The Clinical Pharmacology of Human Immunodeficiency Virus (HIV) Therapy Failure

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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CONTENTS

ABSTRACT

The work in my thesis focuses on developing highly sensitive tests, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the measurement of antiretroviral drugs within plasma, cells, and cerebral spinal fluid from clinical studies evaluating compartmentalised antiretroviral therapy (ART) pharmacokinetics and forgiveness for missed or late dosing.

Firstly I developed and validated a LC-MS/MS assay to quantify the antiretroviral (ARV) drugs, lopinavir, darunavir, ritonavir and raltegravir in peripheral blood mononuclear cells (PBMCs). This intracellular assay included optimising methodology for cell separation to minimise loss of drug (Chapter 4). All drugs eluted within an 8 minute run time. Matrix effects were minimal (-2.9%). Calibration curves were validated over a concentration range of 0.4-150ng/mL. Intra and inter assay variation ranged between 0.01-2.29% for precision and 96.76-102.32% for accuracy.

Secondly I developed and validated a LC-MS/MS assay to simultaneously detect and quantify 10 ARVs; maraviroc (MVC), nevirapine (NVP), rilpivirine (RPV), raltegravir (RAL), atazanavir (ATV), darunavir (DRV), amprenavir (APV), ritonavir (RTV), lopinavir (LPV) and etravirine (ETV) in cerebral spinal fluid (CSF). All drugs eluted within a 10 minute run time. Calibration curves were validated over the following concentration ranges; LPV, MVC, RTV = 0.78-100 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 1.95-250 ng/mL and NVP = 19.5-2500 ng/mL (r^2 values >0.99; quadratic $1/x$). Intra and inter assay variation ranged between 1.59-15% for precision and -10.5-6.4% for accuracy. Carryover was <20% of the lower limit of

quantification for all drugs. The recovery was >70% and the co-efficient of variation (CV%) at low, medium and high concentrations was less than 20% for all drugs.

Thirdly I developed and validated a LC-MS/MS assay to quantify intracellular tenofovir-diphosphate (TFV-DP) and emtricitabine-triphosphate (FTC-TP) within PBMCs. This work was very technically challenging and something that was not being done by any other laboratory within the United Kingdom. Analytes eluted within 12 minutes run-time with adequate separation. Calibration curves were validated over the following range TFV-DP=0.35-10.91 ng/mL, FTC-TP=0.38-103.17 ng/mL (r^2 values >0.99; linear $1/x$). The lower limit of quantification was <20 %, signal to noise was >5% and carryover <0.1%. The precision was; TFV-DP=6.3-11% and FTC-TP=6-18.6%, and accuracy was TFV-DP=97.5-100.8%, FTC-TP=98-100.3%.

Finally, all assays developed and validated were successfully applied to collaborative clinical trials (see Communications; Published Research Papers).

Benefits are expected to accrue from this work in the design of more forgiving therapy regimens for HIV patients, and better drug selection to specifically target HIV replicating within sanctuary sites.

PREFACE

Chapter 1

Presents an introduction and overview of the pathogenesis of HIV and provides an outline of the current ARV therapies. This chapter also provides an introduction to the instrumentation and use of liquid chromatography tandem mass spectrometry (LC-MS/MS) for the development of ARV drug assays described in this thesis.

Chapter 2

Describes the development and validation of a LC-MS/MS assay to detect and quantify the protease inhibitors lopinavir, darunavir, ritonavir and the integrase inhibitor raltegravir within a peripheral blood mononuclear cell (PBMC) matrix.

Chapter 3

Presents clinical trial data investigating the pharmacokinetics of plasma and intracellular levels of ritonavir boosted darunavir and raltegravir (at once and twice daily dosing) when co-administered to HIV-infected subjects.

Chapter 4

Investigates the number of wash steps used in the isolation of PBMC by comparison of methodologies currently used for intracellular ARV pharmacokinetic clinical studies.

Chapter 5

Describes the development and validation of a LC-MS/MS for the simultaneous quantification of 10 ARV drugs within cerebral spinal fluid (CSF), to greater understand their concentrations within this sanctuary site.

Chapter 6

This chapter describes the development, validation and technical challenges of a LC-MS/MS assay to detect and measure the intracellular phosphorylated anabolites of tenofovir (TDF) and emtricitabine (FTC).

Chapter 7

Presents data from a clinical trial to assess the 'pharmacokinetic forgiveness' of intracellular tenofovir- diphosphate (TFV-DP), emtricitabine- triphosphate (FTC-TP), and plasma efavirenz (EFV), over a period of 9.5 days after drug intake cessation to provide information on how best to advise HIV-infected patients on non-adherence (delayed and missed doses).

Chapter 8

Concludes the work carried out in this thesis, highlighting the important findings in LC-MS/MS ARV assay development and the clinical trial pharmacological findings for which they were applied. Areas which could be further investigated are also outlined.

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My NIHR Fellowship award has to be the second greatest achievement in my life, obviously my son Jake is my first, and therefore I would like to thank him, and dedicate this work to him ♥, my little angel (nearly 16 now, so not so little anymore!), for not complaining when I missed his football matches, birthdays, weekends away and this Christmas due to 'Mum's work'. Thank you Jake, you have been my greatest inspiration in life and together we have achieved so much, I love you always ♥♥♥

My special thanks and sincere appreciation also go to my PhD supervisors, Professor Saye Khoo, Professor David Back, who patiently sat and introduced me to pharmacology, and was always there to guide and help me, with complicated PK clinical trials, and everything else my troubled little mind faced, and point me in the right direction with the latest papers via post it notes, thank you, and Professor Andrew Owen. I would also like to thank Dr Marta Boffito, Dr Akil Jackson, Dr Alan Winston, Dr Jose Molto and Dr Aylin Yilmaz.

My time in 'H block' at the University of Liverpool, was emotional to say the least. I would like to thank all the friends and colleagues I have met and made on my PhD journey, with special thanks to Dr John Tjia, you made me smile at least 10 times a day, and the day you retired, I lost a little of my drive, you were the one person every day that I knew that I could depend on, my rock, my friend …my calculation expert!!! I miss our daily banter and crazy mathematics! I would also like to thank Deidre Egan, for being a true friend and colleague, whom I could always confide in... chicken salad pesto and all the 100's of lunches we shared over the talk of lab

issues! Thank you for keeping me sane, you're a true adopted Scouser! My thanks also go to Dr. Laura Dickinson, (for all your help and support during conferences away), but mainly for your expertise in teaching me the black art of the PK analysis programme and also how to drink a 'Cosmo' with style… SJP eat your heart out! Lau you are a true trooper! I would also like to thank Dr Joanne Gamble. When it came to the latter stages of my writing this never ending PhD, you helped and guided me (no matter what time it was!). You have been a true 'HIV research buddy' and a friend I have made for life, Thank you, Joey!

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Finally, and most importantly, I would like to thank my family. My loving parents, (a testament of true love, 55 years of marriage!), Betty and Ted, who had me, their fourth child, a 'massive' surprise! …and what a surprise I was… hahaha! The first to go to university in the family! I know you are as proud of me, as I am of both of you.

My heartfelt thanks also go to Dave, who throughout my studies has always been there as a best friend. In my 'cabin fever moments' you have always been there to encourage me, have faith in me, read through my notes and correct me on my spelling! (you should now have a honorary degree in M.Sc Parasitology and a PhD in Pharmacology!) I truly appreciate everything you do for me.

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COMMUNICATIONS

Published Research Papers

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V. Watson

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V. Watson

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V. Watson

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Prizes and Awards

National Institute for Health Research (NIHR) Doctoral Research Fellowship Award.

NIHR Highly Commended Oral Presentation Prize.

Entitled; *Development and validation of a HPLC-MS/MS assay to quantify the anti-retroviral drugs, lopinavir, darunavir, ritonavir, and raltegravir in peripheral blood mononuclear cells.*

Awarded by Professor Dame Sally Davies at the 1st NIHR Biomedical Research Centre Experimental Medicine Summer School. Ashridge Business School, Hertfordshire, UK. (2010).

NIHR National Media Competition. Round 2. Runner up prize

Media video entitled; *Making a difference.*

Video link: http://www.youtube.com/watch?v=PJ9vjlxBxEA&list=PL7N0D2Uw2l0z-ebaKk3L4J8tpbHwf3VPH

Research experience abroad

Skaggs School of Pharmacy and Pharmaceutical Sciences, Anschutz Medical Campus, University of Colorado, United States of America. Detection of intracellular metabolites of FTC and TFV. Supervision: Associate Professor Peter Anderson. September – October 2012.

ABBREVIATIONS

%	Percentage
>	Greater than
<	Less than
^{o}C	Degree centigrade
3TC	Lamivudine
ABC	Abacavir
ACN	Acetonitrile
aCSF	Artificial cerebral spinal fluid
AIDS	Acquired immune deficiency syndrome
ALB	Albumin
APV	Amprenavir (Fosamprenavir)
ARV	Antiretroviral
ATP	Adenosine triphosphate
ATV	Atazanavir
AUC	Area under the curve
AUC_{0-24}	Area under the curve from 0 to 24 hours
AUC_{0-last}	Area under the curve from 0 hour to last measurable time point
AZT	Zidovudine
BSA	Bovine serum albumin
BHIVA	British human immunodeficiency virus association
cART	Combination antiretroviral therapy
C_oHr	Concentration at 0 hour
$C_{12}Hr$	Concentration at 12 hours
$C_{24}Hr$	Concentration at 24 hours
$C_{36}Hr$	Concentration at 36 hours
$C_{48}Hr$	Concentration at 48 hours
$C_{60}Hr$	Concentration at 60 hours
$C_{84}Hr$	Concentration at 84 hours
$C_{108}Hr$	Concentration at 108 hours
$C_{132}Hr$	Concentration at 132 hours
$C_{156}Hr$	Concentration at 156 hours
$C_{180}Hr$	Concentration at 180 hours
$C_{204}Hr$	Concentration at 204 hours
$C_{228}Hr$	Concentration at 228 hours
CCR5	Chemokine receptor 5
CD4+	Helper T lymphocyte antigen marker
CE	Collision energy
CSF	Cerebral spinal fluid
CI	Confidence interval
CI-ATP	2-chloroadenosine 5′-triphosphate
C_{last}	Concentration at last time point
C_{max}	Maximum concentration
C_{min}	Concentration at minimum concentration
CNS	Central nervous system

Conc	Concentration
CPT	Cell preparation tube
C_{trough}	Trough concentration
CV	Coefficient of variation
CXCR4	CX chemokine receptor 4
C&W	Chelsea and Westminster Hospital
D4t	Stavudine
DDI	Drug-drug interaction
ddI	Didanosine
ddC	Zalcitabine (dideoxycytidine)
DLV	Delavirdine
DMHA	Dimethylhexylamine
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
DRV	Darunavir
DRV/r	Ritonavir boosted darunavir
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
ESI	Electrospray ionisation
ETV	Etravirine
EVG	Elvitegravir
Exp	Experiment
FBS	Foetal bovine serum
FDA	Food and drug administration
FI	Fusion inhibitors
Fig	Figure
fL	Femtolitre
f/mol	Femtomole
FTC	Emtricitabine
FTC-DP	Emtricitabine-diphosphate
FTC-MP	Emtricitabine-monophosphate
FTC-TP	Emtricitabine-triphosphate
g	Gram
g/L	Grams per litre
GCLP	Good clinical laboratory practice
GCP	Good clinical practice
GM	Geometric mean
GMR	Geometric mean ratio
HAART	Highly active antiretroviral therapy
HAND	HIV associated neurological disorders
HBSS	Hanks balanced salt solution
H-ESI	Heated electrospray ionisation
HILIC	Hydrophilic interaction chromatography
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HPLC	High performance liquid chromatography
HQC	High quality control
Hr	Hour
IC	Intracellular

IC_{90}	Concentration of a drug required to inhibit 90% of viral replication
IC_{50}	Concentration of a drug required to inhibit 50% of viral replication
ID	Identification
IDV	Indinavir
IS	Internal standard
ITT	Internal transfer tube
ITC	Internal transfer capillary
L	Litre
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLoD	Lower limit of detection
LLQ	Lower limit of quantification
Log	Logarithmic
LPV	Lopinavir
LPV/r	Ritonavir boosted lopinavir
LQC	Low quality control
MΩ	Mega-ohm
MEC	Minimum effective concentration
MeOH	Methanol
mg	Milligram
$mg/h/L^{-1}$	Milligrams per hour per litre
mg/L^{-1}	Milligrams per litre
µg	Microgram
µL	Microlitre
µm	Micromililitre
µM	Micromolar
Mins	Minutes
mL	Millilitre
mm	Millimetre
Mm	Millimolar
Mol	Molar
MQC	Medium quality control
MRM	Multiple reaction monitoring
MS	Mass spectrometry
mTorr	Millitorr
MVC	Maraviroc
m/z	Mass to charge ratio
n	Number of observations
NaCl	Sodium Chloride
NDP	Nucleo-diphosphate
NFV	Nelfinavir
ng	Nanogram
ng/mL	Nanogram per millilitre
nL	Nanolitre
NMP	Nucleo-monophosphate
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors

NTP	Nucleo-triphosphate
NtRTIs	Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Pre-dose
PGC	Porous graphitic chromatography
pH	Minus $Log_{(base\ 10)}$ of Hydrogen ion concentration
PIs	Protease inhibitors
PK	Pharmacokinetics
PKa	Dissociation constant
pL	Picolitres
p/mL	Picomole
PrEP	Pre-exposure prophylaxis
Q1	First quadrupole
Q2	Second quadrupole
Q3	Third quadrupole
QC	Quality control
r^2	Correlation coefficient
QX	6,7-dimethyl-2,3-di(2pyridyl)-quinoxaline
RAL	Raltegravir
rcf	Relative centrifugal force
Recon	Reconstituted
RLUH	Royal Liverpool University Hospital
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPV	Rilpivirine
RT	Retention time
RTIs	Reverse transcriptase inhibitors
RTV	Ritonavir
s.d	Standard deviation
SPE	Solid phase extraction
SRM	Selective reaction monitoring
SQV	Saquinavir
$T_{1/2}$	Drug half life
$TE_{t\ ½}$	Terminal elimination half life
T20	Enfuvirtide
TDF	Tenofovir disoproxilfumarate
TFV	Tenofovir
TFV-DP	Tenofovir-diphosphate
TFV-MP	Tenofovir-monophosphate
TDM	Therapeutic drug monitoring
TIC	Total ion count
T_{max}	Time taken to reach maximum drug concentration
TP	Triphosphate
TPV	Tipranavir
UGT 1A1	UDP glucuronosyltransferase 1 family, polypeptide A1
UK	United Kingdom
ULQ	Upper limit of quantification
USA	United States of America

V	Voltage
v/v	Volume per volume
WAX	Weak anion exchange
WHO	World health organisation

Chapter 1

General Introduction

CHAPTER 1

GENERAL INTRODUCTION

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1.1 Human Immunodeficiency Virus

1.1 Aetiology of HIV

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), is a *lentivirus*, which belongs to the *retroviridae* family. The primary target of HIV is the immune system, which it destroys, allowing opportunistic infections and cancers to thrive. HIV is transferred by exposure of infected bodily fluids (blood, semen, vaginal fluid, breast milk, pre-ejaculate) and although these routes of infection are well established allowing preventive measures to be implemented (screening of blood for donation, use of condoms etc.), a staggering 6,300 people world-wide contract the virus everyday (nearly 262 new infections every hour) [1]. UNAIDS estimated that in 2012 there were 2.3 million new HIV diagnoses (260,000 under 15 years of age), giving a total of 35.3 million people living with HIV/AIDS world-wide (3.3 million under 15 years of age) [1]. HIV infection is however declining with a 50% reduction in new cases between 2001 and 2012, in adults and children (under the age of 15) [1]. This can be attributed to greater education of preventative measures and the on-going advances and accessibility of combination antiretroviral therapy (cART), which has dramatically reduced HIV morbidity and mortality. The introduction and use of cART, has enabled durable suppression of the virus' ability to replicate and has therefore changed HIV from a fatal disease to a chronic manageable disease [2], if diagnosed and treated in the early stages of infection. In 2010, the United Kingdom HIV statistics showed the one year mortality rate in late diagnoses was 40 per 1,000 cases, which is 8 times greater than that of earlier diagnoses (5 per thousand). It was also found that 1 in 10 late diagnoses in the over 50 age group, died within a year [3].

1.2 Antiretroviral therapy

Since the advent of the first HIV antiretroviral (ARV) drug, zidovudine (AZT) in 1987 [4], there are now 27 drugs approved by the Anti-Infective Advisory Committee to United States Food and Drug Administration (FDA). Table 1 shows all of the ARVs and their class. Table 2 shows the co-formulations of ARVs.

ARV drugs work by interruption of the HIV life cycle within the immune cell. ARVs fall into 5 main pharmacological classes according to their mechanism of action. Each class targets HIV at a different point within its life cycle and ultimately prevents the virus from replicating.

1.2.1 Reverse Transcriptase Inhibitors

The first class of ARV agents to be developed was the nucleoside/ nucleotide reverse transcription inhibitors (NRTIs/ NtRTIs). NRTIs are analogues of the naturally occurring intracellular deoxyribonucleic acid (DNA) building blocks, the purine nucleosides guanine and adenosine and the pyrimidine nucleosides cytidine and thymidine. Once inside the immune cell, NRTI undergo phosphorylation. The addition of these phosphate groups (three phosphate groups are added to NRTI and two phosphates to NtRTI) to their deoxyribose moiety by intracellular kinase enzymes, changes the NRTI in to its active phosphorylated metabolite. This is then able to inhibit the viral enzyme reverse transcriptase which is responsible for the synthesis of viral ribonucleic acid (RNA) into viral DNA. The main mechanism of action of NRTI is that their phosphorylated analogues do not possess the 3'-hydroxyl group, thus preventing the formation of the next 5'-3' phosphodiester bond which is

essential in viral DNA elongation. Therefore viral DNA chain termination occurs and the production of viral DNA is inhibited preventing further viral replication [5]. NRTIs act quickly within the cell, and due to the long duration of their action, a single once daily dose is administered.

1.2.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)

The second pharmacological class of ARV therapy are the non-nucleoside reverse transcriptase inhibitors (NNRTIs). Like the NRTI, NNRTIs primary mechanism of action is to prevent viral RNA being transcribed into viral DNA. However, the molecular structure of NNRTI are not analogues of the naturally occurring cellular nucleosides and therefore do not competitively compete for incorporation into the viral DNA. Instead NNRTI, target the viral reverse transcriptase enzyme which they bind to non-competitively. Once bound, the reverse transcriptase enzyme undergoes a conformational change in its 3-dimensional shape. This shape change, a hyper extension of the p66 thumb domain, reduces the catalytic activity of the enzyme by inhibition of the reverse transcriptase's active site, thus preventing the viral RNA being transcribed to viral DNA [6].

1.2.3 Protease Inhibitors (PIs)

The third pharmacological class of ARV are the protease inhibitors (PIs). HIV protease is an essential enzyme in the life cycle of HIV. It is responsible for the cleavage of viral precursor proteins required for the assembly of mature viral proteins to form new infectious virus particles [7]. PIs mode of action is to bind to

the active site of the protease enzyme, thus disabling its action. This leads to the production of immature HIV virus proteins which are non-infectious.

1.2.4 Integrase Stand Transfer Inhibitors (ISTIs)

The integrase stand transfer inhibitors (ISTI) are the fourth class of ARV drugs. ISTIs are the newest pharmacological class of therapeutic HIV drugs, with raltegravir being approved in 2007 and dolutegravir and elvitegravir in 2013 by the FDA [8]. HIV integrase is an essential viral enzyme which encodes the viral DNA into the host genome (integration) [9]. ISTIs mechanism of action is direct competition with viral DNA to bind to the active site of the integrase enzyme. In doing so, ISTIs block the viral DNA being integrated in the cell nucleus and ultimately prevents viral replication [10].

1.2.5 Entry/ Fusion Inhibitors (FIs)

The fifth and final ARV pharmacological class are the entry/fusion inhibitors (FIs). This class of ARVs differs from the other four classes as the mechanism of action of these drugs is extracellular. FIs prevent the initial attachment of the virus to the cell surface receptors. There are two currently approved entry inhibitors, the first enfuvirtide, is a synthetic biomimetic peptide which is administered subcutaneously due to its lack of bioavailability when given orally. It works by binding to the HIV viral transmembrane protein gp41, preventing the virus from creating an entry pore in the CD4+ cell membrane thus keeping the capsid of the HIV virus out of the cell [7]. The second FI, maraviroc is a CCR5 inhibitor. Its mechanism of action is to

prevent HIV from binding to the cell membrane by blocking the CCR5 chemokine receptor, thus preventing HIV (which has to be tropic to either the beta- chemokine receptor CCR5 or the alpha-chemokine receptor CXCR4) gaining access to the cell [7].

The mechanisms of action for each of the five classes are shown in Figure 1.

Table 1. List of HIV antiretroviral drugs approved by the FDA.

Generic name	Brand name	Abbreviation	FDA approval	Class
Abacavir	Ziagen	ABC	1998	NRTI
Didanosine	Videx & Videx EC	DDI	1994	NRTI
Emtricitabine	Emtriva	FTC	2003	NRTI
Lamivudine	Epivir	3TC	1994	NRTI
Stavudine	Zerit	D4t	1996	NRTI
Zidovudine	Retrovir	AZT	1987	NRTI
Zalcitabine [dideoxycytidine] (no longer marketed)	Hivid	ddC	1992-2006	NRTI
Tenofovir disoproxil fumarate	Viread	TDF	2002	NtRTI
Delavirdine	Rescriptor	DLV	1997	NNRTI
Efavirenz	Sustiva	EFV	2002	NNRTI
Etravirine	Intellence	ETV	2008	NNRTI
Nevirapine	Viramune	NVP	1996	NNRTI
Rilpivirine	Edurant	RPV	2011	NNRTI
Amprenavir, APV (no longer marketed)	Agenerase	APV	1999-2004	PI
Atazanavir	Reyataz	ATZ	2004	PI
Darunavir	Prezista	DRV	2007	PI
Fosamprenavir (Prodrug of APV)	Telzir	APV	2003	PI
Indinavir	Crixivan	IND	1996	PI
Lopinavir/ritonavir	Kaletra	LPV/r	2000	PI
Nelfinavir	Viracept	NFV	1996	PI
Ritonavir	Norvir	RTV	1996	PI (booster)
Saquinavir	Invirase	SQV	2006	PI
Tipranavir	Aptivus	TPV	2005	PI
Enfuvirtide	Fuzeon	T20	2003	FI
Maraviroc	Celsentri	MVC	2007	FI
Elvitegravir	GS-9137	EVG	2013	ISTI
Raltegravir	Isentress	RAL	2007	ISTI
Dolutegravir	Tivicay	DTG	2013	ISTI

Table 2. List of antiretroviral co-formulations approved by the FDA.

Fixed dose combinations	Generic Name	Brand Name	FDA approval
Multi-drug class combinations	efavirenz, emtricitabine and tenofovir disoproxil fumarate	Atripla	2006
	emtricitabine, rilpivirine, and tenofovir disoproxil fumarate	Complera	2011
	elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate	Stribild	2012
NRTI co-formulations	lamivudine and zidovudine	Combivir	1997
	abacavir and lamivudine	Epzicom	2004
	abacavir, zidovudine, and lamivudine	Trizivir	2000
	tenofovir disoproxil fumarate and emtricitabine	Truvada	2004

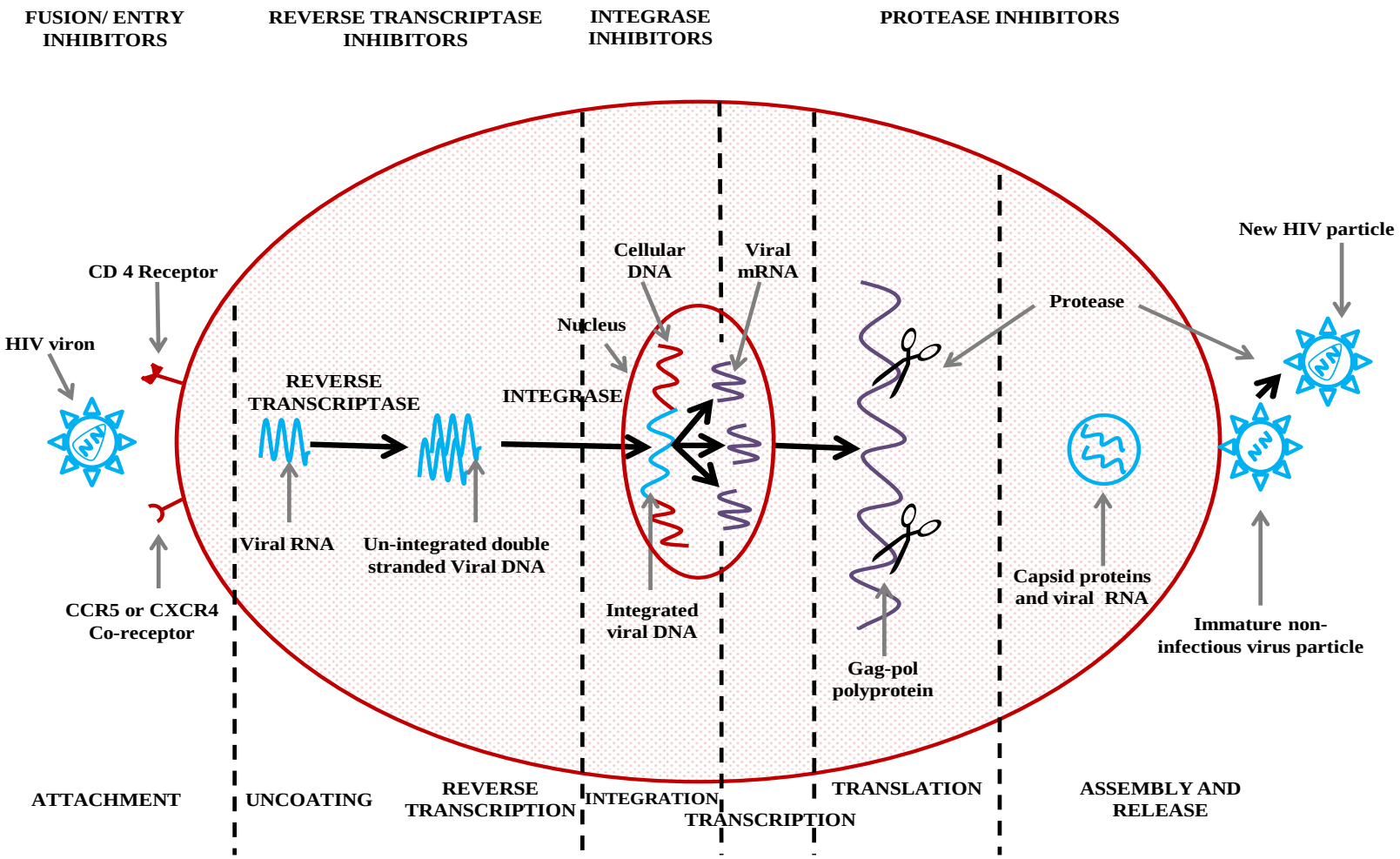

Figure 1. Schematic of HIV replication life cycle and sites of drug action. Modified from Merck Isentress leaflet.

1.2.6 Current Therapy Guidelines

The combination of antiretroviral drugs currently recommended by the British HIV Association (BHIVA) [11] for treatment naïve patients is a ritonavir boosted PI or an NNTRI or a ISTI plus two NRTI drugs, the choice of which are dependent on number of factors including the balance of benefit/risk, age, presence or absence of any drug resistance and co-infection [7]. The BHIVA summary is outlined in Table 3. For treatment experienced patients, the combination is dependent upon their viral genotype and includes three active ARV's from at least two different ARV pharmacological classes [7].

Table 3. BHIVA summary of cART

	Preferred	**Alternative**
NRTI backbone	TDF and FTC	ABC and 3TC*ǂ
Third ARV	ATZ/r	LPV/r
	DRV/r	APV/r
	EFZ	NVP†
	RAL	RPVǂ

*ABC is contraindicated in HLA-B*57:01 positive patients. † NVP is contraindicated if baseline CD4 cell count is greater than 250/400 cells/μL in women/men. ǂThe use is only recommended if the base line viral load is <100 000 copies/ mL: RPV as a third ARV, ABC and 3TC as NTRI backbone [11].

BHIVA recommend initiation of ARV treatment in patients with a CD4 count less than 350 cells/ μL, and should not be delayed in patients approaching this threshold.

1.2.7 Adherence

It is vital to the success of cART that patients adhere to their given ARV regimen to avoid therapy failure. It is well documented that viral rebound, drug resistance, HIV progression to AIDs and mortality are associated with poor ARV adherence [12-14]. BHIVA break down non-adherence into two categories, firstly intentional and secondly unintentional [11]. Intentional non-adherence to ARV therapy is described as a conscious decision which is influenced by beliefs, emotions and patient preferences. Unintentional non-adherence is described as a reduction in the ability to adhere to the intended treatment due to factors limiting the capacity or resources of the ARVs. [11]. In order to avoid non-adherence BHIVA recommends assessment, discussion and support addressing both intentional and non-intentional non-adherence limitations, with patients about their therapy whenever ARVs are prescribed.

1.2.8 Drug- drug interactions (DDIs)

As with any infection requiring therapeutic treatment, drug interactions can cause major clinical problems especially in HIV therapy. Drug interactions can be classified as follows: (i) a physical or chemical interaction between two or more drugs prior to them being absorbed into the body, (ii) pharmacokinetic, in which case one drug will increase or decrease the concentration of the other, (iii) pharmacodynamic, the combination of two or more drugs given together will change the effect of the drug if given alone.

Many studies have looked at various ARVs given in combination with one another and also with a wide range of other pharmaceutical agents, and as a result many online drug interaction web sites have been established, which highlight contraindicated effects of poly-pharmacy in order to circumvent drug-drug interactions.

As well as pharmaco-vigilance, another method of ensuring that a therapy regime is at an optimum for a patient is via therapeutic drug monitoring (TDM). This involves pharmacokinetic (PK) sampling at different time points post drug intake, to ensure that the drug is within its correct concentration range (sub optimal concentrations will cause viral rebound and resistance and too much drug can result in toxicity). Although BHIVA recommend against the use of unselected use of TDM, it plays a key part in the clinical management of some patients (dose adjustments in children, and pregnant women) and also highlights patients who are not fully adherent to their therapy, and in identifying these patients', methods and support can be given to increase their drug intake regime [11, 15].

1.3 Sanctuary sites and viral reservoirs

Despite cART (when adhered to) being highly effective and life-prolonging in HIV-infected individuals, it is only successful in the suppression of HIV viral replication, thus ARVs do not cure HIV infection. It is clear that the virus is not completely eradicated from the body and HIV-1 RNA has the ability to persist in anatomical and cellular compartments, which may or may not be reached by drug in high enough concentrations.

The definition of a viral *reservoir* is described by Blankson *et al*. as "a cell type or anatomical site in association with which a replication-competent form of the virus accumulates and persists with more stable kinetic properties than in the main pool of actively replicating virus." Blankson *et al*. also cite that "a viral reservoir must have a biologically plausible mechanism that allows the virus to escape from biological decay processes or, in the case of a cellular reservoir, from immune effector mechanisms" [16, 17]. Put simply, a viral *reservoir* is a compartment where HIV is present but is in a *latent* state (versus productive infection).

A HIV anatomical sanctuary site is a compartment where HIV is present but ARV drugs are not/ or at least not in a high enough concentration to prevent viral replication. This is due to prevention of sufficient drug penetration into the sanctuary which is influenced by physical barriers, physiochemical characteristics, drug transporters and sequestration. Possible HIV sanctuary sites include the central nervous system (CNS), genital tract, lymphoid tissue, $CD4^+$ T cells (resting/ memory and naïve), $CD8^+$ T cells, monocytes, B cells and dendritic cells [16-20].

The eradication of virus from the possible sanctuary sites is not currently feasible with current cART but a greater understanding of the pharmacokinetics of ARVs in these anatomical compartments may allow us to implement better strategies and identify optimal cART drugs that will enable the greatest viral suppression at each of the sites.

1.4 Liquid Chromatography-Tandem Mass Spectrometry

The assays designed in this thesis to accurately quantify the level of ARVs in various matrices (plasma, CSF and PBMCs), utilise high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The combination of liquid chromatography coupled with tandem mass spectrometry provides an excellent analytical method for separation, detection and quantification of ARVs.

The first step in the LC-MS/MS system is physical separation of the analytes by means of liquid chromatography (LC). This is achieved by the transfer of the sample through two immiscible phases; a mobile phase solution such as acetonitrile with a selected flow rate through the stationary phase within a selected column. Chromatographic separation occurs during binding and release (sorption/ desorption) due to the differences in distribution coefficients of the analytes during their transfer through the stationary phase of the column. The selection of the mobile phases and the column are dependent up on the physical characteristics and molecular structure of the analytes to be separated. Once selected, optimal conditions are assessed for parameters such as, temperature (of column), flow rate (of mobile phases), pH, and mobile phase gradient (isocratic, or step-wise).

The LC system is interfaced to a triple quadrupole tandem mass spectrometer (MS/MS). The mass spectrometer can be divided into three basic parts, (i) the ionisation source, (ii) the analyser and (iii) the detector. For all assays developed and used in this thesis the ionisation source selected was heated electrospray ionisation (H-ESI), and the analyser used was a triple quadrupole. During H-ESI the eluted sample from the LC system is pumped through a heated narrow capillary probe at a set flow rate. The tip of the capillary which is situated in the MS ionisation source

housing, then has a high voltage applied to the exit tip. As a result of this strong electric field, sample entering the source housing unit is dispensed as highly charged aerosol droplets. This process is helped by the introduction of nitrogen used as a nebulising gas, which flows around the outside of the heated capillary. The nitrogen gas directs the aerosol droplets towards the entrance of the MS system. As the droplets travel towards the MS, the warm nitrogen evaporates the solvent eluent, resulting in charged sample ions entering the ion transfer tube (ITT) and being taken into the MS which is held under a high vacuum.

Once the charged precursor parent ions enter the MS they are selected according to their mass-to-charge ratio (*m*/*z*) [molecular related ions $(M+H)^+$ or $(M-H)^-$ depending on ionisation mode selected i.e. positive or negative] within the first mass analyser (quadrupole 1), these selected ions then travel into a collision cell (quadrupole 2) where they are bombarded with gas molecules (argon) which results in the formation of charged fragment (daughter) ions. Finally, the fragment ions are focused into a second mass analyser (quadrupole 3) and the intensity of each fragment ion is measured resulting in a chromatographic peak. The intensity of the fragment ions produced is proportional to the concentration of the sample analyte. The sample concentration can then be determined by comparison to the relevant calibrator of known concentration. The internal travel path of the analytes through the MS/MS system can be seen in figure 2.

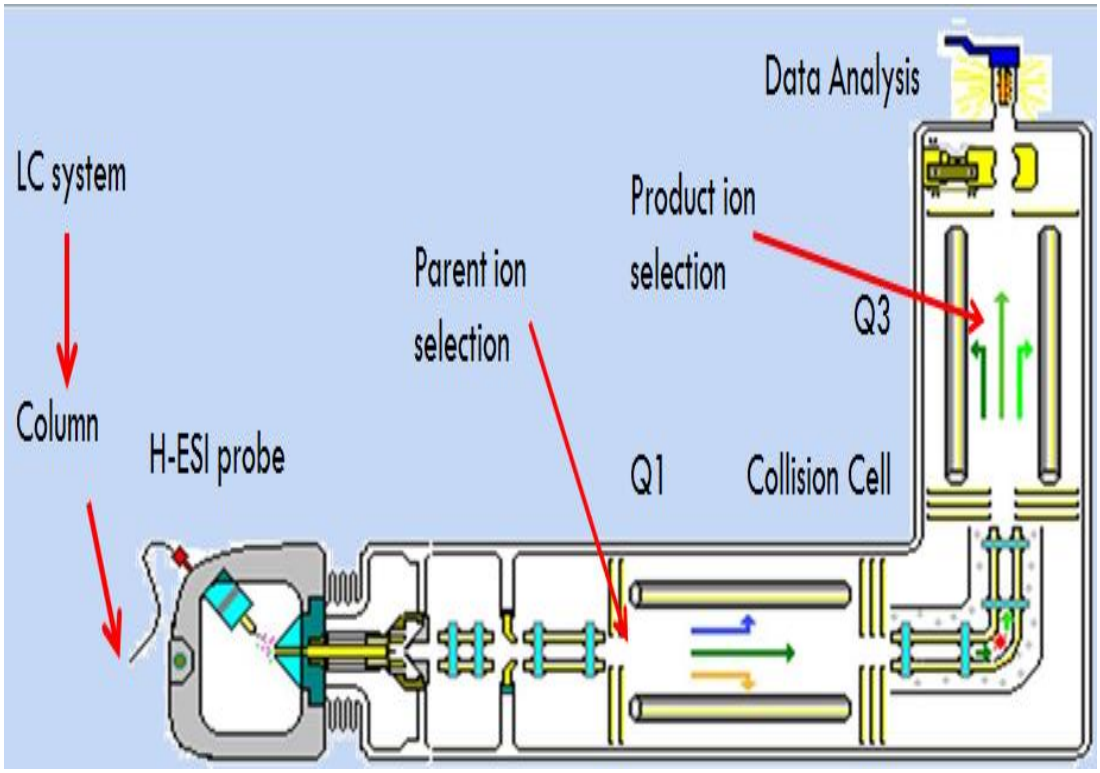

Figure 2. Internal view of the triple quadrupole tandem mass spectrometer modified from a TSQ Thermo Scientific programme screen shot.

1.5 Bioanalytical method validation

In the development of a new bioanalytical methodology for the detection and quantification of HIV antiretroviral drugs and their metabolites, it is essential that the methods used are selective and sensitive and fully validated according to the Food and Drug Administration, Guidance for Industry; Bioanalytical Method Validation guidelines [21]. These guidelines ensure that any quantitative method (in any matrix i.e. plasma, PBMC, CSF etc.) shows reproducibility, reliability and robustness for its intended use. These guidelines were adhered to and followed where appropriate with some modifications. The six main factors in any assay validation procedure are as follows: selectivity and sensitivity, precision, accuracy, reproducibility and stability.

In the context of LC-MS/MS assays developed in this thesis, assays have undergone full validation and in some circumstances partial validation (in that sample matrix has been limited or direct measurement without prior sample preparation has been utilised). When addressing the selectivity of the assays, the ability to differentiate and quantify the ARV in the presence of endogenous and potentially exogenous interferences contained in the sample matrix were investigated and assessed appropriately. Tests were also run of blank (in the absence of any ARV analytes), sample matrix (plasma, PBMC and CSF) from numerous sources or batch numbers in the case of artificial CSF (due to limitation of large CSF blank sample availability), to investigate selectivity. Assessment included the investigation of possible interference at the lower limit of quantification (LLQ) for each individual ARV drug, and also the possibility of carryover, which can occur when a sample of high concentration within the upper limit of quantification of the assay is followed by a sample of a low concentration of the analyte, which could result in the over estimation of the proceeding sample as a response of residual predeceasing sample contamination.

Precision of the analytical methodology described, was also investigated. Precision describes the closeness of individual measurements of the HIV ARV analytes when the methodology was applied repeatedly to multiple individual samples within the matrix. Accuracy was conducted also, and this describes the closeness of the mean analytical test result (actual concentration of the analysed sample) to the nominal value (concentration at which the calibration sample [known sample concentration] of the analyte) concentration. Inter and intra-day variability was assessed also by analysis of 10 or more assays in duplicate carried out on separate days; two of which were carried out on the same day (intra-day). The reproducibility of the assays also

was addressed, but with regards to the *de-novo* nature of the assays described, standard guidelines and 'gold standard' methodology are not currently in place and therefore methodology in this area opens an area which can be enhanced upon.

Stability experiments were conducted to replicate the conditions likely to be faced during sample transfer, handling and analysis. Within this thesis, clinical samples from HIV-infected patients were heat inactivated (58˚C, 40 minutes), stored at -40˚C or -70˚C for preservation of the ARVs and then thawed prior to analysis when appropriate. Therefore, in these circumstances heat and freeze thaw, short term and long term stability was investigated where applicable against quality control samples.

1.6 Hypothesis of this thesis research

Pharmacological characterisation of ARV exposure within plasma, cells and anatomical sanctuary compartments, yields important insight into the effectiveness of HIV therapy, potential eradication of drug from sanctuary sites and forgiveness (the amount of times a drug therapy can be not taken before detecting viral rebound) for missed or late doses.

Aims of this thesis

- To develop and validate sensitive and robust assays for the detection of the ARV drugs; LPV, DRV, RTV and RAL in peripheral blood mononuclear cells (PMBC), MVC, NVP, RPV, RAL, ATV, ETV, LPV, RTV, APV and DRV in cerebral spinal fluid, and the ARV intracellular metabolites TDF-DP and FTC-TP in cells.

- Quantification of ARV and their intracellular metabolites within plasma, cells and cerebral spinal fluid.

- Estimation of the terminal elimination half–life of the ARVs in order to characterise forgiveness for missed/ late dosing.

Chapter 2

Development and validation of a LC MS/MS assay to detect and quantify the antiretroviral drugs, lopinavir, darunavir, ritonavir and raltegravir in peripheral blood mononuclear cells.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF A LC MS/MS ASSAY TO DETECT AND QUANTIFY THE ANTIRETROVIRAL DRUGS, LOPINAVIR, DARUNAVIR, RITONAVIR AND RALTEGRAVIR IN PERIPHERAL BLOOD MONONUCLEAR CELLS.

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2.1 Introduction

Since 1987, when the first anti-retroviral (ARV) drug zidovudine (AZT) was approved by the Food and Drug Administration (FDA), a further 24 ARV's have been licenced and used for the management of HIV infection, which infects an estimated 34 million people worldwide (WHO estimate 2011,[22]). There are five classes of HIV ARVs, each targeting a different stage in the HIV life cycle. The classes comprise of, the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs) and the protease inhibitors (PIs), the entry/fusion inhibitors and the newest class, the strand transfer integrase inhibitors. Highly active ARV therapy (HAART) is a combination of drugs from these different classes. The British Human Immunodeficiency Virus Association (BHIVA) guidelines recommend a standard first line treatment regimen of at least three ARV's. However, it is reported that within the first two years of initiating HAART, there is a 10% failure rate [23]. The failure of HAART is a complex matter of various factors previously discussed in chapter one, which include drug resistance due to viral genome mutations [24], poor adherence [25], pharmacological uptake into sanctuary sites, protein binding factors and sub-therapeutic levels due to pharmacokinetic (PK) drug variability between patients. Therapeutic drug monitoring (TDM) is a helpful tool in the optimisation and improvement of an individual patient response to HAART. The majority of TDM tests rely on measurements of ARVs within the plasma fraction of the blood, giving an individual indication of the drug efficacy. It plays a crucial role in the reduction of toxicity and ensures optimal viral suppression [26]. However, the measurement of the ARVs within the plasma fraction maybe of

limited clinical significance because the major site of action of the ARVs is within the infected cell itself, and only the percentage of drug reaching this intracellular compartment, actually has any impact on preventing the replication of HIV [27]. An effective ARV should therefore be able to penetrate the primary target cells of HIV infection. It is the lymphocytes, CD4+ cells and macrophages of the immune system, which are the target cells for HIV and these cells are collectively known as peripheral blood mononuclear cells (PBMCs). To date there is little data on the intracellular analysis of ARVs compared to the thousands of published research papers on plasma drug levels. This is mainly due to the complexity of intracellular (IC) sampling and analysis. To be able to isolate PBMC from whole blood and take a direct measurement of the IC ARV concentrations enables a greater understanding of the true penetration of HAART into HIV target cells, leading to a greater understanding as to why some patients develop treatment failure despite having optimal ARV plasma concentrations and a fully sensitive virus [28]. It was therefore the primary aim of this chapter to develop a quantitative IC ARV LC-MS/MS assay which was robust and highly sensitive in the accurate measurement of four ARV drugs, to enable a greater insight to the mechanisms underlining HIV drug therapy failure.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Lopinavir (LPV) and ritonavir (RTV) were provided by Abbott Laboratories (Abbott Park, Chicago, IL, USA). Darunavir (darunavir ethanolate, DRV) was provided by Tibotec (Mechelen, Belgium), and raltegravir (RAL) was obtained from Merck

Sharp & Dohme (Haarlem, The Netherlands). Quinoxaline (6,7-dimethyl-2,3-di(2pyridyl)-quinoxaline, QX) was used as the internal standard (IS) and this was purchased from Sigma Aldrich (UK). LCMS grade acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK). HPLC grade methanol (MeOH) was purchased from VWR Laboratory supplies, UK. Formic acid and Hanks balanced salt solution (HBSS) were purchased from Sigma-Aldrich, UK. Deionised water was obtained from an Elga Option-S water purification unit (Elga LabWater, High Wycombe, UK) and then further purified to 18.2 MΩ with a Purelab Ultra unit (Elga LabWater, High Wycombe, UK). Ficoll was purchased from G.E Healthcare (Bucks, UK). Human peripheral blood mononuclear cells (PBMCs) were purchased from the National Blood Service (Liverpool, UK).

2.2.2 Equipment

The high performance liquid chromatography (HPLC) system consisted of a variable loop Accela autosampler (set to a temperature of 15^{o}C) and an Accela LC pump (Thermo Fisher Scientific, Hemel Hempstead, UK). A reverse phase Ascentis™ C_{18} column (3µm: 100 mm×2.1 mm) set at an oven temperature of 30 ^{o}C (Supelco, Dorset, UK), fitted with a 2µm C_{18} Quest column saver, (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to separate all analytes and internal standard. The HPLC system was interfaced with a Thermo Fisher Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer, fitted with a heated-elecrospray ionisation (H-ESI) probe, housed in an ion max source. Two E2M30 rotary vacuum pumps (Edwards High Vacuum International, West Sussex, UK), a NM30LA nitrogen generator (Peak Scientific Instruments, Renfrew, UK), and research grade (99%

pure) argon gas (10L size V, BOC, Manchester, UK) were used. Analyte tuning and optimisation parameters were gained with the use of TSQ Tune software (Thermo Fisher Scientific, Hemel Hempstead, UK). LC Quan ™ software (Version 2.7, Thermo Fisher Scientific, Hemel Hempstead, UK) was used to acquire and process the data.

2.2.3 Chromatographic system

The injection mode was set at full loop (10µl loop), therefore only requiring a sample volume of 10µL. The autosampler tray temperature was set at 15^{o}C and the column oven control at which chromatographic separation took place was set at 30^{o}C. Separation of the antiretrovirals and IS was facilitated by a gradient elution (Table 1). Mobile phase A consisted of 0.2% formic acid, and mobile phase B consisted of 100% ACN. The flow rate was constant at 400 µL/min. At 6.1 minutes the mobile phase ratio was returned back to the starting conditions to allow equilibration of the column. The total run time for the assay was therefore 8 minutes. A wash solution containing ACN: water, 80:20, v/v, was used to clean the injection syringe (2 mL) between each sample injection. Both the mobile phase and the wash solution were de-gassed and mixed by sonication for 20 minutes prior to use. All analytes were scanned for using selective reaction monitoring (SRM) by the TSQ Quantum Ultra operated in positive ionisation mode.

Table 1. Mobile phase gradient.

Time (min)	Mobile Phase A %	Mobile Phase B %	Flow rate (µL/min)
0	95	5	400
2	95	5	400
3.5	20	80	400
6	20	80	400
6.1	95	5	400
8	95	5	400

2.2.4 Mass spectrometric system

Each of the antiretroviral drugs and the IS were prepared in methanol at a concentration of 1µg/mL. This combined solution was then directly injected in to the mass spectrometer using a syringe pump (10 µL/min). The molecular mass (parent mass), for each of the analytes were scanned for in the first quadrupole (Q1). Once detected the tube lens was then optimised for each of the drugs (Fig 1). The parent ions then travelled to the second quadrupole (Q2) which contains inert gas molecules that fragment the ions into their product ions. Collision energy was applied at a range of voltages (v) (Fig. 2) and the product ions for each of the parent masses were scanned for in the selected reaction monitoring (SRM) mode in the third quadrupole (Q3). Two product ions for each of the parent ions were selected. These were the ions which gave the greatest mass to charge ratio (m/z) for their parent ion, with the least amount of background noise. The optimised mass spectrometric parameters are shown in table 2.

