverse transcriptase inhibitors (NtRTI) and lamivudine (3TC), a nucleoside reverse transcriptase inhibitors (NRTI) are frequently prescribed as backbones for the preferred first-line and alternative first-line regimens for people living with HIV/AIDS (2). For many years the first-line therapy for HIV/AIDS in PNG was a fixed-dose combination of 3TC, TDF and efavirenz (EFV); however in 2019 PNG replaced EFV with dolutegravir, an integrase strand transfer inhibitor, following the recommendation of the World Health Organization (2, 3).

Tenofovir disoproxil fumarate (TDF) is the oral prodrug of TNF, which is metabolised intracellularly to the active tenofovir diphosphate, and is mainly excreted unchanged in urine by a combination of glomerular filtration and proximal tubular secretion (4). In patients with normal renal function (creatinine clearance (CrCl) $\geq$ 50 mL min^{-1}) (5) receiving 300 mg of TNF, the plasma median maximum concentration (C_{max}) of 343 ng mL^{-1} is achieved 0.8-1 h following oral dosing, with a terminal half-life of 18 h (6). Overexposure to tenofovir occurs in patients with renal impairment (CrCl < 50 mL min^{-1}) and therefore dose interval extension is recommended, along with clinical response and renal function monitoring (5, 6). Different factors have been associated with higher TNF concentrations, including serum creatinine concentrations (7), estimated glomerular filtration rate (eGFR) (8) and body weight (7, 8). Increased plasma TNF concentrations have also been linked to a higher risk of nephrotoxicity (9-12). Poizot-Martin et al. (2013) found that decreases in eGFR following initiation of a TDF-containing combination antiretroviral treatment (cART) regimen were significantly associated with older age and higher GFR at the time of TDF initiation (in males and females), and that a trough plasma TNF concentration >90 ng mL^{-1} was significantly associated with a decrease in GFR in women but not in men (9).

The majority of 3TC is also eliminated unchanged by the kidneys (~ 70%) via active organic cationic secretion and 5-10% is metabolized to the inactive trans-sulfoxide metabolite, which is also excreted in urine (13). Dose adjustments of 3TC are recommended for patients with CrCl≤ 50 mL min^{-1} (14). 3TC is rapidly absorbed after oral administration, with a median plasma C_{max} of 2600 ng mL^{-1} attained 0.5 - 1.5 hours after dosing with a terminal half-life of 5 to 7 hours (13). Lamivudine is generally well tolerated, however patients with renal impairment are at higher risk of developing adverse effects, such as lactic acidosis, diarrhoea, malaise, headache, fever and neutropenia (14). Previous studies have reported an association between CrCl and 3TC disposition (15, 16), and increased 3TC C_{max} in patients with moderate and severe renal impairment compared to those with normal renal function (16). In contrast to TNF, pharmacokinetic studies on 3TC are limited.

Previous assays for the quantification of plasma 3TC and TNF concentrations have been developed and published (17-19), either simultaneously or with other antiretroviral. However, their stability at high temperatures (60 ºC) had never been previously tested, even though HIV clinics heat the samples just after collection to avoid biological risk in transport and analysis. It has been shown that EFV metabolites lose their stability (up to 94% decrease in plasma concentrations) in patients' samples after this process (20).

In contrast to efavirenz (21), variability in 3TC and TNF pharmacokinetics has never been studied in a PNG population. Therefore, the aims of the present study were to a) validate a method to simultaneously quantify plasma 3TC and TNF concentrations by LC-MS/MS b) assess plasma 3TC and TNF stability at high temperature c) investigate the influence of demographic factors and renal function on 3TC and TNF concentrations, and d) investigate the impact of plasma TNF concentrations on patients' eGFR and CrCl.

2. Methods

2.1 Patients and sample collection

A total of 132 patients receiving a combination antiretroviral therapy (cART) at the HIV Heduru Clinic (Port Moresby General Hospital, PNG) from October 2017 to June 2018 were enrolled in the study, after giving written informed consent. Five were subsequently excluded for the lack of demographic information, and six patients were excluded for being outside the target sampling time range (10 to 20 h post-dose), resulting in 121 patients. Antiretroviral therapy contained 3TC 300 mg, TDF 300 mg and efavirenz (EFV) 600 mg in a single tablet, once daily. Plasma EFV concentrations were reported by us previously (21). Patient information such as gender, age, body weight and co-medication was documented, as well as the dosing time over the three days preceding sampling. The sample collection date/time was also recorded. Serum creatinine concentrations were obtained from the HIV Heduru Clinic. This study was approved by the Medical Research Advisory Committee of the National Department of Health of the Government of Papua New Guinea (MRAC No. 16.32) and the University of Adelaide Human Research Ethics Committee (H-2017-167).

Plasma samples were obtained after centrifuging the lithium heparin collection tubes (BD vacutainers, Melbourne, Australia) containing venous blood collected between 10 and 20 h after dosing. In order to inactivate the HIV virus from the samples, a heating step (60 ºC for 1 h in a water bath) was undertaken before the centrifugation of the tubes. The plasma samples were transported on dry ice from PNG to Adelaide (Australia) and stored at -20 °C until analysis.

2.2 TDF and 3TC plasma concentrations by LC-MS/MS analysis

2.2.1 Stock solutions and chromatography conditions

Stock solutions of TNF, 3TC, 3TC-d3 (Toronto Research Chemicals®), and TNF-d6 (Cayman Chemical®), were prepared by dissolution in either HPLC-grade methanol (TNF

and TNF-d6) or HPLC-grade water (3TC and 3TC-d3) at 1 mg mL^{-1}. Standard solutions were obtained by a mixture of TNF and 3TC at seven concentrations: 0.292, 1.17, 3.5, 7, 14, 28, 84 µg mL^{-1} (concentrations in the standard solutions) in 50% methanol. Labelled internal standard (IS) solutions were prepared by combining and diluting stock solutions to obtain 25 µg mL^{-1} for 3TC-d3 and 10 µg mL^{-1} for TNF-d6 in 50% methanol. Quality control (QC) solutions were prepared in 50% methanol at three concentrations: low (LQC) 0.9 µg mL^{-1}, medium (MQC) 9 µg mL^{-1}, and high (HQC) 81 µg mL^{-1}.

Quantification of the compounds was performed using a Nexera UHPLC system coupled to a LCMS-8040 tandem mass spectrometer (Shimadzu, Tokyo, Japan). Analytes were separated using an ACQUITY® HSS T3 Column, 1.8 µm, 2.1 mm x 150 mm (Waters, Milford, MA, US), at 40 ºC, and a gradient mobile phase at 0.4 mL min^{-1}. Mobile phase consisted of 0.1% formic acid in water (MP-A) and 0.1% formic acid in acetonitrile (ACN) (MP-B), with gradient starting at 3% MP-B held to 0.1 min, followed by a gradient increase to 6% MP-B from 0.1-1.5 min, then gradient increase up to 10% from 1.5-2.8 min and finally, increased to 97% MP-B at 2.8 min and holding for 0.7 min. The column was re-equilibrated with 3% MP-B from 3.5 to 5 min. Retention times for TNF and 3TC were 1.4 and 2.2 min, respectively, with a total run time of 5 min. Electrospray ionization in positive mode was used: nebulizing gas flow 3 L min^{-1}; drying gas flow 15 L min^{-1}; desolvation line temperature 250 °C and heating block temperature 400 °C. The quantification mass transitions (m/z) for TNF, TNF-d6, 3TC and 3TC-d3 were 288.1→136.1, 294.1→136.1, 230.1→112.1 and 233.1→115.1, respectively.

2.2.2 Sample preparation

Plasma samples were prepared by solid phase extraction (SPE) with Oasis MCX µElution 96-well plates (Waters). To prepare QC samples and calibrators, 10 µL of IS solution was

added to spiked plasma (95 µL of plasma + 5 µL of QC or standard solution), followed by 100 µL of 2% formic acid in water. SPE plate wells were conditioned with 200 µL of methanol, followed by 200 µL of 0.5% formic acid in water. Samples (200 uL) were loaded onto the sorbent bed, then washed using 200 µL of 0.5% formic acid in water, followed by 200 µL of 5% methanol in water, using a positive pressure manifold (Presston 1000®, Phenomenex) at 10 psi. The pressure was increased to a maximum of 10000 psi for 5 min in order to dry the cartridges for elution. After drying, samples were eluted with 2 x 25 µL of 5% NH_4OH and eluates evaporated to dryness in a centrifugal vacuum concentrator (Genevac™ miVac, Thermo Fisher Scientific®) at 40 ºC. Extracts were reconstituted in 100 µL of 50:50 MP-A:MP-B and one µL was injected into the system.

2.2.3 General method validation and application

Validation was performed following the FDA bioanalytical method validation guidelines (22), where the acceptance criteria for accuracy are mean estimated concentrations within ± 15% of nominal concentrations and maximum coefficient of variation (CV%) of 15%, except for the lower limit of quantification (LLOQ) (±20%). Tested parameters were precision and accuracy, linearity, sensitivity, selectivity, matrix effect and recovery, processed sample stability, and stability at 60 ºC for one h and room temperature for one h. Stability under other conditions relevant to these samples (e.g. freeze-thaw, long-term (at -20 ºC for 60 d)) was considered acceptable based on previous studies (17-19). Calibration curves were generated using analyte:IS peak area ratios and $1/x^2$ weighted linear regression. Recovery efficiency was assessed comparing the peak area of extracted QCs with extracted blank samples spiked with corresponding QCs post-extraction. Matrix effect on ionization was estimated as percentage difference in analyte peak area of QCs spiked into blank plasma extracts (from five different plasma sources) compared to QCs spiked into mobile phase.

Blank plasma from seven different human sources were processed to determine selectivity. Sensitivity was estimated by performing precision and accuracy tests (n= 30) at the concentration of the lowest calibrator. Intra-day (n= 5) and inter-day (n= 3) precision were determined by analysing three levels of quality control samples (low, medium and high) and were calculated as CV%. Accuracy was evaluated by calculating the percentage difference between nominal concentrations and estimated concentrations from the calibration curve.

Processed sample stability was tested with single LQC, MQC and HQC plasma extracts, stored in the autosampler (15 °C), and analysed every hour for 12 h; a maximum deviation of less than 15% was considered acceptable. To test for bench-top stability, three replicates of each QC (low, medium and high) were prepared and placed at room temperature for one h and then extracted as previously mentioned. HIV blood samples are often heated at 60 ºC for 1 h to avoid biological risk during transport and sample handling; therefore, we evaluated the stability of TNF and 3TC under this condition, with three replicates at each QC concentration, together with a freshly prepared calibration curve. The method was applied to the samples of 121 HIV/AIDS patients from PNG (10 µL of IS solution added 100 µL of patient plasma, extracted and analysed as per standards and QCs).

