Development of Tenofovir Prodrugs as Rectal Microbicides for HIV Prevention

by

Yafei Lyu

Bachelor of Science, Shenyang Pharmaceutical University, 2014

Submitted to the Graduate Faculty of

University of Pittsburgh in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

SCHOOL OF PHARMACY

This thesis was presented

by

Yafei Lyu

It was defended on

July 28th, 2016

and approved by

Lisa C. Rohan, PhD, Professor, Department of Pharmaceutical Sciences

Samuel M Poloyac, PharmD, PhD, Professor, Department of Pharmaceutical Sciences

Vinayak Sant, PhD, Assistant Professor, Department of Pharmaceutical Sciences

Thesis Director: Lisa C. Rohan, PhD, Professor, Department of Pharmaceutical Sciences

DEVELOPMENT OF TENOFOVIR PRODRUGS AS RECTAL MICROBICIDES FOR HIV PREVENTION

Yafei Lyu, M.S.

University of Pittsburgh, 2016

Receptive anal intercourse is common among men who have sex with men (MSM), transgender women, and practiced by women around the world. The risk of becoming infected with HIV through receptive anal intercourse is 20 times greater than vaginal intercourse. Hence, there is an urgent need for the development of rectal specific topical pre-exposure prophylaxis (PrEP) products. In the current study, our lab developed enemas containing tenofovir (TFV) for rectal application, which may provide some advantages with respect to user acceptability and compliance. In addition to evaluation of TFV in enemas, prodrugs of TFV: tenofovir alafenamide fumarate (TAF) and hexadecyloxypropyl (HDP)-tenofovir (CMX157) were evaluated due to their increased potency and better safety profile. In this project, a high performance liquid chromatography (HPLC) method for TAF was developed and qualified. Preformulation studies for TAF including solubility, hydrolytic, thermal, photolytic, and oxidative stability and preservative compatibility were conducted. TAF was found to be susceptible to hydrolytic degradation in acidic pH (pH≤4) and degraded when exposed in an aqueous solution to temperatures greater than 25°C. TAF hypotonic and isotonic enema formulations (1.76 mg/mL) were developed and characterized with respect to pH, osmolality, and drug content. One-week stability of these enemas was assessed in 40°C/75% RH, 30°C/65% RH and 25°C/60% RH environmental chambers. TAF enemas were stable for one week. In addition to the TAF enema, formulation efforts were initiated with CMX157. A preliminary HPLC method for CMX157 was developed. Prototype CMX157 enemas were prepared and pH and osmolality testing conducted. Additional formulation

optimization is required to enhance TAF stability in an enema format. Further investigation of the prototype CMX157 enema developed with respect to its physiochemical characteristics and stability is needed.

TABLE OF CONTENTS

PREFACE ..... **XV**

1.0 INTRODUCTION **1**

1.1 HIV TRANSMISSION THROUGH ANAL INTERCOURSE (AI) **1**

1.2 SEXUAL COLORECTAL HIV-1 TRANSMISSION **1**

1.3 PREVENTION OF HIV-1 SEXUAL TRANSMISSION--PRE-EXPOSURE PROPHYLAXIS (PREP) **3**

1.3.1 The Concept of PrEP **3**

1.3.2 Microbicides **4**

1.4 TENOFOVIR(TFV) AND ITS PRODRUGS **5**

1.4.1 Classification of Anti-HIV Medications **5**

1.4.2 Vaginally and Rectally Applied TFV Gel 6

1.5 TFV PRODRUGS 8

1.5.1 Designing Aim of TFV Prodrugs 8

1.5.2 Tenofovir Alafenamide Fumarate (TAF) 8

1.5.3 Hexadecyloxypropyl-Tenofovir (CMX157) 10

1.6 ENEMAS 11

1.6.1 Highly acceptable HIV prevention strategy among MSM 11

1.6.2 Rationale of a Safe Rectal Enema Design 13

1.7 SCOPE OF WORK 17

1.8 SPECIFIC AIMS 18

2.0 DESIGNING TAF ENEMAS FOR HIV PREVENTION 19

2.1 THE RATIONALE OF CONDUCTING PRE-FORMULATION STUDIES 19

2.2 MATERIALS & METHODS 22

2.2.1 High Performance Liquid Chromatography (HPLC) Method Development 22

2.2.2 Preparations of Solubility Medium and Buffers 22

2.2.3 Preparations of TAF stock solution 23

2.2.4 Hydrolytic Studies 23

2.2.5 Oxidative, Thermal and Photolytic Studies 23

2.2.6 TAF Enema Preparations 24

2.2.7 Preservative Compatibility 25

2.2.8 Statistical Analysis 25

2.3 RESULTS 26

2.3.1 HPLC Method 26

2.3.2 Solubility 28

2.3.3 Hydrolysis 30

2.3.4 Thermal and Photolytic Stability 33

2.3.5 Oxidation 35

2.3.6 Preservative Compatibility 37

2.3.7 One Week Stability 37

2.3.8 The Relationship of Temperature and Degradation Rate 43

2.4 DISCUSSON AND CONCLUSION 44

3.0 DESIGNING CMX157 ENEMAS FOR HIV PREVENTION 49

3.1 THE RATIONAL OF DESIGNING CMX157 ENEMAS 49

3.2 MATERIALS & METHODS 49

3.2.1 HPLC Development 49

3.2.2 CMX157 Enemas Formulas and Preparations 50

3.3 RESULTS 51

3.3.1 HPLC Method 51

3.3.2 CMX157 Enemas Characterizations 52

4.0 MAJOR SIGNIFICANCE, LIMITATIONS AND FUTURE DIRECTIONS 53

5.0 ACKONWLEDGEMENT 57

6.0 APPENDIX A 58

BIBLIOGRAPHY 60

LIST OF TABLES

Table 1 Formulations of TAF isotonic and hypotonic enemas 25

Table 2 Precision results of TAF validation for three days 26

Table 3 Solubility of TAF in different media 28

Table 4 Preservative compatibility. 37

Table 5 pH of hypotonic TAF enema 42

Table 6 pH of isotonic TAF enema 42

Table 7 Osmolality of hypotonic TAF enema 42

Table 8 Osmolality of isotonic TAF enema 43

Table 9 CMX157 gradient elution compositions 50

Table 10 Preliminary formulations of CMX157 isotonic and hypotonic enemas 50

Table 11 Precision results of CMX157 validation for three days 52

Table 12 pH of CMX157 enema..... 52

Table 13 Osmolality of CMX157 enema 52

LIST OF FIGURES

Figure 1 Chemical structures of (A) TFV; (B) TAF and (C) CMX157 11

Figure 2 Representative image of typical tenofovir enema 13

Figure 3 Cartoon of colon anatomy 15

Figure 4 Water and sodium ions absorption in rectum 17

Figure 5 Overlay of the HPLC chromatograms showing repeatability at the low QC level of TAF (4ug/ml). 27

Figure 6 Solubility of TAF at various pH. 29

Figure 7 Solubility of TAF at various ionic strength 30

Figure 8 Overlay of chromatograms of hydrolytic stability test in pH=2 buffer. 31

Figure 9 Hydrolysis in USP buffers (pH=2, pH=4) and 0.1M HCl 32

Figure 10 Linear correlation of hydrolysis peak area with time (h) (left) and TAF peak area at pre-determined time points subtracted by TAF peak area at 0h (mAU·min) (right). 33

Figure 11 Thermal stability study at 40°C and 60°C 34

Figure 12 Oxidation study by 0.03% and 3% H_2O_2 36

Figure 13 Drug recovery of isotonic and hypotonic TAF enemas in 40°C/75% RH environmental chamber 39

Figure 14 Drug recovery of isotonic and hypotonic TAF enemas in 30°C/65% RH environmental chamber 40

Figure 15 Drug recovery of isotonic and hypotonic TAF enemas in 25°C/60% RH environmental chamber 41

Figure 16 HPLC chromatograms overlay of 10 injections of low QC level of CMX157 (5µg/mL) 51

PREFACE

I would like to express my great gratitude to my supervisor Dr. Lisa C. Rohan for the guidance, patience and deep insights through the learning process of this master degree. Her guidance helped me in all the time of research and writing of this master thesis. I could not have imagined having a better advisor for my master study.

Furthermore, I would like to thank Dr. Samuel M. Poloyac and Dr. Vinayak Sant being my committee member, giving instructive suggestions on my thesis.

In addition, I would like to thank my lab mates for your kind help and support on my researches and master thesis. Especially, I am grateful to Taryn Serman for iterative editing this thesis and Mike De Miranda for sharing his work on tenofovir enema formulations.

I would like to thank my family: my parents Chunqin Wang, Guoping Lyu. Your support throughout my life is my motivation to move forward.

1.0 INTRODUCTION

1.1 HIV TRANSMISSION THROUGH ANAL INTERCOURSE (AI)

Although significant strides have been made in the field of HIV treatment and prevention since its initial discovery in 1959, the prevention of new infections continues to be a great challenge [1]. Approximately 2.3 million new HIV infections occur annually, meaning that an average of 6,300 people are newly infected each day [1]. Some groups of people are at higher risk of infection than others, with men who have sex with men (MSM) bearing the greatest burden [2]. Although MSM represent only 4% of the male population in the United States, MSM accounted for 78% of new HIV infections among males and 63% of all new infections in the U.S. in 2010 [2]. Likewise, MSM accounted for 54% of all Americans living with HIV in 2011 [2]. This population is consistently most affected by HIV due to the 18-fold increased risk of HIV transmission through anal intercourse (AI) in comparison to penile-vaginal intercourse. The risk of transmission is increased due to anatomical, physiological, immunological, and histological features of the colorectum [3]. As the practice of AI between both MSM and heterosexual couples has gained popularity, the need for the development of HIV preventative products has also risen.

1.2 SEXUAL COLORECTAL HIV-1 TRANSMISSION

The knowledge of how HIV transmission occurs in the genital and rectal tract is essential for the development of an efficient and safe anti-HIV prevention strategy. Peripheral blood mononuclear cells

(PBMCs), which include lymphocytes (T cells, B cells, natural killer cells), monocytes, and dendritic cells (DCs), are all target cells of HIV viruses [4]. HIV gradually destroys the immune system by binding and killing CD4+ T-cells, which play a central role in cell-mediated immunity [5]. HIV uses the machinery of the CD4+ T-cells to multiply and spread throughout the body [5]. Entry of HIV into the host cell is contingent upon the binding of viral glycoprotein gp120 to the CD4 molecules on the host cell surface, which act as receptors [6]. Additionally, binding to chemokine co-receptors CCR5 or CXCR4 on the host cell surface is required by virus [6]. Several barriers must be overcome for HIV-1 viruses to reach target cells in the genital and rectal tract [7]. Mucus is the first physical barrier that can prevent HIV-1 viruses from crossing mucosal epithelium [7]. Although stratified genital epithelial cells are not susceptible to HIV-1 infection and do not transcytose viral particles, these cells might be able to bind viral particles on their surface and thereby facilitate the infection of other cell types[7]. Although HIV can travel across the mucosal epithelial cell layers and enter the body on its own, damage to the epithelium can facilitate this process [8]. Damage to the epithelium often occurs through physical abrasion/trauma during sex, ulcerations, and inflammation [7]. Unlike the vaginal epithelium, which is composed of multiple layers, the rectal epithelium is only one cell thick and has large surface areas that provide easier access for HIV-1 to access underlying target cells [7]. Likewise, the neutral pH and minimum buffering capacity of rectal fluid provides little nonselective protection against pathogens including HIV[3]. Studies found that the gastrointestinal tract, including the rectum, is populated with HIV target cells such DCs and lymphocytes [9]. In addition, the rectum itself contains abundant microfold cells that are known to initiate mucosal immunity and allow for transport of microbes and particles across the epithelial cell layer from the gut lumen to the lamina propria, where interactions with immune cells can take place [7]. These physiological differences contribute to increased risk of HIV infection through anal intercourse compared to vaginal intercourse [10]. After the HIV virus transfers across the epithelial barrier into the underlying stromal tissues, which are populated with HIV target cells, HIV-localized

mucosal infection is established [7]. HIV viruses can further migrate to draining lymph nodes by migratory dendritic cells, resulting in systemic infection [7].

