2013

Intracellular Quantitation And Pharmacokinetic Modeling Of Nucleoside Analogs In The Female Genital Tract

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INTRACELLULAR QUANTITATION AND PHARMACOKINETIC MODELING OF NUCLEOSIDE ANALOGS IN THE FEMALE GENITAL TRACT

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2013

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ABSTRACT

Human Immunodeficiency Virus (HIV) is one of the leading infectious diseases in the world. In spite of substantial progress in the advancement of antiretroviral therapy for HIV treatment, new infections outpace the number of infected persons initiating ART. Therefore, the prevention of HIV remains a crucial health issue. Pre-Exposure Prophylaxis, (PrEP), which involves using one or more antiretroviral agents to reduce the risk of HIV infection prior to potential exposure, has shown effectiveness at reducing HIV transmission in study volunteers with different sexual orientation around the world. The clinical pharmacology of antiretroviral drugs, specifically tenofovir (TFV) and emtricitabine (FTC), in relation to their ability to prevent HIV transmission is still being evaluated.

The objective of this dissertation was to develop pharmacokinetic models of TFV and FTC in the plasma and eventually peripheral blood mononuclear cells (PBMCs) and the female genital tract (FGT), which can be used to simulate the appropriate dosing interval needed to achieve optimal drug concentrations for PrEP in each compartment.

The development of liquid chromatography/ mass spectrometry (LC/MS/MS) methods resulted in successful quantification of TFV and FTC in plasma, PBMCs, cervical fluid, and endothelial cells of the FGT from 30 HIV-seropositive (HIV+) women. Also, utilizing the LC/MS/MS methods developed, *in vitro* TFV and FTC uptake in non-monocyctic genital tract cells was determined and compared in the presence and absence of hormonal contraceptives. The major results demonstrated 1) CD4+ and squamous epithelia cells exhibited significantly increased uptake relative to control for both antiretrovirals; 2) individually, synthetic estrogen and progesterone significantly altered uptake across all cell lines except squamous epithelium cells for TFV and CD4+ cells for FTC; and 3) CD8+ and dendritic cells demonstrated decreased uptake of TFV and FTC when dosed one hour prior to being dosed with combined hormonal contraceptives.

Development of predictive pharmacokinetic models of TFV and FTC concentration-time profiles in the plasma was achieved using population pharmacokinetic analysis software. Collectively, the results expand upon current knowledge of antiretroviral penetration into the FGT for the use of evaluating PrEP agents and help guide PrEP research on ideal dosing for prevention and transmission of HIV.

Keywords: Human Immunodeficiency Virus (HIV), Pre-Exposure Prophylaxis (PrEP), Female Genital Tract (FGT), Tenofovir (TFV), Emtricitabine (FTC), Population Pharmacokinetic Modeling

DEDICATION

I dedicate this dissertation work to my parents, Mr. Milford Francis James Jr. and Mrs. Dorothy Marie James, who are the most amazing parents in the world. Thank you for helping me reach my dreams. I would also like to dedicate my work to some very important people who are no longer with us, but have never left me; Ms. Charlotte Ann Perry, Ms. Yvonne Marie Harris, Mrs. Barbara Mae James, Mr. William Quarles, Uncle Red, and Ms. Patricia Rae Harris. I hope I have made you all very proud.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Jennifer R. King, for giving me the chance to work on a topic that is near and dear to me and for allowing me into her lab when she was still working very hard to establish herself as an independent principal investigator. Thank you for taking a chance on me. I would also like to extend a special thank you to Dr. Edward P. Acosta, who provided me with a lab home, for overseeing my project, and allowing me to take the lead and expand his lab into new scientific territory. The joint support from both Drs. King and Acosta is invaluable and I am forever indebted to them.

I would also like to thank the members of my committee: Drs. Barnes, Johnson, Ofotokun, and Prichard. Each of you has provided guidance, critical insight, encouragement, and enthusiasm, and dedication to my personal success and my advancement in scientific research. I would like to personally thank Dr. Barnes for fueling the fire on my love for the mass spectrometry and helping me realizing its true relevance and potential. I would like to thank Dr. Johnson for helping me establish my scientific voice and realizing my value as a scientist. I would like to acknowledge Dr. Prichard for his clinical insight and real life application. And I would be remiss if I did not personally thank Dr. Ofotokun for his invaluable collaboration. Dr. Ofotokun and Dr. Anandi Sheth have been essential in my success thus far, by providing the patient samples, critically reviewing my work and the immeasurable encouragement to think critically. I thank you both along with the entire Ofotokun lab group.

I would like to thank former and current members of the King and Acosta lab, including: Kedria Reed-Walker, Corenna Kerstner-Wood, Dr. Chantelle Hood, Dr. Kajal

Buckoreelall-Larson, Daniel Ross, Matthew Smith, and Kimberly Burgess for being my family away from home and for providing me with so much support even when I said, “I CAN’T.” The time I spent here with you ladies and gents I will cherish forever.

I would also like to thank Dr. Louis Dale and the Office of Equity and Diversity. The funding, which was provided to me allowed my experience here to be catered to my academic excellence instead of funding. I would also like to thank the Becky Warnix, Rachelle Hall, and Jennifer Spears for being there for technical support so often. Thanks.

I cannot end this without thanking my family for their continuous support. The James, Harris, Beverly, Bodden, Bulgar, Cabral, Colson, DeMello, Fernald, Gomes, Grace, Hall, Holland, Johnson, Morrissey, Pearson, Perry, Pichard, Senna, Shell, Sloan, Stubbs, Trombly, and Turner families are the source of my strength. I thank you.

I would also like to personally acknowledge my sister, Alisha Marie James, the James’ Girls forever, my brother Milford “Butchie” Francis James III, and my “other” sisters Kharisma James and Shantelle Perry. Special thanks to my Uncle Ray, my cousin Rayana Grace, my besties; Courtney Wilkins, Leanna Pearson, and Dawn Spencer, and my guys; Josh Grimes, Jerome Higgs, Ron Davis, Marquelon Sigler, Carl Frizell, Dwayne Benton, and Aaron Fitzgerald.

I would be remiss not to mention my boys, Curtis Jr., Camren, and Cyle and my beautiful goddaughter, Madelyn Ruby; the sky is your limit. Finally, my church family (CJ, JJ, DW, JW, LY, GS, and TS) at Living Stone Temple, thank you all for your prayers, words of encouragement, continuous prayers, and allowing me to develop an incredible relationship with God through your guidance.

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LIST OF ABBREVIATIONS

3TC	lamivudine
ABC	abacavir
AcN	acetonitrile
ACV	acyclovir
AIDS	Acquired Immunodeficiency Syndrome
APV	amprenavir
ARVs	antiretrovirals
ATV	atazanavir
AUC_N	area under the concentration-time curve at time N
AZT	zidovudine
BLQ	below the level of quantitation
C_{24}	concentration at 24 hours
C_6	concentration at 6 hours
CCR5	Chemokine Receptor 5
CDV	cidofovir
CE	collision energy
CL/F	apparent oral clearance
C_{last}	last observed concentration
C_{max}	maximum/peak concentration
C_{min}	minimum concentration

CMV	cytomegalovirus
CNT	concentrative nucleoside transporter
COCs	combined oral contraceptives
CV	coefficient of variation
CVF	cervicovaginal fluid
CVF_{DA}	CVF collected by Direct Aspiration
CVF_{WS}	CVF collected by TearFlo™ Strips
CVL	cervicovaginal lavage
CXP	collision exit potential
CYP	cytochrome
d4T	stavudine
ddI	didanosine
DMPA	depot medroxyprogesterone acetate
DNA	deoxyribonucleic acid
DP	declustering potential
DRV	darunavir
EE	ethinyl estradiol
EFV	efavirenz
ESI	electrospray ionization
ET	etonogestrel
ETV	etravirine
FA	formic acid
FDA	United States Food and Drug Administration

FGT	female genital tract
FOCEI	first order conditional estimation method with interaction
FPV	fosamprenavir
FSH	follicle-stimulating hormone
FTC	emtricitabine
FTC-DP	emtricitabine-diphosphate
FTC-MP	emtricitabine-monophosphate
FTC-TP	emtricitabine-triphosphate
GCLP	Good Clinical Laboratory Practice
gp	glycoproteins
GVC	ganciclovir
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HPO	hypothalamic-pituitary-ovarian
HQC	high quality control
IC_{50}	effective inhibitory concentration
IDV	indinavir
Ig	immunoglobulim
IPA	isopropanol
IS	internal standards
IV	intravenous
Ka	oral absorption rate constant
KCl	potassium chloride

ke	elimination rate constant
LC/MS/MS	liquid chromatography coupled with Mass Spectrometry
LH	luteinizing hormone
LNG-IUS	levonorgestrel intrauterine system
LPV	lopinavir
MCX	mixed cation exchange
MDRP	multidrug resistance protein
MeOH	methanol
MP	mobile phase
MS	master stock solutions
MVC	maraviroc
NaOAc	sodium acetate
NaOH	sodium hydroxide
NFV	nelfinavir
NH_4OH	ammonium hydroxide
NIH	National Institutes of Health
NK	natural killer cells
NNRTIs	Nonnucleoside Reverse Transcriptase Inhibitors
NONMEN	nonlinear mixed effects modeling
nPEP	nonoccupational post exposure Prophylaxis
NRTIs	Nucleos(t)ide Reverse Transcriptase Inhibitors
NVP	nevirapine
OAT	organic anion transporter

OCT	organic cation transporter
PAR	peak area ratio
PBMCs	peripheral blood mononuclear cells
pcVPC	prediction corrected visual predictive check
PEP	Post Exposure Prophylaxis
P-gp	P-glycoprotein 1
PIs	Protease Inhibitors
PMPA	9-*R*-2 phosphonomethoxypropyl adenine
POPs	progesterone-only pills
PrEP	Pre-Exposure Prophylaxis
Q/F	intercompartmental clearance
QC	quality control
r^2	coefficient of determination
RAL	raltegravir
RNA	ribonucleic acid
RPV	rilpivirine
RT	retention time
RTV	ritonavir
SD	standard deviation
SE	standard error
SQV	saquinavir
STI	sexually transmitted infections
t½	elimination half-life

T-20	enfuvirtide
TDF	tenofovir disoproxil fumarate
TFA	trifluoroacetic acid
TFV	tenofovir
TFV-DP	tenofovir diphosphate
TFV-MP	tenofovir monophosphate
T_{max}	time to maximum concentration
TPV	tipranavir
UFLC-$_{XR}$	ultra-fast liquid chromatography
V	apparent volume
Vc/F	central volume of distribution
Vd	volume of distribution
Vd/F	apparent volume of distribution
Vp/F	peripheral volume of distribution

CHAPTER 1

INTRODUCTION

HIV and Antiretrovirals

Brief Overview of HIV

Human Immunodeficiency Virus (HIV) is a ribonucleic acid (RNA) virus transmitted by biological fluids. HIV causes Acquired Immunodeficiency Syndrome (AIDS), a human autoimmune disease which leads to lethal opportunistic infections (Weiss, 1993). To date, 60 million individuals have been infected with HIV and over 25 million individuals worldwide have died from HIV-related causes since the conception of this epidemic (UNAIDS/WHO, 2008). In 2011, an estimated 34.2 million individuals in the world were living with HIV and 2.5 million of those individuals were newly infected (UNAIDS/WHO, 2009). The largest population of HIV-seropositive individuals is in Sub-Saharan Africa; however, the United States is not immune to this epidemic. Since the early 1990s, the number of new infections per year has remained stable; yet, it is 40% higher than what experts predicted. Intervention on many levels is needed (Supervie, Garcia-Lerma, Heneine, & Blower, 2010). The introduction of antiretroviral (ARV) therapy to the United States in 1995 and globally in 1998 has decreased the rate of AIDS-related mortality and morbidity and improved the lives of individuals who are HIV-seropositive. However, obstacles to successful treatment outcomes, such as availability

to antiretrovirals, adherence to therapy, and resistance to antiretroviral regimens remain (Murphy et al., 2001). HIV is transmitted through the exchange of bodily fluid by: 1) unprotected sex with a person who has HIV, especially unprotected anal sex in comparison to unprotected vaginal sex, 2) sharing needles, syringes, or other drug paraphernalia, and 3) mother to child transmission during pregnancy, childbirth, and breastfeeding ("HIV and Its Treatment: What You Should Know," 2011).

HIV has two subtypes that have been well characterized: HIV-1 and HIV-2. HIV-1 is more lethal, more infective, (Gilbert et al., 2003) and is the cause of the majority of HIV infections globally. HIV-2 is less lethal and is found mainly in Africa. HIV-1 and HIV-2 package their RNA differently, most likely leading to the lower infectivity and fewer cases of infections per exposure with HIV-2 compared with HIV-1. However, once infection with HIV-1 or HIV-2 is achieved, HIV progressively weakens the immune system. Vital immune cells are destroyed, increasing the body's susceptibility to illness, which promotes the acquisition of life-threatening opportunistic infections and cancers. The lethality of HIV is based on the virus's ability to infect vital cells in the human immune system such as CD4+ T cells, macrophages, and dendritic cells (Reeves & Doms, 2002). Invasion of the virus lowers these immune cells by two notable intrinsic cellular mechanisms: increases apoptosis of the infected cells and cell-mediated death triggered by cytotoxic CD8+ lymphocytes of the infected cells.

Cellular HIV replication consists of three major steps: cell entry, followed by transcription and translation, and finally, assembly and release of the HIV virion. HIV contains a complex protein, Env, which consist of two glycoproteins (gp)-120 and gp-41 that are embedded in the viral envelope (Moris et al., 2006). HIV enters the cell by

facilitating a stable interaction between glycoproteins on its surface to chemokine receptors (usually CCR5 or CXCR4) on the target cell. Entry of the HIV particle into the cell is mediated by fusion of the viral envelope gp-120 with the cell membrane's CD4 protein creating a structural change in the viral envelope. Subsequently, the chemokine binding domains of gp120 are exposed, allowing them to interact with the target chemokine receptor. This interaction allows for penetration of gp-41 into the cell membrane and release of the HIV capsid into the cell. Figure 1 highlights the main structural aspects of the HIV-1 virion.

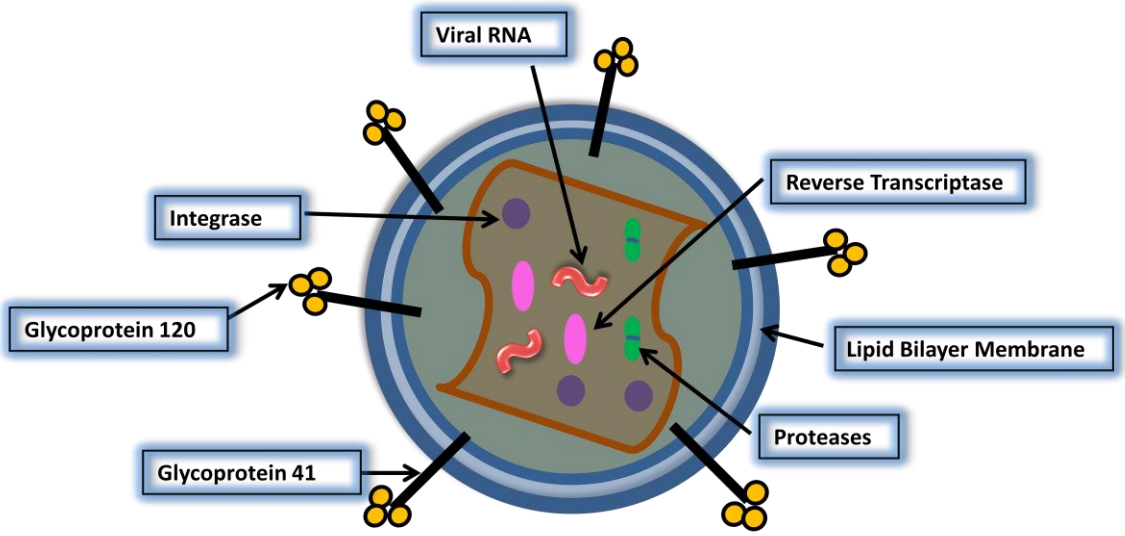


Figure 1. HIV-1 Virion

After entry into the target cell, reverse transcription begins. Reverse Transcriptase is utilized to form viral double-stranded deoxyribonucleic acid (DNA) from the release of viral RNA by viral proteins (Chan & Kim, 1998). This viral DNA is then transported into the target cell's nucleus. Once inside the nucleus, viral integrase facilitates the integration of virus into the target genome and the viral DNA is transcribed

to viral messenger RNA. At this point in the HIV life cycle, the virus can either 1) enter latency, meaning the virus does not affect the continuous function of the target cell or 2) activate which leads to mass replication and production of viral particles.

After the viral mRNA is transcribed, replication continues with the translation and production of viral regulatory and structural proteins. These proteins, specifically Env polyproteins, are essential to new virus production and accumulate in the nucleus of the target cell. (Zheng, Lovsin, & Peterlin, 2005). Viral replication concludes with viral proteases cleaving viral polyproteins into individual functional HIV proteins and enzymes that are packaged into new virus particles. Assembly of new virions begins in the cell membrane of the target cell and ends with maturation and budding of the HIV virion (Frankel & Young, 1998). This mature virus is then able to infect other immune targeted cells. Ultimately, CD4+ T cells are depleted weakening the immune system, which, leads to HIV-related infections and the classification of AIDS.

AIDS, an autoimmune disease caused by HIV, is classified based on 1) a CD4+ cell count lower than 200 cells/μL and 2) the presence of a specific HIV+ related opportunistic infections. The fast progression of HIV to AIDS however has been decreased using Highly Active Antiretroviral Therapy (HAART). Currently, HAART regimens consist of the combinations of at least three drugs belonging to at least two different classes of antiretroviral agents. The main objectives of HAART are to 1) reduce HIV-associated morbidity and prolong the duration and quality of survival, 2) restore and preserve immunologic function, 3) maximally and durably suppress plasma HIV viral load, and 4) prevent HIV transmission. There are currently five classes of antiretrovirals

approved for the treatment of HIV. Each class of antiretrovirals is designed to inhibit a different step of the HIV life cycle and Table 1 summarizes drugs within these classes.

Table 1.

FDA Approved HIV Antiretrovirals

Class	**Drug**
Entry Inhibitors Chemokine Receptor 5 Inhibitors (CCR5) Fusion Inhibitors	maraviroc (MVC) enfuvirtide (T-20)
Nonnucleoside RTIs (NNRTIs)	efavirenz (EFV) nevirapine (NVP) etravirine (ETV) rilpivirine (RPV)
Nucleos(t)ide Reverse Transcriptase Inhibitors (NRTIs)	abacavir (ABC) zidovudine (AZT) didanosine (ddI) **emtricitabine (FTC)** lamivudine (3TC) stavudine(d4T) **tenofovir (TDF)**
Integrase Inhibitors	raltegravir (RAL)
Protease Inhibitors (PIs)	amprenavir (APV) atazanavir (ATV) darunavir (DRV) indinavir (IDV) lopinavir (LPV) ritonavir (RTV) saquinavir (SQV) fosamprenavir (FPV) nelfinavir (NFV) tipranavir (TPV)

Entry Inhibitors are characterized as Chemokine Receptor 5 Antagonists (CCR5 inhibitor) and Fusion Inhibitors. The US Food and Drug Administration (FDA) has approved only two agents categorized as entry inhibitors; maraviroc (MVC) and

enfuvirtide (T-20) (Murakami & Freed, 2000). MVC reversibly binds the CCR5 co-receptor, blocking the V3 loop interaction and inhibiting fusion of the cellular membranes to reduce insertion of the virus into the host cell. T-20 binds to gp41 inhibiting the penetration of the cell membrane by gp41, which limits the fusion of the viral membrane to the target cell.

Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and Non-Nucleoside Reverse Transcriptase inhibitors (NNRTIs), interact with the HIV life cycle by inhibiting the same viral enzyme, reverse transcriptase. NRTIs act competitively against the enzyme reverse transcriptase and are used in combination as the "backbone" of antiretroviral therapy. They incorporate into the replicating viral genome by mimicking endogenous nucleosides and by causing chain formation termination (Saez-Llorens et al., 2008). There are currently seven FDA approved NRTIs that mimic an endogenous nucleoside; Abacavir (ABC) is a guanosine analogue, didanosine (ddI) and tenofovir disoproxil fumarate (TDF) are adenosine analogues, lamivudine (3TC) and emtricitabine (FTC) are cytidine analogues, and zidovudine (ZDV) and stavudine (d4T) are thymidine analogues. All of the nucleoside analogues require phosphorylation by intracellular kinase to their active triphosphate moiety. Tenofovir (TFV), however, only requires double phosphorylation to its active moiety, tenofovir diphosphate (TFV-DP) and therefore is considered a nucleotide analogue (Weller & Williams, 2001). Unlike NRTIs, NNRTIs are not incorporated into viral DNA. NNRTIs instead bind noncompetitively to the p66 subunit of viral reverse transcriptase, which induces a conformational change in the enzyme that alters the active site of the enzyme and limits its activity. Efavirenz

(EFV), nevirapine (NVP), etravirine (ETV) and rilpivirine (RPV) are the four NNRTIs currently approved for use in the treatment of HIV.

In 2007 the FDA approved the newest class of antiretrovirals known as, Integrase inhibitors. Raltegravir (RAL) is the only agent approved in this class. RAL competitively inhibits strand transfer reaction by binding metallic ions to the active site of the viral integrase and restricting viral DNA from covalently linking to the target cell's DNA (Sluis-Cremer, Temiz, & Bahar, 2004).

PIs bind directly to viral protease enzymes preventing cleavage of polypeptides into smaller proteins, which halts maturation and the ability of the virus to infect new cells (Hazuda et al., 2000). There are currently ten PIs approved for use in the treatment of HIV: amprenavir (APV), atazanavir (ATV), darunavir (DRV), fosamprenavir (FPV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and tipranavir (TPV). It should be noted that PIs have limitations, which attribute to theirs adverse events; however, they are still used readily in both treatment-naive and experienced patients. Such restrictions include low barrier to resistance, potential for cross-resistance, high incidence of large number of adverse effects and high pill burden.

In summary, HAART consist of three drugs from at least two different classes (Flexner, 1998). In accordance to the U.S. Department of Health and Human Services as of June 2011 the combination is with a RTV boosted PIs usually EFV or RAL and with a NRTI backbone of TDF/FTC (Llibre et al., 2011). The pharmacological bases of TDF and FTC as the backbone to antiretroviral therapy and potential benefits as preventative agents are described further in the next section.

Pharmacology of TFV and FTC

TDF is chemically identified as 9-*R*-2 phosphonomethoxypropyl adenine (PMPA), and is a potent antiviral inhibitor belonging to the acyclic nucleoside phosphonate family with a molecular weight of 287.213 g/mol (Srinivas & Fridland, 1998). It is an ester prodrug for the antiretroviral, tenofovir (TFV), which is poorly bioavailable because of two negative charges formed by phosphonyl groups. TDF is hydrolyzed by carboxylesterase in the intestinal walls and subsequently hydrolyzed by phosphodiesterase during its first passage through the liver to form TFV (Schinazi et al., 1992). TFV is transported into cells through organic anion transporters (OAT) 1 and 3 (Kearney, Flaherty, & Shah, 2004; Naesens et al., 1998; Shaw et al., 1997). Intracellular phosphorylation occurs to first form tenofovir monophosphate (TFV-MP) from TFV by adenylate kinase. A second conversion occurs by nucleotide diphosphate kinase to form the active chain terminating tenofovir diphosphate (TFV-DP) from TFV-MP. Figure 2 gives the chemical structures of TFV and then the active TFV-DP.

Tenofovir and Tenofovir Diphosphate

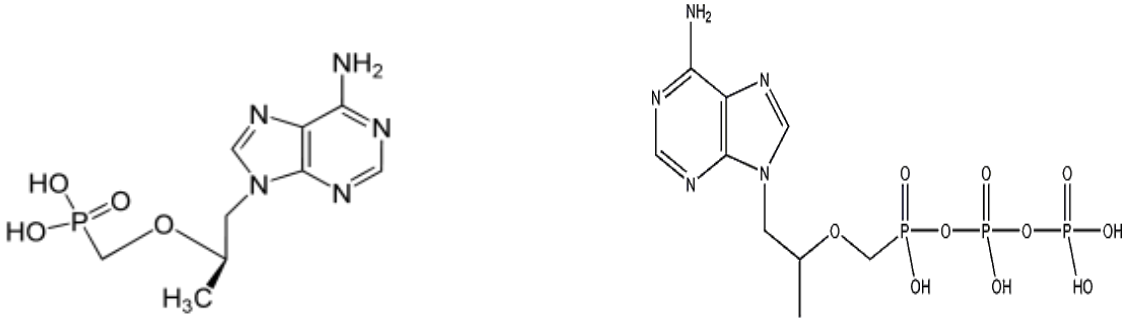


Figure 2. Chemical Structures of Tenofovir and Tenofovir Diphosphate

The chemical name for FTC is β- L-2'-3'-dideoxy-5-fluoro-3-thiacytidine, a 5-fluorinated derivative of 3TC. It is an (–)-enantiomer oxathiolane nucleoside with a molecular weight of 247.22 g/mol (Robbins, Srinivas, Kim, Bischofberger, & Fridland, 1998). TDF and FTC belong to the NRTI class of antiretrovirals although technically, TDF is a nucleotide analog as opposed to a nucleoside analog. Similarly, FTC is also transported into cells using organic cation transporters (OCT) 1 and 3 and concentrative nucleoside transporter (CNT) 1 (Lebrecht et al., 2009; Ray et al., 2006; Rodriguez-Novoa et al., 2009). FTC is readily activated by three different cellular enzymes in a stepwise fashion to form its monophosphate (MP), diphosphate (DP), and triphosphate (TP) forms. FTC is initially phosphorylated by deoxycytidine kinase to emtricitabine-monophosphate (FTC-MP). Endogenous uridylate-cytidylate kinase then converts FTC-MP to emtricitabine-diphosphate (FTC-DP). Finally, 3′-phosphoglycerate kinase forms the active emtricitabine-triphosphate (FTC-TP) from FTC-DP. Figure 3 displays the chemical structure of both FTC and the intracellular active FTC-TP.

Emtricitabine and Emtricitabine Triphosphate

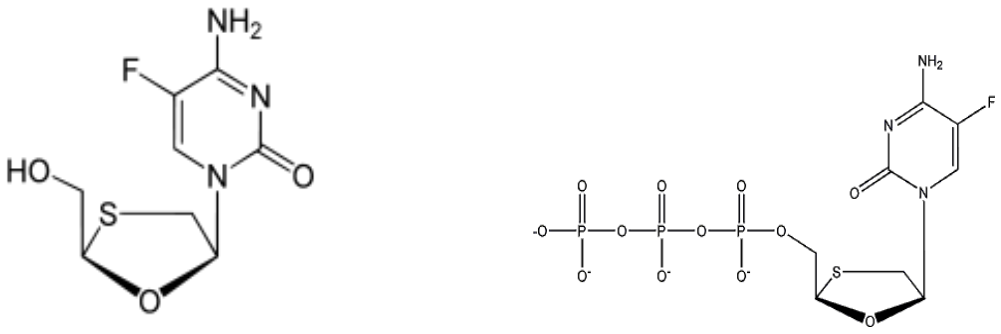


Figure 3. Chemical Structures of Emtricitabine and Emtricitabine Triphosphate

TFV-DP and FTC-TP compete with the naturally occurring nucleotides deoxyadenosine 5'-triphosphate and deoxycytidine 5'-triphosphate, respectively, to inhibit viral reverse transcriptase. Incorporation of TFV-DP and FTC-TP instead of the natural substrates into the viral DNA chain causes termination of DNA elongation and stops further DNA synthesis. TFV-DP and FTC-TP are also weak inhibitors of cellular DNA polymerases α, β, and γ. As a result, incorporation of either active antiretroviral agent into the viral DNA chain has to occur prior to polymerization of the viral DNA to be effective (Anderson et al., 2011). Excess unincorporated TFV-DP and FTC-TP are retained in the cell and dephosphorylated by endogenous phosphatases. Once dephosphorylated, TFV can exit the cell by the transporters multidrug resistance protein (MDRP) 1, 4, & 5 or breast cancer resistance protein, while the efflux of FTC is dictated by equilibrative nucleoside transporter 2 (Ray et al., 2006).

In vitro, TFV and FTC exhibit additive range of activity when combined with certain antiretrovirals. Strong synergism has been seen for TFV with ZDV and NVP, while mild synergism has been noted in combination with ddI, and NFV. In contrast, when TFV is co-administered with ABC, 3TC, FTC, EFV, d4T, and indinavir (IDV) it demonstrates additive inhibition. FTC demonstrated additive-to-synergistic effects with three classes of antiretrovirals, NRTIs (ABC, 3TC d4T, ZDV, and TFV), NNRTIs (EFV and NVP), and PI (SQV, RTV, and NFV) (Saez-Llorens et al., 2008; Suo & Johnson, 1998).

TFV and FTC maintain immunomodulatory effects with minimal cytotoxicity. TFV in HIV-1 infected T- lymphocytes and peripheral blood mononuclear cells (PBMC) from both human and murine cell lines demonstrate effective doses of 22 μmol/L and 29

μmol/L, respectively (Mulato & Cherrington, 1997). However, higher concentrations of FTC are needed to obtain the same immunomodulatory effects as seen in similar cells dosed with TFV. FTC maintains an effective inhibitory concentration (IC_{50}) of 0.0014–0.14 mmol/L for PBMCs, monocytes, and macrophages when infected with laboratory strains of HIV-1 (Van Rompay et al., 2008).

TDF, approved by the FDA as Viread®, is available as a 300 mg tablet that contains 136 mg of TFV, to be taken once daily without regard to meals in adults. Viread® is prescribed to pediatric patients based on weight (150, 200, 250 or 300 mg based on body weight) as an intact tablet or an oral powder dosed at 8 mg/kg, which again can be administered once daily without regard to food (Viread (tenofovir), 2012). FTC is administered under FDA guidelines as Emtriva® also as a once daily pill of 200 mg or oral solution of 240 mg without restriction of food intake. An oral solution of 5 mg/kg FTC is administered to children 3 months to 17 years (Emtriva (emtricitabine), 2012). TFV and FTC are also available as fixed dose tablets with other antiretrovirals: Truvada® (FTC and TFV) Atripla® (EFV, FTC, and TFV), Complera® (RPV, FTC, and TFV), and Stribild® (elvitegravir, cobicistat, FTC, and TFV).

Animal and human *in vivo* studies have determined the absorption, distribution, metabolism, and elimination of TFV and FTC. TFV absorption in Caco-2 intestinal mucosal monolayers of mice and dogs increased 18% and 13%, respectively, with TDF compared with TFV. The bioavailability of TFV increases 14% after a high fat meal compared with fasting (Shaw et al., 1997). Intravenous (IV) TFV dosed at 1 and 3 mg/kg to HIV-seropositive patients produced a volume of distribution (Vd) of 1.3 L/kg and 1.2 L/kg, respectively. Mean steady-state Vd of oral TDF (300 mg) was approximately 800

mL/kg with peak plasma concentrations and area under the concentration-time curve (AUC) of 0.30 ± 0.09 μg/mL and 2.29 ± 0.69 μg•hr/mL, respectively (Naesens et al., 1998). Data suggest that TFV is not highly protein bound to human plasma proteins (1.0%) or to serum proteins (7.2%) over a concentration range of 0.01–25 mg/L. The lack of protein binding and lipophilic characteristics allows the distribution and penetration of TFV into various tissues and fluids after oral administration. TFV is able to enter and distribute into the extravascular compartments, such as the genital tract, by passive or facilitated diffusion and/or active transport due to its small size and degree of ionization (Hazra et al., 2004).

TFV is neither metabolized by the liver nor acts as a substrate for any cytochrome (CYP) 450 enzymes (Hajjar et al., 1998). However, it is extensively and rapidly eliminated renally by a combination of glomerular filtration and active tubular secretion (Cundy et al., 1998). Eighty percent of TFV, when dosed by IV, is recovered as unchanged drug in the urine with a clearance rate of 0.51 L/h/kg. TFV has biphasic elimination with a plasma half-life of 12–18 hours after achieving steady state. The intracellular half-life of the active moiety is 49 hours in activated PBMCs and 150 hours in resting PBMCs (Robbins, Greenhaw, Connelly, & Fridland, 1995). The extremely long intracellular half-life is attributed to trapping of TFV-DP at the site of action even after elimination of the parent drug in plasma (Robbins, Wilcox, Fridland, & Rodman, 2003).

FTC unlike TFV is highly bioavailable (93%) yet demonstrates similar limited binding (<4%) to human plasma proteins and no inhibition of CYP450 enzymes (Barditch-Crovo et al., 2001) Steady state administration of 200 mg once daily produces

mean ± standard deviation (SD) plasma peak concentration (C_{max}) of 1.8 ± 0.7 μg/mL and AUC_{24} of 10.0 ± 3.1 μg·hr/mL. Distribution is linear over a range of 0.02–200 μg/mL and concentration ratios of FTC in plasma and semen leads to high distribution of FTC into extracellular fluids (Bazzoli et al., 2010).

FTC metabolism occurs through hepatic oxidation by the thiol moiety to form the 3'-sulfoxide diastereomers and glucuronidation to form 2'-O-glucuronide (Boyle, 2003). Complete recovery of the drug is achieved in the urine (~86%) and feces (~14%). Finally, similar to TFV, FTC is eliminated renally by both glomerular filtration and active tubular secretion. Plasma half-life is estimated at 10 hours; however, more importantly the intracellular active moiety, FTC-TP, has a t ½ of 39 hours (Rousseau et al., 2001).

Adverse effects associated with TFV and FTC may limit its use in certain patient populations. TDF administered in adult and pediatric populations has shown a 10% incidence of rash, diarrhea, headache, pain, depression, asthenia, and nausea (Wang et al., 2004). TFV administration has also attributed to nephrotoxicity, bone toxicity, and mitochondrial toxicity, which has led to resistance and alternate dosing in renally impaired patients and prepubescent children (Kearney et al., 2004). FTC is relatively safe for longer-term usage. Common adverse reactions(with an incidence more than 10%) were all determined to be minor events such as headaches, diarrhea, nausea, fatigue, dizziness, depression, insomnia, abnormal dreams, rash, abdominal pain, asthenia, increased cough, and rhinitis and skin hyperpigmentation. Nonetheless, TDF and FTC are the most commonly prescribed antiretrovirals for the treatment of HIV.

The frequent and consistent administration of TFV and FTC for the treatment of HIV is based on the ease of use, extensively long safety profile, their low resistance factor, and the drugs' ability to be activity in both resting and active cells. In addition, TDF is being administrated as a treatment for chronic hepatitis B. Both TDF and FTC are also being administrated as Post Exposure Prophylaxis (PEP) and Pre-Exposure Prophylaxis (PrEP) agents based on the same properties that have rendered these drugs the most prescribed antiretrovirals for HIV treatment (Barditch-Crovo et al., 2001; Hazra et al., 2004).