2.3 Pharmacokinetic statistical analysis

Plasma concentrations and patient demographics (age, body weight and gender) were summarized as median and range. Creatinine clearance (CrCl) was calculated using Cockcroft-Gault equation and estimated glomerular filtration rate was calculated using the MDRD equation. The following statistical tests were performed using RStudio cloud [RStudio, PBC].

To rule out a major confounding effect of variable sampling time post-dose requiring adjustment, Spearman correlations between sampling time post-dose and plasma TNF and

3TC concentrations were first checked using the cor.test function of the stats package (stats::cor.test) (23).

Correlations (Spearman) between 3TC and TNF, 3TC and EFV, and TNF and EFV concentrations, and between eGFR and CrCl, were also examined (stats::cor.test).

For regression analyses, the distributions of variables (age, body weight, plasma concentrations, sampling time, creatinine concentrations, CrCl and eGFR) were initially checked using histograms, quantile–quantile plots and Box-Cox power transformations (R packages::functions used were: graphics (23)::hist, stats (23)::qqnorm and qqline, and MASS (24)::boxcox, respectively).

To assess the influence of demographic factors and renal function on 3TC and TNF concentrations, linear regression (stats::lm) analyses were performed separately for 3TC and TNF testing for the main effects of age, gender, body weight, creatinine concentrations, CrCl and eGFR. Independent variables were first analysed separately (univariate) and then in combination (multivariate) with main effects of age, gender, body weight and either creatinine concentrations, CrCl or eGFR. Models incorporating creatinine concentrations, CrCl and eGFR were tested including all samples with creatinine concentrations, as well as within the subset of samples (see Table 1) with creatinine concentrations within eight d before or after the blood collection for plasma drug concentrations (8-day within).

Motivated by the findings of Poizot-Martin et al. (2013)(9), we performed a multivariate analysis with eGFR as the dependent variable, main effects of TNF concentration, age, body weight and gender, and a gender*TNF concentration interaction. In addition, to address potential issues with lateral collinearity (25), we also investigated serum creatinine concentrations as the dependent variable, with main effects of age, body weight and gender, and a gender*TNF concentration interaction.

All regression models were checked for linearity, homoscedasticity, normality and potential high leverage outliers (stats::plot). Multicollinearity was checked by variance inflation factor (VIF car::vif (25, 26)). Relative contributions (R^2) of independent variables in multivariate models were assessed using the averaging over orderings method (relaimpo (27)::calc.relimp).

3. Results

3.1 Patient characteristics

Patient characteristics are summarized in Table 1. One hundred and one patients had complete data for plasma 3TC and TNF concentrations, demographics, serum creatinine concentrations, eGFR and CrCl. CrCl and eGFR were significantly correlated (Spearman rho = 0.71, p= $2.2x10^{-16}$).

3.2 3TC and TNF method validation

The assay showed adequate performance for the quantification of TNF and 3TC in plasma samples. Specificity was confirmed with no interfering peaks detected in the tested blank samples. Calibration curves were linear for both compounds with r^2 ranging from 0.992 to 0.999. Mean recovery efficiencies for TNF and 3TC were 64% and 97%, respectively, and ion suppression for both compounds was between -14.4 to -1.6%. General method validation results (Table 2) demonstrated adequate precision (within- and between-assay imprecision <12.4%) and accuracy (100.7 to 104.1%) for both analytes. TNF and 3TC were stable under the conditions tested, with a maximum variation of 12.7%.

3.3 Interindividual variability in drug concentrations

The median (range) plasma 3TC and TNF concentrations of the 112 patients were 188 (15.5 to 1099) ng mL^{-1} and 64.4 (15 to 251) ng mL^{-1}, respectively (Table 1). Nine patients had

concentrations below the lower limit of quantification (14.6 ng mL^{-1}) for both drugs. Data for EFV plasma concentrations were extracted from our previous study (21), with median (range) being 1634.6 (67.8 to 12951.6) ng mL^{-1}. There was a strong positive correlation (Spearman rho= 0.75, $p< 2.2 \times 10^{-16}$) between plasma 3TC and TNF concentrations, and significant but weak positive correlations between plasma 3TC and EFV (Spearman rho= 0.32, $p= 0.016$) and between TNF and EFV (Spearman rho= 0.24, $p= 0.022$) (see Figure 1).

3.4 Contribution of variables to 3TC and TNF drug concentrations

Whilst sampling times post-dose varied (Table 1), there was no clear relationship between sampling time post-dose and either plasma TNF (Spearman rho= 0.009, $p= 0.92$), nor 3TC (Spearman rho= -0.086, $p= 0.36$) concentrations, and therefore no valid adjustment for differences in sampling time post-dose could be made. Initial distribution and linear regression analyses showed a need for log transformation of plasma TNF and 3TC, body weight, serum creatinine, CrCl and eGFR (with log10 transformation chosen), and square root transformation for age, to meet linear regression model assumptions.

3.5 Univariate Analysis

Analysis of individual variables showed that older age was significantly associated with higher TNF concentrations ($p= 0.048$), whilst gender and body weight had no significant association ($p= 0.28$ and $p= 0.59$, respectively). Lower eGFR (Figure 2) and CrCl were associated with significantly higher TNF concentrations when all patients were included ($p= 0.022$, co-efficient (log10-log10) estimate= -0.51, co-efficient std. error= 0.22; $p= 0.011$, co-efficient (log10-log10) estimate= -0.5, co-efficient std. error= 0.19, respectively). However, they were not significant in the 8-day within cohort ($p= 0.99$, co-efficient (log10-log10) estimate= -0.002, co-efficient std. error= 0.51; $p= 0.87$, co-efficient (log10-log10) estimate= -0.078, co-efficient std. error= 0.47, respectively for eGFR and CrCl) (Figure 2).

Lower CrCl was associated with higher 3TC concentration when all patients were included (p= 0.043, co-efficient (log10-log10) estimate= -0.5, co-efficient std. error= 0.25), but not in the 8-day within cohort (p= 0.59, co-efficient (log10-log10) estimate= 0.3, co-efficient std. error= 0.55). eGFR was not significantly associated with 3TC concentrations when including all patients or just the 8-day within cohort (p= 0.11 and p= 0.43, respectively). Age, gender and body weight were not significantly associated with 3TC concentrations (p= 0.32, p= 0.08 and p= 0.46, respectively).

3.6 Multivariate Analysis

Multivariate model results including eGFR for 3TC and TNF are shown in Table 3a and Table 3b, respectively.

Six percent and 21% of the variability in plasma 3TC concentrations was explained by the models in all patients and the 8-day within cohort, respectively. However, no individual regressor was statistically significant (p> 0.07). Less than 8% and 18% of the variability in plasma TNF concentrations could be explained by the multivariate models in all patients and the 8-day within cohort, respectively, with no significant predictor variables (p> 0.14).

Multivariate models including CrCl instead of eGFR had VIF > 2 for age, weight and CrCl, but are provided in Supplementary material in addition to multivariate models including serum creatinine (in place of eGFR or CrCl).

3.7 Influence of plasma TNF concentrations on estimated glomerular filtration rate and serum creatinine

In multivariate analyses investigating gender differences previously reported (9), older age was associated with significantly lower eGFR (p= 0.0015, when including all patients, and p= 0.004 with the 8-day within cohort), whilst gender, body weight and plasma TNF concentrations were not significantly associated with eGFR, with no significant

gender*TNF concentration interaction (Table 4). For serum creatinine, male gender was associated with significantly higher serum creatinine concentrations (p= 4.9 x 10^{-6}), whilst age, body weight and TNF concentrations were not significantly associated with serum creatinine (p= 0.27, p= 0.35, and p= 0.15, respectively). Similar results were found for the 8-day within cohort, with gender being associated with high serum creatinine (p= 0.0001) and explaining 43 % of serum creatinine variation (Supplementary table 1).

4. Discussion

Several methods have been developed for the quantification of TNF and 3TC, simultaneously or singly (17, 19, 28-39) however, when narrowing down to those specifically validated in human plasma and clinically applied to HIV/AIDS patients there are few reports (32, 34). Furthermore, stability at high temperatures was never investigated. In one of our recent publications we showed that EFV metabolites are not stable under high temperatures (60 ºC) (20) often applied to HIV samples in order to deactivate the virus to avoid biological risk during analysis. In the present study, TNF and 3TC stability was investigated showing that both were stable under higher temperatures, such as 60 ºC. Moreover, the method was shown to be precise, accurate, specific, with a minimum matrix effect which had no impact in the analysis, and it was successfully applied to the 121 PNG HIV/AIDS patient samples.

Tenofovir and lamivudine are generally well tolerated as part of Highly Active Antiretroviral Therapy (HAART), although different degrees of renal toxicity, including proximal tubulopathy, nephritis, acute tubular necrosis, proximal kidney tubular dysfunction (KTD), diabetes insipidus and lactic acidosis have been reported with tenofovir (8, 40-43). These adverse events are often associated with plasma drug concentrations, which are generally related to demographic covariates and individual renal function (10-12, 44). Moreover,

tenofovir renal toxicity can be influenced by changes in drug concentration (8, 9, 12, 45, 46). Rodriguez-Novoa et al. (2010) observed a 4.8 times higher risk of developing KTD in patients with plasma TNF trough concentrations >160 ng mL^{-1} compared to patients below that cut-off value (47). However, in our cohort, only two out of 103 patients had concentrations above this cut-off. Assessment of drug pharmacokinetic variability in specific populations could be useful to learn about factors influencing drug concentrations and renal function, and enable dose optimisation. PNG has the highest frequency of HIV infections in the Western Pacific Region (48), and evidence shows considerable interpatient variability in HIV drug concentrations (7, 12, 47, 49, 50). For example, we recently investigated the variability in EFV concentrations in PNG HIV infected patients and showed high variability being associated with *CYP2B6* polymorphism (21). However, this is the first study to investigate the inter-patient variability in TNF and 3TC plasma concentrations in this population.

As expected, TNF and 3TC plasma concentrations were very strongly correlated as renal excretion is their major route of elimination, whereas their correlations with plasma efavirenz were much less significant as efavirenz is eliminated by metabolism. The different/similar routes of elimination between EFV, TNF and 3TC are most likely to be related to the correlation results, rather than the methods applied for their quantification, considering both assays were developed and validated following official DBS and FDA bioanalytical method validation guidelines.

Regarding the influence of variables on 3TC and TNF plasma concentrations, univariate analysis showed that older age was associated with higher TNF concentrations, corroborating the results of Baxi et al. (2014), where they showed a 1.21 fold increase in TNF concentrations per decade (p= 0.0007) in 101 HIV infected women (11). Examining all patients, lower eGFR and CrCl were significantly associated with higher TNF

concentrations. Similar results were observed in the study by Rungtivasuwan et al. (2015), where low eGFR was independently associated with higher TNF concentrations ($p< 0.001$). Other studies also showed the impact of eGFR on TNF concentrations (11, 12). Previous studies in different ethnic groups showed significant associations between body weight (7, 11, 12, 44) and TNF concentrations, however this was not observed in this PNG cohort.