1.3 PREVENTION OF HIV-1 SEXUAL TRANSMISSION--PRE-EXPOSURE PROPHYLAXIS (PREP)

1.3.1 The Concept of PrEP

PrEP refers to daily medication taken by people at very high risk for HIV infection to lower their chances of infection [11]. To date, dosage forms expolored for administration of PrEP agents includes those involving the oral, injectable, or topical route of delivery. The Food and Drug Administration (FDA) has approved the oral product, Truvada® (Gilead Sciences, Inc., Foster city, CA), for PrEP. Truvada® consists of antiretroviral drugs emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF). An injectable form of PrEP, featuring a two-drug formulation of cabotegravir and rilpivirine, is currently in Phase III clinical trials, while a one drug formulation of cabotegravir has shown promise in HIV prevention among adult males in a Phase II clinical trial [12, 13]. In this thesis work, we focused on topical PrEP, which is also referred to as a "microbicide". Condom use is a well-known and effective strategy for prevention of sexually transmitted diseases (STDs) including HIV/AIDs. However, only 70% effectiveness was achieved with reported consistent use during penile AI among MSM as opposed to 80% among heterosexuals [14]. Studies also revealed that, out of the one-third of US heterosexuals who have engaged in AI, the majority reported that they do not use condoms during AI [15]. The Centers for Disease Control and Prevention (CDC) provided estimates of effectiveness for various strategies to prevent HIV transmission in 2015. They reported that Truvada

offered 92% and 90% effectiveness among MSM and heterosexuals, respectively [16]. So far, six clinical trials have demonstrated the tight relationship between PrEP HIV prevention effectiveness and patient adherence [17]. In other words, effectiveness declines if patients don't take or use PrEP medications regularly [18]. PrEP must be used consistently in order to achieve desired therapeutic effects.

1.3.2 Microbicides

HIV microbicides are prophylactic biomedical products that are self-administered to the rectum or vagina [19]. Microbicides have been developed in a variety of dosage forms, including tablets, gels, creams, films, rings. HIV microbicides are used to prevent the sexual transmission of HIV and give the user control due to the self-dosage [20]. The concept of microbicides was initially based on offering women protection under their own control [21]. Early development of microbicides focused on products that incorporated non-antiretroviral (non-ARV) medicines that have no specific mechanism of action [20]. These products can also work by inactivating pathogens, strengthening the body's natural defenses, or blocking viruses from getting into healthy cells [20]. For example, an early microbicide composed of polyanionic compounds prevented viral attachment to target cells by electrostatic interaction [20]. In addition to secreting medications, microbicide creams and gels also provide lubrication to physical barriers, which can decrease trauma to the mucosal epithelium and thus reduce the risk of HIV transmission [7, 20, 22]. Recently, the development of microbicides has been focused on microbicides containing highly potent and HIV-specific antiretrovirals (ARVs) that can be incorporated into distinctive dosage forms [20]. The CDC reported a 96% reduction in HIV transmission between HIV-positive men and women using antiretroviral therapy and their HIV-negative partners [16]. Therefore, incorporation of ARVs into microbicides is a promising endeavor.

1.4 TENOFOVIR (TFV) AND ITS PRODRUGS

1.4.1 Classification of Anti-HIV Medications

The life cycle of HIV virus offers several specific targets for ARVs. There are several classes of ARV microbicides that are currently under development. Two of these are entry inhibitors and reverse-transcriptase inhibitors [20]. Entry inhibitors can function in several ways. Some impede the binding of the HIV virus to CD4 receptors and CCR5/ CXCR4 coreceptors, thus blocking entry into target cells [7]. Others in this class of ARVs interact with gp120 on the HIV virus. In this project, we focus mainly on nucleotide/nucleoside reverse transcriptase inhibitors (NRTIs), which allosterically and irreversibly bind to reverse transcriptase and thereby prevent HIV replication [23]. Reverse transcriptase (RT) is a viral enzyme used to convert viral mRNA into double-stranded viral DNA, which can then be integrated in the host cell chromosome, allowing host replication of the virus [24]. Therefore, RT is crucial and necessary for HIV replication and spread [24].

NRTIs have been shown to be highly potent in blocking the action of RT. Therefore; this class of ARVs is relevant to microbicide development, as they can maintain sufficient anti-HIV activity throughout the interval between application to the vagina or rectum and the deposition of semen [7, 23]. Among this class, TFV is an acyclic nucleotide that rapidly converts to its active form, TFV-diphosphate (TFV-DP), from TFV monophosphate within cells [25-27]. The active form has a long terminal half-life of up to 17 hours, as it is not subject to intracellular deamination and deglycosylation [26]. TFV has already been approved by the FDA as an oral tablet called Viread® (Gilead Sciences, Inc., Foster city, CA). This tablet incorporates the prodrug version of TFV--TDF, which has higher bioavailability compared to TFV in the treatment of HIV infection and chronic hepatitis B virus infections. TDF is a fumaric acid salt of the bis-isopropoxycarbonyloxymethyl ester of TFV [26]. TDF is readily absorbed by

the gastrointestinal epithelial cells with an oral bioavailability of 25% [26]. Degradation of TDF to its monoester and subsequently to TFV occurs readily in the intestinal mucosa by the action of carboxylesterases and phosphodiesterases, respectively, following oral administration [26].

1.4.2 Vaginally and Rectally Applied TFV Gel

TFV-incorporated microbicides are being developed in many dosage forms, including rings, gels and films for prevention of HIV acquisition through vaginal intercourse. Only the vaginal gel dosage form has been reformulated for safe use in the rectal compartment. CAPRISA 004 provides the first proof of concept study that 1% TFV gel can reduce women's risk of HIV via vaginal sex [28]. This clinical study which focused on evaluating the effectiveness and safety of 1% TFV gel vaginally, showed an estimate of 39% reduction in HIV infection among 444 sexually active, HIV-uninfected 18- to 40-year-old women in South Africa [23]. This study also reported that HIV incidence was 54% lower among high adherers in the TFV gel arm than the placebo gel arm [23]. Another clinical study assessed the safety and tolerability demonstrated by 1% TFV gel used vaginally twice daily and found the gel to be well tolerated, with only mild adverse events observed in the majority of women users [22]. However, this promising TFV vaginal gel cannot be used for rectal application due to its hyperosmolality, which can result in damage to the rectal epithelium [29]. Therefore, further investigation was put into development of a TFV gel that could be rectally applied [3, 29].

Hyperosmolality not only provides HIV with more direct access to CD4 target cells by breaking down the epithelium, but it also causes colonic inflammation due to enhanced luminal permeability [29, 30]. This may lead to poor acceptability among users. A Phase I clinical study (MTN-006) investigating safety and acceptability of vaginal TFV gel when applied rectally reported poor acceptability and some safety concerns [31]. A reduced glycerin TFV gel (RG-TFV gel) with similar overall characteristics

except for osmolality and spreadability/firmness was formulated. In the MTN-007 study this RG-TFV gel showed a 73% lower osmolality, a 29.6% increase in spreadability, and a 27% decrease in firmness compared with vaginal TFV gel [29]. For polarized colorectal and ectocervical tissue, the colorectal and ectocervical epithelium was intact after exposure to RG-TFV gel, in contrast to fractured or sloughed off epithelium after exposure to the vaginal TFV gel [29]. Efficacy testing conducted on tissue explants showed a $3\log_{10}$ decrease and a ≥ 1.5 $\log_{10}$ decrease in HIV-1 p24 release after dosing with TFV gel in colorectal and ectocervical explants, respectively [29]. This suggests that the RG-TFV gel doesn't compromise the effectiveness against HIV infection compared with vaginal TFV gel [29]. In summary, RG-TFV gel, possessing near physiological osmolality, showed improved epithelial integrity and maintained anti-HIV activity equal to vaginal TFV gel [29]. The acidic pH of this formulation remains a potential issue for RG-TFV gel rectal application as it may cause some discomfort associated with adherence, although it is unclear whether frequent rectal use of acidic pH products will impact the gastrointestinal tract in terms of epithelial integrity [29]. This pre-clinical investigation of RG-TFV gel suggests that an isotonic osmolality and a pH value suitable to local environment may be preferable in terms of rectal microbicide product development [29].

Subsequently, TFV rectal specific gel and liquid products were formulated with a neutral pH and slightly above isoosmolality [32]. These formulations retained resected normal human colonic tissue viability and maintained tissue architecture after exposure to HIV-1 [32]. It was also suggested that since it was intended that the liquid products would be ‘‘enema-like’’ and deliver drug further into the rectal compartment, this kind of product would easily spread over the mucosal surface which could provide more protection than viscous gels that require shear forces to spread [32].

1.5 TFV PRODRUGS

1.5.1 Designing Aim of TFV Prodrugs

As introduced previously, some microbicides being developed incorporate anti-HIV pharmaceutical agents that were originally introduced for the treatment of HIV. TFV has high potency both *in vitro* and *in vivo* due to its long intracellular half-life [33]. However, as TFV is a di-anion at physiological pH, it has poor cellular permeability, thus decreasing its bioavailability [33]. Additionally, TFV, which contains a phosphonate moiety, is the substrate of organic anion transporter and rapidly eliminated through proximal tubule cells by efflux transporters such as multidrug resistance-associated proteins [33]. This elimination results in accumulation of TFV in proximal tubule cells due to drug-drug interaction thereby causing renal toxicity [33, 34].

TDF, synthesized as an oral prodrug of TFV, exhibits five-fold higher intracellular levels of TFV after oral administration compared with equivalent TFV injected subcutaneously [33]. This greater intracellular concentration achieved by TDF resulted in a three-fold decrease of HIV viral load in a human clinical trial [33]. However, TDF was designed to release TFV in plasma and has been shown to metabolize 170-fold faster in plasma than in target cells, resulting in a nephrotoxicity issue [33]. Therefore, new classes of ARVs that can circulate systemically as prodrugs and undergo rapid conversion to TFV intracellularly have attracted attention [33].

1.5.2 Tenofovir Alafenamide Fumarate (TAF)

Tenofovir alafenamide fumarate (GS-7340 or TAF) is one of the stereoisomers synthesized from (R)-PMPA (TFV) and (L)-isopropyl alanine ester [33]. Preclinical studies have demonstrated a 200-fold

increase in plasma stability (circulating as prodrug) and a 400-fold increase in accumulation of TFV and its active form (TFV-DP) in lymphatic tissues and PBMCs compared to TDF [26]. TAF has showed a 1000-fold increase in potency *in vitro* over TFV and has shown antiretroviral activity against both wild type and drug-resistant HIV-1 strains with excellent *in vivo* pharmacokinetic properties [26, 27]. The preferential distribution into PBMCs and other lymphatic tissues are hypothesized to be attributed to the increased metabolic activity of these tissues [33]. In addition, by masking the phosphonate group present in the TFV structure, TAF is more hydrophobic than TFV. Therefore, TAF can achieve more cellular uptake as compared to TFV. However, the detailed mechanism for the selective intracellular targeting of TAF is not fully understood [33]. Following rapid target cell uptake, TAF is hydrolyzed at the carboxyl ester bond in lysosomes by the cathepsin A, the serine and cysteine proteases [26]. Then, the unstable product formed during the first-step hydrolysis spontaneously releases phenol by intramolecular cyclization and hydrolysis to a negatively charged, cell impermeable TFV-alanine intermediate. This intermediate is metabolized to parent TFV followed by downstream phosphorylation to TFV-DP [26]. A Phase II clinical study comparing darunavir/cobicistat/emtricitabine/TAF (D/C/F/TAF) and darunavir/cobicistat/emtricitabine/TDF (D/C/F/TDF), all ARVs in HIV-infected treatment-naïve adults, showed orally administered D/C/F/TAF resulted in a 6.5-fold higher TFV-DP exposure in PBMCs with 91% reduced TFV exposure (AUCtau) in plasma than D/C/F/TDF [35]. Patients dosed with D/C/F/TAF showed a smaller increase in serum creatinine, significantly less renal tubular proteinuria, and less change in BMD at both hip and spine compared to patients treated with D/C/E/TDF [35]. All of these studies demonstrate that TAF has improved the bone and renal profile and has increased antiviral activity compared with TDF. Another clinical study comparing TDF 300mg with TAF 50mg and 150mg, respectively, showed TAF at both doses had a significant decrease in HIV-1 RNA [36]. Viral loads were reduced -1.71-log and -1.57-log for 150 mg and 50 mg doses, respectively, compared to 0.94-log for TDF

at day 14 [26]. Phase III studies evaluating D/C/F/TAF versus D/C/F/TDF in treatment-naive adults are ongoing.

1.5.3 Hexadecyloxypropyl-Tenofovir (CMX157)

CMX157, a hexadecyloxypropyl (HDP) prodrug, is a lipid conjugation to TFV that mimics the natural lipid uptake pathway. Similar to TAF, it is not cleaved in the plasma by nonspecific esterase. As it is not a substrate of human organic anion transporters correlated to TFV-induced nephrotoxicity, it has decreased renal excretion and increased intracellular drug exposure compared to TDF and Tenofovir [26, 37]. Free TFV is released intracellularly by hydrolysis to remove HDP lipid by phospholipases [26]. There is also a hypothesized mechanism that CMX157 may hitchhike on the HIV virus by inserting its lipid conjugate into the viral envelope, thus achieving an enhanced intracellular delivery. *In vitro* studies reported that CMX157 is >200-fold more potent than TFV against wild type as well as all major HIV subtypes resistant to current therapies [26]. The amount of TFV and its active form after exposure to CMX157 that accumulated in human PBMCs was shown to be much higher than those accumulated after exposure to TFV [38]. CMX157 has no apparent toxicity when given orally to rats for 7 days at doses of 10, 30, or 100 mg/kg/day [34]. A Phase I clinical study comparing healthy volunteers receiving a single 25mg-400mg dose of CMX157 to healthy volunteers receiving a standard dose of Viread® reported that CMX157 was well tolerated and caused no adverse event trends [37]. TFV -DP was measurable in PBMCs from all patients following a single 400 mg dose of CMX157 [37]. In fact, a once-weekly dosing regimen of CMX157 was recommended after PBMC levels of TFV--DP remained detectable for six days after the ingle 400mg dose [37].