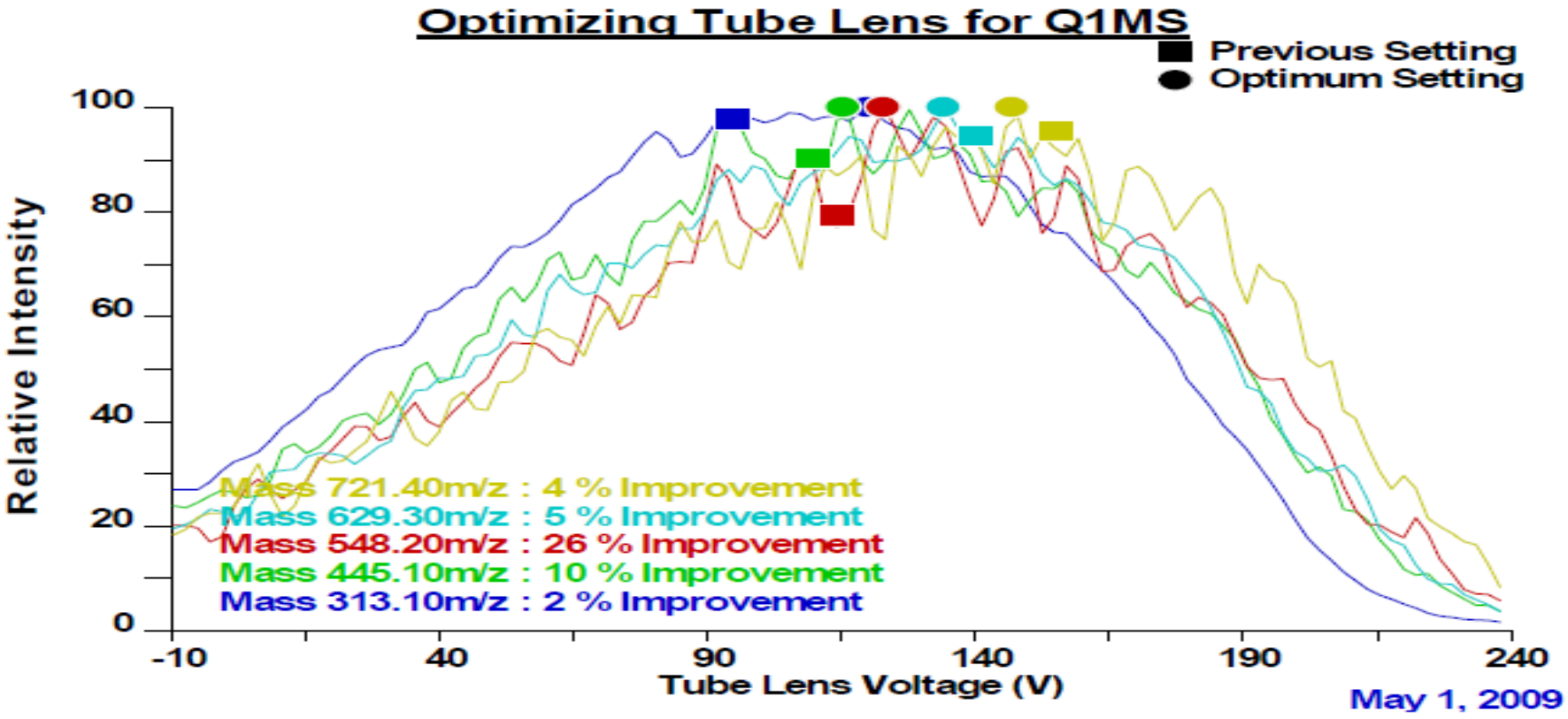

Figure. 1. Optimisation of the tube lens in the first quadrupole. The previous setting show those optimised for an ARV plasma assay. Each ARV is shown in a different colour with its corresponding parent mass. RTV = 721.4 m/z, LPV = 629.3 m/z, DRV = 548.2 m/z, RAL = 445.1 m/z and QX (internal standard) = 313.1 m/z.

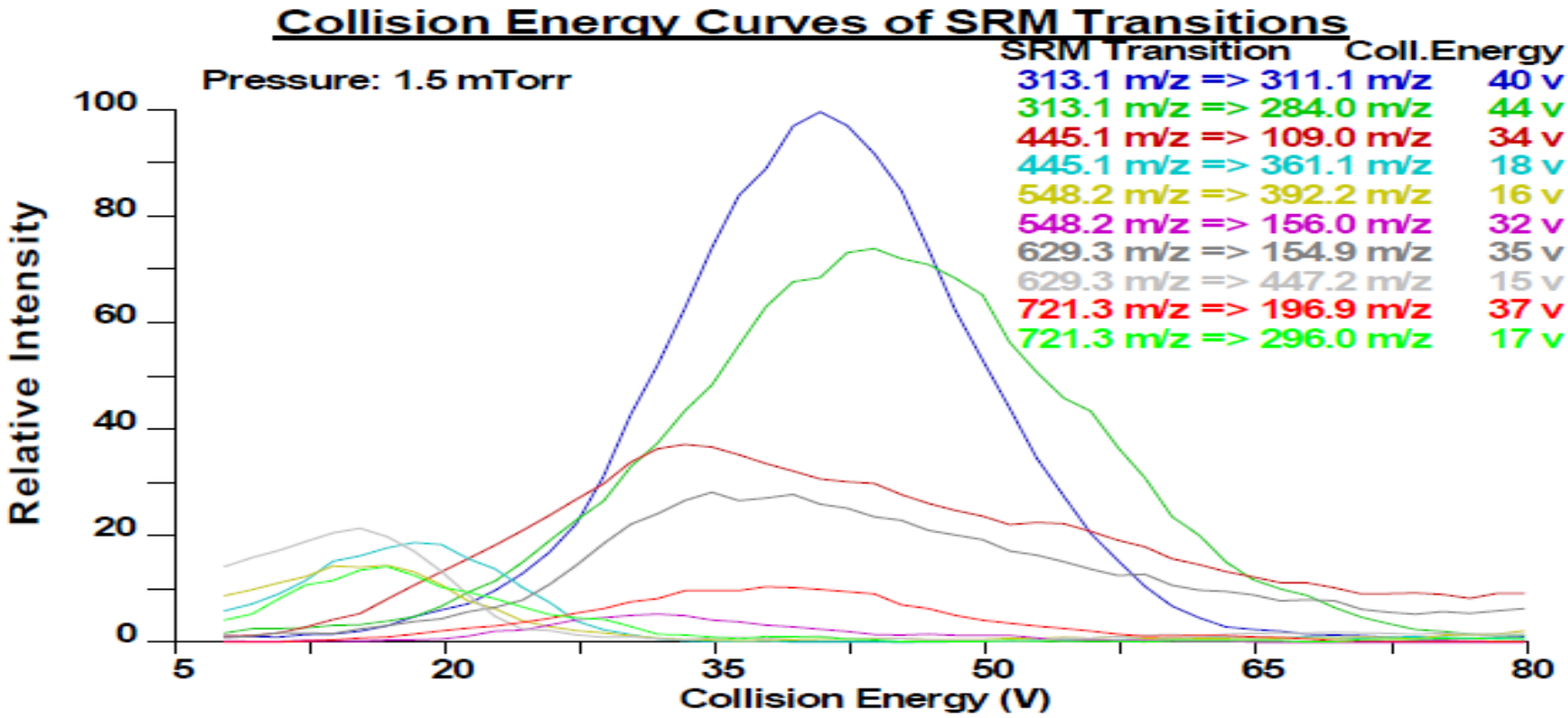

Figure. 2. Optimisation of collision energy in the second quadrupole for each of the analyte parent ions, to fragment into the daughter ions. Each ARV daughter ion is shown in a different colour, outlined in the figure key. Table 2 outlines the ARV parent ions m/z and the fragment ions post collision energy.

Table 2. Optimised conditions for the mass spectrometric detection of each of the analytes in positive ionisation mode.

Drug Name	Retention Time (mins)	Parent ion (m/z)	Product ion (m/z)	Spray voltage	Vap temp	Sheath gas pressure	Aux gas pressure	Capillary temp	tube lens offset	Coll pressure (mTorr)	Collision energy (v)
LPV	4.78	629.3	154.99	5000	350	60	40	300	77	1.5	37
			429.13	5000	350	60	40	300	77	1.5	21
RTV	4.68	721.3	269.01	5000	350	60	40	300	95	1.5	27
			296.01	5000	350	60	40	300	95	1.5	18
DRV	4.35	548.2	155.87	5000	350	60	40	300	95	1.5	33
			392.73	5000	350	60	40	300	95	1.5	14
RAL	4.19	445.1	108.97	5000	350	60	40	300	83	1.5	33
			361.01	5000	350	60	40	300	83	1.5	17
QX	4.3	313.1	246.04	5000	350	60	40	300	109	1.5	42
			284.03	5000	350	60	40	300	109	1.5	45

2.2.5 Source and chromatography maintenance

Concentrated constituents of the intracellular lysate may build up in the column guard, column and LC tubing. In order to circumvent this, the column and column guard were reverse flushed at a flow rate of 400 µL / minute, with 95% ACN and 5% water for 30 minutes after each analytical run. The Ultra source housing unit was removed and cleaned with 50% MeOH after each analytical run. The source cone and ion transfer tube were also removed and cleaned between each assay by immersion in 50% MeOH and sonicated for 20 minutes at room temperature.

2.2.6 Column re-equilibration

Prior to a new assay, the Ascentis™ C_{18} column was re-equilibrated. This was achieved by manually controlling the LC pump conditions to the chromatography starting conditions for the assay (95% mobile phase A and 5% mobile phase B), with a flow rate of 400 µL / minute, for a period of at least 15 minutes. This was also sufficient to raise the source temperature to optimal conditions prior to the samples been run.

2.3 Sample preparation

2.3.1 Isolation and preparation of ARV drug free peripheral blood mononuclear cells (PBMCs).

This method is adapted from that published by Colombo el al. [29]. Blood samples were collected in lithium heparin coated tubes and kept at a temperature of 4^{o}C until

the isolation was carried out. This was done to prevent lysis of the cells. Whole blood (10 mL) was carefully layered over 5ml of ficoll paque™ in a clean sterile universal tube. This was centrifuged at 2000 rpm for 30 minutes at 4^{o}C without break applied. The isolation of the PBMC fraction (containing the lymphocytes and monocytes) was thus achieved on a buoyancy gradient basis as the PBMC's are of a lower buoyancy density than the ficoll paque™. After centrifugation the central band containing the PBMCs was carefully removed using a sterile fine tipped Pasteur pipette and transferred into a clean sterile universal container. The volume was adjusted to 25 mL with ice cold Hanks balanced salt solution (HBSS) and centrifuged (2000 rpm, 5 minutes, at 4^{o}C). This sample wash/spin step was repeated X3. On the final re-suspension only 10 mL (exactly) of HBSS was added and a 50 µL sample of this was transferred into a clean microtube to which 50 µL of each solution A and solution B was added (solutions supplied and used as per manufacture guidelines for Chemometec Nucleocounter cell counting cassettes). From this, 50 µL was drawn into a nucleocounter cassette for a cell count (Nucleocounter NC-100). Once the cell count was known, the cell suspension was centrifuged as before and all the HBSS carefully removed. Methanol (70%) was then added to the cell pellet at a volume calculated to obtain a final PBMC lysed cell density of 4 X 10^{6} cells/ mL. This cell lysate was then sonicated for 10 minutes and followed by centrifugation (5500 rpm, 5 minutes, 4^{o}C, without brake). The sample was then stored overnight at -80^{o}C. The supernatant /cell lysate was then carefully transferred into a new tube and the pellet discarded.

2.3.2 Investigation of PBMC Lysis

It was important to decide the concentration of methanol versus water to use, and also what role the use of sonication of cells played in influencing the greatest lysis yield of ARV from within the cellular compartment. Therefore PBMC were isolated with the use of ficoll paque™ as described above. The PBMC were counted and the cell density corrected to 1 X 10^7 cells/ mL. Cell suspension (500 µL) was aliquoted into labelled 1.5 mL microtubes. Radiolabelled ARV (x 2 activity) was made up in RPMI 1640 media (Sigma, UK) + 15% foetal bovine sera (FBS). 500 µL of this compound was added to the cells and then incubated at 37^{o}C for 30 minutes. The samples were then centrifuged (9000 rpm/ 1 min) and a 100 µL aliquot was removed and placed into a scintillation vial. The remaining media in the sample was removed and the cell pellet was resuspended in 1 mL of ice cold HBSS and then centrifuged (9000 rpm / 1 minute). The supernatant was removed and the wash step repeated (X 2). The cell lysis was then investigated by re-suspending the cell pellets in 100 µL of corresponding lysis solution;

Sonicated	**Non-sonicated**
100 % Methanol	100 % Methanol
70 % Methanol	70 % Methanol
60 % Methanol	60 % Methanol
Distilled water	Distilled water

Samples were then centrifuged (9000 rpm/ 1 minute) and the supernatant removed to a scintillation vial. Scintillation fluid (4 mL) was added to each vial and then capped before placing into the liquid scintillation counter. The counter was run using the appropriate departmental standard operating procedure for the radioactive ARV isotope being investigated. Results are shown in Table 3.

Table 3. Investigation results of PBMC lysis experiment.

	Buffy coat 1	**Cellular count (dpm)**	**Extracellular count (dpm)**	**Cellular Accumulation ratio (CAR)**
Sonicated	Intracellular 100% MeOH	256.01	59863.2	0.21
	Intracellular 70% MeOH	215.72	59084.3	0.18
	Intracellular 60% MeOH	231.73	59396.8	0.2
	Intracellular Distilled water	93.7	61213.6	0.08
Non Sonicated	Intracellular 100% MeOH	298.58	61541.9	0.24
	Intracellular 70% MeOH	207.23	62110.6	0.17
	Intracellular 60% MeOH	264.72	61425.7	0.22
	Intracellular Distilled water	57.47	61069.6	0.05
	Buffy coat 2	**Cellular count (dpm)**	**Extracellular count (dpm)**	**Cellular Accumulation ratio (CAR)**
Sonicated	Intracellular 100% MeOH	289.18	59542.8	0.24
	Intracellular 70% MeOH	254.35	60487.8	0.2
	Intracellular 60% MeOH	181.84	60682.5	0.15
	Intracellular Distilled water	96.85	60319.6	0.08
Non Sonicated	Intracellular 100% MeOH	200.76	61571.3	0.16
	Intracellular 70% MeOH	293.49	61442.2	0.24
	Intracellular 60% MeOH	312.6	60848.2	0.26
	Intracellular Distilled water	88.34	59180.5	0.07
	Buffy coat 3	**Cellular count (dpm)**	**Extracellular count (dpm)**	**Cellular Accumulation ratio (CAR)**
Sonicated	Intracellular 100% MeOH	330.97	69282.3	0.24
	Intracellular 70% MeOH	355.71	68730.5	0.26
	Intracellular 60% MeOH	352.72	66158.3	0.27
	Intracellular Distilled water	313.13	74362.7	0.21
Non Sonicated	Intracellular 100% MeOH	421.57	66237.6	0.32
	Intracellular 70% MeOH	410.12	68500.3	0.3
	Intracellular 60% MeOH	250.45	72211.5	0.17
	Intracellular Distilled water	143.07	81191.4	0.09

We found that the method we had adopted and had been using throughout all intracellular analysis was indeed the method of choice, 70% MeOH with a ten minute sonication.

2.3.3 Preparation of standards and quality controls

Blank PBMC lysate at a cell density of 4 x 10^6 / mL was spiked with LPV, DRV, RTV and RAL at concentrations of 0.5, 5, 10, 20, 50, 100 and 150 ng/ mL, to yield an eight point (including the blank) standard curve. The volume of methanol was adjusted at each concentration to keep the cell density the same throughout. Quality controls (QCs) were generated similarly at concentrations of 5, 50 and 150 ng/ mL (low, medium and high). All standards and QCs were validated in accordance with the Food and Drug Association Guidelines for Industry (FDA 2001[21]). Once the standards and QC were prepared they were stored in the fridge at 4^oC, ensuring that the screw capped bottles were firmly closed to prevent evaporation.

2.3.4 Preparation of internal standard

Quinoxaline (6,7-dimethyl-2,3-di(2pyridyl)-quinoxaline), (QX), was used as the internal standard. A stock solution was prepared at 1 mg/mL in 100% methanol. This was then diluted 1:10 (v/v) with methanol to give a concentration of 100 µg / mL. A working internal standard solution was then prepared. 100 µL of the 100 µg/ mL was added to 4.95 mL of methanol and 4.95 mL of deionised water to give a 1 µg/ mL solution. This was then further diluted to a 100 ng/ mL (1 mL of 100 µL/ mL solution to 4.5 mL of methanol and 4.5 mL of water), to give the final working concentration of internal standard. This was prepared fresh on a daily basis.

2.3.5 Calibration standards and QC sample pre-treatment

Calibration standards and QC samples were removed from the fridge and 100 µL from each was aliquoted into appropriate labelled autosampler vials. To each sample 20 µL of working internal standard QX (100 ng/mL) was added. The vials were then crimp sealed and then vortexed. Aliquots were then centrifuged for 4 minutes at 2500 rpm at room temperature. Sample vials were then loaded into the Accela autosampler ready for analysis.

2.3.6 Preparation of clinical samples

Clinical samples were treated as described earlier (2.3.1 *Isolation and preparation of ARV drug free peripheral blood mononuclear cells (PBMCs)*. They were then stored at -80^{o}C. Prior to testing, the methanol was evaporated to dryness and 500 µL of 70% methanol was added. The sample was then spiked with working internal standard (QX 10µL, 100 ng/ mL in water / methanol [50:50]).

2.3.7 Analyte extraction

No further extraction procedures were carried out. Once the PBMCs had been isolated as described, 100 µL of sample was aliquoted into labelled autosampler vials and analysed by HPLC-MS/MS.

2.3.8 Calculation of the cell-associated concentration of the ARVs

There are two ways in which the results from patient samples could be reported. The first is to express the result in ng/ mL per 10^6 cells (taking in to account the original cell count for the sample) and the second to report the cell associated drug concentration (ng/ mL) assuming that the average volume of a cell is 0.4 pl [30].

2.4 Validation of the LC-MS/MS system

This assay within a lysate matrix using the LC MS/MS system was validated for the drugs LPV, DRV, RTV, RAL and the internal standard QX.

2.4.1 Standard curves

A total of 20 calibration curves (with each level sampled in duplicate) were run and assessed to validate the assay. Each standard curve consisted of seven concentration levels with the addition of a blank lysate sample (level zero), giving 8 levels in total.

Level	Concentration of ARV (ng/mL)
0	0
1	0.5
2	5
3	10
4	20
5	50
6	100
7	150

The assays were also assessed for their linearity, the calibration data was analysed using a quadratic least-square regression of the peak area ratio (drug peak area / IS peak area), versus the nominal concentration of the sample with a weighting factor of 1 / concentration ($1/x$), which achieved co-efficient of correlation (r^2) values greater than 0.98 for all validation assays. Once the calibration values were validated the quality control (QC) samples at low (5 ng/mL), medium (50 ng/mL) and high (150 ng/mL) concentrations were prepared. QC samples at each of the levels were run in duplicate (n = 10) against the standard curves to ensure that all the values (both QC and calibrators) achieved a co-efficient of variation from the target value within +/- 2 standard deviations of the mean target value.

2.4.2 Accuracy and Precision

A inter and intra-day precision and accuracy was determined following the FDA guidelines. Six replicate lysate spiked QC samples containing each of the analytes and the IS at three concentrations (low, medium and high, 5, 50 and 150 ng/mL respectfully) were analysed. Accuracy was determined as the absolute percentage deviation from the theoretically determined concentration (% difference), and the precision of the assay was expressed as the relative standard deviation of the mean as a percentage (% CV) for each of the three concentrations.

2.4.3 Lower limit of quantification

The lower limit of quantification (LLQ) was determined as the lowest concentration which was measured accurately and precisely with the detection not being affected

by interfering influences, i.e. matrix effects or residual analyte carryover. The LLQ concentrations had to be reliable and robust with a co-efficient of variability (CV) % of < 20% for each of the analytes.

2.4.4 Specificity and selectivity

Spectrometric chromatographs for each of the parent ions were assessed and examined to ensure no interference from endogenous compounds was present. The precursor ion chromatographs were examined and then the product ion chromatographs were assessed to ensure the respective ions correlated to their parent masses.

2.5.5 Lysate matrix effects

Lysate matrix effects were assessed by post column infusion of the analytes into the HPLC-MS/MS detector during simultaneous chromatographic analysis of the matrix (PBMC) extract. A standard solution of each of the ARV drugs was infused using a T connection mixer, at the same time as the blank matrix and the mobile phase. Unexpected suppression / enhancement, retention time drift or shift of the analytes chromatographic responses due to any co-eluting intracellular endogenous constituents, were examined to ensure there was no interference at the retention time of each of the ARVs. The ‘absolute matrix effect’ was also investigated. This was to expose any difference in response between the solvent standard (mobile phase) and the sample PBMC lysate matrix [31].

2.5 Results

A successful method has been developed for the detection and quantification of LPV, DRV, RTV and RAL in PBMC lysate by HPLC-MS/MS.

2.5.1 Precursor and product selection for validation

Each of the following chromatographs (Figures 3-12) are the original raw data whilst tuning the mass spectrometer for each of the analytes and the internal standard, at a concentration of 1 µg/ mL by direct injection, with a flow rate of 400 µL/ min. The first chromatographic figures for each drug shows the parent ion (precursor spectrum) from the Q1 cell without any collision energy being applied, followed by the second chromatographs which highlight the daughter ions (product spectrum) in the third quadrupole (Q3) with collision energy applied (see Table 2 for optimised values).

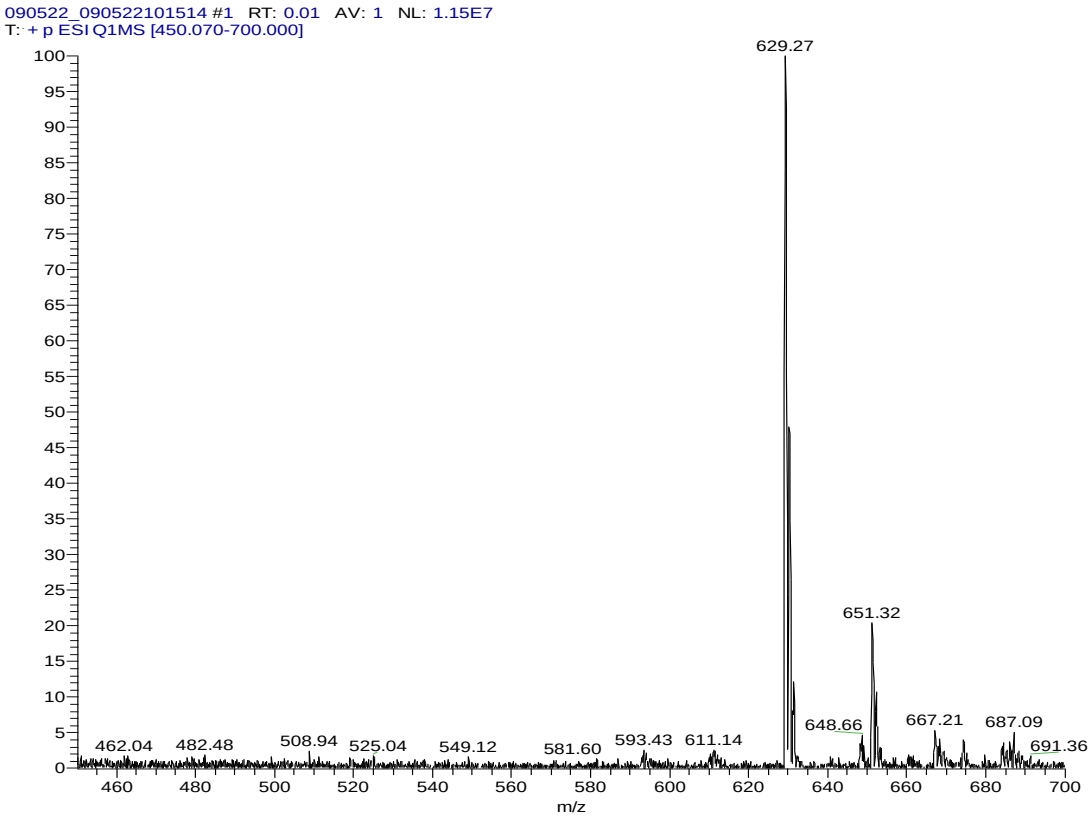

Figure 3. LPV precursor ion spectrum in Q1 LPV + H (629.27)

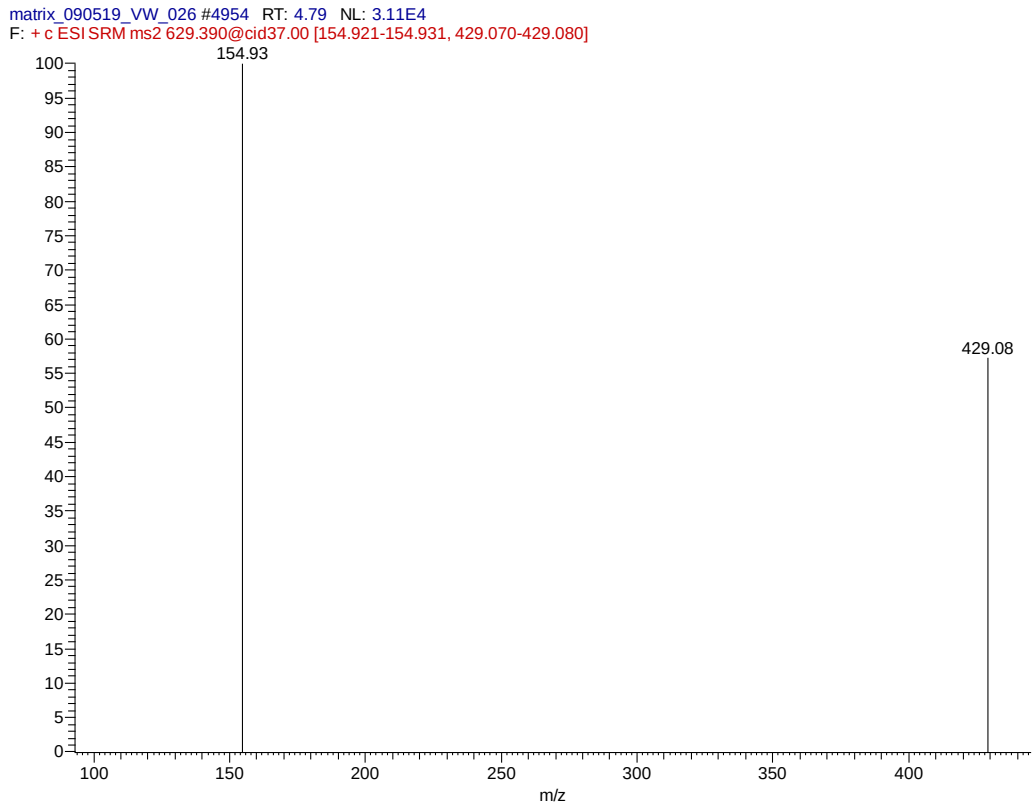

Figure 4. LPV SRM fragment ions (154.93 and 429.08) used for quantification post collision energy application

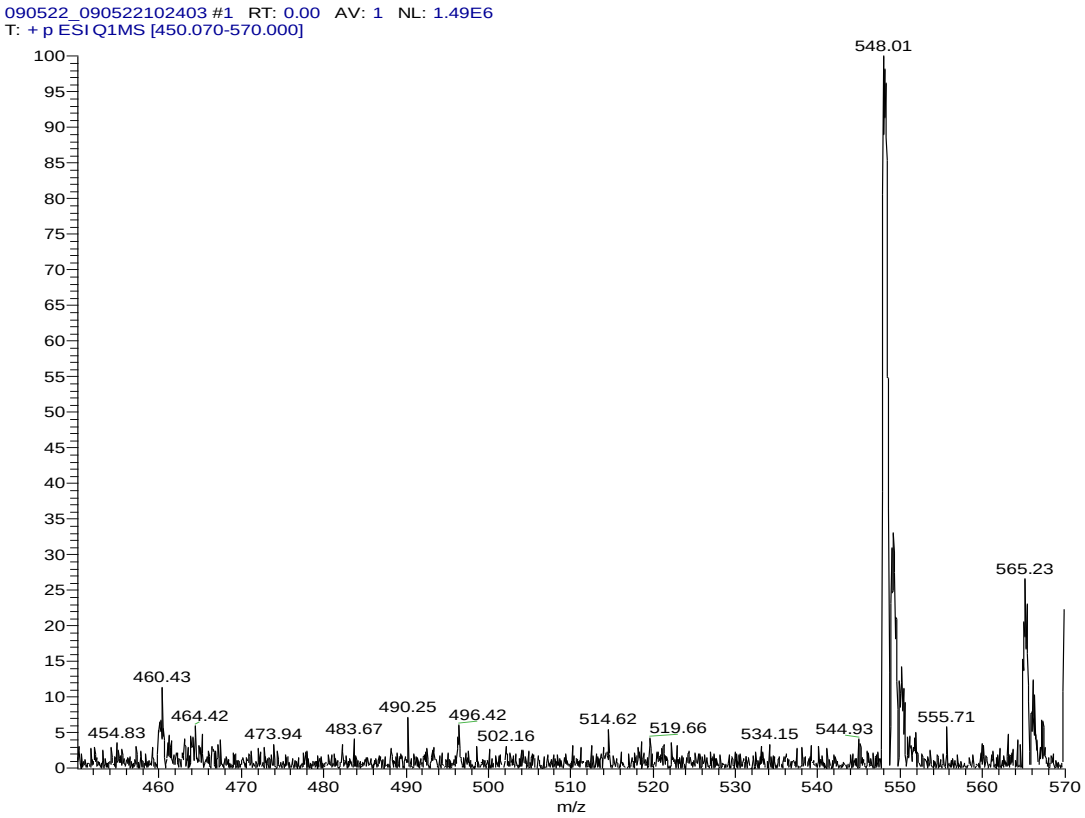

Figure 5. DRV precursor ion spectrum in Q1. DRV + H (548.01)

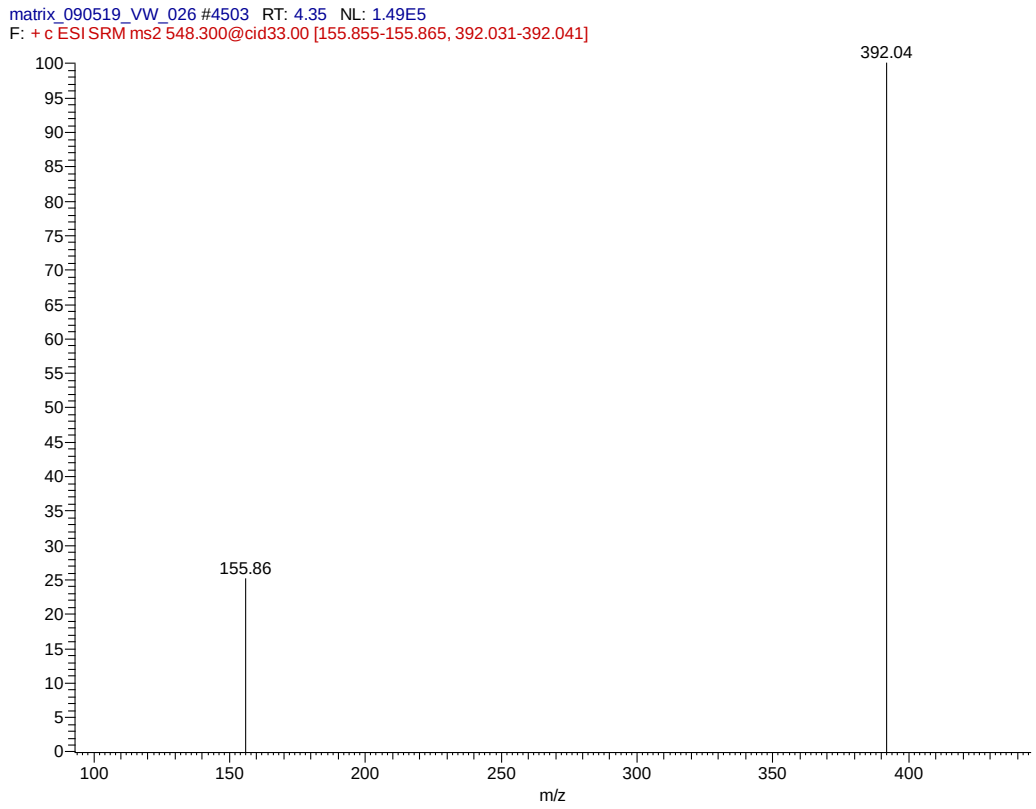

Figure 6. DRV SRM fragment ions used for the quantification (155.86 and 392.04) post collision energy application

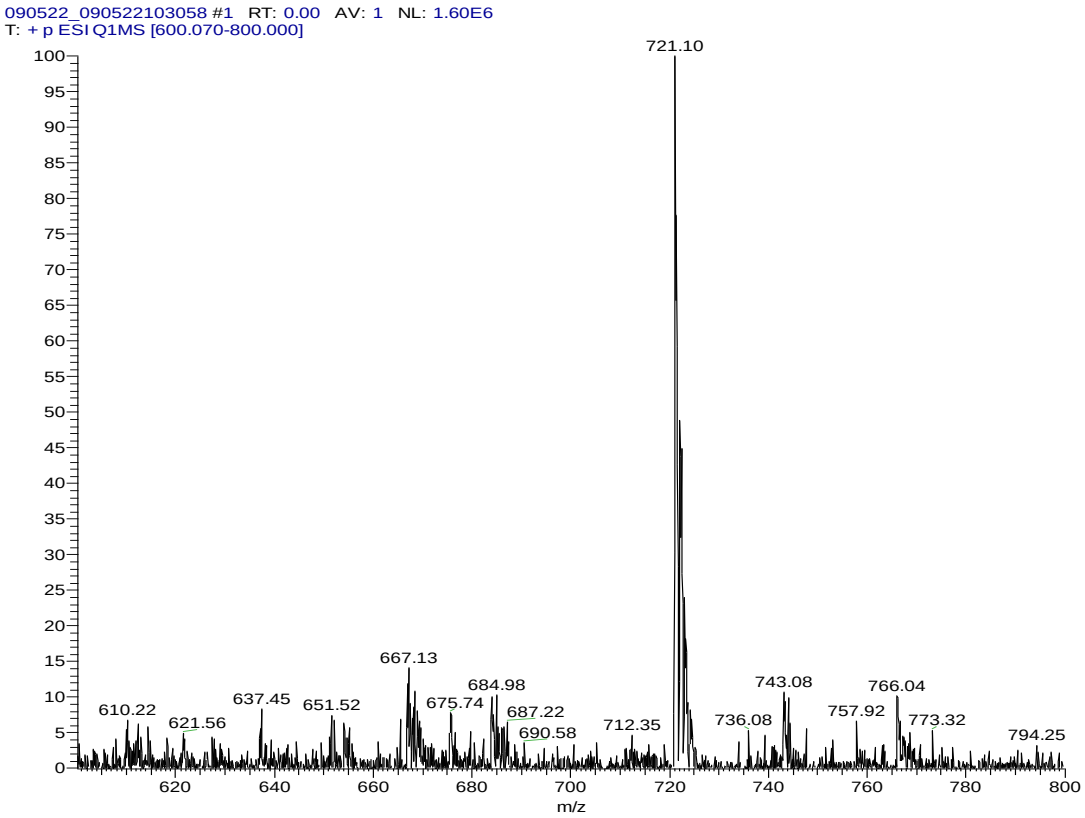

Figure 7. RTV precursor ion spectrum in Q1. RTV + H (721.10)

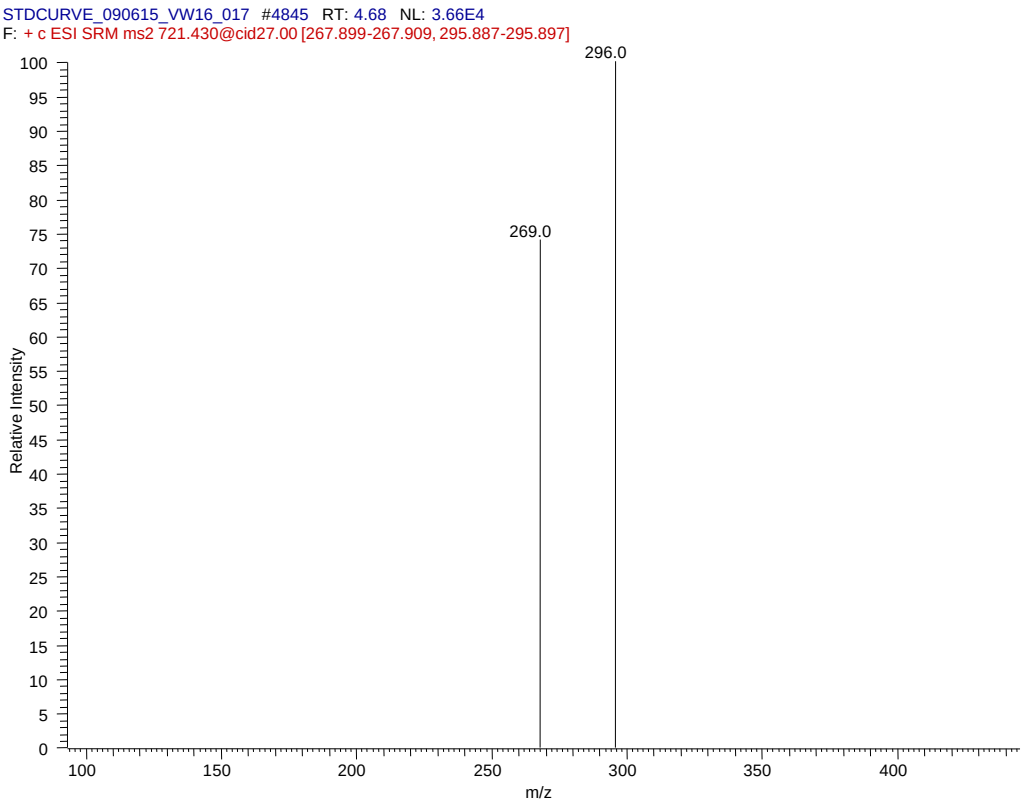

Figure 8. RTV SRM fragment ions used for the quantification (269.0, 296.0) post collision energy application.

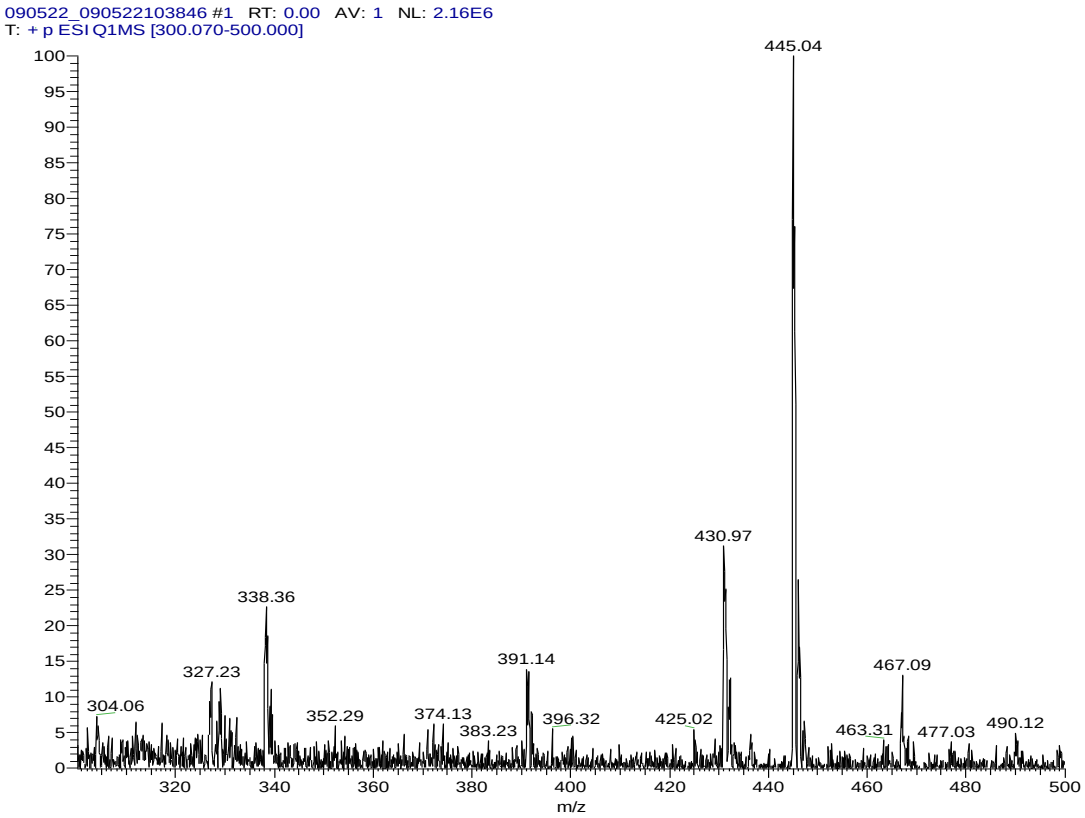

Figure 9. RAL precursor ion spectrum in Q1. RAL + H (445.04).

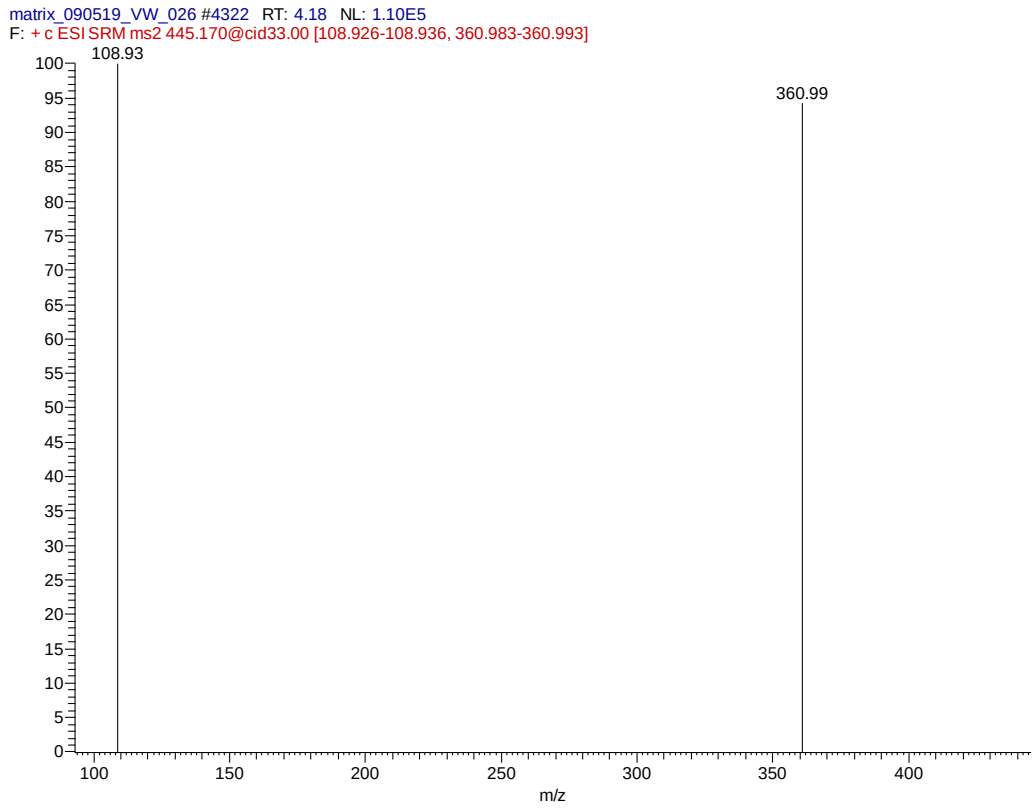

Figure 10. RAL SRM fragment ions used for the quantification (108.93, 360.99) post collision energy application.

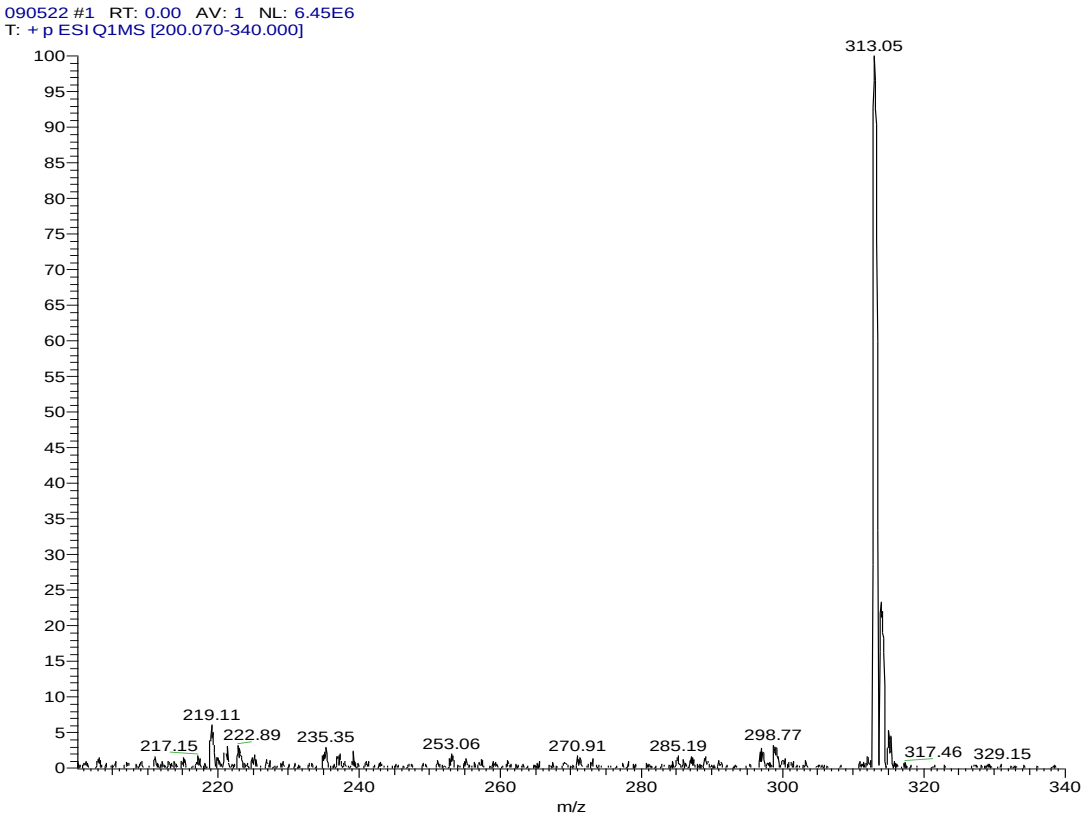

Figure 11. QX precursor ion spectrum in Q1. QX + H (313.05).

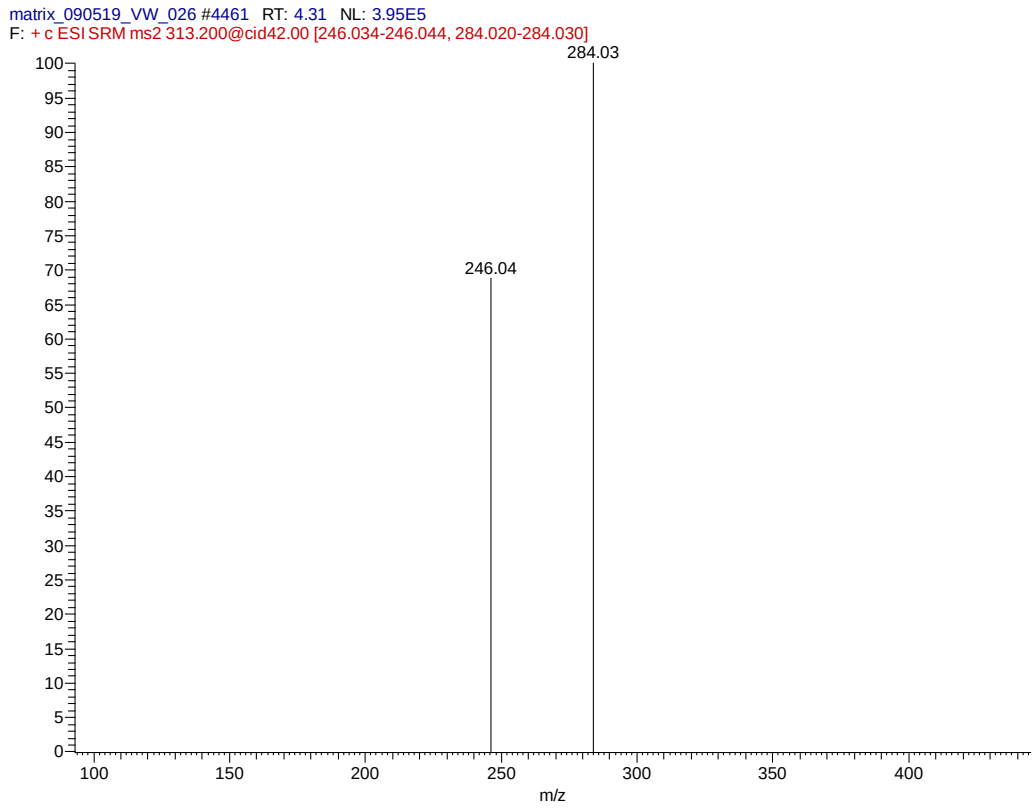

Figure 12. QX SRM fragment ions used in the quantification (246.04, 284.03) post collision energy application.

2.5.2 Optimisation of the chromatographic peaks

Each of the ARVs were tuned and optimised on the HPLC-MS/MS system described. Figures 13-17 show the chromatographic peak and retention time for each of the ARVs and internal standard. Mass spectrometric parameters were controlled through the LC Quan software to provide the optimal conditions which gave way to the greatest resolution (sharpest peaks). Each chromatographic peak used at least 16 individual scanning event points. Confirmation for specificity was achieved using the same software, and on highlighting the chromatographic peak product ions, showing their mass to charge ratio (*m/z*), this was observed and checked. The chromatographs show that the method allows simultaneous determination and quantification of the five analytes (Figure 18).

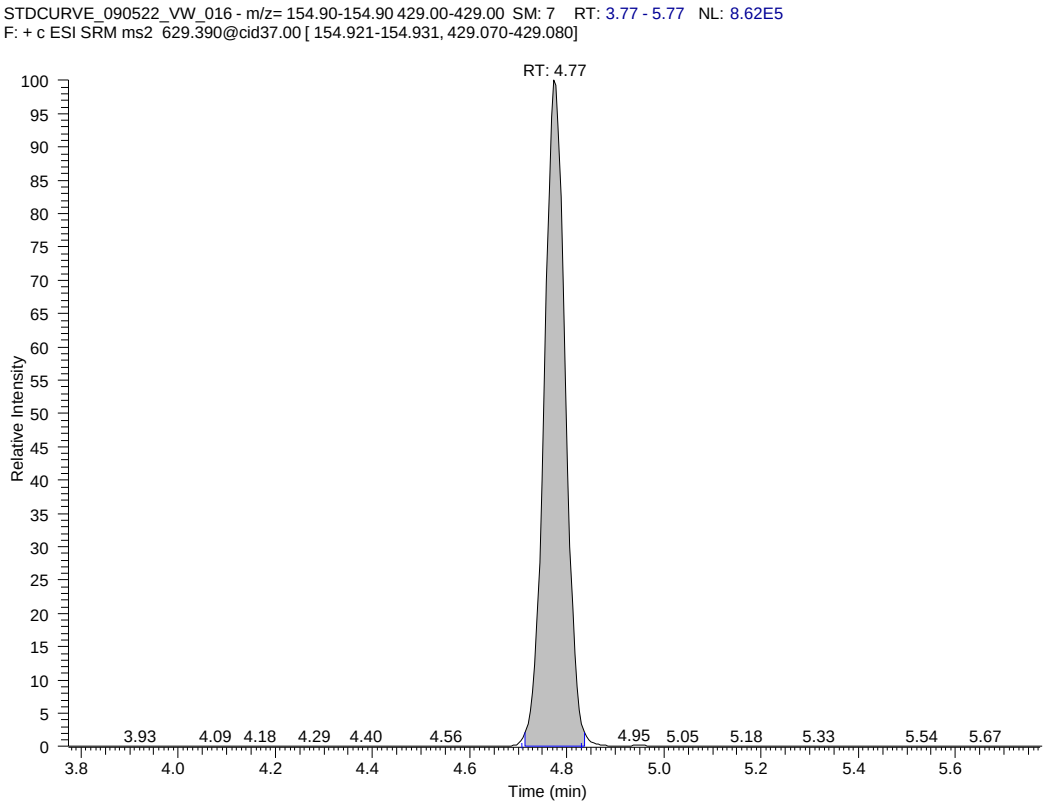

Figure 13. Optimised validation chromatographic peak for LPV (150 ng/mL). Retention time (RT) 4.77 mins.