In contrast to TNF, there is a lack of studies on the influence of variables (including markers of kidney function) on 3TC exposure, even though it is eliminated mainly by the kidneys (approximately 70%) (13). In this study, of all the variables investigated, only lower CrCl was weakly associated with higher 3TC concentrations in the entire cohort. Multivariate models did not reveal any further significant predictors of plasma TNF or 3TC concentrations, with the included variables (eGFR, age, sex, body weight) combined explaining less than 8% of inter-individual variability.

One limitation of this study is that, for most patients, serum creatinine concentrations were not measured on the same day as the blood collection for drug measurements. Therefore, intra-individual variability in kidney function between sampling time points could have confounded eGFR and CrCl associations with plasma drug concentrations. Notably, the relationships between eGFR (and CrCl) and plasma TNF and 3TC concentrations were not significant when limited to patients for whom serum creatinine was quantified within 8 days of sampling for drug analysis. Figure 2 and coefficient estimates within this subset indicate this is not solely due to reduced sample size, nor due to the range of kidney function within this subset of patients compared to the entire cohort (Table 1 and Figure 2) and compared to previous studies demonstrating significant associations between eGFR or CrCl and plasma TNF or 3TC pharmacokinetics. A parameter not included in this investigation that could be contributing to the large unexplained variability is renal drug transporter variants which can

influence TNF concentration (44, 49). The presence and frequency of these alleles in the PNG population is not known.

The impact of TNF concentrations and demographic variables on renal function parameters has also been extensively reported and reviewed (9, 46, 51, 52). Poizot-Martin et al. (2013) reported an association between older age and high TNF concentrations with greater decreases in eGFR levels from time of TDF introduction to time of C_{trough} determination (median of 21 months of TDF exposure) (9). To examine whether a similar gender-dependent association between plasma TNF concentrations and eGFR existed in our cohort, we performed a multivariate analysis investigating the impact of TNF trough concentrations, age, gender and body weight on eGFR levels and serum creatinine. In accordance with this and Scherzer et al. (2012) (53), our results also showed a strong association between older age and lower eGFR. However, no association was found between TNF concentrations and gender, nor a significant gender*TNF concentration interaction. A key difference from Poizot-Martin et al. (2013) is that our study used a point measure of eGFR, whereas Poizot-Martin et al. (2013) used the change of eGFR from treatment initiation, which could explain the different findings (9). An alternative multivariate analysis with serum creatinine as dependent variable (instead of eGFR to avoid lateral collinearity issues with age and gender) also did not identify a significant effect of plasma TNF concentrations.

There are other limitations to the present study. Although we had a moderate sample size, the variability in kidney function compared to other studies was limited, with just a few patients (n= 3) with renal impairment (e.g. CrCl < 50 mL min^{-1}). Other variables that could potentially be associated with variability in TNF and 3TC pharmacokinetics such as reduced function genetic polymorphisms in renal drug transporters were not assessed in this study. In addition, we only used C_{trough} as the pharmacokinetic parameter, whereas others have used

area under the curve (AUC) to show the association between individual variables (e.g. age, body weight, concomitant medications, renal function) and TNF exposure (11).

In conclusion, the variability in plasma TNF and 3TC concentrations in PNG HIV/AIDS patients was assessed for the first time. A specific, precise and accurate method was developed and validated for their simultaneous quantification in human plasma and it was successfully applied to PNG HIV/AIDS patients to reveal large interpatient variability in TNF and 3TC exposure. Older age, and low eGFR and CrCl, were associated with higher TNF concentrations. Lower CrCl was associated with higher 3TC concentrations. However, only a small proportion of pharmacokinetic variability could be explained by the parameters tested. The investigation of polymorphisms in kidney drug transporter genes could help to elucidate further sources of variation.

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Table 1. Demographic data for 121 PNG HIV/AIDS patients receiving 300 mg of each 3TC and TDF per day.

		N
Age (years), median (range)	36 (17-65)	121
Weight (kg), median (range)	61 (38-99)	119*
Female, n (%)	77 (64 %)	121
Serum creatinine (µmol/l), median (range)	74 (31-153)	103*
Days between creatinine and drug sample, median (range)	561 (0 - 4472)	121
Serum creatinine within 8 days of drug sample (µmol/l), median (range)	78 (45-118)	30**
CrCl (mL/min), median (range)	99.3 (44.2-230.1)	103*
CrCl within 8 days of drug sample (mL/min), median (range)	88 (53-149)	30**
eGFR (mL/min/1.73m^2), median (range)	90 (31-221)	103*
eGFR within 8 days of drug sample (mL/min/1.73m^2), median (range)	83 (54-153)	30**
3TC and TNF sampling time post-dose (h), median (range)	14 (11-18)	121
Lamivudine (ng/mL), median (range)	188 (15.5 to 1099)	112[#]
Tenofovir (ng/mL), median (range)	64.4 (15 to 251)	112[#]
Efavirenz (ng/mL), median (range)	1634.6 (67.8 to 12951.6)	112[#]
Co-medications (n)		121
None	114	
Isoniazid	5	
Cotrimoxazole	1	
Cotrimoxazole and Carbimazole	1	

*Data not recorded in all case record forms

**Number of patients with creatinine levels measured within 8 days of the blood collection date for drug measurements.

[#] Excluding 9 patients below lower limit of quantification.

Table 2. Tenofovir and lamivudine LCMS assay precision, accuracy, matrix effect and stability.

QC sample	Analyte	Nominal concentration ng mL^{-1}	Precision (mean CV %)		Accuracy (mean %, n=15)	Matrix Effect (mean %, n= 5)	Stability		
			Inter-day (n=3)	Intra-day (n=5)			Auto-sampler 12 h (% change vs 0 h, n=1)	60ºC 1h (mean %change versus untreated, n=3)	Room T. 1h (mean %change versus untreated, n=3)
LLOQ	TNF	14.6	12.4	3.9	103.7	-	-	-	-
	3TC	14.6	10.1	3.5	100.9	-	-	-	-
LQC	TNF	45	4.0	3.8	104.1	-14.4	-2.7	12.6	8.9
	3TC	45	2.6	5.4	102.9	-14.1	-7.2	2.1	6.1
MQC	TNF	450	5.2	6.2	103.3	-14.1	+12.6	11.3	5.9
	3TC	450	8.1	10.4	101.1	-1.6	+12.4	4.1	4.3
HQC	TNF	4050	4.2	3.5	100.7	-11.5	+7.0	1.1	6.4
	3TC	4050	5.4	1.9	102.5	-8.5	+12.7	6.8	7.7

QC, quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; TNF, tenofovir; 3TC, lamivudine; CV %, coefficient of variation.

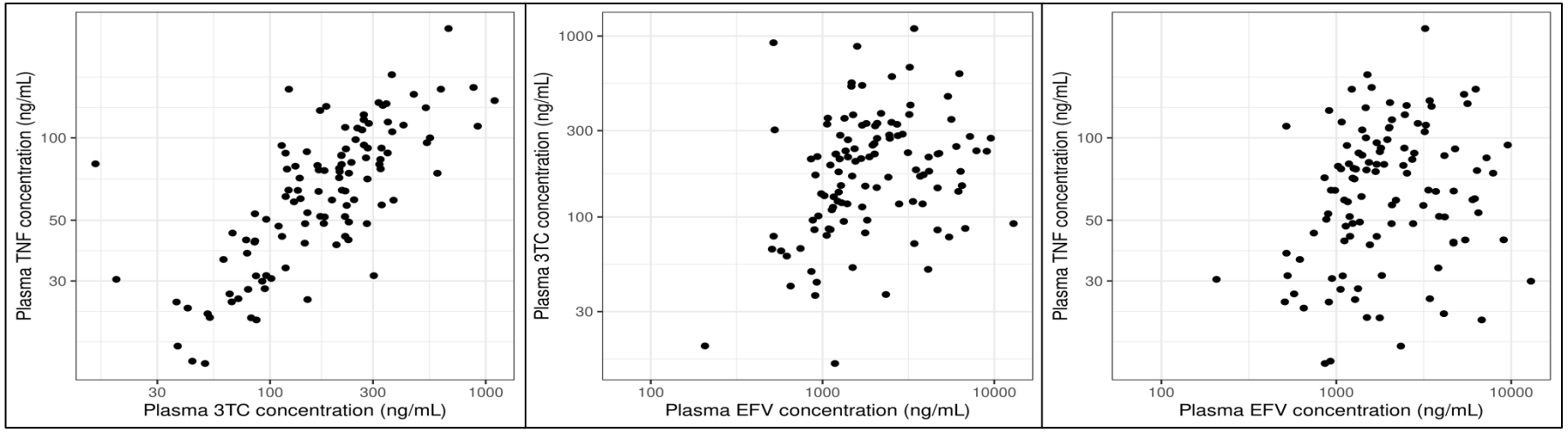

Figure 1. Correlation between lamivudine (3TC), tenofovir (TNF) and efavirenz (EFV) plasma concentrations (n= 112). A, Plasma TNF concentration vs plasma 3TC concentration $p< 2.2 \times 10^{-16}$, Spearman rho= 0.75. B, Plasma 3TC concentration vs plasma EFV concentration, $p=$ 0.016, Spearman rho= 0.32. C, Plasma TNF concentration vs Plasma EFV concentration, $p=$ 0.022, Spearman rho= 0.24. Axes are log10 scale.

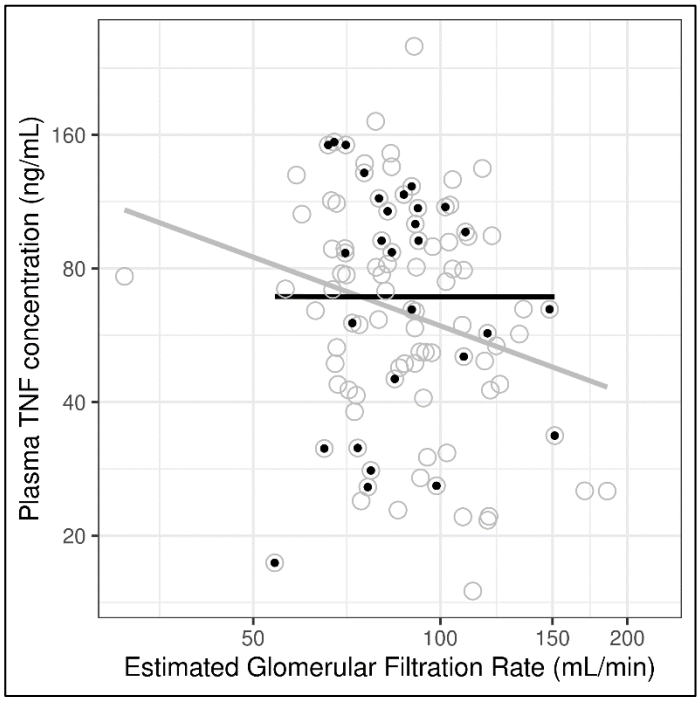

Figure 2. Plasma tenofovir (TNF) concentrations versus estimated glomerular filtration rate for all patients (n= 103, open circles) and the subset of patients with serum creatinine measurements within 8 days of sampling for TNF quantification (n= 30, filled circles). Lines are linear regression best fit of log10(TNF) versus log10(eGFR). Axes are log10 scale.