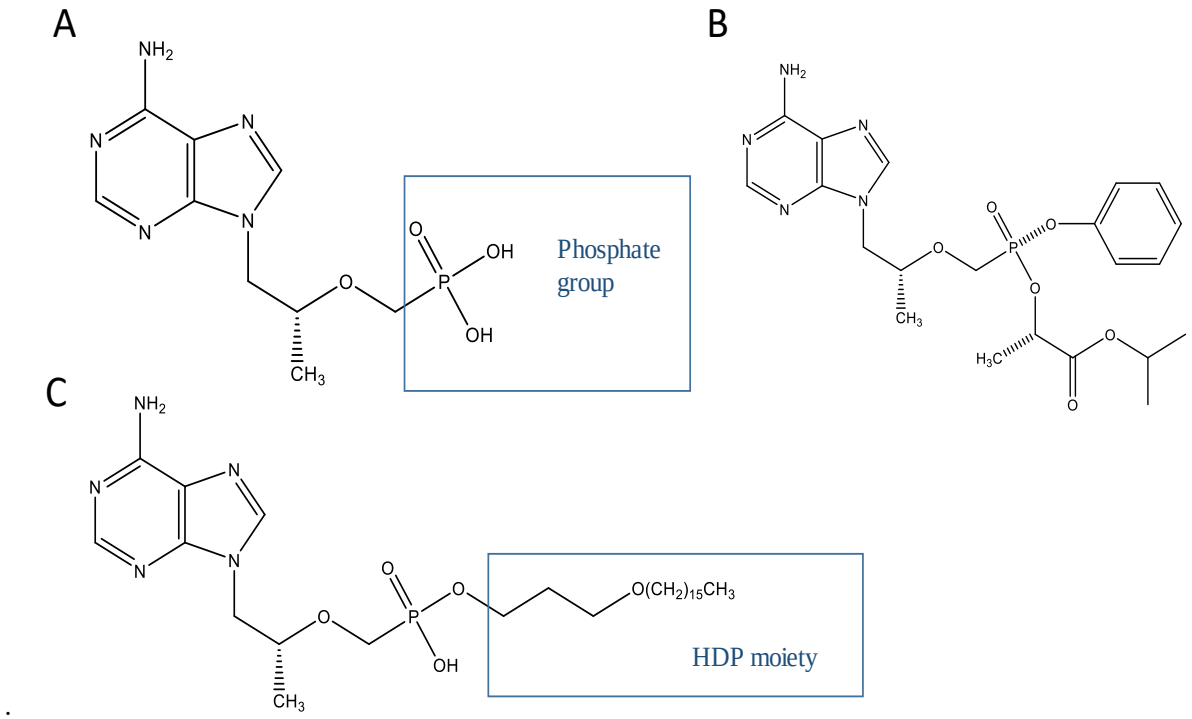

Figure 1 Chemical structures of (A) TFV; (B) TAF and (C) CMX157

Picture by ChemDraw®

1.6 ENEMAS

1.6.1 Highly Acceptable HIV Prevention Strategy among MSM

Enemas, also referred to as rectal douches (Figure 2), are liquid treatments, which is a insertion of fluid into the anus, for medical or hygienic reasons [39]. The main purpose of most commonly used enemas is to help relieve severe constipation or for bowel cleansing, as in the Fleet® enema (Fleet laboratories, Lynchburg, VA) [40]. However, depending on the dissolved active agents, enemas can also

be used to treat diseased rectum, sigmoid, and descending colon [41]. Indications for these enemas include Crohn's disease and ulcerative colitis, which can be treated using a betnesol retention enema [41]. Enemas, depending on their indication, have constituents like saline or phosphate buffer, and some may contain active pharmaceutical ingredients (APIs). Enemas are delivered using a device that can utilize either a nozzle design or spray pattern [42].

Enemas are widely used and highly acceptable among MSM. According to an international survey, among 1339 respondents representing 112 countries, 66% reported they have used rectal douching, among whom, 83% reported high frequency of use before anal intercourse [43]. A study enrolling 105 MSM (78 HIV-negative, 27 HIV-positive) who had unprotected receptive anal intercourse (URAI) in the previous year, 53% of HIV-negative and 96% of HIV-positive men douched in preparation for sex [44]. This popularity of douching before sex among MSM makes it a potential high-compliance dosage form. It should be noted that enemas may cause trauma to the rectum which can increase the risk for HIV and other STD's. There are some behavior studies investigating why men who are engaged in AI douch before or after sex. Studies attributed this behavior preference to the desire for cleanliness prior to sex.

With respect to applying enemas as rectal microbicides, these products if formulated appropriately may decrease the shear during intercourse, possibly providing protection against micro-trauma during intercourse, which provides the HIV virus easier access into the body [3]. Additionally, the large percentage of water in enemas may enhance drug coverage of a sufficient surface area of the colorectum, which could provide potential advantages against HIV transmission, considering the natural poor wetting conditions of the colorectum [3]. However, currently marketed lubricant or rectal douches are hyperosmolar, a trait that may compromise the integrity of colorectal epithelium, leading to increased risk of HIV transmission [45]. Therefore, a safe rectal douche with anti-HIV effects is in urgent need.

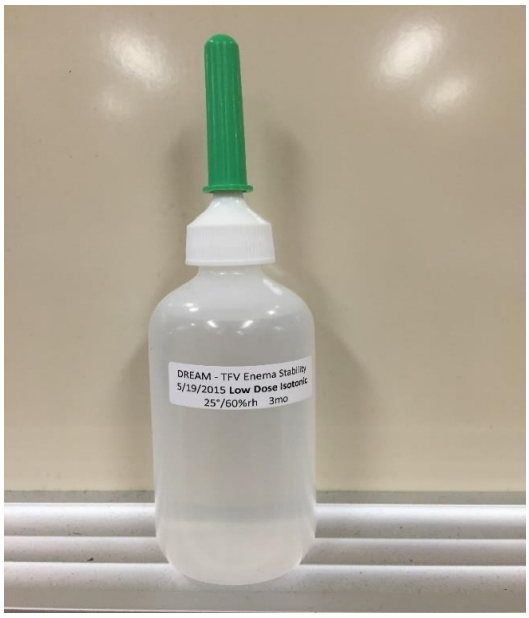

Figure 2 Representative image of typical tenofovir enema

1.6.2 Rationale of a Safe Rectal Enema Design

A successful, rational design for a rectal microbicide relies on the understanding of HIV transmission pathology (distribution and clearance) in the colorectum. In a study conducted on adult males with a history of receptive AI, SPECT/CT imaging showed that cell-free HIV distribution can reach the rectosigmoid colon (Figure 3) and the maximum distribution occurred between 4 and 24 hours [46]. In order to cover all possible temporal distributions of HIV seen in their study, they suggested a microbicide product should be able to distribute into the sigmoid colon and be sustained for a minimum of 24 hours[46]. Findings by the Hendrix group from Johns Hopkins University concluded that a microbicide should cover all the colonic locations during the periods of HIV exposure in order to achieve better efficacy [47]. As discussed previously, the hyperosmolality of enemas has been reported to cause epithelial damage, which can potentially exacerbate HIV transmission [48]. In the absence of an

accessible rectal microbicide, many users tend to use tap water, which is extremely hyposmolar and has been reported to cause epithelial loss [48]. It is crucial to define the osmolality range with a desired distribution profile for rectal application of microbicide enemas. A study conducted by the Hendrix group assessing the distribution, safety, and acceptability of isoosmolar enemas (Normosol™-R, 295mosm/kg) as opposed to hyperosmolar enemas (Fleet®) and tap water showed that isoosmolar enemas had a better proximal colonic distribution (up to splenic flexure). Additionally, this study found that hyperosmolar enemas exhibited sloughing of colon epithelium, as predicted [48]. Hyposmolar enemas exhibited an intermediate distribution between these isoosmolar and hyperosmolar enemas. The pH and buffering capacity in the colorectum are two important parameters that should be taken into consideration when developing rectal microbicides. Extreme pH values may lead to irritation and discomfort, both of which potentially impact user adherence and cause adverse effects [3]. It is advisable to develop a rectal microbicide with a pH range of 7-8, although the effects of pH change to the normal rectal fluid pH on epithelium integrity and HIV transmission is unclear [32].

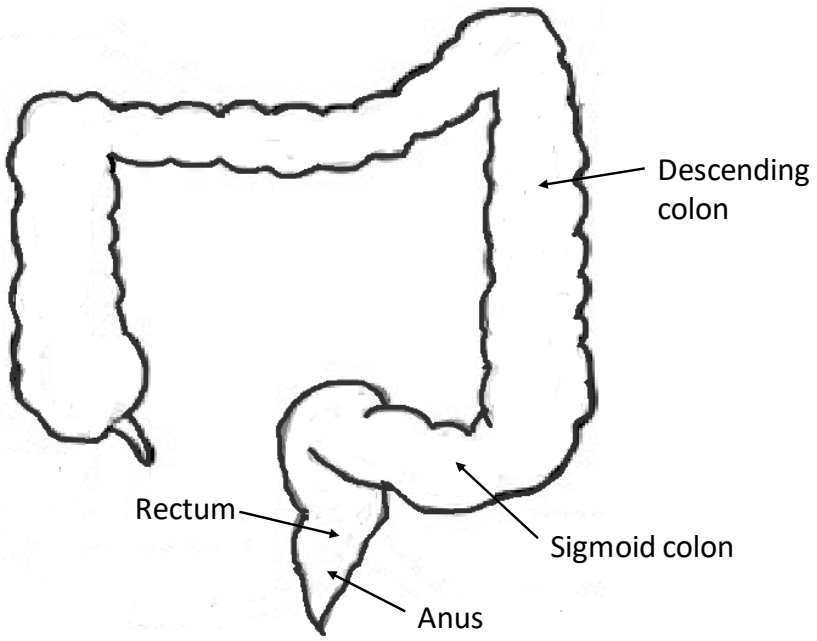

Figure 3 Cartoon of colon anatomy

Previous studies have revealed that by use of an osmotic gradient for fluid absorption and secretion, vaginal drug delivery could be improved to reach the entire epithelial surface, including the deep folds penetrating through the mucus layer [49]. Likewise, an osmotic gradient should be employed in enema development to achieve similar delivery. A study conducted by Johns Hopkins University in the mouse model found that a sodium-based, moderately hypotonic enema containing TFV was able to achieve high local tissue levels of the drug with minimal systemic exposure while providing enhanced local drug retention[50]. It was also found that colorectal tissue coverage was enhanced by hypotonic, absorption-inducing enemas (0.9% saline and deionized water) and bulk fluid absorption only occurred when Na+ was present [50]. This conclusion was based on the physiology of colorectum, since sodium

ion absorption ubiquitous across the human colon and is directly related to water. Water absorption is passive in the colon and is promoted by the osmotic pressure produced by sodium chloride absorption [51].Sodium and chloride absorption can occur without water absorption, due to passive diffusion [52]. Sodium ions passively diffuse along the favorable electrochemical gradient on the apical side of colonocytes and are actively transported on the basolateral membrane of colonocytes [51]. It has also been demonstrated by this study that moderately hypotonic simulated colon solution (SCS, 150msm/kg) and near isotonic tris buffered saline (TBS, 450mOsm/kg) didn't have any detected systemic drug absorption 30mins after administration [50]. But SCS provided optimal tissue drug levels compared with tap water, Fleet® enema and near isotonic TBS [50]. These findings suggest that hypotonic enemas induce water absorption, which may transport drug by bulk fluid flow to epithelial tissue, while remaining mild enough to reach systemic circulation [50]. In terms of epithelial toxicity, tap water and SCS did not induce any detected epithelial damage [50]. Another study assessing the distribution, toxicity, and acceptability of three different types of enema of varying osmolarity in healthy MSM showed that Normosol™-R, an isotonic solution of balanced electrolytes in water for injection, has the greatest luminal and tissue concentrations of radiolabeled enemas with plasma concentrations (AUC and Cmax) lower than distilled water enemas and increased proximal colon distribution (up to splenic flexure) [48]. Normosol™-R also exhibited the highest degree of dose retention compared to fluid-absorption inducing solutions in terms of both luminal and tissue concentrations [48]. As SCS was not used in the study, it is hard to determine whether SCS has a more favorable profile in terms of local and systemic drug concentrations and dose retention as compared to Normosal™-R.