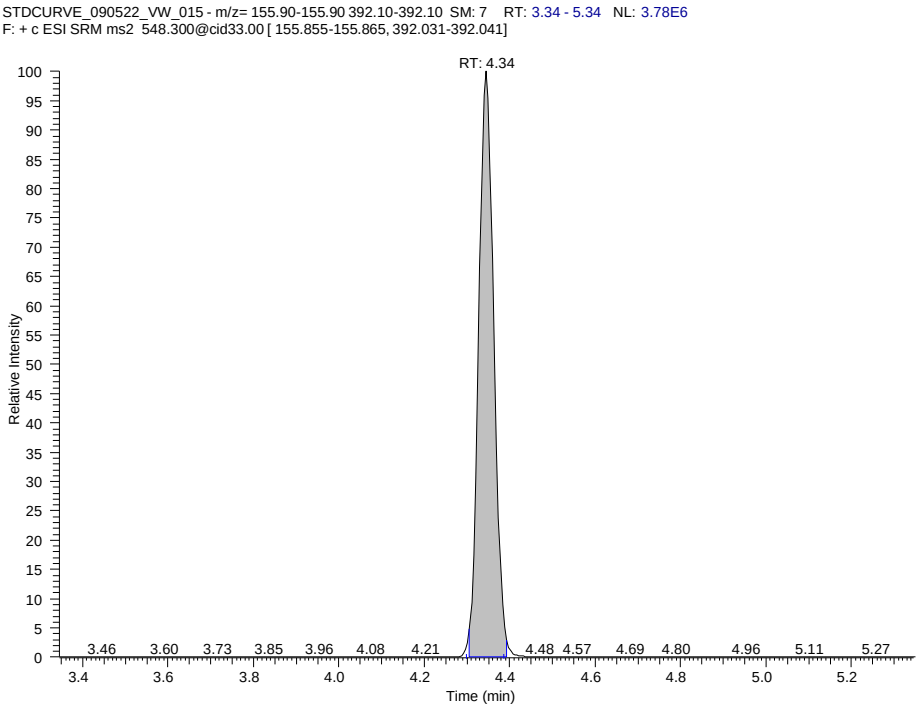

Figure 14. Optimised validation chromatographic peak for DRV (150 ng/mL). Retention time (RT) 4.34 mins.

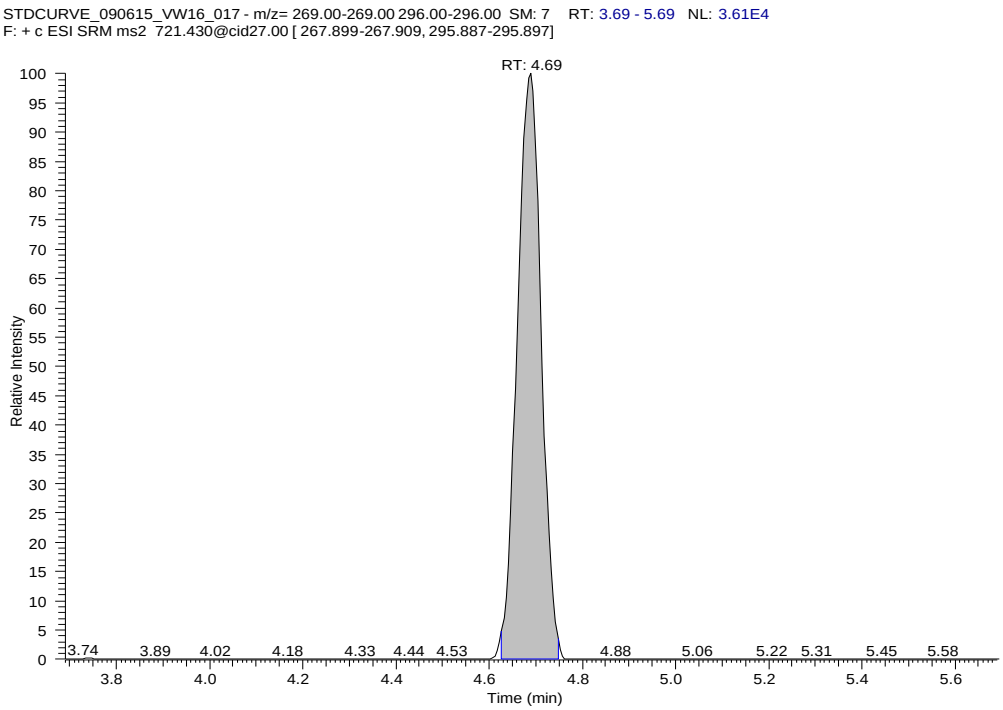

Figure 15. Optimised validation chromatographic peak for RTV (150 ng/mL). Retention time (RT) 4.69 mins.

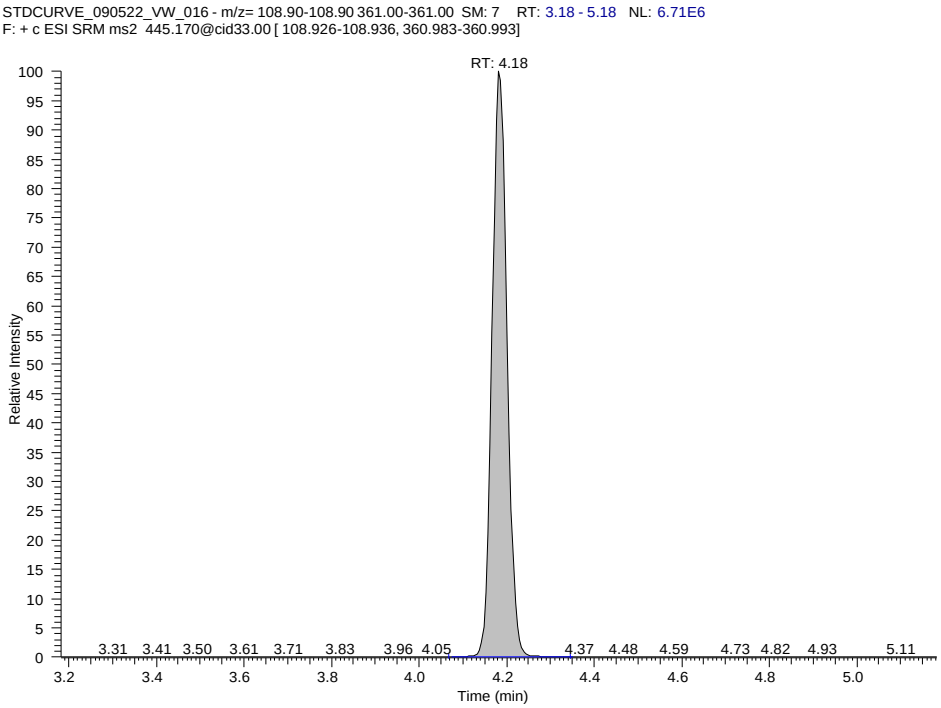

Figure 16. Optimised validation chromatographic peak for RAL (150 ng/mL). Retention time (RT) 4.18 mins.

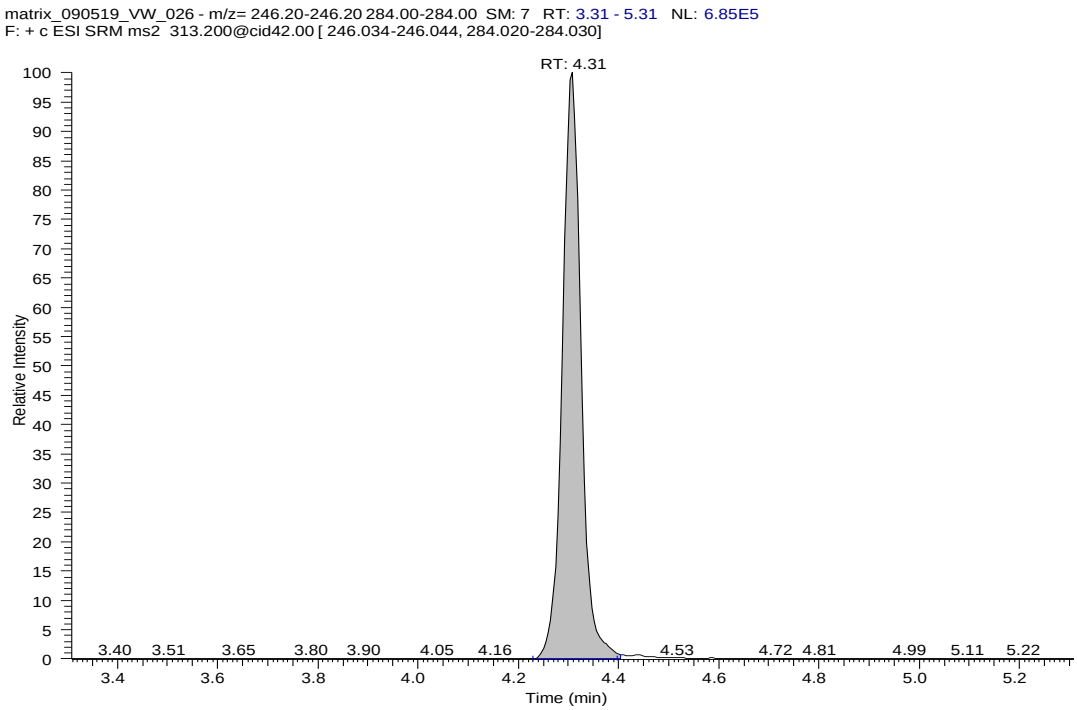

Figure 17. Optimised validation chromatographic peak for internal standard QX (100 ng/mL). Retention time (RT) 4.31 mins.

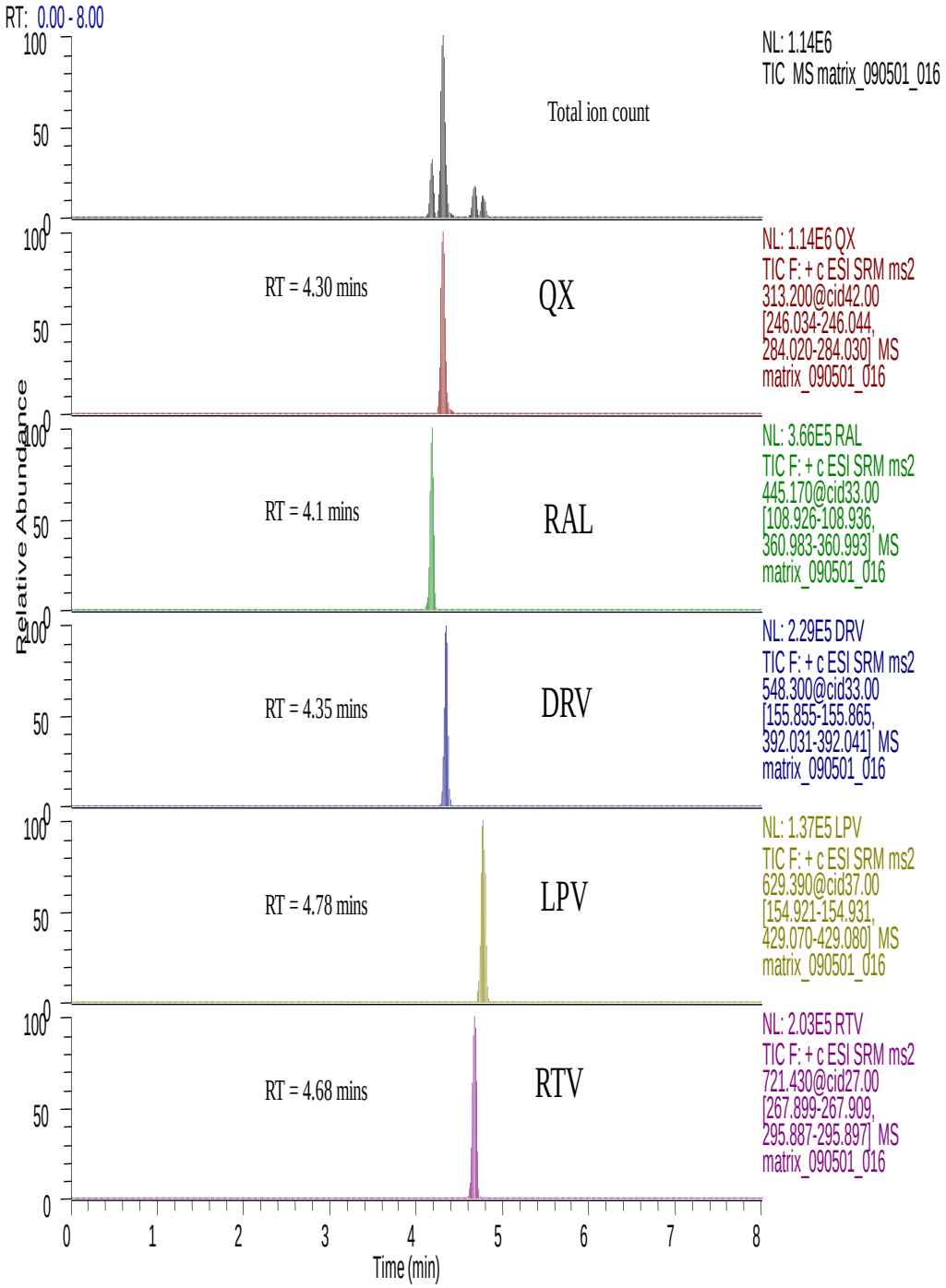

Figure 18.Chromatograph of a calibration standard (150 ng/mL), showing the peak resolution and retention time for each ARV, internal standard QX and the total ion count (TIC).

2.5.3 Accuracy and precision

The precision of this methodology describes the closeness of each individual analyte measured when it is repeatedly applied to multiple samples. The accuracy of this methodology is described by the closeness of the mean test result for each of the individual analytes when compared to the ARV stock concentration prepared. The assay was linear over the concentration range 0.5-150 ng/ mL in PBMC lysate. The inter and intra-assay performance data are presented in Table 3. Accuracy was defined in terms of percentage bias and precision was defined as CV % relative to the standard deviation of the mean. All calculations were derived from the three levels of quality control samples with n = 10. Assays were also assessed for their linearity, the calibration data was analysed using the quadratic least-square regression of the peak area ratio (drug peak area / IS peak area), versus the nominal concentration of the sample with a weighting factor of 1 / concentration (1/x) which achieved a co-efficient of correlation (r^2) values greater than 0.98 for ARV validation assays.

Table.3. Inter and intra-assay performance data for each of the ARVs

Drug	Nominal concentration (ng/ mL)	Inter-assay (n = 6)				Intra-assay (n= 6)			
		Mean (ng/ mL)	Precision (%CV)	Accuracy (% bias)	S.D	Mean (ng/ mL)	Precision (%CV)	Accuracy (% bias)	S.D
DRV									
	7.92	7.88	0.36	99.49	0.0.	7.90	0.18	99.75	0.01
	52.67	52.98	0.41	100.59	0.22	53.88	1.59	102.25	0.86
	130.60	133.25	1.41	101.99	1.87	133.70	1.64	102.32	2.19
		Precision CV % = 0.36-1.41 Accuracy % = 99.49-101.99				**Precision CV % = 0.36-1.41 Accuracy % = 99.49-101.99**			
LPV									
	10.09	10.25	1.10	101.56	0.11	10.13	0.28	100.39	0.00
	52.36	52.48	0.16	100.23	0.08	52.45	0.12	100.17	0.10
	126.16	128.38	1.22	101.73	1.57	128.10	1.07	101.51	1.40
		Precision CV % = .0.16-1.22 Accuracy % = 100.23-101.73				**Precision CV % = 0.12-1.07 Accuracy % =100.17-101.51**			
RAL									0.15
	10.0.1	10.03	0.14	100.20	0.01	9.80	1.52	97.86	0.07
	50.88	51.26	0.52	100.74	0.27	50.78	0.14	99.80	0.59
	128.86	129.00	0.08	100.11	0.10	129.70	0.46	100.65	
		Precision CV % = 0.08-0.52 Accuracy % = 100.11-100.74				**Precision CV % = 0.14-1.52 Accuracy % =97.86-100.65**			
RTV									
	9.56	9.7	1.02	101.44	0.10	9.6	0.29	100.42	0.03
	49.14	49.85	1.01	101.42	0.50	47.6	2.29	96.76	1.09
	125.62	123.9	0.98	98.61	1.22	125.6	0.01	99.98	0.01
		Precision CV % = 0.98-1.02 Accuracy % = 98.61-101.44				**Precision CV % = 0.01-2.29 Accuracy % = 96.76-100.42**			

2.5.4 Matrix effects

The lysate matrix effect was -2.9%, this was calculated using the matrix factor, which is expressed as (peak area response of the cell lysate [n = 20]) divided by the (peak area response of the mobile phase [n = 20]) multiplied by 100. The result being a negative percentage, suggests that there is a slight enhancement of the signal response in the lysate matrix. Therefore, the effect of this for each individual ARV was analysed by post column infusion, which is a more precise method of investigation. Figure 19 shows the typical overlay of the observed signals for a post column infusion injection. Coloured peaks indicate the specific drug of interest, the black line indicates the lysate matrix injection and the grey line indicates the mobile phase injection. A decrease in the relative abundance is known as ion suppression and an increase is ion enhancement. The coloured peaks of the ARVs should therefore ideally not be in either of these areas, which are a result of the co-elution of endogenous cellular components, hence causing the matrix effect.

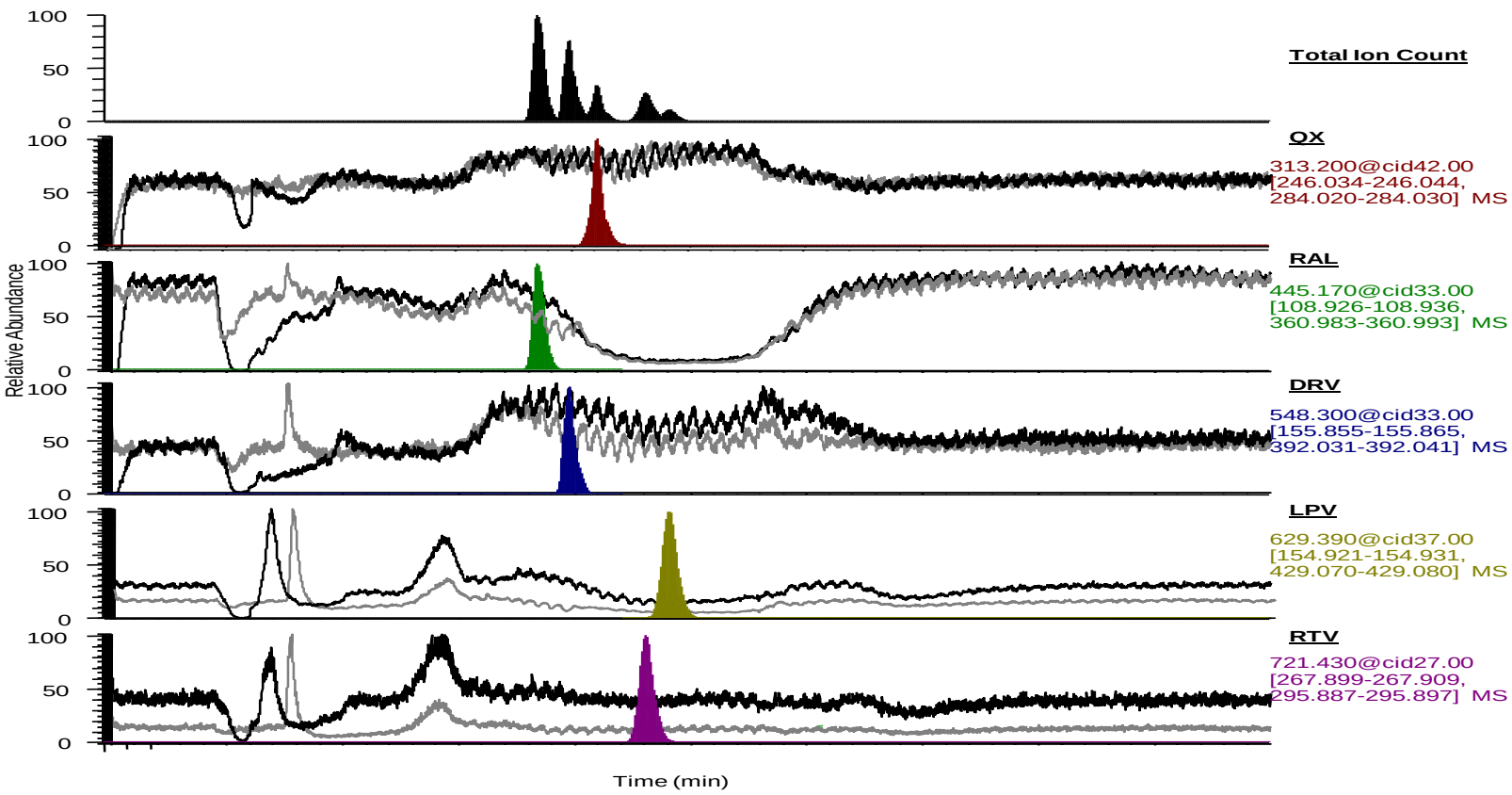

Figure 19. Post column infusion chromatograph overlays of the observed signals of the following injections, (150 ng/ mL of each of the analytes). Coloured peaks indicate the drug of interest, black line indicates the lysate matrix, and the grey line indicates the mobile phase. Eight minute assay run time.

2.5.5 Signal to noise ratio

The signal to noise ratio was investigated to determine the lower limit of quantification (LLQ) for each of the ARVs. The analyte response at the LLQ should be a least five times greater than that of a blank response. The LLQ represents the lowest point on the standard calibration curve and therefore must be measured with accuracy and precision (co-efficient of variation should not exceed +/- 20 %). This was achieved by DRV (mean 6), RAL (mean 31), and RTV (mean 6) at level one (<1 ng/ mL), however, LPV failed to achieve this at the level one standard and therefore level two was LLQ of LPV (mean 12).

2.5.6 Carry over

Carry over is described as a residual drug concentration which is detected in a sample as a result of the preceding injected sample. This can occur when a sample of a high concentration is followed by a sample of very low concentration and therefore the result given is falsely enhanced. To assess the level of carryover for each of the ARV's the highest calibration standard was analysed, followed by five blank samples and comparing the mean signal response value for the high concentration sample to the response given for each set of blank samples. The signal response was extrapolated into a concentration value and carry over was as follows; DRV = 0.04 ng/mL, LPV = 0 ng/mL, RAL = 0.11 ng/mL and RTV = 0.0 ng/mL. Therefore, it was confirmed that during an analytical run, over calculation of an analyte due to a high concentration sample preceding a low concentration sample, carryover did not have significant impact on LLQ results.

2.6 Conclusions

Described here, is the development, optimisation and validation of an LC-MS/MS assay to simultaneously quantify the ARVs, DRV, LPV and RTV which are protease inhibitors and also RAL which is a strand transfer integrase inhibitor, in PBMC lysate. This assay performance was monitored with the use of known concentration quality controls at low, medium and high levels appropriate to the calibration range of the analytes. All ARVs showed good inter assay accuracy, expressed as the % bias (DRV range 99.49-101.99%, LPV range 100.23-101.73%, RAL range 100.11-100.74% and RTV range 98.61-101.44%), and good intra assay accuracy (DRV range 99.49-101.99%, LPV range 100.17-101.51%, RAL range 97.86-100.65% and RTV range 96.76-100.42%). All ARVs also showed good inter assay precision, expressed as the % coefficient of variation (DRV range 0.36-1.41%, LPV range 0.02-1.22%, RAL range 0.08-0.52% and RTV range 0.98-1.02%), and good intra assay precision (DRV range 0.49-101.99%, LPV range 0.12-1.07%, RAL range 0.14-1.52% and RTV range 0.01-2.29%). All analyte calibration data curves illustrated excellent linearity with correlation coefficients (r^2) greater than 0.99, using a quadratic least-square regression equation with a weighting factor of 1/*x*.

The assay run time is only 8 minutes for each sample. This rapid time efficiently allows for a high through put of sample analysis which is especially advantageous when sampling large numbers of samples from clinical pharmacokinetic trials, and also for the use in therapeutic drug monitoring (TDM).

The selection of which ARVs to include in this assay was based solely on two clinical trials which needed to quantify intracellular DRV, LPV, RAL and RTV, and as an intracellular/ plasma ratio would be calculated the internal standard QX was

chosen for this method as it is matched in the established departmental multi ARV plasma assay. At the time of development it was not fully established in the published literature, what concentration ranges would be required for clinical samples. Therefore, the ARV concentration ranges selected were mainly for the purpose of a robust assay development, which could later be altered if needed be. The dynamic range was validated at 0.5-150 ng/mL for DRV, RAL and RTV and 5-150 ng/mL for LPV as this ARV did not show consistency at the LLQ of 0.5 ng/mL. In the initial stages of ARV LC-MS/MS optimisation, up to 6 mass transitions for each analyte were scanned for. From these quantification was carefully selected for by selection of the two best (4 scanned for) fragment ions for each ARV. Fragment ion selection was based on their relative mass intensity. In doing this, all four fragment ions were scanned for and signal intensity monitored. Each combination of the fragment ions was investigated and the duo which gave the greatest signal with the least background noise interference were selected. It is important to have the least background noise possible to enable the greatest assay sensitivity.

One major point of this assay development is that the ARVs are directly spiked in to PBMC lysate. In doing so, the calibration standard levels were constant and were not affected by PBMC batch cell transporters or other cell accumulation factors. This was the only way to regulate and quality control the assay. Therefore, when comparison with clinical samples there are no wash steps involved. The amount of drug spiked into the lysate could therefore not be reduced/ changed depending on various washing procedures. The effects of differences in the clinical sample washing procedures are investigated in chapter 4.

In conclusion, the results of this bio-analytical validation, demonstrate that this LC-MS/MS IC methodology is reliable and reproducible. It is robust, accurate, precise

and sensitive for the simultaneous quantification of DRV, LPV, RAL and RTV in PBMC lysate, enabling a greater insight into the level of ARV which reaches the IC compartment target. To be able to directly quantify IC ARV concentration enables a greater understanding of the true efficacy of HAART and assists in the elucidation of why some patients develop treatment failure despite having optimal ARV plasma concentrations.

Chapter 3

A study investigating plasma and intracellular pharmacokinetics of once daily darunavir / ritonavir and once and twice daily raltegravir in HIV infected individuals.

CHAPTER 3

A STUDY INVESTIGATING PLASMA AND INTRACELLULAR PHARMACOKINETICS OF ONCE DAILY DARUNAVIR/r AND ONCE AND TWICE DAILY RALTEGRAVIR IN HIV INFECTED INDIVIDUALS

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This clinical trial was published in J. Acquir Immune Defic Syndr. 2011 December 15; 58(5): 450-457.

3.1. Introduction

The primary target for antiretroviral (ARVs) drugs is within the cells infected with HIV, therefore the success of a therapy regime can be directly correlated to the amount of drug which penetrates the infected cell. Previous studies have shown links between the activity of ARVs and IC concentrations for protease inhibitors (PIs) and nucleoside reverse transcriptase inhibitors (NRTIs) [26], however the methods used in the measurement of intracellular ARV concentrations is extremely difficult, with each class of ARV measurement having their own challenges, i.e. mass spectrometric ionisation of highly polar drugs in the case of tenofovir. There are currently no 'gold standard' methodologies for the IC measurement of ARVs and as a result present literature shows extensive inconsistency in the reported data, which will be discussed further in this chapter.

There are many different pharmacological considerations which may influence the concentration of IC ARVs, these include but are not limited to, drug oral bioavailability, the percentage of plasma protein binding, drug physiochemical characteristics, and the involvement of multi-drug transport proteins responsible for cellular influx and efflux[26]. Therefore depending on the class of ARV drug, the IC concentration and accumulation can vary greatly.

While PI cellular accumulation has been studied by several research groups previously[26, 29, 32-34], drug interactions involving ARV agents are more commonly studied in formal pharmacokinetic clinical trials investigating drug

concentrations in plasma, again this is mainly due to the more complex nature of IC drug measurement.

Darunavir (DRV) is an antiretroviral used in the combination treatment regime of HIV. It belongs to the class of protease inhibitors and was licenced for use in treatment-naïve patients by the Food and Drug Administration (FDA) at a once daily dose of 800 mg co-administered with 100 mg of ritonavir (RTV) in 2008, and at a twice daily dose of 600 mg/ 100 mg for treatment experienced patients in 2006 [35]. Current data on DRV IC concentrations is constrained to a few heavily pre-treated HIV-infected patients on the 600/100 mg regime taken in combination with RTV, raltegravir (RAL) and etravirine (ETV). Ter Heine reported the DRV IC to plasma ratio to be 1.32 [36]. However, in the same study, Ter Heine also reported that RAL IC concentrations were not detected [36]. This is in contrast to other groups who have investigated IC RAL concentrations and have reported IC to plasma ratios of 0.039, 0.07, and 0.24 [37-39].

The potent ARV raltegravir is the first HIV-1 integrase strand-transfer inhibitor. It was licenced by the FDA for use in the United States of America (USA) in 2007 and then in Europe in 2008 for use in HIV combination therapy, for treatment-experienced patients at a dose of 400 mg twice daily as a result of clinical findings from the BENCHMRK 1 and 2 [40] studies. In 2009, clinical data from the STARTMRK trial, showed that RAL (in combination with Truvada) given as a first line therapy, obtained a level of viral suppression the same as that achieved with an efavirenz based regime, and was therefore given approval for initial therapy also [41].

RAL primary clearance mechanism is UGT1A1- mediated glucoronidation, and as it is not a substrate of cytochrome P450 or an inducer of CYP3A4 it is therefore characterized as having a low drug-drug interaction potential [42].

A once a day dose of RAL (800 mg) has also been investigated by Eron et al, and they concluded that the once daily dose did not show the same level of virological response as the twice daily dosing, especially in patients with a high viral load at base line [43].

The primary objective of this clinical trial was to investigate the plasma and IC PK of DRV/r and RAL (at once and twice daily dosing) when co-administered to HIV-infected subjects.

3.2 Materials and Methods

3.2.1 Subjects

Twenty four adult male HIV-1 antibody positive subjects were recruited. All subjects had a CD4 count greater than 100 cells per cubic millimetre and a viral load less than 400 copies/mL, with no history of virological failure. Subjects were on a stable dual class regimen containing two NRTI's and an NNRTI or a boosted protease inhibitor or an integrase inhibitor. All recruited subjects were aged between 18 and 65 years.

Subjects were excluded based on the following criteria; (1) presence of any currently active clinical disease or AIDS-defining illness, (2) a body mass index greater than 35, (3) clinically relevant drug or alcohol abuse, or (4) the use of any disallowed concomitant therapies.

Approval for the study was granted by the Riverside Research Ethics Committee, UK. All subjects gave written informed consent before any study procedures were undertaken.

3.2.2 Study Design

This trial was a 50 day (excluding screening and follow-up), open label, three phase, pharmacokinetic study, conducted at a single trial site (The Clinical Trial Unit of the St. Stephens AIDS Trust, Chelsea and Westminster Hospital, London, UK), and was carried out in accordance with Good Clinical Practice (GCP), the declaration of Helsinki, and relevant regulatory requirements (EudraCT, 2008-008321-30).

Following successful screening, subjects were randomised 1:1 to Group 1 or Group 2 for phase 2 of the study.

The study treatment regimen was as follows (Figure 1);

Phase I; All subjects (n= 24) received one raltegravir (Insentress) 400 mg film coated tablet twice daily for 21 days. On day 2, subjects underwent an intensive 12 hour pharmacokinetic sampling analysis, with a witnessed morning and evening drug intake.

Phase II; On day 22, all subjects commenced darunavir/ritonavir 800/100 mg once daily. Subjects in Group 1 (n=13) commenced darunavir 800 mg tablets plus one ritonavir (Norvir) 100 mg capsule once daily plus one raltegravir 400 mg tablet twice daily. Subjects in Group 2 (n=11) received darunavir 800 mg tablet plus one ritonavir 100 mg capsule once daily plus two raltegravir 400 mg tablets once daily

for 14 days. On day 35, all subjects underwent an intensive pharmacokinetic sampling analysis.

Phase III; On day 36, all subjects cessed raltegravir intake and continued to take darunavir 800 mg tablet and one ritonavir 100 mg capsule once daily plus their background regime for the following 14 days. A 24 hour concentration sample was collected on day 36 from each subject.

On day 49, all subjects underwent a final intensive pharmacokinetic sampling analysis, and on day 50 a final 24 hour concentration sample was also collected for analysis.

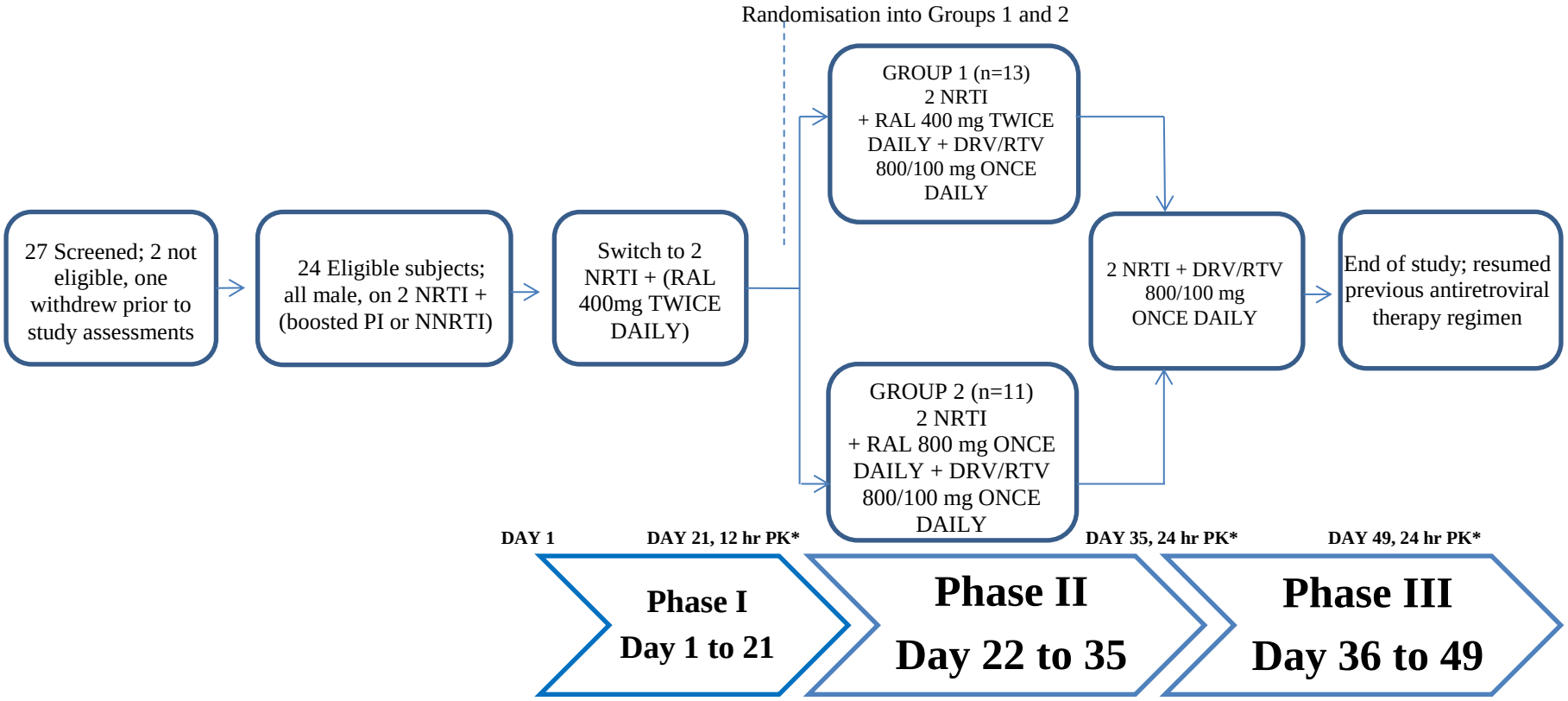


Figure 1. Flow diagram to show the study treatment regimen. *Plasma and intracellular intensive PK profile analysis at 0 (pre dose within 10 minutes before dosing), 1, 2, 4, 6, 8, and 12 hours post dose (and darunavir [DRV] concentrations at 24 hours on days 35 and 49).

3.2.3 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Two 8mL cell preparation tubes (CPT) (Becton-Dickinson Vacutainer; Oxford, United Kingdom) were collected for each time point. The anticoagulant was mixed thoroughly by gently inverting the tubes 8-10 times. CPT were then immediately centrifuged (horizontal rotor) for 20 minutes (1600 relative centrifugal force [rcf]) at room temperature (20°C). Peripheral blood mononuclear cells (PBMCs) were then suspended in the plasma by carefully inverting the CPT. The mixed plasma/cells suspension from both cell preparation tubes were then combined into a single graduated 50 mL conical tube to which isotonic saline (0.9%) was added to bring the total volume to exactly 30 mL. The cell/plasma sample was then gently mixed by inversion, and a 40 μL aliquot was taken for initial cell counting (Digital Bio Adam Microchip Automatic Cell Counter; NanoEnTek, Inc, Seoul, Korea). The cell count was normalised by multiplying by 30 to calculate the actual total cell count in 30 mL and recorded. As there were problems with the cell counter a confirmatory cell count was obtained using a DNA-based method developed by Benech et al[44]. The plasma/cell suspension was then centrifuged for 15 minutes, at 400 relative rcf to pellet the cells. The supernatant was carefully removed to avoid disruption of the cell pellet. In total this cell isolation method used a single cell wash step to remove the supernatant; this contrasts with methods used in other studies, each having a different number of cell washes in the isolation process [36-38]. Ice-cold methanol (precisely 1 mL, 70%) was added to the pellet to lyse the cells and ultimately release the intracellular drugs. The cell lysate was vortexed (3–5 minutes) and stored at −80°C.

3.2.4 Analytical Methods

The concentrations of the antiretroviral drugs RAL, DRV and RTV were determined in a total of 552 plasma samples, paired with a further 552 intracellular samples, collected during the clinical pharmacology study. Concentrations of each antiretroviral was determined by reversed phase high performance liquid chromatography (HPLC) interfaced with a triple quadrupole mass spectrometer.

3.2.5 Plasma Extraction and Analysis

Plasma samples for the analysis of RAL, DRV and RTV were extracted alongside standard calibrators and quality controls (QC) at low, medium and high concentrations. Each sample was extracted in duplicate (100 µL per sample). The internal standard quinoxaline (6,7-dimethyl-2,3-di(2pyridyl)-quinoxaline) [QX] (20 µL of a 1 µg/mL) was added to each sample followed by acetonitrile (500 µL). Samples were then mixed with the use of a hand vortex covered and left to stand, at room temperature for 15 minutes to precipitate the proteins. This was followed by vortexing the samples once again and then centrifuging for 10 minutes at 4000 revolutions per minute (rpm). The resulting supernatant was then carefully transferred into clean glass labelled 5 mL tubes. To each sample 0.05% formic acid (200 µL) was then added and the samples vortexed once again. Samples were then pipetted into glass autosampler vials ready for injection (10 µL) onto the HPLC column. This method described is a modified validated version of that published by Else et al[45] to incorporate the detection and quantification of the RAL.

RAL, DRV and RTV plasma concentrations were extrapolated from validated spiked plasma matrix calibration curves. The range of each calibration curve was as follows; RAL 4.7- 2011 ng/mL, DRV 76-14803 ng/mL, and RTV 13-4993 ng/mL. Calibration curves were obtained using 1/concentration weighted quadratic regression of peak area ratio versus nominal concentration. The lower limit of quantification (LLQ) for each of the ARV's were determined to be; RAL = 4.7, DRV = 76, and RTV = 13ng/mL. LLQ was defined as the lowest concentration for which the percentage deviation from the nominal standard concentration was less than 20 % and signal to noise was greater than 5%.

The precision, expressed as a coefficient of variation [CV%] ((Standard deviation / mean) X 100), of the assays, as determined from the analysis of low, medium and high quality control samples, was as follows; RAL = 6.9-9.3%, DRV = 7.8-10.6%, RTV = 6.7-8.2%.

The accuracy, expressed by percentage bias ((measured value – target value) / measured value) X 100) of the assays, as determined from the analysis of 6 replicates of each quality control samples in relation to the actual target quality control sample concentration for plasma samples, was as follows; RAL = 89.3-100.3%, DRV = 97.5-102.6, RTV = 102.9-105.7%.

3.2.6 Drug Quantification in Peripheral Blood Mononuclear Cells (PBMC)

The methodology for the determination of the intracellular concentration of raltegravir, darunavir and ritonavir is described in chapter 2 of this thesis. Briefly, subject intracellular lysate samples in 1 mL of 70% methanol (MeOH) were removed

from the -80^{o}C freezer. Samples were centrifuged and the cell lysate was transferred into a clean tube. The methanol was evaporated off the lysate samples and they were then reconstituted in 400µL of 70% MeOH, ready for direct analysis by reverse phase HPLC interfaced with a triple quadrupole mass spectrometer.

Calculations to normalize the reading from the LC-MS/MS for intracellular samples were as follows:

A = Raw mass spectrometer result

B = Cell count (cells per 1 mL)

1000 = Normalization to 1 mL

400 = Reconstituted (MeOH) volume for extraction

2.5 x 10^{9}= (PBMC volume 0.4 pL transformed and normalized from ng/pL to ng/mL)

Equation $\frac{\left(\frac{A}{1000}\right)\times 400}{B} \times 2.5 \times 10^{9}$

Example = Raw raltegravir mass spectrometer value for a patient sample was 3.64 ng/mL. However, the number of cells, the volume of liquid which the cells are in and the average volume of a PBMC in that sample must be taken in to account:

$$\frac{3.64 \text{ X } 400}{1000 \qquad \text{X } 2.5\text{x}10^{9}}$$

12755075

= 285 ng/mL

RAL, DRV and RTV intracellular concentrations were extrapolated from validated spiked cell lysate matrix calibration curves. The range of each calibration curve was as follows; RAL = 0.45-146.08 ng/mL, DRV = 0.45-1483 ng/mL, and RTV = 0.48-153.23 ng/mL. Calibration curves were obtained using 1/concentration weighted linear regression of peak area ratio versus nominal concentration. The lower limit of quantification (LLQ) for each of the ARV's were determined to be; RAL = 0.45, DRV = 0.45 and RTV = 0.48 ng/mL. LLQ was defined as the lowest concentration for which the percentage deviation from the nominal standard concentration was less than 20 % and signal to noise was greater than 5%.

The precision, expressed as a coefficient of variation [CV%] (Standard deviation / mean] X 100), of the assays, as determined from the analysis of low, medium and high quality control samples, was as follows; RAL =7.0-9.3%, DRV = 5.1-10.0%, RTV = 7.2-9.5%.

The accuracy, expressed by percentage bias ((measured value – target value) / measured value) X 100) of the assays, as determined from the analysis of 6 replicates of each quality control samples in relation to the target actual target quality control sample concentration for plasma samples, was as follows; RAL = 98.5-104.2 %, DRV = 98.9-100.4 %, RTV = 97.6-100 %.

3.2.7 Pharmacokinetics and Statistical Analysis

The pharmacokinetic parameters calculated for plasma and intracellular RAL, DRV and RTV were C_{trough}, defined as the plasma concentration at hour 12 and 24 after observed drug intake. The maximum concentrations (C_{max}), elimination half-life

($t_{1/2}$), time point at C_{max} (T_{max}), and total drug exposure, expressed as area under the plasma/ intracellular concentration time curve (AUC) from 0-12 (for RAL in both groups in phase I and in Group 1 in phase II) and 0-24 hours (Group 2 in phase II) after drug intake.

Pharmacokinetics were calculated using WinNonLin version 5.2 (Mountain View, CA, USA), by non-compartmental linear-linear trapezoidal analysis. Inter-individual variability in concentration of RAL, DRV and RTV was assessed by calculating the percentage coefficient of variation (%CV = standard deviation/mean × 100). Intracellular accumulation was expressed as the ratio of intracellular drug to plasma drug concentration for each of the pharmacokinetic parameters. Calculation of geometric means (GM), ratios (GMR), and 90% confidence intervals (CIs), were used to assess within-subject changes of drug concentrations (drug alone versus drug combination). Any changes in pharmacokinetic parameters were only considered significant when the 90% CI did not cross the value of 1.

3.3. Results

3.3.1 Demographic and Clinical Characteristics

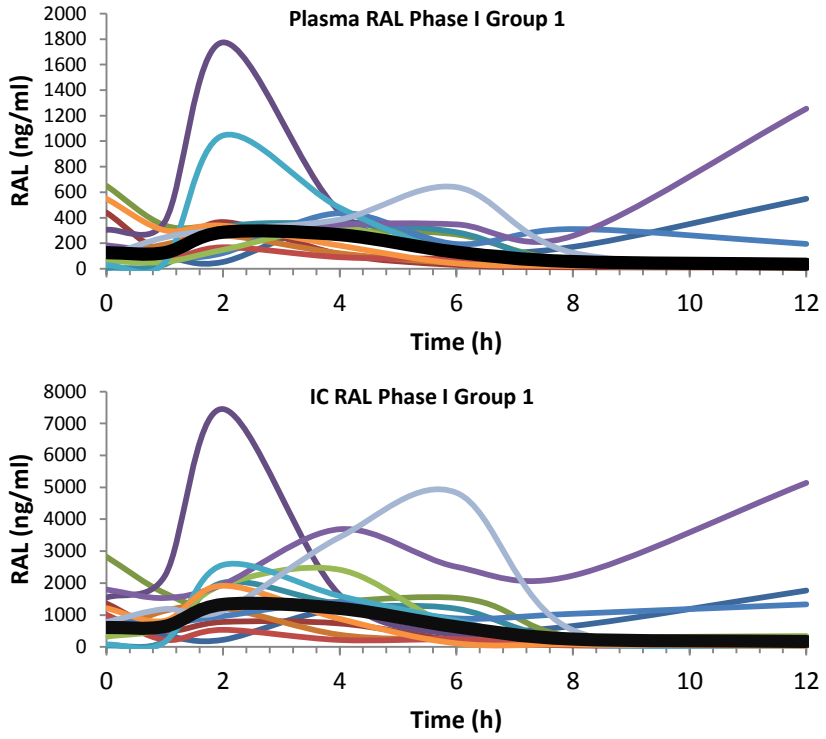
Twenty seven HIV positive patients were screened for the study, two participants were not eligible (one screened positive for drugs of abuse, and the second because of problems gaining venous access). A third participant withdrew for personal reasons, leaving 24 eligible participants (Group 1; n = 13, Group 2; n = 11). Assuming a wide inter individual variability (approximately 60%) in the study drug pharmacokinetics, 24 subjects provided a 90% power to show any effect of an

interaction of the drugs studied to be statistically significant at a 5% level. The median age range of the 24 participants was 37 (range = 21-62) years, body mass index was 26 (range = 19-32) kg/m^2, and CD4 count was 466 (range = 249-849) cells per cubic millimetre. All participants were male (17 white, 4 Asian and 3 black), on a stable antiretroviral therapy (concomitant ARV medications taken during the study included tenofovir [n = 21], emtricitabine [n= 21], abacavir [n = 3] and lamivudine [n = 3]), with viral loads less than 50 copies per millimetre. No grade 3 or 4 adverse effects were reported during the clinical trial and all study drugs were well tolerated.

3.3.2 Raltegravir Pharmacokinetics and Statistical Analysis

Plasma and IC patient pharmacokinetic profiles and parameters for Group 1 and 2 in phase I; RAL in the absence of DRV and phase II; RAL in the presence of DRV are shown in Figures 2, 3, 4 and 5. The plasma and IC pharmacokinetic parameter ratios for Group 1 and Group 2 are shown in Tables 1 and 2 respectively.