HIV and Women

Globally, women are the largest group of newly infected individuals with HIV. In 2009 there were 11,200 new cases of HIV in the United States with an estimated 250,000 women infected with HIV in the United States (Cihlar & Ray, 2010). The disparity of newly diagnosed HIV infection has affected women over the past decade. Most women contract HIV through heterosexual activities, including consensual and forcible, however; transmission can also occur through the use of illicit drug paraphernalia and rarely blood transfusions, as depicted in Figure 4. The risk of sexual transmission is greater from man to woman than from woman to man due to the larger surface area of the female genital tract (FGT) compared with the male genitals. Therefore, a prophylactic method against HIV transmission that can be used by women without the consent of others is needed. These female-controlled methods include the strategic use of FDA approved antiretrovirals. The next section briefly overviews the FGT, effects of antiretrovirals on

decrease HIV replication in the FGT, and the role of hormonal contraceptives in conjunction with antiretrovirals.

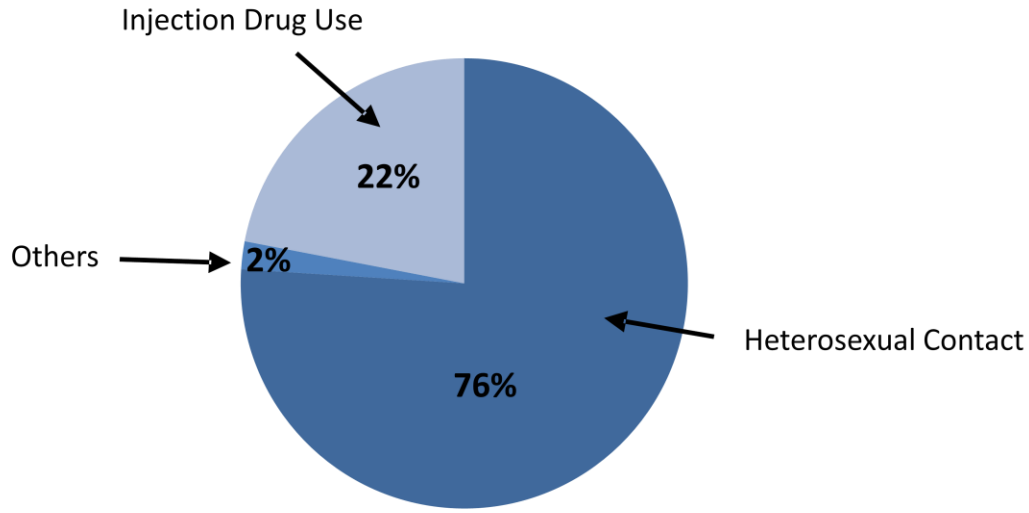


Figure 4. Estimated Percentage of New HIV Infections by Transmission Category in US Women- 2010

The Female Genital Tract

Anatomically, the FGT is divided into internal and external genitalia. Internal genitalia are located in the pelvic cavity and consist of the ovaries, fallopian tubes, uterus, cervix, and vagina. External genitalia, or vulva includes mons pubis, labia majora and minora, clitoris with glands, and structures associated with vestibule. The internal FGT is divided into three compartments: 1) the lower genital tract including the vagina; 2) the ectocervix and endocervix, and 3) the upper genital tract including the endometrium and fallopian tubes (Cole, 2006). Figure 5 demonstrates the internal FGT.

Anatomical Composition of the Internal FGT

This section is focused on the lower internal FGT due to its extensive role in the acquisition of HIV. The vagina and the cervix due to location proximal to the external FGT allow it to be vulnerable to both external and internal destructive mechanisms. The vagina is a thin-walled fibromuscular tube, about 8-10 cm long, extending from the external genitalia to the cervix (Cole, 2006). The upper part of the vagina surrounds the end of the cervix, and produces a vaginal exit point called the vaginal fornix. The fornix allows the physician to collect samples of vaginal secretions during physical examination and is the ideal location to quantify both HIV-1 RNA and antiretrovirals. The vagina helps regulate fertility, hormonal influences, and protection against foreign pathogens, all activities that are dependent upon the physical composition of this region. The vaginal lumen, or inner layer of the vagina, consists of mucosa and is lubricated by mucous of the cervical glands.

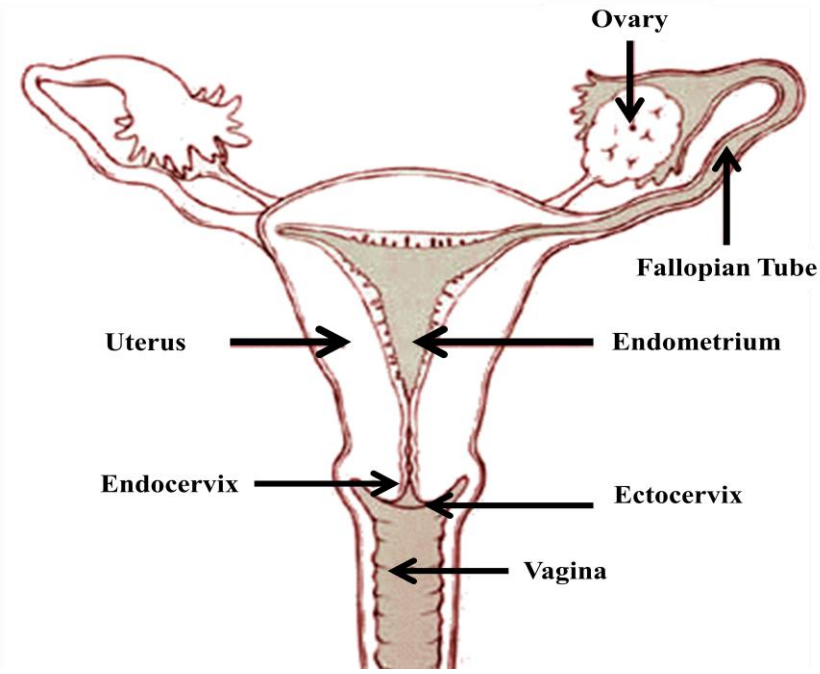


Figure 5. The Human Female Genital Tract

This section of the vagina is most studied in its relationship to HIV transmission and invasion. The vaginal lumen is lined by non-keratinized stratified squamous epithelium, approximately 10-30 cells deep and rests on a thick submucosa known as the lamina propria. These cells contain large amounts of glycogen, which is necessary for the production of lactic acid needed to resist bacterial invasion. The production of glycogen is regulated by estrogen. Squamous epithelial cells of the vagina contain large numbers of estrogen receptors, which respond to ovarian estrogen stimulation by creating a dynamic layer of cells which differ in density, which affect the permeability of pathogens and drugs (Marieb, 2000). In conjunction with the cervix, the vagina is a complex microenvironment that easily changes the FGT cellular make up, secretions, production of microbiota and levels of steroidal hormones (Llewellyn-Jones D, 2004).

The second component of the internal FGT is the cervix. The cavity of the cervix, called the cervical canal, forms a connection between the vagina and the uterine cavity. The cervical canal opens into the vagina via the external orifice and on the opposite end to the uterus, via the internal orifice. The part of the cervical canal, which sticks out into the vagina, is called the ectocervix and the lining of the cervical lumen is called the endocervix. Disparities in cell composition and quantitation of HIV-RNA and Antiretrovirals are seen when collecting samples from the cervix due the vast differences in these two sections.

The ectocervix is lined by a non-keratinizing, stratified, squamous epithelium that continues into the vaginal epithelial layer. These squamous epitheliums of the ectocervix extend to columnar epithelium of the endocervix in an anatomical area referred to as the squamo-columnar cell junction or the transformation zone. This anatomical area is the

most affected section of the FGT by disease associated with cell mutations such as dysplasia and carcinoma *in situ* (Stevens A, 2005). The endocervix is lined by a mucus-secreting single cell layered columnar epithelium (Stevens A, 2005). The mucus acts as a protective barrier, blocking the spreading of bacteria and viruses from the vagina into the endometrial cavity. The secretory ability of the endocervical glands is regulated by estrogens (Kierszenbaum, 2002; Marieb, 2000).

Cellular and Functional Components of the Lower Internal FGT

Cellular secretions from the vagina and cervix also referred to as cervicovaginal fluid (CVF) is the major surrogate used in qualitative and quantitative analysis of the microenvironment , pathogens, and drug concentrations in the FGT. When the CVF is studied, the composition of the fluid and immunological function of these two anatomical sections need to be taken into consideration. CVF is a mixture of several components, including water, ions (Na^+, Ca^{2+}, Cl^-), antimicrobial proteins, polypeptides such as lactoferrin, lysozyme, immunoglobulins, defensins, glycoproteins, lactic acid, acetic acid, glycerol, urea, and glycogen, which vary both in concentration and presence depending on the absolute levels and ratios of estrogens and progesterone, sexual stimulation and the status of the microenvironment (Marieb, 2000; Paavonen, 1983). Additionally, the CVF contains exfoliated cells which may be from the superficial layer or intermediate layer of the vagina and cervix (Paavonen, 1983). The fluid of the FGT is under hormonal control, and changes in viscosity dependent on the menstruation cycle (Gipson et al., 1999; Huggins & Preti, 1981; Wiggins, Hicks, Soothill, Millar, & Corfield, 2001). CVF

contains glycogen and nutrients for lactobacillus, which are the main bacteria in the FGT. Lactobacilli degrade glycogen and create an acidic environment restricting the growth of pathogenic microorganisms (Paavonen, 1983). The ability of bacteria to degrade the major components of the CVF is dictated by microbial enzymes mucinases and sialidases. It is often a fundamental step in disruption of the defensive aspect of the cervicovaginal mucosal barrier, as the major components directly influence the internal and external environments of the FGT (Gipson et al., 1999; Huggins & Preti, 1981).

Immunological Response of the Lower Internal FGT

The immunological response to foreign substances in the FGT is predominantly in the lower section. This section is capable of rapidly eliminating foreign pathogens by local secretion of antimicrobial factors, by enhanced phagocytosis with local macrophages, neutrophils, dendritic cells, natural killer (NK) cells and monocytes, and by activation of inflammation proteins cytokines and chemokines (Holmes, 1999; Seifer DB, 2001). Dendritic cells and macrophages are professional antigen presenting cells. They trigger an early defense against infections or disturbances and also instruct the adaptive immune system to respond (A. Abbas, AH Lichtman, 2006; Janeway, 2005). In reference to HIV-1, this immune response is detrimental to the intrinsic influx of lymphocytes, the target cell of HIV-1 invasion, leaving the FGT vulnerable to infection (A. Abbas, AH Lichtman, JS Pober 2000). Lymphocytes are divided into T and B-lymphocytes. T cells are involved in cell-mediated immune responses, and B cells in secretion of antibodies. T

cells are either cytotoxic CD8+ T cells, or they activate CD4+ T cells, while B cells are characterized as macrophages.

Epithelial cells of the cervix and vagina are the first and major lines of defense against any pathogen, including HIV-1, due to their chemical and physical composition (A. Abbas, AH Lichtman, JS Pober 2000; Fichorova, Cronin, Lien, Anderson, & Ingalls, 2002; Fichorova, Desai, Gibson, & Genco, 2001; Wira, Grant-Tschudy, & Crane-Godreau, 2005). The cellular matrix of each section differs slightly. For example, the endocervix contains the highest numbers of macrophages and T cells. Immunoglobulin M, G, and A (IgM, IgG, IgA), are produced by macrophages in the FGT tissue and transferred into the CVF. The vagina is abundant in CD4+ T cells and the ectocervix has CD4+ T cells, NK cells, and dendritic cells.

The main role of these professional antigen-presenting cells is to block attachment of pathogens and eliminate pathogenic materials (Basso, Gimenez, & Lopez, 2005; Mestecky & Fultz, 1999; Mestecky & Russell, 2000). However, recent data suggests several proteins expressed on the surface of the cervicovaginal epithelial cells and CD4+ T cells may mediate attachment of HIV-1 in spite of the presence of antigens fostering transcytosis of HIV-1 (Mestecky & Fultz, 1999). After initial transmission, which only takes as little as 30 minutes, the more documented and understood pathways in which HIV invade are employed. The initial immune response and the overall protection of the FGT from HIV therefore needs further in depth studying, including a more comprehensive understanding of HIV invasion and the protective effects of antiretrovirals in the FGT.

Impact of ARVs on HIV RNA in FGT

Understanding the impact of antiretrovirals on HIV replication is necessary to reduce transmission and acquisition of the virus. Since HIV is predominantly transmitted through sexual contact, its replication in the FGT and penetration of ARV to this compartment should be studied separately and then systematically together. HIV-seropositive patients treated with HAART regimens effectively suppress viral replication in the plasma, but viral eradication is never fully achieved due to HIV-1 persistence in the cells of the FGT and other anatomical reservoirs such as breast milk and the male genital tract. The prominent cells in these anatomical reservoirs include resting memory CD4+ T-lymphocytes, naïve CD4+ T-lymphocytes, CD8+ T-lymphocytes, monocytes, B cells, and dendritic cells. These HIV vulnerable cell types allow independent replication of HIV, accumulation of trapped virus, and greater kinetic stability compared to the main pool of virus throughout the body. These conditions along with differences in viral evolution and HIV-1 RNA concentrations in the FGT are an ideal environment for sexual transmission of the virus (Blankson, Persaud, & Siliciano, 2002; Bomsel, 1997; Sodora, Gettie, Miller, & Marx, 1998). (Martinez-Picado et al., 2000; Quinn et al., 2000). Understanding the concentration of ARV required in this compartment to reduce viral replication is vital in reducing sexual HIV-1 transmission.

Penetration of ARVs in the FGT

Antiretroviral penetration into the genital tract has been more extensively studied in men than in women although data suggest the degree of penetration appears to be

similar between sexes for NNRTIs and PIs (Chakraborty et al., 2001). It is known that antiretrovirals in the blood stream enter extravascular compartments, such as the FGT, by passive or facilitated diffusion and/or active transport. Passive diffusion is non-specific, non-saturable, not susceptible to competitive inhibition, and does not require transporters (Reddy, Kashuba, Gerber, Miller, & Rondtable Participants, 2003). Facilitated diffusion is regulated by the drug's physical and chemical characteristics, such as molecular weight, degree of ionization, lipid solubility, polarity, and protein binding. Active transport is the movement of drugs across a cellular membrane, mostly through transporters and channels, acting against the concentration gradient. Active transport requires expenditure of energy and is dictated by both the physical properties of the drug and the drug transporter utilized for entrance.

Specifically, TFV and FTC, achieve the highest concentrations in the genital tract, likely due to facilitated diffusion and active transport of these drugs. Facilitated diffusion has characteristics similar to active transport with the specificity, saturation kinetics, and susceptibility to competitive inhibition. Once TFV and FTC enter the FGT, influx is greater than efflux and the drugs begin to accumulate. Drug sequestration helps sustain the accumulation of NRTIs in the FGT by preventing drug efflux through diffusion and/or transport. This process of drug sequestration occurs based on the ionization and/or protein binding of TFV and FTC. Ionization of these drug leads to ion trapping. Ion trapping occurs at equilibrium, when non-ionized concentrations are equal between two compartments while total concentrations (ionized and un-ionized) are unequal. This is achieved when the degree of drug ionization between another extravascular compartments such as the male genital tract or breast milk and the FGT differs which

subsequently is dependent upon the pKa of a drug and the pH of its environment. This concept is important in regards to the FGT since the pH of blood approximates 7.4 and the pH of the vagina approximates 4.0, which is more acidic. In this environment, weak bases will accumulate explaining why TFV and FTC are detected in the FGT.

Amongst all antiretrovirals, PIs and NNRTIs achieve the lowest concentrations in the genital tract and have demonstrated high variability in their degree of penetration. Their high affinity for plasma proteins prevents the compounds from crossing biological membranes. Thus, they are more likely to use passive diffusion and efflux transporters, which have not been characterized in the FGT. Passive diffusion is non-specific, non-saturable, and not susceptible to competitive inhibition and does not require transporters (Taylor & Pereira, 2001). It is regulated by the drug itself and the properties of the compartment. The most well known efflux transporter is p-glycoprotein (P-gp), which can actively transport PIs and NNRTIs out of FGT, limiting their therapeutic efficacy. However, it should be noted that other transporters, which have not been extensively explored, likely contribute to the passage of PIs and NNRTIs in and out of the FGT.

Techniques for ARV Quantitation in the FGT

Studies have shown that after initiation of a HAART regimen, viral load may decline and respond more rapidly in CVF compared to plasma (John et al., 2001). However, the viral load in the FGT is capable of rebounding in the presence of suboptimal ARV concentrations or by evolution of the virus. HIV virions transmitted sexually have been documented to show resistance to NNRTIs, NRTIs and PIs, the

mechanism behind these resistances are still being explored (Taylor & Pereira, 2001). The appearance of drug-resistant mutants in the FGT compared with the plasma is most likely reflections of differences in ARV pharmacokinetics and HIV-1 replication dynamics between anatomical compartments. Therefore, the determination and accurate administration of optimal antiretroviral concentrations in the genital tract must be maintained to prevent the evolution of drug resistant virus. Logistically, the correlation of HIV mutations and ARV penetration differences by anatomical design has led to the need to quantify concentrations of antiretrovirals in the FGT.

Many antiretrovirals, especially those that easily penetrate into the FGT, act intracellularly and current protocols evaluating drug penetration into the FGT marginally consider this aspect. There is a need to measure extracellular and intracellular ARV concentrations in this compartment. Current techniques used for FGT sampling obtain small volumes of secretions, such that sensitive and specific assays often require mass spectrometry technology. Regardless of the sampling technique used, collecting samples can be logistically difficult, especially outside the clinical setting. Finally, current evaluations of ARV concentrations in the FGT usually collected samples at single random time points, which make it difficult to measure total drug exposure. However, an intensive collection process may be difficult. The most common collection method for the FGT is cervicovaginal lavage (CVL) with sterile saline or swabs of the cervix and though it proves informative data about the FGT it does have some drawbacks (Baron et al., 2000; Hajjar et al., 1998; Hart et al., 1999; Philpott et al., 2005; Rasheed, 1998). The first investigation of ARV penetration into the FGT collected CVL samples (Iversen et al., 1998). Obstacles associated with these techniques limit their use. For example, the

small sample volume collected by CVL is diluted with large volumes of lavage fluid that makes it difficult to estimate the true influence of the CVL.

Newer sampling methods include direct aspirates of the cervical mucus, cytobrush, swabbing, and TearFlo Strip™/Sno-Strip® (Coombs et al., 2001; Si-Mohamed et al., 2000). The next FGT collection technique commonly used is swabbing. Swabs provide an imprecise amount of drug in the sample and the sample may be contaminated with blood due to mucosal disruption. Direct aspirates allow precise localization of sample and are useful when collecting both cell-associated and cell-free virus. However, direct aspiration presents problems similar to those for CVL with volume standardization, and is technically more difficult than other collection methods. While cytobrushing is limited by the fact that it mainly collects cells disrupting the mucosal surface, which may lead to inaccurate quantification of antiretrovirals. The Sno-strip® and TearFlo™ strip are ophthalmic fluid collection devices adapted for the collection of CVF. They allow CVF to be collected from fornix of the cervix with little or no disruption of the epithelial surface. One limitation of the ophthalmic test strips is the inability to collect large volumes of genital secretions (~8 μL per Sno-strip® and ~25 μL per TearFlo™ strip). However, this technique allows accurate calculation of the dilution factor, and has recently been shown to be more accurate than CVL for quantifying HIV-1 RNA (Kwara et al., 2008).

Methods to quantify antiretrovirals in CVF and cells of the FGT have enormous variability and need further development. Accurate collection of samples to measure ARV concentrations in the FGT will determine which antiretroviral therapy successfully

penetrates the FGT and decreases cervicovaginal HIV RNA concentrations to reduce the transmission and acquisition of HIV.

Hormonal Contraceptives and ARVs

As modern methods of contraception and the alternative usage of antiretroviral drugs as prophylactic agents expands, the concept of women, HIV, and hormonal contraceptives enter a newly researched area that may contain potential drug interactions, resistance, and worst of all, acquisition vulnerability. Hormonal contraceptives are a preventative form of medicine therefore the long-term use is likely. The novel use of antiretrovirals to prevent the transmission of HIV is also preventative medicine and though both researched separately only recently has focus been giving to the co-administration (NuvaRing (Etonogestrel/Ethinyl estradiol), 2010). Understanding the pharmacokinetics of antiretrovirals and hormonal contraceptives in the FGT is needed.

Hormonal contraceptives are available in the United States include once daily pills known as combined oral contraceptives (COCs) that contain estrogen and a progesterone, progesterone-only pills (POPs), a weekly combined hormonal patch, a monthly combined hormonal vaginal ring, quarterly injectable depot medroxyprogesterone acetate (DMPA), a three-year etonogestrel (ET) (progesterone) implantable rod, and a five-year levonorgestrel intrauterine system (LNG-IUS). All of these methods are highly effective at preventing pregnancy with the typical-use failure rate ranging from 0.1% (LNG-IUS) to 8% (POPs, COCs, and ring) (Cu-Uvin et al., 1999). Emergency hormonal contraceptives are also available, which reduce the risk of

pregnancy by approximately 90% when taken within 72 hours of unprotected sex (Cu-Uvin et al., 1999).

Oral hormonal contraceptives undergo first pass metabolism utilizing the hepatic CYP450 pathway, which is also responsible for the metabolism of many antiretrovirals (Zieman, 2007). Studies have been conducted to understand these interactions. However, novel administration methods of hormonal contraceptives bypass first pass metabolism, and therapeutic doses are achieved with much less estrogen and progesterone then administered in oral contraceptives. The interaction of antiretrovirals with hormonal contraceptives when alternative methods of administration are used still remains unclear (Faculty of Family & Reproductive Health Care Clinical Effectiveness, 2005). Another aspect, which has not been explored, is the effect that endogenous and synthetic estrogens and progesterones has on cellular transporters, which in turn will alter the kinetics of other drugs co-administrated (Hayer-Zillgen, Bruss, & Bonisch, 2002; van den Heuvel, van Bragt, Alnabawy, & Kaptein, 2005; Wielinga et al., 2003). For example, Progesterone up regulates OAT1 and down regulates OCT1 and MDRP 1, 4, and 5. Estrogen down regulates OAT1, OAT3, and OCT1. Co-administration of synthetic estrogen and progesterone with PrEP drugs may be altered and potentially affect clinical outcomes.

Understanding the pharmacokinetics and the mechanism of action of hormonal contraceptives is important in developing a comprehensive picture of hormonal contraceptives in conjunction with antiretrovirals. The major mechanism of action of all hormonal contraceptives is by negative feedback inhibition of the hypothalamic-pituitary-ovarian (HPO) axis, with additional effects on cervical mucus and the endometrium. The

modes of action for synthetic estrogen and progesterone include suppression of the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Ljubojevic et al., 2004; Mahesh, 1985) and ovulation suppression, thinning of the endometrium, and cervical mucus thickening (Hemrika, Slaats, Kennedy, de Vries Robles-Korsen, & Schoemaker, 1993; Ljubojevic et al., 2004). Ethinyl estradiol (EE), the estrogenic component of most currently marketed COCs, is primarily metabolized by hydroxylation by the hepatic enzymes CYP3A4 and CYP2C9. It should be noted that these two enzymes differ greatly in women based on age and race , thus creating a very large variability in EE pharmacokinetics (Speroff, 2005). The progesterone component differs between contraception, resulting in varying metabolism and pharmacokinetics (Guengerich, 1990).

From the many classes of hormonal contraceptives mixed advice about interactions with antiretroviral are reported. The focus here is on oral contraceptives and the vaginal ring, which contains both synthetic estrogen and progesterone and its interaction with Antiretrovirals by classes. Lo/Ovral (EE/norgestrel), Alessa (EE/levonorgestrel), and Ortho Tri-Cyclen (EE/norgestimate) all have labels which mention possible interaction with PIs (Alesse (ethinyl estradiol/levonorgestrel), 2008; Edelman, Cherala, & Stanczyk, 2010; LoOvral (ethinyl estradiol/norgestrel), 2007). While Yaz (EE/drospirenone) and Mircette (EE/desogestrel) have prescribing labels which warn of drugs which induce CYPs, however, the labels never specifically name any antiretrovirals (Ortho TriCyclen (ethinyl estradiol/norgestimate), 2009; Yaz (ethinyl estradiol/ drospirenone), 2010). Decreased pharmacokinetic parameters of both estrogen and progesterone when co-administered with PIs leads providers to recommend that

women use an additional barrier method or rely on nonhormonal contraceptives (Agenerase (amprenavir), 2002; Aptivus (tipranavir), 2011; Kaletra, 2010; Mircette (ethinyl estradiol/desogestrel), 2009; Norvir (ritonavir), 2010; Prezista (darunavir), 2008; Viracept (nelfinavir), 2009).

Three independent studies evaluated NNRTIs with synthetic estrogen and progesterone displayed more than a 20% systemic reduction (AUC and C_{max}) in estrogen and progesterone leading to sub-therapeutic levels of the hormonal contraceptives (Invirase/Fortovase (saquinavir), 2010; Mildvan et al., 2002; Sevinsky et al., 2011). The reduction in synthetic hormones allows for successful ovulation and therefore is not an effective primary form of pregnancy prevention.

NRTIs demonstrate no significant differences in exposure along with no alterations in the pharmacokinetic parameters of the synthetic hormones. However, it is noted that the active intercellular concentrations of NRTIs have not been extensively studied. Therefore, no conclusions can definitely be made on cellular interactions (Scholler-Gyure et al., 2009).

The Centers for Disease Control and Prevention Medical Eligibility Criteria lists all hormonal contraceptive methods (COC, patch, and vaginal ring) as Category 1(a condition for which there is no restriction for use of the contraceptive method) for women with HIV or AIDS (German, 2011). While further detailing the use of hormonal contraceptives methods as Category 2 (a condition for which the advantages of using the method generally outweigh the theoretical or proven risks) for women taking NNRTIs, and finally labeling the use of hormonal contraceptives as Category 3 (a condition for

which the theoretical or proven risks usually outweigh the advantages of using the method) for women taking ritonavir-boosted PIs.

Etonogestrel *Ethinyl Estradiol*

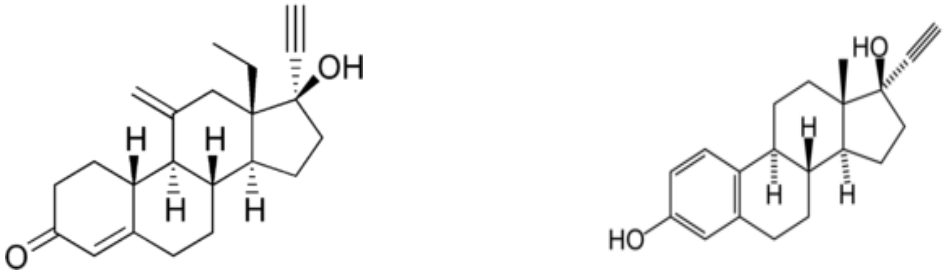


Figure 6. Chemical Structures of Etonogestrel and Ethinyl Estradiol, the Synthetic Hormones of NuvaRing®

Another gap in the relationship of antiretrovirals and hormonal contraceptives is seen in the novel delivery methods, specifically the intravaginal ring. NuvaRing was FDA approved in 2002 and releases 15 µg EE/120 µg etonogestrel (ET) daily. The chemical structure of ET and EE is shown in Figure 6 (CDC, 2010). There is no discussion of how the pharmacokinetics of a vaginally administered hormone might differ from one that is orally administered, and there are no data specific to the novel use of the progesterone, ET in conjunction with antiretrovirals. Previously noted is the thought that the interaction of antiretrovirals with synthetic hormones are most likely related to their metabolism rather than their absorption suggesting no reason to expect different interaction results between vaginal and oral administration. However, recent *in vitro* data has suggested that the uptake of antiretrovirals differs in the presence of

hormonal contraceptives. Due to very few studies offering data for the intravaginal absorption of synthetic hormones with antiretrovirals, the recommendation officially by the CDC considers the combination safe for women with HIV or AIDS.

The data in this section thus far are focused on antiretrovirals and hormonal contraceptives in relation to women who have already acquired HIV. Data addressing the risk of use of hormonal contraceptives in relation to HIV-1 transmission is still limited. Most studies found no statistically significant association between oral hormonal contraceptive use and HIV-1 acquisition, although point estimates varied and several had limited statistical power (indicated by wide 95% confidence intervals). All the studies had limitations that affected data interpretation such as study population, primary study objective, study size, number of seroconverters, or the statistical methods used.

Hormonal contraceptives are effective in preventing pregnancy and will continue to be administered to HIV-seropositive women. The interactions of synthetic estrogen and progesterone with antiretrovirals in HIV-seropositive women seen in altered plasma pharmacokinetics have been documented and restrictions have been suggested. Nevertheless, more research is needed in alternate administration methods of hormonal contraceptives and the co-administration of antiretrovirals. However, the more pressing issues with hormonal contraceptives is the likelihood of increasing acquisition of HIV and the negative effects of co-administration with antiretrovirals as a PrEP agent. For these reasons, detailed research on antiretrovirals co-administered with alternative delivery routes of hormonal contraceptives is vitally needed.

Pre-Exposure Prophylaxis

Pre-exposure prophylaxis (PrEP) is a preventative medical technique using Antiretrovirals to reduce the risk of contracting HIV. HIV-seronegative individuals at high risk of acquiring HIV take Antiretrovirals with the hope to lower their risk of infection if exposed to HIV (Reid et al., 2010). The use of Antiretrovirals, in this setting, is applicable to those patients with a history of substance abuse, mental health issues, unprotected intercourse, or injecting with shared needles (van 't Wout et al., 1998). Furthermore, patients with a history of sexually transmitted infections (STI) may benefit since STIs, especially those acquired through anal intercourse, have been linked with a higher risk of contracting HIV.

Unprotected sexual activities remain the leading cause of HIV transmission and account for approximately 80% of new cases. These sexual activities include but are not limited to; vaginal and anal intercourse seen in homosexual contact and high-risk heterosexual contact. Men globally comprise the majority of HIV-seropositive individuals; however, women between the ages of 15 and 24 years old are becoming the largest group of newly infected individuals. This growth is caused by the lack of personal control a woman has in implementing preventative methods such as condom use, monogamy, and their partner's high risk lifestyle (Galvin & Cohen, 2004).

It is likely that women will benefit the most from the introduction of PrEP globally. PrEP emphasizes individual adherence to antiretrovirals increasing its benefits in a population such as women, whom previously lacked control in using other preventative methods. In the United States alone, there are an estimated 230,000 women

who are infected with HIV; within this estimation 1 out of 5 of these women do not know their status (Buchbinder, 2009; Liu, Grant, & Buchbinder, 2006). The majority of these women contracted HIV during heterosexual activities and was unaware of their partners' status prior to intercourse. Therefore, a method such as PrEP, which 1) can be used without the consent of the other party, 2) is safe for the user, and 3) harmless to others, is warranted (UNAIDS/WHO, 2010). This section briefly explains the history and current clinical trials supporting such method.

History of PrEP

Since the HIV epidemic began almost three decades ago, many different methods have been proposed to curb the spread of this deadly infection. Such methods include abstinence, barrier methods, condoms, male circumcision, microbicides, nonoccupational post exposure prophylaxis (nPEP), PEP, and PrEP (Dumond et al., 2007). Each method has benefits and flaws but only reaches their full potential when used correctly and consistently. Barrier methods, condoms, and male circumcision are methods that have been utilized as the main preventative techniques for the majority of the epidemic. Newer methods, including microbicides, nPEP, PEP, and PrEP, uses FDA approved drugs normally prescribed for the treatment of HIV (Buchbinder, 2009).

PrEP has been the focus of the HIV community over the last decade (2004-2013) as an effective preventative method. The rationalization behind the development and need for PrEP is not to eradicate the use of other preventative methods, but to create a method which can be integrated into other existing preventative programs (Al-Jabri &

Alenzi, 2009). The concept of distributing a medication as a preventative method has been in medical practice since the 1800s, with the first rabies vaccination. In the early 1990s, the concept expanded into the methods and techniques associated with HIV research. One of the first HIV PrEP trials utilized antibodies gp120 v3, which reduced the transfer of infection to cells that had been exposure to the virus. Although promising, this method was not practical in application due to the lack of availability of the antibodies. Therefore, future clinical trials altered the approach and focused on transitioning current antiretrovirals into PrEP agents.

Implementation of an oral or vaginal/anal method that uses FDA approved antiretrovirals has proven in vivo and in animal models to be effective in the treatment of HIV (AVAC, 2009). TFV/FTC has been the most ideal antiretrovirals for PrEP due to their well-documented toxicological and pharmacological history. However, other antiretrovirals have been evaluated and newer antiretrovirals are being considered as future PrEP agents. Majority of PrEP animal studies, preliminary laboratory research, and clinical trials have focused on the use of TFV alone or a combination of TFV/FTC. The selection of these two NRTIs as leaders in PrEP was based on their ease of use, safety profile for long term use, their modes of action, the high genetic barrier, and cost effectiveness (Peterson et al., 2007). Two additional classes of antiretrovirals, integrase inhibitors and entry inhibitors, have shown efficacy as PrEP agents. Their success is linked to the antiretrovirals mechanism of action and their lack of resistance; however extensive PrEP research still needs to be conducted (Abdool Karim et al., 2010; Marcelin et al., 2005; Nunn et al., 2009).

In contrast, other antiretrovirals have not shown much promise as PrEP agents. For example, NVP has potential for resistance by mutant strains of HIV-1 due to its lack of specificity in its mechanism of action to withstand the presence of mutating virus (Rossi, 2010). NVP used as a PrEP agent also had low adherence due to gastintestinal and central nervous system side effects. Two additional Antiretrovirals, ZDV and 3TC, which are being used to reduce the transmission of mother-to-child infection of HIV, were evaluated as potential PrEP agents. They were shown to have low tolerability after extended usage (Gallant et al., 2006; Whitcomb, Parkin, Chappey, Hellmann, & Petropoulos, 2003). Therefore, TFV and TFV/FTC regimens remain vital in PrEP research.

Effectiveness of PrEP Trials

Eight large (> 800 subjects each) randomized, double blind, placebo-controlled, PrEP clinical trials administering TFV or TFV/FTC either orally or vaginally are ongoing or have been recently completed. These eight trials have enrolled almost 20,000 subjects who differ in age, sexual orientation, sex, location, and risk factors. All trials listed in Table 2included monthly study visits with frequent HIV testing, provision of study medication, adherence evaluations, and a package of HIV prevention services relative to the population and location. The major purpose of these trials is to assess TFV or TFV/FTC efficacy for HIV prevention above and beyond standard HIV prevention services. Taken together, these trials will provide a broad overview of HIV PrEP globally.