In summary, it is suggested that slightly hypotonic enemas containing sodium ions are able to achieve the optimal luminal & colonic tissue drug distribution and local tissue drug level with minimal systemic drug exposure.

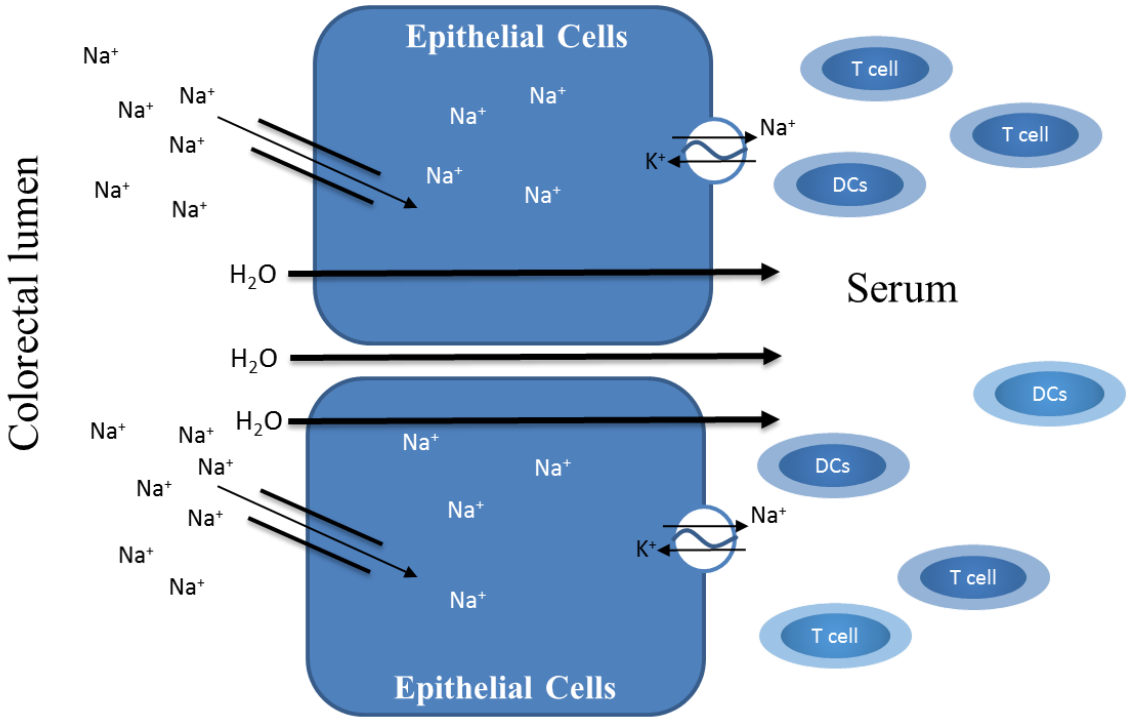

Figure 4 Water and sodium ions absorption in rectum

1.7 SCOPE OF WORK

Rectal enema products containing TFV and its prodrugs may be a promising means to prevent HIV sexual transmission. In the NIAID funded Development of a Rectal Enema as Microbicide (DREAM) Program (Grant # U19AI113127), it is hypothesized that a moderately hypotonic enema incorporating prodrugs of TFV could achieve sufficient distribution and amount of the API in colorectal tissue, with minimal systemic drug exposure. To this end, TFV enema products were developed in our lab. For this TFV enema product, a dose of 220mg/125mL was selected to achieve the same colon tissue

CD4+ cells TFV-DP concentration as a TFV 1% rectal gel dose. Utilization of this dosing level allows for comparison of the 1% TFV gel to the TFV enema formulation. TFV enemas were formulated with isotonic and hypotonic osmolality for comparison of the effect of osmolality on TFV-DP tissue concentration in animal studies. The TFV enemas developed were shown to be stable for over one year. This TFV enema formulation served as the basis for formulation of TFV prodrug enemas described in this thesis.

Specifically, this thesis work focused on developing analytical tools for the analysis of TAF and CMX157, pre-formulation work on stability of TAF under varying conditions, and formulation work to incorporate TAF and CMX157 into buffers within target pH and osmolality. Since little information is available in the literature regarding the physiochemical characteristics of TFV prodrugs, TAF and CMX157, we designed a series of preformulation studies to explore their physiochemical characteristics.

1.8 SPECIFIC AIMS

There are three aims in this thesis project.

Specific Aim 1: Identify degradation pathways for TAF by performing preformation studies under selected conditions

Specific Aim 2: Formulation development of TAF enemas and characterizations of pH, osmolality and drug content

Specific Aim 3: Formulation development of CMX157 enemas and characterizations of pH, osmolality and drug content

2.0 DESIGNING TAF ENEMAS FOR HIV PREVENTION

2.1 THE RATIONALE OF CONDUCTING PREFORMULATION STUDIES

Solubility, the dissolution of solute in solvent to yield a homogenous system, is an important parameter used to achieve the desired concentration of drug for the anticipated pharmacological response [53]. For any drug to be absorbed, the drug must be in solution at the site of absorption [53]. Determination of drug candidate solubility typically occurs during the preformulation stage of pharmaceutical product development. Solubility is also very important in drug discovery and formulation development phases [54]. There are two commonly used terms for solubility: kinetic solubility and intrinsic solubility [54]. Kinetic solubility is the concentration of a compound at the time when an induced precipitate first appears in the solution. It is often investigated in the early phase of drug discovery [54]. On the other hand, intrinsic solubility refers to the maximum quantity of the substance that can be completely dissolved at a given temperature and pressure in a given amount of solvent. This measure is thermodynamically valid as long as a solid phase is in equilibrium with the solution phase [55]. In terms of drug development, the intrinsic solubility is considered more important [54]. To note, the term intrinsic solubility is used to describe the solubility of the un-ionized form in the absence of acid or alkali as pH affects solubility of ionizable compound. Intrinsic solubility is the concentration of a compound in a saturated solution when excess solid is present, and solution and solid are at equilibrium [56].

There are many methods to determine intrinsic solubility, but the saturation shake-flask method is considered to be the classical method. This method uses a sample that is typically prepared by adding an excess amount of solid to the solubility medium to produce a suspension [57]. With an agitation rate adequate to prevent particle agglomeration and to ensure particle contact with the diluent, samples often reach equilibrium quickly (within 24 h) [57]. It is generally accepted that equilibrium has been reached

when multiple samples, assayed after different equilibration time periods, yield the same result for apparent solubility [57]. Currently, there is no standardized way to conduct the shake flask method for determining solubility [54]; therefore, one may find varied stirring and sedimentation times and techniques for collecting aliquots in published papers [58]. These factors all account for the variability among solubility determinations. This may explain why reported solubility of a drug molecule can vary amongst different developers. Besides this, solubility determination among various compounds is affected by other factors including particle size, crystallinity, and molecular features [58]. In our solubility studies, we utilized 1h sonication and room temperature rotation to reach equilibrium up until 24h. This method was applied to promote saturation of TAF in the solubility medium. In addition to procedural effects on the determination of solubility, intrinsic factors must be taken into account when conducting a thorough solubility study. The effects of pH and ionic strength on the solubility of ionizable compounds are extensively examined phenomenon [54]. As TAF is ionizable, solubility experiments with consideration of pH and ionic strength of the solubilizing medium were conducted.

Chemical stability of pharmaceutical agents is highly important for developing a successful formulation, determining appropriate packaging, and indicating proper storage conditions and shelf-life[58]. U.S. Food and Drug Administration (FDA) and the International Council for Harmonisation (ICH) require stability test data, which indicate the quality of the product and stability over time under various environmental factors. Stability testing can be divided into two main parts: forced degradation studies (stress testing) and accelerated degradation studies [58]. Forced degradation studies of novel drug substances products are essential to help develop and demonstrate the specificity and sensitivity of the analytical tools [59]. In addition to demonstrating specificity, forced degradation studies can be used to determine the degradation pathways and products of the active pharmaceutical ingredients (APIs) that could form during storage, and facilitate formulation development, manufacturing, and packaging [59]. For forced degradation studies, conditions beyond temperature, including photolysis, low and high pH,

and oxidation are typically examined. For accelerated conditions, temperature is the most common factor because its relationship with the degradation rate is defined by the Arrhenius equation [60]. In accelerated stability tests, a product is stored at elevated stress conditions [60]. The real-time stability, during which a product is stored at the recommended storage conditions and monitored can be predicted using known relationships between the acceleration factor and the degradation rate [60]. However, the guidelines on conducting preformulation studies, and what conditions to test, are not strictly defined due to variation between drug products [58]. Therefore, designing preformulation studies that seek to determine intrinsic stability must be based on the API's chemical structure and anticipated molecular behaviors.

As stability data should be able to indicate possible degradation during manufacturing and under storage conditions, chosen stress conditions must be indicative of the degradation mechanism of action under real conditions [58]. Some stressed conditions are considered necessary for an overall pre-formulation study: acid and base hydrolysis, thermal degradation, photolysis, and oxidation. Additionally, freeze–thaw and shear are often tested [58]. Selected conditions should force degradation of the drug substance between 5% to 20% in order to validate a stability indicating method [58]. However, a forced degradation study does not have to induce a degradation product [58]. Stability testing can be done over a short period of time under harsh conditions or labile conditions [58]. Optimization of stress conditions is required, as anticipated conditions may not degrade the drug experimentally [58].

The preservatives sodium methylparaben and sodium propylparaben were used in TAF enema formulation to inhibit bacterial growth. These two preservatives are commonly used in rectal enema, liquid, solution or suspension formulations and have adequate solubility in water [61]. The sodium methylparaben and sodium propylparaben preservative concentrations in TAF enemas were at 0.18% and 0.02% respectively in accordance to the guideline and our previous experience with these preservatives [61].

2.2 MATERIALS & METHODS

2.2.1 High Performance Liquid Chromatography (HPLC) Method Development

The HPLC method was developed according to an assay used to analyze metabolites of TAF[62]. TAF was analyzed on a ZORBAX Agilent (Agilent Technologies, Santa Clara, CA) XDB-C18 column (3.5 μm, 100 ×4.6 mm) using the Waters (Milford, MA) HPLC system connected to a diode array detector (Waters 2996 photodiode Array). An isocratic elution was utilized by mobile phase A (5% Acetonitrile in 25mM phosphate buffer and 5mM *t*-butyl ammonium bromide, pH=6) and B (60% Acetonitrile in 25mM phosphate buffer and 5mM *t*-butyl ammonium bromide, pH=6) for 8 min at a flow rate of 2mL/min at ambient temperature in auto-sampler. The UV detector has a PDA setting of 210nm~400nm at 1.2nm. TAF was detectable at the wavelength of 260nm. Sample injection volume was 10μL. TAF was identified by comparison with TFV retention time (t_R=3.7min). The method was then validated by examining precision, accuracy, linearity, and challenged at various pH and oxidative conditions. The precision was performed by testing samples of three concentrations, with 10 injections for each concentration on each day for three days. Samples were diluted by 25% acetonitrile before analysis within validated linearity range and then analyzed on HPLC.

2.2.2 Preparations of Solubility Medium and Buffers

Standard buffer solutions with pH values of 2, 4, 5, 6, 7, and 8 were prepared according to United States Pharmacopeia (USP 26). For the pH-dependent solubility profile, USP citrate and phosphate buffers ranging from pH 4-8 were utilized. The ionic strength-solubility profile was assessed using serial dilutions of purchased 10X PBS (MediaTek, Inc., Hsinchu, Taiwan) ranging from 0.5X—5X PBS. TAF

solubility was tested in varying pH buffers, PBS, water and normal saline. TAF was well suspended in media and rotated for 24h. After rotation, suspension was centrifuged and filtered through a 0.22μm filter. Aliquots was diluted within validation range of the validated HPLC method and assayed on HPLC.

2.2.3 Preparations of TAF Stock Solution

TAF was dissolved in 100% acetonitrile at a concentration of 7mg/mL

2.2.4 Hydrolytic Studies

Hydrolytic studies were conducted in 0.1M hydrochloric acid (HCl, pH=1) and 0.1M sodium hydroxide NaOH, pH=11) USP buffers. The pH of each buffer was measured using an accumet XL600 dual channel pH meter (Fisher Scientific, Pittsburgh, PA), before and after addition of TAF stock solution. Diluents from stock solution were prepared. Samples were stored at room temperature until 24h. 100μL aliquots of each sample were collected and analyzed for drug content at predetermined time points: 0h, 2h, 4h, 6h, 8h, and 24h. Acid and base controls without API were assayed by HPLC at each time point to ensure that change in acid or base would not affect potential TAF degradation. An API control was also performed and assayed at each time point.