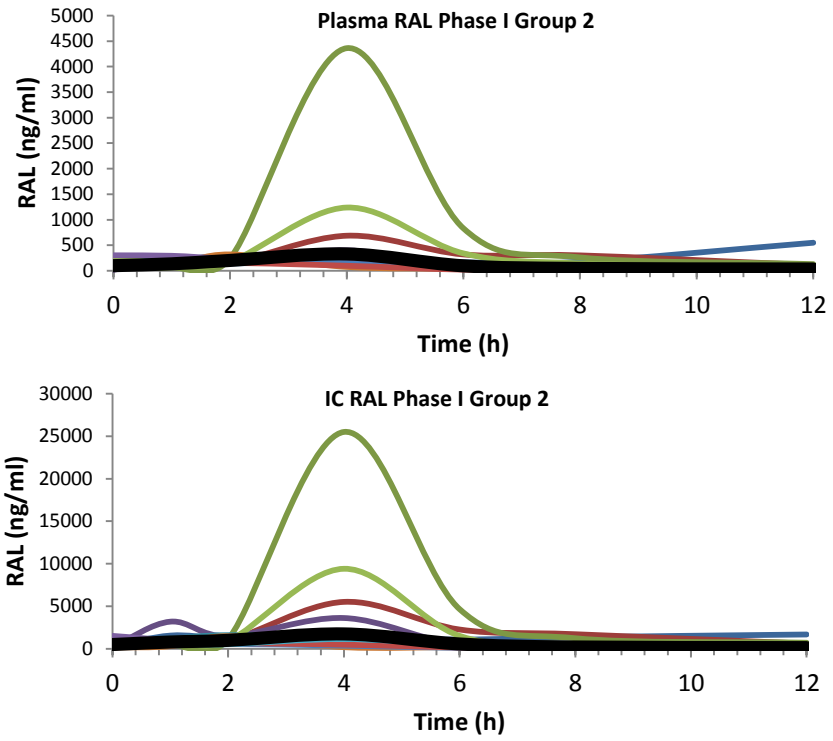
Group 1 plasma and IC RAL PK profiles for Phase I (day 21)



	Day 21 (-DRV)						
	Group 1 Plasma PK parameters RAL 400 mg BID						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-12} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	2.29	3.93	533	23	1944	114	206
low 90%	2.03	2.00	441	9	1686	122	180
up 90%	2.66	5.69	857	62	2623	312	276
Min	1.68	0.00	169	10	942	2	91
Max	3.21	12.00	1774	195	4414	651	425
CV	24	105	70	150	47	95	46
	Day 21 (- DRV)						
	Group 1 IC PK parameters RAL 400 mg BID						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-12} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	2.83	3.67	2306	101	9010	609	44
low 90%	2.11	2.70	1892	24	7852	624	37
up 90%	4.72	6.90	3636	403	13519	1339	70
Min	1.38	2.00	1008	35	2820	36	19
Max	8.26	12.00	7453	1766	21588	2825	142
CV	70	84	69	179	58	80	66

Figure 2. Group 1 plasma and IC RAL, 400 mg twice daily in the absence of DRV (phase I day 21) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

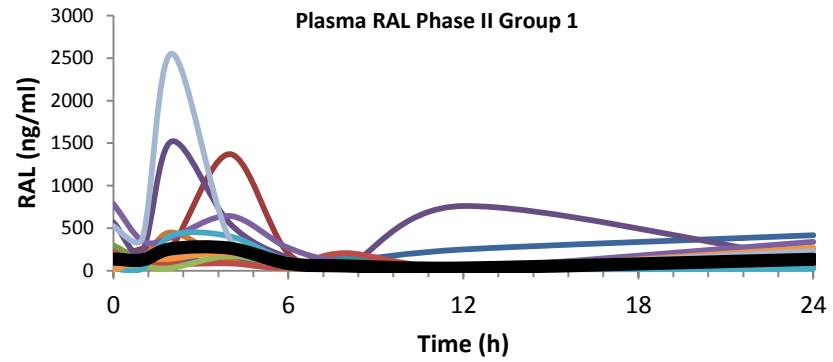
Group 2 plasma and IC RAL PK profiles for Phase I (day 21)

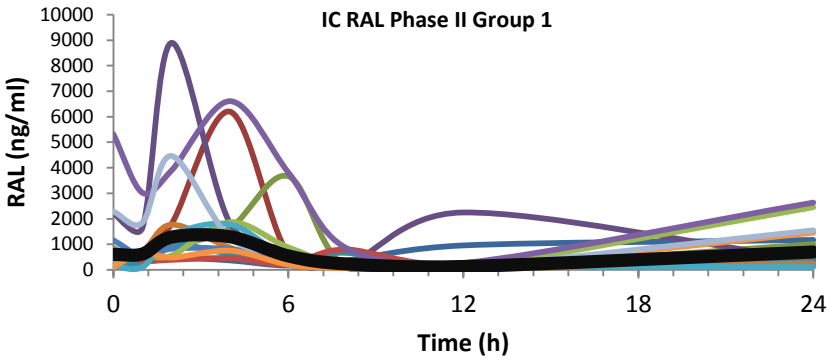


	Day 21 (- DRV)						
	Group 2 Plasma PK parameters RAL 400 mg BID						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-12} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	3.46	3.63	361	28	1635	101	245
low 90%	2.50	2.31	269	20	1311	88	208
up 90%	5.59	5.32	609	66	2663	188	372
Min	2.21	0.00	152	7	638	18	94
Max	11.92	12.00	1239	131	4252	304	627
CV	73	79	74	103	68	73	57
	Day 21 (-DRV)						
	Group 2 IC PK parameters RAL 400 mg BID						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	2.6	3.4	1871	115	8780	511	46
low 90%	2.2	2.4	1276	80	7286	424	38
up 90%	3.3	5.6	4185	345	16400	918	84
Min	1.6	2.00	574	21	2728	164	15
Max	5.3	12.00	9416	723	27218	1529	147
CV	38	75	102	119	77	74	76

Figure 3. Group 2 plasma and IC RAL, 400 mg twice daily in the absence of DRV (phase I day 21) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 1 plasma and IC RAL PK profiles for Phase II (day 35)

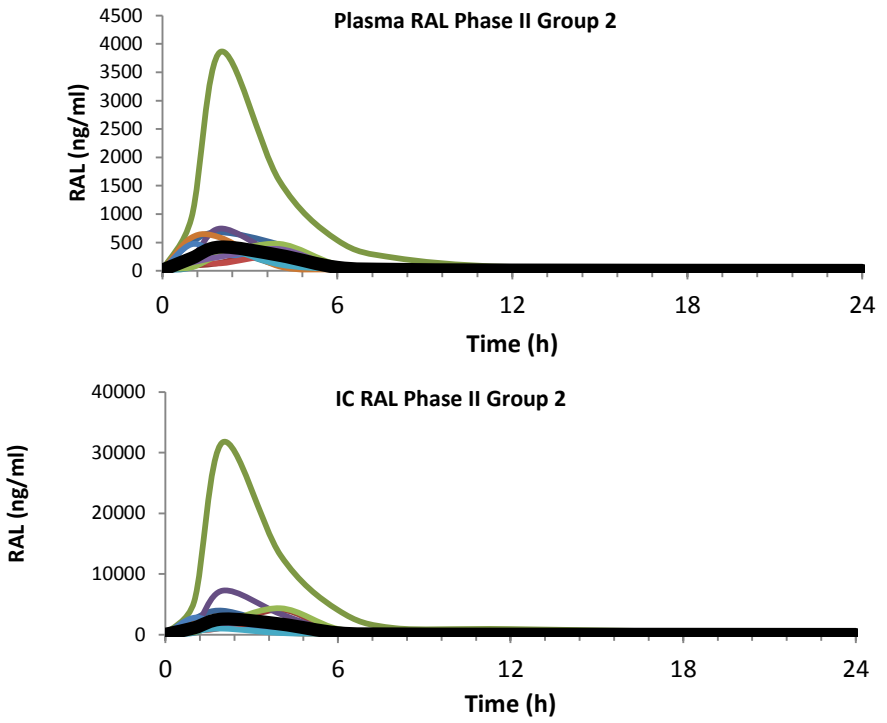




	Day 35 (+ DRV)						
	Group 1 Plasma PK parameters RAL 400 mg BID						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-12} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	3.26	2.72	438	20	1759	146	227
low 90%	2.64	1.25	337	17	1459	144	202
up 90%	4.97	2.75	999	25	2937	362	344
Min	1.87	0.00	174	12	660	20	70
Max	8.74	4.00	2552	34	5676	788	606
CV	62	82	108	34	73	95	57
	Day 35 (+ DRV)						
	Group 1 IC PK parameters RAL 400 mg BID (steady-state)						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-12} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	2.41	3.81	2173	105	9223	605	43
low 90%	2.09	3.09	1846	51	7416	464	39
up 90%	2.90	5.91	4299	295	15694	1791	65
Min	1.65	2.00	754	38	3672	116	11
Max	3.99	12.00	8896	958	35452	5314	109
CV	29	66	87	148	78	129	55

Figure 4. Group 1 plasma and IC RAL, 400 mg twice daily in the presence of DRV 800 mg RTV 100 mg once daily (phase II day 35) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 2 plasma and IC RAL PK profiles for Phase II (day 35)



	Day 35/36 (+ DRV)						
	Group 2 Plasma PK parameters RAL 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	9.08	2.27	556	11	1979	16	404
low 90%	7.25	1.94	273	9	1079	13	365
up 90%	13.63	3.15	1298	20	4318	27	574
Min	3.99	1.00	268	5	1023	8	65
Max	24.79	4.00	3862	35	12373	46	782
CV	58	48	131	75	121	71	45
	Day 35/36 (+DRV)						
	Group 2 IC PK parameters RAL 800 mg OD (steady-state)						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	7.2	2.4	2817	22	11183	54	72
low 90%	3.4	2.1	2289	16	7201	36	59
up 90%	18.8	3.2	4153	59	23547	156	121
Min	2.99	1.00	991	4	3345	8	13
Max	57.06	4.00	7260	146	62495	422	239
CV	139	42	55	114	107	125	69

Figure 5. Group 2 plasma and IC RAL, 800 mg once daily in the presence of DRV 800 mg RTV 100 mg once daily (phase II day 35) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Table 1. Geometric mean ratio, lower and upper 90 % confidence intervals and % co efficient of variance for Group 1 and 2 plasma RAL phase I (day 21 without DRV/r) versus phase II (day 35 with DRV/r).

GROUP 1 Plasma RAL : DAY 21 (RAL 400 mg BID) vs. DAY 35 (RAL 400 mg BID PLUS DRV/r 800/100 mg OD).																				
	Plasma half-life (h)				**Plasma T_{max} (h)**				**Plasma C_{max} (ng/ml)**				**Plasma C_{trough}(ng/ml)**				**Plasma AUC (ng.h/ml)**			
	-DRV	**+DRV**	**ratio**	**log ratio**	**-DRV**	**+DRV**	**ratio**	**log ratio**	**-DRV**	**+DRV**	**ratio**	**log ratio**	**-DRV**	**+DRV**	**ratio**	**log ratio**	**-DRV**	**+DRV**	**ratio**	**log ratio**
Geomean	2.3	3.3	**1.11**		3.9	2.7	**0.77**		532.7	438.2	**0.82**		22.9	19.9	**0.76**		1943.8	1759.1	**0.90**	
low 90%CI	2.0	2.6	1.00	-0.20	3.0	2.3	0.65	-0.23	441.4	337.3	0.62	-0.24	9.0	17.5	0.69	-0.30	1686.4	1459.0	0.73	-0.20
high 90%CI	2.7	5.0	1.95	0.29	7.0	3.5	1.01	0.01	857.1	999.0	1.67	0.10	62.1	24.5	1.12	0.00	2622.6	2937.1	1.44	0.10
	increase by 11%				*decrease by 23%*				*decrease by 18%*				*decrease by 24%*				*decrease by 10%*			
anti-log low 90%CI	*NON sig Difference*			0.64	*NON sig Difference*			0.58	*NON sig Difference*			0.57	*NON sig Difference*			0.50	*NON sig Difference*			0.69
anti-log high 90%CI				1.93				1.03				1.18				1.10				1.19
min	1.7	1.9	0.10		2.0	2.0	0.33		169.0	174.0	0.39		10.0	12.0	0.10		942.0	660.0	0.43	
max	3.2	8.7	2.81		12.0	4.0	1.00		1774.0	2552.0	3.99		195.0	34.0	1.40		4413.5	5675.5	3.24	
cv%	24.3	61.7	58.87		78.3	36.5	34.64		70.0	108.2	99.82		150.1	33.7	45.80		47.5	73.5	71.60	
GROUP 2 Plasma RAL : DAY 21 (RAL 400 mg OD) vs. DAY 35 (RAL 800 mg OD PLUS DRV/r 800/100 mg OD).																				
	Plasma half-life (h)				**Plasma T_{max} (h)**				**Plasma C_{max} (ng/ml)**				**Plasma C_{trough}(ng/ml)**				**Plasma AUC_{0-24} (ng.h/ml)**			
	-DRV/r	**+DRV/r**	**ratio**	**log ratio**	**-DRV/r**	**+DRV/r**	**ratio**	**log ratio**	**-DRV/r**	**+DRV/r**	**ratio**	**log ratio**	**-DRV/r**	**+DRV/r**	**ratio**	**log ratio**	**-DRV/r**	**+DRV/r**	**ratio**	**log ratio**
Geomean	3.5	9.1	**3.10**		3.6	2.3	**0.59**		360.6	555.7	**1.27**		27.5	11.4	**0.42**		1634.7	1979.0	**1.21**	
low 90%CI	2.5	7.3	2.61	0.34	2.7	1.9	0.46	-0.39	269.2	273.0	1.09	0.00	19.9	9.3	0.35	-0.50	1310.8	1079.4	1.03	0.00
high 90%CI	5.6	13.6	4.57	0.64	5.7	3.1	1.02	-0.10	608.8	1297.9	1.82	0.21	65.9	20.4	0.59	-0.30	2662.6	4317.8	1.77	0.20
	increase by 210%				*decrease by 41%*				*increase by 27%*				*decrease by 58%*				*increase by 21%*			
anti-log low 90%CI	*SIG Difference*			2.17	*SIG Difference*			0.40	*NON sig Difference*			0.92	*SIG Difference*			0.30	*NON sig Difference*			0.90
anti-log high 90%CI				4.41				0.86				1.64				0.53				1.60
min	2.2	4.0	0.77		2.0	1.0	0.17		152.0	268.0	0.38		7.0	5.0	0.02		637.5	1022.5	0.47	
max	11.9	24.8	6.65		12.0	4.0	2.00		1239.0	3862.0	2.87		131.0	35.0	0.88		4252.0	12373.0	2.90	
cv%	73.2	58.5	49.48		69.0	47.7	72.32		74.1	131.2	48.50		102.8	75.3	49.40		68.4	120.6	53.10	

Table 2. Geometric mean ratio, lower and upper 90% confidence intervals and % co efficient of variance for Group 1 and 2 IC RAL phase I (day 21 without DRV/r) versus phase II (day 35 with DRV/r).

GROUP 1 IC RAL : DAY 21 (RAL 400 mg BID) vs. DAY 35 (RAL 400 mg BID PLUS DRV/r 800/100 mg OD).																				
	IC half-life (h)				IC T_{max} (h)				IC C_{max} (ng/ml)				IC C_{trough}(ng/ml)				IC AUC_{0-12} (ng.h/ml)			
	-DRV	+DRV	ratio	log ratio	-DRV	+DRV	ratio	log ratio	-DRV	+DRV	ratio	log ratio	-DRV	+DRV	ratio	log ratio	-DRV	+DRV	ratio	log ratio
Geomean	2.8	2.4	**0.82**		3.7	3.8	**0.91**		2306.5	2172.6	**0.94**		101.2	104.7	**0.70**		9009.8	9222.8	**1.02**	
low 90%CI	2.1	2.1	0.47	-0.34	2.7	3.1	0.75	-0.2	1891.5	1846.4	0.68	-0.15	23.7	51.5	0.57	-0.48	7851.6	7415.8	0.81	-0.14
high 90%CI	4.7	2.9	1.75	0.17	6.9	5.9	1.40	0.1	3636.5	4299.1	1.66	0.10	403.2	295.0	2.06	0.26	13519.3	15693.9	1.67	0.10
	decrease by 18%				*decrease by 9%*				*decrease by 6%*				*decrease by 30%*				*decrease by 2%*			
anti-log low 90%CI	*NON sig Difference*			0.45	*NON Sig Difference*			0.64	*NON sig Difference*			0.71	*NON sig Difference*			0.34	*NON sig Difference*			0.78
anti-log high 90%CI				1.48				1.30				1.25				1.43				1.35
min	1.4	1.7	0.22		2.0	2.0	0.33		1008.0	754.0	0.39		35.0	38.0	0.04		2819.5	3672.0	0.42	
max	8.3	4.0	2.89		12.0	12.0	2.00		7453.0	8896.0	4.55		1333.0	958.0	47.85		21587.5	35452.0	3.99	
cv%	69.6	29.4	85.99		83.8	66.0	55.47		69.0	87.2	91.22		178.7	147.6	108.70		58.0	78.3	76.00	
GROUP 2 IC RAL : DAY 21 (RAL 400 mg OD) vs. DAY 35 (RAL 800 mg OD PLUS DRV/r 800/100 mg OD).																				
	IC half-life (h)				IC T_{max} (h)				IC C_{max} (ng/ml)				IC C_{trough}(ng/ml)				IC AUC_{0-24} (ng.h/ml)			
	-DRV/r	+DRV/r	ratio	log ratio	-DRV/r	+DRV/r	ratio	log ratio	-DRV/r	+DRV/r	ratio	log ratio	-DRV/r	+DRV/r	ratio	log ratio	-DRV/r	+DRV/r	ratio	log ratio
Geomean	2.6	7.2	**2.72**		3.4	2.4	**0.68**		1870.8	2816.7	**1.51**		114.6	21.7	**0.19**		8780.0	11183.3	**1.27**	
low 90%CI	2.2	3.4	1.42	0.24	2.4	2.1	0.55	-0.32	1275.8	2289.4	1.18	0.02	80.5	16.2	0.12	-0.95	7285.9	7201.5	1.07	-0.03
high 90%CI	3.3	18.8	6.80	0.63	5.6	3.2	1.08	-0.01	4185.2	4152.6	2.59	0.34	344.7	59.1	0.50	-0.48	16400.2	23547.2	1.94	0.24
	increase by 172%				*decrease by 32%*				*increase by 51%*				*decrease by 81%*				*increase by 27%*			
anti-log low 90%CI	*SIG Difference*			1.74	*SIG Difference*			0.48	*SIG Difference*			1.04	*SIG Difference*			0.11	*NON sig Difference*			0.93
anti-log high 90%CI				4.27				0.97				2.19				0.33				1.74
min	1.6	3.0	0.89		2.0	1.0	0.17		574.0	991.0	0.46		21.0	4.0	0.01		2727.5	3345.0	0.57	
max	5.3	57.1	18.30		12.0	4.0	2.00		9416.0	7260.0	4.84		723.0	146.0	1.28		27217.5	62494.5	2.76	
cv%	37.9	139.4	125.38		74.5	42.5	62.68		102.1	5.4	71.70		119.1	114.5	116.70		77.3	106.9	58.94	

Correlation between IC and plasma PK parameters for RAL in the absence (phase I day 21) and presence of DRV (phase II day 35), for Group 1 and Group 2 are shown in Figures 6 and 7.

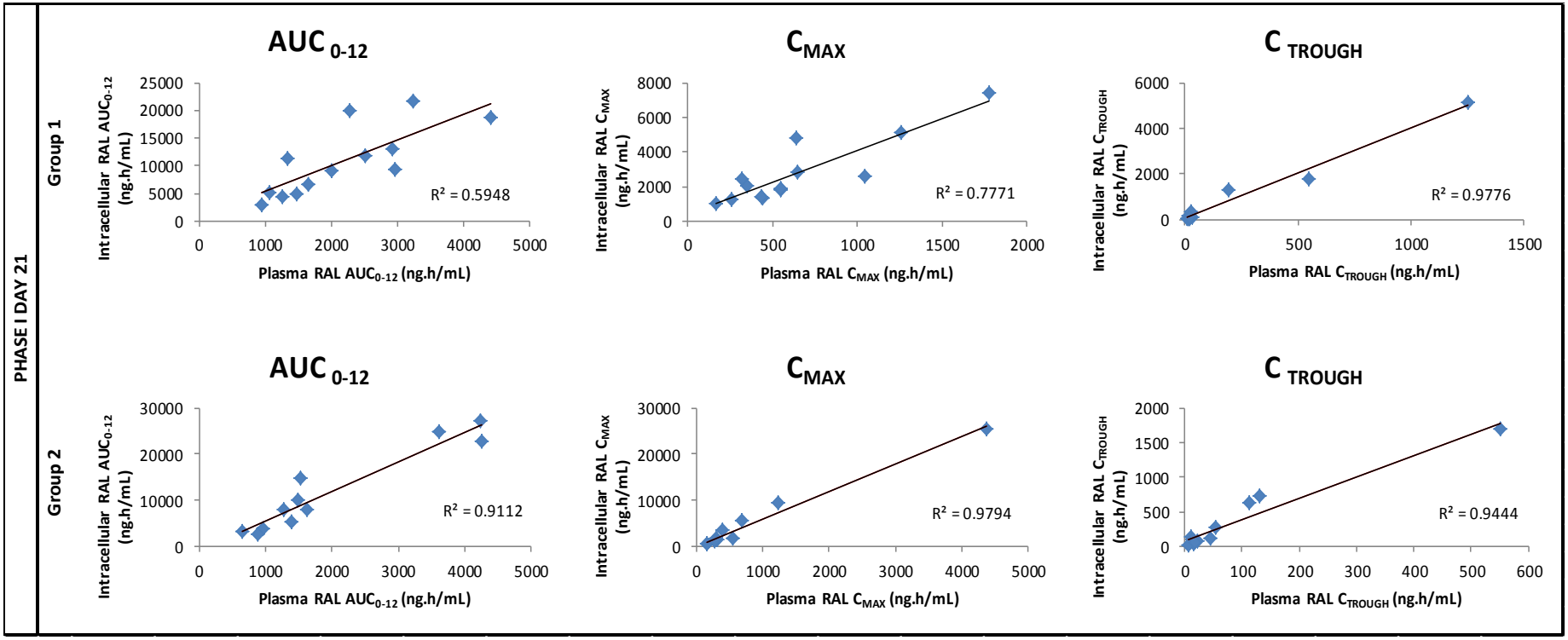


Figure 6. Correlation between IC and plasma pharmacokinetic parameters (AUC $_{0\text{-}12hr,}$ C_{max}, and C_{trough}) for Group 1 and 2, phase I (day 21) RAL 400 mg twice daily in the absence of DRV/r.

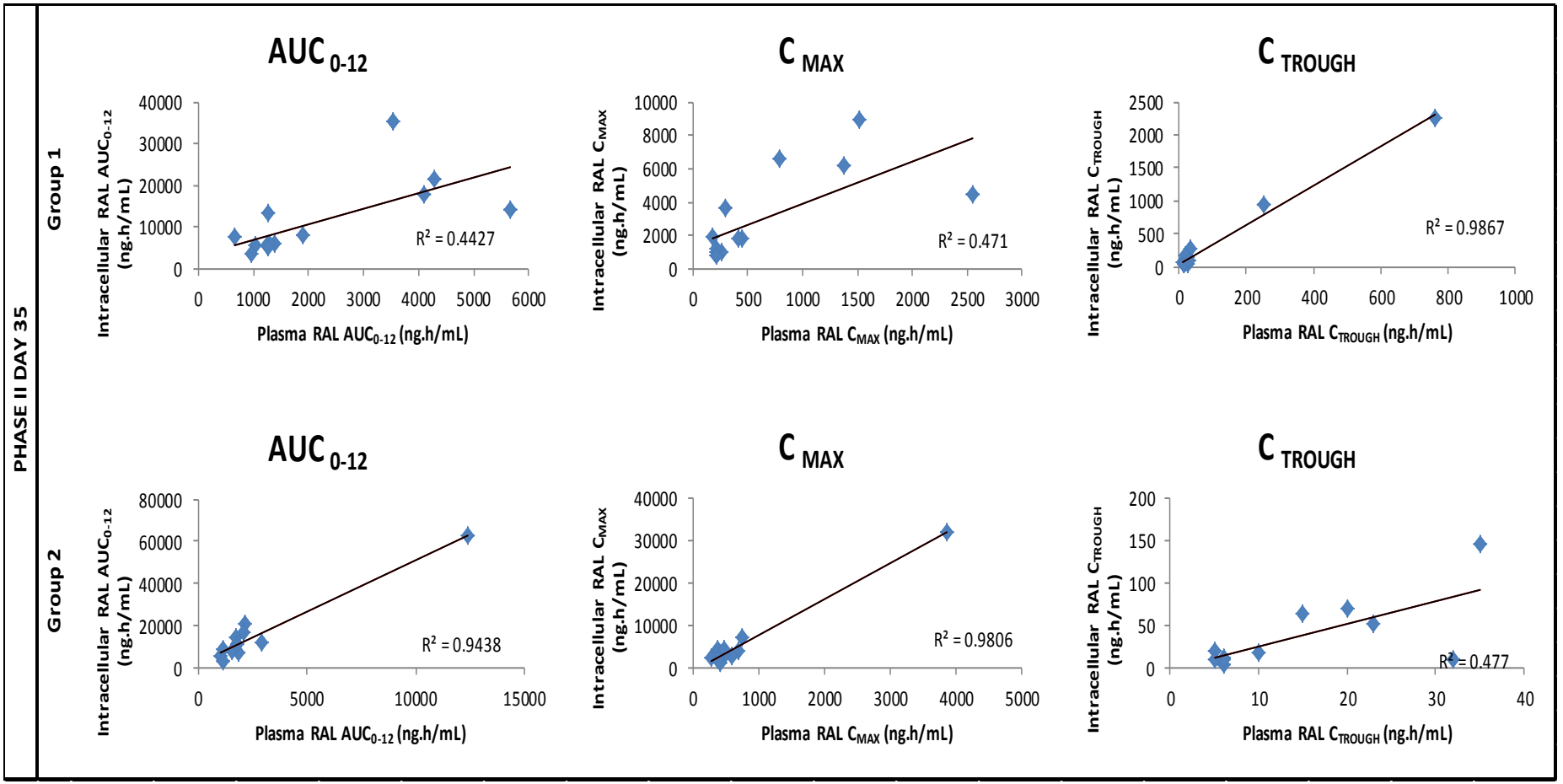
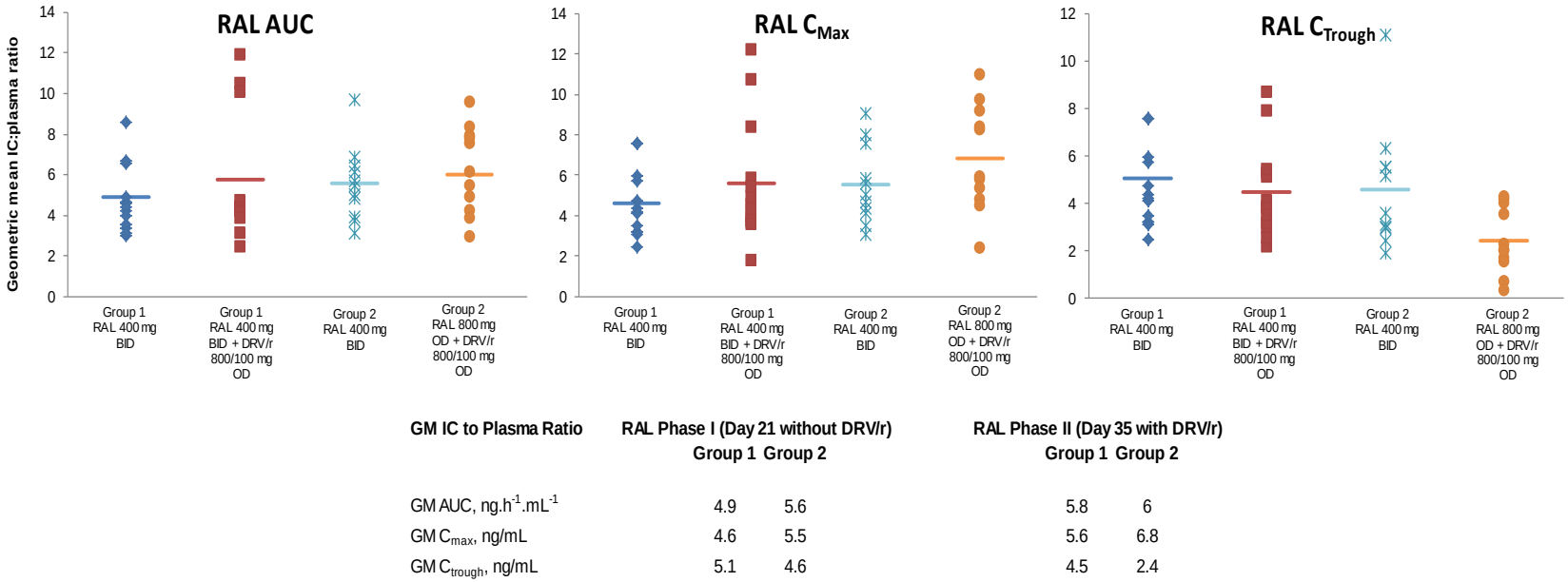


Figure 7. Correlation between IC and plasma pharmacokinetic parameters (AUC $_{0\text{-}12hr,}$ C_{max}, and C_{trough}) for Group 1 and 2, phase II (day 35) RAL 400 mg twice daily (Group 1) RAL 800 g once daily (Group 2) in the presence of DRV/r 800/100 mg once daily.

RAL comparisons of individual patient geometic mean IC to plasma ratio for AUC, C_{max} and C_{trough} are displayed side by side for Groups 1 and 2 during phase I and II (RAL without and with DRV/r).



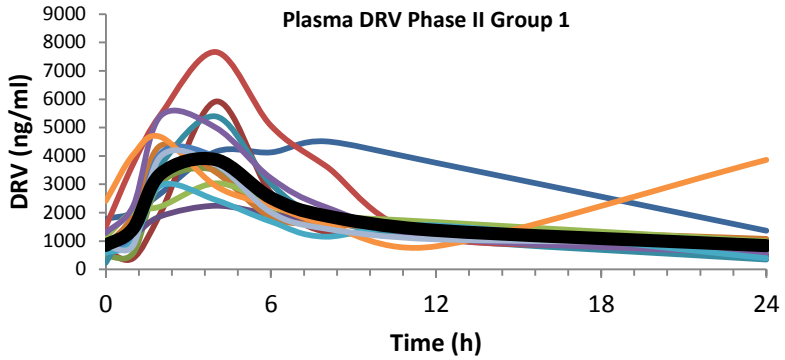
GM IC to Plasma Ratio	RAL Phase I (Day 21 without DRV/r) Group 1	Group 2	RAL Phase II (Day 35 with DRV/r) Group 1	Group 2
GM AUC, $ng.h^{-1}.mL^{-1}$	4.9	5.6	5.8	6
GM C_{max}, ng/mL	4.6	5.5	5.6	6.8
GM C_{trough}, ng/mL	5.1	4.6	4.5	2.4

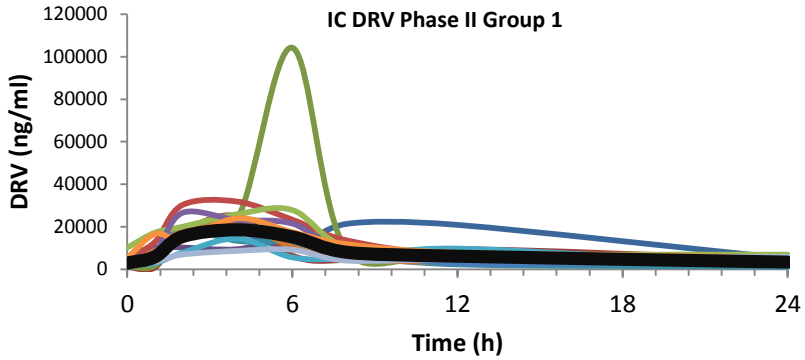
Figure 8. RAL PK parameters comparison of geometric mean IC to plasma ratios for Groups 1 and 2 during phase I and II. For each set of group data the mean ratio is highlighted by a solid horizontal bar. RAL PK parameters showed a high level of inter-individual variability in the plasma (53-220%) and also within the cells (69-202%).

3.3.3 Darunavir Pharmacokinetics and Statistical Analysis

Plasma and IC patient pharmacokinetic profiles and parameters for Group 1 and 2 in phase II; DRV/r in the presence of RAL and phase III; DRV/r in the absence of RAL are shown in Figures 9, 10, 11 and 12. The pharmacokinetic parameter ratios for plasma and IC for Group 1 and Group 2 are shown in Tables 3 and 4 respectively.

Group 1 plasma and IC DRV/r PK profiles for Phase II (day 35)

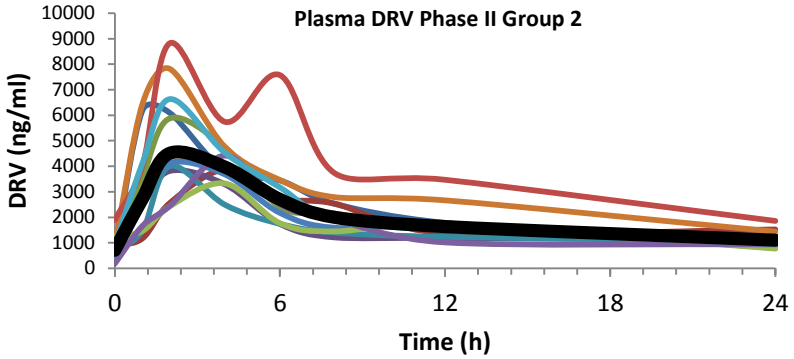


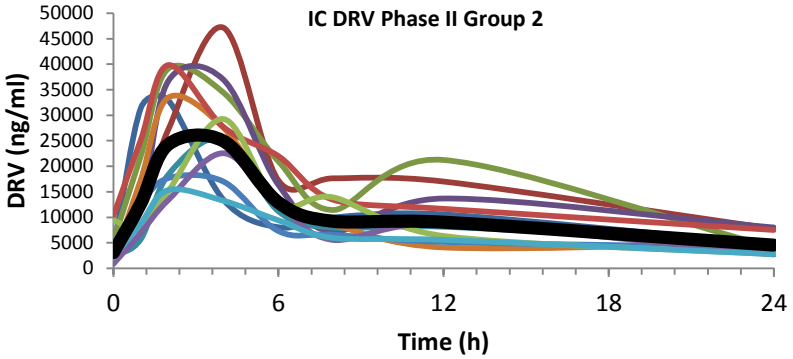


	35/36 (+ RAL)								35/36 (+) RAL						
	Group 1Plasma PK parameters DRV 800 mg OD								Group 1 IC PK parameters DRV 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)		$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	12.67	3.06	4235	697	40064	846	20	**Geomean**	13.46	4.30	20210	3431	202865	2982	4
low 90%	10.58	2.60	3788	616	36905	723	19	**low 90%**	10.17	3.83	18168	3078	181237	2550	4
up 90%	17.98	4.17	5101	877	44709	1280	22	**up 90%**	24.72	5.40	24324	4788	243109	4897	5
Min	6.89	2.00	2244	347	29083	235	13	**Min**	5.65	2.00	9259	1262	136020	837	2
Max	28.10	8.00	7665	1077	63499	2417	28	**Max**	51.23	8.00	31882	6940	348096	10226	6
CV	52	51	32	35	21	61	19	**CV**	84	37	30	47	32	69	29

Figure 9. Group 1 plasma and IC DRV/r, 800/100 mg once daily in the presence of RAL 400 mg twice daily (phase II day 35) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 2 plasma and IC DRV/r PK profiles for Phase II (day 35)

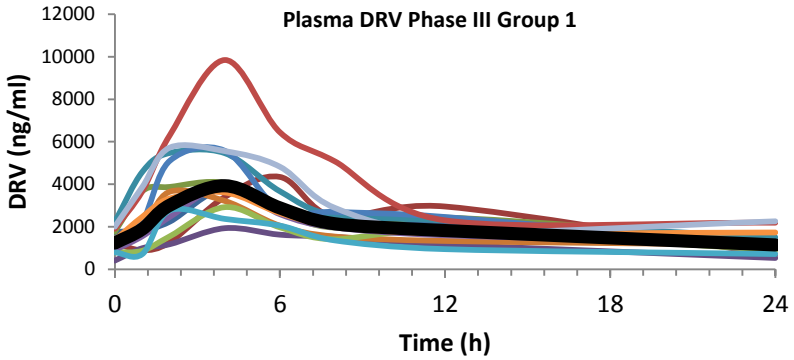


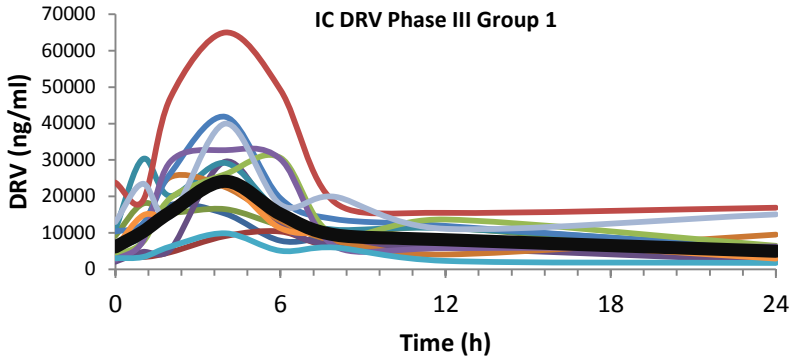


	35/36 (+ RAL)								35/36 (+) RAL						
	Group 2 Plasma PK parameters DRV 800 mg OD								Group 2 IC PK parameters DRV 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)		$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	17.45	2.27	5092	1106	50040	710	16	**Geomean**	13.49	2.7	29293	4555	263148	3078	3
low 90%	13.68	1.94	4443	979	43433	597	14	**low 90%**	11.33	2.4	25965	3914	229991	2412	3
up 90%	24.42	2.97	6266	1313	61161	1053	19	**up 90%**	18.23	3.4	35804	5901	325739	5544	4
Min	9.81	1.00	3328	775	36896	186	8	**Min**	7.49	2.00	15353	2729	157620	864	2
Max	46.95	4.00	8822	1859	94896	1896	22	**Max**	30.40	4.00	47146	8014	422728	10258	5
CV	54	42	34	29	34	56	26	**CV**	47	36	32	41	35	79	34

Figure 10. Group 2 plasma and IC DRV/r, 800/100 mg once daily in the presence of RAL 800 mg once daily (phase II day 35) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 1 plasma and IC DRV/r PK profiles for Phase III (day 49)



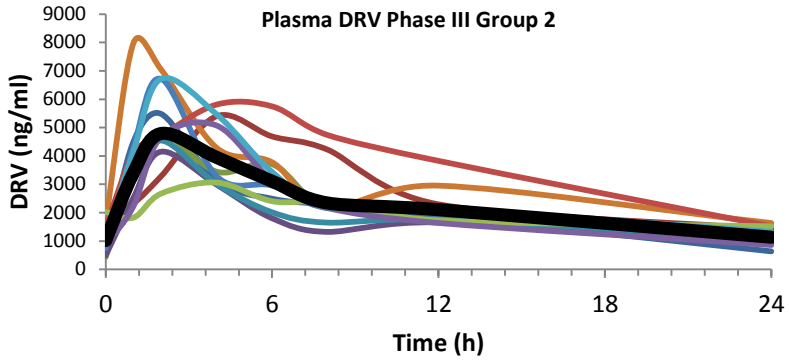


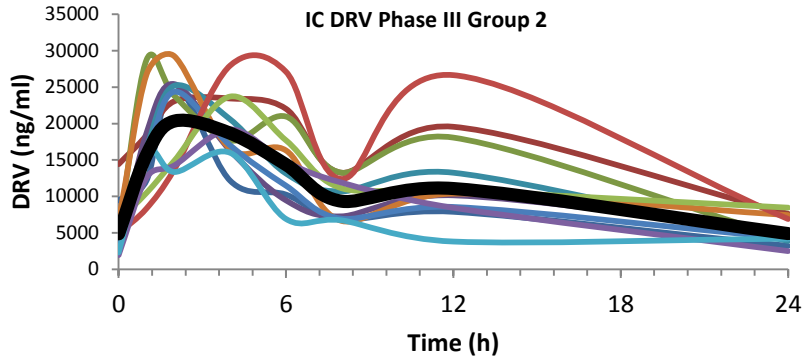
	49/50 (- RAL)						
	Group 1Plasma PK parameters DRV 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	16.84	3.33	4106	1151	49871	1239	16
low 90%	14.21	2.99	3534	1005	44379	1108	14
up 90%	23.83	4.09	5339	1505	60885	1609	20
Min	7.82	2.00	1937	537	27281	414	9
Max	40.50	6.00	9843	2261	93519	2232	29
CV	55	34	44	43	34	40	35

	49/50 (-) RAL						
	Group 1 IC PK parameters DRV 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	14.98	3.09	25433	4984	251471	6170	3
low 90%	11.24	2.80	22050	4112	217731	5065	3
up 90%	24.33	4.28	35538	8525	338135	10500	4
Min	8.95	1.00	9899	1765	91576	2110	1
Max	62.65	6.00	64976	16912	609933	23820	9
CV	80	46	51	76	47	76	53

Figure 11. Group 1 plasma and IC DRV/r, 800/100 mg once daily in the absence of RAL (phase III day 49) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 2 plasma and IC DRV/r PK profiles for Phase III (day 49)





	49/50 (- RAL)						
	Group 2 Plasma PK parameters DRV 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	14.55	2.42	5272	1135	57055	1019	14
low 90%	12.52	2.08	4755	1017	51386	874	13
up 90%	18.59	3.19	6109	1339	65626	1424	16
Min	8.92	1.00	3061	636	41255	433	9
Max	26.96	4.00	7977	1634	88921	2026	19
CV	39	42	25	27	24	48	22

	49/50 (-) RAL						
	Group 2 IC PK parameters DRV 800 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	13.12	2.3	24082	4910	270194	4878	3
low 90%	10.30	1.9	22446	4259	239620	3944	3
up 90%	19.06	3.1	26371	6254	323007	7554	4
Min	7.07	1.00	16025	2510	159065	1960	2
Max	38.85	4.00	29222	8464	433264	14425	5
CV	60	48	16	38	30	63	30

Figure 12. Group 2 plasma and IC DRV/r, 800/100 mg once daily in the absence of RAL (phase III day 49) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Table 3. Group 1 and 2 pharmacokinetic parameter ratios for plasma DRV/r 800/100 mg once daily in the presence of RAL 400 mg twice daily (Group 1) and RAL 800 mg once daily (Group 2), (phase II day 35) versus DRV 800 mg once daily in the absence of RAL (phase III day 49). Note: N= 2 AUC results 0-12 Hr only.

GROUP 1 Plasma DRV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 400 mg BID) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	Plasma half-life (h)				Plasma T_{max} (h)				Plasma C_{max} (ng/ml)				Plasma C_{trough}(ng/ml)				Plasma AUC_{0-24} (ng.h/ml)			
ID	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio
Geomean	12.7	16.8	**1.27**		3.1	3.3	**1.09**		4235.1	4106.4	**0.97**		697.0	1150.6	**1.62**		40064.3	49870.5	**1.24**	
low 90%CI	10.6	14.2	1.05	-0.03	2.6	3.0	0.95	-0.05	3787.9	3534.0	0.88	-0.06	615.8	1005.5	1.32	0.10	36905.3	44378.8	1.13	0.00
high 90%CI	18.0	23.8	1.95	0.24	4.2	4.1	1.43	0.13	5101.5	5338.6	1.11	0.03	877.1	1505.0	2.33	0.30	44708.9	60884.5	1.45	0.20
	increase by 27%				*increase by 9%*				*decrease by 3%*				*increase by 62%*				*increase by 24%*			
anti-log low 90%CI	*NON sig Difference*		0.92		*NON sig Difference*		0.88		*NON sig Difference*		0.87		*SIG Difference*		1.26		*NON sig Difference*		1.10	
anti-log high 90%CI			1.74				1.34				1.08				2.10				1.40	
min	6.9	7.8	0.31		2.0	2.0	0.50		2244.0	1937.0	0.72		347.0	537.0	0.80		29082.5	27280.5	0.90	
max	28.1	40.5	3.30		8.0	6.0	2.00		7665.0	9843.0	1.43		1077.0	2261.0	4.35		63499.0	93519.0	1.90	
cv%	52.0	55.3	60.22		50.5	33.9	43.78		32.3	44.4	24.22		35.2	43.5	55.70		20.9	34.3	27.80	
GROUP 2 Plasma DRV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 800 mg OD) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	Plasma half-life (h)				Plasma T_{max} (h)				Plasma C_{max} (ng/ml)				Plasma C_{trough}(ng/ml)				Plasma AUC_{0-24} (ng.h/ml)			
ID	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio
Geomean	17.4	14.5	**0.80**		2.3	2.4	**1.07**		5092.2	5271.6	**1.04**		1106.0	1134.7	**1.03**		50040.2	57054.9	**1.14**	
low 90%CI	13.7	12.5	0.67	-0.18	1.9	2.1	0.91	-0.05	4443.1	4755.3	0.93	0.00	979.2	1016.9	0.89	0.00	43432.5	51386.3	1.07	0.00
high 90%CI	24.4	18.6	1.06	0.00	3.0	3.2	1.36	0.11	6265.6	6108.7	1.20	0.07	1313.4	1339.2	1.24	0.07	61160.7	65625.9	1.24	0.09
	decrease by 20%				*increase by 7%*				*increase by 4%*				*increase by 3%*				*increase by 14%*			
anti-log low 90%CI	*SIG Difference*		0.65		*NON sig Difference*		0.88		*NON sig Difference*		0.92		*NON sig Difference*		0.90		*SIG Difference*		1.06	
anti-log high 90%CI			0.99				1.28				1.17				1.17				1.23	
min	9.8	8.9	0.47		1.0	1.0	0.50		3328.0	3061.0	0.66		775.0	636.0	0.80		36895.5	41255.0	0.88	
max	47.0	27.0	1.71		4.0	4.0	2.00		8822.0	7977.0	1.63		1859.0	1634.0	1.96		94896.0	88921.0	1.46	
cv%	54.0	39.2	42.87		42.2	42.5	39.80		34.2	25.0	25.20		29.3	27.5	32.19		34.1	24.5	15.20	

Table 4. Group 1 and 2 pharmacokinetic parameter ratios for IC DRV/r 800/100 mg once daily in the presence of RAL 400 mg twice daily (group 1) and RAL 800 mg once daily (group 2), (phase II day 35) versus DRV 800 mg once daily in the absence of RAL (phase III day 49).

GROUP 1 IC DRV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 400 mg BID) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	IC half-life (h)				**IC T_{max} (h)**				**IC C_{max} (ng/ml)**				**IC C_{trough}(ng/ml)**				**IC AUC_{0-24} (ng.h/ml)**			
	+ RAL	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**
Geomean	13.5	15.0	**1.02**		4.3	3.1	**0.72**		20209.5	25433.3	**1.11**		3431.2	4984.4	**1.45**		202865.4	251471.3	**1.24**	
low 90%CI	10.2	11.2	0.85	-0.12	3.8	2.8	0.64	-0.28	18167.7	22050.2	0.95	-0.12	3078.4	4112.4	1.22	0.02	181237.1	217731.5	1.07	-0.01
high 90%CI	24.7	24.3	1.55	0.14	5.4	4.3	1.09	-0.01	24323.8	35538.0	1.94	0.21	4788.3	8525.5	2.41	0.30	243108.7	338134.8	1.73	0.20
	increase by 2%				*decrease by 28%*				*increase by 11%*				*increase by 45%*				*increase by 24%*			
anti-log low 90%CI	*NON Sig Difference*			0.76	*SIG Difference*			0.53	*NON sig Difference*			0.76	*SIG Difference*			1.06	*NON sig Difference*			0.98
anti-log high 90%CI				1.38				0.99				1.61				2.00				1.57
min	5.7	9.0	0.35		2.0	1.0	0.17		9259.0	9899.0	0.17		1262.0	1765.0	0.53		136019.5	91576.0	0.55	
max	51.2	62.6	2.57		8.0	6.0	2.00		31882.0	64976.0	4.32		6940.0	16912.0	4.63		348096.0	609933.0	2.95	
cv%	83.8	80.4	58.79		37.0	45.6	57.72		30.0	51.2	74.27		47.5	76.3	71.43		32.0	47.3	51.72	
GROUP 2 IC DRV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 800 mg OD) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	IC half-life (h)				**IC T_{max} (h)**				**IC C_{max} (ng/ml)**				**IC C_{trough}(ng/ml)**				**IC AUC_{0-24} (ng.h/ml)**			
	+RAL	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**
Geomean	13.5	13.1	**0.97**		2.7	2.3	**0.83**		29293.0	24082.1	**0.82**		4555.1	4909.9	**1.08**		263148.1	270193.5	**1.03**	
low 90%CI	11.3	10.3	0.63	-0.16	2.4	1.9	0.69	-0.18	25964.6	22445.7	0.73	-0.14	3914.1	4258.7	0.86	-0.06	229990.8	239620.4	0.94	-0.04
high 90%CI	18.2	19.1	1.95	0.14	3.4	3.1	1.13	0.01	35803.6	26371.0	0.96	-0.03	5901.2	6253.8	1.53	0.13	325739.3	323007.4	1.16	0.06
	decrease by 3%				*decrease by 17%*				*decrease by 18%*				*increase by 8%*				*increase by 3%*			
anti-log low 90%CI	*NON Sig Difference*			0.69	*NON Sig Difference*			0.66	*SIG Difference*			0.72	*NON sig Difference*			0.87	*NON sig Difference*			0.92
anti-log high 90%CI				1.37				1.03				0.94				1.34				1.15
min	7.5	7.1	0.52		2.0	1.0	0.50		15353.0	16025.0	0.50		2729.0	2510.0	0.64		157620.0	159065.0	0.69	
max	30.4	38.8	5.06		4.0	4.0	2.00		47146.0	29222.0	1.38		8014.0	8464.0	3.06		422727.5	433263.5	1.29	
cv%	47.0	60.0	103.49		35.9	47.7	48.06		32.0	16.2	27.14		40.7	38.1	57.01		34.6	29.8	20.82	

Correlation between IC and plasma PK parameters for DRV in the presence (Phase II day 35) and absence of RAL (Phase III day 49), for Group 1 and Group 2 are shown in Figures 13and 14.