Table 3a. Predictors of log10-transformed plasma lamivudine (3TC) concentrations in PNG HIV/AIDS patients.

Variable	**Co-efficient estimate** (standard error)	**P-value**	**Contribution to model** (R^2)	**Variance inflation factor**
All patients *(n=101)*				
sqrt (Age) (years)	0.03 (0.04)	0.4	0.013	1.28
Male gender	0.05 (0.07)	0.44	0.006	1.23
Log10(Body Weight) (kg)	-0.52 (0.38)	0.17	0.015	1.61
Sampling time (h)	-0.02 (0.023)	0.33	0.008	1.05
Log10(eGFR) (mL/min/1.73 m^2)	-0.38 (0.31)	0.23	0.018	1.21
Subset of patients with serum creatinine measured with 8 days of 3TC quantification *(n=30)*				
sqrt (Age) (years)	0.08 (0.08)	0.31	0.02	1.61
Male gender	0.11 (0.15)	0.48	0.02	1.68
Log10(Body Weight) (kg)	0.36 (0.86)	0.67	0.006	1.78
Sampling time (h)	-0.08 (0.04)	0.07	0.12	1.26
Log10(eGFR) (mL/min/1.73 m^2)	1.06 (0.77)	0.18	0.04	1.72

Co-efficient estimates are for log10-transformed plasma 3TC concentrations. Sqrt = Square root transformation.

Table 3b. Predictors of log10-transformed plasma tenofovir (TNF) concentrations in PNG HIV/AIDS patients.

Variable	**Co-efficient estimate** (standard error)	**P-value**	**Contribution to model** (R^2)	**Variance inflation factor**
All patients *(n=101)*				
sqrt (Age) (years)	0.04 (0.03)	0.2	0.03	1.28
Male gender	0.009 (0.06)	0.88	0.0009	1.23
Log10(Body Weight) (kg)	-0.44 (0.31)	0.15	0.02	1.61
Sampling time (h)	-0.003 (0.019)	0.85	0.0004	1.05
Log10(eGFR) (mL/min/1.73 m^2)	-0.38 (0.25)	0.14	0.03	1.21
Subset of patients with serum creatinine measured with 8 days of 3TC quantification *(n=30)*				
sqrt (Age) (years)	0.09 (0.07)	0.19	0.05	1.61
Male gender	0.13 (0.13)	0.32	0.05	1.68
log10(Body Weight) (kg)	0.09 (0.74)	0.9	0.005	1.78
Sampling time (h)	-0.05 (0.04)	0.18	0.06	1.26
Log10(eGFR) (mL/min/1.73 m^2)	0.68 (0.66)	0.32	0.02	1.72

Co-efficient estimates are for log10-transformed plasma TNF concentration; eGFR, estimated glomerular filtration rate; sqrt, square root transformation.

Table 4. Predictors of log10-eGFR in PNG HIV/AIDS patients.

Variable	**Co-efficient estimate** (standard error)	**P-value**	**Contribution to model** (R^2)	**Variance inflation factor**
All data *(n=101)*				
sqrt(Age) (years)	-0.04 (0.01)	0.0015**	0.12	1.16
Male gender	0.012 (0.02)	0.62	0.004	51.8[a]
Log10(Body Weight) (kg)	-0.12 (0.13)	0.35	0.012	1.18
Log10(TNF) (ng/mL)	-0.06 (0.04)	0.14	0.03	1.05
Log10(TNF):Gender	-0.03 (0.08)	0.74	0.001	52.8[a]
Subset *(n=30)*				
sqrt(Age) (years)	-0.06 (0.02)	0.004**	0.27	1.23
Male gender	0.17 (0.23)	0.24	0.1	56.8[a]
Log10(Body Weight) (kg)	-0.08 (0.2)	0.67	0.05	1.51
Log10(TNF) (ng/mL)	0.13 (0.09)	0.33	0.012	3.07
Log10(TNF):Gender	0.12 (0.12)	0.35	0.017	62.4[a]

Co-efficient estimates are for log10-transformed eGFR; eGFR, estimated glomerular filtration rate; sqrt, square root transformation. **P<0.01.
[a]High VIF is expected for interaction and component terms.

Supplementary table 1. Predictors of log10-Creatinine in PNG HIV/AIDS patients.

Variable	**Co-efficient estimate** (standard error)	**P-value**	**Contribution[a] to model** (R^2)
All data *(n=101)*			
sqrt (Age) (years)	0.05 (0.21)	0.27	0.04
Male gender	0.1 (0.02)	$4.9x10^{-6}$	0.23
Log10(Body Weight) (kg)	0.11 (0.11)	0.35	0.03
Log10(TNF) (ng/mL)	0.06 (0.04)	0.15	0.02
Subset *(n=30)*			
sqrt (Age) (years)	0.02 (0.01)	0.17	0.09
Male gender	0.15 (0.03)	0.0001	0.43
Log10(Body Weight) (kg)	0.09 (0.17)	0.59	0.12
Log10(TNF) (ng/mL)	-0.05 (0.05)	0.32	0.008

Co-efficient estimates are for log10-transformed creatinine; sqrt, square root transformation.

Chapter 6: Development and clinical application of a method to quantify DTG in DBS and VAMS

Statement of Authorship

Title of Paper	Determination of dolutegravir in dried blood spots and volumetric absorptive microsampling (VAMS) by LC-MS/MS
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	To be submitted

Principal Author

Name of Principal Author (Candidate)	Natalia Bordin Andriguetti		
Contribution to the Paper	Study design, performed experiments and statistical analysis, data interpretation, wrote the first manuscript draft and will act as the corresponding author.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/09/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Background Dolutegravir has been prescribed widely for HIV infection since 2019. Accurate quantification of dolutegravir pharmacokinetic variability is required to investigate the potential contribution to clinical efficacy and side effects. Conventional plasma sampling can be a challenge for remote patient populations due to cold chain transportation requirements to reference laboratories. Microsampling devices are an alternative for sample collection and transportation, not requiring refrigeration. **Aim** The present study aimed to develop and validate a LC-MS/MS method to quantify dolutegravir in dried blood spots (DBS) and volumetric absorptive microsampling devices (VAMS). **Methods** Dolutegravir was extracted from DBS and VAMS with a simple one-step solvent sample preparation, followed by reverse-phase chromatographic separation in a C18 column with a run time of 4.1 min, and detection by tandem mass spectrometer. Validation was performed following official DBS and FDA bioanalytical method validation guidelines. The method was applied to matching DBS and VAMS samples from 15 HIV/AIDS patients from Papua New Guinea. **Results** The assay was linear in the range of 0.025 to 8 μg mL^{-1} for both devices. Inter-day and intra-day precision show ed a maximum variation of 4.3% and 6.1% for DBS and 4.4% and 2.9% for VAMS, respectively. Accuracy was in the range of 98.2 to 105.4% for DBS and 93.8 to 101.4% for VAMS. The method had minimal matrix effect, with less than 10% deviation. Mean recoveries at a haematocrit of 40% were 70-71% for both devices. For DBS, haematocrits of 20 and 50% had mean dolutegravir recovery of 52%, and there was an increase in estimated concentrations at a haematocrit of 50% (15-35% higher than nominal concentrations). Dolutegravir was stable in both devices at room temperature for up to 14 days and extracted samples were stable in the autosampler for 12 h. When kept at 60 ºC for 48 h, 30% and 55% decreases in dolutegravir concentrations were observed for DBS and VAMS devices, respectively. Volcano effect test for DBS showed no difference in accuracy

between central and peripheral punch. Patients' samples had mean dolutegravir concentrations of 0.938 µg mL^{-1} (range 0.168 to 2.08 µg mL^{-1}) for DBS and 1.2 µg mL^{-1} (range 0.279 to 2.44 µg mL^{-1}) for VAMS. There was a positive correlation between DTG DBS and VAMS concentrations (Pearson r= 0.96, p= $9.8x10^{-8}$), with a bias (-0.17 µg mL^{-1}) toward lower concentrations in DBS. **Conclusions** For the first time, a method for the quantification of dolutegravir in DBS and VAMS was developed and applied to HIV patients' samples. DBS and VAMS presented similar assay performance, however as a fixed volume device, VAMS did not require extra haematocrit experiments, simplifying method validation. DBS and VAMS results in clinical samples were highly correlated.

1. Introduction

Dolutegravir (DTG), an integrase strand transfer inhibitor (INSTI), was approved by the US FDA in 2013 (1) and is currently recommended worldwide as the first-line treatment for HIV (2). This class of antiretroviral acts by blocking the viral integrase enzyme, which is responsible for insertion of the viral genome into the host DNA (3). DTG is administered orally (50 mg), once a day, in a co-formulated combination tablet (1).

The administration of DTG often leads to the development of side effects with a high incidence (~90%), however they are mostly mild reactions that cease with time (4, 5). Most common side effects are diarrhoea, fatigue, headache and nausea (1, 6). Most recent studies have reported neuropsychiatric toxicities, such as peripheral neuropathy, sleep disturbances, depression and anxiety (7, 8). The tolerability to these side effects is partially related to pharmacokinetic (PK) and pharmacogenetic differences between individuals (9, 10).

The PK profile of DTG has been assessed in healthy and HIV-infected adults. The variability in DTG PK has shown to be moderate, with a coefficient of variability (CV%) of 25-40% in area under the curve (AUC) and maximum plasma concentration (C_{max}), and 30-65% in

trough concentrations (C_{trough}). However, most studies were in the clinical trial setting (10-13), and there is a lack of studies on DTG pharmacokinetic variability and impact on long-term therapeutic effects in "real world" patients. A therapeutic range has not been established, however mean C_{trough} previously reported were between 1000-4000 ng mL^{-1} (14-17), and development of neurological toxicity related to DTG use was reported at concentrations of 6060 ng mL^{-1} (18). Therapeutic drug monitoring (TDM) could be useful to optimise antiretroviral therapy, reducing the occurrence of side effects while maintaining clinical efficacy (18, 19). TDM is dependent on the availability of reliable and specific bioanalytical assays, mostly involving chromatographic techniques coupled with mass spectrometry (MS) (20).

The conventional sampling strategy to estimate drug PK for TDM purposes requires plasma separation from blood after phlebotomy, and several analytical methods to quantify DTG in human plasma have been published (15-17, 21-23). However, this conventional sampling method requires cold transport of samples which is not ideal or suitable for remote sites such as Papua New Guinea (PNG). The use of microsampling, such as dried blood spots (DBS) and volumetric absorptive microsampling devices (VAMS) could be an alternative for sample transportation from distant sites to reference laboratories, due to simplified logistics, not requiring refrigeration (24, 25). Other advantages with these devices are the possibility of self-sampling, less invasive sampling and increased biosafety after drying (24, 26). VAMS has an advantage over DBS related to sample volume accuracy and not requiring haematocrit testing (25). To date, there are no published validated methods for the quantification of DTG in DBS or VAMS.