2.2.5 Oxidative, Thermal and Photolytic Studies

The same stock solution and dilution procedure was used for oxidative, thermal, and photolytic studies. For oxidative studies, 3% and 0.03% hydrogen peroxide (H_2O_2) was diluted from 30% H_2O_2 (Fisher Scientific, Pittsburgh, PA) and 3% H_2O_2 (Target, Minneapolis, MN) respectively with water. TAF

diluents in 3% H_2O_2 were assayed at predetermined time points: 0h, 2h, 4h, 6h and 24h. At 0.03% H_2O_2, 100µL aliquots were taken and assayed at predetermined time points: 0h, 2h, 4h, 6h, 24h, and 72h.

For thermal studies, TAF stock solution prepared was diluted in water and diluents were placed in 40°C and 60°C water baths, 100µL aliquots were taken on predetermined time points: 0h, 2h, 4h, 6h, 8h, 24h, 72h and assayed immediately on HPLC.

For photolytic studies, TAF was dissolved directly in water and well sealed in 2ml plastic vials; All the samples were stored in a 25°C/60% relative humidity environmental chamber (Caron 6010, Caron Products & Services, Inc.). Aliquots were taken on time zero and 72h.

2.2.6 TAF Enema Preparations

Isotonic and hypotonic 1.76mg/ml TAF enemas were prepared according to the formulas (Table 1), 100mL purified water was weighed into a 250mL beaker, and stirred for 5mins, followed by the addition of sodium chloride, sodium parabens, and TAF. The solution was then sonicated until all components were dissolved. After sonication, 5% hydrochloric acid (HCl) diluted (Fisher Scientific, Pittsburgh, PA) was added to the solution, with pH measurement. The osmolarity and drug content were then measured immediately after preparation. The prepared TAF enema was separated and placed in three different environmental chambers (Caron 6010, Caron Products & Services, Inc.): 25°C ± 2°C/60% ± 5% RH, intermediate 30°C ± 2°C/65% ± 5% RH, and accelerated 40°C ± 2°C/75% ± 5% RH, as described previously. Aliquots were taken to measure for pH, osmolality, and drug content at day 0, day1, day2, day3, day5 and day7. The instruments used for osmolarity were a Model 3320 osmometer (Advanced Instruments, Inc., Norwood, MA).

Table 1 Formulations of TAF isotonic and hypotonic enemas

Component	Hypotonic TAF enema Amount Per Batch	Isotonic TAF enema Amount Per Batch	Function
TAF	176mg	176mg	**API**
Sodium Methylparaben	0.18g	0.18g	**Preservative**
Sodium Propylparaben	0.02g	0.02g	**Preservative**
NaCl, USP	0.370g	0.793g	**Osmogen**
5% HCl Diluted from 37% HCl	610µL	610µL	**pH modifier**
Purified Water	100mL or 100g	100mL or 100g	**Solvent**

2.2.7 Preservative Compatibility

Three independent, isotonic TAF enemas (1.76mg/ml) were prepared with no addition of preservatives. Aliquots were taken to measure pH and drug content. Following this, the preservatives were added and assayed. Monitoring drug content results assessed the stability of TAF as an indicator of compatibility.

2.2.8 Statistical Analysis

All results are presented as the mean ± standard deviation (SD). Pairwise or unpairwise differences were determined by Student's *t* test. One-way ANOVA with Bonferroni post-hoc test was conducted comparing more than two groups. A p value <0.05 was considered statistically significant. In some of the forced degradation studies and stability studies, drug concentration determined over time is reported as TAF percent recovery (% of time zero) ± SD.

2.3 RESULTS

2.3.1 HPLC Method

As part of the preformulation studies, an HPLC analytical method was developed and validated. The retention time of TAF was observed at 3.73±0.08 minutes. The limit of detection (LOD) was estimated to be 0.17 µg/mL, and the limit of quantification (LOQ) was 0.5µg/mL based on signal-to-noise. A linear standard curve was obtained over a range of 0.5-150µg/Ml. No TAF degradants were detected during the analysis. The perfect overlay among the 10 TAF chromatograms affirms the repeatability of samples at the lowest quality control (QC) level (4ug/mL) (Figure 5). This developed HPLC method was applied to quantify solubility of TAF in different solvents and drug concentrations in samples collected throughout the stability studies. The regression coefficients of all calibration curves were greater than 0.999 in three days. The coefficient of variation (CV), reported as relative standard deviation (RSD), is as below (Table 2):

Table 2 Precision results of TAF validation for three days

RSD (%)	Low Conc.	Middle Conc.	High Conc.
Precision	3.82%	4.23%	3.66%

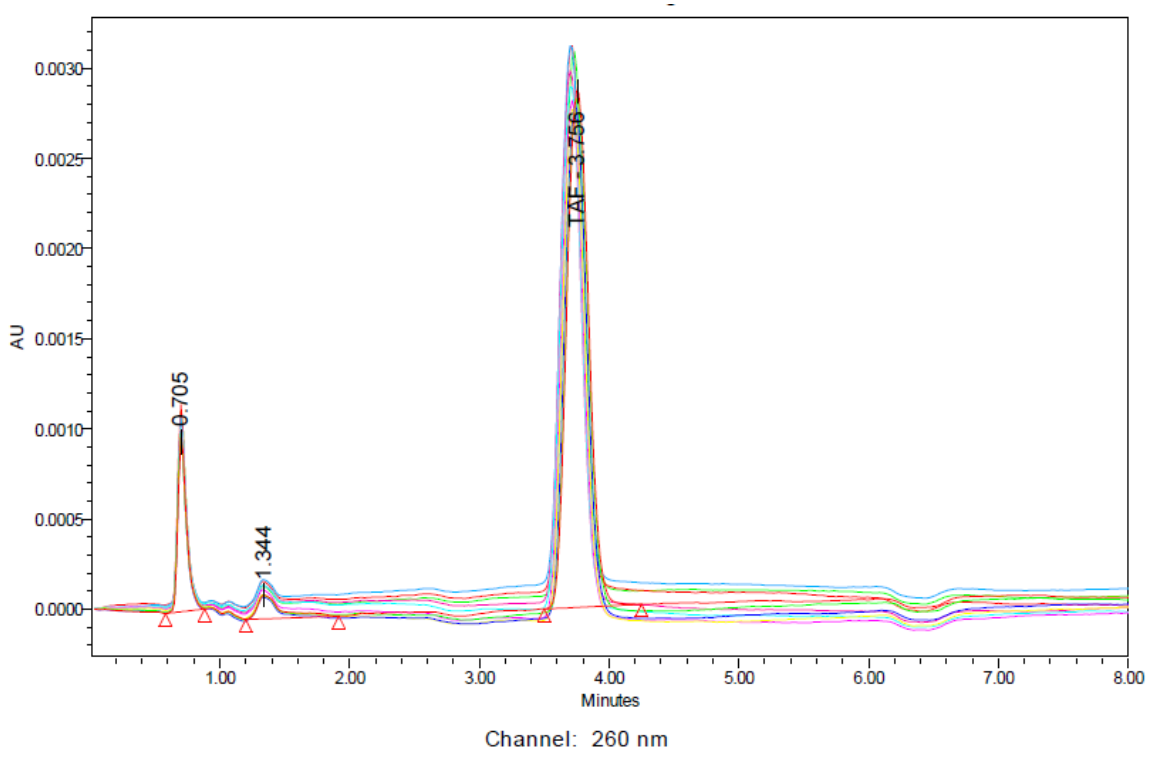

Figure 5 Overlay of the HPLC chromatograms showing repeatability at the low QC level of TAF (4ug/ml).

10 injections of each QC level were made on each day of three days. RSD was 0.981% for injections shown above. TAF peaks were consistent on each day.

2.3.2 Solubility

Saline (0.9% sodium chloride solution) and phosphate buffer solution (PBS) were used as solubility mediums since both are commonly used buffers in solubility studies and possess a neutral pH and desirable osmolarity. Based on TAF's ionizable features and the hydrophilicity of TFV, high solubility in these buffers was expected.

According to the pKa calculated by ChemAxon®, the pKa of TAF is 5.12 and 11.36 in its acidic and basic form, respectively. At neutral pH, TAF is both negatively and positively charged, with a net charge of zero. The compound in its non-ionized form always has a minimal solubility, whereas the charge on the compound increases its solubility. The charge on TAF is dependent on varying pH, and therefore affects solubility. As TAF is an ionizable compound, its solubility was predicted to be impacted by ionic strength as well.

TAF solubility in different buffers is indicated below (Table 3). TAF solubility decreased linearly with increasing pH (4-8) (Figure 6) and increasing ionic strength (150-1500 mosm/kg). (Figure 7)

Table 3 Solubility of TAF in different media

Solubility Medium	Equilibrium solubility (mg/ml)
Water	7.61 ± 0.42
1XPBS	5.06 ± 0.11
0.9% Saline	7.95 ± 0.70

TAF was well suspended in media, rotated for 24h, centrifuged and then filtered by 0.22μm filters. The data represents the mean ± SD (n=3) of triplicates determinations.

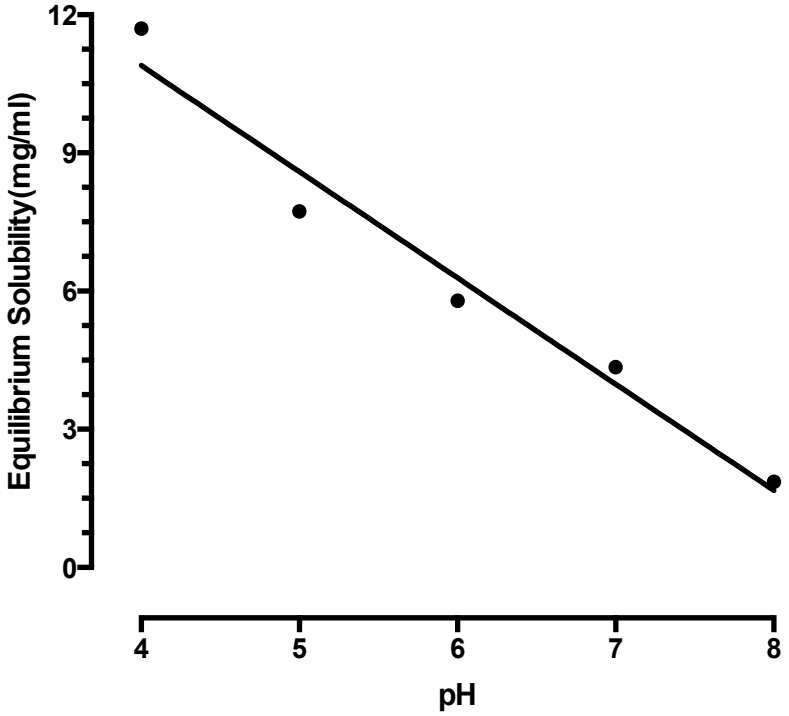

Figure 6 Solubility of TAF at various pH.

UPS citrate buffer (pH=4 and 5) and phosphate buffer (pH=6, 7 and 8) were used to control pH. TAF was well suspended in media, rotated for 24h, centrifuged and filtered by 0.22μm filters. The data represents the average of each vial of triplicates determinations (n=1).

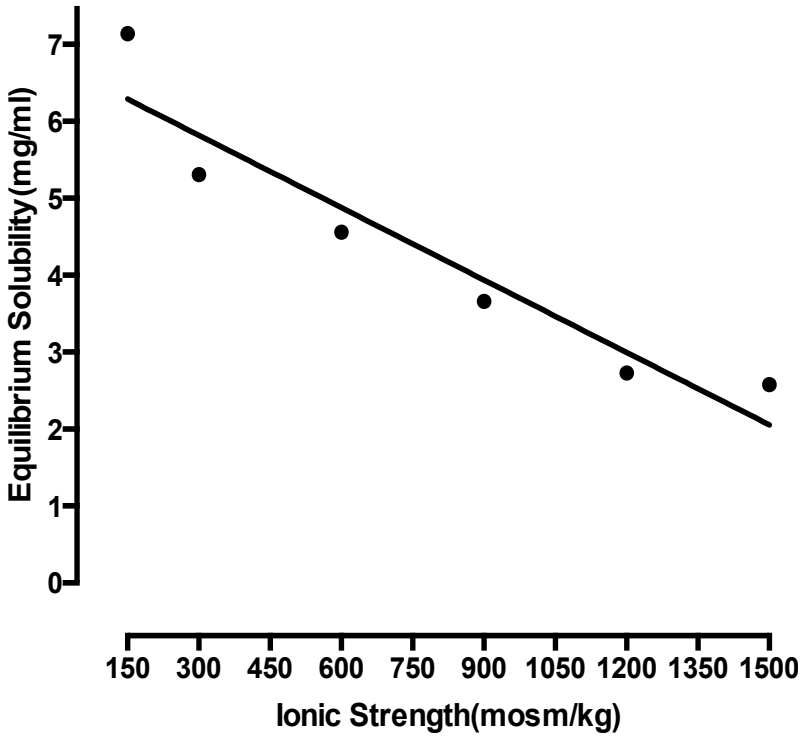

Figure 7 Solubility of TAF at various ionic strength

TAF was suspended in PBS buffer with various osmolality (150-1500mosm/kg), rotated for 24h, centrifuged and filtered by 0.22μm filters. The samples were analyzed using HPLC. The data represents the average of each vial of triplicates determinations (n=1).