CAPRISA 004 (Centre for the AIDS Programme of Research in South Africa), enrolled 889 HIV-seronegative women between 18 and 40 years of age from Durban and rural KwaZulu-Natal, South Africa to test the efficacy of TFV orally and vaginally. (Hosseinipour, Cohen, Vernazza, & Kashuba, 2002). This study employed both an original oral dosing strategy and application of a 1% TFV vaginal gel compared to other studies, which only used an oral once daily dosing. The application of the 1% TFV gel was designed uniquely to follow an intermittent event orientated strategy. This strategy, referred to as BAT24, consist of the application of 1% TFV gel both **B**efore and **A**fter sex, not to exceed **T**wo doses in **24** hours. The 1% TFV gel users compared to the randomized placebo users had a 39% reduction in risk of HIV acquisition (Hosseinipour et al., 2002). TFV users were further divided into subgroups where reduction of HIV acquisition was greater. TFV user subgroup 1 had greater than 80% adherence to the 1% TFV gel with a 54% efficacy, while the second subgroup of TFV users had a 74% efficacy when CVF TFV concentrations were greater than 1000 ng/mL (Abdool Karim et al., 2010). This study was a proof of concept it connected the use of an intermittent TFV gel and its significant ability to reduce HIV risk in a very high incidence population.

FACTS 001 (Follow-on African Consortium for Tenofovir Studies), mimicked the CAPRISA 004 BAT24 trial and was a randomized placebo-controlled trial among a larger population of women from diverse sites across South Africa (Karim, Kashuba, Werner, & Karim, 2011). The purpose of this study was to gather more data on the efficacy and safety of a 1% TFV gel using the BAT24 strategy. Efficacy of the 1% TFV gel will be determined and compared to efficacy data obtained from its parent study,

CAPRISA 004 (39-74% efficacy). Once this efficacy is proven national and international proceeding will begin for the medical approval of the 1% TFV gel as a PrEP agent.

Another trial, which is evaluating on vaginal application of PrEP is VOICE (Vaginal and Oral Interventions to Control the Epidemic study). The VOICE trial is an ongoing five-arm study among 5,021 HIV-seronegative women from South Africa, Uganda, and Zimbabwe. The five arms are: 1) daily 1% TFV gel, 2) daily oral TDF, 3) daily oral FTC/TDF, 4) placebo gel, and 5) oral placebo. The objective of the study is to evaluate the safety and effectiveness of treatment arms to the placebo arm. Medical ethic and review authorities however, have advised the discontinuation of certain arms within the study due to lack of effectiveness. The Data Safety Monitoring Board for the VOICE trial recommended the discontinuation of the oral TDF arm in September 2011 (FACTS001, 2010) and of the daily vaginal 1% TFV gel arm in November 2011 due to futility (Celum & Baeten, 2012). The remaining arms of the study are still ongoing and complete effectiveness and safety data will be available in 2013.

FEM-PrEP, which also had futility problems and was terminated early, assessed some critical concepts. The study enrolled 2,021 high-risk HIV-seronegative women from Kenya, South Africa, and Tanzania into a placebo controlled trial of daily oral TDF/FTC (Celum & Baeten, 2012). There were 68 cases of HIV infection observed equaling only a 6% efficacy rate; 35 infections were among those randomized to placebo and 33 infections were among those randomized to TDF/FTC. An Independent Data Monitoring Committee in April 2011 terminated the study due to lack of efficacy by equal numbers of infections being seen in each of the two study arms. However, the more important data which was gathered from this study was the understanding of the

importance of adherence (only about 25% of the women had detectable levels of TFV) and the perception of risk of HIV acquisition (70% of the women reported that they perceived themselves to have little or no chance of acquiring HIV).

The next two studies have similar populations, both conducted African nations, and have proven to be efficacious. The first study, the TDF2 study, was a placebo-controlled trial of daily oral TDF/FTC. It enrolled 1,200 heterosexual HIV-seronegative men and women between the ages of 18 and 39 years old from Botswana. The study reported 63% efficacy (95% CI 22–83, $p = 0.01$) for HIV protection among those randomized to TDF/FTC PrEP compared to placebo (Van Damme et al., 2012). The trial was relatively small thus, the ability to demonstrate differences by gender was limited. However, among men, the antiretrovirals applied a protection of 80% overall ($p = 0.03$) and 82% in the subgroup receiving PrEP ($p = 0.06$). While PrEP agents among women, were shown to protect an overall 49% ($p = 0.1$) and 76% in the subgroup receiving TDF/FTC ($p = 0.02$). Nonetheless, efficacy estimates suggested protection for both men and women.

The second study, Partners PrEP, was a three arm placebo trial of daily oral TDF, oral FTC/TDF, and control among 4,758 HIV-serodiscordant couples from Kenya and Uganda in which the HIV-seropositive partner was not eligible for antiretroviral therapy according to national guidelines at the time of enrollment (Thigpen et al., 2012). The HIV-seronegative partners were randomized to receive PrEP agents or placebo, however, the Data Safety Monitoring Board discontinued the placebo arm (Baeten et al., 2012). Nevertheless, there was an overall 67% efficacy of TDF (95% CI 44–81, $p < 0.001$) and 75% efficacy of TDF/FTC (95% CI 55–87, $p < 0.001$) compared with placebo in men

and women. Breaking down the efficacy between men and women the reduction resulting from TDF was 63% ($p = 0.01$) for men and 71% ($p = 0.002$) for women, and the reduction results from TDF/FTC were 84% ($p < 0.001$) for men and 66% ($p = 0.005$) for women. Therefore, this study, similar to the TDF2 study showed efficacy for both men and women (Mujugira et al., 2011).

This next study, iPrex, randomized 2,499 seronegative men who have sex with men from the North and South America, Thailand, and South Africa to daily oral TDF/FTC and placebo. The study patients were classified as being at higher risk for HIV acquisition by anal sex and by fitting at risk behavioral patterns which included 1) anal sex with four or more male partners, 2) a diagnosis of a sexually transmitted infection, 3) transactional sex activity, or 4) condomless anal sex with a partner who was HIV-infected or of unknown infection status in the prior six months. Overall, the iPrEx study demonstrated that TDF/FTC reduced HIV acquisition risk by 44% (Bangsberg D, 2012). Adherence in the study population was only moderate throughout the study yet, higher adherence was correlated to age >25 years, participants from US sites, and recent unprotected anal receptive sex (Grant et al., 2010).

The last clinical trial, the Bangkok Tenofovir Study, also has a unique subject population; it enrolled seronegative injection drug users. The trial enrolled 2,413 participants with daily oral TDF with anticipated results to demonstrated efficacy only. It should be noted that majority of participants were also enrolled in methadone replacement programs, which is where they receive their PrEP medicine (L. J. Anderson P, Buchbinder S, et al. , 2011; Suntharasamai et al., 2009). Results are pending but do seem promising.

Table 2. Summary of PrEP Trials

Trial/Study	Sites	Population	Intervention	Status: % Efficacy	Reference
CAPRISA 004	South Africa	889 heterosexual women	Topical TDF gel; BAT24	Completed: Overall 39% efficacy	Karim et al, 2010
FACTS 001	South Africa	1200 + heterosexual Women	Topical TDF gel; BAT24	Ongoing	Karim et al, 2011
VOICE	South Africa, Malawi, Uganda, Zimbabwe	5000+ heterosexual women	Daily oral TDF or TDF/FTC or daily topical TDF gel	Ongoing: Discontinuation of daily oral and topical TDF arms	Baeten et al, 2012
FEM-PrEP	Kenya, Malawi, South Africa, Tanzania, Zambia	2000+ heterosexual women	Daily oral TDF/FTC	Terminated:>6% efficacy	Celum & Baeten, 2012
TDF2	Botswana	1200 heterosexual men and women	Daily oral TDF/FTC	Completed: Overall 63% efficacy	Van Damme et al, 2012
Partners PrEP	Kenya, Uganda	4700+ serodiscordant heterosexual couples	Daily oral TDF or TDF/FTC	Completed: Overall 67% efficacy with TDF and 75% efficacy with TDF/FTC	Thigpen et al, 2012
iPrEx	Peru, Ecuador, Brazil, South Africa, Thailand, United States	2,500 men who have sex with men	Daily oral TDF/FTC	Completed: Overall 44% efficacy	Grant et al, 2010
Bangkok TFV	Thailand	2400 injecting drug users	Daily oral TDF	Complete: Results Pending	Suntharasamai et al, 2009

Together, these eight trials provide a comprehensive picture of the global HIV epidemic and the potential for PrEP to reduce HIV incidence in key at-risk groups. This is, however, no way a completed story. The results from ongoing trials and future trials will continue to bring forward new information on dosing regimen, dosing, strategies, and the overall success of PrEP agents. However, these initial trials demonstrate proof of concept of HIV ARV PrEP and suggest focal points about site of action dosing and dosing intervals, which can be targeted in future PrEP studies.

Rationale and Objectives

To date, a multitude of TFV and FTC PrEP clinical trials have been completed, are enrolling subjects, or are planned. Yet, there are no extensive and specific publications on the clinical pharmacology of these agents in the FGT relative to their ability to prevent HIV transmission. TFV/FTC were evaluated as PrEP agents due to their ease of use, safety profile for long term use, high genetic barrier, and activity in resting cells. However, this application of TFV/FTC as a PrEP agent may have pharmacological outcomes not seen when administered in the traditional setting as a form of treatment. The lack of a thorough understanding of the clinical pharmacology of PrEP agents at their effective dosing strategies or by their penetration into compartments that are subject to HIV transmission represents a fundamental gap in our knowledge. Due to gaps in the information known about TFV/FTC as PrEP agents, these trials are misinformed in terms of the pharmacologic aspect of PrEP dosing, therefore their results may be misleading. We hypothesized that a thorough understanding of drug

concentrations in plasma and the FGT is pivotal in the design of optimal doses and dosing intervals for PrEP. While the plasma pharmacokinetics of TFV/FTC is well characterized, little is known about relevant concentrations in the FGT to prevent infection in this compartment. There is also no precise correlation between relevant TFV/FTC plasma concentrations used for prevention and those concentrations determined in the FGT.

All current PrEP studies using oral TFV/FTC have utilized doses approved for HIV treatment. However, a different dose and/or dosing interval may be required to achieve necessary drug concentrations in the FGT. If adequate parent and intracellular drug concentrations are indeed achieved in this compartment at the standard dose, then dose and dosing interval are likely to inhibit infection. However, if lower concentrations are adequate to prevent infection, then lower doses and/or less frequent dosing intervals could be explored. This change in dose and dosing interval could result in lower drug costs, diminished adverse events, and possibly a lower likelihood of the development of drug resistance.

The major goal of this project was to expand upon our current knowledge of ARV penetration into the FGT for the use of evaluating PrEP agents using pharmacokinetic modeling and simulations. We accomplished this goal by using novel collection methods to obtain samples from the FGT and by the development of novel assays to quantify drugs from the FGT. The collection methods and analytical assays were validated against current collection techniques and assays for drug quantitation. This data created a base population pharmacokinetic plasma model that will be combined with covariant data and used in the development of pharmacokinetic models to describe penetration of PrEP

agents into the systemically and by compartment (i.e. FGT). The models will be used in simulation experiments to identify optimal dosing, dosing intervals, and compartmental transfer rates of other future PrEP agents. The combination of state-of-the-art pharmacokinetic modeling with a novel quantification assay has improved the interpretation and understanding of drug penetration into each compartment. These findings can be applied to other compartments such as the male genital tract and rectal compartments when taking into account parameters specific to those PrEP drugs and the compartments.

The project was able to contribute new data to PrEP research by addressing and understanding the following three related and overlapping specific aims: 1) quantify TFV and FTC concentrations in the plasma, PBMCs, CVF and endothelial cells of the FGT; 2) determine the uptake of TFV and FTC by cells of the FGT; and 3) develop the parameters needed for a predictive pharmacokinetic models which will help achieve optimal drug concentrations in the plasma and FGT.

AIM 1. Develop analytical and quantitative methods to measure TFV and FTC in plasma, PBMCs, CVF, and endothelial cells of the FGT using liquid chromatography/ mass spectrometry (LC/MS/MS). The determination and validation of optimal, reproducible, and efficient settings and condition for the liquid chromatography attached to a triple quadruple mass spectrometer was achieved. With fine-tuning of the equipment, the quantitation of TFV and FTC were linear over a range much lower than previously reported for these drugs. The aim then focused on the development and validation of extraction techniques for the recovery of TFV and FTC in plasma, PBMCs, CVF collected by direct aspirate and on TearFlo™ Strips, and

endothelial cells of the FGT collected by cytobrushing. The completion of these techniques allowed for effectively determination of TFV and FTC concentrations in the patient samples which comprised of plasma, PBMCs, and the FGT fluid and cells.

AIM 2. Determine *in vitro* TFV and FTC uptake in non-monocytic genital tract cells in the presence and absence of hormonal contraceptives and the comparison of TFV and FTC concentrations in the FGT using different collection techniques. The differences in uptake of TFV and FTC by representative cells of the FGT were determined to ensure collection techniques and quantitation methods from the FGT considered and accounted for all cell types and fluids, which are likely to exist in the FGT. Various methods are used to collect samples from the FGT; however, no technique has been validated by a standardized protocol. No method has been validated due to the CVF variation compositions seen in women. However, certain cells, nutrients, and fluid are consistently present in the FGT, therefore, should be the major component of a surrogate matrix for laboratory usage.

The cellular components likely collected by each technique were achieved by literature review. These identified cell types include: squamous epithelium, CD8+, CD4+, dendritic cells: and macrophages. These cell types were represented by the following cell lines, Ect1/E6E7, BC-3, HeLa, TF-1, and THP-1 cells. The determination of uptake of TFV and FTC by these cell lines were quantified using LC/MS/MS. Additionally, the uptake of TFV and FTC was determined in the presence of the two major synthetic compounds in hormonal contraception, etonogestrel and ethyl estradiol.

Moreover, a complete statistical comparison of the CVF collection techniques was performed. This aim planned to shed light on the novelty of FGT ARV concentrations in both cell based and fluid based collection techniques.

AIM 3. Develop predictive pharmacokinetic models describing antiretroviral concentration-time profiles in the plasma and potentially in the FGT. Using NONMEN and WinNonLin pharmacokinetic analysis software, data obtained from patient samples, which, were analyzed using techniques developed in Aim 1 and clarified in Aim 2, were modeled. The models fit data from TFV and FTC plasma concentrations. Simulations were performed to ensure the model fit the data for a general population. Additional simulations will be done as the complex model of TFV and FTC systemically is developed. The likelihood of clinical success for not just TFV and FTC but also other antiretrovirals, in the future are improved with the model and simulation of the data with detailed covariant data, which allows for a specific and thorough model to be developed.

CHAPTER 2

METHODS

Supplies and Equipment

TFV and FTC were obtained for laboratory use only from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Pathogenesis and Basic Research Branch Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (Germantown, MD, USA). Isotopically labeled analogs of TFV and FTC known as internal standards (IS) are used for all assays and are added to each sample to minimize inter-sample variability related to sample extraction and injection (TFV-IS and FTC-IS). Carbon and nitrogen Labeled TFV-IS and FTC-IS were purchased from Moravek, Inc. (Brea, CA, USA). The phosphorylated forms of TFV and FTC, TFV-DP and FTC-TP, were also purchased from Moravek, Inc. (Brea, CA, USA). Additionally, EE was purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA) and ET was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) for the use during the *in vitro* dosing experiments only.

Methanol (MeOH), acetonitrile (AcN), and isopropanol (IPA), were HPLC grade and purchased from Fisher Scientific, Inc. (Fairlawn, NJ, USA). Glacial acetic acid, ammonium hydroxide (NH_4OH), sodium acetate (NaOAc), trifluoroacetic acid (TFA), formic acid (FA), were certified ACS plus. Sodium hydroxide (NaOH) and potassium

chloride (KCl) were ACS certified and finally acid phosphatase (sweet potato, type XA) was purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). Water was deionized and distilled on-site using Millipore UltraFiltration System (Billerica, MA, USA).

Drug-free human plasma (K+EDTA and heparin) was purchased from Biological Specialties Corporation (Colmar, PA, USA). Cell lysate was made in house by the lysing of five different cell lines (THP-1, HeLa, BC-3, TF-1, and Ect1/E6E7). The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in media supplemented with 0.05 mM final volume of β-mercaptoethanol, 10% of final volume of fetal bovine serum, 1% non-essential amino acids and other media supplementary nutrients, and 1% of final volume of Tri-antibiotic (Penicillin, Streptomycin, & Neomycin) in a free standing incubator at 37°C with 5% CO_2. The cells were passaged between 5 and 12 times at 95-100% confluence. Confluent monolayers of adherent cell lines were subcultured by treatment with 0.35% trypsin containing EDTA while confluent monolayers of suspension cell lines were subcultured with a 1 to 3 splitting technique. Cells were harvested by removing the media and were pelleted using centrifugation (13,000 x g; 10 min). The pellet was re-suspended in 70:30 methanol: water, vortexed, placed on ice for 15 minutes, and centrifuged again. The supernatant, representing the cellular matrix, was collected and stored at -80°C until analysis.

Validation and consumable materials used in the extraction included Waters Oasis MCX (mixed cation exchange) 30mg (1cc) SPE cartridges, Waters Accell Plus 60 mg (3cc) QMA (anion exchange) cartridges, and Waters Oasis HLB (hydrophilic lipophilic balance) 60mg (3cc) Reverse phase SPE cartridges purchased from Waters Corporation

(Milford, MA, USA). The TearFlo™ Diagnostic Test Strips were purchased from Sigma Pharmaceuticals, LLC (Monticello, IA, USA).

Liquid chromatography coupled with mass spectrometry was performed using a Shimadzu (Columbia, MD, USA) Prominence Ultra-Fast Liquid Chromatography ($UFLC_{XR}$) system coupled to a MDS Scienx Applied Biosciences (Toronto, ON, Canada) API 5000 triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface. All data were recorded and stored by the Analyst 1.5.1® software and quantitated with Applied Biosystem MultiQuant® software. Complementary to the LC/MS/MS was an Atlantis dC18 analytical columns (2.0 x 100 mm; 3µm particle size) purchased from Waters Corporation and was used for the chromatographic separation of all analytes and ISs in all matrices. A C18 SecurityGuard cartridge (4.0 x 20mm) was purchased from Phenomenex (Torrance, CA, USA) to extend the life of the Atlantis analytical column. Other essential equipment used was the Fisher Scientific Isotemp Incubator (Fairlawn, NJ, USA) which was maintained at 37°C with 5% CO_2 and the Fisher Scientific Isotemp Water Bath.

Determination of TFV and FTC in Various Matrices by LC/MS/MS

Assay Validation

To ensure each assay was functioning correctly a validation was performed. The validation of this assay followed Good Clinical Laboratory Practice (GCLP) and FDA standards. The standards accounted and ensured the assay was selectivity, had minimum

matrix effects, high extraction efficiency, was both precise and accurate (determined by quality control (QC) performance), and was linear and sensitive (determined by the calibration curve). Each validation assay contained one of each of its calibration standard, a blank matrix sample without the addition of IS, and three of each QC standard used to determine within and between assay performance. Plasma, PBMC, and CVF_{WS} (CVF collected by TearFlo™ Strip) assay had validations, which included each of the listed criteria. While, FGT cells utilized the PBMC assays in their entirety and the CVF_{DA} (CVF collected by Direct Aspiration) assay had its own QCs but, calibrated its QCs using the PBMC calibration curves. A detailed description of each validation criteria is as followed:

Specificity of All Matrices

Assay specificity is the ability of the analytical method to distinguish and quantify the analytes of interest in the presence of other components in the sample. Interferences can include metabolites or decomposition products and concomitant medications. Initially, the specificity is tested by screening different lots of blank matrix. Each blank sample was tested for interfering matrix components using the extraction technique and chromatographic conditions developed for TFV and FTC in each matrix. Next, we analyzed for several medications commonly administered with antiretrovirals under the specific chromatographic conditions used for TFV and FTC quantitation. The retention times of these agents were then compared with the analytes of interest.

Matrix Effects and Recovery

Mass spectrometry is becoming more commonly used for drug quantitation. It provides specificity because of its ability to monitor selected mass ions, sensitivity because of the enhanced signal-to-noise ratio, and speed due to less intensive sample cleanup procedures and shorter analysis times. However, LC/MS/MS is not without problems related to interference. Therefore, during the development of mass spectrometry methodologies, matrix effects and analyte recovery are also investigated. There are two types of matrix effects, relative and absolute. These are defined as direct or indirect alteration or interference in analyte response due to the presence of unintended analytes or other interfering substances in the sample. Ion suppression is the most common effect and can result from the presence of less volatile compounds in the sample such as salts, ion pairing agents, endogenous matrix compounds, and/or other drugs and metabolites. These less volatile materials can change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector. The relative matrix effect was determined by comparing the difference in the precision of peak area and peak area ratio (PAR) (peak area analyte/peak area IS) for sample set 1 (unextracted sample) and set 2 (post-extracted sample). A difference of less than 15% reveals a lack of significant relative matrix effect. Absolute matrix effect is assessed by comparing the mean peak areas in sample sets 1 and 2. A matrix effect value of 100% indicates the absence of absolute matrix effect, while a value greater than 100% indicates ionization enhancement and a value less than 100% indicates ionization suppression.

Also during the development of these LC/MS/MS methodologies analyte recovery is evaluated. Analyte recovery is a measurement of the analyte extraction efficiency from the sample matrix and is determined by comparing the ratio of mean peak areas in normally extracted samples (set 3) to those spiked with analyte post-extraction (set 2). Samples are injected as three replicates at each concentration being investigated, which is the low, mid, and high QC samples. The recovery of each analyte most likely will not reach 100%, however, at a minimum, absolute recovery should be 50% and should be consistent using the proposed extraction methodology across all concentration levels tested.

Accuracy and Precision: QC Performance

Inter-day and intra-day precision and accuracy for each assay were illustrated using three levels of QC standards for each calibration curve and sample type and was analyzed on separate days. Precision is the measure of the degree of repeatability and is defined as the CV (coefficient of variation), which is calculated as the SD (standard deviation) divided by the mean, expressed as a percent (% CV = (SD/mean) x 100). Accuracy measures the exactness of the calculated to the true concentration of the analyte, which is calculated using the following equation; % deviation = ([mean determined concentration – theoretical concentration)/theoretical concentration] x 100). The mean, SD, precision, and accuracy were calculated from the replicates over all days for the inter-day values, and on each individual day (n=3) for intra-day values. The criteria describe the precision and accuracy calculations and it should be noted that all

validation samples should fall within ± 15% of the theoretical value to be considered accepted. In addition, in order for any run (validation or routine) to be considered valid, two-thirds of the QC samples at each concentration must be within range.

Linearity and Sensitivity: Calibration Curve Performance

Each assay's linearity and sensitivity were assessed using matrix matched calibration curves. A calibration curve was created by determining the best fit of the PAR versus the predetermined concentration, and fitted to the equation $y = mx + b$ using a weighted ($1/concentration^2$) linear regression. The resultant calibration curve equations are used for quantification of TFV and FTC in the QCs and unknown samples based on PAR values obtained for these samples. The calibration curve is considered valid when more than three-quarters of all the back-calculated calibration points fall within ± 15% of the theoretical value. Points outside of these acceptance criteria are omitted from the curve and the calibration curve is recalculated, however no two consecutive points may be removed from the curve. Moreover, the calibration curve coefficient of determination (r^2) should be a minimum of 0.980 for the curve to be accepted.

In Vitro: Intracellular Uptake of TFV and FTC in FGT Cells

This study was conducted with three major sections; 1) the identification and selection of cell types which represented the FGT, 2) the comparison of the uptake of TFV and FTC among the five cell lines previously chosen to represent the FGT, and 3)

the determination of TFV and FTC uptake by these five cell lines in the presence of hormonal contraceptives.

Identification and Selection of FGT Cells

To provide clinical equivalence, which can improve the methods used to quantitate TFV and FTC concentrations in Tearflo™ Strips, direct aspiration, and cytobrushing collected in the FGT, identification of cell types of the FGT, which successfully represent each compartment were evaluated. Two major criteria were considered when identifying cell types from the FGT and when determining which cell lines sufficiently represented these cell types (Suntharasamai et al., 2009). The first criterion was the identification of the most abundant cellular components in the FGT. This criterion lead to the identification of the cell lines which easily and readily represented the abundant cell types (Blankson et al., 2002). The second major criterion was distinguishing relevant representation by anatomical compartments of each cell type in the FGT (Hladik et al., 2007; Timmer & Mulders, 2000). The difference in abundance of each cell type in the FGT is dependent on age, disease states, and menstrual cycle. Therefore, cell selection took into consideration the clinical study's collection techniques, the study population's age, disease state, and menstrual cycle along with the use of hormonal contraceptive intervention.

Intracellular Uptake of TFV and FTC in the Presence and Absence of Hormonal Contraceptives

The determination of the uptake of TFV and FTC was done after the selection of five distinct cell lines, which represented the cell types of the FGT. Each cell line was dosed (n=5) with either 0.3 µg/mL of TFV or 1.8 µg/mL of FTC, which represented the C_{max} recorded in literature for adult plasma samples (Emtriva (emtricitabine), 2012; Viread (tenofovir), 2012). After one hour, the cells were harvested by removing the dosed media and the cells were collected by centrifugation at 13,000 x g for 20 minutes. The pellet was re-suspended in 70:30 methanol: water, vortexed, placed on ice for 15 minutes, and centrifuged again. The supernatant was collected and stored at -80°C until analysis using the PBMC assay. The baseline uptake of TFV and FTC only was determined.

Next, the uptake of TFV and FTC was determined in the presence of hormonal contraceptives. Each cell line was dosed (n=5) with either 0.3 µg/mL of TFV or 1.8 µg/mL of FTC, again, along with dosing with the progesterone and estrogen components of NuvaRing®, EE and/or ET. The concentrations of EE (34.7 pg/mL) and ET (1716 pg/mL) used for dosing represented the plasma C_{max} recorded in literature for the NuvaRing® contraceptive (NuvaRing (Etonogestrel/Ethinyl estradiol), 2010). Hormonal contraceptive dosing experiments were conducted in conjunction with additional TFV and FTC only dosing experiments. Each cell line was dosed with either TFV or FTC one hour prior to, simultaneously, or one hour after dosing with the hormonal contraceptives. In each case, the hormones were allowed to incubate with each ARV drug for one hour, the dosing schedule is shown in Table 3. In addition to the initial doses of 0.3 µg/mL of

TFV or 1.8 µg/mL of FTC doses, cells were also dosed with a concentration representing a plasma minimum concentration (C_{min}) (0.06 µg/mL for TFV and 0.09 µg/mL for FTC) and a 25-fold increase from the C_{min} (1.5 µg/mL for TFV and 2.25 µg/mL for FTC).

All cell lines were then harvested to form a pellet, which was re-suspended in 70:30 methanol: water, vortexed, placed on ice for 15 minutes, and centrifuged as previously reported. The supernatant, representing the cellular matrix, was again collected and stored at -80°C until analysis. Cell viability was checked prior to dosing by visual inspection and using Trypan Blue and the cell count was checked and recorded prior to washing the cells from the dosed media and used in the analysis.

The comparison of the uptake with and without hormonal contraceptive was calculated using Microsoft Excel and statistical parameters were derived using GraphPad Prism 5.04 (La Jolla, CA). The THP-1 cells were used as the control cell line (Mikovits et al., 1990) for statistical comparisons of the TFV and FTC only experiments using an unpaired nonparametric *t*-test. The uptake from each cell line dosed with EE and/or ET prior to, simultaneously, or after TFV and FTC administration were compared to that cell line's TFV and FTC only controls and were also evaluated using an unpaired nonparametric *t*-test. Tests were two-sided with significance set at 0.05. Data are reported as a mean with standard deviation and associated *p*-values.

Table 3.

Dosing Regimen for Each Cell Line (n =5)

1st Dose	2nd Dose	Dosing Time
TFV FTC EE ET	Cells were Harvested	Control
TFV	EE	ARVs dosed prior to Hormonal dosing
TFV	ET	
TFV	EE,ET	
FTC	EE	
FTC	ET	
FTC	EE,ET	
EE	TFV	ARVs dosed after Hormonal dosing
EE	FTC	
ET	TFV	
ET	FTC	
EE,ET	TFV	
EE,ET	FTC	
TFV,EE TFV,ET TFV,EE,ET FTC,EE FTC,ET FTC,EE,ET	Cells were Harvested	Simultaneous Dosing of ARVs and Hormones

All remaining cells were harvested after the 2nd Dose.

Clinical Study Overview

This prospective cohort study recruited and enrolled HIV-seropositive women between the ages of 21 and 60 years old at the Emory University affiliated Grady Infectious Diseases Outpatient HIV Clinic (Grady IDP). The use of HIV-seropositive women eliminated study drug cost along with avoiding the need to wait additional time to ensure steady state concentrations. The pharmacokinetics of TDF and FTC do not differ between HIV-seronegative or seropositive women and therefore generalizations can be concluded. Subjects were all receiving Atripla prior to enrollment as part of their antiretroviral regimen for at least four weeks to be certain steady-state had been achieved. Atripla is a fixed dose tablet of TDF (300 mg), FTC (200 mg), and EFV (600 mg). Subjects were evaluated for acceptable study criteria and provided written informed consent and to be confined to the clinic as required by the protocol. Exclusion criteria for the study included subjects who were breast feeding or pregnant, subjects with active STIs with ulcerative genital lesions or friable cervix that bled easily, and subjects receiving concomitant medications that may interact with the study medications.

Subjects, whom provided written informed consent and were determined by screening and eligibility criteria, underwent two additional visits. Both visits occurred about five to seven days apart during the first (follicular) phase of the menstrual cycles to minimize the influence of menstrual changes on the physiology of the FGT, which may have altered study outcomes. The first visit was a 24 hr intensive pharmacokinetic study at the Grady satellite of the Emory University Atlanta Clinical and Translational Science Institute (ACTSI). For visit one, subjects were admitted in the morning after one week of morning dosing of Atripla. Five different sample types were collected from four different

anatomical compartments; plasma, PBMCs, CVF (collected by both direct aspiration (CVF_{DA}) and TearFlo™ Strips (CVF_{WS})), and FGT cells collected by cytobrushing.

The plasma samples were an intensive pharmacokinetic sampling where blood samples (4 mL each) were drawn prior to the morning dose (within 15 minutes) for time 0 and then at 1, 2, 3, 4, 6, 8, 12 and 24 hrs post dose. The PBMC samples were obtained at 0, 4, and 24 hrs post dose. 10 mL of whole blood was collected and processed by centrifugation of the Accuspin System-Histopaque-1077 tubes and the cell count and volume was determined. TearFlo™ samples were obtained from the posterior fornix of the vagina at 0, 12, and 24 hrs post dose. CVF samples were obtained at 0, 12, and 24 hrs post dose from the posterior fornix of the vagina via direct aspiration with a volumetric vaginal aspirator (Rovumeter; Recipe Pharmaceuticals, Munich, Germany) and TearFlo™ Strips. The last sample collected at visit one was FGT cells, which were collected by cytobrush sampling at 24 hrs post dose, after all other genital samples required during this visit, have been collected to avoid contamination of the other specimens (CVF_{WS} and CVF_{DA}) with blood following trauma from cytobrushing. The brush was placed in 1 mL of PBS solution to remove the cells; the cells were counted by hemacytometer, and prepared for quantitation. The second clinical study visit occurred 5 to 7 days after the first clinical visit and occurred between 4 and 12 hrs post dose. At this visit, all five sample types were collected; plasma, PBMCs, CVF via Tearflo™ Strips and Direct Aspiration, and FGT cells from cytobrushing. The samples were all stored at -80°C and prepared for shipping to the University of Alabama at Birmingham Clinical Pharmacology Laboratory, where quantitation and analysis occurred.

LC/MS/MS Assay for Human Plasma

Calibration Standard Curve, Quality Controls, and Internal Standard

Master stock solutions (MS) of TFV and FTC (0.1 mg/mL) were prepared by dissolving approximately 1 mg each of TFV and FTC powder in 10 mL of deionized water. The tubes were capped, and vortexed for 1 minute or until all of the TFV and FTC powder had dissolved. The solutions were parafilmed tightly and stored at -80°C for up to 1 year. The MS of TFV-IS and FTC-IS (1.0 mg/mL) were prepared by reconstituting each IS powder with deionized water inside its original container. Again, the tubes were capped and vortexed for 1 minute or until all of the TFV-IS or FTC-IS powder has dissolved. Stability is also known for 1 year at -80°C.

Calibration standards were prepared by non-serial dilution, beginning with the TFV-MS and FTC-MS. In 15-mL plastic centrifuge tubes, (to allow for expansion of the aqueous solution during the freezing process) 7.5 mL of each standard was made. The actual concentration of the prepared standard solution was ten times stronger in concentration then the calibration level. The standard is diluted at a 1:10 ratio by the addition of the plasma during the extraction procedure, producing the calibration concentrations displayed in Table 4. Each calibration standard was vortexed for 30 seconds before preparing subsequent standards. Stability was valid for up to 1 year at –80°C.

QCs displayed in Table 5 were prepared in 15-mL centrifuge tubes by serial dilution at three different concentration levels; low (11 ng/mL), mid (dle) (220 ng/mL), and high (2200 ng/mL). The high quality control (HQC) was gently mixed by rotation

for one hour and then serially diluted to the subsequent quality control levels, which were also rotated for one hour. Stability was valid when stored in 1-2 mL aliquots for up to 1 year at –80°C.

Table 4.

TFV and FTC Plasma Calibration Curve

Standard Identification	Concentration (ng/mL)
H	2500
G	1000
F	500
E	100
D	50
C	25
B	10
A	5

Table 5.

TFV and FTC Plasma Quality Controls

Identification	Concentration (ng/mL)
High	2200
Mid(dle)	220
Low	11

Sample Preparation

Adaptations from an in house SPE technique developed for TFV only was used to prepare clinical plasma samples, along with the calibration standards and QC samples. Each tube contained 100 µL of either QC sample, 100 µL of blank plasma spiked with calibration working standard, or 100 µL of unknown patient sample, with 10 µL of the IS working solution at 200 ng/mL and 500 µL of 0.5% TFA. The mixture was then briefly vortexed. SPE was performed by the Oasis MCX SPE cartridges due to their ability to bind the very positively charged TFV and FTC. Prior to loading the samples to the cartridge, the cartridge was conditioned by washing with 1 mL of MeOH followed by 1 mL of deionized water. After the sample was loaded, cartridges were subsequently washed with 1 mL of 0.5% TFA and again with 1 mL of deionized water and 1 mL MeOH in order to remove all non-specifically bound components from the cartridge. Sample waste and wash containing negatively charged and neutral compounds were discarded.

TFV, FTC, TFV-IS, and FTC-IS, which are positively charged compounds, were eluted using 500 µL of 5% NH4OH in MeOH. This elute was then evaporated to dryness at 40°C under a gentle stream of nitrogen. The dried residue was reconstituted in 100 µL of deionized water, vortex mixed briefly, and transferred to clean autosampler vial inserts. For each sample, 10 µL aliquots were subject to separation and detection on the LC/MS/MS system. The LC/MS/MS parameters were configured for optimal specificity and sensitivity using declustering potential (DP), collision energy (CE), collision exit potential (CXP), polarity, temperature, gas flow, mobile phase (MP), and scan type ideal for TFV, FTC, TFV-IS, and FTC-IS.