Considering the potential advantages of this method application to remote sites for the study of pharmacokinetics and TDM of HIV drugs, this study aimed to develop and validate a

method for the quantification of DTG in DBS and VAMS, and apply it to DBS and VAMS samples of HIV/AIDS patients, based on FDA and DBS guidelines criteria.

2. Experimental

2.1 Reagents, materials and standards

DTG and labelled DTG (DTG-d5) were purchased from Toronto Research Chemicals (North York, Canada). LCMS-grade water, formic acid, acetonitrile (ACN) and methanol (MeOH) were acquired from Thermo Fisher Scientific (Australia). Whatman 903® paper was obtained from GE Healthcare (Westborough, USA). VAMS® devices (10 µL tip) were purchased from Neoteryx (Torrance, USA).

2.2 Preparation of solutions

Stock (1000 µg mL^{-1}) and working (0.5, 2, 10, 20, 50, 100, 160 µg mL^{-1}) solutions of DTG were prepared by dissolution in 50% MeOH. Quality control (QC) working solutions were prepared from DTG stock solution at low (LQC, 1.5 µg mL^{-1}), medium (MQC, 12 µg mL^{-1}) and high (HQC, 120 µg mL^{-1}) concentrations in 50% MeOH. The working solutions were prepared at concentrations 20 times higher than the target calibration and quality control levels for further dissolution in whole blood. Stock and intermediate solutions of DTG-d5 internal standard (IS) were prepared at 1000 µg mL^{-1} and 10 µg mL^{-1}, respectively in 50% MeOH. DBS and VAMS extraction solution was prepared as a mixture of MeOH and ACN (90:10, v/v), containing DTG-d5 at 20 ng mL^{-1}.

2.3 Chromatography and mass spectrometry conditions

DTG DBS and VAMS samples were analysed using a Nexera binary UHPLC system coupled to a LCMS-8040 tandem mass spectrometer (Shimadzu, Tokyo, Japan). Chromatographic separation was performed with a C18 Acquity Premier HSS T3 1.8 µm,

2.1x150 mm column (Waters, Milford, USA) at 40ºC and gradient mobile phase at 0.5 mL min^{-1}. The mobile phase comprised 0.1% formic acid in water (MP-A) and 0.1% formic acid in ACN (MP-B), starting at 50% MP-B, follow by a gradient increase to 90% MP-B from 0.01 to 2.1 min, held at 90% MP-B 2.1 to 3.1 min, then back to 50% MP-B at 3.11 min and held at 50% until the end at 4.1 min. DTG and DT-d5 retention time was 1.5 min with a total run time of 4.1 min. Mass spectrometry (MS) conditions were as follows: electrospray ionization in positive mode, desolvation line temperature 300ºC; heating block temperature 450ºC; nebulizing gas flow 1.5 L min^{-1}; drying gas flow 20 L min^{-1}. Precursor and product ion scans were applied to find the transitions (*m/z*) for DTG and DTG-d5. The selected multiple reaction monitoring (MRM) *m/z* for quantification of DTG were 420.1 → 277.05, and for DTG-d5 were 425.1 → 277.05.

2.4 Preparation of DBS and VAMS samples (calibrators and quality control samples)

Calibrators and QC samples were prepared by diluting DTG working solutions with venous blood (HT 40%, unless otherwise specified) in the proportion of 1:20 (v/v) in 1.5 mL polypropylene tubes. The tubes were gently mixed by inversion and 50 µL of blood of each calibrator and QC were pipetted onto the Whatman 903® paper and kept at room temperature for at least 3 h to dry before extraction. VAMS tips were gently touched to spiked blood for 2 seconds and slowly removed and left to dry for a minimum of 3 h before extraction.

2.5 DBS and VAMS sample preparation

Six millimetre diameter punched discs from DBS (cut in half) and VAMS tips from calibrator or QC samples were transferred to a 2 mL polypropylene tube, followed by the addition of 500 µL of extraction solution containing IS. The tubes were agitated at 500 rpm for 60 min at room temperature (ThermoMixer, Eppendorf). Extraction solution (480 µL) was then transferred to a 1.5 mL polypropylene tube and evaporated in a vacuum centrifuge

(Genevac^{TM} miVac − Thermo Fisher Scientific) at 50 ºC for 30 min and reconstituted with 150 µL of mobile phase (50%MP-B). The tubes were agitated (500 rpm) for 1 min, then filtered in 0.22 µm filter plate wells (ISOLUTE® filter plates, Biotage), transferred to glass vials and finally 2 µL was injected into the LC-MS/MS system.

2.6 Linearity

Calibration DBS and VAMS samples had concentrations of 0.025, 0.1, 0.5, 1, 2.5, 5 and 8 µg mL^{-1} and were analysed on 5 different days. Calibration curves were constructed by weighted least-squares linear regression of nominal concentrations of the calibration samples against the peak area ratios of DTG/DTG-d5, and were evaluated through their coefficients of correlation (r) and cumulative percentage relative error (Σ%RE). Weighting factors evaluated were unweighted, $1/x$, $1/x^2$, $1/x^{0.5}$, $1/y$, $1/y^2$ and $1/y^{0.5}$.

2.7 Precision and accuracy

QC samples had concentrations of 0.075 (LQC), 0.60 (MQC) and 6.0 µg mL^{-1} (HQC) and were analysed in quintuplicate (DBS and VAMS), on each of 3 days. Accuracy was described as the percentage of the nominal concentration of the estimated concentration from the calibration curve. Within and between-assay precision was expressed as coefficient of variation (CV%). The acceptance criteria were a maximum CV of 15% and mean inaccuracy ± 15% of the nominal concentrations (26, 27).

2.8 Sensitivity

Five replicates of the lower limit of quantification (LLOQ, 0.025 µg mL^{-1}) were included in precision and accuracy experiments (DBS and VAMS), on each of 3 separate days. Acceptance criteria were inaccuracy <20% and imprecision <20% CV (26, 27).

2.9 Selectivity

Blank capillary blood from six different human sources were used to prepared DBS and VAMS. Criteria of participants inclusion was: healthy adults (25 to 72 years old), not taking DTG, and written informed consent (University of Adelaide Human Research Ethics Committee H-2022-158). The samples were processed as described above, without IS, to verify the absence of chromatographic peaks that might interfere with DTG and DTG-d5 quantification.

2.10 Carryover

Zero (free of drug and IS) and a blank (with IS) DBS and VAMS samples were injected immediately after the highest calibrator on five of the analysis days for evaluation of chromatographic carryover effect. Peak areas ≤ 20% of the LLOQ and ≤ 5% (IS) were accepted (26, 27).

2.11 Matrix effect and recovery

For estimation of matrix effect, a post-extraction spike method was performed (28). Blank venous blood samples from the six different human sources (same participants as for selectivity experiments) (DTG free) and two levels of QCs (low and high) were used for this experiment (University of Adelaide, Ethics approval: H-2022-158). For DBS, it is known that HT will impact spot size and homogeneity, and possibly extraction recovery and matrix effect, so it is essential to evaluate recovery and matrix effect at different haematocrit values. The blood from one of the donors was prepared in three different HT (20, 40 and 50%), combining appropriate volumes of cells and plasma as per (29). For the evaluation of matrix effect, zero DBS sample (whole spot containing 10 µL of venous blood without DTG) extracts spiked post-extraction with DTG and IS were compared to mobile phase spiked with DTG and IS concentrations equivalent to complete recovery. Recovery efficiency was estimated by comparing DBS samples (whole spot containing 10 µL of venous blood) spiked

with DTG before spotting and extraction (with IS added post-extraction) to blank DBS (whole spot containing 10 μL of venous blood) extracts spiked post-extraction with DTG and IS. The same process was performed for VAMS for evaluation of recovery and matrix effect, except that for VAMS there was no need to evaluate different haematocrit values (30).

2.12 Stability in the autosampler

Single low and high QC DBS and VAMS samples were extracted as described above and injected under the conditions of a normal analytical run at one hourly intervals for 12 h. Peak areas ratios of DTG/DTG-d5 between the beginning and end of series were compared. A maximum deviation of ± 15% in peak area ratios from the beginning to the end of the series was considered acceptable (26, 27).

2.13 Stability of DBS and VAMS maintained at different temperatures

Low and high QC DBS and VAMS samples were prepared, stored at 25 and 60 ºC and analysed on days 2, 7 and 14 (only at 25 ºC), in quintuplicate. Five replicates of freshly prepared LQC and HQC samples were extracted on day zero to use as reference. Stability was considered acceptable if all results were within 100 ± 15% of the QC sample concentrations measured on day zero (26).

2.14 DBS-specific validation parameters

2.14.1 Influence of haematocrit on accuracy

Whole venous blood was prepared at three different HT (20, 30, 50%) as per (29). DTG was added to achieve the concentrations of LQC and HQC and extraction was performed as described above (see section 2.5). Five replicates of each QC concentration for each HT were analysed. The influence of the HT on DTG measurements was determined as the

percentages of nominal concentrations that were measured in the DBS with HT 40%. Acceptance criteria were values in the range of 85–115% (26).

2.14.2 Impact of spotted blood volume on accuracy

LQC and HQC were prepared with blood HT 40% and pipetted onto Whatman 903® paper at the volumes of 30, 40 and 55 µL, consistent with finger prick blood drops. The samples were dried at room temperature for 3 h then analysed as described above and DTG was quantified with a standard calibration curve (HT 40%, 50 µL blood spots). The influence of the spotted volume on DTG measurements was determined as the percentages of concentrations that were measured in the HT 40%, 50 µL DBS. Acceptance criteria were values in the range of 85–115% (26).

2.14.3 Volcano effect

DBS homogeneity (volcano effect) was assessed at three different HT levels (30, 40 and 50%). LQC and HQC spots of 50 µL of blood were placed on paper and central and peripheral punches were analysed, five of each QC of each HT% level. The samples were compared with a calibration curve with HT% 40 and average volume of blood spot (50 µL) of which a central punch was used for extraction. Acceptance criteria were "peripheral punches" values with a deviation of ≤ 15% of "central punches" (26).

2.15 Application of the method

The methods were applied to 15 DBS and 15 VAMS samples from Papua New Guinean HIV subjects who were being treated with dolutegravir (50 mg once daily). This study was conducted under the ethics approval by the University of Adelaide Human Research Ethics Committee (H-2017-167) and Medical Research Advisory Committee of the National Department of Health Government of Papua New Guinea (MRAC No. 16.32). Patients provided written informed consent. Following lancing with E.Z. lancet (Macquarie Medical

Systems), one drop of blood from the patient finger was placed onto Whatman 903® paper without touching the surface of the paper. The VAMS tip was then touched on the blood drop formed on the patient finger for 2 seconds. The samples were left in room temperature for the minimum of 3 hours. The samples were transported to Adelaide in plastic bags containing desiccants, in a carton box and maintained at ambient temperature until analysis.