2.3.3 Hydrolysis

Hydrolysis is one of the most common degradative chemical reactions over a wide range of pH. Hydrolysis peaks were observed on the HPLC chromatogram and did not interfere with the TAF main

peak at around t_R=1.05min (Figure 8). Hydrolysis of TAF at various pH was expected at room temperature, due to its labile carboxyl ester bond.

The rate of TAF hydrolysis increased with decreasing pH with statistical significant differences between each condition (p<0.05) (Figure 9). Using pH=2 forced degradations as an example, the hydrolytic degradant peak area was positively correlated with decreasing TAF peak area over time (Figure 10). The calculated half-life of TAF forced by pH=2 buffer was 22h (R square was 0.99 and equation was shown in Figure 9). In 0.1M HCl, the TAF peak was not detected after 24h, suggesting the concentration is less than 0.17µg/ml. The TAF peak could not be detected once 0.1M NaOH was added. The figure below showed consistent hydrolysis peaks increased over time with TAF peaks decreased in pH=2 buffer (Figure 8).

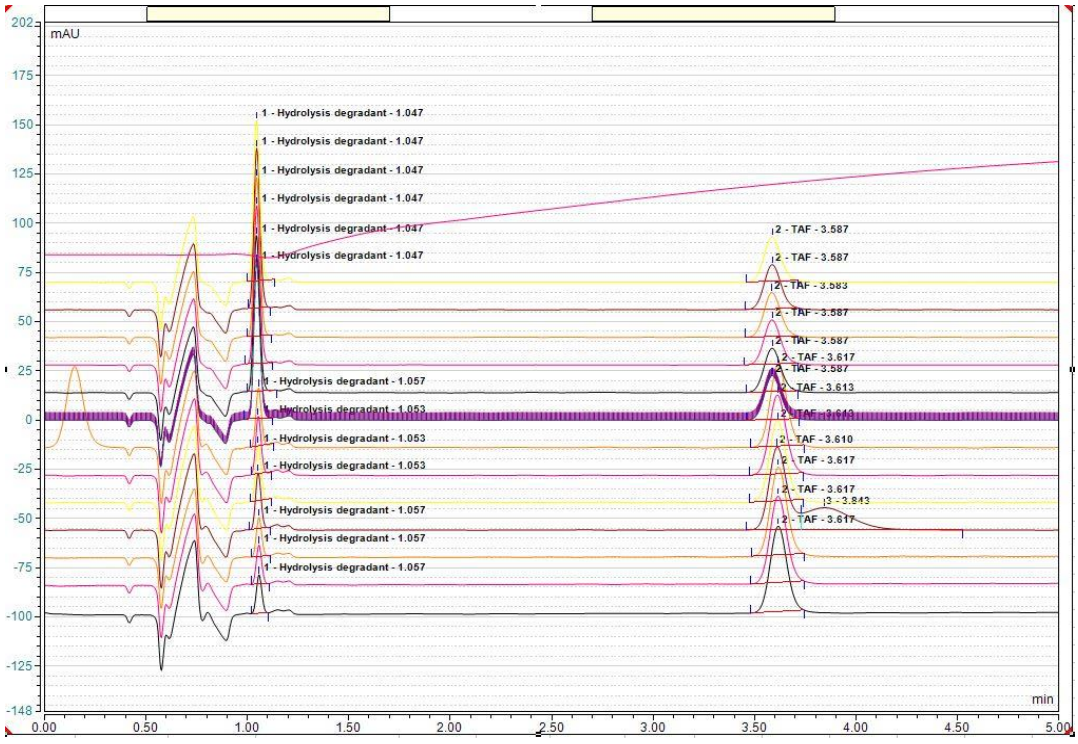

Figure 8 Overlay of chromatograms of hydrolytic stability test in pH=2 buffer.
Hydrolysis peaks increased over time with TAF peaks decreasing.

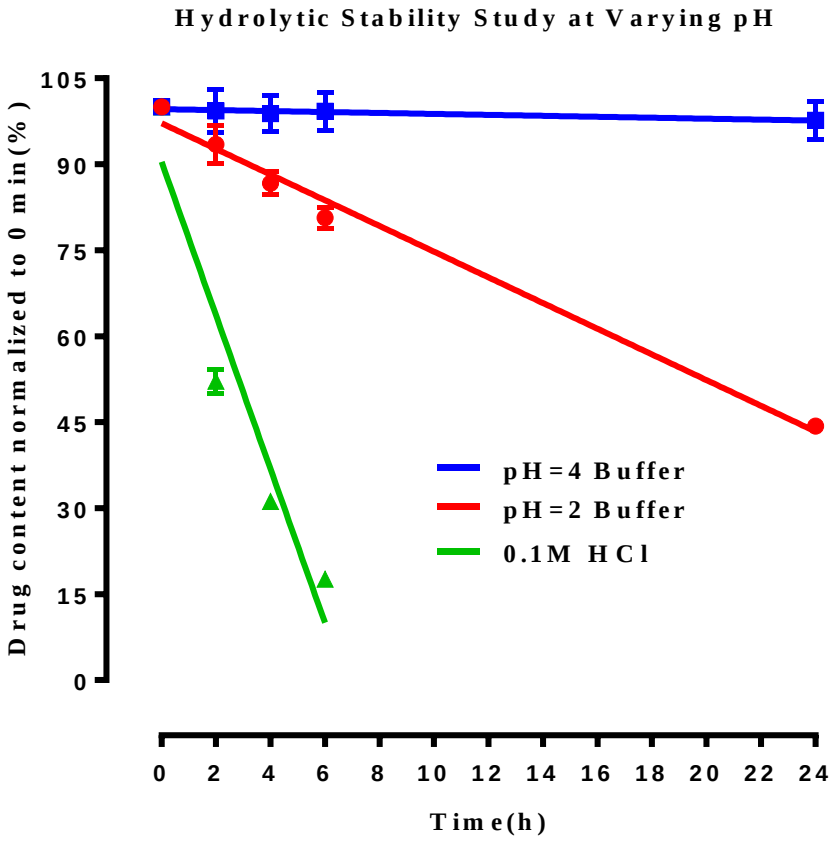

Figure 9 Hydrolysis in USP buffers (pH=2, pH=4) and 0.1M HCl

TAF stock solution prepared in 100% acetonitrile (7mg/mL) were diluted by hydrochloric buffer (pH=2) and citrate buffer (pH=4) to 70µg/mL and stored at room temperature. Drug content was measured on HPLC at predetermined time points for up to 24h. Data is reported as mean ± SD, n=3.

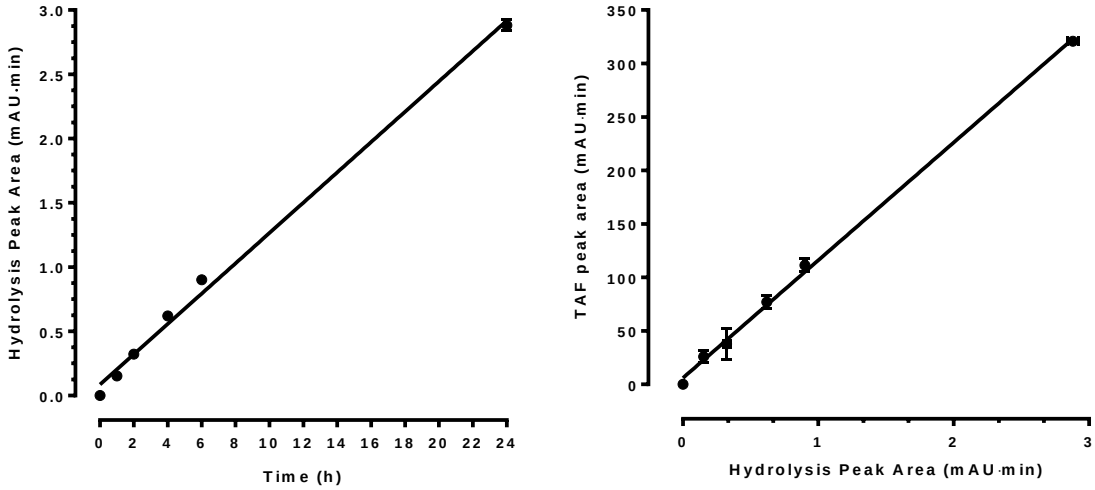

Figure 10 Linear correlation of hydrolysis peak area with time (h) (left) and TAF peak area at pre-determined time points subtracted by TAF peak area at 0h (mAU·min) (right).

TAF stock solution prepared in 100% acetonitrile were diluted by hydrochloric buffer (pH=2). TAF hydrolysis peak was measured on HPLC at predetermined time points for up to 24h. The peak areas (mAU·min) were plotted against time (h) and hydrolytic degradant peak areas (mAU·min). The equation of linear regression in Figure 10 (left) is Y=0.118*X+0.086, and the R square is 0.995. The equation of linear regression in Figure 10 (right) is Y=0.009*X-0.053, and the R square is 0.998 (n=3).

2.3.4 Thermal and Photolytic Stability

The calculated half-life of TAF thermal degradation was 13 days under 60°C based on linear regression (R square and equation were shown in Figure 11). A 10% loss was investigated at 65h under 60°C (Figure 11).

In photolytic studies, TAF was prepared directly in water at 1mg/mL and diluted as 120µg/mL. Only 1% loss was observed at 72h.

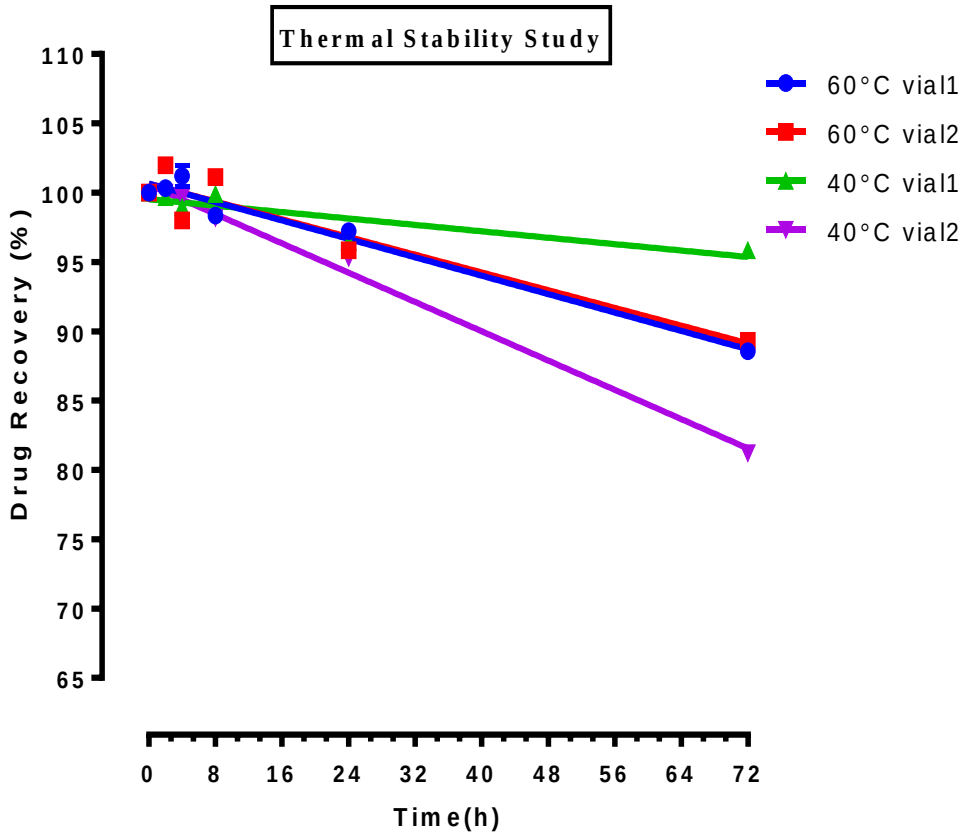

Figure 11 Thermal stability study at 40°C and 60°C

TAF stock solution prepared in 100% acetonitrile were diluted to 70 µg/mL with water and stored in amber glass vials, sealed and put in 40°C and 60°C water baths and monitored for 72h. 100 µL samples were taken on predetermined time points and drug contents were measured on HPLC. The equation of linear regression at 60°C is Y = -0.164*X + 101, and the R square is 0.987. The average of each vial was reported with triplicates determinations (n=1).