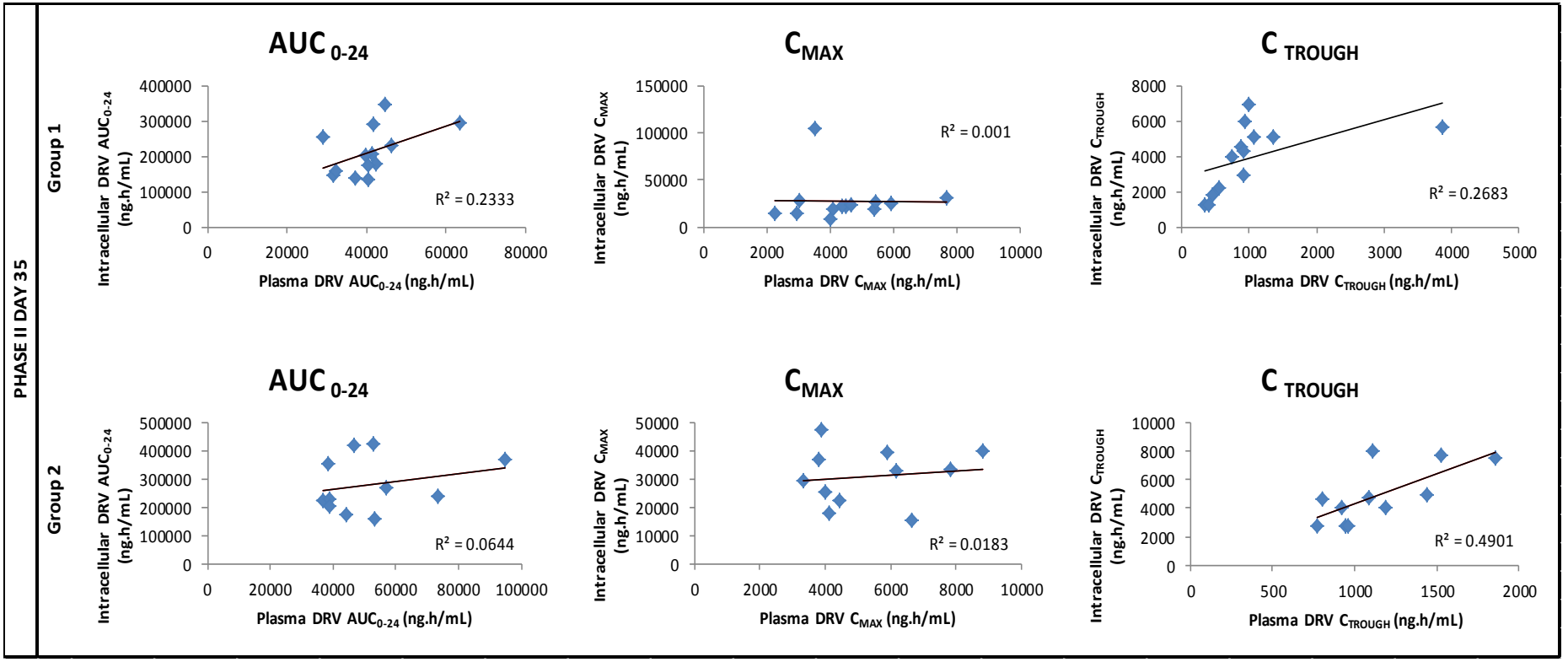


Figure 13. Correlation between IC and plasma pharmacokinetic parameters (AUC $_{0\text{-}12hr,}$ C_{max}, and C_{trough}) for Group 1 and 2, phase II (day 35) DRV/r 800/100 mg once daily in the presence of RAL 400 mg twice daily(Group 1) and RAL 800 mg once daily (Group 2).

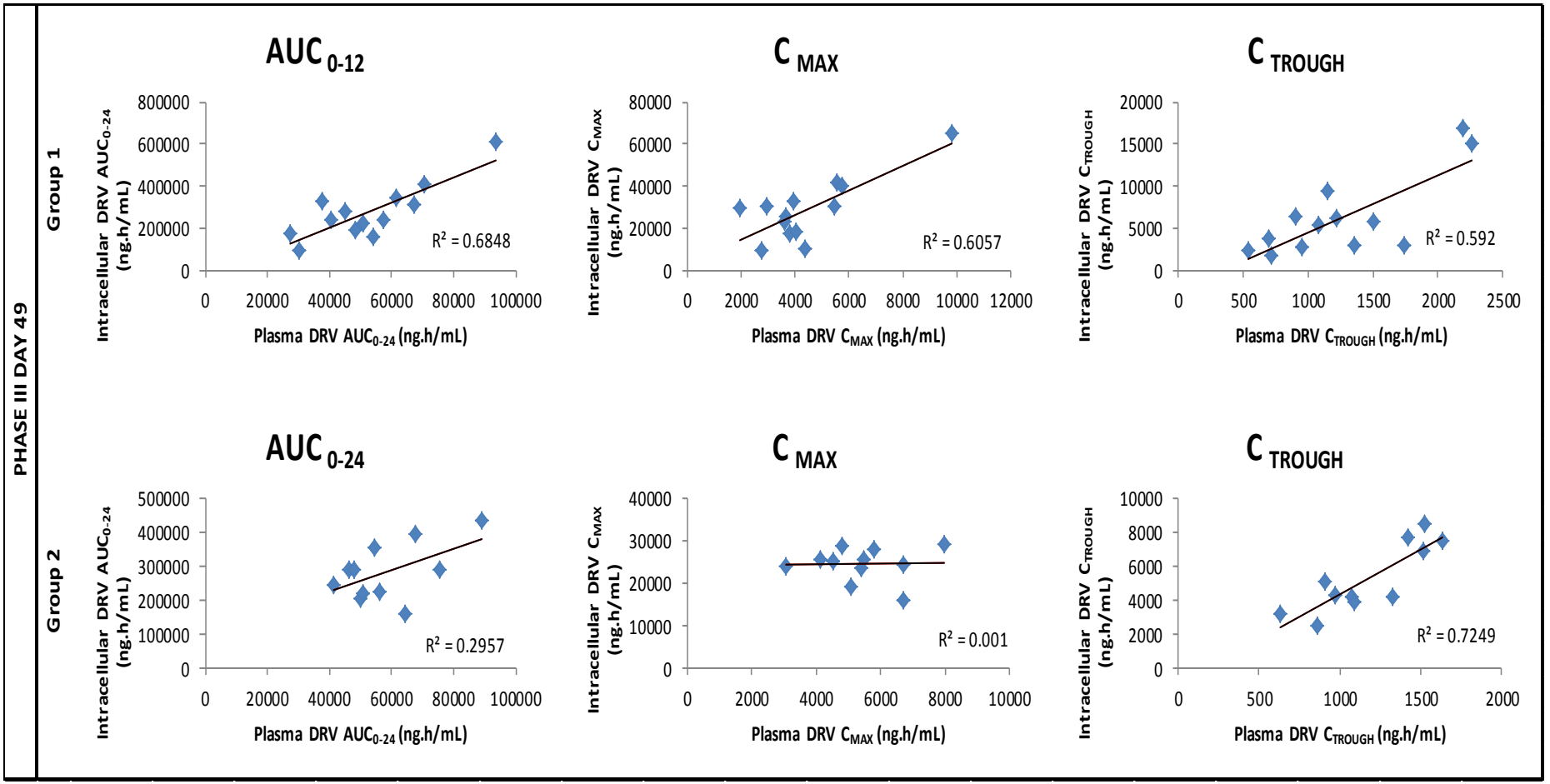
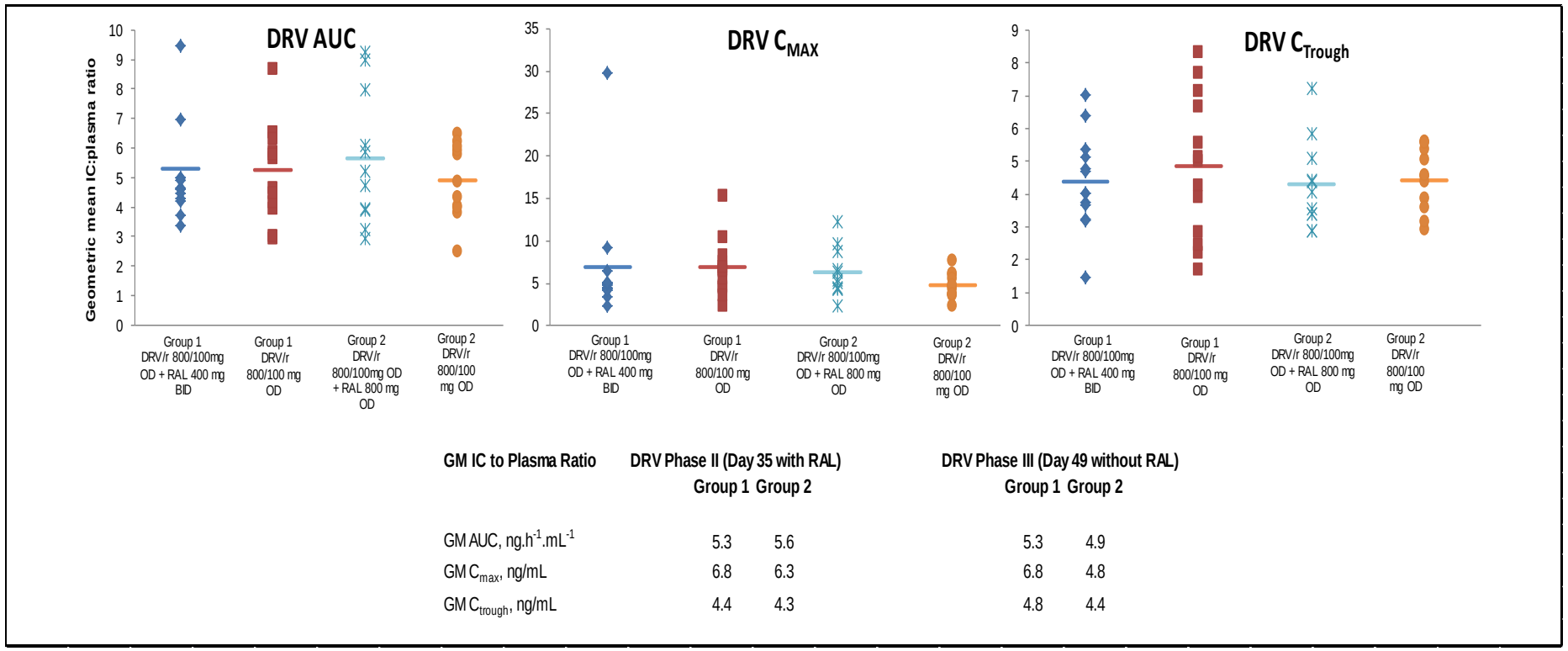


Figure 14. Correlation between IC and plasma pharmacokinetic parameters (AUC $_{0\text{-}12hr,}$ C_{max}, and C_{trough}) for Group 1 and 2, phase III (day 49) DRV/r 800/100 mg once daily in the absence of RAL.

DRV/r comparisons of individual patient geometic mean IC to plasma ratio for AUC, C_{max} and C_{trough} are displayed side by side for Groups 1 and 2 during phase II and III (DRV/r with and without RAL).



GM IC to Plasma Ratio	DRV Phase II (Day 35 with RAL)		DRV Phase III (Day 49 without RAL)	
	Group 1	Group 2	Group 1	Group 2
GM AUC, $ng.h^{-1}.mL^{-1}$	5.3	5.6	5.3	4.9
GM C_{max}, ng/mL	6.8	6.3	6.8	4.8
GM C_{trough}, ng/mL	4.4	4.3	4.8	4.4

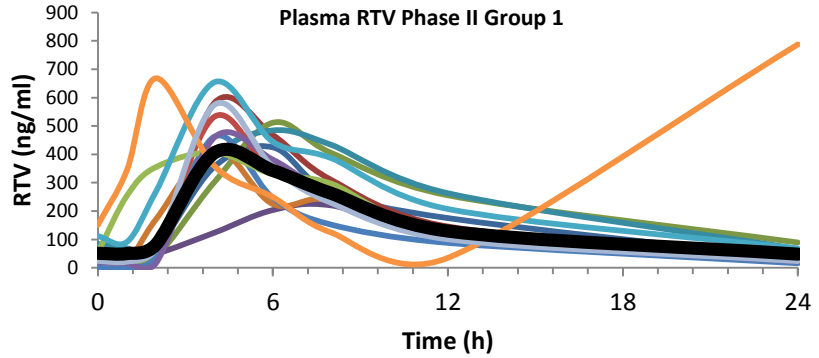
Figure 15. DRV PK parameters comparison of geometric mean IC to plasma ratios for Groups 1 and 2 during phase II and III. For each set of group data the mean ratio is highlighted by a solid horizontal bar.

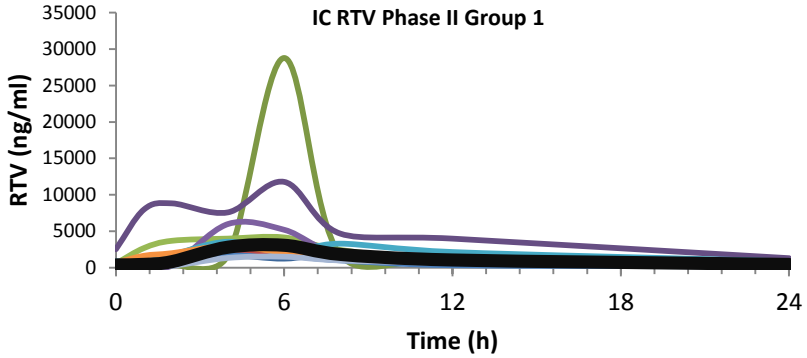
DRV PK parameters showed a high level of inter-individual variability in the plasma (32-87%) and also within the cells (32-86%). During phase III (PK day 49) in the absence of RAL, the plasma AUC was marginally increased in both Group 1 and 2 (24 % and 14 % respectively). Group 1 also showed a 37 % increase in the plasma C_{trough} concentration in the absence of RAL whereas no change was seen in Group 2. An increase of 45 % in C_{trough} was observed in the IC concentrations of DRV for Group 1, in the absence and presence of RAL and for Group 2 a decrease of 18 % in C_{max} was shown (Table 4).

3.3.4 Ritonavir Pharmacokinetics and Statistical Analysis

Plasma and IC patient pharmacokinetic profiles and parameters for Group 1 and 2 in phase II; RTV in the presence of DRV (800 mg) and RAL (800 mg) and phase III; RTV in the presence of DRV (800 mg) and the absence of RAL are shown in Figures 16, 17, 18 and 19 respectively. The pharmacokinetic parameter ratios for plasma and IC for Group 1 and Group 2 are shown in Tables 5 and 6 respectively.

Group 1 plasma and IC RTV PK profiles for Phase II (day 35)



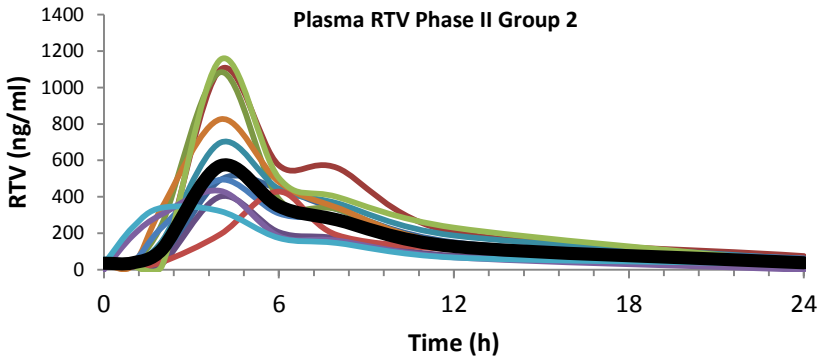


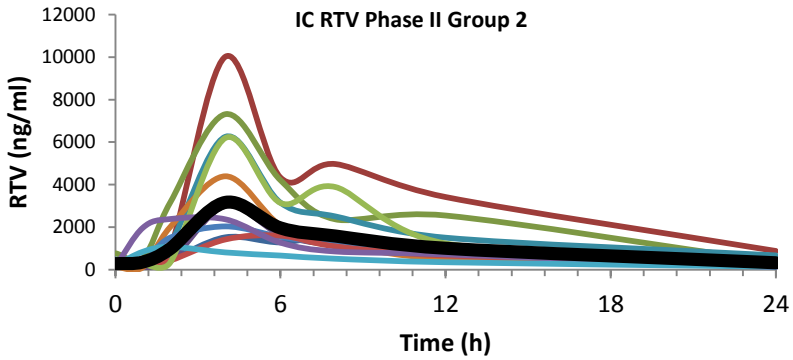
	35/36 (+RAL)						
	Group 1 Plasma PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	5.48	5.32	483	48	4299	45	23
low 90%	5.11	3.81	439	5	3823	39	21
up 90%	5.97	8.81	564	195	5176	76	28
Min	4.44	4.00	220	16	2747	10	12
Max	7.44	24.00	788	788	8017	151	36
CV	16	87	27	207	33	71	29

	35/36 (+RAL)						
	Group 1 IC PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	7.9	4.9	3883	458	33789	394	3
low 90%	7.2	4.5	2392	388	27362	282	3
up 90%	8.8	5.7	9163	676	53255	868	4
Min	5.9	4.0	1518	174	14788	97	1
Max	11.8	8.0	28767	1312	115160	2539	7
CV	20.3	26.0	128	59	70	111	52

Figure 16. Group 1 plasma and IC RTV 100 mg once daily in the presence of DRV 800 mg once daily plus RAL 400 mg twice daily (phase II day 35) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 2 plasma and IC RTV PK profiles for Phase II (day 35)



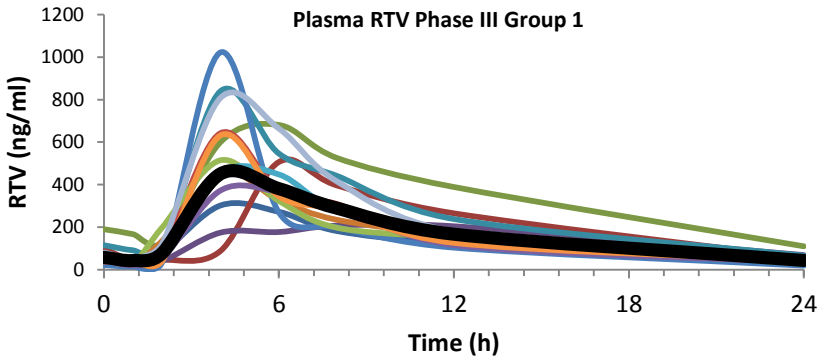


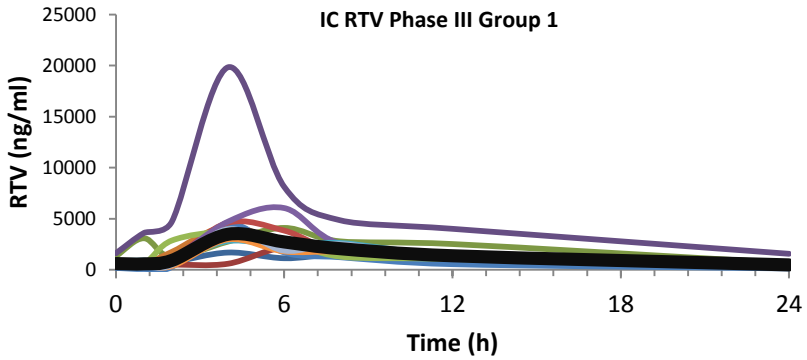
	35/36 (+RAL)						
	Group 2 Plasma PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	5.55	3.90	617	34	4359	32	23
low 90%	5.09	3.56	522	29	3816	28	20
up 90%	6.21	4.44	832	49	5443	42	29
Max	4.26	2.00	341	11	2570	10	14
Min	7.77	6.00	1153	75	7255	57	39
CV	20	22	46	51	35	42	37

	35/36 (+RAL)						
	Group 2 IC PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	7.15	3.9	3289	329	28683	286	3
low 90%	6.45	3.4	2719	269	23318	236	3
up 90%	8.22	5.0	5615	489	43702	430	5
Min	4.99	2.00	1079	123	10786	139	1
Max	9.53	8.00	10018	885	78422	776	9
CV	23	40	70	58	61	59	56

Figure 17. Group 2 plasma and IC RTV 100 mg once daily in the presence of DRV 800 mg once daily plus RAL 800 mg once daily (phase II day 35) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 1 plasma and IC RTV PK profiles for Phase III (day 49)



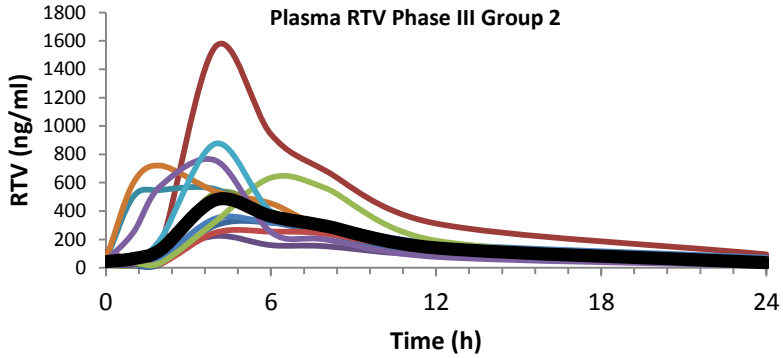


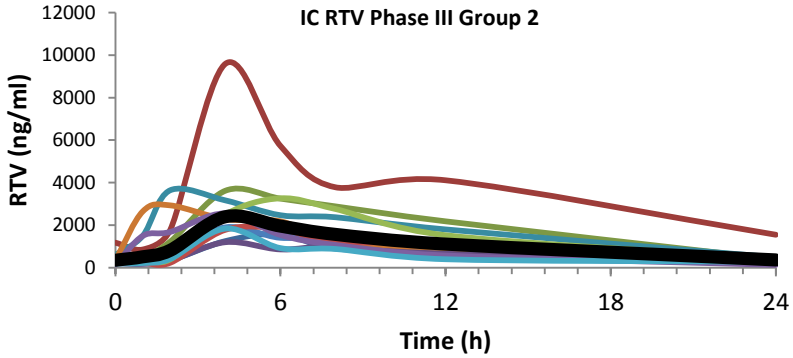
	49/50 (-RAL) Group 1 Plasma PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	5.77	4.49	528	44	4568	59	22
low 90%	5.40	4.04	468	38	4070	48	20
up 90%	6.26	5.19	678	60	5427	89	25
Min	4.82	4.00	208	20	3087	22	12
Max	7.72	8.00	1022	110	8367	191	32
CV	16	27	40	49	31	66	25

	49/50 (-RAL) Group 1 IC PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	7.77	4.39	4041	478	36769	607	3
low 90%	7.17	4.06	2858	399	29157	518	2
up 90%	8.70	4.86	7075	718	53037	879	3
Min	4.89	4.00	1688	129	21776	180	1
Max	10.75	6.00	19836	1565	123457	1614	5
CV	21	20	93	62	63	57	34

Figure 18. Group 1 plasma and IC RTV 100 mg once daily in the presence of DRV 800 mg once daily and the absence of RAL (phase III day 49) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Group 2 plasma and IC RTV PK profiles for Phase III (day 49)





	49/50 (-RAL) Group 2 Plasma PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	5.48	4.19	527	36	4516	43	22
low 90%	4.99	3.76	427	30	3798	36	19
up 90%	6.18	4.96	806	54	5986	63	29
Min	4.17	2.00	225	13	2275	21	10
Max	8.12	6.00	1563	94	10475	114	44
CV	21	28	62	57	45	55	40

	49/50 (-RAL) Group 2 IC PK parameters RTV 100 mg OD						
	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)	C_{trough} (ng/ml)	AUC_{0-24} (ng.h/ml)	Pre-dose (ng/ml)	CL/F (L/h)
Geomean	7.81	3.9	2607	369	27652	354	4
low 90%	6.99	3.5	1936	263	21621	276	3
up 90%	9.18	4.9	4240	645	42997	572	5
Min	5.46	2.00	1203	136	14019	145	1
Max	11.41	6.00	9589	1552	88347	1181	7
CV	27	34	75	85	66	70	48

Figure 19. Group 2 plasma and IC RTV 100 mg once daily in the presence of DRV 800 mg once daily and the absence of RAL (phase III day 49) PK patient profiles. The mean concentrations are highlighted by the black line. The geometric mean, lower and upper 90% confidence intervals, minimum and maximum concentrations and the coefficient of variation for each of the PK parameters are shown below each PK profile set.

Table 5. Group 1 and 2 pharmacokinetic parameter ratios for plasma RTV 100 mg once daily plus DRV 800 mg once daily in the presence of RAL 400 mg twice daily (group 1) and RAL 800 mg once daily (group 2), (phase II day 35) versus RTV 100 mg and DRV 800 mg once daily in the absence of RAL (phase III day 49). Note: N= 2 AUC results 0-12 Hr only.

GROUP 1 Plasma RTV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 400 mg BID) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	Plasma half-life (h)				Plasma T_{max} (h)				Plasma C_{max} (ng/ml)				Plasma C_{trough}(ng/ml)				Plasma AUC_{0-24} (ng.h/ml)			
ID	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio
Geomean	5.5	5.8	**0.95**		5.3	4.5	**1.18**		482.8	528.0	**0.91**		48.2	44.0	**1.09**		4299.1	4568.3	**0.94**	
low 90%CI	5.1	5.4	0.90	0.00	3.8	4.0	0.80	0.00	439.4	468.4	0.82	-0.10	5.3	37.7	-0.03	-0.10	3823.1	4069.6	0.83	-0.10
high 90%CI	6.0	6.3	1.00	0.00	8.8	5.2	2.10	0.20	564.1	677.8	1.11	0.00	195.4	59.6	4.63	0.20	5175.6	5426.7	1.16	0.00
	decrease by 5%				*increase by 18%*				*decrease by 9%*				*increase by 9%*				*decrease by 6%*			
anti-log low 90%CI	*SIG Difference*			0.89	*SIG Difference*			0.93	*SIG Difference*			0.78	*SIG Difference*			0.73	*SIG Difference*			0.81
anti-log high 90%CI				1.01				1.51				1.07				1.65				1.09
min	4.4	4.8	0.70		4.0	4.0	0.70		220.0	208.0	0.50		16.0	20.0	0.50		2747.0	3087.0	0.60	
max	7.4	7.7	1.10		24.0	8.0	6.00		788.0	1022.0	1.40		788.0	110.0	19.20		8016.5	8366.5	1.90	
cv%	15.7	15.5	11.90		86.7	27.3	96.70		27.2	39.9	32.50		207.1	49.3	221.40		32.8	31.2	36.30	
GROUP 2 Plasma RTV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 800 mg OD) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	Plasma half-life (h)				Plasma T_{max} (h)				Plasma C_{max} (ng/ml)				Plasma C_{trough}(ng/ml)				Plasma AUC_{0-24} (ng.h/ml)			
ID	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio	+ RAL	- RAL	ratio	log ratio
Geomean	5.6	5.5	**1.01**		3.9	4.2	**0.93**		616.6	527.4	**1.17**		34.4	36.3	**0.95**		4359.0	4516.1	**0.97**	
low 90%CI	5.1	5.0	1.00	0.00	3.6	3.8	0.80	-0.10	534.8	441.8	1.05	0.00	30.0	31.0	0.86	-0.10	3881.2	3885.2	0.88	-0.10
high 90%CI	6.2	6.1	1.10	0.00	4.4	4.9	1.20	0.00	819.9	790.3	1.56	0.20	48.2	52.8	1.09	0.00	5377.4	5898.8	1.13	0.00
	increase by 1%				*decrease by 7%*				*increase by 17%*				*decrease by 5%*				*decrease by 3%*			
anti-log low 90%CI	*SIG Difference*			0.95	*SIG Difference*			0.79	*SIG Difference*			0.91	*SIG Difference*			0.85	*SIG Difference*			0.84
anti-log high 90%CI				1.08				1.09				1.50				1.06				1.11
min	4.3	4.2	0.80		2.0	2.0	0.50		341.0	225.0	0.40		11.0	13.0	0.60		2569.5	2274.5	0.60	
max	7.8	8.1	1.30		6.0	6.0	2.00		1153.0	1563.0	2.10		75.0	94.0	1.40		7255.0	10475.0	1.40	
cv%	20.0	21.4	13.70		22.4	27.6	38.90		46.0	61.8	42.20		50.7	57.0	25.10		35.3	45.0	28.00	

Table 6. Group 1 and 2 pharmacokinetic parameter ratios for IC RTV 100 mg once daily plus DRV 800 mg once daily in the presence of RAL 400 mg twice daily (group 1) and RAL 800 mg once daily (group 2), (phase II day 35) versus RTV 100 mg and DRV 800 mg once daily in the absence of RAL (phase III day 49).

GROUP 1 IC RTV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 400 mg BID) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	IC half-life (h)				**IC T_{max} (h)**				**IC C_{max} (ng/ml)**				**IC C_{trough}(ng/ml)**				**IC AUC_{0-24} (ng.h/ml)**			
	+ RAL	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**	**+ RAL**	**- RAL**	**ratio**	**log ratio**
Geomean	7.9	7.8	**1.02**		4.9	4.4	**1.12**		3883.4	4041.2	**0.96**		457.6	478.3	**0.96**		33788.9	36768.9	**0.92**	
low 90%CI	7.2	7.2	0.90	0.00	4.5	4.1	1.00	0.00	2391.7	2858.5	0.56	-0.20	387.8	399.0	0.82	-0.10	27362.2	29157.3	0.82	-0.10
high 90%CI	8.8	8.7	1.20	0.10	5.7	4.9	1.40	0.10	9163.3	7074.9	2.15	0.10	675.8	718.4	1.32	0.10	53254.8	53037.2	1.11	0.00
	increase by 2%				*increase by 12%*				*decrease by 4%*				*decrease by 4%*				*decrease by 8%*			
anti-log low 90%CI	*SIG Difference*			0.90	*SIG Difference*			0.97	*SIG Difference*			0.69	*SIG Difference*			0.77	*SIG Difference*			0.78
anti-log high 90%CI				1.15				1.30				1.34				1.19				1.08
min	5.9	4.9	0.70		4.0	4.0	0.70		1518.0	1688.0	0.40		174.0	129.0	0.50		14787.5	21775.5	0.50	
max	11.8	10.7	1.90		8.0	6.0	2.00		28767.0	19836.0	7.00		1312.0	1565.0	2.20		115160.0	123456.5	1.50	
cv%	20.3	21.0	28.90		26.0	19.7	32.90		128.1	92.8	127.80		59.2	62.5	51.00		70.2	63.5	32.60	
GROUP 2 IC RTV: DAY 35/36 (DRV/r 800/100 mg OD PLUS RAL 800 mg OD) vs. DAY 49/50 (DRV/r 800/100 mg OD)																				
	IC half-life (h)				**IC T_{max} (h)**				**IC C_{max} (ng/ml)**				**IC C_{trough}(ng/ml)**				**IC AUC_{0-24} (ng.h/ml)**			
	+RAL	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**	**+RAL**	**-RAL**	**ratio**	**log ratio**
Geomean	7.8	7.2	**0.91**		3.9	3.9	**0.99**		2606.7	3288.9	**1.26**		368.7	328.6	**0.89**		27652.0	28682.7	**1.04**	
low 90%CI	7.0	6.5	0.80	-0.10	3.5	3.4	0.80	-0.10	1936.5	2718.9	1.08	0.00	263.0	269.5	0.78	-0.10	21621.4	23317.7	0.94	0.00
high 90%CI	9.2	8.2	1.00	0.00	4.9	5.0	1.30	0.10	4239.5	5614.9	1.67	0.20	645.3	488.7	1.10	0.00	42997.0	43701.8	1.18	0.10
	decrease by 9%				*decrease by 1%*				*increase by 26%*				*decrease by 11%*				*increase by 4%*			
anti-log low 90%CI	*NON Sig Difference*			0.80	*NON Sig Difference*			0.78	*NON Sig Difference*			1.01	*NON sig Difference*			0.75	*NON sig Difference*			0.93
anti-log high 90%CI				1.03				1.25				1.57				1.06				1.16
min	5.5	5.0	0.60		2.0	2.0	0.50		1203.0	1079.0	0.60		136.0	123.0	0.50		14018.5	10786.0	0.80	
max	11.4	9.5	1.30		6.0	8.0	2.00		9589.0	10018.0	2.50		1552.0	885.0	1.60		88347.0	78422.0	1.50	
cv%	27.3	23.1	23.90		33.5	39.8	47.00		75.0	69.9	43.10		84.6	58.1	35.60		66.5	61.1	22.10	

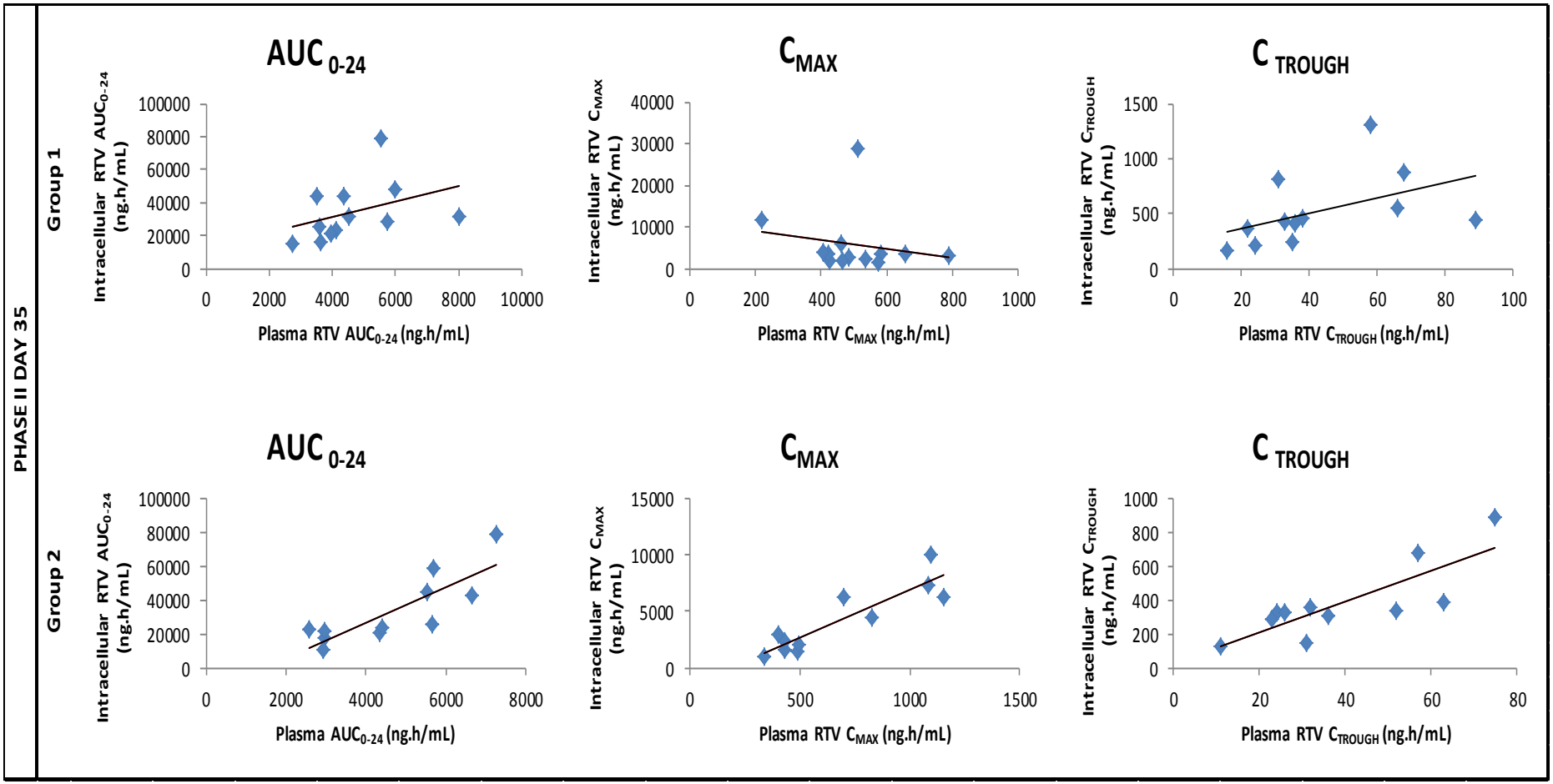


Figure 20. Correlation between IC and plasma pharmacokinetic parameters (AUC $_{0\text{-}12hr,}$ C_{max}, and C_{trough}) for Group 1 and 2, phase II (day 35) RTV 100 mg plus DRV 800 once daily in the presence of RAL 400 mg twice daily(Group 1) and RAL 800 mg once daily (Group 2).

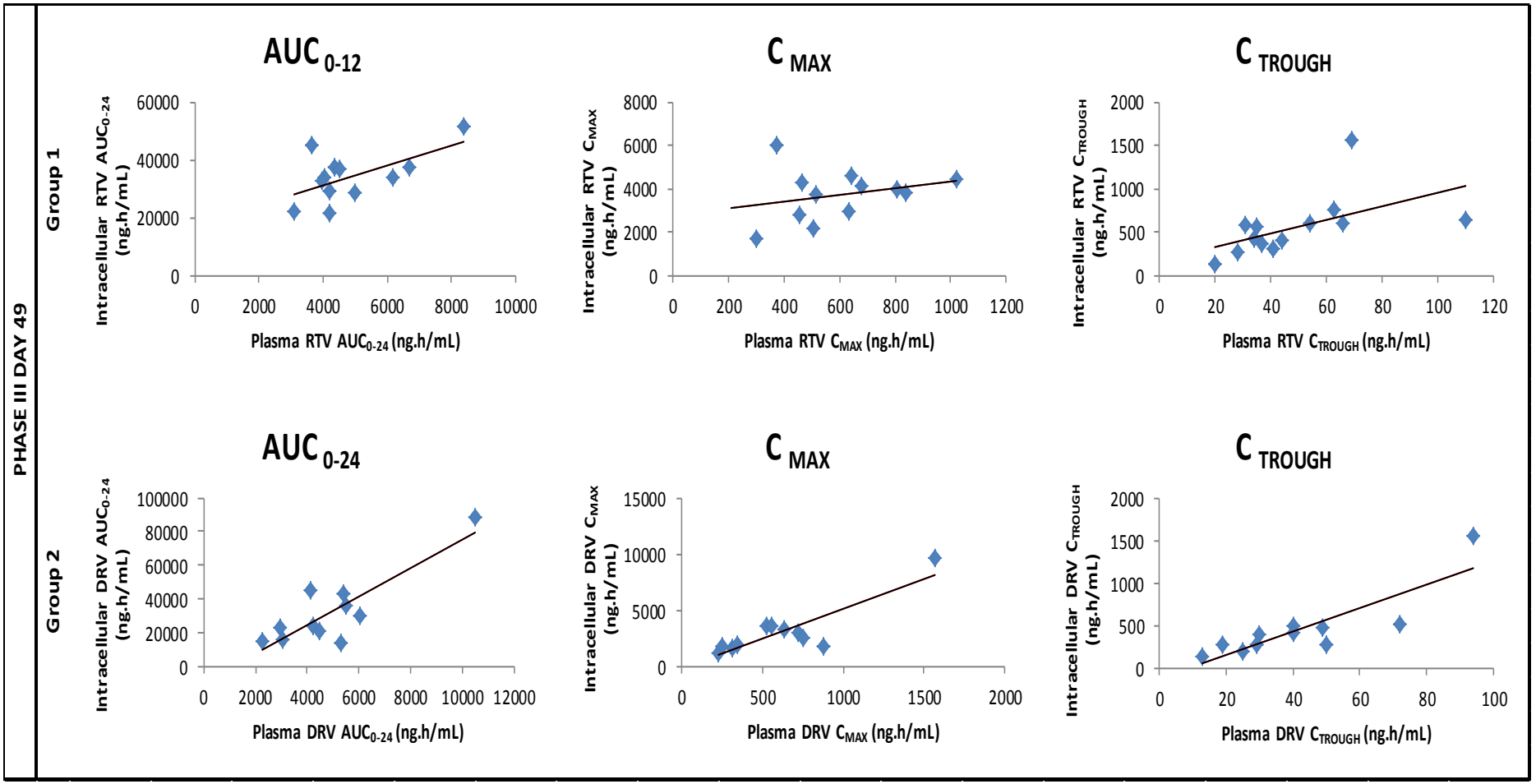
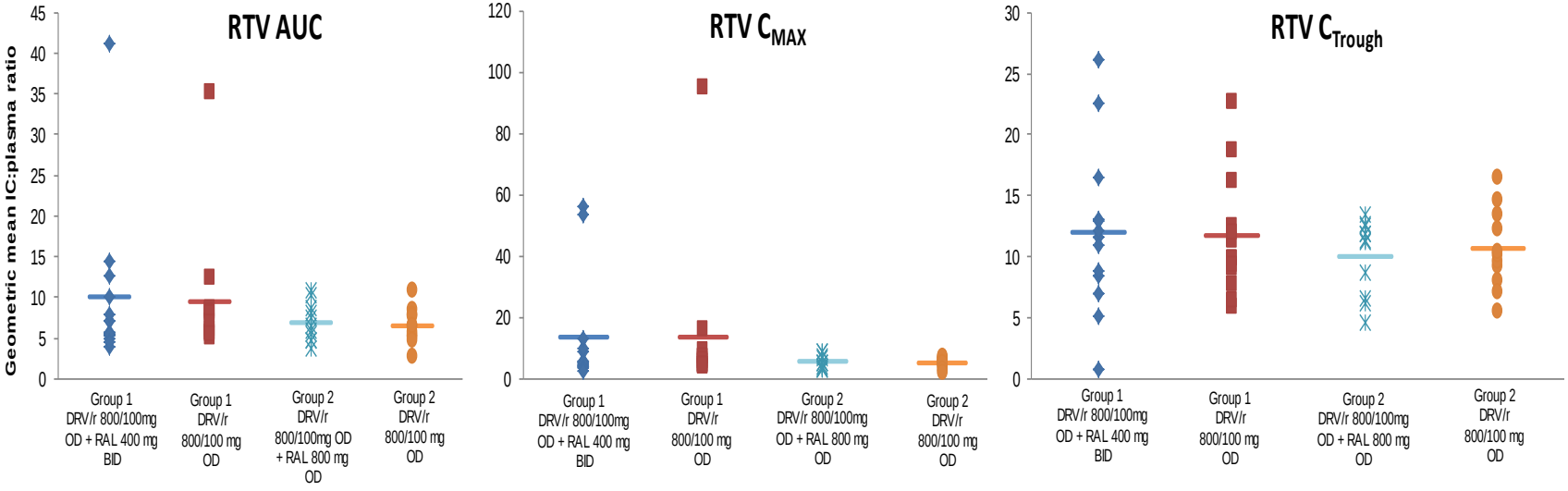


Figure 21. Correlation between IC and plasma pharmacokinetic parameters (AUC $_{0\text{-}12hr,}$ C_{max}, and C_{trough}) for Group 1 and 2, phase III (day 49) RTV 100 mg plus DRV 800 once daily in the absence of RAL.



GM IC to Plasma Ratio	RTV Phase II (Day 35 with RAL)		RTV Phase III (Day 49 without RAL)	
	Group 1	Group 2	Group 1	Group 2
GM AUC, $ng.h^{-1}.mL^{-1}$	10	6.9	9.5	6.5
GM C_{max}, ng/mL	13.8	5.7	13.5	5.2
GM C_{trough}, ng/mL	12	10.1	11.7	10.6

Figure 22. RTV PK parameters comparison of geometric mean IC to plasma ratios for Groups 1 and 2 during phase II and III. For each set of group data the mean ratio is highlighted by a solid horizontal bar. The plasma and IC RTV PK parameters were equivalent in both phases I and II when administered with and without RAL.

3.4. Discussion and Conclusion

This clinical trial reports the pharmacokinetics of the co-administration of the ARV drugs DRV/r and RAL during steady-state within plasma and the IC compartment of HIV infected subjects. DRV/r was studied at the recommended dose of 800/100 mg once daily in the presence and absence of RAL, whereas RAL was examined at two different doses; the currently recommended dose of 400 mg twice daily, and also 800 mg once daily. Both doses were studied in the presence and absence of DRV/r. During the study all patients were monitored and all drugs were well tolerated. No major adverse effects were reported.

Data investigating any drug interaction with the use of DRV/r co-administered with RAL once daily and RAL IC concentrations do not exist, therefore this study was the first to investigate the two ARVs from different classes in plasma and within the intracellular compartment.

The DRV AUC concentrations in the absence of RAL for both group 1 and 2 was higher than the concentrations seen in the presence of RAL (geometric means; group 1 minus RAL = 49871 ng.h/mL, plus RAL = 40064 ng.h/mL, and group 2 minus RAL = 57055 ng.h/mL, plus RAL 50040 ng.h/mL). In light of the intracellular AUC concentrations, we seen again no significant changes, therefore, we do not believe that the DRV plasma decrease in the presence of RAL to be of any clinical significance. The group 1 plasma C_{trough} concentration in the absence of RAL (1151 ng/mL) was 62% higher than that seen in the presence of RAL (697 ng/mL) but in group 2 there was only an increase of 3% in the absence of RAL, therefore although it may appear statistically significant we do not believe that this to have any clinical significance on the efficacy of DRV, but instead a result of the high level of inter

individual patient variability in the plasma and IC concentrations found. The confirmation of a drug-drug interaction, leading to a reduced DRV concentration when co-administrated with RTV and RAL, remains uncertain. Given that DRV and RAL use two different metabolic pathways the potential for a drug-drug interaction was characterised as low. Further studies may need to be conducted with larger populations to determine if there is indeed a drug-drug interaction of any clinical significance. A study published by Taiwo et al, describes 112 treatment naïve, HIV infected subjects who received DRV/r 800/100 mg once daily plus RAL 400 mg twice daily. They concluded that the co-administration of DRV/r and RAL were well tolerated in most subjects however they reported sub-optimal efficacy, viral failure and integrase resistance was found to be common especially in subjects with a viral load greater than 100,000 copies per mL at base line analysis [46].Fabbiani et al [47] discuss the question of a drug interaction further and highlight other clinical trials which have also shown a reduction in DRV concentrations when co-administered with RAL [48-50], However, Fabbiani et al also point out that DRV plasma C_{trough} concentration levels far exceed the IC_{50} (0.550 μg/mL) for PI resistant virus [35], therefore the modest reduction in DRV concentration when co-administered with RAL is probably negligible and of no clinical significance in the majority of patients taking combined ARV therapy. Further studies are important to address this. One possibility for the reduction could be transporter competition in the uptake of the drugs in the gastrointestinal tract and a future clinical trial investigating this mechanism of reduction at the transporter level may do this.

The RAL PK profiles for group 1 and 2, showed a marked level of inter individual patient variability in both the plasma and the IC results. However, those patients who showed higher plasma C_{max} concentrations mirrored this with higher IC C_{max} levels.

In Figure 2, one patient concentration increases significantly at the 12 hour PK analysis, this is probably due to the sample being taken post the second dose of RAL and not pre-dose as it should have been.

Group 1 plasma RAL AUC (geometric mean) in the absence of DRV/r (1944 ng.h/mL) and in the presence of DRV/r (1759 ng.h/mL), showed no significant difference. Group 2 RAL AUC (geometric mean) in the absence of DRV/r (1635 ng.h.mL) and in the presence of DRV/r (1979 ng.h/mL) also showed no significant difference.

However, in a previous study by Fayet Mello et al, involving 10 HIV-infected patients, a reduction in plasma AUC by 33% and a 29% decrease in C_{max} were observed. Six of the ten study patients were also taking DRV/r twice daily as part of their combination therapy [37].

The methodology used for the measurement of intracellular drugs is extremely complex. There is currently no 'Gold Standard' for PBMC isolation and this as a result means that different research groups are using different approaches, i.e. ficol, CPT or magnetic bead isolation, and then further to this, the number of washes each group conducts varies greatly[29, 36, 44]. This lack of standardization is perhaps the cause of the variation in plasma to IC drug concentrations. Our group and others have previously published data on the IC measurements of PIs and their accumulation [32, 34, 51, 52]. Here we report a DRV geometric IC to plasma ratio of 5.3 to 5.6 in the presence of RAL and 4.9 to 5.3 in the absence of RAL, this ratio is greater than that previously reported by Ter Heine et al [36] but consistent with other PI concentrations. The RTV IC concentrations reported here are also slightly higher compared to those reported by Ter Heine, RTV geometric IC to plasma ratio of 6.9

to 10.0 in the presence of RAL and 6.5 to 9.5 in the absence, compared to 7.72 [36]. However, overall the IC to plasma ratios reported here for PI measurements are effected very minimally by the reduced cell washing. This however is not the case when we compare the integrase inhibitor concentrations for IC RAL. The results presented and found here are much higher than those reported from four other research groups. Ter Heine et al reported that IC levels of RAL were undetectable and therefore were unable to give a ratio [36], Molto et al [38] reported a plasma to IC ratio of 0.07 (range 0.05-0.10), Fayet Mello et al reported 0.039 (range 0.007-0.177) [37] and Wang et al reported 0.24 (range 0.06-0.58) [39].

These differences reported are most certainly due to the techniques used in the PBMC isolation procedures. IC measurements are always going to vary when different isolation methodologies are applied and I will discuss this further in chapter 4. Due to the rapid efflux of RAL from cells (demonstrated in a parallel study involving isolation of PBMC with varying number of washes [53]), our methodology here used only one cell washing step. This clearly reduced the amount of RAL effluxing from the cells during the isolation procedure and ultimately gave us the higher plasma to IC RAL concentration compared to other group findings who used more washing steps.

This study has shown a wide inter-individual variation in all the studied drugs for both IC and plasma concentrations especially for RAL. The reasoning for this could be due to a number of factors previously discussed in addition to drug intake with food (increased RAL concentrations with the intake of food [54]), genetic variability of drug efflux/influx transporters responsible for drug metabolism, and differences in PBMC isolation methodologies. However, Arab-Alameddine at al reported in their

population PK analysis of RAL, that a large majority of RAL pharmacokinetic variability remains unexplained [55].

To conclude, reported here are the findings of a clinical trial investigating the co-administration of DRV/r and RAL plasma and IC concentrations given to HIV–infected subjects. This was also the first time that a dose of RAL 800 mg once daily has been studied and measured within the cellular compartment. The results found here increase our knowledge of IC drug measurements and highlight the importance in the need for consistency between groups by means of a gold standard method for the isolation of PBMC for each class of ARV drugs.

Chapter 4

Investigation of variability of reported intracellular raltegravir concentrations: Contribution of peripheral blood mononuclear cell (PBMC) isolation methodology.