DBS and VAMS methods were compared by Pearson correlation, Bland-Altman analysis and Passing-Bablok regression.

3. Results and discussion

3.1 General validation method

Of the weighting factors evaluated, $1/x^2$ was selected for the analysis for having the highest R^2 and lowest Σ%RE. The method was linear within the interval of 0.025-8 µg mL^{-1} with coefficients of determination (R^2) ranging from 0.996 to 0.999 for both DBS and VAMS. No interfering peaks were detected in the blank samples. Chromatograms of dolutegravir in DBS and VAMS are represented in Figure 1, including LLOQ, HQC and patient samples. The parameters of precision, accuracy, sensitivity, matrix effect and recovery are presented in Table 1. Inter-day and intra-day imprecision showed a maximum variation of 4.3% and 6.1% for DBS and 4.4% and 2.9% for VAMS, respectively. Accuracy was in the range of 98.2 to 105.4% for DBS and 93.8 to 101.4% for VAMS. The lower limit of quantification was 25 ng mL^{-1}, and presented a maximum variation for inter- and intra-assay imprecision of 6% for both DBS and VAMS, and accuracy of 97.6% and 104.4% for DBS and VAMS, respectively. The method had a minimal matrix effect, with a maximum deviation of 9.9%, for DBS and -9.6% for VAMS. Mean recovery rates were 70% for DBS and 71% for VAMS. For both HT 20 and 50% mean recovery rate for DBS was 52%.

Results for stability experiments are present in Table 2. DTG was stable in DBS and VAMS after 14 days at 25 ºC, with a maximum variation from the nominal concentration of -7.6% and -5.5%, respectively. However, both devices had a decrease in concentration by 30% (DBS) and 55% (VAMS), after 2 days in an incubator at 60 ºC. The stability of extracted DTG kept in the autosampler for 12 h was acceptable, with a maximum loss in concentration of 8.9% for DBS and 6.5% for VAMS at the end of the series. Stability test at high temperature was conducted at the extreme temperature for sample transportation, however the results were unacceptable. For this reason, further stability at high temperatures under more likely conditions of transport are desirable (low and high QC DBS and VAMS samples stored at 35 and 45 ºC, analysed on days 1, 2 and 7).

3.2 Specific DBS method validation

Blood viscosity increased with HT, resulting in higher volumes in the 6 mm disk (less spread) at high HT levels and smaller volumes (more spread) in the disk at low HT levels. Therefore, the impact of HT on accuracy was evaluated on LQC and HQC samples at HT ranging from 20 to 50%. Results were acceptable for HT 20 and 30%, 95.7-105.2% and 90.5-96.5%, respectively (Table 3). However, for HT 50% an increase in mean concentration was observed (15-35% increase from the nominal concentration). The HT range that needs to be evaluated depends on the target population (26); there are no published data on HT ranges in the PNG HIV/AIDS patient population, and HT of patients in this study was unknown. An alternative in this situation is the use of VAMS, which was designed to take up a fixed volume by capillary action, therefore minimising issues associated with the volume of blood analysed with different HT.

No significant impact of blood spot volume (30-55 µL) on the accuracy of DBS DTG measurements was observed (Table 4), with DTG measured concentration in the range of

88.5 to 97.4% of nominal levels. When comparing results from peripheral to central areas (Table 5), no unacceptable volcano effect on DBS accuracy was observed, with a range of 89.3-109.9%.

3.3 Method application

Mean DTG DBS concentration of the 15 samples analysed was 0.938 µg mL^{-1} (range 0.168 to 2.08 µg mL^{-1}). Mean DTG VAMS concentration of the 15 samples analysed was 1.2 µg mL^{-1} (range 0.279 to 2.44 µg mL^{-1}). Two patients had concentrations below LLOQ for both devices.

There was a strong positive correlation between the two devices (Pearson r = 0.96, p= $9.8x10^{-8}$), see Figure 2. Bland-Altman analysis identified a bias of -0.17 µg mL^{-1} (upper 95% CI: -0.08, lower 95% CI: -0.26) toward lower concentration in DBS.

The Passing-Bablok regression equation, comparing DTG measured in DBS and DTG measured in VAMS, presented a slope coefficient of 1.09 with 95% confidence interval of 0.8841 to 1.3799 and 0.09 with 95% coefficient interval of -0.1270 to 0.3066 for intercept (Figure 3). Since 1 is included in the confidence interval for the slope and 0 is included in the confidence interval for intercept, the methods are considered overall comparable.

Previous studies found DTG (50 mg daily) plasma concentrations between 0.65 and 3.8 µg mL^{-1} (22, 31, 32). DTG shows minimum binding to blood cells, with a mean blood/plasma concentration ratio of 0.441 to 0.535 (33). Hence, for a patient with the mean VAMS DTG concentration of 1.2 µg mL^{-1} the estimated plasma concentration would be approximately 2.4 µg mL^{-1}. Therefore, although the HT and plasma concentrations for these patients are unknown, results are within the expected range.

4. Conclusions

A method for the determination of DTG in DBS and VAMS devices was developed and validated using LC-MS/MS. DTG was stable at room temperature for up to two weeks, indicating the possibility of short-term storage if required. Loss of DTG concentrations was observed when kept for 2 days at 60 ºC. It is unlikely that samples would be transported at this temperature, but attention should be given when shipping is required and additional experiments testing the stability at 45 ºC (usual temperature inside transport trucks) should be performed. DBS and VAMS presented similar peak areas (same sample volume of 10 µL and similar recovery) and results for general validation parameters, although lower limits of detection and quantification were not evaluated below 25 ng mL^{-1}. DBS measurements were significantly affected by HT, highlighting the main advantage of VAMS (in addition to simpler validation not requiring the additional HT experiments).

The method was successfully applied to PNG HIV/AIDS patient DBS and VAMS samples, however, unfortunately, plasma samples of the same patients were not available to complete the clinical validation with the translation of DBS and VAMS concentration results to plasma values. It is important to emphasise that the conventional blood sampling (venous blood) and plasma preparation is a big challenge in remote sites, where the samples need to be transported to long-distance reference laboratories for analysis, thus the use of microsampling comes as a great alternative.

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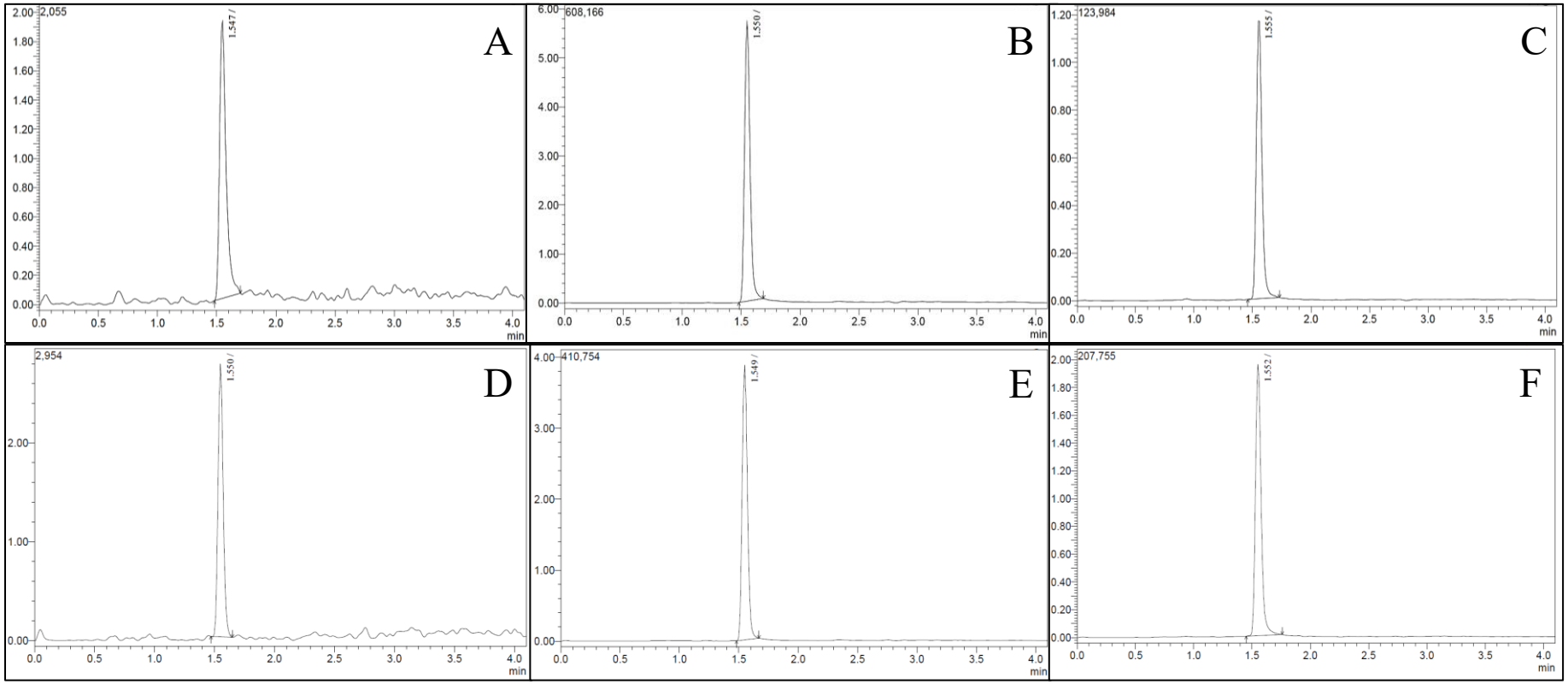

Figure 1. Chromatograms of dolutegravir in DBS (A, B, C) and VAMS (D, E, F) samples. A, LLOQ of dolutegravir (25 ng mL^{-1}); B, HQC of dolutegravir (6000 ng mL^{-1}); C, Patient sample (1300 ng mL^{-1}); D, LLOQ of dolutegravir (25 ng mL^{-1}); E, HQC of dolutegravir (6000 ng mL^{-1}); F, Patient sample (2000 ng mL^{-1}). Auto-scale to intensity signal. Figures C and F are DBS and VAMS samples from the same patient.

Table 1. Dolutegravir determination in DBS and VAMS: precision, accuracy, matrix effect and recovery.