LC/MS/MS Assay for PBMCs

Sample Collection

PBMCs have become a key compartment in measuring the concentration of intracellularly active drugs. PBMC were harvested from whole blood of patients by first gentle mixing the whole blood CPT tubes and then centrifuging the tubes at 1,500 x g for 20 minutes. After the top two layers are mixed, the layer above the gel is decanted into a 50 mL conical tube. The volume was recorded and a small aliquot was used for cell counting with a hemacytometer. These values were used for a total cell count measurement, which was recorded and used during analysis. The decanted solution is then centrifuged for 10 minutes at 400 x g to form a pellet. The cell pellet was then resuspended and transferred to a 1.5 mL microcentrifuge tube and centrifuged for 10 minutes at 400 x g. The cell pellet supernatant was removed and the cells were lysed with cold 70:30 methanol: water by vortexing the solution and placing it on ice for 15 minutes. The lysate solution was then centrifuged again for 1 minute at 13,000 x g and the supernatant was transferred to a fresh 1.5 mL cryovial ready for analysis.

Sample Preparation

The PBMC assay followed the same quantitation parameters on the LC/MS/MS as the plasma assay; however, the validation was performed with calibration standards and QCs specific to the sample type. The complete range of the calibration standards for the PBMC assay was from 0.1 fmol – 2500 fmol per 10^6 cells for both TFV and FTC,

with one curve ranged from 0.1 – 15 fmol per 10^6 cells and then the other curve ranged from 10-2500 fmol per 10^6 cells. Table 6 shows the nonserial dilution scheme from the 0.1 mg/mL TFV-MS and FTC-MS solutions used to make the calibration standards. The QCs were made to mimic actual patient PBMC samples therefore, were made from a MS (5000 fM) of the intracellular phosphorylated TFV-DP and FTC-TP. There were two sets of serially diluted QCs, one for each curve; QC set 1 was 0.3 fmol per 10^6 cells (low), 5 fmol per 10^6 cells (mid), and 10 fmol per 10^6 cells (high) while QC set 2 was 11, 220, and 2200 fmol per 10^6 cells respectively for low, mid, and high. Both sets of QCs are shown in Table 7. The combined TFV-IS and FTC-IS maintained a concentration of 7 fmol per 10^6 cells for the calibration curve A and 40 fmol per 10^6 cells for calibration curve B.

Table 6.

TFV and FTC PBMC, FGT Cells, and CVF_{DA} Calibration Curves

A.	Standard Identification	Concentration (fmol/10^6 cells)	B.	Standard Identification	Concentration (fmol/10^6 cells)
	H	15		**H**	2500
	G	10		**G**	1000
	F	5		**F**	500
	E	2.5		**E**	100
	D	1		**D**	50
	C	0.5		**C**	25
	B	0.25		**B**	10
	A	0.1		**A**	5

Sample Extraction

TFV and FTC are phosphorylated within the cell to their respective NRTI-triphosphate forms, which are the active and respectively the more essential concentration needed to determine the efficacy of the drugs. Four major steps occurred to successfully quantify intracellular levels of TFV and FTC. First aspect was the establishment of the cellular matrix needed for the calibration standards and QCs. It was determined that THP-1 cells would be the surrogate cell line for PBMCs throughout this project and therefore these cells were grown to confluence. A small volume was removed from each cellular flask for cell counting with a hemacytometer and the total cell count measurement was used to determine how much 70:30 methanol: water was needed to make a cell lysate with 10^6 cells per 100 µL. The THP-1 cells were then collected and the tubes were processed using a similar method as used for the PBMC patient samples to form the blank cellular matrix.

Table 7.

TFV and FTC PBMC, FGT Cells, and CVF_{DA} Quality Controls

A. Identification	Concentration (fmol/10^6 cells)	B. Identification	Concentration (fmol/10^6 cells)
High	10	**High**	2200
Mid(dle)	5	**Mid(dle)**	220
Low	0.3	**Low**	11

The next major aspect of the assay was the dephosphorylation of TFV-DP and FTC-TP to TFV and FTC, which are the successfully measurable analytes for the LC/MS/MS systems. This was done by subjecting 100µL of the calibration curve, QCs, and unknown samples to an anion exchange SPE with a Waters QMA cartridge utilizing a 24 step KCl salt gradient (5 mM to 1 M) to isolate TFV-DP, FTC-TP, TFV-IS, and FTC-IS. TFV-DP and FTC-TP were then dephosphorylated to TFV and FTC using a sweet potato acid phosphatase/acetate buffer. Next, using Waters HLB reverse phase cartridge, the solution containing TFV, FTC, TFV-IS, and FTC-IS was desalted using MeOH and 1% TFA washes. The sample was then concentrated by evaporating it to dryness at 40°C under a gentle stream of nitrogen. The dried residue was reconstituted in 50 µL of deionized water, vortex mixed briefly, and transferred to clean autosampler vial inserts. For each sample, 40 µL aliquots were subject to separation and detection on the LC/MS/MS system.

LC/MS/MS Assay for FGT Cells

Cytobrush samples collected FGT cells, which were proximal to the cervix. The patient samples were then placed in tubes and washed with 1 mL of 1x PBS. After gentle mixing, the tubes are centrifuged at 1,500 x g for 20 minutes at 21°C. The volume is recorded and a small aliquot is used for cell counting with a hemacytometer and used during analysis. The total cell count measurement was recorded. The solution is then centrifuged for 10 minutes at 400 x g. The cells are resuspended and transferred to a 1.5 mL microcentrifuge tube and centrifuged for 10 minutes at 400 x g. The supernatant is

removed and the cells are lysed with a cold lysis solution of 70:30 methanol: water. After vortexing, the cells were allowed to sit for 15 minutes in an ice bath. The lysate solution is centrifuged for 1 minute at 13,000 x g at and the supernatant is transferred to a fresh 1.5 mL cryovial. These unknown samples are quantitated exactly the same as PBMC samples (See section: PBMC Sample Extraction). The same calibration curve, QCs, shown in Tables 5 and Table 6 were used along with the LC/MS/MS parameters were maintained and reused for this assay.

LC/MS/MS Assay for CVF_{DA}

Sample Preparation

Direct Aspirated CVF (CVF_{DA}) was collected from the posterior fornix of the vagina using a CVF collection device. The fluid was then transferred to a 1.5 mL cryovial. Although cells may have been disturbed by the procedure, the assay only measures TFV and FTC and does not account for phosphorylated TFV and FTC from within cells. However, untreated CVF was not available for purchase, therefore, the surrogate matrix for the calibration curve and QCs was lysed THP-1 cells. The calibration curve for the CVF assay was the same calibration curve used for the PBMC and Cytobrush samples and ranged from 0.1 fmol – 2500 fmol per 10^6 cells for both TFV and FTC. However, unlike the PBMC and cytobrush assay the QCs for the CVF assay were made of TFV and FTC only. Two sets of QCs were made. Set 1 had QCs at 0.3 fmol per 10^6 cells (low), 5 fmol per 10^6 cells (mid), and 10 fmol per 10^6 cells (high) and

set 2 had QCs at 11 fmol per 10^6 cells (low), 220 fmol per 10^6 cells (mid), and 2200 fmol per 10^6 cells (high).

Sample Extraction

The extraction of the CVF mimics the extracts of plasma. A 100 µL aliquot of sample is acidified with 0.5% TFA and IS solution is added. The sample is then filtered through the Waters MCX cartridges which where pretreated with deionized water and MeOH. Then, the sample loaded cartridges were then carefully washed with deionized water, 0.5% TFA and MeOH, and the drugs were eluted off with 5% NH_4OH. The CVF sample was then evaporated under nitrogen at 40°C and then reconstituted in 50 µL of deionized water vortex mixed briefly, and transferred to clean autosampler vial inserts. For each sample, 40 µL aliquots were subject to separation and detection on the LC/LC/MS system previously determined.

LC/MS/MS Assay for CVFws

Sample Collection and Extraction

Using the novel technique of collecting CVF on Tearflo™ Strip led to the quantitation of TFV and FTC from the wicking stripped CVF sample (CVF_{WS}). CVFws patient samples were collected using Tearflo strips that were attempted to be completely saturated during cervix swabbing (saturation occurs with 25 µL of CVF). The surrogate for CVF for the calibration curve and QCs again was lysed THP-1 cellular matrix. The calibration standards ranged slightly different from the previous curves, extending from

0.5-1000 fmol per 10^6 cells as shown in Table 8. The extraction method for TearFlo™ Strip was simple and only consisted of the strips, the extraction solution (100% cold MeOH) and TFV-IS/FTC-IS. The calibration curve was prepared for extraction by spiking 25 µL of blank THP-1 cellular matrix on each strip. The strip was then allowed to dry and stored at -80°C until needed. The QCs shown also in Table 8 were made by spiking cellular matrix with TFV and FTC concentrations of 1.5 (low), 400 (mid), and 800 (high) fmol per 10^6 cells. Then 25 µL of the QC cellular matrix was aliquoted onto Tearflo™ Strips and allowed to dry and stored at -80°C to mimic patient samples.

Table 8.

TFV and FTC CVF_{WS} Calibration Curve and Quality Controls

Standard Identification	Concentration (fmol/10^6 cells)
H	1000
G	500
F	100
E	50
D	10
C	5
B	1
A	0.5
High	800
Mid(dle)	400
Low	1.5

The extraction method consisted of taking the entire strip; the blank cell lysed strips for the calibration curve, the QCs, or unknown samples, and placing each strip in a 3 mL glass tube. Then 2 mL of the extraction solution was added into each tube ensuring to cover the entire strip. Each tube had 10 µL of the TFV-IS/FTC-IS (at 40 fmol/106 cells) added to the extraction solution. Specifically for the calibration standards an additional 10 µL of the concentrated standards was spiked into the extraction solution. The strips are then allowed to shake vigorously for one hour. The extraction solution and the TearFlo™ Strips were then evaporated under nitrogen at 40°C. The Strip was removed and the residue was reconstituted in 50 µL of deionized water vortexed briefly, spun down by centrifugation, and then transferred to clean autosampler vial inserts. For each sample, 40 µL aliquots were subject to separation and detection on the LC/MS/MS system, which has been previously described.

Pharmacokinetic Analysis of Clinical Samples

Pharmacokinetic parameters of TFV and FTC were determined using non-compartmental methods (WinNonLin version 5.2; Pharsight Corp., Mountain View, CA). The TFV dose of 136 mg in a 300-mg dose of TDF was used in all calculations of TFV parameters, while 200 mg dose was used for FTC. The AUC was calculated using the linear-up/log-down trapezoidal rule. Maximum concentration (C_{max}), minimum concentration (C_{min}), the last observed concentration (C_{last}), the concentration at 6 hours (C_6), the concentration at 24 hours (C_{24}), and time to maximum concentration (T_{max}) were taken directly from the observed concentration-time data. Apparent oral clearance

(CL/F) was calculated as dose/AUC. Terminal apparent distribution volume (Vz/F) was calculated as dose divided by the product of the elimination rate constant (ke) and AUC. The elimination rate constant (ke) was determined by linear regression of the terminal elimination phase concentration-time points; elimination half-life (t½) was calculated as ln (2)/ke. Measured samples below each assay's limit of quantitation (below the level of quantitation (BLQ), [plasma BLQ: 5 ng/mL] [PBMC, FGT Cells, CVF_{DA} BLQ: 0.1 fmol/10^6 cells] [CVF_{WS} BLQ: 0.5 fmol/10^6 cells]) were assigned the value 2.5 ng/mL, 0.05 and 0.25 fmol/10^6 cells, respectively. Each pharmacokinetic parameter reported the number of patients used in the calculation and also determined each parameters' mean, SD, standard error (SE), geometric mean, range (minimum and maximum), median and the CV%.

Comparison of TFV and FTC Concentrations by CVF Collection Techniques

CVF_{DA} and CVF_{WS} samples were extracted differently and quantified with assays, which were validated for these samples types. CVF_{DA} were obtained from the posterior fornix of the vagina using a volumetric vaginal aspirator. The CVF_{DA} samples were transferred to a 1.2 mL cryovial and were frozen at -80˚C and shipped for analysis. The TearFlo™ Strips were stored in cryovials at – 80˚C after collecting CVF from pressing the Tearflo™ Strip against the cervix.

The two sample types were analyzed, quantified, and then the mean concentrations for both TFV and FTC collected by direct aspirate and wicking strip were

compared. Statistical calculations, included correlations between samples types, were performed using Wilcoxon Matched-pairs Signed Rank test in GraphPad Prism.

Development of Predictive Pharmacokinetic Models

Pharmacokinetic modeling was accomplished using a nonlinear mixed effects approach. A first-order conditional estimation method with interaction (FOCEI) using the nonlinear mixed effects modeling (NONMEM) system (NONMEM Version 7 and Wing for NONMEM; Perl speak to NONMEM, GloboMax LLC (Lindbom, Ribbing, & Jonsson, 2004)) was used and specified with ADVAN4 and TRANS4. The TFV and FTC models were parameterized with CL/*F*, central volume of distribution (*VC*/*F*), intercompartmental clearance(s) (Q/*F*), peripheral volume of distribution(s) (*VP*/*F*), and oral absorption rate constant (Ka). R program Xpose4 (Jonsson & Karlsson, 1999) was used for goodness-of-fit assessment and model evaluation. Models were investigated to characterize TFV and FTC plasma concentrations separately. Classical one-, two-, and three-compartment models were evaluated. Selection of the structural pharmacokinetic model was driven by the data and based on goodness-of-fit plots and the objective function value.

The prediction-corrected visual predictive checks (pcVPC) (Bergstrand, Hooker, Wallin, & Karlsson, 2011) were simulated by NONMEM. The interindividual variability

for each structural parameter of the basic model was modeled using the exponential error model:

$$P_i = P_{TV} \times Exp\ (\eta_i)$$

Where *Pi* is a parameter in an *i*th individual, P_{TV} is a typical parameter value for the modeled population, and η_i is an interindividual random variable, which is assumed to be distributed with a mean of zero and a variance of ω^2. The residual variability was evaluated (include additive error, proportional error, combined additive model) to describe the intraindividual variability. The additive, proportional, and combined additive and proportional error model, based on the following equation best described the residual variability:

$$Additive:\ C_{ij} = C_{pred,ij} + \varepsilon_{add,\ ij})$$

$$Proportional:\ C_{ij} = C_{pred,ij} \times (1 + \varepsilon_{prop,\ ij})$$

$$Combination\ Additive\ and\ Proportional:\ C_{ij} = C_{pred,ij} \times (1 + \varepsilon_{prop,\ ij}) + \varepsilon_{add,\ ij}$$

Where *C*ij is the jth observed value in the ith subject, *C*pred,ij is the jth predicted value in the ith subject, and εprop, ij is the residual intraindividual variability with mean of zero and variances of σpro2. The pcVPC was used as an informative diagnostic tool to examine model appropriateness over time. A total of 1000 simulations were done for VPC for both plasma models.

CHAPTER 3

RESULTS

Determination of TFV and FTC in Various Matrices using LC/MS/MS

Method development was determined with the optimization of chromatographic conditions including column type, mobile phase composition, and mass spectrometer setting optimization. Various aqueous and organic mobile phase components were investigated including 0.2% and 0.1% FA, 0.1% TFA, AcN, and MeOH. The aqueous part of the mobile phase was 0.1% TFA in water combination with AcN (90:10) to produce the best analyte separation and peak shape. The final mobile phase composition was then tested with extracted plasma and PBMC samples on two different C18 columns, the Water Atlantis and XBridge. The Atlantis dC18 column provided better selectivity as well as superior peak shapes. The optimal conditions for maximal recovery of analyte with minimal interference by endogenous matrix components were determined using various extraction techniques and solvents including: AcN, TFA, MeOH, KCl and a simple MCX, HLB, QMA SPE techniques.

Chromatography

Determination of TFV and FTC in plasma, PBMCs, CVF, and endothelial cells of the FGT were separated via reversed-phase liquid chromatography. Analytes were separated on an Atlantis dC18 analytical column (2.1 x 100 mm; 3μm particle size) using an isocratic binary mobile phase consisting of 90% 0.1% TFA buffer and 10% AcN (v/v). As shown in Figure 7, separation of a mid-concentration extracted plasma sample was achieved with a 5 minute run time. TFV, FTC, TFV-IS and FTC-IS were eluted at retention times of 2.1, 3.2, 2.2 and 3.2 min, respectively.

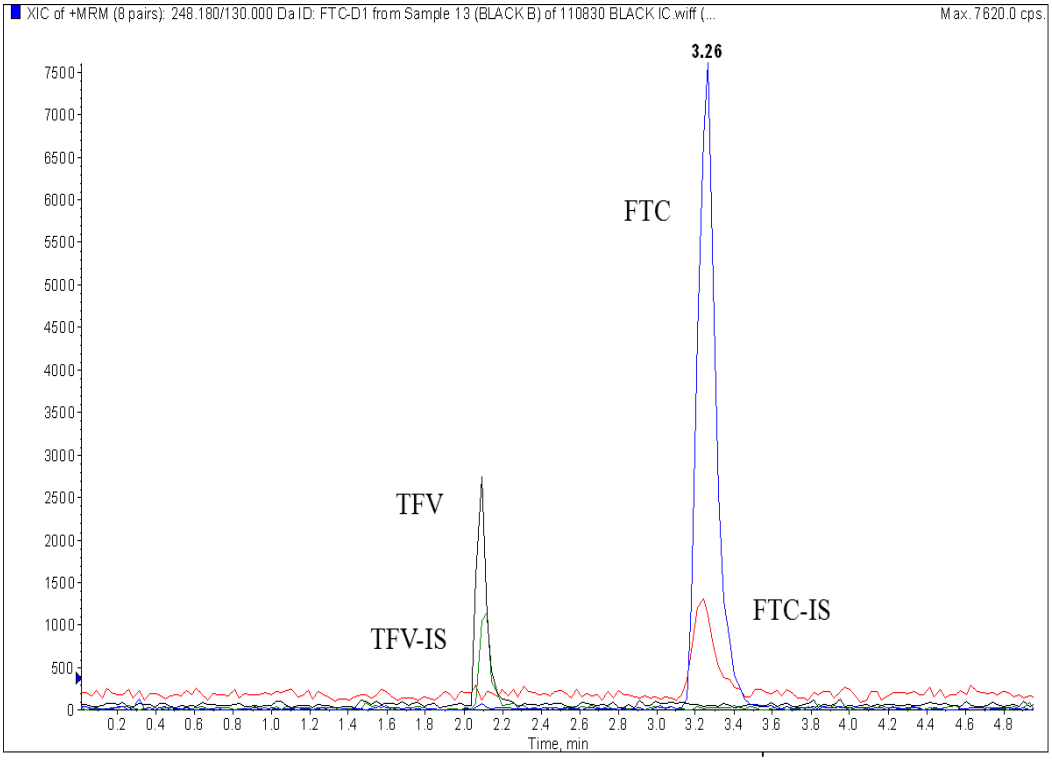
In order to optimize MRM conditions for TFV, FTC, TFV-IS, and FTC-IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra revealed peaks at m/z TFV 288→176.1 and 288→159, *m/z* TFV-IS 293→181, *m/z* FTC 248.1→130 and 248.1→113, *m/z* FTC-IS 251→133. A syringe pump was used to optimize the mass spectrometer operating parameters for TFV, FTC, TFV-IS and FTC-IS by directly infusing one of the working solutions into the source (of the Mass Spectrometer) at a flow rate of 10 μL/min. During mass spectrometer optimization, gas flow (curtain, gas 1, and gas 2), source temperature, and ion source were adjusted during direct analyte infusion. Parameters were set at levels that produced the greatest signal intensity. Final operating parameters were as follows: curtain (nitrogen) 20 L/hr, gas1 (nitrogen) 55 L/hr, gas2 (nitrogen) 50 L/hr, source temperature 500°C, ion source 5500 kV, and a dwell time of 0.5 s. Additionally, the Declustering Potential (DP), Collision Energy (CE), and Collision Exit Potential (CXP) all changed and were optimized for each drug and IS and are listed in Table 9.

Table 9.

MS Optimization Settings for TFV and FTC in All Matrices

Ion Name	Q1 Mass (parent ion)	Q3 Mass (daughter ion)	DP	CE	CXP
TFV-1	288.198	176.100	16	35	18
TFV-2	288.198	159.000	16	47	20
FTC-1	248.180	130.000	46	17	18
FTC-2	248.180	113.00	46	53	14
TFV-IS	293.198	181.000	131	35	24
FTC-IS	251.092	132.937	66	23	14

Figure 7. Representative Chromatogram of TFV and FTC in Plasma



Analytes were separated on an Atlantis dC18 analytical column (2.1 x 100 mm; 3µm particle size) using an isocratic binary mobile phase consisting of 90% 0.1% TFA buffer and AcN

Assay Validation

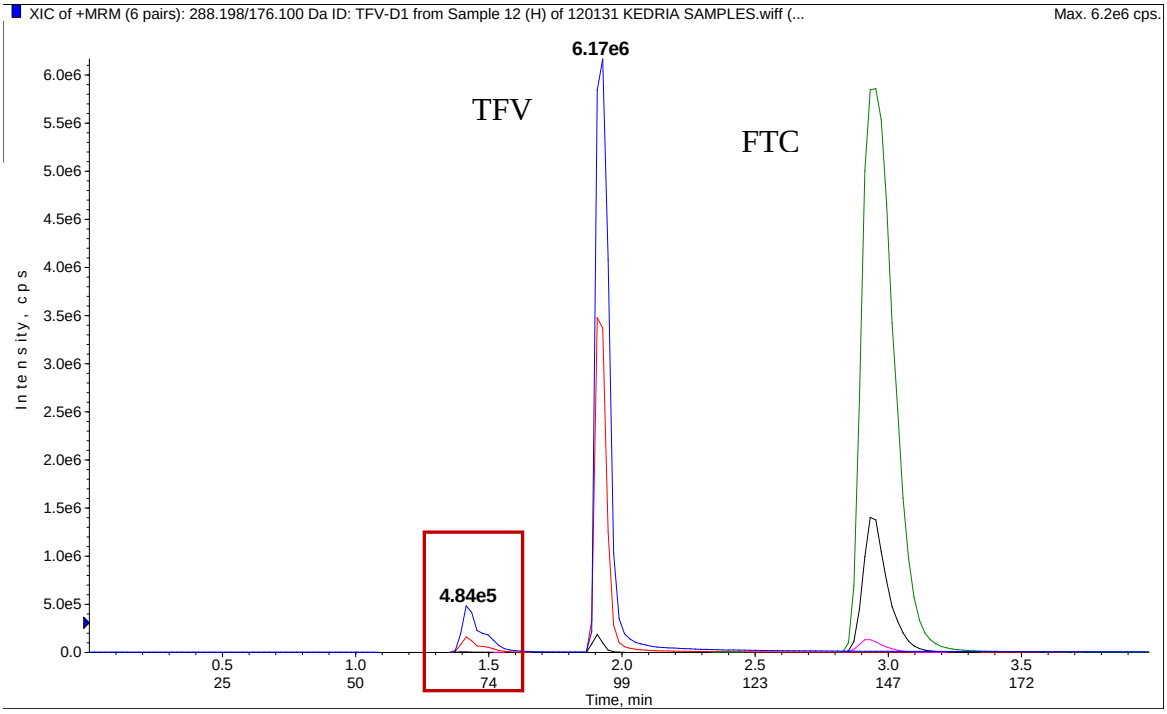
All TFV and FTC assays were validated according to the FDA and GCLP guidance for the validation of bio analytical methods. This validation included an evaluation of specificity of analytes, matrix effect, recovery of analytes from each matrix of interest, precision and accuracy (determined by QC performance), and linearity and sensitivity of the calibration curves for each matrix. Each validation assay was performed three times, in secession, and contained one of each: a calibration curve, a blank matrix sample without the addition of IS, and three of each QC to determine within and between assay performance.

Separate extraction methods for plasma, PBMC, and wicking strips, which are described in the Methods section, were paired with our LC/MS/MS parameters. It should be noted that the PBMC method contained two calibration curves with two sets of QCs. Two sets of calibration standards and QCs ensured that the calibration range expanded to successfully quantitate all samples. The PBMC extraction method was used for the extraction of TFV and FTC from clinically obtained PBMCs, endothelial cells from the FGT collected by cytobrushing, and all the *in vitro* work. In addition, the CVF_{DA} samples utilized the calibration curves designated for PBMCs, however, had their own QCs. CVF_{DA} was extracted by the plasma extraction method. Routine assays for the analysis of all clinical samples require a calibration curve, including a blank sample, and three sets of each QC for each run once the method was validated.

Specificity for All Matrices

In order to evaluate assay specificity, three different lots of drug-free human plasma along with three different batches of lysed THP-1 cellular matrices were tested for interfering components. The various lots of drug-free blank human plasma showed a small endogenous matrix peak under the conditions used in the assay. However, the peaks maintained retention times away from the analytes and ISs, and did not interfere with quantitation (Figure 8). The three batches of lysed THP-1 cellular matrices showed no endogenous matrix peaks and provided clean and consistent signal.

Figure 8. TFV and FTC Chromatogram- Matrix Effect



Analytes were separated on an Atlantis dC18 analytical column (2.1 x 100 mm; 3µm particle size) using an isocratic binary mobile phase consisting of 90% 0.1% TFA buffer and AcN (v/v). The first peak (shown in the red box) demonstrates endogenous particles; however, their presence does not interfere with quantitation of TFV or FTC.

As an additional specificity test, potential concomitant medications, which are listed in Table 10, were put through the TFV and FTC plasma and PBMC extractions in conjunction with high concentrations of TFV, FTC, TFV-IS, and FTC-IS. All of these compounds were analyzed under the current chromatographic conditions. The concomitant drugs investigated during specificity experiments included other currently prescribed antiretroviral agents, acyclovir (ACV), used to treat herpes co-infections, ganciclovir (GCV), also used to the treatment of herpes simplex virus and cytomegalovirus (CMV), and cidofovir (CDV) an antiviral used in the treatment of CMV in HIV-seropositive patients. High concentrations of both the concomitant medications and our drugs of interest were tested in order to maximize the potential for interference. The parent and daughter ion channels on the mass spectrometry for TFV, FTC, TFV-IS, and FTC-IS were monitored, and none of the drugs listed demonstrated a measurable peak at any of our analytes of interest retention time. It should be noted that GCV, ACV, and APV had retention times within 0.3 minutes of TFV and its IS. While IDV, 3TC, NFV, and AZT were eluted from the column under the conditions described previously at retention times within 0.3 minutes of FTC and its IS. All concomitant drugs' retention times (RT) are shown in Table 10. These experiments verify the high specificity of the current method with regard to possible endogenous matrix components as well as commonly co-administered medications.

Table 10.

TFV and FTC LC/MS/MS Assay Specificity

Concomitant Medications	**RT (min)**
abacavir (ABC)	1.1
acyclovir (ACV)	2.56
amprenavir (APV)	1.82
atazanavir (ATV)	3.8
cidofovir (CDV)	<1
didanosine (ddI)	3.7
darunavir (DRV)	3.99
efavirenz (EFV)	>5
emtricitabine (FTC)	**analyte**
ganciclovir (GCV)	2.50
indinavir (IDV)	3.1
lamivudine (3TC)	3.00
lopinavir (LPV)	>5
nelfinavir (NFV)	3.5
nevirapine (NVP)	<1
raltegravir (RAL)	3.74
ritonavir (RTV)	4.05
saquinavir (SQV)	4.00
stavudine (d4T)	>5
tenofovir (TFV)	**analyte**
zidovudine (AZT)	3.34

RT (Retention time) Run time was 5 mins

Matrix Effects and Recovery

Ionization suppression or enhancement during the ionization process of quantitation can be a problem when developing LC/MS/MS assays and extractions. The probability of interference increases with the use of more than one analyte, and with enriched matrices that differ from each other in texture. Our LC/MS/MS assay for the quantitation of TFV and FTC is paired with the extraction of TFV and FTC from three distinct matrices: plasma, cellular lysate (PBMCs), and wicking strip. The calculations for matrix effects, as well as absolute recovery were carried out following the procedures described previously in the methods section. Two distinct comparisons were used for the matrix effect determination. The first comparison, relative matrix effect, indicates the significance of the matrix effects by the difference in the precision of PAR for each sample sets 1 and 2 (CV (post-extracted) - CV (unextracted)). While the second comparison, absolute matrix effect, evaluated ion suppression or enhancement by comparing the mean peak areas in sample sets 1 and 2. Absolute recovery, determined by comparing the ratio of mean peak areas in normally extracted samples (set 3) to those spiked with analyte post extraction, indicated extraction efficiency from the sample matrix.

Plasma. The matrix effect and absolute recovery data for TFV determined in three different lots of blank human plasma are presented in Table 11. Average absolute recoveries for TFV were 93, 100, and 97% for QC samples at concentrations of 11, 220, and 2200 ng/mL, respectively. The CV% for all recoveries throughout the entire

concentration range evaluated were less than 12.6%, demonstrating method consistency in multiple lots of human plasma. Differences in PAR ranged from 1.7 to 2.6 revealing high precision and lack of significant matrix effect. Similarly, shown in Table 11, the average matrix effect for TFV was 88, 90, and 106% for QC samples at concentrations of 11, 220, and 2200 ng/mL, respectively. It should be noted, a matrix effect value of 100% indicates the absence of an absolute matrix effect, while a value greater than 100% indicates ionization enhancement and a value less than 100% indicates ionization suppression. No significant peak area differences were observed, signifying an absence of suppression or enhancement of TFV ionization.

Table 11.

Matrix Effect and Absolute Recovery for TFV and FTC in Human Plasma (n=3)

	Nominal Concentration (ng/mL)	Relative Matrix Effect (CV%)	Absolute Matrix Effect (%), Mean ± SD	Absolute Recovery (%), Mean ± SD
TFV	11	2.3	88 ± 4.1	93 ± 4.0
	220	1.7	90 ± 8.1	100 ± 3.5
	2200	2.6	106 ± 1.6	97± 4.0
FTC	11	-5.5	108 ± 3.2	92 ± 5.6
	220	3.6	98 ± 5.8	107 ± 7.9
	2200	0.4	107 ± 2.2	110 ± 2.9

Table 11 also illustrates the matrix effect and absolute recovery data for FTC in the same three different lots of blank human plasma used for TFV. The average absolute recoveries for FTC were 92, 107, and 110% for QC samples at concentrations of 11, 220, and 2200 ng/mL, respectively. The CV% for all recoveries throughout the entire

concentration range evaluated were less than 9.1%, which is more variable than FTC, but still falls within ±15% to demonstrate the method consistency in multiple lots of human plasma. Differences in PAR ranged from -5.5 to 3.6 revealing high precision and lack of significant matrix effect. Again, shown in Table 11, the average matrix effect for FTC was 108, 98, and 107% for QC samples at concentrations of 11, 220, and 2200 ng/mL, respectively. Similar to TFV in plasma, no absence of suppression or enhancement of FTC was observed.

PBMC (Set A). *T*he matrix effect and absolute recovery data for TFV determined in three different batches of lysed THP-1 cellular matrices are presented in Table 12. Average absolute recoveries for TFV were 102, 92, and 93% for QC samples at concentrations of 0.3, 5, and 10 fmol/10^6 cells, respectively. The CV% for all recoveries throughout the entire concentration range (0.1 -15 fmol/10^6 cells) evaluated were less than 14.7%, demonstrating method consistency in multiple batches of lysed cells. Differences in PAR ranged from -12.3 to 13.9 revealing high precision and lack of significant matrix effect. Similarly, shown in Table 12, the average matrix effect for TFV was 88, 80, and 88% for QC samples at concentrations of 0.3, 5, and 10 fmol/10^6 cells, respectively. No significant (±15%) peak area differences were observed, signifying an absence of suppression or enhancement of TFV ionization.

Table 12.

Matrix Effect and Absolute Recovery for TFV and FTC Lysed THP-1 Cells (Set A) (n=3)

	Nominal Concentration (fmol/10^6 cells)	Relative Matrix Effect (CV%)	Absolute Matrix Effect (%), Mean ± SD	Absolute Recovery (%), Mean ± SD
TFV	0.3	-12.3	88 ± 8	102 ± 4.0
	5	13.9	80 ± 10.0	92 ± 13.5
	10	2.3	88 ± 4.2	93± 4.0
FTC	0.3	5.0	77 ± 10.7	77 ± 9.6
	5	-2.5	95 ± 7.7	102 ± 5.3
	10	10.2	100 ± 15.0	99 ± 14.5

Also in Table 12 are the matrix effect and absolute recovery data for FTC in three different batches of lysed THP-1 cellular matrices. The average absolute recoveries for FTC were 77, 102, and 99% for QC samples at concentrations of 0.3, 5, and 10 fmol/10^6 cells, respectively. The CV% for all recoveries throughout the entire concentration range evaluated were less than 14.5%, which is more variable than FTC, but still falls within ±15% to demonstrate the method consistency in multiple batches of lysed cells. Differences in PAR ranged from -2.5 to 10.2 revealing high precision and lack of significant matrix effect. Again, shown in Table 12, the average matrix effect for FTC was 77, 95, and 100% for QC samples at concentrations of 0.3, 5, and 10 fmol/10^6 cells, respectively. Similar to TFV in PBMC at this lower concentration range, no absence of suppression or enhancement of FTC was observed.

PBMC (Set B). The matrix effect and absolute recovery data for TFV determined in three different batches of lysed THP-1 cellular matrices are presented in Table 13. Average absolute recoveries for TFV were 80, 96, and 94% for QC samples at concentrations of 11, 220, and 2200 fmol/10^6 cells, respectively. The CV% for all recoveries throughout the entire concentration range (5 – 2500 fmol/10^6 cells) evaluated were less than 13.8%, demonstrating method consistency in multiple batches of lysed cells. Differences in PAR ranged from -1.2 to 12.0 revealing high precision and lack of significant matrix effect. Similarly, shown in Table 13, the average matrix effect for TFV was 105, 106, and 111% for QC samples at concentrations of 11, 220, and 2200 fmol/10^6 cells, respectively. No significant peak area differences were observed, signifying an absence of suppression or enhancement of TFV ionization; however, it should be noted that all values were greater than 100% indicates ionization enhancement. The enhancement, however, is not statistically significant by GCLP and FDA standards.

Table 13.

Matrix Effect and Absolute Recovery of TFV and FTC in Lysed THP-1 cells (Set B) (n=3)

	Nominal Concentration (fmol/10^6cells)	Relative Matrix Effect (CV%)	Absolute Matrix Effect (%), Mean ± SD	Absolute Recovery (%), Mean ± SD
TFV	11	2.5	105 ± 5.4	80 ± 5.1
	220	-1.2	106 ± 3.9	96 ± 2.6
	2200	12.0	111 ± 7.6	94± 10.9
FTC	11	-6.1	88 ± 3.9	106 ± 2.2
	220	6.8	88 ± 5.0	102 ± 6.4
	2200	2.3	87 ± 4.1	93 ± 4.0

Table 13 illustrates, again, the matrix effect and absolute recovery data for FTC in three different batches of lysed THP-1 cellular matrices. The average absolute recoveries for FTC were 106, 102, and 93% for QC samples at concentrations of 11, 220, and 2200 fmol/10^6 cells, respectively. The CV% for all recoveries throughout the entire concentration range evaluated were less than 14.7%, which is more variable than FTC, but still falls within ±15% to demonstrate the method consistency in multiple batches of lysed cells. Differences in PAR ranged from -6.1 to 6.8 revealing high precision and lack of significant matrix effect. Again, shown in Table 13, the average matrix effect for FTC was 88, 88, and 86% for QC samples at concentrations of 11, 220, and 2200 fmol/10^6 cells, respectively. In contrary to TFV in PBMCs at this high concentration range, suppression of FTC was observed, however it was not significant and do not warrant any additional tests.