2.3.5 Oxidation

Oxidation is a common chemical process that entails the loss of electrons and the transfer of electrons between reactive groups. Amines and phenols present in TAF's structure are susceptible to electron transfer oxidation to yield N-oxides, hydroxylamine, and sulfones [63, 64]. Benzylic carbons and tertiary carbons, present in the TAF benzene ring and carbon chain, contain labile hydrogen and are susceptible to oxidation, forming hydro peroxides or ketones [64, 65]. Therefore, it was expected that TAF could be oxidized under hydrogen peroxide at room temperature.

TAF diluents forced by 0.03% H_2O_2 had no significantly statistical drug loss within 3 days (p=0.06). 99.85% drug recovery was obtained at 24h forced by 0.03% H_2O_2, as opposed to 77.68% drug recovery forced by 3% H_2O_2 (Figure 12). No oxidative degradant peak was observed on HPLC chromatographs

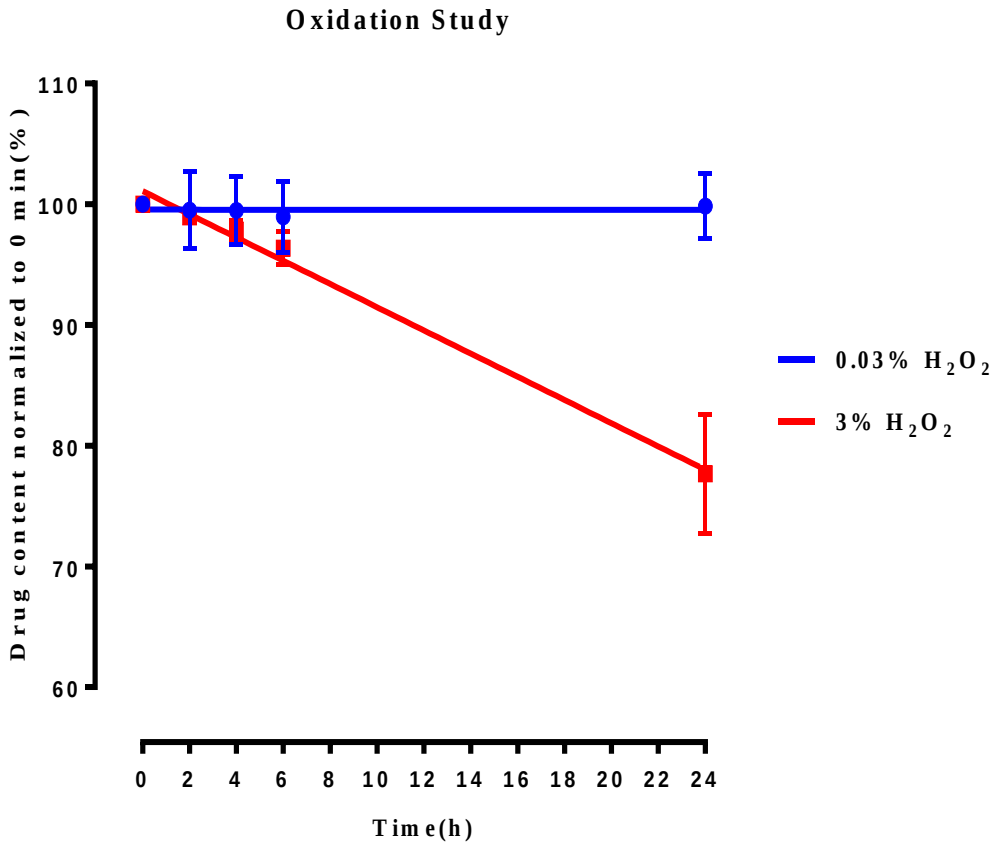

Figure 12 Oxidation study by 0.03% and 3% H_2O_2

TAF stock solution were diluted with 30% H_2O_2 and 3% H_2O_2 solution by water and stored at room temperature for 24h of 3% H_2O_2 solution and 72h of 0.03% H_2O_2 solution. Drug content were measure on HPLC at predetermined time points Y-axis is drug content at predetermined time points normalized to drug content at time zero (100%). The equation of linear regression at 3% H_2O_2 is Y= -0.961*X+101 and R square is 0.992. Drug content at 0.03% H_2O_2 had no statistically significant decrease within 72h (p=0.06). Data is reported as mean ± SD, n=3.

2.3.6 Preservative Compatibility

The average recovery without parabens was 98.8%±0.6% and after addition of parabens was 98%±0.53%. There is no statistical difference (p=0.097) before and after addition of sodium parabens concerning drug content recovery ratio (normalized to actual weighed amount divided by volume). Table 4 below shows the concentrations of each independent TAF enema during preparation. All enemas followed the same preparation procedure (see 2.2.6)

Table 4 Preservative compatibility.

	Theoretical Concentration (mg/ml)	Actual Concentration without Parabens (mg/ml)	Actual Concentration after Addition of Parabens (mg/ml)	Recovery without Parabens (%)	Recovery after Addition of Parabens (%)
1	1.8	1.77±0.020	1.77±0.006	98.2	98
2	1.81	1.79±0.005	1.76±0.009	99	97.4
3	1.76	1.75±0.006	1.74±0.020	99.2	98.6

TAF enemas were first prepared according to preparation procedure without preservative, analyzed by HPLC and then preservative was added followed by drug content analysis. Data is reported as mean ± SD, n=3. The recovery ratio is the mean of actual concentrations divided by the mean of theoretical concentrations. The results of three independent experiments are reported separately.

2.3.7 One-Week Stability

Short-term stability testing of drug product formulation was carried out in three conditions: long (25°C ± 2°C/60% RH ± 5% RH), intermediate (30°C ± 2°C/65% RH ± 5% RH), and accelerated (40°C ± 2°C/75% RH ± 5% RH), according to the International Conference on Harmonization (ICH) Q1A (R2).

The specification of drug content is within 10%, pH within 6-8 and osmolality within ±5 (Isotonic: 290mosm/kg and hypotonic: 150mosm/kg).

Results show that higher temperatures forced faster degradation. For isotonic and hypotonic TAF enemas conducted at 40°C (Figure 13), 30°C (Figure 14), 25°C (Figure 15), the chemical degradations forced by thermal conditions followed zero-order kinetics at 30°C and 25°C (R square were all greater than 0.98). The percent of drug remaining was plotted as a function of time. The calculated half-life was based on linear regression equations (equations shown in Figure 14 and Figure 15). The calculated half-life for isotonic enemas was 17 days and 27 days at 30°C and 25°C, respectively. For hypotonic TAF enemas, the half-life was 19 days and 33 days, respectively. When comparing isotonic and hypotonic TAF enemas, there was no obvious difference in degradation rate. Isotonic TAF enemas at 25°C had 10% loss of drug content at day 5 in comparison with hypotonic TAF enemas at day 7. For pH and osmolality, they were both within the specification range within 7 days. A slight decrease in pH was observed over 7 days (Table 5 and Table 6). Osmolality did not change significantly over time. (Table 7 and Table 8)

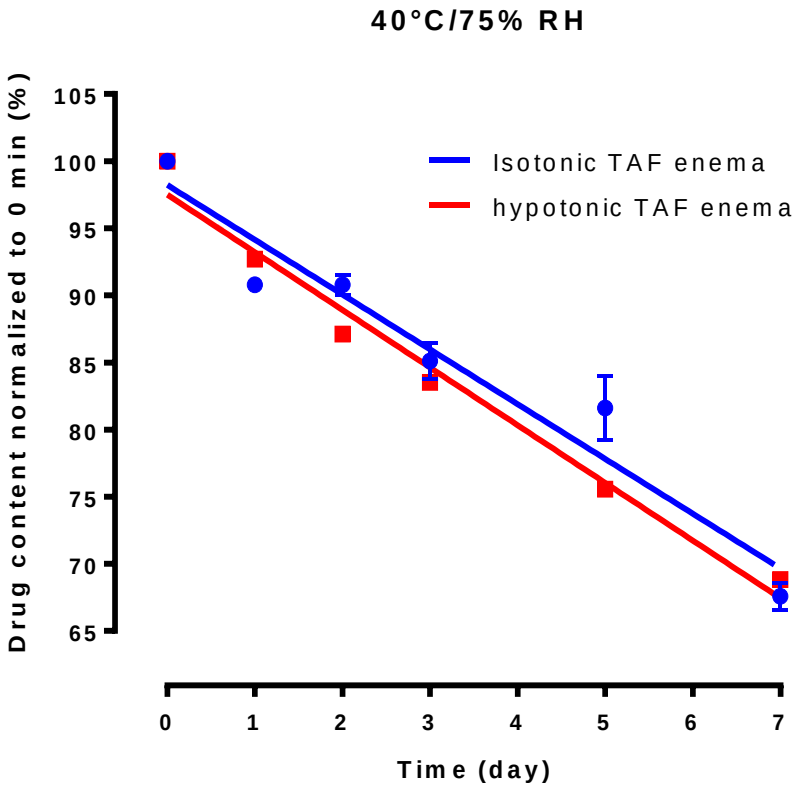

Figure 13 Drug recovery of isotonic and hypotonic TAF enemas in 40°C/75% RH environmental chamber

Isotonic and hypotonic TAF enemas in 30mL capped glass bottle were stored in 40°C/75%RH environmental chamber. 1mL aliquots were taken each time, diluted by 25% acetonitrile and drug contents were analyzed by HPLC at predetermined time points. The equation of the linear regression of isotonic TAF enema is Y= -4.082X+98.22, R square is 0.942 and the equation of the linear regression of hypotonic TAF enema is Y= -4.295*X+97.5, R square is 0,980. The data represents the average of each vial of triplicates determinations (n=1).

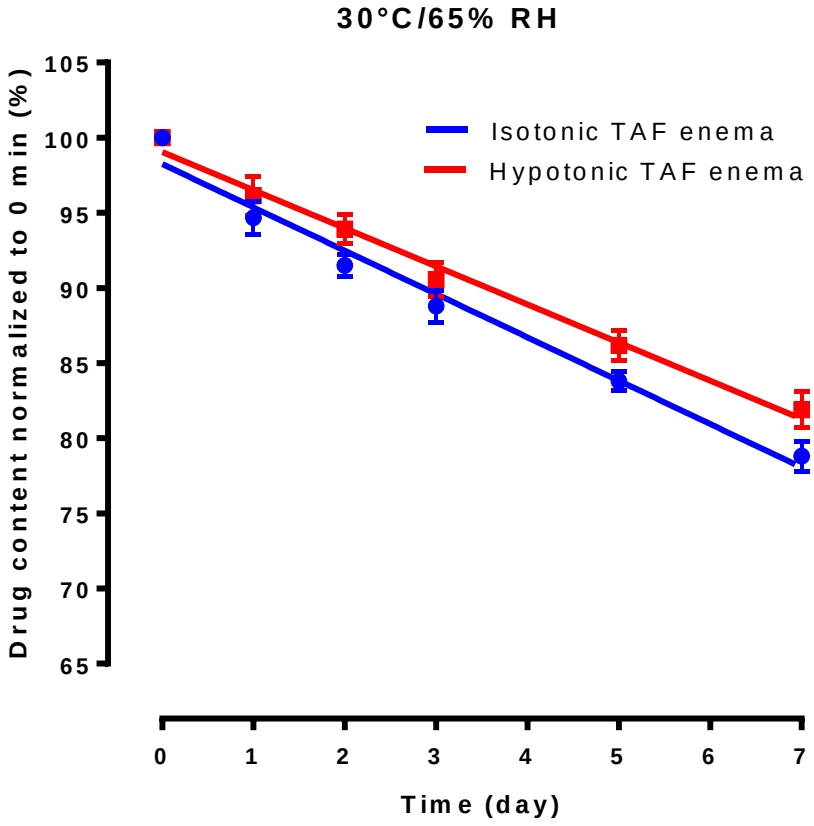

Figure 14 Drug recovery of isotonic and hypotonic TAF enemas in 30°C/65% RH environmental chamber

Isotonic and hypotonic TAF enemas in 30mL capped glass bottle were stored in 30°C/65%RH environmental chamber. 1mL aliquots were taken at each time point, diluted by 25% acetonitrile and drug content was analyzed by HPLC at predetermined time points. The equation of the linear regression of isotonic TAF enema is Y= -4.082X+98.22, R square is 0.942 and the equation of the linear regression of hypotonic TAF enema is Y= -4.295*X+97.5, R square is 0,980. The data represents the average of each vial of triplicates determinations (n=1).