CHAPTER 4

INVESTIGATION OF VARIABILITY IN REPORTED INTRACELLULAR RALTEGRAVIR CONCENTRATIONS: CONTRIBUTION OF PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION METHODOLOGY

4.1 Introduction

4.2 Methods

4.2.1 Comparisons of PBMC isolation methods

4.2.2 Ficoll gradient cell preparation method (Liverpool)

4.2.3 Vacutainer cell preparation tube (CPT) method (London)

4.2.4 Ficoll gradient cell preparation method (Spain)

4.3 Investigation of methodology and results

4.4 Discussion and concluding comments

This work was presented at the 12th International Workshop on Clinical Pharmacology of HIV Therapy. 13-15th April 2011, Miami, Florida, USA.

4.1 Introduction

The primary site of action for highly active antiretroviral drug therapy (HAART) in the treatment of HIV is within the infected immune cell. Previously therapeutic drug monitoring (TDM) to study the pharmacokinetics (PK) of HAART has focused on measurement within the plasma fraction of the blood but with advances in analytical instrumentation, namely LC-MS/MS it is now possible to achieve much greater levels of sensitivity, thus in turn leading to more intracellular PK clinical trials being conducted. This knowledge of cellular penetration and intracellular (IC) pharmacokinetics should aid understanding of virological response and toxicity.

In order to accurately measure IC drug concentrations the peripheral blood mononuclear cells (PBMC) have to be isolated from the patient whole blood. To do this there are currently two main methodological approaches used by TDM research groups. The first method uses self-contained Vacutainer® cell preparation tubes™ (CPT). Cells are isolated by buoyancy gradient centrifugation (total 20 minutes) at room temperature (18-25^{o}C), with zero to four washing steps. The second most commonly used method is the use of Ficoll Paque, which again works on the principle of buoyancy gradient centrifugation but is carried out at a much lower temperature (4^{o}C), with a longer centrifugation time (30 minutes), with one to four washing steps. The number of washes in both methodologies varies in different research groups.

Recently Bazzoli et al [7] summarised findings from PK studies on IC concentrations of antiretrovirals and highlighted clear limitations, e.g. small

numbers, poor design and methodological aproaches. The determination of IC drug quantification is indeed challenging. Here I address methodological issues particularly cell collection and washing procedures. The impetus was the observation of marked differences in raltegravir (RAL) IC disposition in 5 publications [36, 38, 56-58], ranging from undetectable to IC/plasma ratios of 0.07, 0.05, 0.24 and 5.11 respectively (i.e. a difference of > 100-fold). Since I was involved in the analysis of two of the studies (Barcelona [38] and London [58]) generating different data it seemed prudent to investigate the underlying causes of the disparity. As the LC-MS/MS assay used was the same in both studies, I decided to investigate the pre-analysis isolation step by direct comparison of CPT tubes versus Ficoll paque separation to try to pin-point why such variation was occurring. My primary hypothesis was that in this initial cell isolation procedure step, the number of washes, temperature and length of time to final PBMC isolation had an integral influence on the quantification of the RAL IC drug concentration.

4.2. Methods

4.2.1 Comparisons of PBMC isolation methods:

Initial investigation was to look at the PBMC isolation methodology used by different research groups.

4.2.2 Ficoll gradient cell preparation method (Liverpool)

The method routinely used at the Liverpool Bioanalytical Facility

5 mL of Ficoll paque solution is measured into a sterilin. Using a pipette, 8 mL of whole blood is very carefully trickled down the side of the plastic container so that it

rested on top of the Ficoll paque solution. This is then spun in a pre-cooled centrifuge (4^{o}C) for 30 min at 2000 rpm. The top plasma layer is then carefully removed using a Pasture pipette and discarded. The interface layer containing the cells is then transferred into a fresh 20 mL sterilin. Ice cold Hanks balanced salt solution is then added to bring the total volume to exactly 20 mL. This is then gently inverted to mix the cells/ Hanks evenly. A 50 µL sample is taken for counting. The cell count is recorded. The sterilin is then centrifuged at 2000 rpm for 5 min at 4^{o}C. The supernatant is carefully removed. 1 mL of ice cold Hanks is added to the cell pellet, mixed well and a further 9 mL of Hanks is added to bring the cell pellet back into solution. This is then further centrifuged at 2000 rpm for 5 min. The supernatant is carefully removed taking care not to disrupt the cell pellet. 1 mL of ice cold 70% methanol is then added to the cell pellet to re-suspend the cells. The cells are then vortexed and placed in to the freezer (-40^{o}C) for 24 hours to allow complete lysis of the cells.

4.2.3 Vacutainer cell preparation tube (CPT) method (London)

The method supplied by C&W hospital London was used as follows [58];

8 mL of whole blood was measured into a CPT. The tube was then gently inverted 8-10 times to mix the anticoagulant thoroughly. The CPT was then immediately centrifuged (horizontal rotor) at room temperature (20^{o}C) for 20 minutes at a speed of 1600 RCF (relative centrifugal force). After centrifugation the CPT was gently inverted to suspend the PBMCs in the plasma. The plasma/cells were then transferred into a sterilin using a disposable Pasture pipette. Ice cold Hanks balanced salt solution was then added to bring the total volume to exactly 15 mL. This was inverted gently to mix the plasma/cells/ Hanks evenly. A 50 µL sample was taken for

counting. The cell count was recorded. The sterilin was then centrifuged for 15 minutes at 400 RCF to pellet the cells. The supernatant was carefully removed taking care not to disrupt the cell pellet. 1 mL of ice cold 70% methanol was then added to the pellet to re-suspend the cells. The cells were then vortexed and placed in the freezer (-40^{o}C) for 24 hours to allow complete lysis of the cells.

4.2.4 Ficoll gradient cell preparation method (Spain)

The method supplied by Barcelona hospital Spain was used as follows [38];

Blood samples for the PK measurements of RAL concentrations were collected into potassium and EDTA 10 mL tubes, and were processed within 1 hour after collection, at 4^{o}C. Samples were first centrifuged at 390 g for 10 minutes. The supernatant was then transferred to 10 mL tubes and centrifuged at 1090 g for 10 minutes, and the isolated plasma was stored at -80^{o}C until analysis. The remaining cellular fraction was diluted with 0.9 % sodium chloride (NaCl) at 4^{o}C up to a total volume of 10 mL. Subsequently, it was transferred to a 50 mL conical tube containing 5 mL of Ficoll paque at 4^{o}C, and centrifuged at 720 g for 30 minutes. Thereafter, the PBMC layer was washed twice using 0.9% NaCl solution at 4^{o}C, and the final cell pellet was stored at -80^{o}C for further cell analysis.

Note a cell count was not taken at the time of isolation and therefore a DNA detection procedure substantially based on the method described by Benech et al [44] was used.

The above methods are the pre-analysis methods used for the two studies (Barcelona [38] and London [58]) generating very different RAL data (IC / plasma ratio; 0.07 versus 5.11 respectively) on the same LC-MS/MS at the Liverpool Biomedical

Research Centre. Table 1, summarises these methods alongside three other IC RAL PK studies.

Table 1. Comparison of IC RAL PK studies.

Reference	**Ter Heine R et al [36]**	**Fayet-Mello A et al [56]**	**Molto J et al [38]**	**Wang L et al [57]**	**Jackson A et al [58]**
Amount of whole blood	8 mL	8 mL	Not given	Not given	8 mL
CPT/ Ficoll paque	CPT for patients, Ficoll used for standard curves	CPT for patients, Ficoll used for standard curves	Ficoll (post removal of plasma) diluted in 0.9% NaCl. Ficoll used for standard curves	Not given	CPT for patients, Ficol used for standard curves
Number of washes	X 2 in PBS with 0.1% ice cold BSA	X 3 in PBS with 2% ice cold FBS	X 2 in 0.9% NaCl at $4^{o}C$	Not given	No wash
Cell count method	Cell-Dyn 4000 differential counter	Cell-Dyn 3500 coulter counter	DNA detection method [44]	Not given	Digital Bio Adam Microchip Automatic cell counter
Cell counts for standard curves	5 x 10^6 cells	4-10 x 10^6 cells	4 x 10^6 cells	Not given	4 x 10^6 cells
Extraction solution	50% Methanol	50% Methanol	70% Methanol	Not given	70% Methanol
Lysis method	Sonicated for 10 minutes	3 Hr on shaker and sonicated for 5 minutes	Vortexed and overnight	Not given	Vortexed and overnight
IC/ Plasma ratio	Not detected	0.05	0.07	0.24	5.11

PBS = Phosphate buffered saline. BSA= Bovine serum albumin. FBS= Foetal bovine serum.

4.3 Investigation of methodology and results

Due to this disparity in IC /plasma ratios the following RAL drug quantification experiments comparing the use of CPT tubes versus Ficoll paque, and the number of wash steps was compared.

This was carried out in two ways; The first experiment was conducted with a known concentration of RAL (1000 ng/mL) being spiked in to whole blood, which was subsequently tumbled for 1 hour at room temperature (*ex vivo*). This was carried out to allow the uptake of RAL into the PMBCs. 8 mL of spiked blood was then placed into CPT tubes or layered over Ficoll paque. The number of wash steps varied from zero to four and both cell lysate and wash supernatant were analysed. Figure 1, shows a diagrammatic representation of the experiment.

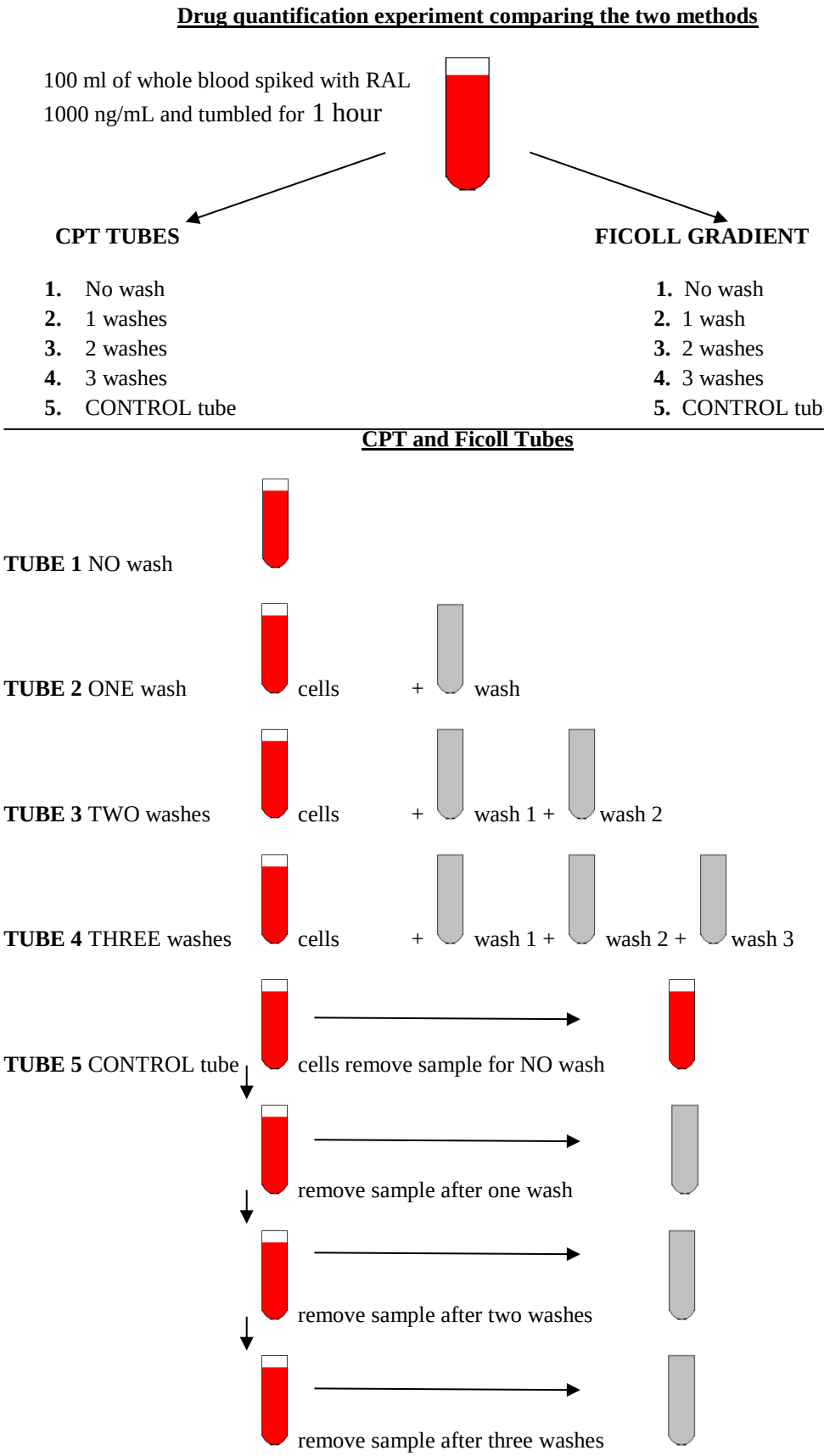


Figure 1. RAL drug quantification experiment comparing methodology.

All 1 mL cell lysate samples were removed from the freezer and spun at 13,000 g for 5 minutes to remove the cell debris. The samples were then transferred in to clean glass tubes and dried in a rotary evaporator. 400 µL of 70% methanol was added to each tube and vortexed thoroughly. 150 µL of each sample was pipetted into an autosampler vial and 20 µL of 100 ng/mL internal standard was added. The aliquots were then capped, mixed carefully and then analysed by LC-MS/MS for RAL quantification. Results are shown in Table 2.

Table 2. Colour scale: The darker blue is equal to more wash steps. Red type indicates quantification below the validated limit of quantification for the assay (<0.47 ng/mL). Time between each CPT wash = 20 minutes. Time between each Ficoll wash = 5 minutes. CPT room temp/ Ficoll 4^{o}C. Note. The first wash supernatants were not analysed as they were too 'dirty' to put through the LC-MS/MS system without the use of a validated extraction method.

Calculated raw Conc (ng/mL)	Sample ID	Final RAL ng/mL (EXP 1)	Final RAL ng/mL (EXP 2)	
752.710	Cell CPT 1 NO WASH	78530.0	15364.0	
7.339	Cell CPT 2 ONE WASH	691.1	995.9	
2.166	Cell CPT 3 TWO WASH	204.0	122.9	
1.138	Cell CPT 4 THREE WASH	150.6	303.3	
0.621	Cell CPT 5 FOUR WASH	73.8	95.5	
19.916	Cell Ficoll Tube 1 NO WASH	1899.5	7868.3	
1.053	Cell Ficoll Tube 2 ONE WASH	109.4	342.8	
0.639	Cell Ficoll Tube 3 TWO WASH	91.1	129.2	
0.469	Cell Ficoll Tube 4 THREE WASH	64.4	135.0	
0.338	Cell Ficoll Tube 5 FOUR WASH	112.2	112.6	
13.423	CPT tube 2, wash 2	201.34		Total RAL in 10 mL of wash solution
9.275	CPT tube 3, wash 2	139.12	338.9	
0.648	CPT tube 3, wash 3	9.72	4.6	
12.401	CPT tube 4, wash 2	186.01	200.3	
0.496	CPT tube 4, wash 3	7.44	4.6	
0.324	CPT tube 4, wash 4	4.86		
12.814	CPT tube 5, wash 2	192.21	224.2	
0.808	CPT tube 5, wash 3	12.12	3.9	
0.269	CPT tube 5, wash 4	4.04		
0.259	CPT tube 5, wash 5	3.89		
1.336	Ficoll tube 2, wash 2	20.05	47.7	
1.648	Ficoll tube 3, wash 2	24.72	41.4	
0.291	Ficoll tube 3, wash 3	4.36		
1.362	Ficoll tube 4, wash 2	20.43	24.3	
0.257	Ficoll tube 4, wash 3	3.86		
0.254	Ficoll tube 4, wash 4	3.81		
1.929	Ficoll tube 5, wash 2	28.94	40.2	
0.308	Ficoll tube 5, wash 3	4.62		
0.250	Ficoll tube 5, wash 4	3.75		
0.251	Ficoll tube 5, wash 5	3.77		

These results shows an (n) of 2 (EXP 1 and EXP 2) which were carried out on separate days. The results show the lysate sample results and also a sample taken from each of the wash supernatants (which usually gets discarded). The results show a much higher drug level in the CPT cell sample tubes with no wash. After the first wash there is a much greater loss of the drug into the supernatant, this is suggestive that drug is efluxed out of the cell over the greater centrifugal time (20 minutes) at room temperature.

The second approach in this investigation was to use HIV infected patients blood who were receiving a RAL-containing regime (n = 4; four time points collected, *in vivo*). Figure 2 shows a diagrammatic representation of the experimental procedure.

Calculation of the cell count for each method was taken at the initial step of addition of Hanks balanced salt solution in each instance. A 1 mL sample from the CPT or Ficoll tube was also removed, centrifuged at 4000 rpm for 5 minutes and the supernatant removed carefully as not to disrupt the pellet. The cell pellets were then re-suspended in 70% methanol, vortexed and then stored overnight at – 40^{o}C to allow cell lysis.

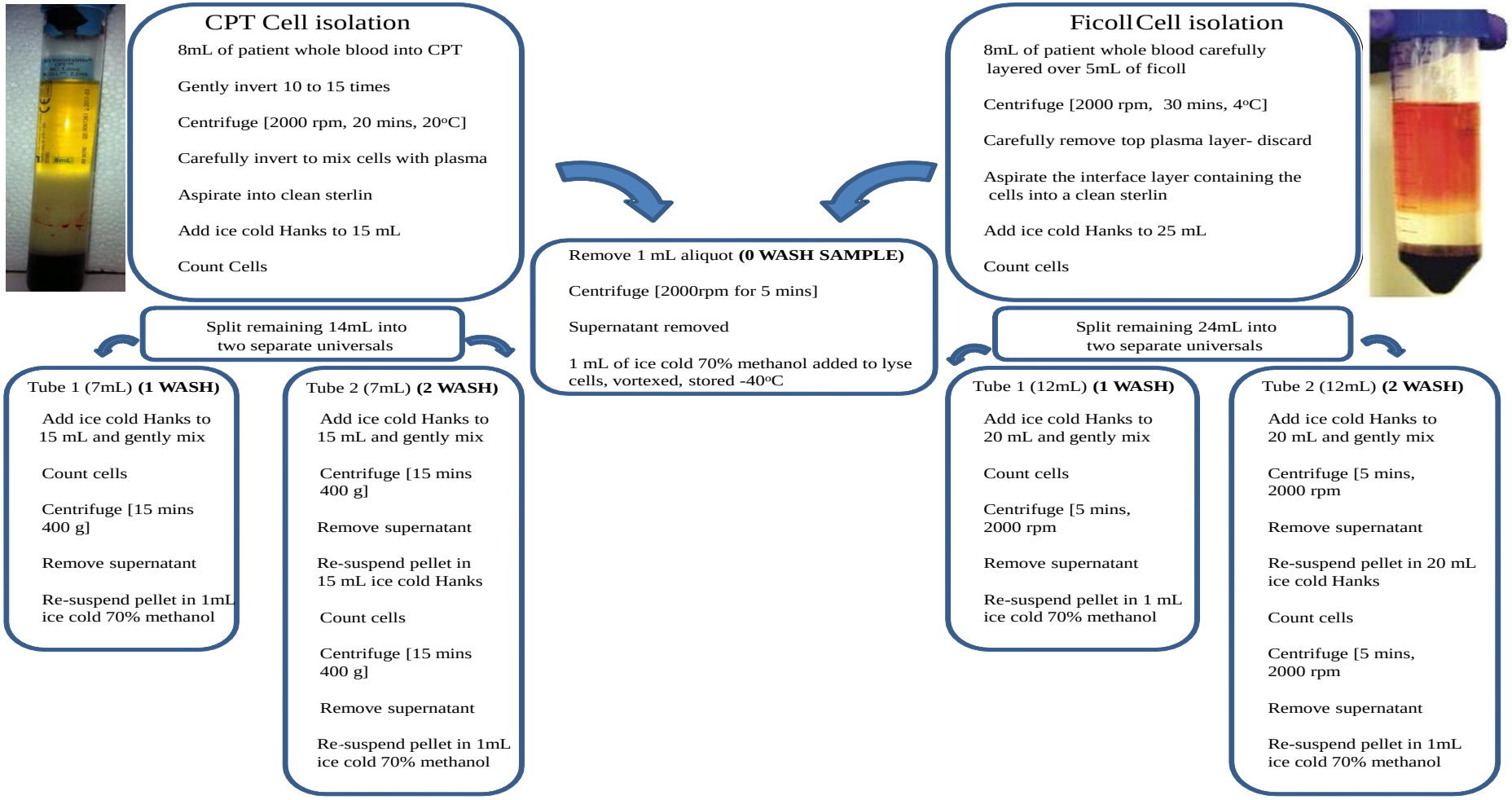
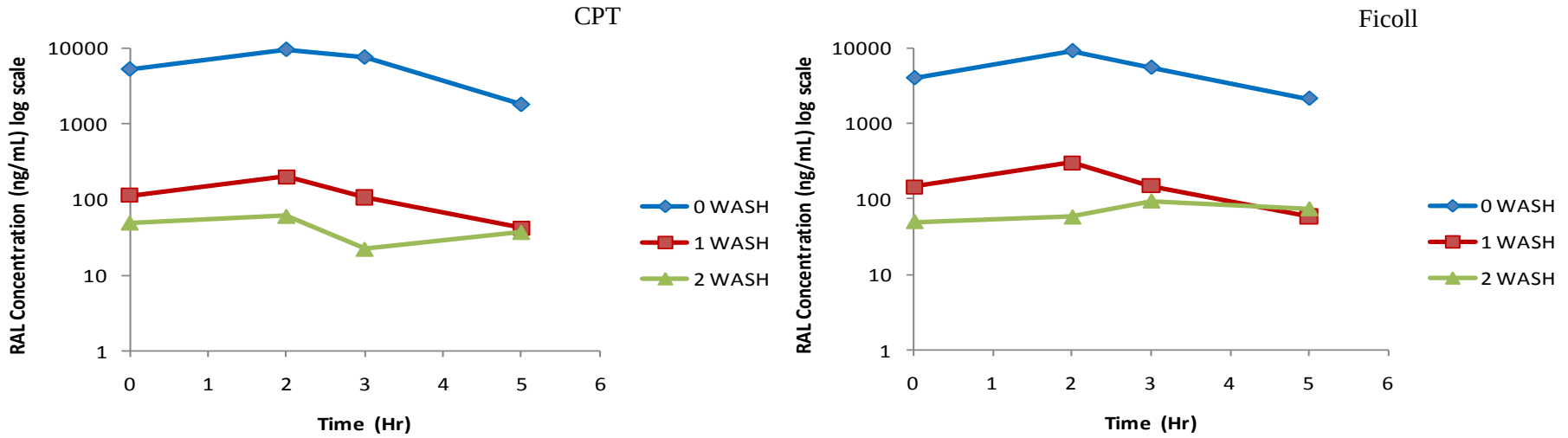


Figure 2. Flow diagram of the methodology used with patient samples to investigate number of washes in CPT versus Ficoll.

Figure 3. Representive HIV + patient graphs to show the IC RAL concentration after 0, 1 or 2 washes in CPT versus Ficoll separation.



CPT	WASH	RAL ng/mL	% loss
0 HOUR	0 WASH	5239	
	1 WASH	114	97.8
	2 WASH	50	99.0
2 HOUR	0 WASH	9453	
	1 WASH	200	97.9
	2 WASH	61	99.4
3 HOUR	0 WASH	7507	
	1 WASH	107	98.6
	2 WASH	22	99.7
5 HOUR	0 WASH	1816	
	1 WASH	42	97.7
	2 WASH	37	97.9

FICOLL	WASH	RAL ng/mL	% loss
0 HOUR	0 WASH	4014	
	1 WASH	145	96.4
	2 WASH	50	98.8
2 HOUR	0 WASH	9170	
	1 WASH	304	96.7
	2 WASH	58	99.4
3 HOUR	0 WASH	5528	
	1 WASH	149	97.3
	2 WASH	94	98.3
5 HOUR	0 WASH	2141	
	1 WASH	59	97.2
	2 WASH	74	96.5

Table 3. IC RAL % loss post 1 and 2 washes in CPT and Ficoll cell isolation methodology.

4.4 Discussion and Concluding comments

Cell counts following the CPT and Ficoll methodologies of cell separation were comparable. However, the number of washes markedly impacted the final intracellular RAL concentration. Results from both the *ex-vivo* and *in-vivo* samples show a decrease in the intracellular concentration of RAL as the number of washes increases (Table. 3). The greatest difference was seen between no wash and the first wash (e.g. *ex-vivo* in CPT tubes had a RAL loss of approximately 95% and in Ficoll the RAL loss was approximately 95% also). In the *in-vivo* collection there was a greater reduction in intracellular RAL concentration after the first wash with samples layered over Ficoll (>95% reduction) than with CPT (approximately 70% loss). Cells for the CPT collection showed a greater loss after the second wash in comparison with the cells from the Ficoll collection method.

Bazzoli et al. [7] have previously discussed some of the methodological issues in relation to intracellular measurements of ARVs. Differences between studies can be attributed to, i) cell isolation by either conventional Ficoll paque gradient centrifugation or Vacutainer cell preparation tubes (CPT), ii) the timing between blood sampling and cell isolation, iii) the number of cell isolation washing steps, and iv) temperature at which isolation is undertaken.

In conclusion, the number of cell isolation washes has a marked impact on the quantification of intracellular RAL concentration. The most notable effect was that the washing reduced the intracellular concentration by > 96% (in a single wash) and more thereafter in both methods investigated. The marked difference between the data from Jackson et al. [58] (IC/ plasma ratio of 5.11) and other studies (which show no drug- to IC/ plasma ratio of 0.24) is directed related to the number of

washes used in the PBMC isolation. The absence of a washing step (as shown in the London clinical trial [58], potentially over-estimates the true intracellular RAL concentration by inclusion of residual plasma drug contamination and drug bound to cell membrane. RAL has also shown that it is rapidly effluxed out of the cellular compartment during isolation washing.

Currently the ‘optimal’ numbers of washes for intracellular determination of RAL is unclear. It is important to have consensus if studies from different research groups are to be compared and a ‘gold standard’ of cell isolation methodology should be internationally decided up on and implemented under governed regulations for the isolation of intracellular RAL. Further analysis is required to investigate the impact of cell isolation washing steps for other ARVs.

Chapter 5

Development and validation of a LC-MS/MS assay to quantify 10 antiretroviral (ARV) drugs in cerebral spinal fluid (CSF).

CHAPTER 5

DEVELOPMENT AND VALIDATION OF A LC-MS/MS ASSAY TO QUANTIFY 10 ANTIRETROVIRAL DRUGS IN CEREBRAL SPINAL FLUID (CSF).

5.1 Introduction

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5.3.4 Recovery and matrix effects

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5.4 Conclusions

This work was presented as a poster at the 13th International Workshop on Clinical Pharmacology of HIV Therapy. 16-18th April 2012, Barcelona, Spain.

5.1. Introduction

The use of combination antiretroviral therapy (cART) has resulted in a dramatic decline in HIV morbidity and mortality [59]. However, in an ageing HIV-infected population, HIV-associated neurocognitive disorders (HAND) remain a concern [60]. HIV may reside outside of the systemic circulation in anatomical sanctuary sites, including the central nervous system (CNS). There is some evidence that antiretrovirals (ARV) exhibiting high CNS penetration effectiveness (CPE) score, suppress compartmentalised viral replication and improve neurocognitive functioning [61, 62], however the 2014 BHIV treatment guidelines debate the clinical relevance of the CPE score in relation to clinical outcome [23]. Ineffective penetration of ARVs into the CNS can result in sub-therapeutic concentrations and the possibility of viral resistance, whereas excessive levels can potentially lead to neurotoxicity. A greater understanding of ARV pharmacokinetics (PK) in the CNS may influence the timing of cART initiation, in order to preserve neurocognitive functioning. In order to gain better understanding of ARV penetration into anatomical sanctuary sites we need sensitive and specific analytical methods. Here I describe the validation of a LC-MS/MS method for the quantification of 10 ARV agents in cerebral spinal fluid (CSF).

5.2 Materials and Methods

5.2.1 Chemicals

LPV and RTV were kindly donated by Abbott Laboratories Ltd (Chicago, IL, USA), APV by GlaxoSmithKline (Middlesex, UK), ATV (atazanavir sulphate) by Bristol-Myers Squibb (Hounslow, UK), DRV (darunavir ethanolate), RPV (rilpivirine hydrochloride) and ETV by Janssen-Cilag Ltd (Mechelen, Belgium). NVP was kindly contributed by Boehringer Ingelheim Pharmaceuticals Inc (Berkshire, UK), RAL by Merck (New Jersey, USA) and MVC by Pfizer (Sandwich, Kent, UK). The internal standards, [$^{13}C_6$]-Rilpivirine was obtained from Alsachim (Strasbourg, France) and quinoxaline (6,7-dimethyl-2,3-di(2pyridyl)-quinoxaline (QX) was obtained from Sigma-Aldrich, UK. Formic acid (minimum 95% pure) and human serum albumin (ALB) were also obtained from Sigma-Aldrich, UK. LC-MS grade acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK) and artificial CSF from Harvard Apparatus, Kent, UK. LC-MS grade methanol was purchased from VWR Laboratory Supplies (Poole, UK). De-ionised water (LC-MS grade) was produced by an Elga Option-S water purification unit and further purified to 18.2 MΩ by a Purelab Ultra (Elga Labwater, High Wycombe, UK).

5.2.2 Equipment

The HPLC system consisted of a variable loop Accela autosampler (set at a temperature of 4^{o}C) and an Accela LC pump (Thermo Fisher Scientific, Hemel Hempstead, UK). A reverse phase Fortis^{TM} C_{18} column (3µm: 150 mm x 2.1 mm) set at an oven temperature of 30^{o}C (Fortis Technologies Ltd, Cheshire, UK) fitted with a 2 µm C_{18} Quest column saver (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to separate all analytes and internal standards. The HPLC system was interfaced with a Thermo Scientific TSQ Quantum Access triple stage quadrupole

mass spectrometer fitted with an ion MAX source with a heated-electrospray ionization (H-ESI) probe. One E2M30 rotary vacuum pump (Edwards High Vacuum International, West Sussex, UK), a Nitro Flow nitrogen generator (Parker, Etten-Leur, Netherlands) and 99% pure argon gas (10L size V, BOC Gases, Worsely, Manchester, UK) were used. Analyte tuning and optimisation parameters were gained with the use of TSQ Tune Software (Thermo Fisher Scientific, Hemel Hempstead, UK). LC Quan^{TM} software (Version 2.7, Thermo Fisher Scientific, Hemel Hempstead, UK) was used to acquire and process the data.

5.2.3 Chromatography and mass spectrometry

All analytes and internal standards were measured simultaneously. Chromatographic separation was achieved by a gradient elution method. Mobile phase A: water with 0.1% formic acid and mobile phase B: 100% ACN were run as follows: from 0 to 5 minutes 95% A. At 5 minutes 20% phase A was held for 2 minutes and then increased back to the starting conditions for 3 minutes to equilibrate the column, giving a total run time of 10 minutes. The flow rate was 400 µL/min. Samples were injected (20 µL) on to the column via a partial loop injection. A wash solution containing ACN: water (0.1% formic acid), 80:20, v/v, was used to clean the injection syringe (2 mL) between each sample. Both the mobile phases and wash solution were de-gassed by sonication (20 minutes) prior to use. All analytes were scanned for using selective reaction monitoring (SRM) by the TSQ Quantum Access operated in positive ionisation mode.

5.2.4 Source and liquid chromatography maintenance

Concentrated constituents of the CSF may build up in the column guard, column and LC-tubing if the CSF sample is particularly viscous due to infection. In order to circumvent this, the column and column guard were reverse flushed at a flow rate of 400 µL/ minute, using 95% of mobile phase A and 5% mobile phase B. The MAX source housing unit was removed and cleaned after each analytical run with 50% methanol and then 100% ultra pure water. The ion MAX source cone and internal transfer tube were also removed and cleaned between each assay (50% methanol, sonicated for 30 minutes).

5.2.5 Column re-equilibration

Prior to each assay, the Fortis C_{18} column was re-equilibrated. This was achieved by manually controlling the LC pump conditions to the chromatography starting conditions for the assay (95% mobile phase A and 5% mobile phase B, with a flow rate of 400 µL/ minute) for 15 minutes. This was also sufficient timing to raise the source temperature to optimal conditions prior to an analytical run.

5.2.6 Sample preparation

5.2.7 Standard curves

Stock solutions (1 mg/mL) of each of the analytes (as base) were prepared in 100% ultra pure deionised water (with the exception of ETV [50/50 ACN/water]). Methanol could not be used as this caused thermal decomposition of the artificial CSF and compromised the matrix. The 1 mg/mL stock solutions were further diluted and combined to give a master mix stock solution containing all of the analytes.

To a clean glass tube 250 µL of NVP stock, 25 µL of RAL, APV, ATV, RPV, ETV, DRV stock and 10 µL of LPV, MVC and RTV stock were added (total volume 440 µL). To this, 1560 µL of 50/50 ACN/0.1% formic acid was added to gain the master mix stock solution. The master mix stock solution was then further diluted in artificial CSF containing 0.2 g/L of human serum albumin (ACSF + ALB) to produce the level 9 standard (upper limit of quantification [ULQ]); NVP = 2500 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 250 ng/mL, LPV, MVC and RTV = 100 ng/mL. The level 9 standard was then serially diluted with ACSF + ALB to produce a nine point standard calibration curve (including blank containing only ACSF + ALB). The lower limits of quantification (LLQ) were as follows; NVP = 19.53 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 1.95 ng/mL, LPV, MVC and RTV = 0.78 ng/mL. Each of the levels were aliquoted (250 µL) into labelled screw cap tubes and stored at -20^{o}C until use.

Fresh stock solution of each analyte was prepared similarly as before to prepare three internal quality control levels. The low quality control (LQC) was prepared at a concentration within three times that of the lower limit of quantification for each of the analytes; NVP = 50 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 5 ng/mL, LPV, MVC and RTV 2 ng/mL. Medium quality control (MQC) samples were prepared at; NVP = 250 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 25 ng/mL, LPV, MVC and RTV 10 ng/mL. High quality control (HQC) samples were prepared

at; NVP = 1250 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 125 ng/mL, LPV, MVC and RTV 50 ng/mL. Labelled screw cap aliquots (250 µL) of each of the QC levels were stored at -20^{o}C until use.

A 1 mg/mL stock solution of the internal standard quinoxaline (QX) was prepared (10 mg of QX dissolved in 10 mL of ultra pure deionised water [stable for 6 months at 4^{o}C]). This was further diluted with ultra pure water to a daily working concentration of 1 µg/mL. A 1 mg/mL stock solution of the internal standard $^{13}C_6$-RPV was also prepared in the same way as QX, however, the daily working concentration was diluted to 80 ng/mL and as the compound is light sensitive, the glass tube containing the stock solution was wrapped in aluminium foil.

5.2.8 Sample pre-treatment

Standard calibrators and QC samples were removed from the -20^{o}C and allowed to thaw at room temperature (18^{o}C). After centrifugation (4000 rpm, 5 minutes) samples were pipetted in duplicate (100 µL) into labelled clean glass tubes. The internal standards QX and $^{13}C_6$-RPV were then added to each sample (20 µL of each daily working concentration, 1 µg/mL and 80 ng/mL respectively) prior to protein precipitation with 50:50 ACN/0.1% formic acid (200 µL). Samples were then vortexed and left to stand at room temperature for 10 minutes. Samples were once again vortexed and then centrifuged (4000 rpm, 10 minutes) to pellet any precipitated proteins and then aliquoted into autosampler vials.

5.2.9 Validation of the standard calibrators and quality controls

Two separate stock solutions were prepared, one for the standard calibrators and the other for the QC. Calibration curves consisted of ACSF + ALB spiked with analytes as described earlier. The level 1 calibrator which was free of analytes, was extracted both with and without external standards. Ten calibration curves were run on separate days to assess the linearity of the standard samples. Each standard was run in duplicate (two separate extractions). Calibration curves of the standard samples were constructed by calculation of the peak area ratio for each analyte to the internal standard QX (with the exception of RPV, internal standard used was $^{13}C_6$-RPV). These ratios were plotted against the nominal concentration for each standard point, from which unknown analyte concentrations could be extrapolated. The equation for each standard curve was determined using the best fitted equation model for least squares regression analysis. The validation acceptance criteria for the calibration curves were calculated using the co-efficient of correlation (r^2). For a valid assay this was equal to or greater than 0.98 and +/- 20% deviation of the nominal standard value for the lower limit of quantification (LLQ) and +/- 15% deviation of the other standard nominal concentration for each of the analytes. The LLQ was the lowest calibration standard level which met the FDA guidelines for industry [21]. The LC-MS/MS signal response at the LLQ for each analyte was at least five times greater than the response obtained for the blank sample. The analyte response (chromatographic peak) was also identifiable, discrete and reproducible with a precision value within 20% and an accuracy value between 80-120%. To determine the LLQ ten samples independent of the standard curve samples were assayed. Analyte carryover was also examined at this point to ensure that the LLQ and any low concentration sample proceeding a high concentration sample were not

compromised. This was assessed by running six extracted level 9 standards, each followed by four extracted blank matrix samples.

Quality control was validated by running ten QC samples (LQC, MQC, and HQC) in duplicate (separate extractions) with the ten standard curves run on different days. The QC samples were treated as unknown concentrations and compared to their target values to determine the inter-assay precision and accuracy for each analyte. An additional calibration curve was run which included six QC samples at each level to assess the intra-assay precision and accuracy. Inter and intra assay variation for the QC samples were expressed by the percentage co-efficient of variation [(CV% = standard deviation / mean)*100]. To comply with the FDA guidance for industry, the CV% for each QC was within +/- 15% of the nominal QC value for each analyte.

5.2.10 Recovery and matrix effects

The percentage recovery (extraction efficiency) of each of the analytes and the internal standards after extraction via protein precipitation was determined by comparing analyte chromatographic peak area extracted from the CSF + ALB matrix to those of non-extracted standard solutions spiked in mobile phase (95% mobile phase A and 5% mobile phase B). The response data for each analyte in mobile phase provided a relative 100% response value and the corresponding response data for extracted samples containing the analyte highlighted any loss in signal which was attributable to the sample extraction process and the sample matrix (CSF +ALB).

Recovery experiments were carried out by processing six QC samples (at each level, LQC, MQC and HQC) and comparing these with pure solution (spiked mobile

phase) at concentrations representing a 100% extraction efficiency of QC sample concentrations (non-extracted). The percentage recovery = (peak area extracted from CSF + ALB / peak area of directly injected spiked mobile phase)*100.

The recovery acceptance criteria of an analyte need not be 100 %, but the extent of recovery of each analyte and internal standard has to be consistent, precise and reproducible (FDA guidance for industry). This was assessed by examining the mean % recovery at the LQC, MQC and HQC concentrations and % CV of the mean % recovery for all analytes at the LQC, MQC and HQC concentrations.

Matrix effects (ion suppression or enhancement) can be caused by co-eluting CSF + ALB matrix components which can suppress or enhance the ion intensity signal of the analytes and therefore compromise the reproducibility and accuracy when quantifying analytes from multiple patients or artificial CSF batches. Therefore, an absolute matrix effect was determined for each of the analytes by comparison of chromatographic peak areas of the analytes spiked into blank extracted CSF + ALB to the peak areas obtained from the same concentration of analyte spiked into mobile phase.

The matrix acceptance criteria were calculated using the absolute matrix effect % CV equal to or less than 20 %.

Absolute matrix effect experiments were carried out by processing six QC samples (at each level, LQC, MQC and HQC) and comparing these with pure solution (spiked mobile phase) at concentrations representing a 100% extraction efficiency of QC sample concentrations (non-extracted). The percentage matrix effect = (peak area of analyte spiked in blank CSF + ALB extract / peak area of spiked analyte in mobile phase)*100.

5.2.11 Stability

Stability experiments were carried out to reflect the conditions likely to be encountered during sample transfer, handling and analytical analysis for clinical samples. Prior to sample analysis both patient and healthy volunteer CSF samples are heat inactivated (58^{o}C for 40 minutes in a water bath), stored at -40^{o}C and then thawed ready for extraction. Thus heat / freeze thaw, short term and long term stability was investigated.

5.2.12 Stock solution stability

Stock solution stability was determined by comparing the freshly prepared stock solutions with old stock solutions. A medium concentration (dependant on each analyte equal to the MQC) of each of the analytes was prepared from freshly prepared stock (1 mg/mL). This was repeated using old (previously prepared under the same conditions) analyte stock solutions. Replicates (n=6) of each were injected and analysed and the mean response of fresh stock to old stock was compared. The percentage difference had to be within +/- 15% (range 85-115%).

5.2.13 Heat / freeze thaw stability

Blank CSF + ALB was spiked at low, medium and high concentrations: LQC (LPV, MVC and RTV = 2 ng/mL, RAL, APV, ATV, RPV, ETV and DRV = 5 ng/mL,

NVP = 50 ng/mL) MQC (LPV, MVC and RTV = 10 ng/mL, RAL, APV, ATV, RPV, ETV and DRV = 25 ng/mL, NVP = 250 ng/mL) HQC (LPV, MVC and RTV = 50 ng/mL, RAL, APV, ATV, RPV, ETV and DRV = 125 ng/mL, NVP = 1250 ng/mL). These concentrations were chosen to match the predicted therapeutic ranges in the published literature. Samples were then analysed in triplicate under the following three treatment conditions; i). Freshly prepared (serving as the test control). ii). Heat inactivated (58^{o}C, 40 minutes). iii). Three freeze/thaw cycles (samples were heat inactivated and frozen overnight and allowed to thaw at room temperature for 1 hour the following morning and then refrozen). This freeze/thaw cycle was repeated and analysed on the third cycle. All samples were then extracted under normal assay conditions. The peak areas obtained from samples undergoing the heat inactivation process and the freeze/thaw cycles were compared to corresponding peak areas (and internal standard adjusted peak area ratios) for each individual analyte obtained under the control sample conditions.

5.2.14 Bench top and processed sample stability

Bench top stability was performed to assess the stability of each of the analytes at room temperature over the time period equal to and beyond that of the extraction procedure. This was investigated by leaving 6 aliquots of QC samples (at LQC, MQC and HQC) on the laboratory bench top for 4-24 hours. These were then analysed against a freshly prepared calibration curve.

Processed sample stability was performed to assess the stability of analytes after 24 hours and to establish that the reinjection of the samples kept in the autosampler at a controlled temperature (4^{o}C) would have no effect on the result reproducibility. This

would allow extracted sample to be re-injected in the incidence of unexpected delay in the analysis such as mechanical (LC-MS/MS instrument) failure. This was investigated by extraction of 6 aliquots of QC samples at LQC, MQC and HQC concentrations. The extracted samples were then left in the autosampler (4^{o}C) for 24 hours and analysed against a freshly prepared calibration curve.

For both the bench top and processed sample stability acceptance, a minimum accuracy of 67% of the QC results had to be within +/- 15% of the respective nominal value and additional to this 50% of the QC samples at each level (LQC, MQC and HQC) also had to be within +/- 15% of the respective nominal value.

5.2.15 Short term stability

Short term stability (1 month) was assessed by comparing the first calibration curve response ratios with later (1 month) calibration curve response ratios. Heat inactivation and freeze/ thaw processes were also investigated.

5.2.16 Dilution integrity

Dilution integrity was investigated by extraction of a calibration curve and QC samples. Duplicate LQC samples were extracted as normal with no dilution factor. The MQC and HQC samples were also extracted in duplicate as normal with the addition of duplicates of each level diluted with CSF + ALB at 1:2 and 1:4. The samples were run as normal and the results back calculated with the appropriate dilution factor against the calibration curve. Diluted QC samples should have precision of <15% and accuracy should be within +/- 15% of the nominal value.

5.2.17 Data analysis

All data analysis for inter and intra- assay variation, carried out at the LQC, MQC and HQC levels was expressed as the percentage co-efficient of variation (% CV = [standard deviation / mean] x 100). All assays were conducted under good clinical laboratory practice (GCLP) conditions and in accordance with the FDA guidelines for industry and QC assessed with a modification of the Westgard regulations used in the clinical diagnostic setting. All calibration standards and QCs had a mean target concentration of +/- 15% of their nominal target concentration with the exception of the LLQ which had a mean target concentration of within +/- 20% of its nominal concentration for each individual analyte. The % CV concentrations followed the same rules. All data was analysed using Microsoft Excel 2007 (Microsoft Corporation, USA).

5.3 Results and Discussion

5.3.1 Detection and Chromatography

Firstly, each of the analytes had to be tuned into the tandem mass spectrometer. This was done by direct injection with a T-piece fixture which allowed a continuous flow of the mobile phase to enter the source at the same time, simulating the conditions which would later be applied in sample analysis. The tuning process allows

optimisation of the mass spectrometric performance for the detection of each of the analytes. This was achieved by investigating and gaining the best probe position, gas flow, lens element and collision energy which detected optimum mass spectral performance for each of the analytes. Tuning was carried out on the following parameters; *the source*, a stable spray was obtained by optimising the probe position, the flow of the AUX and sheath gas flow rates, and the temperature of the internal transfer capillary (ITC) until a variation of <5% was achieved. *Ion optics*, the voltages for the ion optics components were adjusted to optimise the transmission of the analyte to the analyser. Finally, the *Mass spectrometer collision cell (MS^2)*, the collision energy and collision pressure were then optimised for each analyte. Each parent analyte was scanned for four (daughter) fragment ions, and from these, the two with the greatest mass to charge ratio (m/z) and signal to noise ratio were selected for quantification analysis, however NVP gave a better response using only one fragment ion and RTV, LPV and ETV gave better signal with all four fragments. The parameters for each of the analytes are shown in Table 1.

Table 1. Optimised LC-MS/MS tune parameters for each of the analytes and internal standard.

Drug	Drug class	Parent ion (m/z)	Fragment ions (m/z)	Tube lens (V)	Collision energy (eV)	Retention time (mins)
Maraviroc	CCR5 antagonist	514.3	280, 389	103	31, 20	3.02
Nevirapine	NNRTI	267.1	226,	96	25	3.61
$^{13}C_6$ Rilpivirine	IS	372.1	192, 208	124	41, 34	3.71
Rilpivirine	NNRTI	367.1	195, 207	116	34, 33	3.72
Raltegravir	Integrase inhibitor	445.1	109, 361	83	32, 18	4.61
Quinoxalone	IS	313.1	246, 284	105	34, 45	4.83
Atazanavir	PI	705.4	168, 335	129	41, 29	4.94
Darunavir	PI	548.2	156, 392	115	31, 13	5.13
Amprenavir	PI	506.2	156, 245	106	28, 17	5.14
Ritonavir	PI	721.3	140, 171, 197, 268	120	54, 33, 39, 26	5.81
Lopinavir	PI	629.3	155, 183, 429, 447	103	37, 21, 21, 14	5.98
Etravirine	NNRTI	435	144, 162, 273, 303	131	36, 32, 31, 40	6.48

All analytes eluted over a ten minute assay run time, which was divided in to three segments shown in Table 1. Depending on the retention time of the analyte on the column, this was done to enhance the sensitivity of the assay. The mass spectrometer was operated in positive ionisation mode though out and acquired the analyte data by selected reaction monitoring (SRM) in centroid mode. The scan width and time for each analyte was 0.01 m/z and 0.01 sec respectively. As described in our previous paper [45], I applied a low pass chromatography filter which is an application in the real time data software (LC Quan^{TM} software. Version 2.5.6, Thermo Fisher Scientific, Hemel Hempstead, UK). This filter improves peak resolution and smoothing by reducing any background noise/ interference at the baseline of each analyte chromatographic peak at the point of data acquisition. This is much more accurate than using a post-acquisition smoothing approach and reduces the likelihood of artificial chromatographic peak tailing [63]. Figure. 1 illustrates a typical chromatograph showing the total ion count and each chromatographic peak for all ten ARVs plus the two internal standard analytes obtained from extracted CSF.

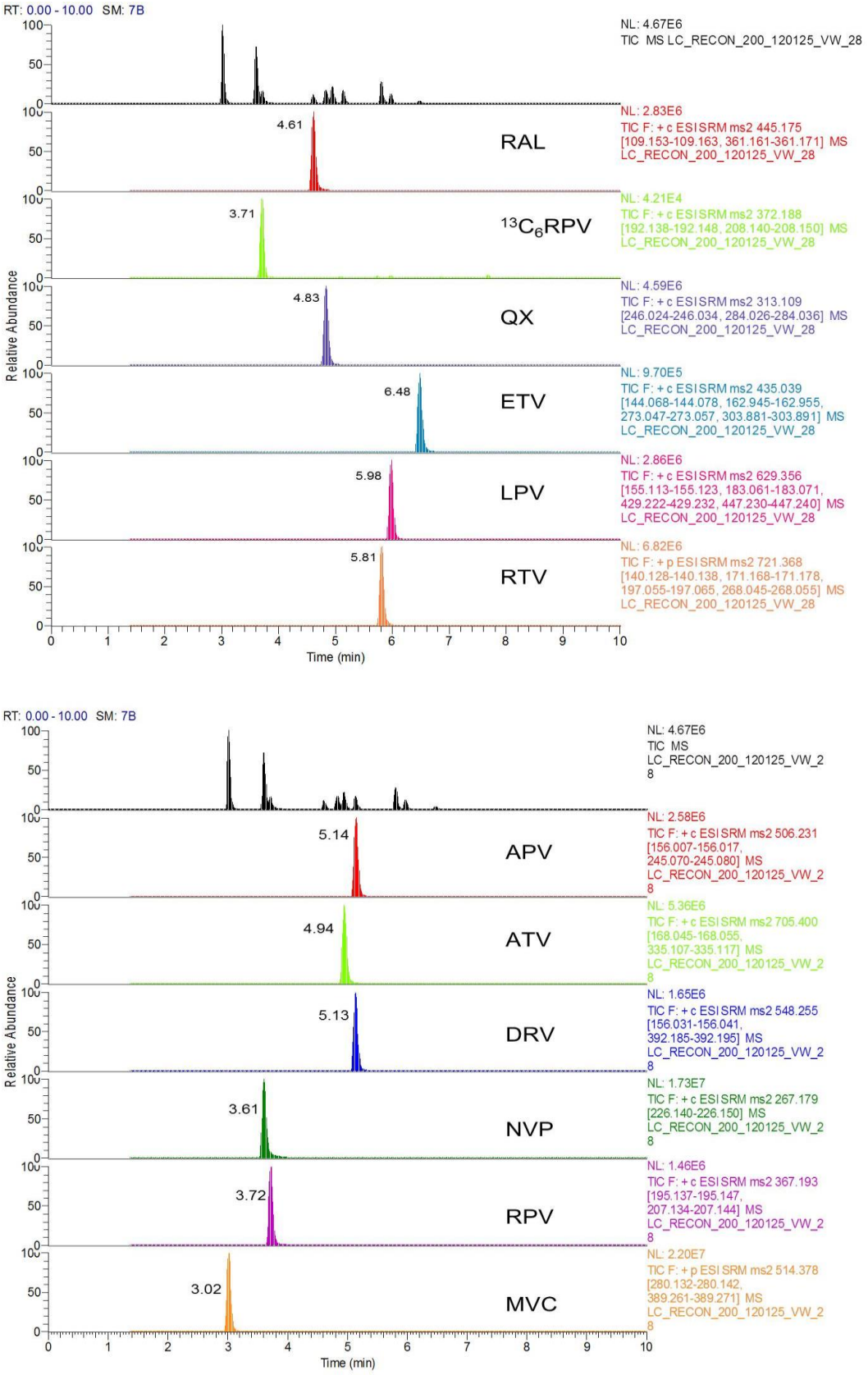

Figure.1 CSF LC-MS/MS chromatograph showing the total ion count (TIC) in solid black and each of the ARVs and IS retention times, and their respective parent and fragment ions scanned for during the 10 minute assay run time.