CVF$_{WS}$. The matrix effect and absolute recovery data for TFV determined in three different batches of wicking strips spiked with THP-1 lysate are presented in Table 14. Average absolute recoveries for TFV were 103, 99, and 99% for QC samples at concentrations of 1.5, 400, and 800 fmol/10^6 cells, respectively. The CV% for all recoveries throughout the entire concentration range (0.5 – 1000 fmol/10^6 cells) evaluated were less than 14.3%, demonstrating that the method was consistent in multiple batches of strips. Differences in PAR ranged from –0.9 to 1.9 revealing high precision and lack of significant matrix effect. Similarly, shown in Table 14, the average matrix effect for TFV was 81, 94, and 103% for QC samples at concentrations of 1.5, 400, and 800

fmol/10^6 cells, respectively. No significant peak area differences were observed, signifying an absence of suppression or enhancement of TFV ionization.

Table 14.

Matrix Effect and Absolute Recovery for TFV and FTC on Wicking Strips Spiked with Lysed THP-1 Cells (n=3)

	Nominal Concentration (fmol/10^6cells)	Relative Matrix Effect (CV%)	Absolute Matrix Effect (%), Mean ± SD	Absolute Recovery (%), Mean ± SD
TFV	1.5	-1.9	81 ± 8.7	103 ± 0.8
	400	-0.9	94 ± 5.0	99 ± 2.4
	800	0.0	103 ± 19.3	99 ± 1.4
FTC	1.5	-5.5	108 ± 1.3	115 ± 6.2
	400	3.6	98 ± 7.0	107 ± 10.6
	800	0.4	107 ± 3.9	110 ± 3.1

Along with Table 14 illustrating data for TFV, it also contained the matrix effect and absolute recovery data for FTC in three different batches of spiked wicking strips. The average absolute recoveries for FTC were 115, 107, and 110% for QC samples at concentrations of 1.5, 400, and 800 fmol/10^6 cells, respectively. The CV% for all recoveries throughout the entire concentration range evaluated were less than 9.1%, which is more variable than FTC, but still falls within ±15% to demonstrate the method consistency in multiple batches of strips. Differences in PAR ranged from -5.5 to 3.6 revealing high precision and lack of significant matrix effect. Again, shown in Table 14, the average matrix effect for FTC was 108, 98, and 107% for QC samples at concentrations of 1.5, 400, and 800 fmol/10^6 cells, respectively. Similar to TFV on spiked wicking strips, no absence of suppression or enhancement of FTC was observed.

Accuracy and Precision: QC Performance

Accuracy and precision for TFV and FTC in plasma, PBMC, CVF_{DA} and CVF_{WS} QCs were determined. Each set of QCs determined their accuracy and precision by calculating the inter-day and intra-day variability of three or more sets of QCs from three different assay runs. The mean ± SD of the precision and accuracy were calculated from the replicates over all three assay runs to provide inter-day values while intra-day values were calculated from the triplicate of each QC on each individual run.

Plasma. Precision and accuracy data for TFV and FTC determined in 11 (low), 220 (mid) and 2200 ng/mL (high) QCs are presented in Table 15. The overall inter-assay precisions of the low, mid, and high TFV QCs were 3.5, 3.6, and 1.7%, respectively, while the overall accuracies were 0.3, 6.3, and 1.8%, respectively. The intra-assay precisions of the low, mid, and high TFV QCs ranged from 1.8 to 3.7%, 1.7 to 6.1%, and 1.4 to 2.0% respectively, while the accuracies ranged from -2.1 to 3.7%, 5.7 to 7.2%, and 0.8to 2.9%, respectively. Similarly, the overall inter-assay precisions of the low, mid, and high FTC QCs were 5.6, 6.9, and 2.9%, while the overall accuracies were 7.8, 7.8, and 0.6%, respectively. The intra-assay precisions of the low, mid, and high FTC QCs ranged from 2.3 to 9.4%, 1.5 to 8.8%, and 1.8 to 2.9%, while the accuracies ranged from 5.6 to 9.8%, 0.3 to 11.9%, and –1.7 to 2.9%, respectively. All TFV and FTC plasma QC samples fell within ± 15% of the theoretical value required for acceptance and therefore, validation was achieved.

PBMC (Set A). Precision and accuracy data for TFV and FTC determined in 0.3 (low), 5 (mid), and 10 fmol/10^6 cells (high) PBMC QCs are presented in Table 16. The overall inter-assay precisions of the low, mid, and high TFV QCs were 4.4, 8.3, and 7.7%, respectively, while the overall accuracies were -11.5, 6.0, and 3.5%, respectively. The intra-assay precisions of the low, mid, and high TFV PBMC QCs ranged from 0.9 to 7.0%, 1.6 to 4.9%, and 3.8 to 12.6% respectively, while the accuracies ranged from -13.6 to -9.3%, -3.5 to 14.7%, and -1.1 to 8.1%, respectively. Similarly, the overall inter-assay precisions of the low, mid, and high FTC QCs were 5.2, 5.8, and 7.9%, while the overall accuracies were 2.4, 7.4, and 7.0%, respectively. The intra-assay precisions of the low, mid, and high FTC QCs ranged from 2.7 to 6.6%, 2.1 to 4.5%, and 1.8 to 13.5%, while the accuracies ranged from -1.3 to 6.1%, 1.2 to 13.6%, and 4.5 to 9.6%, respectively. All TFV and FTC PBMC set A QC samples fell within ± 15% of the theoretical value required for acceptance and therefore, validation was achieved.

PBMC (Set B). Precision and accuracy data for TFV and FTC determined in 11 (low), 220 (mid), and 2200 fmol/10^6 cells (high) PBMC QCs are presented in Table 17. The overall inter-assay precisions of the low, mid, and high TFV QCs were 6.5, 5.2, and 8.5%, respectively, while the overall accuracies were -2.3, 4.6, and 3.0%, respectively.

Table 15.

Inter-day and Intra-day Precision and Accuracy for the Analysis of TFV and FTC in Human Plasma Quality Control Samples

Nominal Concentration (ng/mL)	TFV			FTC		
	Measured Concentration (ng/mL), mean ± SD	CV (%)	Accuracy (%)	Measured Concentration (ng/mL) mean ± SD	CV (%)	Accuracy (%)
Interday (=9)						
11	11.0 ± 0.4	3.5	0.3	11.9 ± 0.7	5.6	7.8
220	234.0 ± 8.4	3.6	6.3	237.1 ± 16.3	6.9	7.8
2200	2238.9 ± 37.7	1.7	1.8	2213.4 ± 64.1	2.9	0.6
Intraday (n=3)						
11	10.8-11.4 ± 0.2 - 0.4	1.8 - 3.7	-2.1 - 3.7	11.6-12.1 ± 0.3 - 1.1	2.3 – 9.4	5.6 – 9.8
220	233-236 ± 3.9 - 14	1.7 - 6.1	5.7 - 7.2	221-246 ± 3.6 - 20	1.5 – 8.8	0.3 – 11.9
2200	2217 -2264 ± 30 - 46	1.4 - 2.0	0.8 - 2.9	2163 -2264 ± 41 - 62	1.4 - 2.0	-1.7 - 2.9

The intra-assay precisions of the low, mid, and high TFV PBMC QCs ranged from 2.8 to 7.0%, 1.6 to 8.3%, and 3.8 to 12.6% respectively, while the accuracies ranged from -9.0 to 0.9%, 2.6 to 7.2%, and -1.7 to 7.8%, respectively. Similarly, the overall inter-assay precisions of the low, mid, and high FTC QCs were 8.9, 4.5, and 9.0%, while the overall accuracies were 1.1 2.9, and 0.8%, respectively. The intra-assay precisions of the low, mid, and high FTC QCs ranged from 3.4 to 6.6%, 2.0 to 4.5%, and 1.8 to 13.5%, while the accuracies ranged from -5.0 to 11.6%, -1.4 to 6.8%, and -5.0 to 1.8%, respectively. All TFV and FTC PBMC set B QC samples fell within ± 15% of the theoretical value required for acceptance and therefore, validation was achieved.

CVF_{WS}. Precision and accuracy data for TFV and FTC determined in 1.5 (low), 400 (mid), and 800 fmol/10^6 cells (high) CVF_{WS} QCs are presented in Table 18. The overall inter-assay precisions of the low, mid, and high TFV QCs were 7.2, 2.9, and 1.2%, respectively, while the overall accuracies were -5.8, 4.9, and 1.2%, respectively. The intra-assay precisions of the low, mid, and high TFV CVF_{WS} QCs ranged from 0.4 to 5.5%, 1.8 to 4.0%, and 1.3 to 1.4% respectively, while the accuracies ranged from -11.2 to 1.9%, 3.6 to 6.3%, and 0.8 to 1.6%, respectively. Similarly, the overall inter-assay precisions of the low, mid, and high FTC CVF_{WS} QCs were 6.1, 4.1, and 2.4%, while the overall accuracies were -4.6, 8.4, and 0.8%, respectively. The intra-assay precisions of the low, mid, and high FTC QCs ranged from 1.4 to 4.2%, 1.5 to 4.9%, and 1.7 to 2.6%, while the accuracies ranged from -9.0 to 2.2%, 4.3 to 10.8%, and -0.7 to 2.9%, respectively. All TFV and FTC CVF_{WS} QC samples fell within ± 15% of the theoretical value required for acceptance and therefore, validation was achieved.

Table 16.

Inter-day and Intra-day Precision and Accuracy for the Analysis of TFV and FTC in Set A of PBMCs Quality Control Samples

Nominal Concentration (fmol/10^6cells)	TFV			FTC		
	Measured Concentration (fmol/10^6cells) mean ± SD	CV (%)	Accuracy (%)	Measured Concentration (fmol/10^6cells) mean ± SD	CV (%)	Accuracy (%)
Interday (=9)						
0.3	0.27 ± 0.01	4.4	-11.5	0.31 ± 0.02	5.2	2.4
5	5.30 ± 0.44	8.3	6.0	5.37 ± 0.31	5.8	7.4
10	10.35 ± 0.80	7.7	3.5	10.70 ± 0.85	7.9	7.0
Intraday (n=3)						
0.3	0.26 - 0.27 ± 0.002 - 0.018	0.9- 7.0	-13.6 - -9.3	0.30 - 0.32 ± 0.08 - 0.02	2.7 - 6.6	-1.3 - 6.1
5	4.82 - 5.74 ± 0.08 - 0.26	1.6 - 4.9	-3.5 - 14.7	5.06 - 5.68 ± 0.11 - 0.26	2.1 - 4.5	1.2 - 13.6
10	9.89 - 10.81 ± 0.41 – 1.25	3.8 - 12.6	-1.1 - 8.1	10.45 - 10.96 ± 0.20 -1.41	1.8-13.5	4.5 - 9.6

Table 17.

Inter-day and Intra-day Precision and Accuracy for the Analysis of TFV and FTC in Set B of PBMCs Quality Control Samples

Nominal Concentration ($fmol/10^6 cells$)	TFV			FTC		
	Measured Concentration ($fmol/10^6 cells$) mean ± SD	CV (%)	Accuracy (%)	Measured Concentration ($fmol/10^6 cells$) mean ± SD	CV (%)	Accuracy (%)
Interday (=9)						
11	10.9 ± 0.8	7.6	-1.3	11.1 ± 1.0	8.9	1.1
220	230.1 ± 12.0	5.2	4.6	226.4 ± 10.3	4.5	2.9
2200	2265.5 ± 193.2	8.5	3.0	2218.3 ± 199.3	9.0	0.8
Intraday (n=3)						
11	10.0 – 11.5 ± 0.3 - 0.8	2.8 - 7.0	-9.0 - 4.3	10.5 - 12.3 ± 0.4 - 0.7	3.4 - 6.6	-5.0 – 11.6
220	226 - 236 ± 3.7 - 19	1.6 – 8.3	2.6 – 8.3	217 - 235 ± 4.7- 10.2	2.0 - 4.5	-1.4 – 6.8
2200	2163- 2372 ± 81 – 299	3.8 - 12.6	-1.7 – 7.8	2090 - 2338 ± 43 - 281	1.8-13.5	-5.0 – 6.3

Table 18.

Inter-day and Intra-day Precision and Accuracy for the Analysis of TFV and FTC on Wicking Strips Spiked with Lysed THP-1 Cells Quality Control Samples

Nominal Concentration (fmol/10^6cells)	TFV			FTC		
	Measured Concentration (fmol/10^6cells) mean ± SD	CV (%)	Accuracy (%)	Measured Concentration (fmol/10^6cells) mean ± SD	CV (%)	Accuracy (%)
Interday (=9)						
1.5	1.41 ± 0.1	7.2	-5.8	1.43 ± 0.09	6.1	-4.6
400	419.4 ± 12.3	2.9	4.9	433.5 ± 17.6	4.1	2.4
800	809.9 ± 10.0	1.2	1.2	806.8 ± 19.4	2.4	0.8
Intraday (n=3)						
1.5	1.33 – 1.53 ± 0.01 - 0.08	0.4 – 5.5	-11.2 – 1.9	1.36 – 1.53 ± 0.02 - 0.06	1.4 – 4.2	-9.0 – 2.2
400	415 - 425 ± 7.7 - 17	1.8 – 4.0	3.6 – 6.3	417 - 443 ± 6.5- 20	1.5 - 4.9	4.3 – 10.8
800	806 - 813 ± 11 – 11	1.3 - 1.4	0.8 – 1.6	794 - 823 ± 13 - 21	1.7-2.6	-0.7 – 2.9

CVF_{DA} (Set A). Precision and accuracy data for TFV and FTC determined in 0.3 (low), 5 (mid), and 10 fmol/10^6 cells (high) CVF_{DA} QCs are presented in Table 19. It should be noted that the precision and accuracy of the CVF_{DA} QCs were based off their quantitation by the PBMC calibration curves that corresponded with the CVF_{DA} QC by Set Identification. The overall inter-assay precisions of the low, mid, and high TFV CVF_{DA} QCs were 3.0, 7.0, and 4.7%, respectively, while the overall accuracies were -11.1, 8.7, and 4.3%, respectively. The intra-assay precisions of the low, mid, and high TFV QCs ranged from 1.5 to 5.1%, 3.0 to 6.7%, and 1.3 to 8.3% respectively, while the accuracies ranged from -12.6 to -10.4%, 1.7 to 12.2%, and 1.2 to 5.8%, respectively. Similarly, the overall inter-assay precisions of the low, mid, and high FTC QCs were 3.9, 3.6, and 7.8%, while the overall accuracies were 3.0, 6.4, 6.6%, respectively. The intra-assay precisions of the low, mid, and high FTC QCs ranged from 2.4 to 4.5%, 2.1 to 3.2%, and 4.6 to 10.4%, while the accuracies ranged from 0.6 to 4.3%, 4.3 to 10.5%, and 5.8 to 8.3%, respectively. All TFV and FTC CVF_{DA} set A QC samples fell within ± 15% of the theoretical value required for acceptance and therefore, validation was achieved.

CVF_{DA} (Set B). Precision and accuracy data for TFV and FTC determined in 11 (low), 220 (mid), and 2200 fmol/10^6 cells (high) CVF_{DA} QCs are presented in Table 20, noted again that these curves were measured with the corresponding PBMC calibration curve. The overall inter-assay precisions of the low, mid, and high TFV QCs were 2.7, 5.0, and 5.6%, respectively, while the overall accuracies were -4.0, 4.1, and 2.1%, respectively. The intra-assay precisions of the low, mid, and high TFV PBMC QCs

Table 19.

Inter-day and Intra-day Precision and Accuracy for the Analysis of TFV and FTC in Set A of CVF_{DA} Quality Control Samples

Nominal Concentration ($fmol/10^6 cells$)	TFV			FTC		
	Measured Concentration ($fmol/10^6 cells$) mean ± SD	CV (%)	Accuracy (%)	Measured Concentration ($fmol/10^6 cells$) mean ± SD	CV (%)	Accuracy (%)
Interday (=9)						
0.3	0.27 ± 0.01	3.0	-11.1	0.31 ± 0.02	3.9	3.0
5	5.43 ± 0.38	7.0	8.7	5.32 ± 0.20	3.6	6.4
10	10.43 ± 0.49	4.7	4.3	10.66 ± 0.83	7.8	6.6
Intraday (n=3)						
0.3	0.26 - 0.27 ± 0.004 - 0.013	1.5- 5.1	-12.6 - -10.4	0.30 - 0.31 ± 0.07 - 0.01	2.4 – 4.5	0.6 – 4.3
5	5.08 - 5.61 ± 0.16 - 0.38	3.0 – 6.7	1.7 - 12.2	5.22 - 5.52 ± 0.11 - 0.18	2.1 – 3.2	4.3 – 10.5
10	10.12 - 10.58 ± 0.14 – 0.84	1.3 – 8.3	1.2 – 5.8	10.58 - 10.83 ± 0.50 -1.11	4.6 – 10.4	5.8 – 8.3

Table 20.

Inter-day and Intra-day Precision and Accuracy for the Analysis of TFV and FTC in Set B of CVF_{DA} Quality Control Samples

Nominal Concentration ($fmol/10^6 cells$)	TFV			FTC		
	Measured Concentration ($fmol/10^6 cells$), mean ± SD	CV (%)	Accuracy (%)	Measured Concentration ($fmol/10^6 cells$) mean ± SD	CV (%)	Accuracy (%)
Interday (=9)						
11	10.6 ± 0.3	2.7	-4.0	11.4 ± 0.4	3.6	3.8
220	229.0 ± 11.5	5.0	4.1	227.7 ± 5.5	2.4	3.5
2200	2246.9 ± 125.9	5.6	2.1	2199.8 ± 182.1	8.3	0.0
Intraday (n=3)						
11	10.6 – 10.6 ± 0.1 - 0.5	1.3 – 5.0	-4.0 - -3.9	11.4 – 11.5 ± 0.3 - 0.6	2.2 – 4.8	3.3 – 4.1
220	227 - 232 ± 6.8 - 15	3.0 – 6.7	3.3 – 5.6	226 - 231 ± 4.8- 6.9	2.1 – 3.0	2.7 – 5.1
2200	2212- 2317 ± 43 – 221	1.9 – 9.5	0.5 – 9.5	2158 - 2283 ± 110 - 229	4.8-10.6	-1.9 – 3.8

ranged from 1.3 to 5.0%, 3.0 to 6.7%, and 1.9 to 9.5% respectively, while the accuracies ranged from -4.1 to -3.9%, 3.3 to 5.6%, and 0.5 to 5.3%, respectively. Similarly, the overall inter-assay precisions of the low, mid, and high FTC QCs were 3.6, 2.4, and 8.3%, while the overall accuracies were 3.8, 3.5, 0.01%, respectively. The intra-assay precisions of the low, mid, and high FTC QCs ranged from 2.2 to 4.8%, 2.1 to 3.0%, and 4.8 to 10.6%, while the accuracies ranged from 3.3 to 4.1%, 2.7 to 5.1%, and -1.9 to 3.8%, respectively. All TFV and FTC CVF_{DA} set B QC samples fell within ± 15% of the theoretical value required for acceptance and therefore, validation was achieved.

Linearity and Sensitivity: Calibration Curve Performance

The linearity and sensitivity of the calibration curves for TFV and FTC in each assay (plasma, PBMCs, and CVFws) were assessed using calibration curves prepared from working calibration standards designated by sample type. The calibration curve's range for each assay was chosen to encompass the expected C_{max} for each sample type quantitated as well as providing sufficient sensitivity to allow quantitation of very low drug concentrations.

Plasma. The linearity and sensitivity of TFV and FTC determined by the performance of the calibration curve is presented in Table 21. The assay demonstrated linearity in the concentration range (5 to 2500 ng/mL), with a mean r^2 of 0.9970 and 0.9973 for TFV and FTC, respectively. The lowest standard (LLOQ) obtained for both

Table 21.

Calibration Curve Results for TFV and FTC in Human Plasma (n=8)

	TFV			FTC		
	Back-Calculated Concentration (ng/mL), mean ± SD	CV (%)	Accuracy (%)	Back-Calculated Concentration (ng/mL), mean ±SD	CV (%)	Accuracy (%)
Calibration Standards (ng/mL)						
STD A (5)	4.9 ± 0.1	1.3	-1.2	5.1 ± 0.2	4.3	2.8
STD B (10)	10.2 ± 0.5	4.9	1.6	8.8 ± 1.2	13.4	-12.1
STD C (25)	25.2 ±2.3	9.0	0.9	24.3 ± 0.6	2.5	-2.6
STD D (50)	50.9 ± 2.0	3.9	1.7	53.2 ± 3.4	6.3	6.3
STD E (100)	100.6 ± 5.0	5.0	0.6	102.1 ± 4.9	4.8	2.1
STD F (500)	513.1 ± 21.6	4.2	2.6	506.7 ± 19.6	3.9	1.3
STDG(1000)	1000.4 ± 30	3.0	0.0	990.9 ± 40	4.0	-0.9
STDH(2500)	2385.9 ± 84	3.5	-4.6	2476.9 ± 99	4.0	-0.9
	Mean ±SD	CV (%)	Accuracy (%)	Mean ±SD	CV (%)	Accuracy (%)
Calibration Curve						
Slope	0.014 ± 0.0004	3.1	N/A	0.012 ± 0.001	11.3	N/A
r^2	0.9970 ± 0.003	0.28	N/A	0.9973 ± 0.002	0.2	N/A

TFV and FTC was 5.0 ng/mL in plasma using a sample volume of 100 μL. The mean ± SD LLOQ for TFV and FTC was 4.9±0.007 and 5.1±0.22 ng/mL, respectively, indicating that the TFV and FTC assay was both linear and sensitive over a concentration range of 5-2500 ng/mL in extracted plasma samples.

PBMC (Set A). The linearity and sensitivity of TFV and FTC determined by the performance of the calibration curve is presented in Table 22. The assay demonstrated linearity in the concentration range (0.1 to 15 fmol/10^6 cells), with a mean r^2 of 0.9957 and 0.9980 for TFV and FTC, respectively. The LLOQ obtained for both TFV and FTC was 0.1 fmol/10^6 cells in lysed THP-1 cells using a sample volume of 100 μL. The mean ± SD LLOQ for TFV and FTC was 0.0947 ±0.0012 and 0.100±0.0266 fmol/10^6 cells, respectively, indicating that the TFV and FTC assay was both linear and sensitive over a concentration range of 0.1 – 15 fmol/10^6 cells in extracted lysed THP-1 cells samples.

PBMC (Set B). The linearity and sensitivity of TFV and FTC determined by the performance of the calibration curve is presented in Table 23. The assay demonstrated linearity in the concentration range (5 to 2500 fmol/10^6 cells), with a mean r^2 of 0.9964 and 0.9987 for TFV and FTC, respectively. The LLOQ obtained for both TFV and FTC was 5 fmol/10^6 cells in lysed THP-1 cells using a sample volume of 100 μL. The mean ± SD LLOQ for TFV and FTC was 5.6 ±0.37 and 4.8±0.54 fmol/10^6 cells, respectively, indicating that the TFV and FTC assay was both linear and sensitive over a concentration range of 5 – 2500 fmol/10^6 cells in extracted lysed THP-1 cells samples.

Table 22.

Calibration Curve Results for TFV and FTC in Set A of PBMCs (n=3)

	TFV			FTC		
	Back-Calculated Concentration (fmol/10^6cells), mean ± SD	CV (%)	Accuracy (%)	Back-Calculated Concentration (fmol/10^6cells), mean ±SD	CV (%)	Accuracy (%)
Calibration Standards (fmol/10^6cells)						
STD A (0.1)	0.095 ± 0.001	1.2	-5.3	0.100 ± 0.027	13.5	0.3
STD B (0.25)	0.215 ± 0.062	14.9	-13.9	0.276 ± 0.040	14.4	10.2
STD C (0.5)	0.563 ± 0.073	13.0	12.5	0.507 ± 0.038	7.5	1.4
STD D (1.0)	0.961 ± 0.082	8.5	-3.9	1.03 ± 0.494	8.1	2.7
STD E (2.5)	2.48 ± 0.038	1.6	-0.9	2.57 ± 0.308	12.0	2.7
STD F (5.0)	5.29 ± 0.418	7.9	5.9	4.40 ± 0.414	9.4	-11.9
STD G (10)	9.63 ± 0.331	3.4	-3.7	9.56 ± 0.206	2.2	-4.4
STD H (15)	16.6 ± 1.09	6.5	10.8	16.8 ± 0.950	5.6	12.3
	Mean ±SD	CV (%)	Accuracy (%)	Mean ±SD	CV (%)	Accuracy (%)
Calibration Curve						
Slope	0.0027 ± 0.0015	5.5	N/A	0.011 ± 0.0008	6.8	N/A
r^2	0.9957 ± 0.0013	0.13	N/A	0.9980 ± 0.002	0.16	N/A

Table 23.

Calibration Curve Results for TFV and FTC in Set B of PBMCs (n=3)

	TFV			FTC		
	Back-Calculated Concentration (fmol/10^6cells), mean ± SD	CV (%)	Accuracy (%)	Back-Calculated Concentration (fmol/10^6cells), mean ±SD	CV (%)	Accuracy (%)
Calibration Standards (fmol/10^6cells)						
STD A (5)	5.6 ± 0.4	6.7	11.5	4.8 ± 0.5	11.3	-4.3
STD B (10)	11.4 ± 1.3	11.4	13.6	9.5 ± 0.5	4.7	-4.9
STD C (25)	24.8 ± 2.3	9.1	-0.7	25.1 ± 1.6	6.4	0.3
STD D (50)	56.3 ± 1.2	2.1	12.7	55.6 ± 5.6	10.1	11.1
STD E (100)	92.4 ± 9.4	10.1	-7.6	109.1 ± 9.2	8.4	9.1
STD F (500)	429.7 ± 102.6	13.9	-14.1	513.3 ± 39.4	7.7	2.7
STD G (1000)	1031.6 ± 140.5	13.6	3.2	1017.7 ± 48.8	4.8	1.8
STD H (2500)	2526.2 ± 366.2	14.5	1.0	2487.2 ± 253.7	10.2	-0.5
	Mean ±SD	CV (%)	Accuracy (%)	Mean ±SD	CV (%)	Accuracy (%)
Calibration Curve						
Slope	0.0027 ± 0.0015	5.8	N/A	0.011 ± 0.0008	6.8	N/A
r^2	0.9964 ± 0.001	0.11	N/A	0.9987 ± 0.002	0.16	N/A

CVF$_{WS}$. The linearity and sensitivity of TFV and FTC determined by the performance of the calibration curve is presented in Table 24. The assay demonstrated linearity in the concentration range (0.5 to 1000 fmol/10^6 cells), with a mean r^2 of 0.9952 and 0.9971 for TFV and FTC, respectively. The LLOQ obtained for both TFV and FTC was 0.5 fmol/10^6 cells on the wicking strip using a sample volume of 100 μL. The mean ± SD LLOQ for TFV and FTC was 0.4968 ±0.0038 and 0.5144±0.0124 fmol/10^6 cells, respectively, indicating that the TFV and FTC assay was both linear and sensitive over a concentration range of 0.5 – 1000 fmol/10^6 cells on the wicking strips samples.

Table 24.

Calibration Curve Results for TFV and FTC for CVF_{WS} (n=3)

	TFV			FTC		
	Back-Calculated Concentration (fmol/10^6cells), mean ± SD	CV (%)	Accuracy (%)	Back-Calculated Concentration (fmol/10^6cells), mean ±SD	CV (%)	Accuracy (%)
Calibration Standards (fmol/10^6cells)						
STD A (0.5)	0.497 ± 0.004	0.8	-0.6	0.514 ± 0.012	2.4	2.9
STD B (1)	1.03 ± 0.006	0.6	3.5	0.954 ± 0.069	7.3	-4.6
STD C (5)	4.97 ± 0.038	0.8	-0.6	5.14 ± 0.124	2.4	2.9
STD D (10)	10.3 ± 0.063	0.6	3.5	9.54 ± 0.692	7.3	-4.6
STD E (50)	51.1 ± 2.55	5.0	2.2	51.2 ± 3.66	7.1	2.4
STD F (100)	98.3 ± 6.33	6.4	-1.7	101.4 ± 4.47	4.4	1.4
STD G (500)	532.2 ± 26.9	5.1	6.4	521.4 ± 25.7	4.9	4.3
STD H (1000)	1008 ± 20.1	2.0	0.8	1023 ± 42.9	4.2	2.3
	Mean ±SD	CV (%)	Accuracy (%)	Mean ±SD	CV (%)	Accuracy (%)
Calibration Curve						
Slope	0.014 ± 0.0004	3.5	N/A	0.013 ± 0.0003	2.5	N/A
r^2	0.9952 ± 0.004	0.43	N/A	0.9971 ± 0.002	0.15	N/A

In Vitro: Intracellular Uptake of TFV and FTC in FGT Cells

Identification and Selection of FGT Cells

Selection of immortal cell lines to represent the cells of the FGT was based on 1) cell types most abundant in the FGT, 2) the anatomical location of these cell types and 3) the likelihood of HIV invasion into the cell type. Anatomical locations of the FGT can be divided into the vagina, the cervix, which includes the ectocervix and endocervix, and the transformation zone, as listed in Table 25. The transformation zone contains a small area located in the upper portion of the FGT and is rarely sampled for detection of HIV-1 RNA. Instead, the vagina and cervix are more widely sampled to obtain clinical information. Therefore, the most abundant cell types present in these anatomical locations were identified. These cell types included but were not limited to epithelial (squamous, columnar, luminal, glandular), macrophages, T cell- lymphocytes, CD4+, CD8+, CD45R, CD103, TIA1, CLA+, CD68+, dendritic cells, HLA-DR+, CD62L-selectin, CD57+, CD56+, granulocytes, and CD1a+ cells. Table 25 displays the five most common cell types of the FGT and the immortal cell lines chosen to represent those cell types. Cell line selection was based on the similarity of each cell line to the major genotypic and phenotypic characteristics (such as abundance in CD4 receptors or keratinized epithelial cells) of the cell type in which they were represented.

Table 25.

Selected Cell Types of the FGT

Cell Type	Representative Cell Line	FGT Location
Macrophages	THP-1	Peripheral blood
CD4+	HeLa	Cervix
CD8+	BC-3	Endocervix; lymphocytes
Dendritic Cells	TF-1	Vagina
Squamous Epithelium	Ect1/E6E7	Ectocervix; Cervix

Intracellular Uptake of TFV and FTC in the Presence and Absence of Hormonal Contraceptives

Uptake of TFV and FTC Uptake in the Absence of Hormonal Contraceptives

The validated intracellular TFV and FTC method for PBMCs as described in Chapter 2 and 3 was used for quantitation of TFV and FTC uptake in all cellular uptake studies. TFV and FTC uptake into each cell line of the FGT were initially compared with TFV and FTC uptake by THP-1 cells. In other words, THP-1 cells served as a control since it is the most characterized immortal cell line for HIV-1 infectivity (Mikovits et al., 1990). Antiretroviral concentrations in each cell line relative to concentrations in THP-1 cells were statistically compared by a student t test.

Intracellular concentrations of TFV and FTC dosed with 0.3 and 1.8 µg/mL, respectively, in the five distinct cell types are shown in Table 26. The data suggest an inconsistency in the uptake of TFV and FTC between the cell lines and THP-1 cells. Compared with FTC, TFV concentrations were higher throughout the analysis. Further, the average uptake of TFV by the five distinct cell lines representing the FGT was 4.85% of the initial dose, which was much higher than FTC uptake of 0.07% of the initial dose.

Table 26.

Uptake of TFV and FTC in Selected Cell Lines

Cell Type (Cell line)	Uptake of TFV (fmol/10^6 cells)	P Value	Uptake of FTC (fmol/10^6 cells)	P Value
Macrophages (THP-1)	402.55 ± 74.77	-	18.07± 4.88	-
CD4+ (HeLa)	13021.60 ± 1838.51	<0.0001	1151.70 ± 239.51	0.0008
CD8+ (BC-3)	484.11± 53.18	0.4156	19.50 ± 5.01	0.8428
Dendritic Cells (TF-1)	573.61 ± 227.30	0.4592	44.10 ± 15.00	0.1299
Squamous epithelium (Ect1/E6E7)	10778.09 ± 1515.74	<0.0001	1142.62 ± 237.56	0.0008

Concentration: Mean ± SD

The mean ± SD intracellular concentrations of TFV and FTC in THP-1 cells were 402.55 ± 74.77and 18.07 ± 4.88 fmol/10^6 cells, respectively. A 27-fold increase in TFV intracellular concentrations and a 63-fold increase in FTC intracellular concentrations

were noted in Ect1/E6E7 cells compared with THP-1 cells. Similarly, HeLa cells demonstrated a 32-fold and 63-fold increase in TFV and FTC uptake, respectively, compared to the THP-1 cells. Concentrations of TFV and FTC in TF-1 cells and BC-3 cells were similar to THP-1 cells.

Uptake of TFV and FTC Uptake in the Presence of Hormonal Contraceptives

The validated intracellular TFV and FTC method for PBMCs as described in Chapter 2 was also used for quantitation of TFV and FTC uptake in the presence of hormonal contraceptives, ethinyl estradiol (EE) and etonogestrel (ET). The media for each cell line was dosed with 34.7 pg/mL of EE and/or 1716 pg/mL of ET. Since the focus of our work centered on antiretroviral exposure in the context of prophylaxis, intracellular concentrations of EE and ET were not determined in any of the experiments. In these studies, antiretroviral uptake into each cell line of the FGT in the presence of hormones was compared with the drug's uptake by the same cell line in the absence of hormones. In other words, each cell line served as its control and comparisons were made using student t test.

TFV and FTC Uptake Prior to Dosing with EE and ET. Intracellular TFV and FTC concentrations when dosed prior to administration of hormonal contraceptives in the five cell lines are described in Table 27. TFV and FTC Intracellular concentrations, when dosed one hour prior to exposure of EE and/or ET, decreased in two cell types,

specifically TF-1 and BC-3 cells. However, no significant differences in TFV or FTC uptake in the absence or presence of EE and/or ET were noted for THP-1, HeLa, or Ect1/E6E7 cells when dosed prior to EE or ET. TF-1 and BC-3 cells treated with TFV followed by EE demonstrated an 87% and 78% decrease in uptake, respectively, compared with their TFV only controls. Similarly, when dosed with TFV followed by ET, the same cell lines demonstrated an 87% and 79% decrease, respectively, in TFV uptake compared with their control. When dosed with TFV followed by EE/ET, TFV uptake by TF-1 cells decreased 85%, while uptake by BC-3 cells decreased to a lesser degree of 83%.