QC sample	Microsampling device	Nominal concentration µg mL^{-1}	Precision (mean CV %)		Accuracy (mean %)	Matrix effect (mean %)	Recovery (mean %)
			Inter-day	Intra-day			
LLOQ	DBS	0.025	5.2	2.8	97.6	-	-
	VAMS	0.025	6.0	2.7	104.2	-	-
LQC	DBS	0.075	2.2	1.8	105.4	1.0	69
	VAMS	0.075	4.4	1.0	101.4	-3.3	68
MQC	DBS	0.60	2.1	6.1	106.4	-	-
	VAMS	0.60	3.2	2.9	101.6	-	-
HQC	DBS	6.0	4.3	5.5	107.6	6.1	72
	VAMS	6.0	3.9	2.7	107.9	3.7	75

QC, quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; DBS, dried blood spot; VAMS, volumetric absorptive microsampling; CV%, coefficient of variation.

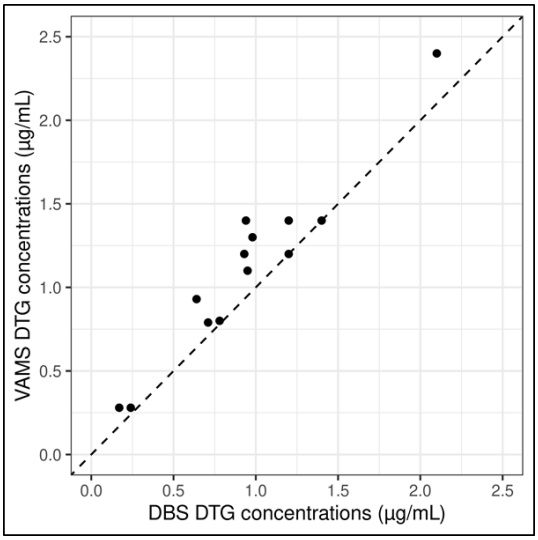

Figure 2. Correlation between DBS and VAMS DTG concentrations (Pearson's r= 0.96, p= $9.8x10^{-8}$) in 13 PNG HIV/AIDS patients. Dashed line= line of equality.

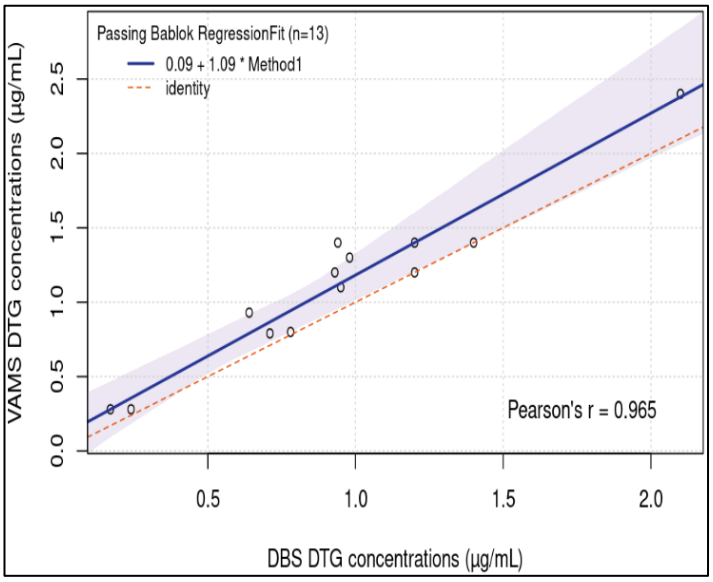

Figure 3. Passing-Bablok comparison of DTG concentrations measured in DBS and DTG concentrations measured in VAMS in 13 PNG HIV/AIDS patients. Dashed line= line of equality/identity. Blue coloured area= confidence interval (95%).

Table 2. Stability of DTG at different temperatures (percentage of nominal concentration).

QC sample	Microsampling device	Nominal concentration (µg mL^{-1})	Temperature (ºC)	Day 2	Day 7	Day 14
LQC	DBS	0.075	21	95.5	95.1	92.4
	DBS	0.075	60	62.1	70.4	-
	VAMS	0.075	21	101.7	92.3	99.6
	VAMS	0.075	60	63.3	46.4	-
HQC	DBS	6.0	21	86.5	94.2	92.5
	DBS	6.0	60	56.7	67.8	-

	VAMS	6.0	21	98.1	86.8	94.5
	VAMS	6.0	60	62.3	46.4	-

QC, quality control; LQC, low quality control; HQC, high quality control; DBS, dried blood spot; VAMS, volumetric absorptive microsampling.

Table 3. Evaluation of the influence of HT on accuracy (HT effect) in DTG measurements.

HT%	QC sample	Nominal concentration (µg mL^{-1})	Accuracy (%)
20	LQC	0.075	105.2
	HQC	6.0	95.7
30	LQC	0.075	96.5
	HQC	6.0	90.5
50	LQC	0.075	135.1
	HQC	6.0	115.1

n= 5 each control, at each HT%; HT%, haematocrit; QC, quality control; LQC, low quality control; HQC, high quality control (n= 5 each control sample, at each HT% value).

Table 4. Evaluation of the influence of spotted volume on the accuracy (volume effect) of DTG measurements in DBS.

Volume (µL)	Nominal concentration (µg mL^{-1})	Accuracy (%)
30	0.075	89.4
	6.0	88.5
40	0.075	93.6
	6.0	93.3
55	0.075	97.4
	6.0	91.6

n= 5 for each control, at each volume.

Table 5. Evaluation of DTG homogeneity (volcano effect) in DBS.

HT%	QC sample	Nominal concentration µg mL^{-1}	Accuracy (%)
30	LQC	0.075	106.6
	HQC	6.0	109.9
40	LQC	0.075	100.9
	HQC	6.0	103.8
50	LQC	0.075	89.3
	HQC	6.0	112.2

HT%, haematocrit; QC, quality control.

Chapter 7: Discussion and Future Perspectives

Even though manageable, HIV continues to be a major public health problem, affecting millions of people around the world, particularly in underdeveloped countries, such as PNG. In addition, PNG has the highest prevalence of HIV in the Western Pacific Region, the majority of people living with HIV who know their status are receiving treatment. The treatment involves a combination of antiretroviral drugs that often leads to the development of side effects and in some cases discontinuation of their treatment (205). Moreover, it has been shown that the response to the treatment is highly variable between and within individuals (76, 206-210). Maintaining drug concentrations within a therapeutic range helps to avoid side effects and achieve efficacy. For the quantification of drug concentrations in the context of TDM, precise, accurate and reliable analytical methods are required (211). Therefore, the main aim of this study was to develop bioanalytical methods to identify and quantify some of the main drugs used for HIV treatment, such as tenofovir, lamivudine, efavirenz and dolutegravir (recommended first-line treatment for HIV – by WHO) in plasma and in microsampling devices (especially convenient for sampling in remote sites, e.g. PNG). In addition, to apply the methods to PNG HIV/AIDS patients to assess the variability of drug concentrations between patients and to explore the possible causes for these variations.

Study contributions

- **Stability of EFV metabolites under sample pre-treatment conditions**

As introduced in sections 2.2.2 and 2.2.4 in Chapter 1, EFV metabolites do not contribute to antiretroviral activity, however they have been associated with neuronal damage in a dose-dependent manner in a study *in vitro* (71), suggesting that the study of the metabolites could be important because their accumulation could contribute to CNS side effects. Therefore, in Chapter 2, I tested the stability of EFV, 8-OH-EFV, 7-OH-EFV and 8,14-diOH-EFV

under the conditions HIV samples are usually treated after collection and before analysis in order to avoid biological risk during transport and analysis (heating the samples for one hour immediately after the blood is taken, at 60 ºC). FDA guidelines for bioanalytical method validation recommends testing drug stability under expected sampling conditions before receipt at the analytical site as well as during receipt and analysis. Therefore, when validating a method to quantify HIV drugs it is crucial to check if the patients' samples were treated with high temperatures for the inactivation of the virus, and so a stability test should be designed to cover the same conditions. This virus inactivation protocol is used in PNG clinics for sample handling safety.

In addition to stability, a full validation assay (including stability under other conditions) was performed and applied to five PNG HIV/AIDS patients. I observed a maximum decrease of 95% in plasma for 7-OH-EFV and 8,14-diOH-EFV metabolites concentrations after being kept at 60ºC for one hour, while EFV and 8-OH-EFV were stable under this condition. This was the first report of tests of stability under high temperatures and loss of drug concentration after the usual treatment performed after HIV blood collection. The loss of concentration was confirmed in clinical samples when HIV/AIDS patients' samples had concentrations below the validated LLOQ, even though the method I developed and validated had sufficient sensitivity to quantify these metabolites.

Heat is the most effective method for inactivating HIV, however for PK studies, this should be avoided in order to ensure the reliability of the results for HIV drugs concentrations. Alternatively, the drug stability at high temperatures should be tested prior blood sampling. A new study design should start with a dialogue with the clinic that performs the blood collection with enough evidence to sustain the action of not heating the samples. Alternatively, the analytical laboratory could ensure the use of personal protective equipment (PPE) during sample collection, handling and analysis. Storage of samples should

also be treated with care with the use of sealed containers with warning labels. Ultimately, during analysis, physical containment control, such as Biosafety Cabinets (Class II) should be used to ensure minimum contact with the analyst. After using biosafety cabinets, it is crucial to clean it with sodium hypochlorite and ethanol 70% (212). Alternatively, employ microsampling devices for the blood collection, such as DBS and VAMS, as it represents minimal biological risk after the samples are dried.

- **Contribution of CYP2B6 c.516G>T genotype to plasma EFV and 8-OH-EFV variability in PNG HIV/AIDS patients**

As introduced in section 2.2.3.2 in Chapter 1, *CYP2B6* c.516G>T missense variant is the most frequently studied and clinically relevant for EFV metabolism. This variant has been extensively associated with a decrease in CYP2B6 enzyme activity and increased plasma EFV concentrations in different populations. Of all populations studied to date, PNG has the highest frequency of this variant. Even though the high frequency is known in this population, Chapter 3 brings the first report of the investigation of *CYP2B6* c.516G>T genotype *vs* EFV and main metabolite 8-OH-EFV plasma concentrations. A high frequency of *CYP2B6* c.516T allele was found (53%), corroborating previous studies in the same population and all patients carrying this variant allele achieved plasma EFV concentrations above the therapeutic range (> 4000 ng mL^{-1}). Genotype *per se* explained 38% of plasma EFV concentration variability and 50% of metabolic ratio variability (8-OH-EFV/EFV). Demographic factors (age, gender and weight) made no significant contribution to EFV or metabolite plasma concentration variations. The residual variability may be due to other single nucleotide polymorphisms (SNPs) in *CYP2B6*, such as 983T>C, and or in other genes involved in EFV metabolism (but to a lesser extent), such as *CYP3A5*, *CYP1A2* and *CYP2A6*, which could not be addressed in this study due to the initial study design. No association between *CYP2B6* c.516G>T genotype and side effects was found in this study,

possibly due to the self-reporting of side effects, which has the potential for bias in the interpretation of the data (213).

Another plausible explanation for the lack of association between genotype and side effects is the development of tolerance. The cohort of the patients studied had been under treatment from 3 months to 13 years. A study showed that central nervous system side effects were significantly associated with 516TT genotype at week 1 of the treatment but not at week 24 (73). They hypothesised development of tolerance to the side effects with the chronic use of EFV. Interestingly, they observed that tolerance to central nervous system side effects occurred despite persistently greater plasma efavirenz exposure in the patients that reported side effects only in the week 1.