5.3.2 Validation of calibrators and quality controls

To determine the calibration curve upper and lower levels of quantification, the stock solutions of the analytes were serially diluted, covering the expected published (if any) ranges shown in Table 2, to produce 13 levels including the blank.

Level	LPV, MVC, RTV (ng/mL)	RAL, APV, ATV, RPV, ETV, DRV (ng/mL)	NVP (ng/mL)
1	0	0	0
2	0.049	0.122	1.22
3	0.098	0.244	2.44
4	0.195	0.49	4.88
5	0.39	0.98	9.77
6*	**0.78**	**1.95**	**19.5**
7	1.56	3.91	39.1
8	3.125	7.81	78.1
9	6.25	15.63	156
10	12.5	31.25	313
11	25	62.5	625
12	50	125	1250
13	100	250	2500

Table. 2. Serial dilution of the analyte stock solutions to produce calibration curve. * Level at which all validation criteria were met.

The 13 level calibration curve was then run 4 times in duplicate to determine the lower limit of quantification (LLQ) for each of the analytes. It was found that at level 6, all analytes were within +/- 20% of their respective target value. Below this level some of the analytes did not meet the criteria. Each analyte response was also

compared to the response seen in the blank level 1 samples to ensure that the LLQ gave a signal 5 times greater than that of any background noise observed in the blank sample. Once this was known 10 standard curves were run with level 6 becoming level 2 (level 1 = blank) the LLQ for the assay (which now consisted of 9 levels including the blank). Assays were also analysed for their linearity, the calibration data was analysed using a 1/ concentration (1/*x*) weighted quadratic equation which achieved co-efficient of correlation (r^2) values greater than 0.98 for all validation assays. This gave the following final calibration ranges for each of the analytes; LPV, MVC, and RTV = 0.78-100 ng/mL, RAL, APV, ATV, RPV, ETV and DRV = 1.95-250 ng/mL and NVP = 19.53-2500 ng/mL. Once the LLQs were identified, the quality controls (QC) could be prepared. The low quality control (LQC) concentration had to be within 3 times the concentration value of the analytes LLQ. The values from the QC concentrations at low, medium and high points of the calibration curve were as follows; LPV, MVC, and RTV = 2, 10, 50 ng/mL, RAL, APV, ATV, RPV, ETV and DRV = 5, 25, 125 ng/mL and NVP = 50, 250 and 1250 ng/mL. QC samples at each of the levels were then run ten times against standard curves to ensure that all values (both QC and calibrators) achieved a co-efficient of variation from the target value within +/- 15% (with exception of the LLQ which was within +/- 20%). Carryover was also assessed by running a level 9 (the upper limit of quantification ULQ) sample, followed by 5 extracted blank samples (repeated X 3) and comparing the mean signal response value for the ULQ to the response given for each set of blank samples as a percentage. Carryover range was 0.000 – 0.1 % (Table. 3) and therefore confirmed that during an analytical run, over calculation of analyte concentration due to a high concentration sample preceding a low concentration sample would not occur.

5.3.3 Accuracy and precision

The precision of this methodology describes the closeness of each individual analyte measured when it is repeatedly applied to multiple samples. The accuracy of this method is described by the closeness of the mean test result for each of the individual analytes when compared to the target (actual) concentration of the analyte. This was carried out at each of the QC levels (low, medium and high) in a total of 10 replicates. The results for each of the analyte ranges (spread over the QC levels) are shown in Table.3.

Table. 3. Precision, accuracy and carry over results for each of the analytes. Precision expressed as the co-efficient of variation [% CV (standard deviation/ mean) x 100]. Accuracy expressed as the % bias [((mean concentration – target concentration) / mean concentration) x 100].

Drug	**Accuracy (% bias)**	**Precision (% CV)**	**Carry over (%)**
MVC	-2.91 to 0.86	5.31 to 12.03	0.000
NVP	-10.5 to 5.96	3.82 to 6.36	0.003
RPV	-3.04 to 1.96	5.06 to 8.84	0.037
RAL	-6.64 to -3.8	3.97 to 7.86	0.007
ATV	2.54 to -2.17	4.32 to 7.34	0.004
APV	-7.7 to -1.45	8.89 to 15	0.091
DRV	-8.5 to -5.43	4.75 to 7.69	0.017
RTV	0.04 to 4.48	6.10 to 9.84	0.055
LPV	1.2 to 4	5.69 to 7.67	0.035
ETV	4.6 to 6.4	5.83 to 8.46	0.034
Range	**-10.5 to 6.4**	**3.82 to 15**	**0.000 to 0.091**

5.3.4 Recovery and matrix effects

A recovery experiment to assess how much drug is lost during the protein precipitation extraction procedure was carried out (n=6). The signal response of six extracted QC samples at each level, were compared to the signal response of pure drug (spiked into mobile phase) at the same concentration of those extracted (representing 100% drug recovery). The % CV of the mean % recovery for each analyte was then calculated. In all experiments these values were consistent, precise and reproducible. Recovery % CV results are shown in Table. 4. During the assay co-elution of CSF components may result in a suppressive or an enhancement effect on the ion intensity of the analytes, which may compromise the reproducibility and accuracy when analysing patient samples. Therefore the absolute matrix effect of CSF was determined for all analytes. This was carried out by comparison of the chromatographic peak area of analytes spiked into blank extracted CSF to the peak area of mobile phase spiked at the same concentrations (% matrix effect = [peak area of analyte spiked in blank CSF extract / peak area of analyte in mobile phase]*100). The absolute CSF matrix effect of 100% would be indicative of zero effect of the matrix, however, a value below or above 100% would indicate suppression or enhancement of any of the analytes, results are show in Table. 4. Although matrix suppression and enhancement was shown (range 87% - 120 %), it was within 20 %, reproducible and reliable and therefore should not cause a significant effect on the quantitation of samples.

Table.4. Analyte recovery expressed as % CV and % CSF matrix effects.

Analyte	Recovery (% CV)	% Matrix effect
MVC	14.2	87
NVP	4.3	115
RPV	1.7	119
RAL	1.9	119
ATV	10.8	114
APV	12.5	94
DRV	9.3	101
RTV	18.5	114
LPV	18.6	119
ETV	16.7	120

5.3.5 Stability

Stability experiments to replicate the conditions which samples are exposed to (sample transfer, processing, storage and analysis), were carried out. Clinical samples (from both patients and healthy volunteers) which arrive in the laboratory are heat inactivated (water bath, 58^{o}C for 40 mins), frozen and stored at -40^{o}C and thawed before they are analysed. Therefore, we replicated these conditions with QC samples at each level which had undergone heat inactivation and three freeze/ thaw cycles against freshly prepared samples as a control. The results for each analyte are shown in Table. 5.

Short term stability (1 month) was assessed by comparing the first calibration curve response ratios with later (1 month) calibration curve response ratios, the results showed no significant fluctuations in analyte peak response and drug concentrations

did not deviate by +/- 15%, therefore samples were shown to be stable when stored at -40^{o}C for a 1 month period. Sample stability when stored at 4^{o}C overnight and stability of samples left in the auto-sampler for 24 hours (in the case of mechanical/ equipment failure) were also assessed and again showed no deviation +/- 15% of the same samples analysed under normal conditions.

Table. 5. Stability analysis for analytes analysed against control samples (freshly prepared), after heat inactivation and with three freeze/thaw cycles.

Drug	Low QC	Medium QC	High QC
MVC			
Control	100	100	100
Heat inactivated	104 (9)	100 (7)	111 (9)
Heat -freeze/thaw	99 (10)	99 (5)	111 (4)
NVP			
Control	100	100	100
Heat inactivated	107(3)	103 (1)	106 (3)
Heat -freeze/thaw	106 (3)	97 (2)	102 (4)
RPV			
Control	100	100	100
Heat inactivated	103 (8)	98 (6)	109 (2)
Heat -freeze/thaw	96 (15)	93 (7)	103 (2)
RAL			
Control	100	100	100
Heat inactivated	100 (8)	99 (3)	109 (3)
Heat -freeze/thaw	93 (5)	100 (3)	105 (2)
ATV			
Control	100	100	100
Heat inactivated	103 (6)	100 (1)	115 (3)
Heat -freeze/thaw	96 (7)	98 (5)	108 (2)
APV			
Control	100	100	100
Heat inactivated	101 (10)	101 (2)	114 (2)
Heat -freeze/thaw	102 (3)	97 (4)	110 (2)
DRV			
Control	100	100	100
Heat inactivated	104 (9)	100 (5)	111 (3)
Heat -freeze/thaw	99 (8)	99 (5)	111 (2)
RTV			
Control	100	100	100
Heat inactivated	114 (6)	110 (7)	127 (2)
Heat -freeze/thaw	87 (15)	99 (5)	100 (4)
LPV			
Control	100	100	100
Heat inactivated	118 (3)	113 (2)	124 (1)
Heat -freeze/thaw	91 (9)	99 (6)	95 (2)
ETV			
Control	100	100	100
Heat inactivated	81 (10)	115 (10)	95 (4)
Heat -freeze/thaw	116 (20)	106 (14)	86 (5)

For each level of QC six samples were analysed. Peak areas of the analytes are expressed as a mean percentage (%CV).

5.6 Conclusions

Described here is the development and validation of an LC-MS/MS assay to simultaneously quantify 10 antiretroviral agents from different therapeutic classes, in cerebral spinal fluid. This assay performance was monitored with the use of known concentration quality controls at low, medium and high levels appropriate to each analytes concentration range, and showed good accuracy (% bias -10.5 to 6.4) and precision (% CV 3.82 to 15) for analyte analysis. All analyte calibration data curves illustrated excellent linearity with correlation coefficients (r^2) greater than 0.998.

The use of artificial CSF for the matched matrix was selected as the large volumes of CSF required to establish a fully validated method were not available to purchase. Unlike some research groups who 'make' their own artificial CSF in-house, the use of artificial CSF bought from Harvard Apparatus, ensured that there was quality and batch control and therefore a level of standardisation. The artificial CSF is made up of the following; high purity water, sodium 150 mM, potassium 30 mM, calcium 1.4 mM, magnesium 0.8 mM, phosphorus 1.0 mM and chlorine 155 mM, it did not however contain any protein or albumin. During initial development of this assay it was quickly observed that there was a rapid loss of analyte signal (some more than others) over a short period of time (post two weeks) and we postulate that this drop in signal was due to the analytes having nothing to bind to (protein or albumin) in the artificial CSF mixture and therefore binding to the plastic microtubes in which they were stored. Therefore, the addition of human serum albumin at a concentration of 0.2 g/L (which is within the normal clinical range found in CSF) was added to the artificial CSF. It was also observed when developing the extraction method that the addition of methanol into the artificial CSF caused thermal decomposition of the

artificial CSF and therefore methanol was replaced with ACN. The combination of the addition of human serum albumin and removal of methanol stabilised the assay.

The described extraction method uses only 100 µL of patient sample. Given that CSF is a sample which requires an invasive lumbar puncture procedure to obtain a small amount, this low sample volume required was advantageous. The assay run time is only 10 minutes per sample. This rapid time efficiently allows high though put sample analysis which is needed when sampling large numbers of samples from pharmacokinetic trials, and for therapeutic drug monitoring (TDM). Given that ARV therapy consists of a combination of drugs, the ability of this assay to measure ten different ARVs, allows multiple drugs from one patient sample to be simultaneously quantified, reducing cost of test, analysis time and volume of sample required.

This method was developed with each ARVs dynamic range in mind. The assay shows high sensitivity for all drugs (LLQ; LPV, MVC, RTV = 0.78 ng/mL, RAL, APV, ATV, RPV, ETV, DRV = 1.95 ng/mL and NVP = 19.5 ng/mL). This was achieved and optimised in the tandem mass spectrometric stage of development, in that, for each analyte up to 6 mass transitions were screened for, and from these quantification was achieved by careful selection of between one and four fragment ions. Selection of these daughter ions was carried out based on their relative signal to noise intensity. In doing this, all four fragment ions where scanned and signal intensity monitored, then each possible combination of the fragment ions was investigated. In some cases it was apparent that even when the signal is at its greatest from a particular fragment ion, the background noise was also at its greatest. A high level of background noise then affects the sensitivity of the analyte and therefore the selection of the fragment ions chosen for detection was a fine balance between which

gave the best signal with the least background noise, ultimately giving the greatest sensitivity.

In conclusion, the results of this bio-analytical validation, demonstrate this new methodology is reliable and reproducible. It is robust, accurate, selective and highly sensitive for the simultaneous quantification of 10 ARVs within CSF, enabling a greater insight into ARV concentrations within this sanctuary site. This developed assay has successfully been applied to clinical CSF samples collected for pharmacokinetic trials [61, 64-68].

The direct quantification of an ARV in CSF is one important aspect of seeking a greater understanding of why some people develop HIV-associated neurocognitive disorders despite having optimal ARV plasma levels.

Chapter 6

Development and validation for a LC-MS/MS assay to detect and quantify the intracellular phosphate metabolites of tenofovir and emtricitabine.

CHAPTER 6

DEVELOPMENT AND VALIDATION FOR A LC-MS/MS ASSAY TO DETECT AND QUANTIFY THE INTRACELLULAR PHOSPHATE METABOLITES OF TENOFOVIR AND EMTRICITABINE.

6.1 Introduction

6.2 Materials and methods

6.2.1 Chemicals

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6.2.4 Source and liquid chromatography maintenance

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6.3 Results and Discussion

6.3.1 Detection and chromatography

6.3.2 Accuracy and precision

6.3.3 Signal to noise ratio

6.4 Concluding remarks

This work was presented as a poster at the 11th International Congress on Drug Therapy in HIV infection. 11-15th November 2012, Glasgow, United Kingdom.

6.1. Introduction

This chapter describes the development and validation of a LC-MS/MS assay to detect and measure the intracellular phosphorylated anabolites of tenofovir (TDF) and emtricitabine (FTC). TDF and FTC are first line antiretrovirals which require intracellular phosphorylation to their active moieties (Figure 1).

Tenofovir disoproxil fumarate (TDF) is a prodrug which first requires hydrolization into tenofovir (TFV) and then phosphorylation into tenofovir diphosphate (TFV-DP) [Figure 1]. It belongs to the ARV drug class known as the nucleotide reverse transcriptase inhibitors (NtRTI). NtRTI are analogues of the cellular deoxynucleotides. TFV once phosphorylated into its active phosphate anabolite TFV-DP is an analogue of adenosine however it does not possess a 3'-hydroxyl group on the deoxyribose moiety like its naturally occurring cellular competitor deoxyadenosine 5'-triphosphate. TFV-DP competes with the host cellular deoxyadenosine 5'-triphosphate during viral DNA replication. When the analogue is successfully incorporated into the viral DNA the anabolites lack of the 3'-hydroxyl group prevents the formation of the next 5'-3' phosphodiester bond which is essential in viral DNA elongation. Therefore DNA chain termination occurs and the production of viral DNA is inhibited preventing further viral replication.

Emtricitabine (2',3'-dideoxy-5-fluoro-3'-thiacytidine; FTC) belongs to the ARV drug class known as the nucleoside reverse transcriptase inhibitors (NRTI). NRTI are analogues of the naturally occurring cellular deoxynucleosides. FTC once phosphorylated into its active phosphate anabolite emtricitabine-triphosphate (FTC-

TP) [Figure 1] is an analogue of cytidine. Again like TFV-DP its mechanism of action is to compete against its naturally occurring intracellular competitor deoxycytidine 5′-triphosphate during viral replication to prevent viral DNA replication and cause DNA chain termination.

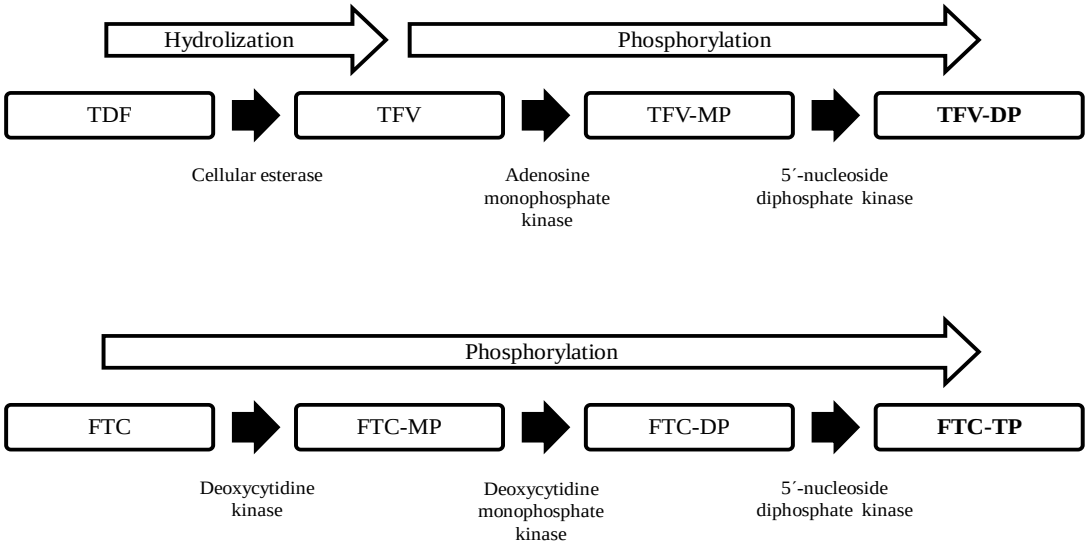

Figure 1. Flow diagram of the intracellular enzymatic phosphorylation steps for TDF to TFV-DP and FTC to FTC-TP.

The intracellular concentration of the phosphorylated anabolites is dependant upon various factors, including the immune cell type and its activation state (it may be latent), the amount of uptake transporters on the cell membrane, and the amount of intracellular kinase enzymes available to phosphorylate the parent drug into the deoxynucleotide triphosphate (dNTP) active state [7].

The ability to measure these intracellular phosphorylated anabolites (which show very different pharmacokinetic profiles from plasma ‘parent drug’ concentrations) will greatly aid understanding of the pharmacology of treatment failure. FTC shows a plasma half-life of between 8-10 hours compared to that of its intracellular half-life which is 39 hours [69]. TFV in the plasma has a half-life of approximately 14 hours

and a far longer intracellular half-life of between 150-180 hours [70]. As for all antiretrovirals, high inter-patient variability has been observed [71].

Here I describe the considerable technical challenges in developing a robust assay for measuring intracellular TFV and FTC anabolites. Broadly the choice of extraction methodology can be divided into two main approaches, i) an indirect method which uses solid phase extraction (SPE) utilises fractionation of the phosphorylated anabolites, followed by removal of the bonded phosphates (dephosphorylation), and finally a desalting step to purify the anabolite back into its parent form by conventional LC-MS/MS measurement. During a research secondment I observed that this process is extremely labour intensive, introducing multiple processing steps with the potential for error. ii) The alternative approach is the direct measurement of the phosphorylated anabolites by LC-MS/MS. The major drawback with this approach is the difficulty in the adequate separation of the phosphorylated anabolites chromatographically. Conventional methods in the direct methodological approach rely upon the use of ion pairing agents such as dimethylhexylamine (DMHA), but the use of such agents can lead to major mass spectrometry instrument contamination issues when not cleared from the system adequately. Other direct approaches include the use of weak anion exchange (WAX), hydrophilic interaction (HILIC) and porous graphitic carbon (PGC) chromatography. Both approaches are illustrated very simplistically in Figure 2. which is adapted from Jansen et al. [5].

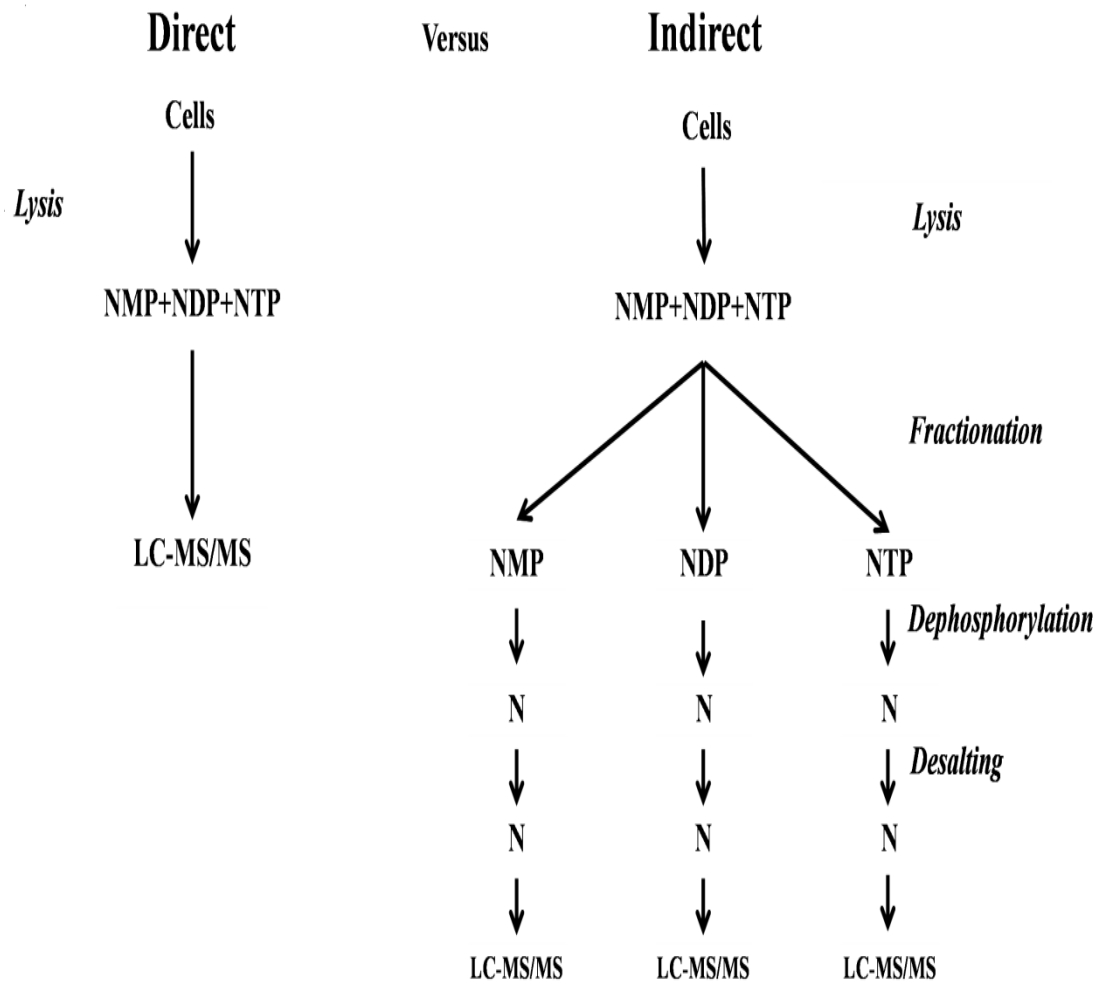

Figure 2. Comparison of direct versus indirect methodological approaches to measure phosphorylated nucleotides. Nucleo (N) mono (M) di (D) tri (T) phosphates (P). Diagram adapted from Jansen et al. [5]

I selected the direct approach by weak anion exchange chromatography. This standard operation procedure of WAX chromatography is a modification of the Jansen et al [72] methodology which is based on the findings of Shi et al [73]. Shi et al showed that the charge of the basic functional groups (pKa) of a WAX column could be changed by applying a pH gradient (6-10.5) via the mobile phases. As a consequence the capacity of the column decreased at a higher pH, thereby eluting anionic nucleotides. Therefore, as opposed to classical anion-exchange methods, this

method does not require high concentrations of involatile competing ions for elution, making it directly applicable to MS detection.

A LC-MS/MS assay has been developed, documented and validated for the specific quantitative determination of TFV-DP and FTC-TP in human peripheral blood mononuclear cells (PBMC's). This assay was developed to assist in their measurement in a clinical trial outlined in the following chapter.

6.2 Materials and Methods

6.2.1 Chemicals

TDF was obtained from Toronto Research Chemicals Inc. Canada. TFV-DP was obtained from Gilead, California, USA. FTC was obtained from Toronto Research Chemicals Inc. Canada. FTC-TP was obtained from Gilead, California, USA. 2-chloroadenosine 5′-triphosphate (Cl-ATP), used as the internal standard was obtained from Sigma, U.K. Ammonium acetate (1 M), and formic acid (minimum 95% pure) were obtained from Sigma-Aldrich, UK. LC-MS grade acetonitrile (ACN) and acetic acid were purchased from Fisher Scientific (Loughborough, UK). LC-MS grade methanol and ammonia were purchased from VWR Laboratory Supplies (Poole, UK). De-ionised water (LC-MS grade) was produced by an Elga Option-S water purification unit and further purified to 18.2 MΩ by a Purelab Ultra (Elga Labwater, High Wycombe, UK).

6.2.2 Equipment

The HPLC system consisted of a variable loop Accela autosampler (set at a temperature of 4^{o}C, to keep the samples chilled) and an Accela LC pump (Thermo Fisher Scientific, Hemel Hempstead, UK). A Thermo BioBasic AX column (5 µm 50 mm x 1 mm) set at an oven temperature of 40^{o}C, fitted with a 2 µm Quest column saver (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to separate all analytes and the internal standard. The HPLC system was interfaced with a Thermo Scientific triple stage quadrupole (TSQ) Quantum Access mass spectrometer, fitted with an ion MAX source with a heated-electrospray ionization (H-ESI) probe. One E2M30 rotary vacuum pump (Edwards High Vacuum International, West Sussex, UK), a Nitro Flow nitrogen generator (Parker, Etten-Leur, Netherlands) and 99% pure argon gas (10 L size V, BOC Gases, Worsely, Manchester, UK) were used. Analyte tuning and optimisation parameters were gained with the use of TSQ Tune Software (Thermo Fisher Scientific, Hemel Hempstead, UK). LC Quan ™ software (Version 2.7, Thermo Fisher Scientific, Hemel Hempstead, UK) was used to acquire and process all the data.

6.2.3 Chromatography and mass spectrometry

A mobile phase gradient was used at a flow rate of 250 µL/ min, onto a BioBasic AX column, which was maintained at a temperature of 40^{o}C. Mobile phase A consisted of 10 mM ammonium acetate in ACN/ ultra pure water (30:70, v/v) adjusted to pH 6 with acetic acid and mobile phase B consisted of 1 mM ammonium acetate in ACN/ ultra pure water (30:70, v/v) adjusted to pH 10.5 with ammonia (100%). The optimised step-wise gradient is outlined in Table 1.

Table 1. Chromatographic conditions during the 12 minute step-wise gradient assay run time.

Time (mins)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (µL/min)
0.0	90	10	250
0.50	90	10	250
0.51	50	50	250
1.75	50	50	250
1.76	0	100	250
6.50	0	100	250
6.60	90	10	250
12.0	90	10	250

The wash solution (2mL) between each sample injection consisted of ultra pure water / ACN (80:20, v/v) with 0.1% ammonium hydroxide. A 2 mL flush volume was also injected between each injection. Both mobile phases and wash solution was sonicated for 20 minutes prior to use.

The Thermo TSQ tandem mass spectrometer was operated in positive ionisation mode and was optimised for the analytes at the following settings: H-ESI spray voltage 5000 V, vap temperature 400^{o}C, sheath gas pressure 30, AUX gas pressure 5 and heated capillary temperature 325^{o}C. The molecular mass (parent mass), for each of the phosphorylated anabolites and the internal standard were scanned for in the first quadrupole (Q1) of the instrument. Once detected the tube lens was then optimised for each of the drugs. The parent ions then travelled to the second quadrupole (Q2) which contains inert gas molecules that fragment the ions into their product ions. Collision energy was applied at a range of voltages (V) and the product ions for each of the parent masses were scanned for in the selected reaction monitoring (SRM) mode in the third quadrupole (Q3). Two product ions for each of the parent ions were selected. These were the ions which gave the greatest mass to

charge ratio (m/z) for their parent ion, with the least amount of background noise. The optimised mass spectrometric parameters are shown in table 2. The method was programmed with an acquire time of 12 minutes, during which the divert value was used as follows: inject/waste (0 min) > load/detector (1.5 mins) > inject/waste (10 mins). The scanning width for the analytes was 0.010, with a scan time of 0.025 for each. Data was acquired in centroid mode. The collision gas was 1.5 mTorr and the chrom filter was applied and used at a setting of 3.0 s.

Table 2. Mass spectrometry parameters for each of the phosphorylated anabolites using the Thermo TSQ in positive ion SRM mode.

Analyte	**Precursor ion (m/z)**	**Product ion (m/z)**	**Collision Energy (V)**	**Tube lens**
TFV-DP	447.98	270.0	28	101
		350.0	19	
FTC-TP	487.91	130.0	32	144
		229.9	15	
2-CI ATP	541.92	169.9	31	140

6.2.4 Source and liquid chromatography maintenance

Due to the highly concentrated samples, constituents of the PBMC lysate matrix can build up in the column saver, column frit, column and LC instrument tubing. To circumvent and avoid ‘blockages’ the column was ‘back flushed’ daily at a flow rate of 250 µL / min, using 90% of mobile phase A and 10% mobile phase B.

The source housing unit was also removed daily after each analytical assay and cleaned internally with 50% methanol and thoroughly dried. The source cone and the internal transfer tube (ITT) were also removed and cleaned by emersion into 50% methanol and sonicated for 30 minutes.

6.2.5 Column re-equilibration

Prior to each analytical assay the BioBasic AX chromatography column was equilibrated with the starting instrument method mobile phase conditions (90% mobile phase A and 10% mobile phase B) with the oven temperature set at 40^{o}C, for a period of 15 mins (approximately 22 column volumes; column volume = 173 µL).

6.2.6 Sample preparation

6.2.7 Isolation and preparation of peripheral blood mononuclear (PBMC) cells for lysate matrix

This method is adapted from that published by Colombo el al [29]. Sample blood was collected in Lithium heparin coated tubes and kept at a temperature of 4^{o}C until the isolation was carried out. This was done to prevent lysis of the cells. Whole blood (10 mL) was carefully layered over 5 mL of Ficoll paque™ in a clean sterile universal tube. This was centrifuged at 2000 rpm for 30 minutes at 4^{o}C without break applied. The isolation of the PBMC fraction (containing the lymphocytes and monocytes) was thus achieved on a buoyancy gradient basis as the PBMC's are of a lower buoyancy density than the Ficoll paque™. After centrifugation the central band containing the PBMCs was carefully removed using a sterile fine tipped Pasteur pipette and transferred into a clean sterile universal container. The volume

was adjusted to 25 mL with ice cold Hanks balanced salt solution (HBSS) and centrifuged (2000 rpm, 5 minutes, at 4^{o}C). This sample wash/spin step was repeated X 3. On the final re-suspension only 10 mL (exactly) of HBSS was added and a 50 µL sample of this was transferred into a clean microtube to which, 50 µL of each solution A and solution B was added (solutions supplied and used as per manufacture guidelines for Chemometec Nucleocounter cell counting cassettes). From this, 50 µL was drawn into a nucleocounter cassette for a cell count (Nucleocounter NC-100). Once the cell count was known, the cell suspension was centrifuged as before and all the HBSS carefully removed. Methanol (70 %) was then added to the cell pellet at a volume calculated to obtain a final PBMC lysed cell density of 4 X 10^6 cells/ mL. This cell lysate was then sonicated for 10 minutes and stored at 4^{o}C overnight. Finally, the cell lysate was centrifuged (5500 rpm, 5 minutes, 4^{o}C, without brake) and the supernatant / cell lysate (debris free) was then carefully transferred into a new tube and the pellet discarded.

6.2.8 Standard curves and quality controls

FTC-TP and TFV-DP stock solution (1 mg/mL) were prepared in ultra pure water. These were then diluted further in ultra pure water to concentrations of 10 µg/ mL, 5 µg/ mL and 0.1 µg/ mL solutions which were used ‘fresh’ to prepare the standard calibrators and quality control samples. Drug-free cell lysate (4 X 10^6 cells/ mL) was spiked with working concentration FTC-TP and TFV-DP solutions (range FTC-TP = 0.38-103.47 ng/ mL, TFV-DP= 0.35-10.91 ng/ mL). The amount of methanol was adjusted at each concentration to keep the cell density the same throughout. Quality controls (QCs) were generated similarly at low, medium and high concentrations

across the range of the standard curves. Once the standards and QC were prepared they were aliquoted into screw top microtubes labelled appropriately and stored in the freezer at -80^{o}C.

6.2.9 Sample pre-treatment

Samples were removed from the -80^{o}C freezer. 1 mL of LC-MS-grade ACN was added to the calibrators, QC and clinical samples. Samples were then microfuged at 13,000 g for 6 minutes. The supernatant was then transferred into glass tubes and internal standard, 2-chloroadenosine 5′-triphosphate (Cl-ATP) [20 µl; 4 µg/ mL] was added. The samples were then evaporated to dryness using a rotary evaporator (20°C). Once dry, samples were then resuspended in 150 µL of 5 mM ammonium formate. From this, 25 µL was transferred to an auto sampler vial and centrifuged at 2000 rpm for 5 minutes, ready for analysis (20 µL; no waste) by LC-MS/MS. The remaining 125 µL of each sample was also placed into glass autosampler vials, capped, labelled and stored at -80^{o}C.

6.2.10 Validation of the standard calibrators and quality controls

A total of 20 calibration curves (with each level sampled in duplicate) were run and assessed to validate the assay. The assays were also assessed for their linearity, the calibration data was analysed using a linear regression of the peak area ratio (drug peak area / IS peak area), versus the nominal concentration of the sample with a weighting factor of 1 / concentration (1/*x*), which achieved co-efficient of correlation (r^2) values greater than 0.99 for all validation assays. Once the calibration values

were validated the quality control (QC) samples at low, medium and high concentrations were prepared. QC samples at each of the levels were run in duplicate (n = 10) against the standard curves to ensure that all the values (both QC and calibrators) achieved a co-efficient of variation from the target value within +/- 2 standard deviations of the mean target value.

6.2.11 Accuracy and precision

The inter and intra-day precision and accuracy was determined following the FDA guidelines. Ten freshly prepared lysate spiked QC samples containing each of the analytes and the IS at three concentrations for TDF-DP (low, medium and high) and at four QC concentrations for FTC-TP (ultra-low, low, medium and high), were analysed in duplicate. Accuracy was determined as the absolute percentage deviation from the theoretically determined concentration (% difference), and the precision of the assay was expressed as the relative standard deviation of the mean as a percentage (% CV) for each of the three concentrations.

6.2.12 Lysate matrix effects

Lysate matrix effects were assessed by post column infusion of the analytes into the LC-MS/MS detector during simultaneous chromatographic analysis of the matrix (PBMC) extract. A standard solution of each of the phosphorylated anabolites was infused using a T connection mixer, at the same time as the blank PBMC lysate matrix and the mobile phase. Unexpected suppression / enhancement, retention time drift or shift of the phosphorylated anabolites chromatographic responses due to any

co-eluting intracellular endogenous constituents, were examined to ensure there was no interference at the retention time of each of the ARVs.

6.2.13 Interpretation and calculation of clinical sample concentrations

The concentration calculation for the clinical intracellular samples, requires the exact number of cells in each patient sample to be known. It is crucial that the cell count is accurate and it should be performed and recorded at the point of cell isolation. Calculations to normalize the reading from the LC-MS/MS for intracellular samples were as follows:

A = Raw mass spectrometer result

B = Sample cell count (cells per 1 mL)

C = 10^6 (per million cells)

D = Molecular weight of analyte (TFV-DP = 447, FTC-TP = 487)

E = ng / 10^6 cells

F = pmol / 10^6 cells

G = fmol / 10^6 cells

Equation 1 $\frac{A}{(\frac{B}{C})}$ = E

Equation 2 (E / D)*1000 = F

Equation 3 F*1000 = G

This allows back calculation to pmol or fmol per million cells.

6.3 Results and Discussion

A successful direct WAX method has been developed for the detection and quantification of TFV-DP and FTC-TP in PBMC lysate by LC-MS/MS.

6.3.1. Detection and Chromatography

Each of the phosphorylated anabolites and the internal standard were tuned and optimised on the HPLC-MS/MS system described. Figure 3 shows the chromatographic peak and retention time for each of the ARVs, the internal standard and the total ion count (TIC). Mass spectrometric parameters were controlled through the LC Quan software to provide the optimal conditions which gave way to the greatest resolution (sharpest peaks). Each chromatographic peak used at least 16 individual scanning event points. Confirmation for specificity was achieved using the same software, and on highlighting the chromatographic peak product ions, showing their mass to charge ratio (*m/z*), this was observed and checked.

Figures 4-11 show the individual chromatographic peaks for blank lysate samples, LLQ, ULQ samples and clinical patient samples for both FTC-TP and TFV-DP.

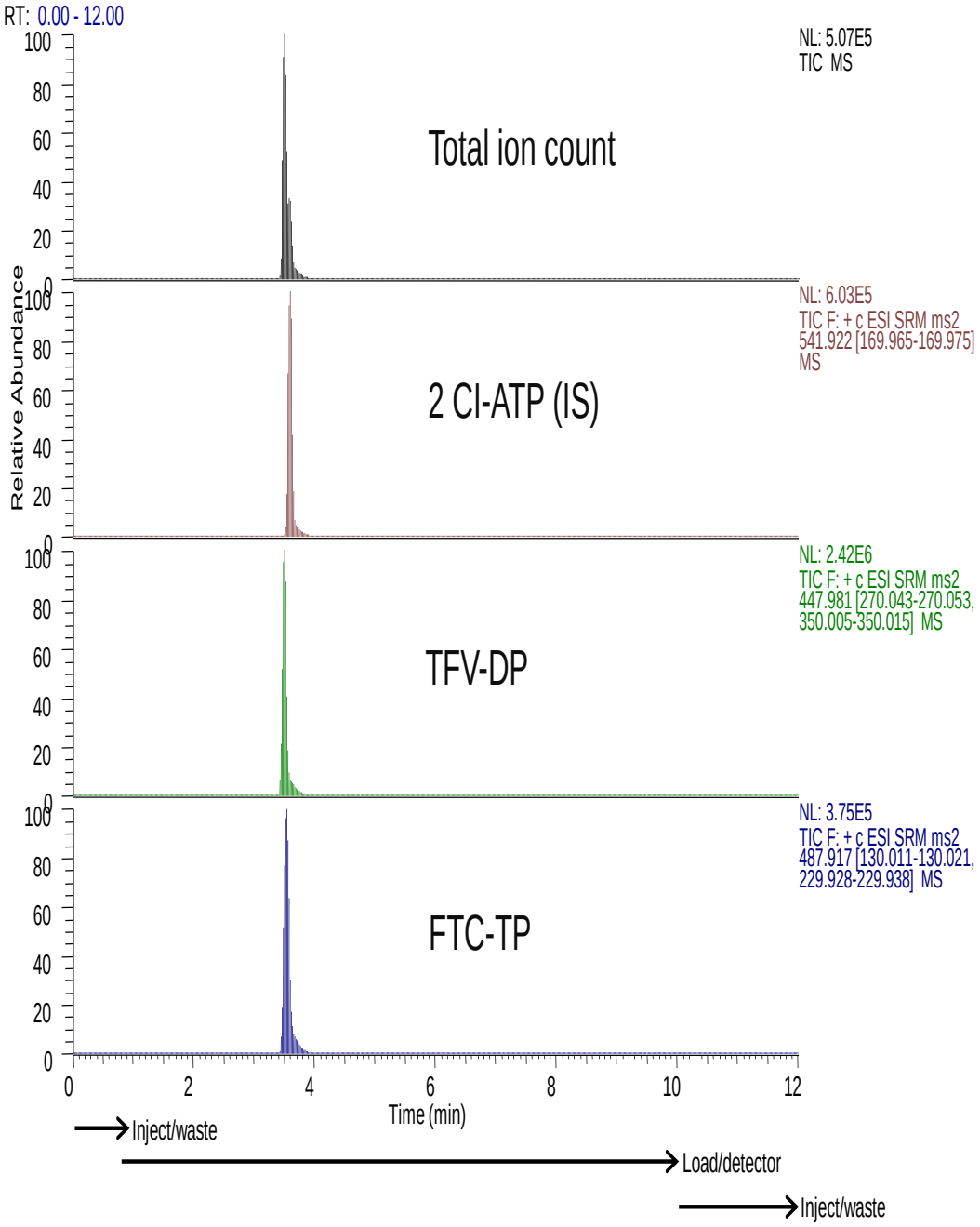

Figure 3. Chromatograph of a calibration standard showing the peak resolution for 2 Cl-ATP (internal standard), TFV-DP, FTC-TP and the total ion count (TIC). Also shown is the divert valve usage though out the assay run time.

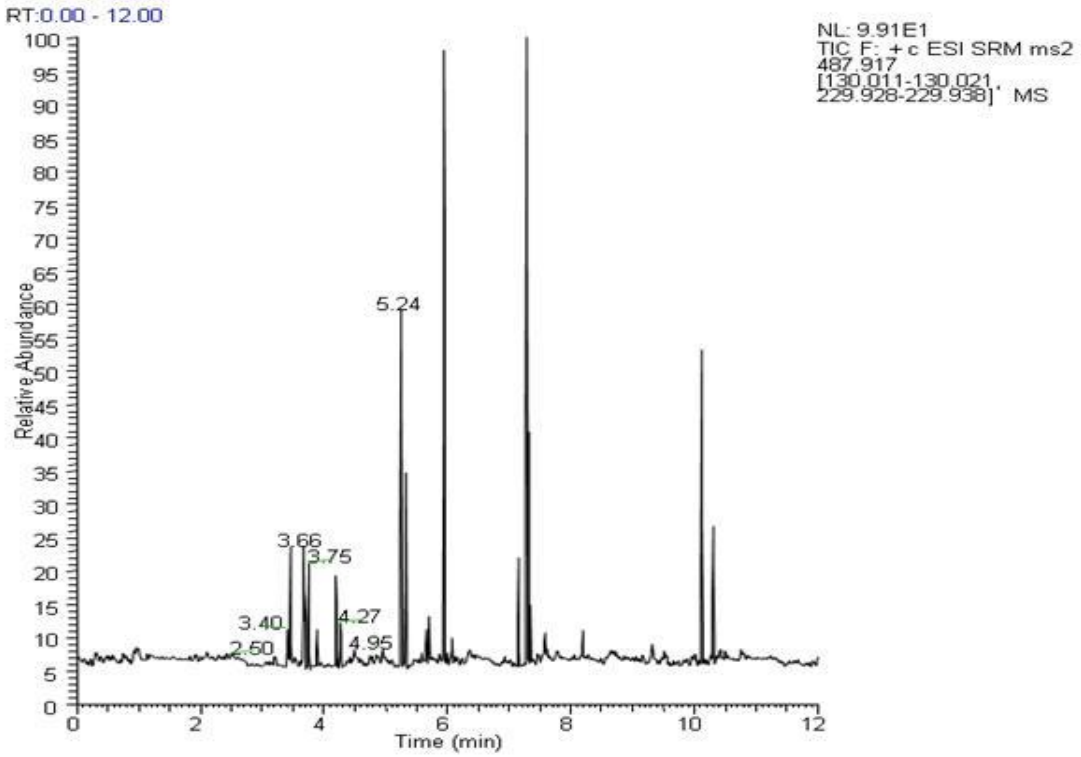

Figure 4. Chromatograph of a FTC-TP blank lysate sample.

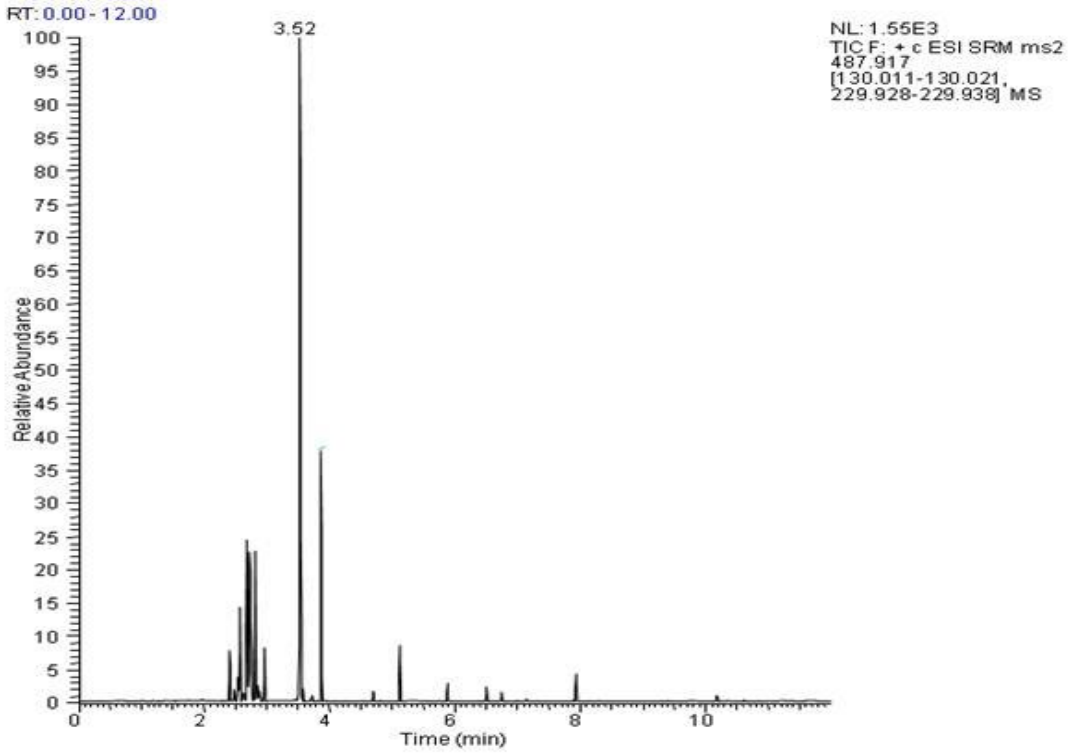

Figure 5. FTC-TP. Chromatograph of a low level (LLQ) calibrator sample (0.38 ng/mL).

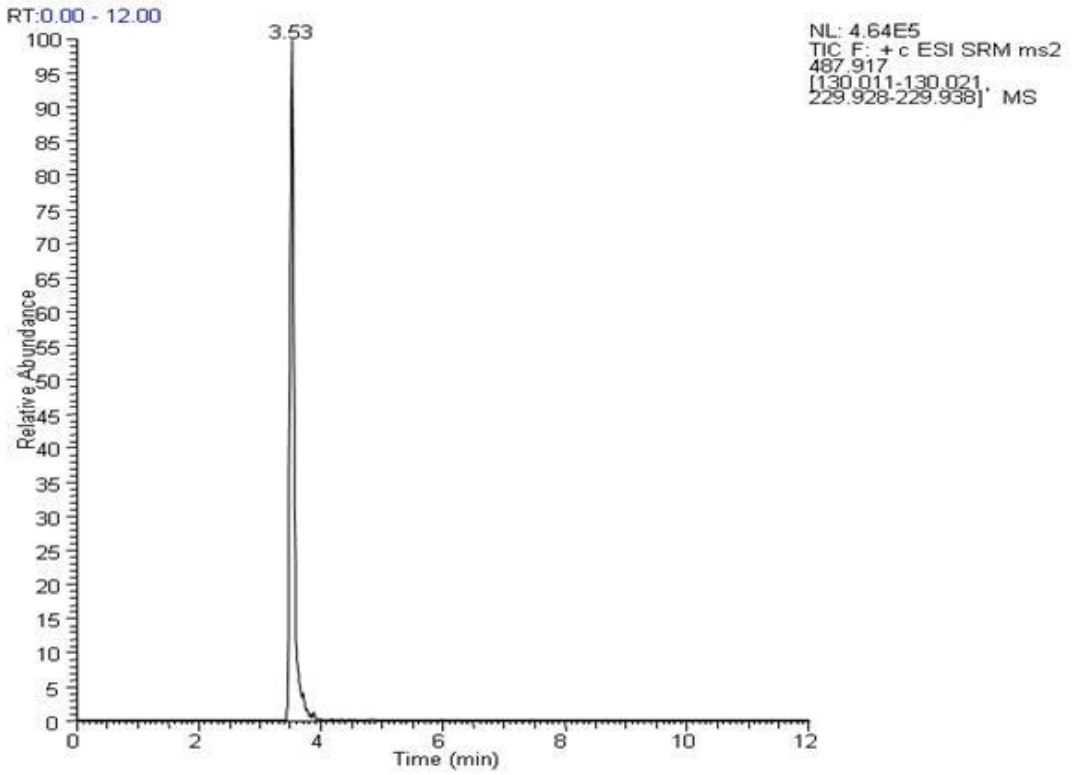

Figure 6. FTC-TP. Chromatograph of a high level (ULQ) calibrator sample (103.17 ng/mL).