TF-1 and BC-3 cells treated with FTC followed by EE demonstrated an 85% and 79% decrease in uptake, respectively, compared with their FTC only controls. Similarly, when dosed with FTC followed by ET, the same cell lines demonstrated an 96% and 69% decrease, respectively, in FTC uptake compared with their control. When dosed with FTC followed by EE/ET, FTC uptake by TF-1 cells decreased 93%, while uptake by BC-3 cells decreased to a lesser degree of 65%. Overall, TFV dosed prior to EE and/or ET, resulted in a trend towards lower intracellular concentrations in all five cell lines. In contrast, results for FTC varied.

TFV and FTC Uptake after Dosing with EE and ET. Intracellular TFV and FTC concentrations when dosed after administration of hormonal contraceptives in the five cell lines are described in Table 28. Four of five cell lines dosed with TFV after EE, demonstrated a significant decrease in cellular uptake. TFV uptake decreased in THP-1

cells, BC-3 cells, TF-1 cells, and Ect1/E6E7 cells by 21, 17, 38, and 21%, respectively. THP-1 cells, BC-3 cells, and TF-1 cells also demonstrated a significant decrease in intracellular TFV concentration by 25, 29, and 47%, respectively, when TFV was dosed after ET. Only TF-1 cells, however, demonstrated a significant decrease of 38% when dosed with TFV after the administration of EE/ET.

Two of five cell lines dosed with FTC after EE, demonstrated a significant decrease in cellular uptake. FTC uptake, however, demonstrated 1.9 fold increase of intracellular FTC concentrations in BC-3 cells when FTC dosing followed EE dosing. Similarly, two of the five cell lines dosed with FTC after ET demonstrated a significant change in cellular uptake. BC-3 cells demonstrated a significant increase in intracellular FTC by 148% and TF-1 cells demonstrated a significant decrease by 63%. When dosed with EE/ET followed by FTC, two trends were observed. FTC uptake by BC-3 cells increased 100%, similar to the increase in uptake seen in BC-3 cells with EE then FTC. While uptake by TF-1 cells decreased by 55%, similar to the decrease observed in the uptake of ET then FTC in TF-1 cells. Overall, TFV dosed after EE and/or ET, resulted in a trend towards lower intracellular concentrations in all five cell lines. In contrast, results for FTC varied, only showing limited trends based on cell line.

TFV and FTC Uptake with Simultaneous Dosing of EE and ET. The uptake of TFV and FTC when dosed simultaneously with EE and/or ET was highly variable across the cell lines. The comparisons of simultaneous dosing for both TFV and FTC with the hormones are shown in Table 29. When dosed simultaneously with EE, TFV uptake was

Table 27. Intracellular TFV and FTC Concentrations by FGT Cell Lines When Dosed Prior with EE and ET (n=6)

Cell Lines	Uptake of TFV (fmol/10^6 cells) (mean± SD)			
	Alone	**EE**	**ET**	**EE & ET**
THP-1	393.58 ± 19.93	374.70 ± 7.30	377.11 ± 8.88	386.39 ± 13.72
HeLa	18455.65 ± 2293.62	71680.05 ± 41797.78	22597.32 ± 6523.80	31713.17 ± 7993.78
BC-3	469.65 ± 14.64	61.90 ± 1.50 *	63.18 ± 1.74 *	79.68 ± 1.67 *
TF-1	505.68 ± 16.39	111.80 ± 2.84 *	108.44 ± 2.47 *	77.48 ± 2.42 *
Ect1/E6E7	11912.34 ± 720.35	12551.22 ± 757.81	14945.01 ± 3053.54	11900.22 ± 500.46
	Uptake of FTC (fmol/10^6 cells) (mean± SD)			
	Alone	**EE**	**ET**	**EE & ET**
THP-1	74.79 ± 2.60	78.80 ± 2.99	72.53 ± 3.49	76.46 ± 3.01
HeLa	2001.31 ± 123.08	2086.73 ± 73.66	1877.10 ± 61.44	1788.86 ± 53.23
BC-3	35.73 ± 2.53	7.73 ± 0.23 *	11.07 ± 0.55 *	12.04 ± 0.53 *
TF-1	75.14 ± 4.47	11.05 ± 0.58 *	3.20 ± 0.31 *	5.49 ± 0.40 *
Ect1/E6E7	1357.29 ± 211.86	1228.09 ± 54.60	1110.06 ± 38.09	1156.33 ± 28.10

*p-value <0.05; Data presented as mean ± SD

Table 28. Intracellular TFV and FTC Concentrations by FGT Cell Lines After Dosing with EE and ET (n=6)

Cell Lines	Uptake of TFV (fmol/10^6 cells) (mean± SD)			
	Alone	**EE**	**ET**	**EE & ET**
THP-1	393.58 ± 19.93	309.87 ± 7.87 *	295.65 ± 3.75 *	588.90 ± 157.90
HeLa	18455.65 ± 2293.62	38199.23 ± 8500.58	82369.23 ± 36250.32	32356.63 ± 9232.94
BC-3	469.65 ± 14.64	387.25 ± 12.60 *	331.65 ± 15.41 *	458.55 ± 10.64
TF-1	505.68 ± 16.39	315.64 ± 10.70 *	266.31 ± 11.00 *	313.65 ± 8.30 *
Ect1/E6E7	11912.34 ± 720.35	9457.02 ± 687.17 *	9076.08 ± 1207.35	11151.24 ± 917.85
	Uptake of FTC (fmol/10^6 cells) (mean± SD)			
	Alone	**EE**	**ET**	**EE & ET**
THP-1	74.79 ± 2.60	64.52 ± 2.51 *	77.42 ± 2.99	79.81 ± 2.89
HeLa	2001.31 ± 123.08	2011.31 ± 33.43	1870.21 ± 84.93	1822.00 ± 70.49
BC-3	35.73 ± 2.53	69.11 ± 3.88 *	88.54 ± 4.67 *	70.82 ± 4.53 *
TF-1	75.14 ± 4.47	62.02 ± 4.21	27.97 ± 2.31 *	33.85 ± 7.30 *
Ect1/E6E7	1357.29 ± 211.86	1279.83 ± 189.06	1186.31 ± 43.64	1383.10 ± 168.69

*p-value <0.05; Data presented as mean ± SD

Table 29. Intracellular TFV and FTC Concentrations by FGT Cell Lines with Simultaneous Dosing of EE and ET (n=6)

Cell Lines	Uptake of TFV (fmol/10^6 cells) (mean± SD)			
	Alone	**EE**	**ET**	**EE & ET**
THP-1	393.58 ± 19.93	412.72 ± 12.27	358.94 ± 18.29	319.19 ± 18.82 *
HeLa	18455.65 ± 2293.62	7998.66 ± 2959.33 *	6494.60 ± 420.16 *	16610.79 ± 5299.61
BC-3	469.65 ± 14.64	489.70 ± 18.51	869.36 ± 16.21 *	641.28 ± 17.47 *
TF-1	505.68 ± 16.39	465.16 ± 13.32	330.62 ± 10.02 *	490.00 ± 13.15
Ect1/E6E7	11912.34 ± 720.35	11538.9 ± 283.58	11986.71 ± 1790.68	11106.54 ± 933.25
	Uptake of FTC (fmol/10^6 cells) (mean± SD)			
	Alone	**EE**	**ET**	**EE & ET**
THP-1	74.79 ± 2.60	64.08 ± 1.60 *	54.84 ± 2.11 *	62.56 ± 2.41 *
HeLa	2001.31 ± 123.08	2078.31 ± 87.82	1861.14 ± 116.17	1679.95 ± 74.85
BC-3	35.73 ± 2.53	304.89 ± 14.66 *	88.70 ± 5.01 *	28.96 ± 1.43 *
TF-1	75.14 ± 4.47	74.20 ± 4.45	33.70 ± 2.44 *	81.08 ± 3.36
Ect1/E6E7	1357.29 ± 211.86	834.20 ± 153.75	837.72 ± 150.81	1114.10 ± 93.88

*p-value <0.05; Data presented as mean ± SD

significantly different from its control in HeLa cells only, with a decrease in uptake of 57%. When dosed simultaneously with ET, TFV uptake was significantly different from its control in HeLa, TF-1, and BC-3 cells. TFV uptake decreased 65% in HeLa cells and 35% in BC-3 cells but increased 85% in TF-1 cells.

When TFV was co-administered with EE/ET, TFV uptake significantly decreased in THP-1 by 19% and significantly increased in BC-3 cells by 37%. When dosed simultaneously with EE, FTC uptake significantly decreased 14% in THP-1 cells and increased 148% in BC-3 cells. When dosed simultaneously with ET, FTC uptake was significantly different from its control in THP-1, BC-3, and TF-1 cells. FTC uptake decreased 27% in THP-1 cells and 55% in TF-1 cells but increased 148% in BC-3 cells. When FTC was co-administered with EE/ET, FTC uptake significantly decreased in THP-1 by 16% and significantly decreased in BC-3 cells by 20%.

Additional Dosing Concentrations of TFV and FTC with EE and ET in Selective Cell Lines. Low, mid, and high intracellular TFV (0.06, 0.3, 1.8 µg/mL) and FTC (0.09, 1.8, 2.25 µg/mL) doses prior to or after EE/ET exposure were also evaluated in BC-3 cells and TF-1 cells. Results from these two cell lines for the three doses of TFV and FTC are shown in Table 30 and Table 31. These experiments evaluated the percent change in TFV and FTC uptake over a 25-fold range of antiretroviral doses.

Mean intracellular TFV concentrations for BC-3 cells increased 275.39% with a 400% increase in TFV dose and further increased 371.7% with an additional 400% increase in TFV dose. Similar percent increases in intracellular TFV concentrations were

noted in the presence of EE/ET, regardless of the time of dosing. Thus, these data suggest that TFV dosed with EE/ET does not alter dose-dependent uptake of TFV into BC-3 cells. In contrast, intracellular FTC concentrations in BC-3 cells increased 200.6% with a 1900% increase in dose and further increased 34.9% with an additional 25% in dose. However, FTC dosed one hour prior to EE/ET increased concentrations only 2.5% and 53.2%, respectively, with increase in FTC dose. These data suggest that the presence of EE/ET alters uptake of FTC into BC-3 cells and may explain the discrepancy in FTC uptake in BC-3 cells as reported in Table 30.

Mean intracellular TFV concentrations for TF-1 cells increased 330% with a 400% increase in dose and further increased 324.2% with a 400% increase in dose. Similar percent increases in intracellular TFV concentrations were noted in the presence of EE/ET, regardless of the time of dosing. Thus, these data suggest that TFV dosed with EE/ET does not alter dose-dependent uptake of TFV into TF-1cells. Intracellular FTC concentrations in TF-1 cells increased 24.4% with a 1900% increase in dose and increased 227.8% with a 25% increase in dose. However, FTC dosed with EE/ET, regardless of dosing times, resulted in highly variable changes in intracellular concentrations that cannot be explained.

Table 30. Intracellular TFV and FTC Concentrations in BC-3 Cells When Dosed Prior to and After EE/ET (n =12)

		Uptake of TFV (fmol/10^6 cells) (mean± SD)					
		TFV Only		*TFV 1 hr Prior*		*TFV 1 hr After*	
Dose	% Change	Concentration	% Change	Concentration	% Change	Concentration	% Change
Low (0.06 µg/mL)	400	125.2 ± 43.6	275.39	80.7 ± 31.5	457.6	79.9 ± 12.1	285.3
Mid (0.3 µg/mL)		469.9 ± 35.2		449.7 ± 47.4		308.0 ± 66.6	
High (1.5 µg/mL)	400	2216.4 ±296.9	371.7	1623.7± 372.9	261.1	1445.3 ±111.9	369.3
		Uptake of FTC (fmol/10^6 cells) (mean± SD)					
		FTC Only		*FTC 1 hr Prior*		*FTC 1 hr After*	
Dose	% Change	Concentration	% Change	Concentration	% Change	Concentration	% Change
Low (0.09 µg/mL)	1900	13.2 ± 5.5	200.6	57.3 ± 7.6	2.5	11.5 ± 1.3	233.5
Mid (1.8 µg/mL)		39.8 ± 2.8		58.8 ± 7.7		38.4 ± 8.4	
High (2.25 µg/mL)	25	53.7 ± 8.1	34.9	90.0 ± 17.0	53.2	158.4 ± 26.9	312.5

Table 31. Intracellular TFV and FTC Concentrations in TF-1 Cells When Dosed Prior to and After EE/ET (n =12)

		Uptake of TFV (fmol/10^6 cells) (mean± SD)					
		TFV Only		*TFV 1 hr Prior*		*TFV 1 hr After*	
Dose	% Change	Concentration	% Change	Concentration	% Change	Concentration	% Change
Low (0.06 µg/mL)	400	120.6 ± 75.6	330.0	54.7 ± 26.0	474.8	45.1 ± 28.1	753.5
Mid (0.3 µg/mL)		518.5 ± 150.0		314.4 ± 97.8		385.1 ± 155.8	
High (1.5 µg/mL)	400	2199.5±1075.8	324.2	4105.0±2034.2	1205.8	5093.4 ± 895.1	1222.7
		Uptake of FTC (fmol/10^6 cells) (mean± SD)					
		FTC Only		*FTC 1 hr Prior*		*FTC 1 hr After*	
Dose	% Change	Concentration	% Change	Concentration	% Change	Concentration	% Change
Low (0.09 µg/mL)	1900	64.4 ± 17.2	24.4	22.1 ± 3.7	19.8	30.8 ± 11.3	94.6
Mid (1.8 µg/mL)		80.1 ± 13.2		17.7 ± 6.0		60.0 ± 19.6	
High (2.25 µg/mL)	25	262.6 ± 49.0	227.8	84.4 ± 6.8	376.3	81.5 ± 5.7	35.8

Clinical Study Overview

Demographics

Thirty HIV-seropositive women were enrolled and completed the study between March 2010 and June 2012. Data from one subject were excluded from the plasma and PBMC analyses due to problems with intravenous access and production of blood. Fifteen subjects experienced FGT bleeding during sample collection but all samples were used in the analyses. Demographic information is presented in Table 32. The racial distribution of the 30 subjects was 87% African American, 10% Caucasians, and 3% other or mixed. The median (range) age of the study population was 49 years (29–62 years). The median (range) HIV-1 RNA was 0 (0-310) copies/mL and CD4+T-cell count was 486 (108–1024) cells/mm^3 at study entry. In addition to Atripla® (TDF, FTC, and EFV), concomitant medications included antibiotics (n=8), antivirals (n= 4), antihistamines (n= 21), antidepressants (n= 14), antipsychotics (n=8), acid reducing agents (n=18), anti-asthmatics (n=10), pain relievers (n= 21), antihypertensives (n=17), multivitamins (n=18), and hormonal contraceptives (n= 2). None of these drugs were thought to alter TFV or FTC pharmacokinetics. Seven percent of the women were on oral hormonal contraceptives, 50% had hysterectomies, and 43% of the women were using other forms of contraceptive means (62% Postmenopausal and 38% Barrier Protection). Subjects tolerated the study medication well.

Table 32.

Characteristics of Study Patients (n=30)

Parameters	**Results** Median (range)
Age (yrs)	49 (29-62)
Body wt. (kg)	82.5(56-128)
Height (in)	64 (60-167)
Race	
African American	26
Caucasian	3
Hispanic	1
HIV-1 RNA Levels (copies/mL)	0 (0-310)
$CD4^+$ lymphocyte (count/mm^3)	486 (108-1024)
Birth Control	
Oral Hormonal Contraceptives	3
Topical Hormonal Contraceptives	0
Implantation	0
Surgery	15
Others *	12

* Vaginal Ring and Female and Male Condoms

Pharmacokinetic Analysis of Clinical Samples

Plasma Samples

TFV and FTC pharmacokinetic parameters are summarized in Table 33 and 34, respectively. Furthermore, the pharmacokinetic parameters for each antiretroviral from a previous study are also presented for comparison. TFV and FTC concentration- time plots for all 29 patients are presented in Figure 9.

Table 33.

TFV Plasma Pharmacokinetic Parameters (n=29)

Parameters	Present Study (n=29) [a]	Literature Data (n=17)[b]
AUC_{24}(hr*mg/L)	2.42(26)	2.80(17)
C_{max} (mg/L)	0.28 (33)	0.288(22)
C_{min} (mg/L)	0.043(41)	0.054(20)
T_{max} (hr)	1.54(60)	2.40(38)
$t_{1/2}$ (hr)	17.08(93)	15.9(24)
CL/F (L/hr)	60.4(30)	49.74 (18)
Vd/F (L)	1430.4(77)	NA

Data presented in mean (CV%); CV, coefficient of variation; [a] 300 mg TDF/200 mg FTC/ 600 mg EFV once daily; [b] 300 mg TDF/200 mg FTC once daily

Table 34.

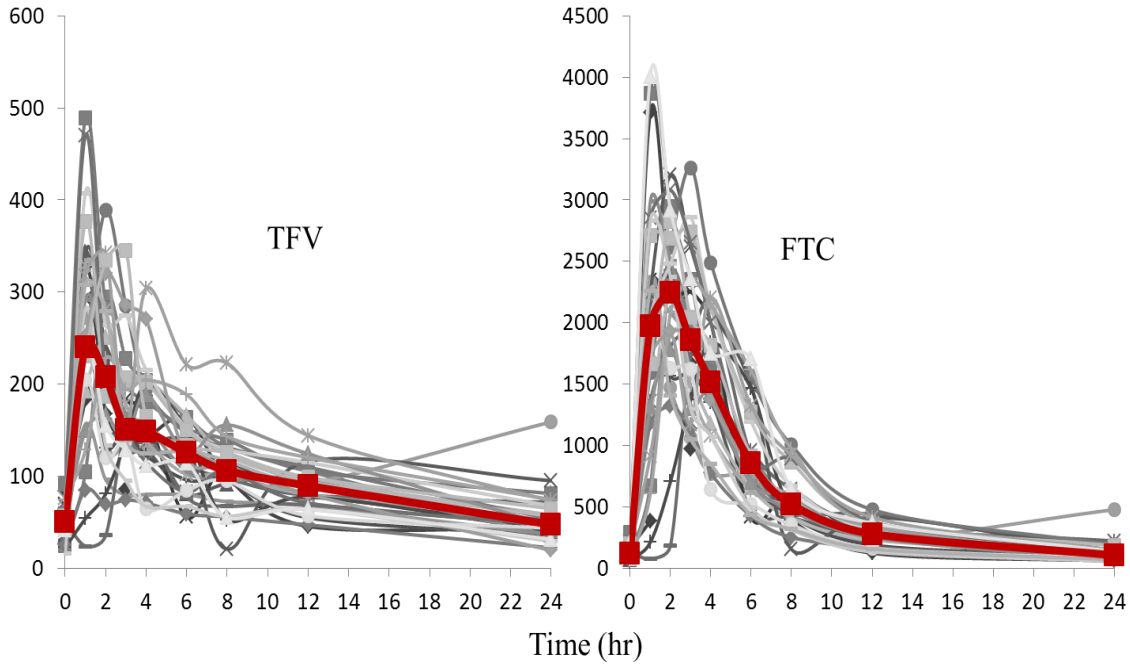
FTC Plasma Pharmacokinetic Parameters (n=29)

Parameters	Present Study (n=29) [a]	Literature Data (n=17)[b]
AUC_{24} (hr*mg/L)	14.57(24)	10.7(11)
C_{max} (mg/L)	2.53 (25)	1.69(18)
C_{min} (mg/L)	0.61(24)	0.075(22)
T_{max} (hr)	1.99(50)	2.98(20)
$t_{1/2}$ (hr)	5.18 (24)	10.7(16)
CL/F (L/hr)	14.5(25)	18.96 (12)
Vd/F (L)	109.1(36)	NA

Data presented in mean (CV%); CV, coefficient of variation; [a] 300 mg TDF/200 mg FTC/ 600 mg EFV once daily; [b] 300 mg TDF/200 mg FTC once daily

Figure 9.

Plasma Concentration-Time Profiles



TFV and FTC plasma concentrations were measured for 24 hrs post dose. Data shown in gray are the individual concentration time profiles from 29 patients with the median concentration from each time point plotted in red.

Mean ± SD AUC_{24}, C_{max}, and $t_{1/2}$ for TFV were 2.42± 0.63 mg x h/L, 0.28±0.09 mg/L, and 17.08 ± 15.85 h, respectively. FTC mean ± SD for AUC_{24}, C_{max}, and $t_{1/2}$ were 14.57± 3.50 mg x h/L, 2.53±0.65 mg/L, and 5.18±1.25 h, respectively. TFV and FTC plasma pharmacokinetic parameters were similar compared to previously reported data (Blum, Chittick, Begley, & Zong, 2007)

PBMC Samples

All 29 women provided four PBMC samples for a total of 116 samples. The LC/MS/MS assay for quantifying TFV and FTC levels in PBMCs reports concentrations in fmol/µL. These concentrations require conversion to a "mol per cell volume" unit in order to adequately compare it to data in the literature. The conversion equation takes into consideration that 100 µL is equivalent to 10^6 cells (based upon growth patterns of THP-1 cells, which served as a surrogate PBMC matrix). In other words, 100 µl of lysed THP-1 contained 10^6 cells. The conversion also takes into consideration the individualized cell count and sample volume. For example, the volume of PBMCs obtained varied between patients based upon the amount of whole blood collected. Therefore, the final concentration takes into account each patient's cell count per volume of lysed PBMCs. In order to compare TFV and FTC concentrations in PBMCs with plasma, results required units of ng/mL. The conversion equation used to determine the TFV and FTC PBMC concentrations in ng/mL is shown in Figure 10.

Figure 10. Cell Conversion Equation

$$\text{Raw data (fmol)} \left\{ \frac{10^6 \text{cells}}{\#\text{ of cells in 100µL of patient sample}} \right\} \left\{ \frac{\text{actual fmol}}{10^6 \text{ cells}} \right\} \left\{ \frac{\text{molecular weight in fg/fmol}}{} \right\} \left\{ \frac{1 \text{ pg}}{10^3 \text{ fg}} \right\} \left\{ \frac{1 \text{ng}}{10^3 \text{ pg}} \right\} \left\{ \frac{10^6 \text{cells}}{100 \mu\text{L}} \right\} \left\{ \frac{100 \mu\text{L}}{0.1 \text{ mL}} \right\} = \text{ng/mL}$$

The equation is designed to adjust the concentration of any drug, which concentrations are measured intracellularly. The data needed prior to using the equation are the raw concentration data in fmol and the molecular weight of the drug. The equation adjusts the raw data to a mass per volume concentration.

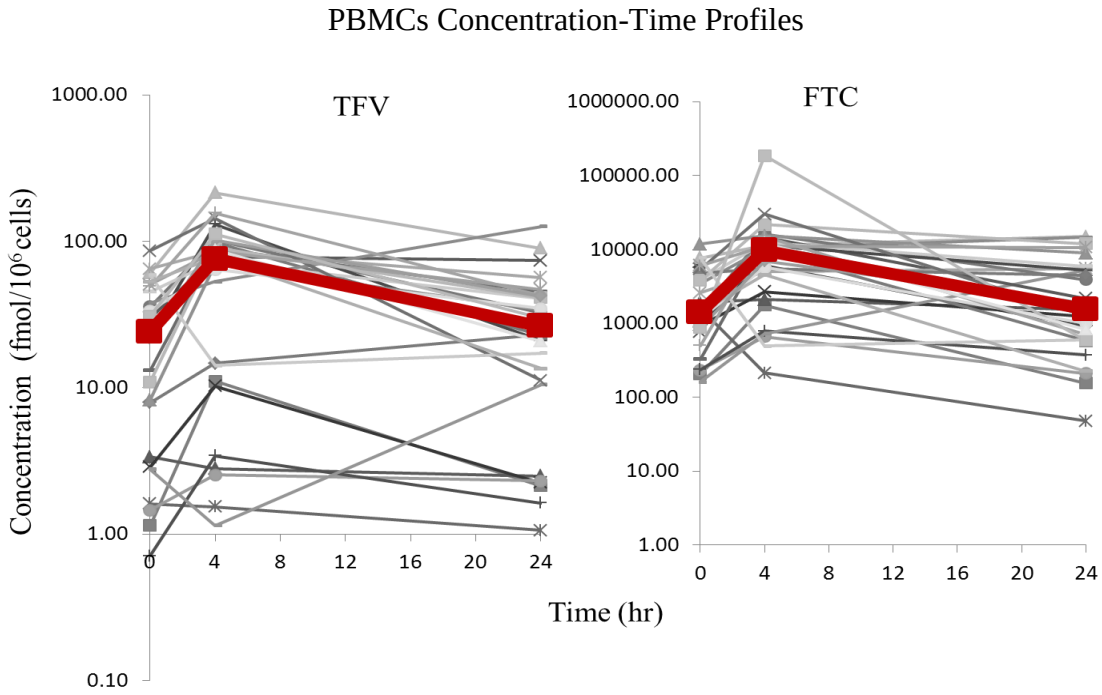
Pharmacokinetic parameters for TFV and FTC in PBMCs are presented in Table 35. Median (range) TFV PBMC C_{min} and C_{max} were 14.2 (0.03- 50.5) fmol/10^6 cells and 89.9 (1.64- 214.07) fmol/10^6 cells, respectively, while the median (range) FTC PBMC C_{min} and C_{max} were 854.2 (47.7- 8892.7) fmol/10^6 cells and 11337.6 (1597.7- 186184.6) fmol/10^6 cells, respectively. These values are comparable to recorded median (range) TFV and FTC PBMC concentrations obtained on days 20 and 30 at accumulated various steady state time points over 24 hours in 17 HIV-seronegative adults receiving TDF/FTC for 30 days. TFV and FTC PBMC median (range) concentrations were 99 (49-158) fmol/10^6 cells and 5000 (4100-6500) fmol/10^6 cells, respectively (L. J. Anderson P, Buchbinder S, et al. , 2011; Anderson Peter, 2012). The authors concluded that the TFV and FTC concentrations in PBMCs were similar to those in HIV-seropositive patients in whom antiviral effect had been established; thus, the concentrations achieved were adequate for use as prophylaxis against HIV transmission in men who have sex with men (MSM). Taken together, similarities between our data and those from Anderson et al, supports the use of TFV and FTC as prophylaxis against HIV.

Table 35. PBMC Pharmacokinetic Parameters (n=29)
(mean ±SD)

Parameters	TFV	FTC
AUC_{24} (hr*mg/L)	0.0032± 0.0023	0.324± 0.232
C_{max} (mg/L)	0.0002±0.00002	0.041±0.083
C_{last} (mg/L)	0.00009±0.00008	0.018±0.014
T_{max} (hr)	4.26±3.99	7.13±10.59
$t_{1/2}$ (hr)	27.13±39.21	47.47±67.12
CL/F (L/hr)	205000±356000	2201±4839
Vd/F (L)	1.09E+7 ± 2.97E+7	7.99E+4 ± 1.30E+5

Concentration-time profiles for TFV and FTC are illustrated in Figure 11. Overall, TFV concentrations in PBMCs peaked around four hours post dose and subsequently declined. Mean ± SD TFV PBMC C_0 (28.0± 24.3 fmol/10^6 cells) was not statistically different from C_{24} (31.7 ± 28.2 fmol/10^6 cells) (p=0.58620). Thus, collecting PBMCs for TFV determination in patients could be conducted pre- or post- dosing. Similarly, the mean ± SD FTC PBMC C_0 (2640.3 ± 2861.5 fmol/10^6 cells) was not statistically different from C_{24} (3627.1 ± 4378.3 fmol/10^6 cells) (p= 0.3057), therefore our data suggest that the clinical utility of collecting PBMCs for FTC determination could also occur pre- or post- dosing.

Figure 11.



TFV and FTC PBMC concentrations were measured (3 samples) for 24 hrs post dose. Data shown in gray are the individual concentration time profiles from 29 patients with the median concentration from each time point plotted in red.

FGT Cells Samples

At both study visits, thirty women provided a cytobrush sample containing endothelial cells of the FGT for a total of 60 collected samples. Sample collection at study visit 1 occurred 24 hours post dose and at study visit 2, occurred at approximately 6 hours post dose. Samples from the same woman are referred to as paired samples. Quantitation of antiretrovirals in some samples was limited by low cell count, contamination with red blood cells, and small sample volume. Therefore, TFV could be quantitated in only 14 paired samples while FTC could be quantitated in only 23 paired samples. Quantitation of TFV and FTC in FGT cells utilized the LC/MS/MS assay designated for TFV and FTC quantitation in PBMCs. Concentrations of TFV and FTC in FGT cell samples also required conversion from fmol/μL to a "mol per cell volume" unit for easy comparison with literature data. Again, the conversion equation used is shown in Figure 10.

Although samples were collected on different days at different times post dose, concentrations at these time points represent steady-state concentrations in the FGT. TFV and FTC C_6 and C_{24} for TFV and FTC are presented in Table 36. TFV median (range) concentrations at 6 and 24 hours post dose were 28.25(0.1208-1908.2) fmol/10^6 cells and 11.75 (0.35-240.75) fmol/10^6 cells, respectively. FTC median (range) concentrations at the same time points were 1651.2 (3.77-188457.5) fmol/10^6 cells and 282.6 (2.38-74459.6) fmol/10^6 cells, respectively. Median intracellular TFV concentration in the FGT cells was higher 6 hours post dosed compared to 24 hours post dose (3-fold difference), which was dissimilar to the pattern seen in PBMCs. FTC

intracellular concentrations in FGT samples were higher at 6 hours post dose compared with 24 hours post dose demonstrated by 5-fold difference.

Table 36.

Median (range) TFV and FTC Concentrations in FGT Cell Samples

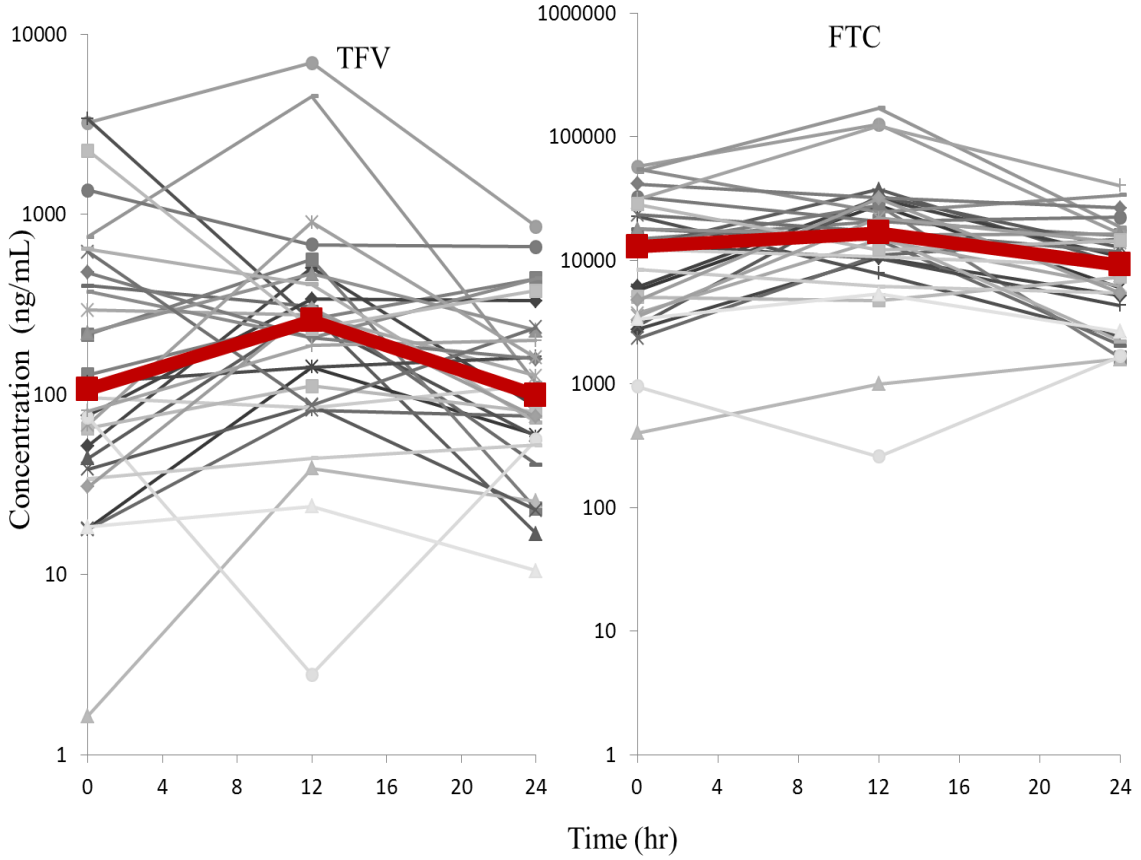
Parameters	**TFV** (fmol/10^6 cells)	**FTC** (fmol/10^6 cells)
C_6	28.25(0.1208-1908.2)	1651.2 (3.77-188457.5)
	n =23	n =27
C_{24}	11.75 (0.35-240.75)	282.6 (2.38-74459.6)
	n =17	n =24

CVF_{DA} Samples

Four CVF samples were collected by direct aspiration (CVF_{DA}) from each patient; three samples were collected at study visit 1 and one sample at study visit 2. A total of 120 samples were collected. Concentration-time profiles for TFV and FTC from the three samples collected at study visit 1 are shown in Figure 12. Both TFV and FTC concentrations increased until 12 hours post dose, and then decreased at 24 hours post dose to concentrations similar to those observed at time 0. Similar to plasma, FTC concentrations in CVF_{DA} were higher than TFV concentrations in CVF_{DA}. However, this magnitude of increase was higher for CVF_{DA} (25 times) compared to plasma (10 times). Differences between TFV and FTC concentrations in CVF_{DA} are likely a result of sequestration of drugs in the FGT. In other words, physical properties of each drug (i.e. ionization and polarity) would trap the drug in this anatomical compartment and produce higher antiretroviral concentrations in the FGT.

TFV and FTC pharmacokinetics parameters in CVF_{DA} are displayed in Table 37. The mean TFV $t_{½}$ of 15.5 hr and FTC $t_{½}$ of 23.3 hr in CVF_{DA} were higher than the mean TFV $t_{½}$ of 17.08 hr and FTC $t_{½}$ of 5.18 hr reported in plasma. The longer half-life in CVF_{DA} suggests slower movement of drug from the anatomical sites back into the blood stream. Thus, it is possible that adequate TFV and FTC concentrations required to inhibit viral replication in this compartment are maintained for a longer period of time compared with plasma.

Figure 12. CVF_{DA} Concentration-Time Profiles



TFV and FTC CVF_{DA} concentrations were measured (3 samples) for 24 hrs post dose. Data shown in gray are the individual concentration time profiles from 30 patients with the median concentration from each time point plotted in red.