A suggested design in case of a new study evaluating side effects would include sampling patients for drug measurements at the beginning of the treatment (< 1 week) and a follow-up after 12 and 24 weeks. A discussion with the physicians about side effects being the possible explanation for the patients who had virtually no EFV in their plasma would help to confirm noncompliance and action accordantly. In addition, the study of other SNPs in *CYP2B6* and in other enzymes (*CYP3A5*, *CYP1A2* and *CYP2A6*) could bring some clarification on the remaining unexplained variability on EFV disposition.

- **Simultaneous analysis of TNF, 3TC and DTG by LC-MS/MS**

As mentioned in section 2.4 in Chapter 1, in PNG 2019, DTG replaced EFV as the first-line drug for the treatment of HIV in a combination therapy with 3TC and TNF. An assay that quantifies these drugs simultaneously is preferred in order to reduce analysis cost and time. In Chapter 4, I attempted to develop an assay to quantify DTG, 3TC and TNF in plasma by LC-MS/MS. Many challenges were faced, from poor chromatography resolution to the presence of drug carry-over in blank samples. To address these problems and develop the

method, several experiments were performed; optimization of MS parameters, changes in mobile phase composition and flow, optimization of interface parameters and testing PEEK tubing. To date, there are no published methods of the simultaneous analysis of these three compounds in human plasma. These analytes differ substantially regarding their chemical properties and so they need different conditions to be eluted through the chromatographic column. After 11 months working on this, I concluded that it was not practical to develop a method to simultaneously quantify these three drugs, so they were separated into two assays (Chapter 5 – TNF and 3TC quantification in human plasma) and (Chapter 6 – DTG quantification in DBS and VAMS). The method for quantification of TNF and 3TC was developed and validated in plasma samples because that was the samples I had access to. DTG measurements was part of a new patient cohort, therefore I could design the experiments for the validation of microsampling devices, and also organise all the collection kits and instructions for DBS and VAMS collections to be sent to PNG.

- **Assessment of the influence of demographic factors and renal function on plasma 3TC and TNF variability in PNG HIV/AIDS patients**

As introduced in sections 2.3.1 and 2.3.2 in Chapter 1, TNF and 3TC variability between and within patients can be influenced by demographic characteristics, such as body weight and sex, concomitant use of other medications and by individual renal function (serum creatinine, CrCl and eGFR). To be able to quantify these drugs in patients' samples I developed and validated a method for the simultaneous quantification of 3TC and TNF by LC-MS/MS, which is described in Chapter 5. Method validation included the first report on high temperature stability testing for TNF and 3TC, which both showed to be stable at 60 ºC for one hour. The method was clinically applied to 121 PNG HIV/AIDS patients. Chapter 5 also brings the investigation of the influence of sex, weight, age, sampling time, creatinine levels, CrCl and eGFR on 3TC and TNF plasma concentrations in a PNG HIV/AIDS cohort.

Only 30 patients (n=121) had creatinine levels measured close (within 8 days) to the time of blood collection for plasma drug concentrations, therefore a subgroup was incorporated ("8-day within") for statistical testing. Older age, lower eGFR and CrCl were independently associated with higher TNF concentrations in univariate analysis. When performing multivariate analysis, all parameters together only explained a very small percentage of TNF (8%) and 3TC (6%) variability, and no individual predictor was statistically significant when all patients were included. For the 8-day within patients group, all the parameters together explained 21% and 18% of the variability on 3TC and TNF concentrations, respectively. However, the 8-day within group had a smaller sample size and the co-efficients reveal that the association with renal function is not greater in this group. The unexplained variability in 3TC and TNF concentrations may be due to other factors, for example, polymorphisms in kidney drug transporter genes, such as *ABCC2* c.3463 T>A and 1249 G>A. Variability between patients can also be due to variations in absorption.

- **Development and validation of a method to quantify DTG in DBS and VAMS by LC-MS/MS**

As introduced in section 3 and 3.1 in Chapter 1, microsampling strategies are alternatives for the standard blood collection, with advantages such as simplified sample transportation, in particular for collection in remote sites, such as PNG. Therefore, in Chapter 6, I described the development and validation of the first method to quantify DTG in two of the most commonly used microsampling devices in the context of TDM. In addition, I applied the method to 15 PNG HIV/AIDS patients' samples. The method was precise and accurate for both devices. It had good sensitivity and no significant matrix effect. DTG was stable at room temperature for up to 14 days, but it showed a loss in stability when the devices were kept at 60 ºC. In the dried blood spots (DBS) specific experiments, I observed an inaccuracy in DTG measurements for blood with haematocrits (HT) of 50%. Volumetric absorptive

microsampling (VAMS) brings the advantage over DBS regarding its fix volume collection, eliminating the HT effect that can sometimes be found when using DBS. Patients' samples were analysed using both devices and the results were highly correlated, however VAMS provided slightly higher concentrations than DBS for the same patients, potentially with HT having an effect on DBS concentrations.

Limitations of the study

Study limitations were summarised in Chapters 3, 5 and 6. In Chapter 3, there was moderate sample size sufficient to demonstrate genotype effects on plasma EFV concentrations; however, the sample size for the reported side effects was relatively small, resulting in limited power to establish significant associations between side effects and plasma concentrations or genotype. In addition, self-reported side effects can create bias in the interpretation of the data (214). The lack of information on serum creatinine levels at the time of the blood collection for drug concentrations for all patients, single point measure eGFR and the low variability in kidney function compared to other studies brought some limitations for the assessment of variability in 3TC and TNF concentrations in Chapter 5. Ideally, eGFR should be assessed and interpreted, in regards to its contribution to drug variability, using at least 2 points of measurement before and after the initiation of treatment.

For the majority of drugs, concentration measurements in the context of TDM is based on references ranges established in plasma samples. Even though DTG therapeutic range has not been established yet, all the studies conducted to date have been measuring its concentrations, and associating with side effects, in plasma samples. Therefore, as per DBS guidelines (174), to complete a microsampling device method validation the comparison and translation of the concentrations measured in the microsampling devices to estimated plasma concentrations (or in the biological sample the therapeutic range for the specific drug is

established in) is mandatory. Besides the microsampling devices, we only had access to the PNG patients' frozen whole blood, so the separation of the plasma was not possible and for this reason, the completion of the method validation for Chapter 6 was unfeasible. Additional experiments comparing whole blood concentrations with DBS and VAMS can be useful to show the correlation between venous and capillary blood.

Strengths of the study

Even though several methods for the quantification of EFV and metabolites have been published, this study brings the first report of lack of stability of the metabolites when the HIV samples are treated by the standard protocol after blood collection, suggesting that an alternative procedure, such as use of PPE and physical containments controls, should be performed to avoid biological risk between sample collection and analysis. This study confirms the highest frequency (of all populations) of *CYP2B6* c.516T allele in PNG patients and its strong association with increase EFV concentrations (above therapeutic range), suggesting that a dose adjustment (lower dose) should be consider for patients with 516GT and 516TT genotypes (53). For the first time, the variability in 3TC and TNF drug concentrations was studied and associated with demographic factors and kidney function in PNG HIV/AIDS patients. This study also brings the first report of a development of a method to quantify DTG in microsampling devices and its application to PNG HIV/AIDS patients. Results from Chapter 3 and 5 showed that some patients had EFV, 3TC and TNF below the limit of quantification of the methods developed suggesting drug noncompliance. In some cases, the patients cease the use of antiretrovirals because of developing toxicities (58). Therefore, the identification of these patients through drug concentration measurements is very important. In cases where noncompliance is identified, the physicians prescribing the drugs to the patients should be contacted, so measures can be taken.

As mentioned before, PK studies were conducted for the first time in PNG HIV patients bringing important information about treatment for this population that has the highest prevalence and incidence of HIV in the Western Pacific Region. In addition, the association of PK with pharmacogenetics (PGx) showed interesting results regarding the need to review the alternative possibilities for patients carrying the genetic variant associated with higher EFV exposure. Although, with the EFV discontinuation as first-line HIV drug in PNG, future studies should focus in investigating the chronic effects of DTG use.

The studies performed in this thesis could potentially be applied to other diseases affecting PNG population, such as tuberculosis (TB) and malaria. Even though the treatment for TB often leads to a cure, it also causes side effects in some patients and possibly the cessation of the therapy. Therefore, TDM associated with PGx would be useful for TB drugs as well.

Future perspectives

The treatment for HIV improves constantly with the development of new drugs aiming to reduce the occurrence of side effects and to enhance efficacy. However, very often after the clinical trials, the long-term effects of the drug start to be noticed by patients. Therefore, the pharmacokinetics of the drug should always studied in cohorts of patients under treatment. TDM is implemented in order to avoid side effects and improve efficacy maintaining the concentrations in a desired range. Ultimately, TDM can be a part of a strategy for personalised medicine with individualization of drug therapy. Moreover, polymorphisms in genes encoding enzymes responsible for drug metabolism and transport have also been linked to drug disposition and also response. The combined study of PK and PGx in patient populations from different ethnicity groups brings great understanding of individual response to drug treatment and ultimately improving the quality of life for the HIV-positive individuals.

Dolutegravir has been introduced as a preferred first-line drug only three years ago, so its possible drawbacks are still to be discovered. In order to facilitate TDM in remote sites, e.g. PNG, blood collection methods using microsampling devices should be developed and validated. Besides DBS and VAMS, other microsampling techniques have been used to facilitate sampling in remote sites and other advantages. For example, microneedles (215), dried plasma spots (DPS) and capillary microsampling (CMS) (216). Perhaps the more useful device for drugs that have therapeutic ranges established in plasma would be DPS, which contains a spreading layer and a separation membrane to remove blood cells by a combination of adsorption and filtration. After 3 min of a drop of whole blood on top of the card, the blood cells are retained in the paper membrane and the final sample is plasma. Although very effective, the amount to plasma collected is very restricted (2.5 µL) requiring highly sensitive methods. In addition, the cost of these devices is sometimes higher than most facilities can afford.

Besides providing information to maximize therapeutic effect with minimal risk of toxicity, measuring drug concentrations can provide information about adherence and also reduce in overall cost of treatment for the providers with better treatment managements for the patients.

Conclusion

In conclusion, *CYP2B6* c.516G>T significantly affected EFV variability in PNG HIV/AIDS patients but it was not associated with side effects. Methods for the quantification of HIV drugs in plasma and microsampling devices were developed, validated and clinically applied to HIV/AIDS patients. Kidney function and patient demographics did not explain a significant portion of 3TC and TNF variability. Future work can focus on further investigation of polymorphisms and other factors that could be associated with drug

variability and ultimately with the development of side effects. In addition, the lack of studies on DTG PK, PGx and side effects should be addressed in different populations.

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