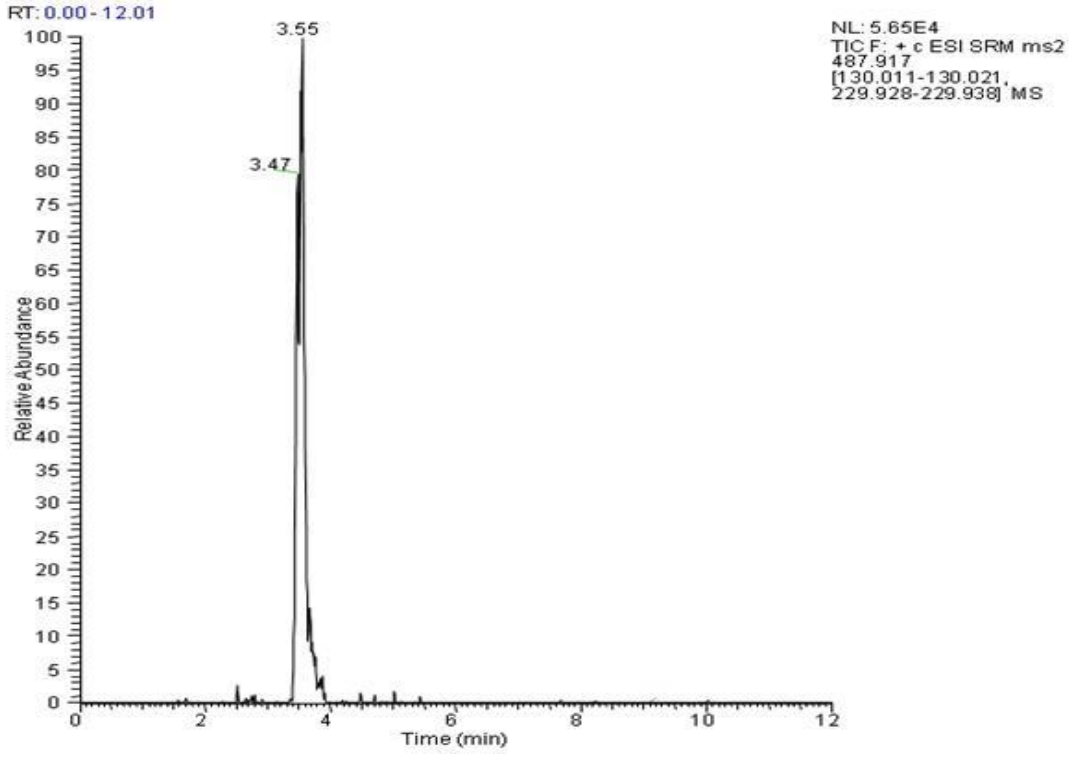

Figure 7. FTC-TP. Chromatograph of a patient sample (2.57 x 10^7 cells).

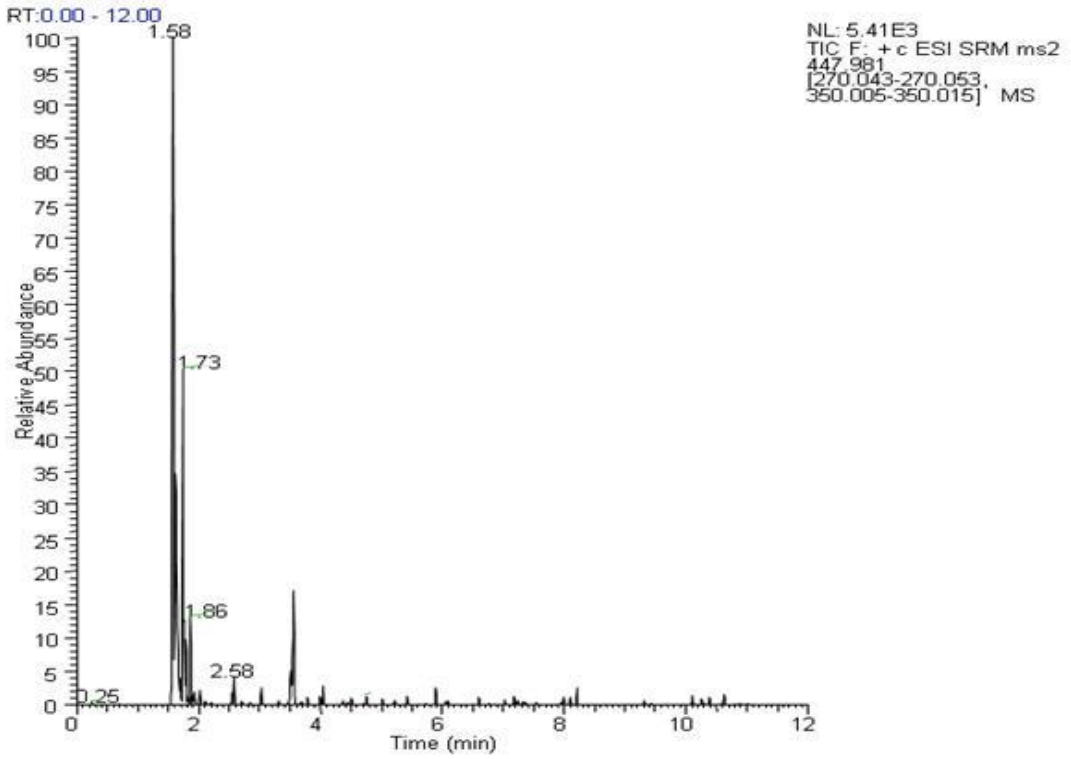

Figure 8. Chromatograph of a TFV-DP blank lysate sample.

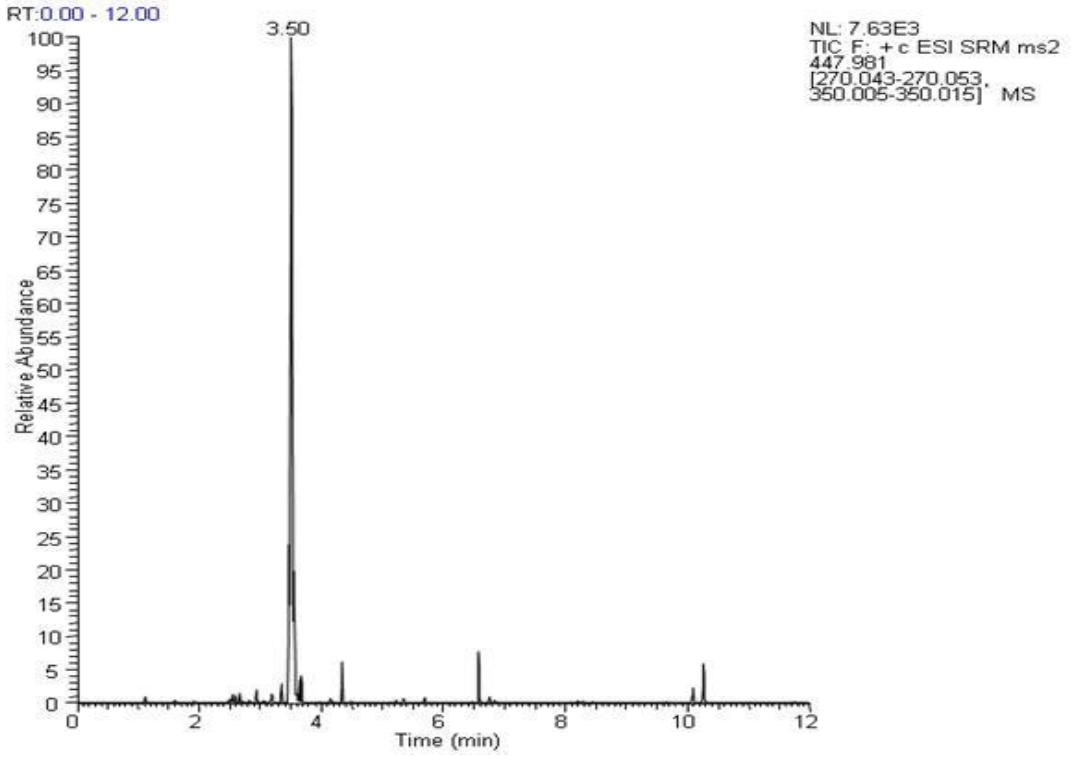

Figure 9. TFV-DP. Chromatograph of a low level (LLQ) calibrator sample (0.35 ng/mL).

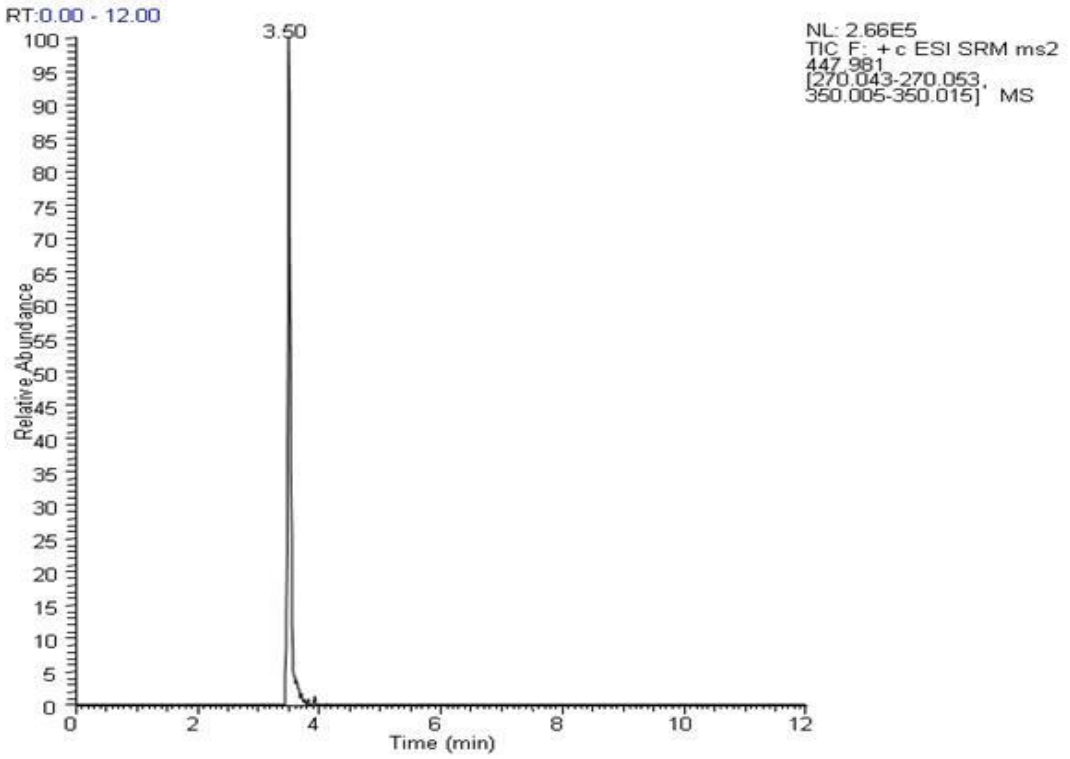

Figure 10. TFV-DP. Chromatograph of a high level (ULQ) calibrator sample (10.91 ng/mL).

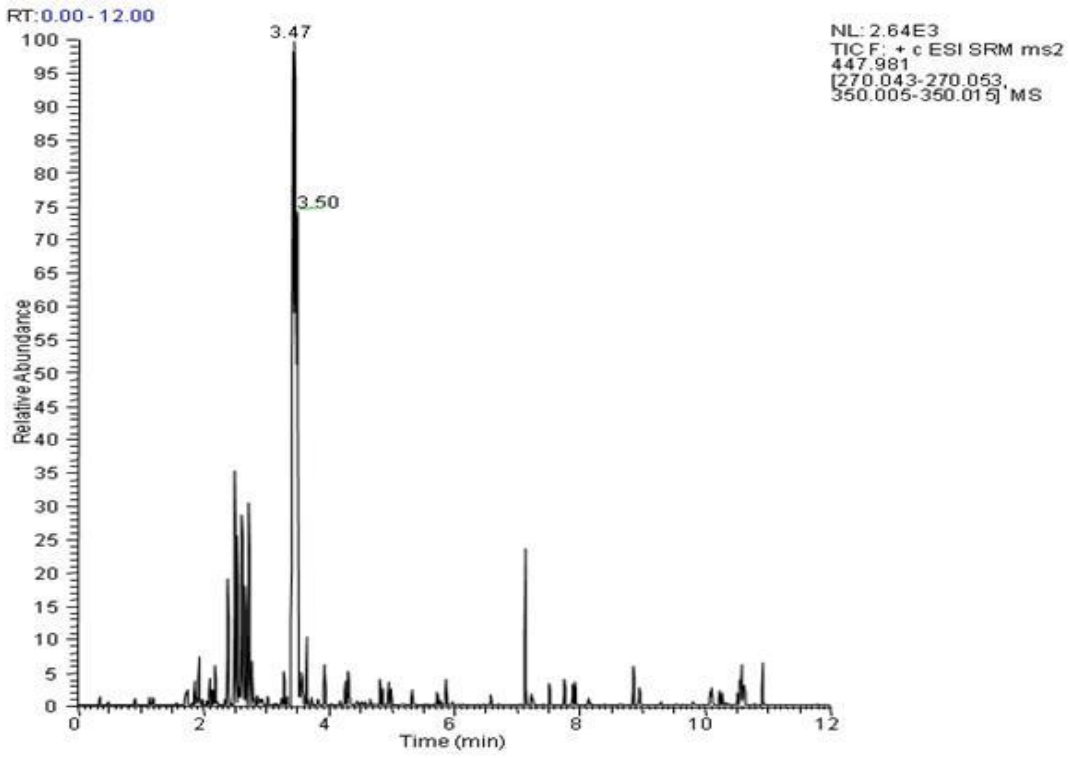

Figure 11. TFV-DP. Chromatograph of a patient sample (2.57 x 10^7 cells).

6.3.2 Accuracy and precision

The precision of this methodology describes the closeness of each individual analyte measured when it is repeatedly applied to multiple samples. The accuracy of this methodology is described by the closeness of the mean test result for each of the individual analytes when compared to the ARV stock concentration prepared. The assay was linear over the concentration ranges for both TFV-DP and FTC-TP in PBMC lysate.

The inter and intra-assay performance testing data is presented in Table 3. Accuracy was defined in terms of percentage bias and precision was defined as CV % relative to the standard deviation of the mean. All calculations were derived from quality control samples with n = 20. Both precision and accuracy was found to be below 20% for TFV-DP and FTC-TP.

Table 3. Precision expressed as the co-efficient of variation [% CV; (standard deviation / mean) x 100]. Accuracy expressed as the % bias [(mean concentration – target concentration) / (mean concentration) x 100].

	LLQC	**LQC**	**MQC**	**HQC**
FTC-TP (ng/mL)	1.39	5.44	26.64	62.01
% CV (Precision)	10.6	6.3	5.4	8.3
% Bias (Accuracy)	103	99	100	99

	LQC	**MQC**	**HQC**
TFV-DP (ng/mL)	1.17	5.39	10.41
% CV (Precision)	11	6.3	8.2
% Bias (Accuracy)	98	98	101

Assays were also assessed for their linearity, the calibration data was analysed using the linear regression of the peak area ratio (drug peak area / IS peak area), versus the nominal concentration of the sample with a weighting factor of 1 / concentration ($1/x$) which achieved a co-efficient of correlation (r^2) value greater than 0.990 for all ARV validation assays.

6.3.3 Signal to noise ratio

The signal to noise ratio was investigated to determine the lower limit of quantification (LLQ) for each of the phosphorylated anabolites. The analyte response at the LLQ should be a least five times greater than that of a blank sample response. The LLQ represents the lowest point on the standard calibration curve and therefore must be measured with accuracy and precision (co-efficient of variation should not exceed +/- 20 %). The LLQ (sensitivity) of TFV-DP was 0.35 ng/ mL and FTC-TP was 0.38 ng/ mL. Giving standard calibration curve ranges for TFV-DP and FTC-TP of 0.35-10.91 ng/ mL and 0.38-103.17 ng/ mL respectively. When using the back calculation to determine patient sample results with an accurate individual patient cell count, both TFV-DP and FTC-TP LLQ was ~30fmol/10^6 cells. Matrix effect analysis was also carried out using a varying concentration of PMBC per mL in lysate samples (as this would be representative of patient sample variability) and no significant enhancement or suppression of the analytes was shown.

6.4 Concluding remarks

A direct highly sensitive intracellular anabolites, WAX LC-MS/MS assay has been developed and validated which elutes analytes and internal standard rapidly (12 minutes) and has been successfully applied to patient samples from a pivotal trial (EudraCT 2009-018055-16), the results of which are presented in the following chapter and play a crucial part in our understanding of antiretroviral drug adherence and missed dose forgiveness [74].

Chapter 7

Tenofovir, emtricitabine intracellular and plasma, and efavirenz plasma concentration decay following drug intake cessation: Implications for HIV treatment and prevention.

CHAPTER 7

TENOFOVIR, EMTRICITABINE INTRACELLULAR AND PLASMA, AND EFAVIRENZ PLASMA CONCENTRATION DECAY FOLLOWING DRUG INTAKE CESSATION: IMPLICATIONS FOR HIV TREATMENT AND PREVENTION.

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7.3.1 Demographic and clinical characteristics

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7.3.4 Tenofovir-DP and Emtricitabine-TP intracellular pharmacokinetics

7.4 Discussion and Conclusion

Declaration of transparency: The following work was published in the Journal of Acquired Immune Deficiency Syndromes. Volume 62, Number 3, March 1, 2013. My contribution to the publication was as follows; plasma and intracellular analytical methods for all drugs, plasma and intracellular pharmacokinetic analysis for all drugs, visualisation of data, preparation and corrections of manuscript with the other authors.

7.1 Introduction

The introduction of combination antiretroviral therapy (cART) to patients infected with HIV has led to a dramatic reduction in AIDS-related morbidity and mortality [59]. The British HIV Association (BHIVA) recommends the administration of two nucleoside reverse transcriptase inhibitors (NRTI) in combination with a non-NRTI, a ritonavir boosted protease inhibitor (PI), or with the integrase inhibitor raltegravir, to therapy naïve patients [75].

As an initial first line therapy, in patients whom have a high viral load and absence of primary ARV resistance, the combination of tenofovir (TDF), emtricitabine (FTC) and efavirenz (EFV) has been shown to be highly effective [76, 77].

The success of cART is very much dependant on patient adherence to their prescribed regime each and every day, to ensure that a drug steady-state is maintained at the correct concentration level, which is neither too high causing toxicity, and not too low giving way to resistance [78].

It has been demonstrated that the lower the patient pill burden, the greater the adherence [78], therefore the arrival of Atripla® as a combined medication of TDF,

FTC and EFV given as a one tablet, once a day dose (245 mg, 200 mg and 600 mg respectively), has made adherence for the patient much easier, compared to the earlier complicated regimes consisting of many different medications taken at different times of the day, some with food, and others without. Importantly, the three components of Atripla® each show sustained half-lives, which may allow for a reduced dosing frequency and therefore increase patient adherence. The 'Five-days-On, Two-days- Off' (FOTO) study which showed HIV infected patients, taking TDF/FTC/EFV five days a week, followed by a two day interruption (or 'holiday' period), successfully maintained virological suppression in an equal number of patients taking the drug for seven days a week [79]. This data suggests that either the effects of the drugs or the actual drugs themselves, persist with a sustained efficacy for a significant duration following drug intake cessation, and may allow for a reduced intake drug regime, greater adherence, a reduced drug toxicity and a lower financial cost. However, the persistence time post 48 hours and the inter-individual variability of this efficacy for each of the components of Atripla® were not clearly defined.

The ARV drug tenofovir (TDF) is administrated as tenofovir disproxil fumerate which requires hydrolization by cellular esterase during the absorption phase to its lipophilic and cell permeable pro-drug tenofovir disproxil (TFV). Both TFV and FTC require intracellular (IC) phosphorylation to their active IC metabolites. TFV is firstly phosphorylated to its monophosphate (TFV-MP) form by adenosine monophosphate kinase and then to its active diphosphate form (TFV-DP) by 5'-nucleoside diphosphate kinase. FTC is phosphorylated firstly to its monophosphate (FTC-MP) by deoxycytidine kinase, then to its diphosphate form (FTC-DP) by deoxycytidine monophosphate kinase and then to its active triphosphate (FTC-TP)

by 5'-nucleoside diphosphate kinase. TFV-DP and FTC-TP are the IC active metabolites which inhibit viral reverse transcriptase and ultimately HIV replication. The IC metabolites show different pharmacokinetic profiles compared to those of their parent drugs within the plasma compartment, with IC concentrations characterised by a much longer prolonged half-life (TFV-DP and FTC-TP; 150 [70] and 39 [69] hours respectively, compared to TFV 14 hours [80] and FTC 8-10 hours [69]). Importantly, plasma concentrations alone may not be enough to interpret the intracellular metabolite concentrations, as the patient antiviral response of exposure to IC NRTI is not only dependent upon the amount of DP and TP formed, but on the concentrations of the endogenous deoxyribonucleotide triphosphates (dNTPs) that the metabolites competes against with, for incorporation into to the proviral deoxyribonucleic acid (DNA) [76, 81].

In this study we have assessed the 'pharmacokinetic forgiveness' of IC TFV-DP, FTC-TP, and plasma EFV over a period of 228 hours (9.5 days) after drug intake cessation in HIV-negative healthy volunteers. The pharmacokinetic results should help to provide information on how to advise HIV-infected patients on non-adherence (delayed and missed doses).

7.2 Materials and Methods

7.2.1 Participants

Healthy male and non-lactating females were determined as eligible upon medical history, physical examination, 12 lead electrocardiogram, and clinical laboratory evaluations all being satisfactory. Participants had to provide written informed

consent and were all aged between 18 and 65 with a body mass index within 18-35 kg/m^2 inclusive.

Participants were not eligible if they met any of the following exclusion criteria; any significant acute or chronic medical illness, evidence of any organ dysfunction, a positive blood test for hepatitis B surface antigen and or hepatitis C antibodies, positive blood test for HIV 1 or 2 antibodies, any current or recent gastrointestinal disease, positive urine drug screen or clinically relevant alcohol use/ or history of drug/ alcohol use which the investigator deemed could interfere with trial compliance, previous exposure (within 3 months) of investigational trial drugs or placebos, use of any co-medications including over the counter and herbal remedies unless approved by the trial investigator, and knowledge of any previous allergies to any of the trial drug constituents.

7.2.2 Study Design

This study was conducted at the Pharmacokinetic Unit of the St. Stephen's Centre, Chelsea and Westminster Hospital, London, UK, and was carried out in accordance with Good Clinical Practice (GCP), the declaration of Helsinki, and relevant regulatory requirements (EudraCT, 2009-018055-16). Ethics and study protocol were approved by the Institute of Child Health/Great Ormond Street Hospital Research Committee, UK. All participants gave written consent.

The study was a 24-day (excluding screening and follow up), open label, single treatment arm, PK clinical trial.

Day -28 to 0 comprised of screening the participants. Eligibility criteria were checked and a clinical assessment and laboratory analysis was undertaken. Upon successful screening, participants were administered one tablet of Atripla®, once a day (taken in the evening) for a total of 14 days. Intensive PK blood sampling (for plasma and intracellular analysis) was carried out 10 minutes before the final dose on the evening of day 14, and then 2, 4, 8, and 12 hours postdose whilst participants were admitted overnight at the unit. Participants were discharged after the 12 hour postdose samples were taken and returned to the unit for further sampling over the following 10 days (post cessation of Atripla®) at 24, 36, 48, 60, 84, 108, 132, 156, 180, 204, and 228 hours.

7.2.3 Study Objectives

The primary object of this trial was to assess the PK of plasma tenofovir and emtricitabine, and their active intracellular metabolites, namely tenofovir-diphosphate and emtricitabine-triphosphate, and also efavirenz plasma PK over a ten day period following drug intake cessation. To be carried out in healthy HIV negative volunteers.

Secondary objectives of this trial included the assessment of inter-individual variability in the concentrations of plasma tenofovir, emtricitabine and efavirenz and also TFV and FTC intracellular metabolites after drug intake cessation.

The safety and tolerability of Atripla® intake in healthy HIV negative volunteers over an administration period of 14 days was also assessed.

7.2.4 Plasma Collection for Tenofovir, Emtricitabine, and Efavirenz Analysis

For each scheduled PK time point, approximately, 12 mL of whole blood was collected into two Vacutainer® collection tubes (lithium heparin) from each participant. Both tubes were immediately inverted several times and then stored on ice or refrigerated before centrifugation. Blood samples were centrifuged within a 30 minutes time window, at 1200 g for 10 minutes at 4^{o}C to separate the plasma. Plasma was then carefully transferred into three (patient labelled) 2.0 mL screw top microtubes (Sarstedt Germany) before being stored at -20^{o}C. Samples were then transferred on dry ice to the Liverpool Bioanalytical Facility.

7.2.5 Peripheral Blood Mononuclear Cell Isolation for TFV-DP and FTC-TP Analysis

Two 8 mL cell preparation tubes (CPT) (Becton-Dickinson Vacutainer; Oxford, United Kingdom) were collected for each time point. The anticoagulant was mixed thoroughly by gently inverting the tubes 8-10 times. CPT were then immediately (time allowance within 60 minutes to maximize cell yield) centrifuged (horizontal rotor) for 20 minutes (1600 relative centrifugal force [rcf]) at room temperature (18-25°C). Peripheral blood mononuclear cells (PBMCs) were then suspended in the plasma by carefully inventing the CPT. The mixed plasma/ cells suspension from both cell preparation tubes were then combined into a single graduated 50 mL conical tube to which isotonic saline (0.9%) was added to bring the total volume to exactly 30 mL. The cell/ plasma sample was then gently mixed by inversion, and a 40 µL aliquot was taken for initial cell counting (Digital Bio Adam Microchip Automatic Cell Counter; NanoEnTek, Inc, Seoul, Korea). The cell count was

normalised by multiplying by 30 to calculate the actual total cell count in 30 mL and recorded. The plasma/ cell suspension was then centrifuged for 15 minutes, at 400 relative rcf to pellet the cells. The supernatant was carefully removed to avoid disruption of the cell pellet. Ice-cold methanol (precisely 1 mL, 70%) was added to the pellet to re-suspend and lyse the cells and ultimately release the intracellular drugs. The cell lysate was vortexed (3–5 minutes) and stored at −80°C, before being transported on dry ice to the Liverpool Bioanalytical Facility.

7.2.6 Quantification of Tenofovir and Emtricitabine from Plasma

Plasma samples (300 mL) were prepared in formic acid (300 mL; 0.02%) containing an internal standard, 2-chloroadenosine (Cl-A; 5 mg/ mL; 20 mL), and loaded onto a 100-mg SPE BondElut C18 column (Agilent Technologies, UK Ltd., Berkshire, UK). Columns were conditioned with methanol (1 mL) and 0.02% formic acid (1 mL; pH 3). Samples (400 mL) were loaded onto the column and rinsed with 0.02 % formic acid (200 mL). Analytes were eluted with methanol (100 mL), evaporated to dryness, reconstituted in 100 µL of mobile phase, and injected (10 µL) onto the high-performance liquid chromatography (HPLC) column. Chromatography was performed on a Synergi polar C18 column (4mm: 150 mm· 2.0 mm; Phenomenex, Cheshire, UK). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B, 0.1% formic acid in acetonitrile (ACN). Initial conditions consisted of 99% mobile phase A, increasing in organic content to 40% B in 0.7 minutes maintained over 1.3 minutes and equilibrated back to the initial conditions over a total run time of 6 minutes. The flow rate was 400 mL/ minute. The triple quadrupole mass spectrometer (TSQ Quantum Access; Thermo Electron Corporation, Hemel

Hempstead, UK) was operated in positive ionization mode, and detection and quantification was performed using multiple reaction monitoring. The assay was validated over a calibration range of 0.52–996 ng/mL (TFV) and 0.47–5368 ng/mL (FTC). Inter-day precision (coefficient of variation [CV %]) based on quality control (QC) samples was between 9 %–12.2 % (TFV) and 4.6 %–6.9 % (FTC), and accuracy (% bias) was 97.5 %–100.8 % (TFV) and 107 %–114.2 % (FTC). The percentage recovery for both analytes at the low medium and high levels were consistent, precise, and reproducible.

7.2.7 Quantification of Efavirenz from Plasma

Plasma EFV concentrations were quantified by a validated protein precipitation extraction method coupled with reverse phase HPLC–MS/MS, as previously described [76]. The assay was validated over a calibration range of 8.58–10,203 ng/mL. Inter-day precision (CV %) based on quality control (QC) samples was between 2.9 % and 5.3 % and accuracy (% bias) was between 93.2 % and 105.1 %. The percentage recovery at the low medium and high levels were consistent, precise, and reproducible.

7.2.8 Quantification of IC Tenofovir-Diphosphate (TFV-DP) and Emtricitabine-Triphosphate (FTC-TP).

IC TFV-DP and FTC-TP concentrations were quantified by a validated protein precipitation method coupled with weak anion exchange HPLC–MS/MS. ACN (1 mL) was added to each of the samples of IC lysate (1 mL in 70% methanol). The samples were vortexed and centrifuged (13,000 g, 6 minutes). The sample

supernatant was transferred into glass tubes and internal standard, 2-chloroadenosine 5-triphosphate (Cl-ATP; 4 µg/ mL; 20 mL), was added. Samples were evaporated to dryness and reconstituted in 150 mL of ammonium formate (5 mM), vortexed, and injected (20 mL) onto the HPLC column. Chromatography was performed on a BioBasic AX column (5 mm: 50 mm x 1 mm; Thermo Scientific, Hertfordshire, UK) with a pH gradient. Mobile phase A consisted of 10 mM ammonium acetate in ACN/water (30:70, v/v), pH 6, and mobile phase B consisted of 1 mM ammonium acetate in ANC/water (30:70, v/v), pH 10.5. Initial conditions consisted of 90 % mobile phase A, increasing in organic content to 50 % B in 0.51 minutes maintained over 1.25 minutes, then increased again to 100 % B, and maintained for 4.75 minutes then equilibrated back to the initial conditions over a total run time of 12 minutes. The flow rate was 250 mL/ min. The triple quadrupole mass spectrometer (TSQ Quantum Access MAX; Thermo Electron Corporation) was operated in positive ionization mode, and detection and quantification was performed using selective reaction monitoring. The assay was validated over a calibration range of 0.35–10.91 ng/ mL (TFV-DP) and 0.38–103.47 ng/ mL (FTC-TP). Inter-day precision (CV %) based on quality control (QC) samples was between 6.3 %–11 % (TFV-DP) and 6 % – 18.6 % (FTC-TP) and accuracy (% bias) was 97.5 % – 100.8 % (TFV-DP) and 98 % – 100.3 % (FTC-TP). The percentage recovery for both analytes at the low medium and high levels were consistent, precise, and reproducible.

7.2.9. Pharmacokinetic and Statistical Analysis

The calculated PK parameters for plasma TFV, FTC, EFV, and IC TFV-DP and FTC-TP, were the concentration measured 24 hours after the observed dose ($C_{24}h$),

the maximum observed concentration (C_{max}), and the area under the concentration–time curve (AUC) from 0 to t (in hours). The half-life was determined from the elimination phase within the normal dosing interval of 0–24 hours and as a terminal elimination half-life to the last measurable concentration. All PK parameters were calculated using actual blood sampling times and non-compartmental modelling techniques (WinNonlin Phoenix, version 6.1; Pharsight Corp, Mountain View, CA). Descriptive statistics, including geometric mean (GM) and 90 % confidence intervals (CI) were calculated for plasma TFV, FTC, EFV, and IC TFV-DP and FTC-TP PK parameters. Inter-individual variability in drug PK parameters was expressed as a coefficient of variation [CV = (SD/mean) x 100].

7.3 Results

7.3.1 Demographic and Clinical Characteristics

A total of 16 (six female in total) participants were recruited and completed the study. The median (range) age was 33 (22-58) years, weight was 80 (54–109) kg, and body mass index was 25 (20–34) kg/ m2. Ethnicity was as follows; 12 white, 2 black, 1 of Asian origin, and 1 defined himself as other. All study drugs were well tolerated with no grade 3 or 4 adverse events being reported.

7.3.2 Tenofovir and Emtricitabine Plasma Pharmacokinetics

Plasma TFV and FTC PK parameters are illustrated in Table 1 and concentration–time curves in Figure 1A, B, respectively. GM and 90% CI plasma terminal elimination half-life to 228 hours for TFV was 31 hours (24–45 hours) and longer

than the 0–24 hour half-life (14 hours). GM and 90% CI plasma terminal elimination half-life to 228 hours of FTC were 37 hours (33–43 hours) and was longer than the 0–24 hour half-life (6 hours). There was a marked inter-individual variability in both plasma TFV and FTC PK parameters (range, 25 %–56 %).

7.3.3 Efavirenz Plasma Pharmacokinetics

Plasma EFV PK parameters are illustrated in Table 1 and concentration–time curves in Figure 1C. GM and 90 % CI plasma terminal elimination half-lives to 24 hours was 28 hours (21–47 hours), to 48 hours was 51 hours (43–74 hours), and to 228 hours was 92 hours (84–108 hours). Five of 16 participants had concentrations below the suggested MEC of 1000 ng/mL 48 hours postdose and 50 % maintained concentrations greater than the suggested MEC 84 hours postdose. There was high inter-individual variability in EFV PK parameters (range, 40 %–77 %).

Table 1. Plasma tenofovir (TDF), emtricitabine (FTC), efavirenz (EFV) and tenofovir-diphosphate (TFV-DP) and emtricitabine-triphosphate (FTC-TP) pharmacokinetic parameters

		Plasma		
PK parameter		**TFV, GM (90% CI)**	**FTC, GM (90% CI)**	**EFV, GM (90% CI)**
C_{24}	ng/mL	39 (35-47)	89 (75-121)	1834 (1530-2957)
C_{MAX}	ng/mL	181 (157-237)	1538 (1422-1748)	3747 (3345-4661)
AUC_{0-24}	ng/h/mL	1699 (156-2060)	10,768 (9899-12,208)	58,367 (49,856-84,841)
AUC_{0-last}	ng/h/mL	2895 (2600-3491)	13,863 (12,709-15,876)	231,569 (187,361-409,449)
TE $t_{1/2}$	hours	31 (24-45)	37 (33-43)	92 (84-108)
		Intracellular		
PK parameter		**TFV-DP, GM (90% CI)**		**FTC-TP, GM (90% CI)**
C_{24}	fmol/10^6 cells	41 (39-78)	pmol/10^6 cells	4 (3-6)
C_{MAX}	fmol/10^6 cells	62 (57-107)	pmol/10^6 cells	7 (6-12)
AUC_{0-24}	fmol/h/10^6 cells	925 (853-1712)	pmol/h/10^6 cells	100 (90-63)
AUC_{0-last}	fmol/h/10^6 cells	4717 (4501-8591)	pmol/h/10^6 cells	289 (261-440)
TE $t_{1/2}$	hours	164 (152-190)	hours	39 (36-45)

AUC_{0-24}, area under the curve over 24 hours; $AUC_{0-last,}$ area under the curve to the last measureable concentration within 228 hours; TE $t_{1/2}$, terminal elimination half-life to the last measurable concentration within 228 hours; TFV, tenofovir; FTC, emtricitabine; EFV, efavirenz; TFV-DP, tenofovir-diphosphate; FTC-TP, emtricitabine-triphosphate.

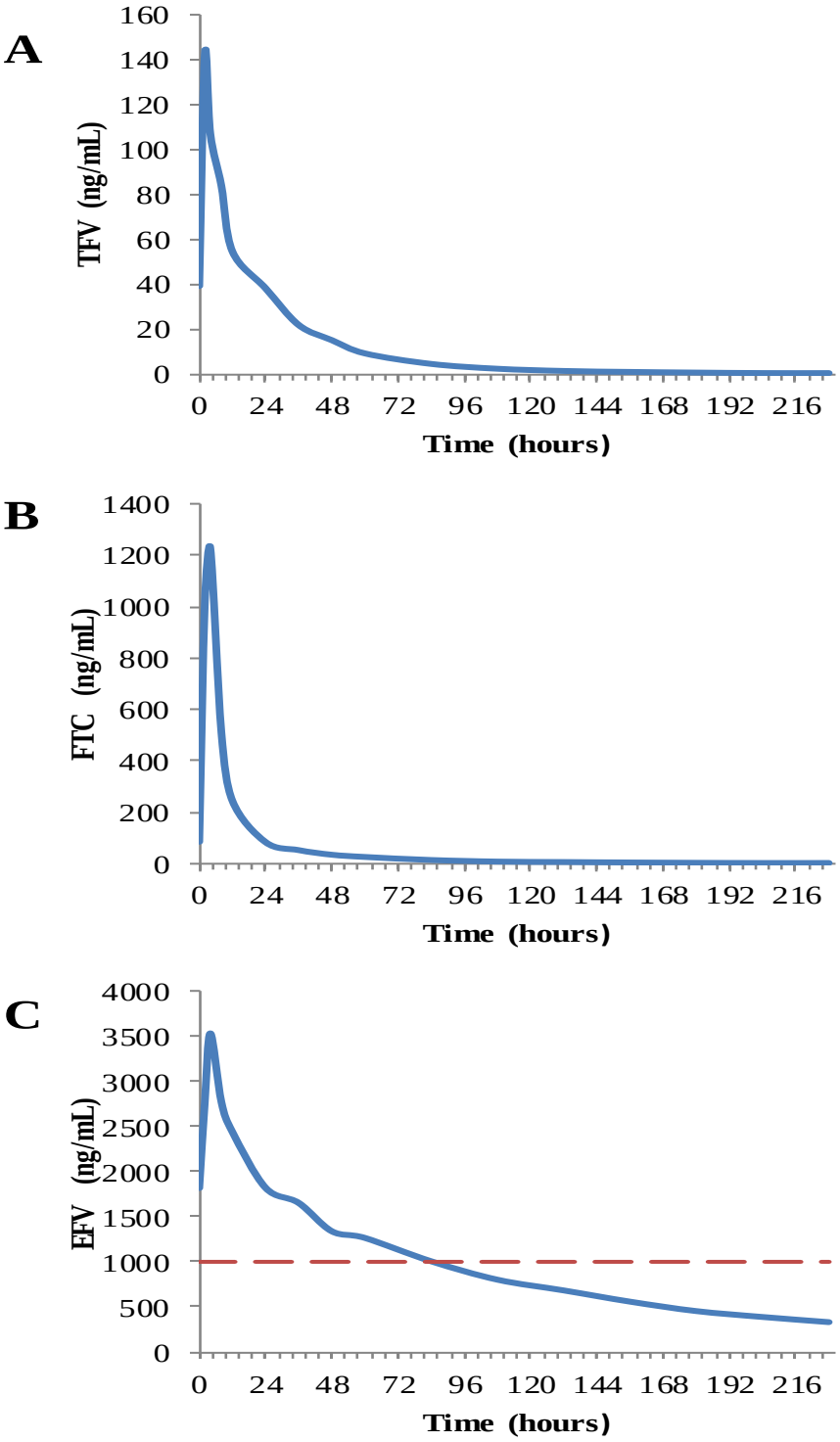

Figure 1. Geometric mean (GM) steady-state plasma concentrations 228 hour post drug intake cessation for (A) tenofovir, (B) emtricitabine and (C) efavirenz. The dashed line on the efavirenz graph illustrates the minimum effective concentration (MEC) of efavirenz which is 1000 ng/ mL.

7.3.4 Tenofovir-DP and Emtricitabine-TP Intracellular Pharmacokinetics

IC TFV-DP and FTC-TP PK parameters are illustrated in Table 1 and concentration–time curves in Figure 2 A, B, respectively. TFV-DP GM and 90 % CI IC terminal half-life to 228 or the last measured concentration was 164 hours (152– 190 hours). FTC-TP GM and 90 % CI IC terminal half-life to 228 hours of FTC-TP was 39 hours (36–45 hours). There was marked inter-individual variability in TFV-DP and FTC-TP PK parameters (range, 64 %–79 %).

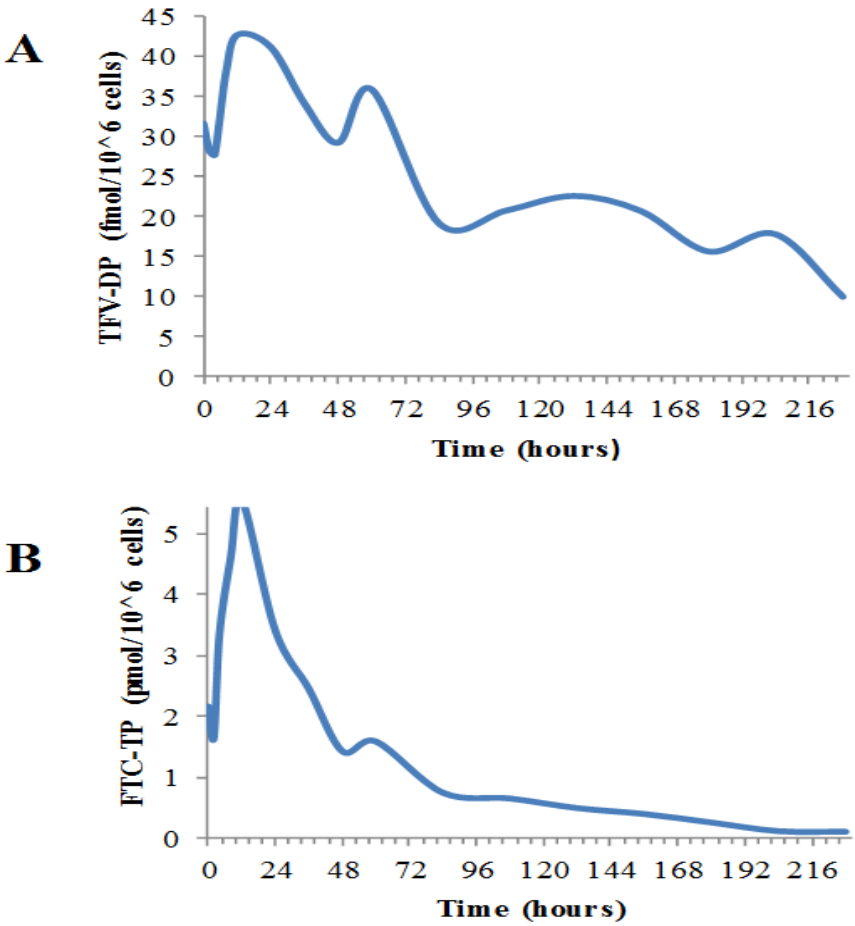

Figure 2. Geometric mean (GM) steady-state intracellular concentrations for 228 hours post drug intake cessation for (A) tenofovir-diphosphate and (B) emtricitabine-triphosphate.

7.4 Discussion and Conclusions

Here reports the PK concentrations of the antiretroviral drugs TFV, FTC and EFV in a total of 256 plasma samples, and TFV-DP and FTC-TP paired intracellular samples after the administration of the co-formulated antiretroviral drug Atripla® (TDF/FTC/EFV, 300/200/600 mg once daily, respectively). Both plasma and IC sampling from 16 healthy HIV-negative participants (male and female) was carried out over a period of 228 hours (9.5 days) after drug intake cessation. This was to investigate the decay of each of the individual drugs when given in the co-formulation Atripla®. No other study has previously looked at the PK decay of these ARVs post 10 days drug cessation, therefore this study is the first to fully characterise the PK data for missed or late dosing (forgiveness) of TFV/FTC/EFV.

The IC metabolite concentrations of both TFV and FTC determine their clinical therapeutic effect. This trial showed both are characterised by prolonged terminal half-lives (164 and 39 hours respectively). Given that EFV is also characterised by a long terminal half-life (92 hours), the co-formulated Atripla® tablet, does indeed allow for missed or late dosing in non-adhering patients, thus providing a PK regimen which is 'forgiving'. However, the current published data does not adequately describe the optimal target concentrations for IC TFV-DP and FTC-TP in successful HIV therapy and prevention (TFV and FTC are currently given as a HIV chemoprophylaxis; more commonly referred to as pre-exposure prophylaxis [PrEP] [82, 83]), and therefore, there is a need for further understanding of their clinical pharmacology.

The results here showed that EFV concentrations had a high level of inter-individual variability during the decay phase. This suggests, that in some patients with

prolonged EFV terminal half-lives, it is a result of a CYP2B6 polymorphism causing slower metabolism of EFV [84].

Importantly, this data also showed a slightly longer geometric mean EFV terminal half-life to that which was previously described during its development (multiple dose terminal half-life 40-55 hours) [85], and previously published PK data in HIV-infected patients stopping EFV intake [83, 86]. Within this area of interest, and impact on HIV treatment, the data suggests that this could be beneficial in cases of non or sub-optimal adherence, and in particularly when cases are not straight forward i.e. initiation of therapy in a patient with a high viral load. These long terminal half-lives may also be the reason why the combination shows greater superiority to other ARV combinations in published trials [77].

In non-adherent patients the concentration of ARVs are reduced. As a result of this, the ARV minimal effective concentration (MEC) at which the drug is known to suppress the virus can become compromised thus allowing the virus to once again replicate. This viral rebound accelerates the rate of viral replication which is HIV-drug-resistant, therefore, non-adherence can be directly related to ARV drug resistance [83, 87]. The level of non-adherence is crucial in knowing how many doses can be missed before this occurs. As we are aware, multiple missed doses can cause serious pharmacological effects, but we are still unaware of the impact of delayed / missed doses or controlled reduced drug doses can have on the long term effect of ARV therapy. Therefore the data presented here, indicates the level of drug persistence post drug cessation, for EFV and the IC phosphorylated metabolites of TFV and FTC, increasing our understanding of their PK forgiveness. A greater knowledge of PK forgiveness plays an important part in HIV prevention when we begin to look at the use of ARVs in PrEP. This is because for an ARV drug to be an

idea candidate for PrEP use, it must show a PK profile which is forgiving and allows for reducing dosing whilst sustaining a virological efficacy.

The study data shows a wide level of inter-individual variability in the concentrations of the IC phosphorylated metabolites TFV-DP and FTC-TP. This may be due to individual variation in the level of cell transporters which are required to uptake the drugs into the cells where they are then phosphorylated to their active form, it may also be due to lack or response of the kinases within the cell which are ultimately responsible for the steps in the phosphorylation of the TFV and FTC into their active forms [82, 88]. At present, this variability cannot be assessed in the terms of efficacy because one of the limitations of this study highlights the lack of PK knowledge known. We are aware of EFV decay, which allows us to establish an MEC (1000 ng/ mL), and this enables us to understand clinical therapeutic drug monitoring (TDM) for patients taking EFV. However, it is still unclear as to what the target concentrations of the IC diphosphate and triphosphates should be, and therefore, no MEC has been established. In order to play a key role in PrEP, further PK research is required to investigate IC accumulation and persistence of these drugs in vaginal and rectal tissue.

This study enrolled HIV-negative participants to investigate Atripla® cessation. Rather than enrolling HIV-infected patients, HIV-negative participants avoided withholding established ARV therapy whilst we examined the effect that this would have. The involvement of HIV-negative patients may be seen as a limitation of the study as the data may or may not be influenced by absence of the virus [69, 77, 81, 89]. A final limitation of this study was the amount of time participants were given Atripla® for (14 days). It may be that a period of greater length is needed to reach a PK steady-state. However published data demonstrates that after only a few doses,

concentrations of IC TFV-DP and FTC-TP achieve a steady accumulation of level which plateaus [89, 90].

In conclusion, this study looked at the cessation of the co-formulated drug Atripla®, and highlighted high concentration and prolonged terminal half-lives of the study drugs. Although further investigations are required to determine exactly how many reduced doses may result in therapeutic failure, this data increases our understanding of PK forgiveness for Atripla® when non-adherence occurs. This information also helps us to better understand the role of Atripla® in HIV prevention.

Chapter 8

Concluding Comments

The work conducted in this thesis shows the feasibility to pharmacologically characterise ARV drugs and their metabolites exposure within plasma, PBMCs and anatomical sanctuary sites through the use of the newly developed LC-MS/MS assays. The data presented yields an important insight into the effectiveness of current HIV antiretroviral therapy, a greater understanding of drug sanctuary sites and guidance for missed or late dosing.

The LC-MS/MS assays developed and validated facilitate the pharmacology of ARV drugs in proposed sanctuary sites, namely PBMCs and CSF, shown in the numerous collaborative clinical trials. The interpretation of the clinical relevance of this analysis and the results obtained here rely upon the production of the standard operating procedures produced in the validation of each LC-MS/MS assay methodology. The simultaneous detection and quantification of ARVs within the produced assays also allow the investigation of whole drug regimes in combination therapy instead of single drug analysis. Benefits are expected to accrue in design of more forgiving ARV therapy regimes for HIV patients, and better drug selection to specifically target HIV replicating within sanctuary sites.

Whilst developing and validating the IC metabolite assay, I further incorporated the dNTPs to enhance the assay. However, time boundaries did not allow for the full vigorous validation procedures to be completed and therefore were not presented here. This is an area which should be researched and conducted in the future.

Further future work from this thesis would also include looking at the transfer of the described LC-MS/MS assays to other antiretroviral agents used in the therapy for viral diseases such as hepatitis C (HCV) and cytomegalovirus (CMV). The design of the LC-MS/MS assays could also be applied and modified to other matrices, mainly

genital tract (vaginal tissue) and seminal fluid in order to investigate ARVs in the use of pre-exposure prophylaxis (PrEP) and further our knowledge of HIV prevention measures and transmission studies.

Future use of the CSF LC-MS/MS could also be applied to more clinical studies investigating cognitive impairment. The ability to measure the concentration of each ARV gaining access to cerebral fluid and the brain may allow more informed clinical prescribing in prevention of HIV associated neurological disorders.

In conclusion, the methodologies developed in this thesis will play an essential role in future studies of HIV ARV therapy as well as the treatment of other chronic diseases.

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APPENDICES

Published Research Papers

(See list at start of thesis)