The ratios of TFV and FTC concentrations in CVF_{DA} to concentrations in plasma over a period of 24 hr were calculated for 114 paired samples obtained under steady-state conditions and demonstrated in Table 38. The collective median (range) CVF_{DA}: plasma concentration ratio from the concentrations obtained at 0, 6, 12, and 24 hrs for TFV and FTC were 0.019 (0.009 to 0.339) and 0.19 (0.00 to 1.65), respectively, indicating that FTC concentration similar to TFV concentrations are much lower in CVF_{DA}. Also shown in Table 38 determined by the CV%s was the pattern of variability within the ratios. Greater interindividual variability in TFV concentration ratios occurred during the first half of the dosing interval than during the last half of the dosing interval (CVs were 37%–188% at 0–12 h, vs. 27%–235% at 12–24 h) compared to more variability in FTC concentrations occurring during the second half of the 24 hour dosing interval (CVs were 47%–368% at 0–12 h, vs. 32%–135% at 12–24 h). The fluctuations in the concentration ratios indicate that there is not a single CVF_{DA}: plasma TFV and FTC concentration ratio that adequately predicted the total TFV and FTC exposure. Therefore, CVF_{DA}: plasma AUC_{24} ratios were also calculated. For TFV, the mean CVF_{DA}: plasma AUC_{24} of 0.01 indicates that the CVF exposure was approximately 1% that of plasma. The mean CVF_{DA}: plasma AUC_{24} ratio for FTC of 0.07 or 7% also indicated very low exposure of FTC in the FGT compared to the plasma.

Table 37.

CVF Pharmacokinetic Parameters (n = 30)

	TFV		FTC	
Collection Technique	Direct Aspirate	Wicking Strip	Direct Aspirate	Wicking Strip
Parameters				
AUC_{24} (hr*mg/L)	0.029± 0.054	0.024± 0.028	1.21± 1.31	0.27± 0.22
C_{max} (mg/L)	0.003±0.004	0.002±0.002	0.081±0.098	0.017±0.014
C_{min} (mg/L)	1.00E-6 ± 2.00E-6	1.00E-6 ± 1.00E-6	3.00E-5 ± 3.50E-5	6.00E-6 ± 5.00E-5
T_{max} (hr)	9.84±7.82	9.83±7.81	7.38±6.69	10.68±7.36
$t_{1/2}$ (hr)	15.53±21.73	26.43±47.65	23.33±37.11	24.71±31.17
CL/F (L/hr)	2.02E+4 ± 2.86E+4	13707.5 ± 13350.6	382.7 ± 620.7	1632.4 ± 1855.2
Vd/F (L)	8.30E+5 ± 1.94E+6	8.49E+5 ± 2.25E+6	8027.2 ± 11913.5	74515.0 ± 136648.4

Data presented as mean ± SD

Table 38.

CVF_{DA} : Plasma Ratios for TFV and FTC

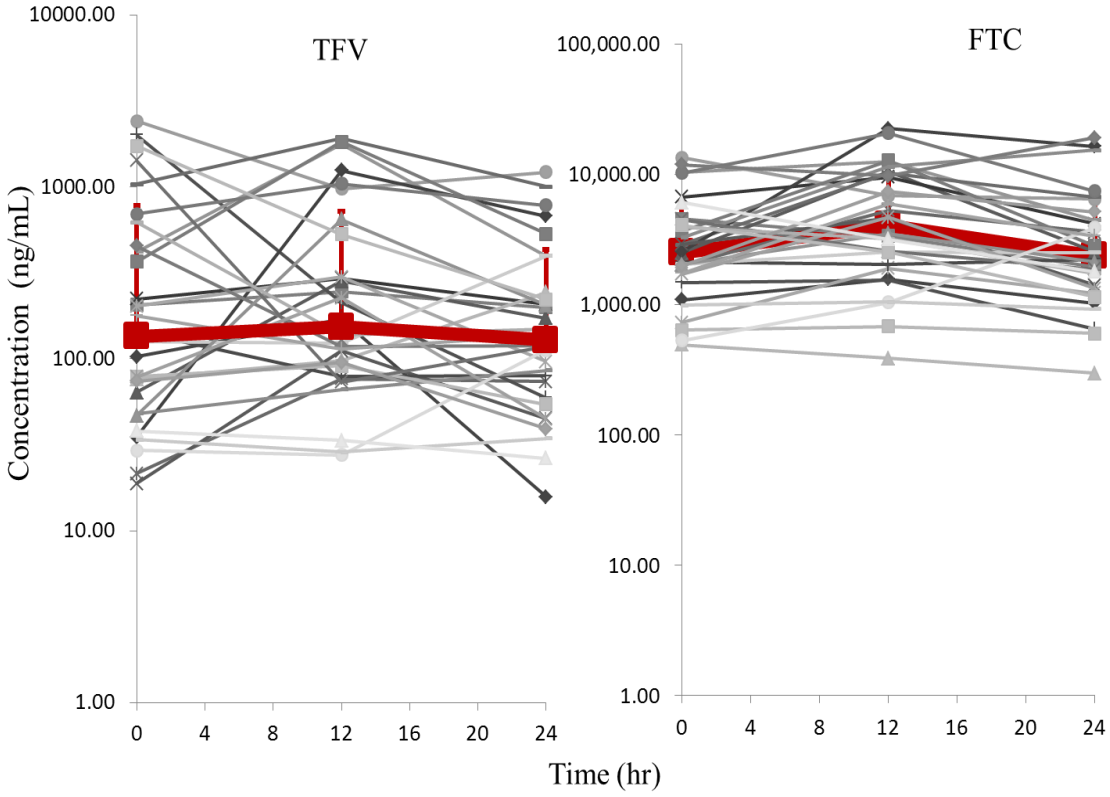
Ratio	TFV		FTC	
	Median (range)	CV%	Median (range)	CV%
C_0	0.012 (0.000 – 0.330)	188	0.39 (0.02 - 1.65)	97
C_6	0.003 (0.000 – 0.024)	105	0.01(0.00 – 0.41)	320
C_{12}	0.007 (0.000 – 0.214)	235	0.14 (0.00 – 1.57)	134
C_{24}	0.006 (0.000 – 0.036)	101	0.21 (0.04 – 1.28)	110
$C_{0\text{-}24}$	0.019 (0.000 - 0.330)	184	0.19 (0.00 - 1.65)	116
AUC_{24}	0.005 (0.000 – 0.082)	174	0.048 (0.05 – 0.33)	102

CVF_{WS} Samples

Four CVF samples were collected by a wicking strip (CVF_{WS}) from each patient; three samples were collected at study visit 1 and one sample at study visit. A total of 120 samples were collected. TFV and FTC concentrations were determined using the novel TearFlo™ Strip method previously described in the method section. Concentration-time profiles for TFV and FTC in samples collected from study visit 1 are illustrated in Figure 13. Data demonstrate similar concentrations at all three time points. TFV and FTC pharmacokinetic parameters in CVF_{WS} are shown in Table 37. TFV and FTC concentrations in CVF_{WS} followed similar trends as observed in plasma and CVF_{DA} samples.

Figure 13.

CVF_{WS} Concentration-Time Profile



TFV and FTC CVF_{WS} concentrations were measured (3 samples) for 24 hrs post dose. Data shown in gray are the individual concentration time profiles from 30 patients with the median concentration from each time point plotted in red.

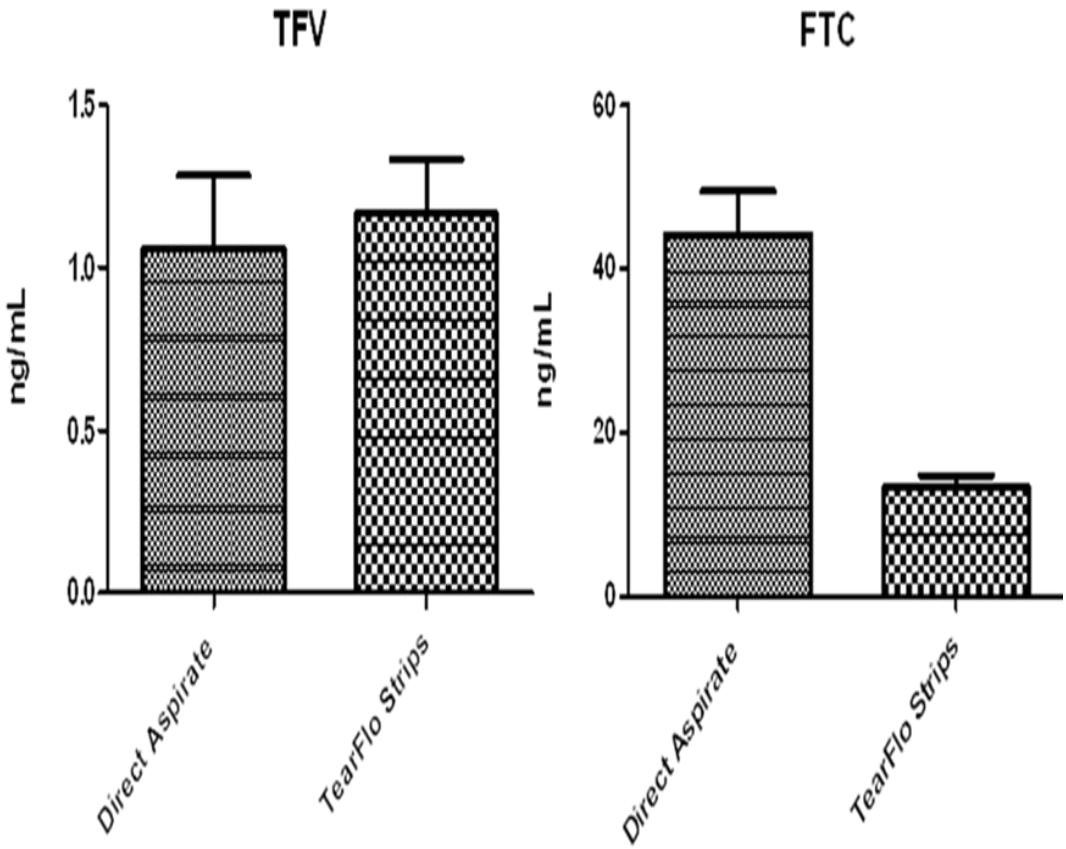
Comparison of TFV and FTC Concentrations by CVF Collection Techniques

Concentrations of TFV and FTC in CVF_{DA} and CVF_{WS} were compared to assess the interchangeability of techniques. All 240 samples (120 for each technique) were included in the analysis. The Wilcoxon Matched-pairs Signed Rank test was used in

GraphPad Prism. Mean ± SD of TFV concentration in CVF_{DA} was not statistically different from CVF_{WS} (1.057 ± 2.496 vs. 1.164 ± 1.844 ng/mL; p=0.0658). Figure 14 demonstrates mean TFV concentrations for each collection technique. Mean ± SD FTC concentration in CVF_{DA} was significantly higher compared with CVF_{WS} (44.24 ± 58.08 vs. 13.40 ± 14.42 ng/mL; p = <0.0001). Our study was not designed to test the superiority of one technique over another. However, the inconsistency of FTC concentrations between CVF collection techniques, as shown in Figure 14, suggests that comparisons of drug concentrations in this compartment can only be made between studies using the same collection technique.

Figure 14.

Comparison of the mean ± SD TFV and FTC Concentrations from CVF_{DA} and CVF_{WS}



Predictive Pharmacokinetic Models

The observed plasma TFV and FTC profiles for each patient are shown in Figure 15 A and B, respectively. A two-compartment model with first-order absorption best described TFV and FTC plasma concentrations. The exponential error model successfully described interpatient random effects. Residual error was modeled as a proportional error model. This model was implemented using ADVAN4 and TRANS4. The population parameter estimates of the two-compartment models and for TFV and FTC with CL/F, VC/F, Q/F, VP/F, and Ka are illustrated in Table 39. In the basic model, the mean (RSE %) population estimate values were 58.5 (5.1) for CL/F, 114 (141.2) for Vc/F, 255 (27.4) for Q/F, 1040 (17.3) for Vp/F and 0.699 (043.6) for Ka in the TFV model. While the FTC model's OFV was significantly reduced by the mean (RSE %) population estimate values of 14.5 (4.6) for CL/F, 18.5 (17.7) for Vc/F, 4.3 (10.4) for Q/F, 142 (12.3) for Vp/F and 0.318 (3.7) for Ka. There is no Ka for the interindividual variability for FTC due the consistency of the Ka among the patient population, therefore estimating a change in Ka among the patients does not influence the base models OFV.

Table 39.

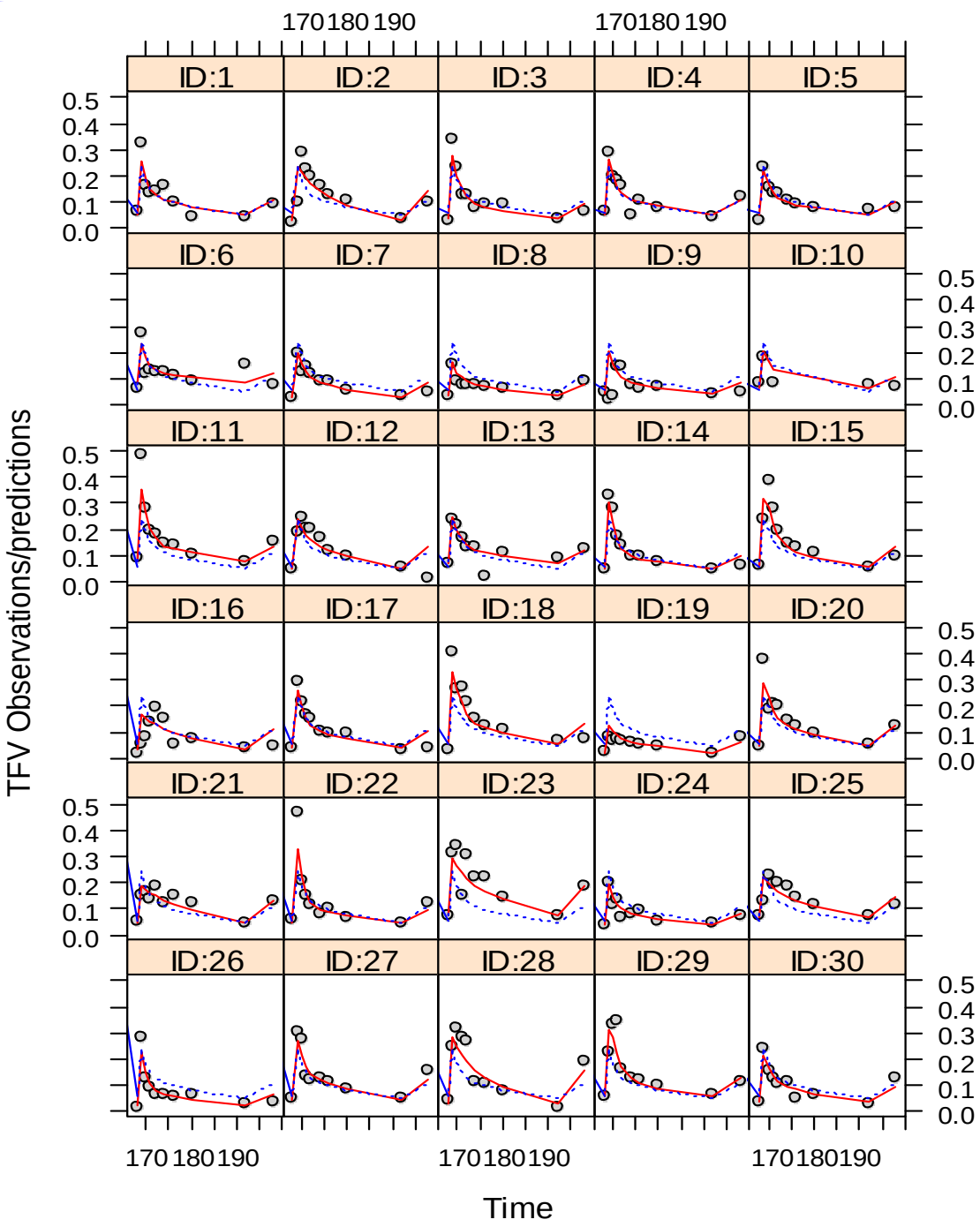
TFV and FTC Plasma Parameters for Base Model

Parameters	Base Model TFV		Base Model FTC	
	Estimate (RSE%)	95% CI	Estimate (RSE%)	95% CI
CL/F (L/h)	58.5 (5.1)	52.6-64.4	14.5 (4.6)	13.20 – 15.80
Vc/F (L)	114 (141.2)	-201.56-429.56	18.5 (17.7)	12.07 – 24.93
Q/F (L/h)	255 (27.4)	118.19 –391.81	4.3 (10.4)	43.42 – 5.18
Vp/F (L)	1040 (17.3)	687.2 – 1392.8	142 (12.3)	107.7 – 176.3
Ka (h^{-1})	0.699 (0.436)	0.101 -1.297	0.318 (3.7)	0.295 -0.341
Interindivdual Variability	Estimate (RSE%)		Estimate (RSE%)	
CL/F (L/h)	57.1 (34.9)		0.0346 (22.7)	
VC/F (L)	143 (623.1)		0.419 (58.7)	
Q/F (L/h)	241 (91.3)		0.124 (76)	
VP/F (L)	228 (49.1)		0.366 (59.6)	
Ka (h^{-1})	28.2 (570.9)		--	

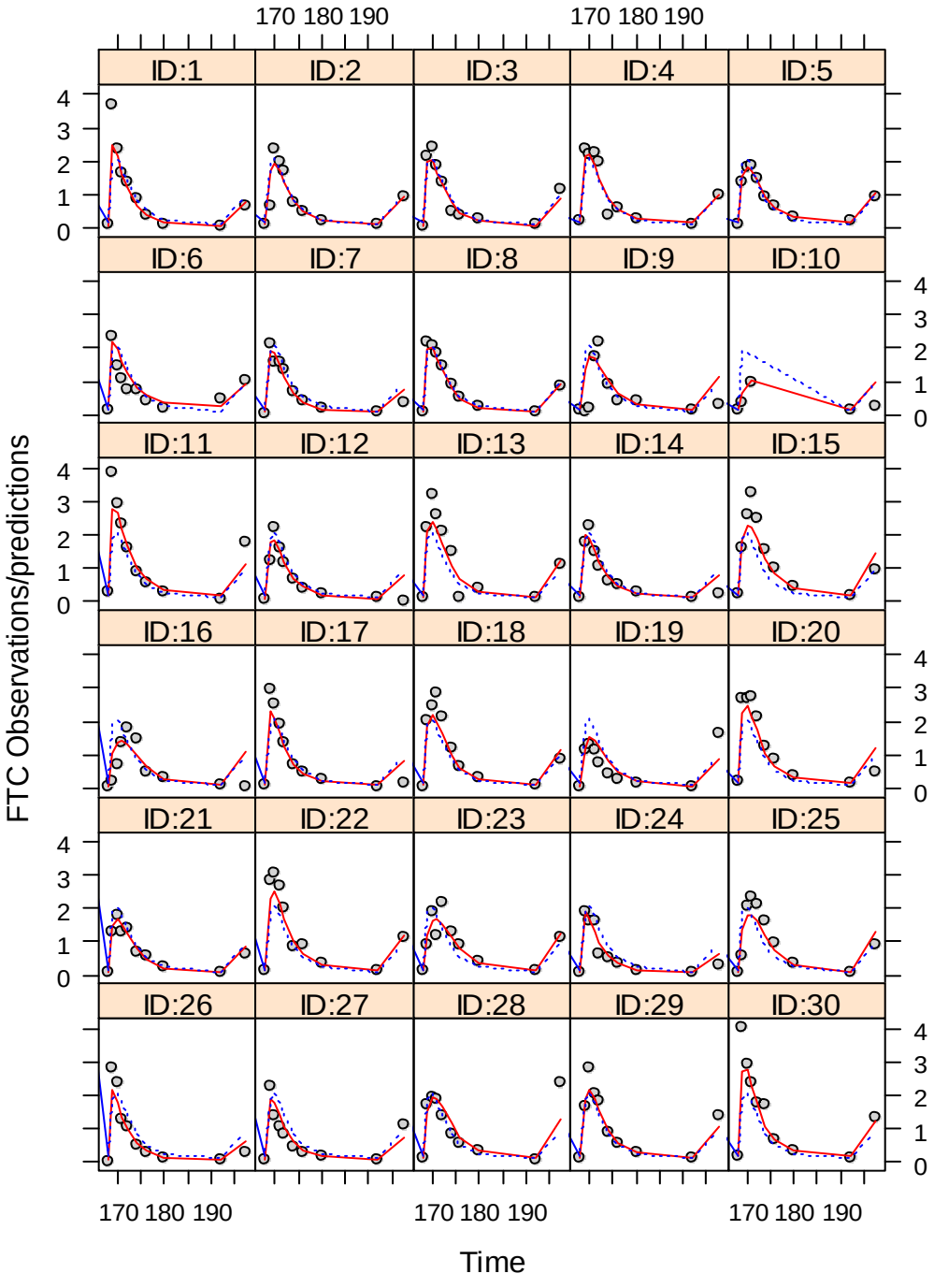
Figure 15.

Individual TFV (A) and FTC (B) Concentration-Time Profiles with Model Predictions

A. TFV



B. FTC



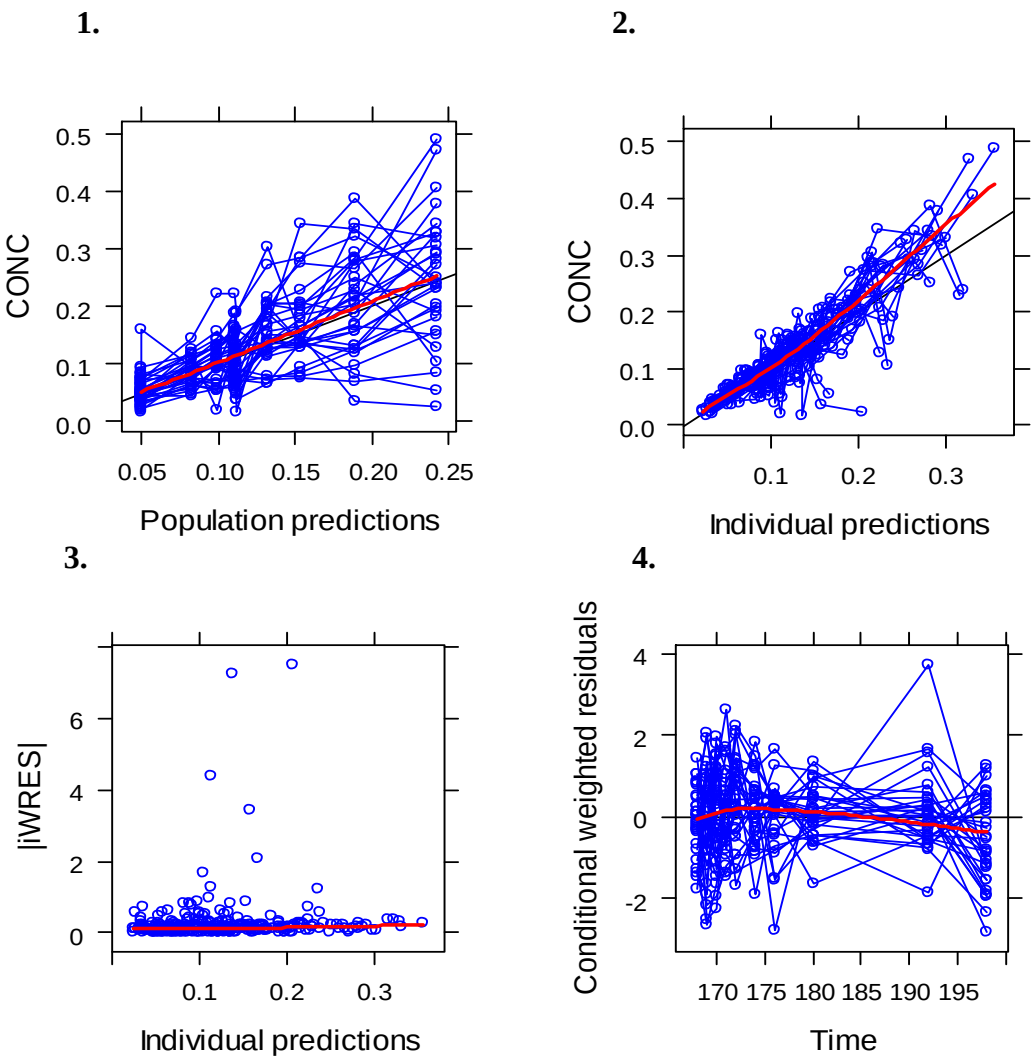
The symbol O indicates the measured concentrations at each time point; The red line indicated the indivdual prediction; blue dotted line indicated the population prediction

Goodness-of-fit plots from both TFV and FTC base model are presented in Figure 16 A and B, respectively. The goodness of fit for the models illustrated the good correlations of the population predictive value (PRED), individual predictive value (IPRED) and observed value (OBS) and their closeness to the trend line.

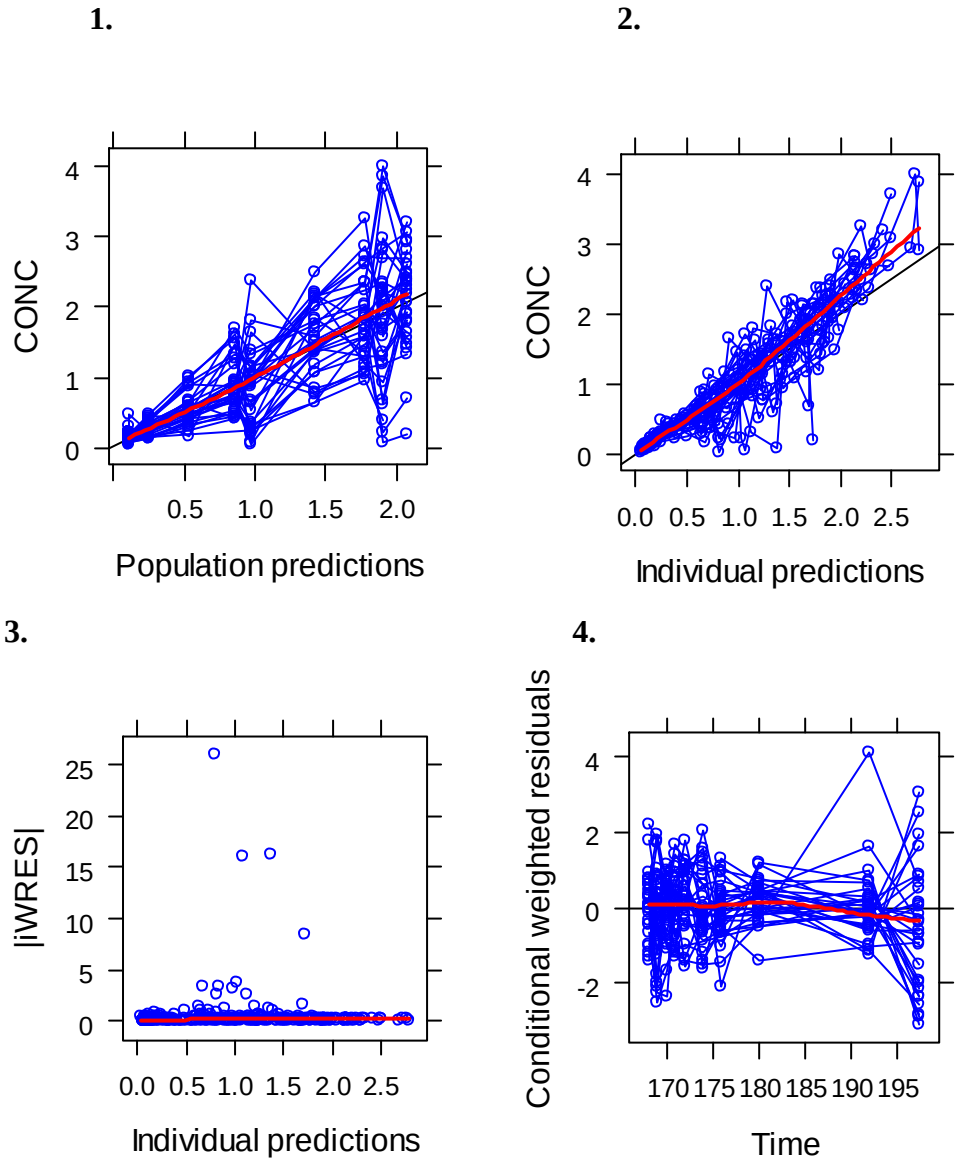
Figure 16.

Goodness-of-Fit Plots for TFV (A) and FTC (B) Plasma Concentrations

A. TFV



B. FTC



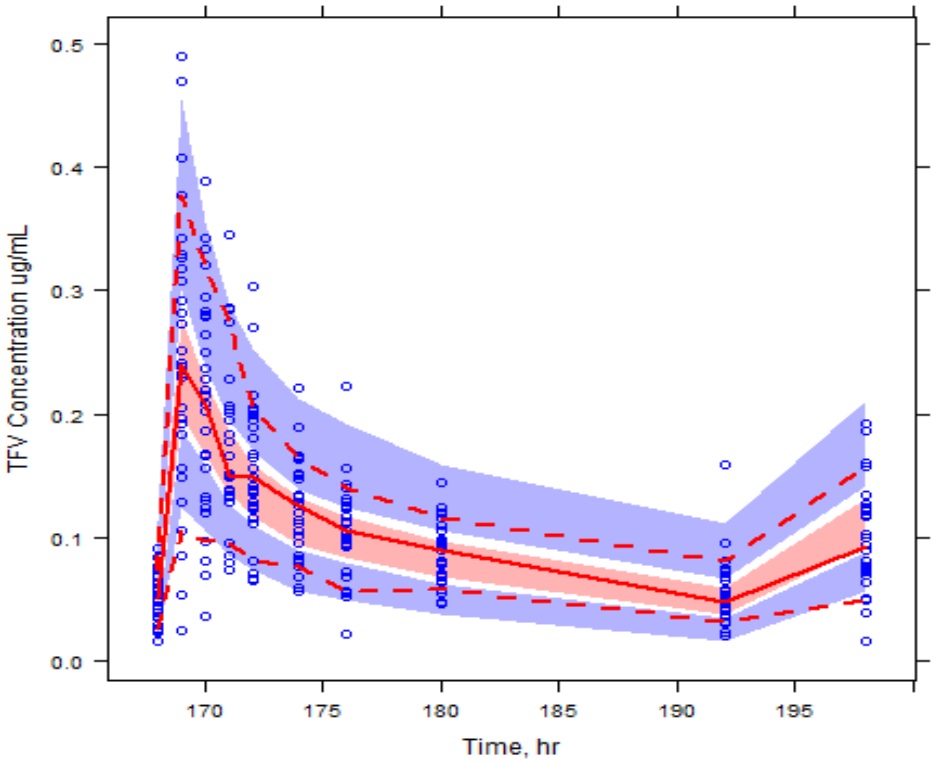
Final model goodness of fit plot of TFV and FTC. (1) Population predicted concentration versus observed concentration. (2) Individual predicted concentration versus observed concentration. (3) Conditional weighted residuals versus population predicted concentration. (4) Conditional weighted residuals versus time. The red lines are the nonparametric regression line. The black line in (1) and (2) represent the line of identity whereas in (3) and (4) are the position where conditional weighted residual equal 0.

Figure 17 shows the results of the pcVPC using 1000 Monte Carlo simulations. The 10th, 90th and median lines of observed concentration were located near the middle of the shadow area, which is the 95% confidence interval of simulated data. This suggest that the sufficiency of the predictive power of the model.

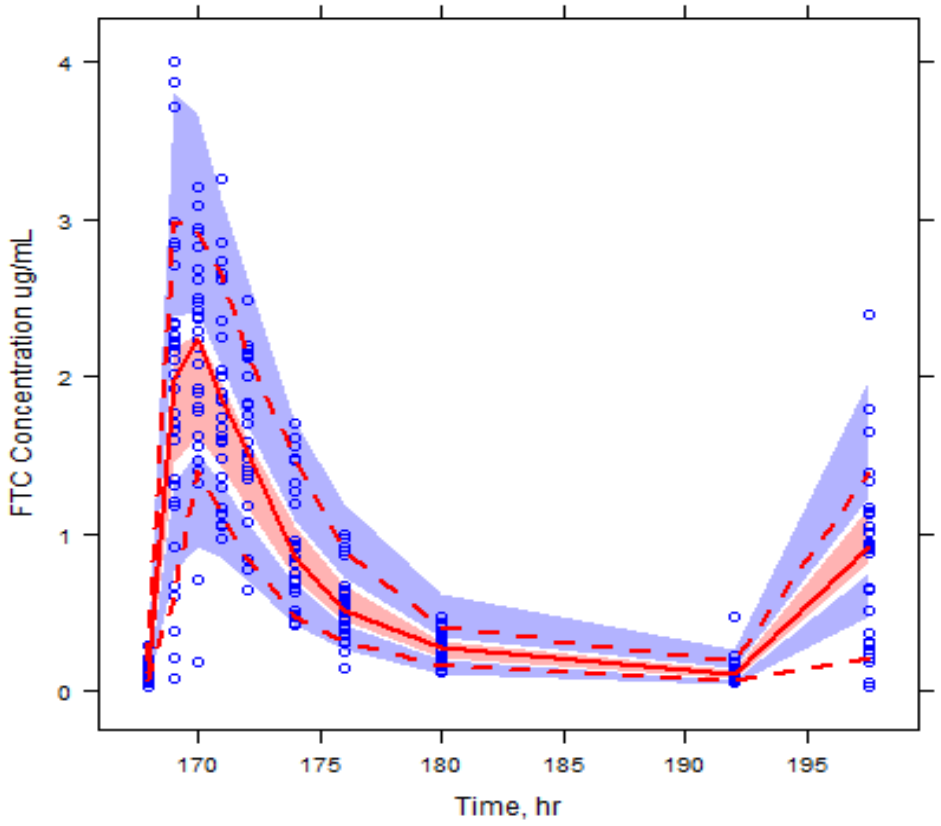
Figure 17.

Visual Predictive Checks for TFV (A) and FTC (B) Plasma Models

A. TFV



B. FTC



The prediction corrected visual predictive checks for the (A) TFV model and (B) FTC model. Open circles represent observed TFV or FTC concentrations, the solid line and the dashed line represents the median and the 95% CI of observation, respectively. The middle red shadow areas represent the 95% confidence intervals of median for the results of 1000 times simulation of final model and the blue shadow areas represent the 95% confidence intervals of the 10^{th} and 90^{th} percentiles of the results of 1000 times simulation of final model.

CHAPTER 4

DISCUSSION

To the best of our knowledge, our study was the first to simultaneously collect four different biological matrices from HIV-seropositive women, specifically plasma, PBMCs, CVF and FGT cells, and successfully quantitate TFV and FTC in these samples using novel LC/MS/MS methods. Additionally, we were the first to evaluate, *in vitro*, the uptake of TFV and FTC in the presence and absence of hormonal contraceptives in five representative FGT cell types, distinguishing that TFV and FTC uptake differs by cell line and hormonal presence. Pharmacokinetic models of TFV and FTC in plasma were also created, in an attempt to establish baseline parameters for the development of complex pharmacokinetic and pharmacodynamic models of TFV and FTC concentrations in PBMCs, CVF, and FGT cells. Knowledge of drug concentrations in these fluids and compartments is essential in understanding drug penetration. Taken together, work presented in this thesis should assist in expanding our knowledge on the selection of appropriate doses and dosing intervals required to achieve optimal drug concentrations for PrEP.

Determination of TFV and FTC in Various Matrices using LC/MS/MS

Assay Quantitation and Validation

Quantitation of TFV and FTC in clinically obtained plasma, PBMCs, CVF, and endothelial cells of the FGT were performed using developed and validated LC/MS/MS methods upheld by the standards set by GCLP and FDA validation guidelines. The development of one set of optimization setting for the LC/MS/MS, which was suitable for five different samples, is to our knowledge the first of its kind. The extraction for TFV and FTC from plasma was an in-house adaptation of a MCX SPE; however, due to adjustments, it was able to quantitate TFV and FTC as low as 5 ng/mL. An extensive double SPE for PBMCs and endothelial cells of the FGT was designed and to our knowledge is able to quantitate lower than any other method of its kind, with an LLOQ of 0.1 fmol/10^6 cells. Lastly, a novel extraction for CVF_{WS} samples was developed. This simple extraction can be useful for the development of future extractions of other antiretrovirals from these collection strips and other paper-like sample collection products.

Our laboratory participates in a blinded external proficiency testing program for the measurement of ARV drug concentrations, which is sponsored by the AIDS Clinical Trials Group. The developed method for plasma TFV and FTC has been successful in determining TFV and FTC concentrations in these blinded proficiency-testing samples. In addition, blind internal proficiencies were made for TFV and FTC in PBMCs and on wicking strips. Following the same GCLP and FDA guidelines for validation, successful

quantitation of these samples assured that good specificity, sensitivity, precision and accuracy were achieved in all our assays.

In Vitro: Intracellular Uptake of TFV and FTC in FGT Cells

Traditionally, laboratories quantitating antiretrovirals and HIV-RNA in clinical samples from the FGT have utilized a surrogate cell line, typically THP-1cells, as a control. However, our study was the first of its kind to determine that representation of the FGT by a single cell line does not take into account shifts in uptake related to differences in cellular makeup and differences in expression of drug transporters likely influenced by the presence of exogenous hormones. Therefore, a combination matrix of multiple cell types of the FGT should be used in laboratory settings when quantitating intracellular ARV concentrations.

Compared with the THP-1 control cell line, which represents macrophages, intracellular TFV and FTC concentrations were increased in HeLa cells, which represent CD4+ T-cells, and Ect1/E6E7 cells, a surrogate for squamous epithelial cells. Previous work has shown these two cell lines are more rapidly invaded by HIV-1(Coombs et al., 2001; Hladik et al., 2007). In the presence of exogenous hormones, the most significant and consistent effect was observed for both TFV and FTC when dosed one hour prior to EE and ET together in BC-3 and TF-1 cells. These results indicate that differential uptake and phosphorylation of TFV and FTC exists across major cellular subtypes in the FGT, and that sex hormones can influence cellular uptake and/or phosphorylation rates of these nucleoside analogs.

Identification and Selection of FGT Cells

Our initial identification of cell types and, therefore, cell lines to represent the FGT was based on data from literature on the FGT, cell types of the FGT, HIV-1 transmission, invasion and acquisition in the FGT, and other confounding factors involved in women's health such as age, race, disease state, and menstrual cycle. Appropriate cell selection was essential for designing optimal qualitative and quantitative methods of intracellular antiretrovirals in the FGT. Two major criteria were considered to define the optimal cell lines to represent cell types of the FGT (Blankson et al., 2002). First, the best cell lines would represent cell types that were well characterized and abundant in the FGT (Pudney, Quayle, & Anderson, 2005). The cervix, specifically the endocervix and ectocervix, and the vagina were identified as the major sections of the FGT with the best characterized cells relevant to antiretroviral drug quantification (Reddy et al., 2003). The difference in abundance of each cell type in the FGT is dependent on age, disease states, and menstrual cycle. Second, the best cell lines to represent the cell types were those cell types that are most plentiful in these sections of FGT (Hladik et al., 2007; Pudney et al., 2005). Five cell types, specifically, macrophages, dendritic cells, epithelial cells, CD4+ and CD8+ T-lymphocytes, were determined to be most plentiful. One cell line, representing each cell type of the FGT was evaluated. THP-1 cells, which are macrophage-like, were selected as the control cell line because of their ability to convey the latency and upregulation of HIV-1, in addition to their use as a control in determining the uptake of antiretrovirals in FGT tissues (Coombs et al., 2001; Hajjar et al., 1998; Iversen et al., 1998; Mikovits et al., 1990). Determining TFV and FTC uptake in only one surrogate FGT cell line and in immortal cells were limitations of this study.

Our findings should be validated against additional cell lines representing these and other cell sub-types from the FGT and by primary FGT cells obtained in a clinical setting.

Intracellular Uptake of TFV and FTC in the Absence of Hormonal Contraceptives

Our study was the first to evaluate the intracellular concentrations of TFV and FTC across five surrogate FGT cell lines. Compared with the THP-1 control cell line, intracellular TFV and FTC concentrations were increased in Ect1/E6E7 and HeLa cells, while concentration in BC-3 and TF-1 cells were similar to the control line. Previous work has shown that Ect1/E6E7 and HeLa cells are more rapidly invaded by HIV-1 (Coombs et al., 2001; Hladik et al., 2007) compared with the other cell lines. Thus, increased TFV and FTC concentrations in Ect1/E6E7 and HeLa cells suggest that these antiretrovirals could potentially reduce invasion of HIV-1 into prominent cells of the FGT.

The five distinct surrogate FGT cells lines we evaluated achieved median intracellular TFV and FTC concentrations of 579 and 40 fmol/10^6 cells, respectively. Although clinical studies evaluating adequate antiretroviral concentrations in the FGT to reduce HIV-1 transmission are ongoing, these data have not been published. However, our data can be compared with steady-state TFV-DP concentrations of 337 fmol/10^6 cells and FTC-TP concentrations of 4300 fmol/10^6 cells from HIV-1 negative cervical cell samples (M. A. Anderson P, Zheng J-H, et al. , 2012). *In vitro*, TFV concentrations in FGT cell lines were similar to those reported in cervical samples from HIV-seronegative patients. However, FTC concentrations in these cell lines were much lower. Even

across the two highest uptake cell lines (HeLa and Ect1/E6E7), the median FTC-TP concentration was only 934 fmol/10^6 cells, which is almost 5 times lower than reported in patient samples. The disparity between *in vitro* and *in vivo* uptake of TFV and FTC data demonstrates that our *in vitro* results are not necessarily comparable to clinical data. These differences may be due to the exposure of patient samples to steady-state concentrations of TFV and FTC while cell lines were exposed to TFV and FTC concentrations for a maximum of 1 hour. Furthermore, antiretroviral concentrations in the body likely fluctuate to some degree as a result of drug distribution and elimination. Cell lines in our *in vitro* study were exposed to only one concentration of the drugs. Although our *in vitro* experiments were not able to adequately mimic physiologic processes that produce variability in antiretroviral exposure, the findings illustrate the need to establish optimal antiretroviral quantitation methods for the FGT. Specifically, these methods should encompass each major cell type of the FGT in hopes to alleviate the chances of under or over-quantitating antiretroviral penetration into this compartment.

Intracellular Uptake of TFV and FTC in the Presence of Hormonal Contraceptives

To our knowledge, this is the first *in vitro* evaluation of TFV and FTC uptake in the presence of exogenous hormones. Our findings of TFV and FTC uptake from five distinct cells lines representing the FGT achieved mean baseline concentrations of 5051.99 fmol/10^6 cells and 475.20 fmol/10^6 cells, respectively. Again, these concentrations are significantly higher than those reported to adequately reduce viral

replication and transmission by 90% in PBMCs (15.6 fmol/10^6 cells for TFV-DP)(Anderson Peter, 2012).

The most significant and consistent finding from our study was the decreased TFV and FTC uptake in the presence of exogenous hormones when dosed 1 hour prior to EE /ET exposure in both BC-3 and TF-1 cells. Penetration of NRTIs into the genital tract of females and males is dictated by facilitated diffusion and active transport (Taylor & Pereira, 2001), leading to one explanation for differences in TFV and FTC uptake among the cell lines. Various cell types in the FGT express transporters differently, and these transporters likely have varying degrees of functional activity. Data suggest that TFV uptake occurs through OAT 1 and 3 while FTC uptake occurs through OCT 1 (Cihlar et al., 2007; Nakatani-Freshwater & Taft, 2008). However, transporter expression and/or activity in FGT cells vulnerable to HIV-1 invasion have not been extensively studied. There are no data on transporter expression and/or activity in the presence of both exogenous hormones and antiretrovirals. However, the effects of antiretrovirals and exogenous hormones on transporters, separately, have been reported. Transporters are manipulated by cyclic nucleotides and steroids (Hayer-Zillgen et al., 2002; Ljubojevic et al., 2004; Wielinga et al., 2003). Progesterone, represented in our study by ET, upregulates OAT1, which TFV uses for intracellular entry, and downregulates OCT1, which FTC utilizes for cell entry. Progesterone also downregulates MDRP 1, 4, and 5, all of which promote TFV and FTC efflux. In the presence of ET, MDRP downregulation inhibits TFV and FTC cellular efflux thus, potentially increasing the intracellular concentrations of both TFV and FTC. However, downregulation of OCT1 by ET should decrease influx of FTC, while upregulation of OAT1 should increase TFV

cellular uptake. Taken together, these alterations in transporter expression likely explain the overall lower concentrations seen in FTC compared to TFV in our study. Meanwhile, estrogen, represented by EE in our study, downregulates OAT1 and 3 and OCT1, which TFV and FTC require for cellular uptake. This downregulation most likely lowered intracellular concentrations of these antiretrovirals.

Co-administration of EE and ET with TFV and FTC may alter antiretroviral cellular uptake and potentially affect clinical outcomes. Our findings, although *in vitro*, report a trend in increased uptake of TFV in the presence of EE/ET based on increasing TFV cellular dosage. However, the percent uptake of FTC in the presence of EE/ET varied as FTC doses increased. Taken together, our data support the need for future *in vivo* studies to evaluate the clinical significance of administering these antiretrovirals with exogenous hormones.

The effect of exogenous hormones on the intracellular phosphorylation of TFV and FTC has not been directly evaluated. Studies, however, have been conducted with some of the intracellular enzymes responsible for phosphorylation and dephosphorylation of NRTIs in the presence of exogenous hormones. Differences in cellular uptake of TFV and FTC in the presence of exogenous hormones can possibly be explained by these mechanisms. For example, human adenylate kinase is responsible for the phosphorylation of TFV to TFV-MP, however, estrogen does not alter the enzyme activity (Roghmann, Skinner, & Hilf, 1987). This lack of effect indicates that differences in uptake and phosphorylation of TFV are most likely regulated by transporters responsible for drug movement or by the enzyme (nucleotide diphosphate kinase) responsible for the second stage of phosphorylation. The effect of exogenous hormones

on activity of the three enzymes, specifically deoxycytidine kinase, uridylate-cytidylate kinase, and 3′-phosphoglycerate kinase, responsible for the phosphorylation of FTC has not been evaluated. However, activity of the enzyme 5'-nucleosidase, which is responsible for the dephosphorylation of FTC-MP to FTC, has been shown to be altered in homogenated rat brain and liver when estradiol and progesterone are co-administered (Garcia, Cabezas, & Perez-Gonzalez, 1985). Enzymatic activity in the liver was decreased in the presence of both exogenous hormones, while the brain demonstrated increase activity of the enzyme in the presence of estradiol. Due to the varying patterns of enzymatic activity by organs and species, it is most likely that enzymatic activity is cell type dependent. Thus, potentially explaining why antiretroviral uptake differs between cell lines in our study. Another study that focused on endogenous hormone activity also supports our findings. This study demonstrated that high physiological concentrations of progesterone decreased (25-68%) the phosphorylated forms of NRTIs, while estrogen caused no significant changes (Anderson, King, Zheng, & MaWhinney, 2008). Consistent with our *in vitro* data, these clinical data suggest intracellular TFV and FTC concentrations may be altered by the effect of exogenous progesterone on enzymatic activity, but the direction and magnitude of the change is highly variable.

Although there are several limitations to this study, we believe that our *in vitro* findings provide insight on the use of TFV and FTC for PrEP. First, antiretroviral uptake was determined in only one immortal cell line representing each cell type. Further validation of our findings should be made in additional cell lines representing these and other cell sub-types from the FGT. In addition, our results should be compared with the uptake of TFV and FTC by primary FGT cells matched to our cell types.

Second, our experiments were conducted with a one hour incubation time for all dosing regimens. Earlier studies evaluating mechanistic and kinetic activities of antiretrovirals or hormones suggest that one hour incubations are sufficient for uptake of antiretroviral and exogenous hormones (Harms et al., 2001; Okada et al., 1997; Si-Mohamed et al., 2000). However, additional incubation times may be needed to account for any changes in cellular antigen, receptor, and/or transporter activity potentially caused by the addition of TFV, FTC, EE, or ET.

Third, only one concentration of EE and ET was dosed into the cell media. Evaluating various concentrations of TFV and FTC with various concentrations of exogenous hormones dosed into the media would determine if TFV and FTC cellular uptake into the surrogate FGT cells are dependent on the functional activity of the transporters, which are regulated by EE and ET. The concentrations of EE and ET used represented the plasma mean C_{max} in humans, which differ from the concentrations seen in the FGT (Roumen & Dieben, 2006). However, it should be noted that the goal of study was to potentially mimic their co-administration as both oral and intravaginal PrEP agents. Additional doses that represent the mean FGT concentrations (from the cervical region) of EE and ET after 1 cycle (17-21 days) of continuous dosing could be considered. For example EE, after 17-21 days of dosing, was reported to obtain tissue concentrations of 68 pg/0.5 g cervical tissue, which assuming 1 g is equivalent to 1 mL, is approximately 75% higher than the concentration used in this study (34.7 pg/mL). Meanwhile, the mean ET concentration was 327 pg/0.5 g of cervical tissue, which is approximately 62% lower compared to our study (1716 pg/mL).

Finally, ET, which is uniquely found in the hormonal contraceptive NuvaRing®, was the only progesterone used in this study. Most hormonal contraceptives use a combination of EE and different synthetic progesterone such as norethindorne, norgestimate, or levonorgestrel. The effect of these progesterones on TFV and FTC should be evaluated. However, the mechanism of action of ET is very similar to other progesterones suggesting a similar effect on TFV and FTC cellular uptake.

In conclusion, our data suggest that TFV and FTC intracellular concentrations differ across surrogate cell types of the FGT and these concentrations may be altered in the presence of exogenous hormones. These findings also advocate quantitating antiretrovirals in complex compartments with more sensitive analytical tools that utilize a compartmental matrix consisting of multiple FGT cell types. Quantitation of TFV and FTC in multiple cell types of the FGT will ensure accurate reporting of antiretroviral concentrations and a more precise analysis of this compartment. The design of future PrEP studies should also factor in patient use of exogenous hormones, which our findings suggest alter the uptake of TFV and FTC in FGT cells. Exogenous and endogenous hormones can change the cellular makeup of the cervical mucus and endometrium in the FGT (Rivera, Yacobson, & Grimes, 1999). These changes shift the prevalence of certain cell types in the FGT while increasing the abundance of the cells present in the vagina and cervix. As a result, cellular uptake of antiretrovirals is altered, possibly requiring different antiretroviral dosages to achieve optimal concentrations needed to reduce transmission in the identified target cell types and cellular regions. Furthermore, the potential effects of exogenous hormones on TFV and FTC uptake in the FGT should be considered when designing, quantitating, analyzing and reporting PrEP data. Correctly,

accounting for the presence of exogenous hormones and varying cellular uptake of TFV and FTC by distinct cell types will enhance our understanding of the pharmacologic consequences of antiretroviral therapy as a PrEP agent in women receiving hormonal contraception.

Clinical Study Findings

Our study was the first of its kind to evaluate two different samples types from the FGT, while pairing it to intensive plasma pharmacokinetic samples coupled with sparse PBMC sampling. The study's intentions were to expand upon our current knowledge of antiretroviral penetration into the FGT by developing predictive pharmacokinetic-pharmacodynamic models. The study also was designed to determine the validity of conventional drug measurements in the FGT using novel methods. These goals were accomplished by the evaluation of pharmacokinetic data analyzed from 30 women.

Plasma and PBMC samples from this study were comparable to literature reported C_{max}, C_{last}, C_{min}, ke, t½, AUC_{24}, Vd, T_{max}, and CL/F values for both TFV and FTC. Pharmacokinetic parameters from samples collected by cytobrushing, $CVF_{DA,}$ and CVF_{WS}, resembled pharmacokinetic parameters previously reported for TFV and FTC in the FGT (L. J. Anderson P, Buchbinder S, et al. , 2011; Dumond et al., 2007). The pharmacokinetic parameters complied from these four different samples types are used in the development of a pharmacokinetic model discussed further below. Limited mainly by sample size, the population driven data configured pharmacokinetic parameters in three major compartments; plasma, intracellular, and the FGT.

Central Compartment Exposure

Plasma Samples

Our study was designed to develop a complete plasma pharmacokinetic profile for both TFV and FTC, which would be a major contributor in the dataset utilized for the development of pharmacokinetic models and simulations. The initial pharmacokinetic parameters were established from a nine point 24-hour intensive sampling for both TFV and FTC. Twenty nine out of the 30 patients were able to contribute complete profiles. TFV and FTC pharmacokinetic parameters were equivalent to those parameters reported for TFV and FTC at steady-state in the literature. In previous studies, where TFV, FTC, and EFV were co administered to healthy volunteers, TFV and FTC plasma exposure was approximately equal to that measured in our study. Mean TFV and FTC AUC_{24} was approximately 4% and 13% higher, respectively, in our patients compared with healthy volunteers (Deeks, 2010). Pharmacokinetic results from our study participants suggest steady state concentrations were achieved prior to study sampling. More importantly, the plasma dataset was also used in the base models of the population pharmacokinetic analysis described further below.

PBMC Samples

The active moieties of TFV and FTC are formed inside cells. Quantitating intracellular concentrations of these antiretrovirals can be complicated. Ideally, plasma concentrations would be used to predict TFV and FTC concentrations in PBMCs.

Although these definite relationships have not been established, many studies have attempted to gain a better clinical understanding of intracellular antiretroviral concentrations. Previously, intracellular TFV-DP has been evaluated mostly in small populations of HIV-seropositive adults, with the majority of studies focusing on TFV exposure in men. Eight different intracellular studies obtained steady-state concentrations that exhibited significant interindividual variability (CV% of ~50%) (Durand-Gasselin, Da Silva, Benech, Pruvost, & Grassi, 2007; Hawkins et al., 2005; Kiser, Aquilante, et al., 2008; Kiser, Fletcher, et al., 2008; Pruvost et al., 2005; Pruvost et al., 2009; Vourvahis et al., 2008). Data from these studies also had varying TFV half-lives in PBMCs, ranging from 2.5 to 7.5 days, consistent with our TFV half-life in PBMCs of 0.22 to 7.9 days (Hawkins et al., 2005; Pruvost et al., 2005). Similarly, active FTC, although evaluated less often in studies, reported concentration in PBMCs of 1000 to 6000 fmol/10^6 cells and a terminal half-life of approximately 47.5 hours (Darque et al., 1999; Wang et al., 2004). Mean FTC concentrations in PBMCs from our patients was 7044.0 fmol/10^6 cells, also similar to previously reported data.

Extracellular concentrations of TFV and FTC are represented by the concentrations obtained in plasma. The intracellular TFV and FTC concentrations are represented by concentrations in PBMCs. Coupling extracellular and intracellular TFV and FTC concentrations allows for the understanding of drug exposure in two major physiologically relevant compartments. A brief comparison of extracellular and intracellular TFV and FTC pharmacokinetics in our study and those in the literature is illustrated in Table 40.

Table 40.

Extracellular and Intracellular Data for TFV and FTC

	Literature Data	Our Data
TFV		
TFV-DP: fmol/10^6 cells	80 to 160	0.7 to 214
TFV-DP half-life: days; range	2.5 to 7.5	0.25 to 7.9
TFV plasma: µg·h/mL	2 to 4	1.1 to 4.0
TFV plasma half-life: days	0.5 to 0.6	0.3 to 4.0
FTC		
FTC-TP: fmol/10^6 cells	1000 to 6000	47 to 186185
FTC-TP half-life: days; range	1.6	0.18 to 10
FTC plasma: µg·h/mL	8 to 11	8 to 22
FTC plasma half-life: days	0.3 to 0.4	0.2 to 0.5

Overall, TFV and FTC concentrations in PBMCs were lower compared with plasma concentrations, although no correlation was found between the two concentrations. TFV and FTC PBMC AUC_{24} were approximately 200% lower than their matched plasma AUC_{24}. The lack of correlation between concentrations in plasma and PBMC, in our study, demonstrates the need for further evaluation of the data. The goal is to establish a parameter that has the ability to predict intracellular concentrations based upon knowledge of plasma concentrations. Until this parameter is determined, a link will remain missing in fully understanding TFV and FTC exposure-response relationships.

Understanding the quantity of TFV and FTC needed to achieve adequate concentrations of the active moiety is pivotal in designating an appropriate dose of these antiretrovirals for prophylactic use. Although intracellular exposure is lower than plasma, the terminal half-lives of the intracellular moieties, determined again by our data, are much longer than those of TFV and FTC in plasma. Therefore, active TFV and FTC may be accumulating and/or penetrating into tissues that were not sampled in this study for antiretroviral quantitation. Knowledge of an extended half-life of the active drugs can be exploited to manipulate alternate dosing regimens and dose adjustments for adherence issues if TFV and FTC are administered as PrEP agents.

FGT Exposure

Movement of antiretrovirals throughout the FGT is best characterized by understanding both extracellular and intracellular concentrations from this compartment. These concentrations from CVF samples and endothelial cells of the FGT will provide understanding of the movement of TFV and FTC in the FGT. The degree of penetration of TFV and FTC is dependent on the chemical and physical properties of TFV, FTC and the FGT. These properties determine whether adequate concentrations to decrease HIV replication or HIV acquisition are allowed to penetrate this site. Concentrations of TFV and FTC in these intracellular and extracellular matrices are essential for the development of a predictive pharmacokinetic model to predictive dosing and dosing intervals of TFV and FTC as PrEP agents.

CVFws and CVF_{DA} Samples

CVF samples were collected at paired time intervals by two distinctly different collection techniques, CVF_{DA} and CVF_{WS}. Collection of CVF samples allowed us to quantitate drug in this compartment while potentially adding pharmacokinetic data about the extracellular exposure of TFV and FTC in the FGT to a complex pharmacokinetic model of the FGT. Two different collection techniques were used to validate the quantitation of TFV and FTC in this compartment and to determine if these collection techniques were equivalent. Equivalence between collection techniques would allow data collected in different studies to be compared and potentially combined to aid in the development of a robust FGT pharmacokinetic model.

CVF_{DA} and CVF_{WS} samples collected in our study produced pharmacokinetic parameters within the range of reported pharmacokinetic parameters of TFV and FTC in CVF collected after steady-state dosing in 5 to 10 healthy women (Dumond et al., 2007; Patterson et al., 2011; Schwartz et al., 2011). TFV and FTC AUC_{24} and C_{last} in CVF as reported in our patients are compared with these data in Table 41. Furthermore, our TFV and FTC CVF C_{last} were above the adequate TFV and FTC concentrations, 10 ng/ml and 2 ng/ml, respectively, needed to inhibit viral replication (Kearney et al., 2004; Wang et al., 2004). This similarity indicates that the CVF may achieve TFV and FTC concentrations sufficient to inhibit HIV replication in the FGT.

TFV and FTC AUC_{24} in both CVF sample types were much lower than TFV and FTC CVF AUC_{24} range reported in previous data. The variability in AUC_{24} between studies could be explained by the differences in sample size, both the number of patients

in each study along with the number of samples collected and used in the analysis. The variability of our studies median (range) recorded for TFV and FTC AUC_{24} could also be attributed to inconsistency in collection techniques or other technical issues and are demonstrated with CV% for TFV and FTC AUC_{24} ranging from 80% to 184% for our CVF samples. However, a comparison of all the TFV and FTC C_{last} among these CVF samples reported in Table 41 demonstrates that FTC maintains higher concentration at the end of the concentration profile compared to TFV. The differences in TFV and FTC concentrations may be related to sequestration of these drugs into cells of the FGT or the movement of these drugs from the blood to the FGT. Nonetheless, additional studies need to be conducted which are focused on the movement and uptake of TFV and FTC into the FGT along with the sequestration of TFV and FTC in the FGT.

Table 41.

CVF Data in Literature and Current Study Data

	Our Data CVF_{DA}	Our Data CVF_{WS}	Literature Data (Dumond et al)	Literature Data (Patterson et al)
TFV				
$AUC_{24:}$ hr*mg/L	0.029 (0.001 -0.277)	0.024 (0.002 – 0.098)	1.75 (0.60-3.13)	6.02 (3.62 – 30.17)
C_{last}: ng/mL	19.87 (2.45 – 116.51)	34.01 (5 – 350)	68.4 (28.2 - 112.6)	69 (57–586)
FTC				
AUC_{24}: hr*mg/L	1.21 (0.0465 – 5.419)	0.027 (0.002 – 0.084)	30.8 (25.6- 52.6)	58.68 (55.42 – 87.14)
C_{last}: ng/mL	544 (247 – 4219)	512 (115 – 822)	596 (537.3 - 644.2)	1,183 (638–2,277)

Data reported as median(range)

Contrary, to previously reported data, our data suggests lower exposure of TFV and FTC in CVF compared to plasma. The TFV CVF AUC_{24} for both collection methods was approximately 196% lower than that seen in plasma. Similarly, FTC CVF AUC_{24} was 169% to 192% lower than that seen in plasma. However, TFV and FTC C_{last} measured in CVF were similar to the recommended plasma trough concentrations necessary for efficacy in patients with HIV, therefore, sufficient concentrations to reduce HIV invasion may still be obtained (Baeten et al., 2012; de Lastours, Fonsart, Burlacu, Gourmel, & Molina, 2011; Uglietti, Zanaboni, Gnarini, & Maserati, 2012).

The collection of CVF_{DA} and CVF_{WS} samples had limitations. Direct aspiration obtains CVF that contains free and cell based matrices and covers a larger portion of the vagina and cervix than any other collection technique. Direct aspiration also collects a homogenous sample, which is difficult to separately cellular. As a result, measuring antiretroviral concentrations in the extracellular fluid of the FGT using direct aspiration may be over or underestimated. Wicking strips, however, contain a smaller amount of CVF sample than other FGT collection techniques, and can only be used to quantitate antiretrovirals once, leaving no room for error in the analysis. Of note, both methods are being utilized frequently because of the need for simple, precise, and easily comparable collection techniques of the FGT.

Our study also evaluated the validity and equivalence of collecting sample with direct aspiration and wicking strips by comparing TFV and FTC CVF concentrations between the two techniques. Development of extensively validated analytical methods to quantitate TFV and FTC from samples obtained from these collection types were described previously. The precision and accuracy that were unique to each quantitation

technique assures us that TFV and FTC concentrations reported are correct. Therefore, if these two CVF samples are matched by concentration time point or by overall exposure they should be equivalent. However, they are not equivalent as illustrated Table 42 by the discrepancies between the two sample collection technique ratios. The inconsistency between CVF collection techniques, therefore, suggests that comparisons of drug concentrations in this compartment can only be made between studies using the same collection technique.

Table 42.

CVF Mean Concentration and AUC_{24} Ratios

Parameters	CVF_{DA}	CVF_{WS}	Ratio CVF_{DA}: CVF_{WS}
TFV			
C_0	1.5E-03	1.2E-03	1.17
C_6	4.8E-04	1.5E-03	0.33
C_{12}	1.8E-03	1.2E-03	1.44
C_{24}	5.1E-04	7.2E-04	0.71
AUC_{24}	0.029	0.024	1.2
FTC			
C_0	0.042	0.009	4.56
C_6	0.034	0.019	1.82
C_{12}	0.073	0.015	4.75
C_{24}	0.028	0.010	2.79
AUC_{24}	1.21	0.027	44.82

C_n in mg/L; AUC_{24} in hr*mg/L

In conclusion, TFV and FTC appear to reach adequate concentrations in the female genital tract to reduce viral replication. Previous data on antiretroviral concentrations in this were measured using various techniques including cervical swabs, cervical lavage and direct aspiration with a volumetric aspiration device. Our findings

suggest that comparing these data across studies should only occur when the same collection technique is used. Furthermore, differences in our results from previously reported data suggest that the same analytical methods should be used to compare results.

FGT Cell Samples

Measuring extracellular and intracellular antiretroviral concentrations in the FGT can be challenging. Nonetheless, the collection of intracellular FGT samples will account for the dynamic movement of TFV and FTC into endothelial cells of the FGT similar to PBMCs concentrations accounting for TFV and FTC movement from the plasma. Due to the complexity of collecting endothelial cells from the FGT, only two samples at 6 and 24 hours post dose were obtained. However, we were able to compare our results to previous studies quantitating intracellular TFV and FTC in cervical cells of healthy volunteers. Previously reported intracellular TFV C_{24} in the FGT ranged from 10 to 119 fmol/10^6 cells, which were similar to our findings of 0.35 to 240.75 fmol/10^6cells. Similarly, intracellular FTC C_{24} in the FGT of our patients ranged from 2.4 to 74,459.6 fmol/10^6cells with the literature data ranging from 170 to 11,090 fmol/10^6 cells (M. A. Anderson P, Zheng J-H, et al. , 2012; Patterson et al., 2011). Results from our study demonstrate adequate penetration of intracellular TFV and FTC into the FGT of HIV-seropositive women, which we believe supports continuous use of TFV and FTC as a PrEP agents. Although our intracellular TFV and FTC concentrations in the FGT 24 hours post dose are low in comparison to the C_{24} in plasma, PBMC, and CVF, adequate concentrations of TFV and FTC essential for the reduction of HIV replication may still be

achieved in this intracellular compartment. The comparison of TFV and FTC concentrations in FGT cells to the other sample types and techniques from our study are demonstrated in Table 43. Collectively, the pharmacokinetic parameters configured from the five sample types representing three different anatomical compartments provides new insights into achievable concentrations of TFV and FTC and can be utilized for initial benchmarks in PrEP studies. However, more detailed pharmacological studies of all anatomical sites are required to better evaluate adequate TFV and FTC concentrations sufficient for the prevention of HIV-1.

Table 43.

Intracellular FGT C_{24} Compared to C_{24} of Study Samples

Sample Type	**TFV**	**FTC**
Plasma	↑ 200%	↑ 197%
PBMCs	↑ 70%	↑ 182%
CVF_{DA}	↑ 151%	↑ 188%
CVF_{WS}	↑ 164%	↑ 155%

Predictive Pharmacokinetic Models

Data collected from this study were used to model plasma TFV and FTC by a population approach. Results demonstrate that a two-compartmental model with first order absorption was sufficient to characterize the plasma concentration-time data for both TFV and FTC and are similar to previously reported data (Jullien et al., 2005; King

et al., 2011). The FTC base plasma model, however, was designed without Ka. The removal of Ka from the FTC model did not affect the OFV. The lack of effect FTC Ka had on the overall base FTC model can be attributed to small interindividual variability. However, if this FTC base plasma model were to be used for another population group, reintroduction of this parameter into the model should be examined. In addition, attempts were made to co-model plasma TFV and FTC with their respective concentrations in PBMCs, CVF, and endothelial cells of the FGT. However, a model with a good fit has not been found for either antiretroviral. The inability to easily fit a model for our data is likely caused by extreme variability of TFV and FTC concentrations in our study patients. A robust dataset acquired through additional sampling or the addition of more study patients is needed to demonstrate relationships between the plasma, PBMCs, CVF, and endothelial cells of the FGT. Future studies that build a complex TFV or FTC model encompassing pharmacokinetic parameters in plasma, PBMCs, CVF, and endothelial cells of the FGT would enhance our understanding of the role of these drugs in HIV prevention.

Conclusion

New ideas applicable to PrEP studies can be obtained from both *in vitro* and *in vivo* studies. *In vitro*, we acknowledged novel connections discerning the differences of TFV and FTC uptake by FGT cell lines and in the presence of exogenous hormones. The disparity between uptake of TFV and FTC with exogenous hormones, lead us to believe that future PrEP studies, especially those with topical application of these drugs, need to take into consideration their co-administration. Our study simultaneously collected four

different biological matrices from HIV-seropositive women, specifically plasma, PBMCs, CVF and FGT cells, and successfully quantitated TFV and FTC in these samples using novel LC/MS/MS methods. These TFV and FTC concentrations will be used to build a complex pharmacokinetic model of both the systemic and FGT exposure of these drugs. As a whole, this knowledge will be used in the selection of appropriate does and dosing intervals required to achieve optimal drug concentrations for PrEP.

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APPENDIX A

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

June 1, 2011

To Whom It May Concern:

The IRB reviewed protocol X100805010, "Antiretroviral Quantitation and Modeling in the Female Genital Tract" on August 31, 2010. The principal investigator for this project is Dr. Edward Acosta.

The initial submission of this project included Amanda James as an investigator on the project, but did not specify that she would be using his work as her dissertation project. The IRB has since been informed of this. Her thesis project has the same title as the above referenced protocol title.

Please accept this letter as confirmation that Ms. James' work on this project was reviewed and approved by the IRB prior to the work beginning.

Sincerely,

Cari Oliver
Assistant Director
OIRB

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UAB THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on October 26, 2010. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines.

Principal Investigator: ACOSTA, EDWARD P

Co-Investigator(s):

Protocol Number: **F100805010**

Protocol Title: *Antiretroviral Quantitation and Modeling in the Female Genital Tract*

The IRB reviewed and approved the above named project on 8/25/2010. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received FULL COMMITTEE review.

IRB Approval Date: 8/25/2010

Date IRB Approval Issued: 8/31/10

Identification Number: IRB00000726

Julius Linn, MD

Julius Linn, M.D.
Acting Chair of the Institutional
Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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THE UNIVERSITY OF
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Institutional Review Board for Human Use

MEMORANDUM

TO: Edward P Acosta, PharmD
Principal Investigator

FAX: 4-6201 (E Acosta)

FROM: Margie Lawson, CIP, CIM
On behalf of IRB 02

Margie Lawson

DATE: August 31, 2010

RE: X100805010 (formerly F100805010)
Antiretroviral Quantitation and Modeling in the Female Genital Tract

The IRB 02 met on August 25, 2010 and approved the protocol referenced above. **This approval will expire and no longer be valid on August 25, 2011.**

Please note the following as related to this review:

- The IRB reviewed this research and determined that it does not involve greater than minimal risk. Therefore, the Office of the IRB has reassigned this protocol for expedited review. Please note that the protocol number now begins with an "X" to indicate this status.
- You will need to maintain documentation of IRB approval from the IRB at Emory, where participants are enrolled.
- All staff listed on UAB IRB protocols must complete the UAB online course about informed consent before December 31, 2010 for Continuing IRB Training. For complete details, please see http://www.uab.edu/irb/2009.

ML/jlg

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