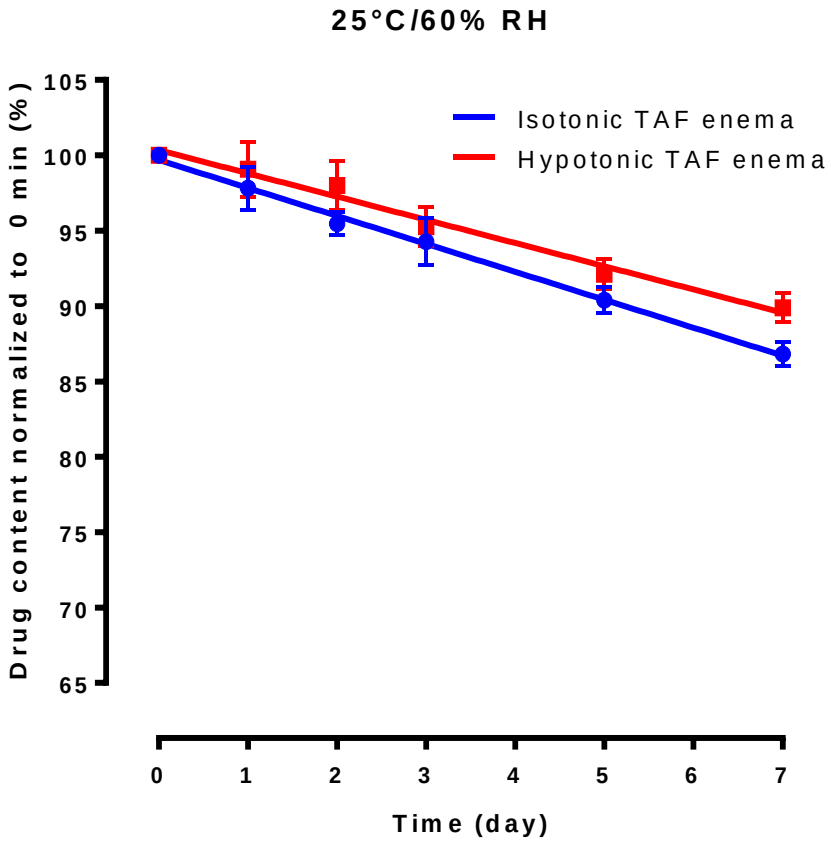

Figure 15 Drug recovery of isotonic and hypotonic TAF enemas in 25°C/60% RH environmental chamber

Isotonic and hypotonic TAF enemas in 30mL capped glass bottle were stored in 25°C/60%RH environmental chamber. 1mL aliquots were taken at each time point, diluted by 25% acetonitrile and drug content was analyzed by HPLC at predetermined time points. The equation of the linear regression of isotonic TAF enema is Y= -4.082X+98.22, R square is 0.942 and the equation of the linear regression of hypotonic TAF enema is Y= -4.295*X+97.5, R square is 0,980. The data represents the average of each vial of triplicates determinations (n=1).

Table 5 pH of hypotonic TAF enema

pH	Time0	Day2	Day3	Day5	Day7
40°C	6.84	6.81	6.8	6.67	6.59
30°C	6.84	6.89	6.85	6.74	6.78
25°C	6.84	6.88	6.89	6.83	6.84

Hypotonic TAF enemas were stored in environmental chambers with temperature of 40°C, 30°C, 25°C and pH was tested at predetermined time points, n=1.

Table 6 pH of isotonic TAF enema

pH	Time0	Day2	Day3	Day5	Day7
40°C	6.76	6.79	6.69	6.59	6.55
30°C	6.76	6.87	6.81	6.75	6.68
25°C	6.76	6.92	6.89	6.84	6.86

Isotonic TAF enemas were stored in environmental chambers with temperature of 40°C, 30°C, 25°C and pH was tested at predetermined time points, respectively, n=1.

Table 7 Osmolality of hypotonic TAF enema

Osmolality (mosm/kg)	Time0	Day1	Day2	Day3	Day5	Day7
40°C	155	154	157	156	161	159
30°C	155	152	157	155	156	158
25°C	155	155	155	155	156	156

Hypotonic TAF enema were stored in environmental chambers with temperature of 40°C, 30°C, 25°C and osmolality was tested at predetermined time points, respectively, n=1.

Table 8 Osmolality of isotonic TAF enema

Osmolality (mosm/kg)	Time0	Day1	Day2	Day3	Day5	Day7
40°C	288	288	290	290	294	294
30°C	288	286	288	286	287	288
25°C	288	287	291	286	290	294

Isotonic TAF enemas were stored in environmental chambers with temperature of 40°C, 30°C, 25°C and osmolality was tested at predetermined time points, respectively, n=1.

2.3.8 The Relationship of Temperature and Degradation Rate

For thermally accelerated temperature, the most commonly used kinetic model used to interpret the effect of temperature on reaction rate is the Arrhenius equation [66, 67]. The Equation 1 is shown below.

$$k = A \exp(-E_a/RT)$$

Equation 1 Arrhenius equation

k = reaction rate constant

A = Arrhenius factor (y-intercept constant)

E_a = the energy of activation for the reaction, cal/mole (1000 cal = 1 kcal)

R = the ideal gas constant, 8.3144598 J mol^{-1} K^{-1}

T = the absolute temperature (degrees Kelvin)

The degradation rate at each temperature is firstly estimated, then using proper weighting, the logarithm of degradation rate is plotted as a function of reciprcal absolute temperature [67]. For many reactions, This linear relationship can be obtained between the inverse of temperature (in degrees Kelvin) and the natural log (ln) of the measured rate constant (k), determined by the Arrhenius equation [66]. This

linear relationship means that the effect of the temperature on the reaction rate is maintained which indicates the reaction mechanism has no change over the range of temperature [66]. In our study, linear relationships were obtained between time and drug content at 25°C, 30°C and 40°C which indicated that degradation were all zero-order. The Equation 2 is the linear form of Equation 1 for graphical presentation.

$$\ln k = -E_a/RT + \ln A$$

Equation 2 y=mx+b format of Arrhenius equation

Based on the content of linear regression equations of TAF enemas under three conditions (25°C/60% RH, 30°C /65% RH, 40°C/75% RH), the relationship between temperature and degradation rate of isotonic and hypotonic TAF enemas can be defined by the equations are $k=984609\times exp^{-10.26/RT}$ and $k=23207824\times exp^{12.29/RT}$ respectively. The unit of E_a is Kcal/mole.

Then we can use the model to calculate the shelf-life at any other combination of temperatures and storage intervals at each temperature [67]. For zero-order reaction, $\alpha(t)=At\times exp^{-Ea/RT}$ can be used to calculate the fraction degraded at any time t and any temperature T [67].

The limitation of this model is when monitoring the generation of a primary degradation product, if a secondary degradation reaction happening during the reaction can introduce error to the calculation of the primary rate constant, meaning the reaction rate will not be a constant under this condition [66].

2.4 DISCUSSON AND CONCLUSION

The overall goal of the preformulation studies was to evaluate the physicochemical properties of the API and its stability profile under various conditions. In the current work, preformulation studies have

shown that TAF is a promising microbicide candidate. This API is slightly soluble in water and other commonly used media at various pH. There was no statistical difference between solubility of TAF in 0.9% saline and water and they were both significantly higher than solubility in PBS from one-way ANOVA Bonferroni post-hoc test (p=0.0018 and p=0.009 for comparisons of water and 0.9% saline with PBS, respectively). All solubility results indicated that TAF is able to reach the target concentration of 1.76mg/ml without aide of solubilizing excipients. This solubility is desirable for incorporation into many dosage forms such as aqueous gels, suppositories, and enemas. The reason why PBS, 0.9% saline and water were chosen as the solubility media was that it was desired to have the enema formulated in a water-based medium. Secondly, 0.9% saline and PBS have different ionic compositions that might affect solubility. In addition, the pH difference between these three media would give us hints on whether pH would affect the solubility of TAF. As enemas are largely composed of water and are less viscous resulting in their potential enhanced coverage of the large surface area of colorectal epithelium when applied, high solubility of TAF can allow drug delivery into the colorectal lumen. Delivery to this location can provide sufficient dosing and a long dose regimen.

However, the task of formulating TAF into an enema product remains challenging due to its hydrolysis at acidic pH conditions or at temperatures of 25°C and higher. In order to progress TAF enemas into animal studies and prolong the shelf life, the stability of TAF enemas must be enhanced.

In the solubility studies conducted, the solubility of TAF decreased with increased pH and ionic strength. Therefore, incorporating higher concentrations of TAF into enemas within pH 6-8 might be problematic.

In the hydrolysis preformulation study, TAF hydrolysis rate increased with decreasing pH (p<0.0001 for three groups comparison), which was consistent with the knowledge that extreme pH will promote hydrolysis. Hydrolysis was found to contribute to the overall loss in drug content, as a linear relationship was determined after plotting hydrolysis peak area with TAF peak area decrease at

corresponding time points. Comparing hydrolysis tests in the preformulation studies with pH=6-8 in the stability tests at 25°C, TAF in pH=4 buffer was predicted to have 90% of claimed drug label at 5 days, as opposed to approximately 6 days with pH close to 7.

In the thermal preformulation study, two storage conditions, 40°C and 60°C, were chosen and utilized as accelerated temperatures to facilitate the degradation rate. Data showed that both 40°C and 60°C promoted degradation. The data obtained at 40°C at 72h showed a large variation so the degradation rate at 40°C could not be calculated. It is commonly agreed that higher temperature accelerates degradation based on Arrhenius equation. More experiments are needed to confirm the degradation rate at 40°C and the comparisons between temperatures can be made. In the stability test, three conditions (25°C/60% RH, 30°C /65% RH, 40°C /75% RH) were chosen to assess the shelf life of TAF enemas. The calculated shelf life of 90% claimed drug label is 5 days, 6 days and a half for isotonic TAF enemas and hypotonic TAF enemas within 95% confidence interval, respectively. The calculated shelf lives of the products were both the lower confidence limit of the estimated time. In terms of thermal stability, isotonic or hypotonic enemas were similarly affected by varying temperatures.

During the process of formulation, storage, handling and application *in vivo*, many sources of oxidation exist, including the use of organic solvents and excipients during formulations and H_2O_2 produced by lactobacillus in the female reproductive tract. Studies have showed that H_2O_2-producing vaginal lactobacillus species in the rectum may contribute to the maintenance of vaginal microflora and serve as a reservoir for vaginal colonization by lactobacilli, especially after a disturbance of the ecology that follows douching or sexual intercourse [68]. In the current study, 3% hydrogen peroxide was applied to test TAF oxidative stability, revealing a 10% loss by day 11^{th}. However, lactobacillus *in vivo* secrete only 0.03% H_2O_2, and. no statistically significant (p=0.063) drug loss was observed in this study under 0.03% H_2O_2 conditions after 3 days. It is unlikely that the microbicide product in the rectum will experience a higher H_2O_2 percentage, and therefore, stability in the rectal tract can be ensured. Although

rectal exposure to H_2O_2 should not affect TAF stability, environmental H_2O_2 may still cause degradation. Antioxidants are commonly used additives in food and drug fields may increase oxidation stability if incorporated into liquid based formulations. Antioxidant vitamins, for example, vitamin C (ascorbic acid) which is a water-soluble free radical scavenger or ethylenediaminetetraacetic acid (EDTA) might be able to incorporate in liquid based formulations. Further investigation of suitable antioxidants is needed to stabilize TAF enemas against oxidative environment.

As discussed above, hydrolysis is a major issue affecting stability of TAF enemas. Hydrolytic degradants might be ionizable and affect pH and osmolality. The pH and osmolality were monitored during the stability test. Strategies to prevent hydrolysis of drug-like compounds that have been employed include intercalation into liposomes, solubilization in micro-emulsions, entrapment in microspheres, and formation of inclusion complexes [69]. It has been reported that encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis by cationic surfactants cetyl trimethylammonium bromide (CTAB) and dodecyl trimethylammonium bromide (DTAB) [70]. When in alkaline solution, cationic surfactants entrapped curcumin and prevented contact with water, thus largely suppressing its hydrolysis in alkaline solution [70]. Carboxyl ester, organophosphate and phosphoamide groups present in TAF structure are subject to acidic and alkaline hydrolysis [40]. Once hydrolyzed, negatively charged species form. The surfactants CTAB and DTAB may be suitable for incorporation into TAF enemas to form micelles and suppress hydrolysis of TAF. Further investigation is needed to explore the compatibility, effects, and toxicity of these chosen and whether addition prevents hydrolysis.

Sodium parabens are freely dissolvable in water and have no impact on TAF drug content. Therefore, it can be concluded that sodium methylparaben and propylparaben are compatible with TAF in enemas. However, the antibacterial effects of these two parabens in TAF enemas remain unknown and require further exploration.

In conclusion, the preformulation evaluation preformed in this chapter affirmed that TAF is a promising drug candidate for the development of rectal water-based microbicides. The studies in this chapter also reveal that hydrolysis under pH and thermal stress conditions represent major challenges for the formulation development of TAF enemas. Additionally, oxidation may be an issue during storage, thus it is important that packaging options which can protect from oxidation be explored. The use of pharmaceutical strategies that can encapsulate and protect TAF as well as the utilization of protective pharmaceutical inactive ingredients must be studied further to improve TAF stability against stress-induced hydrolysis.

3.0 DESIGNING CMX157 ENEMAS FOR HIV PREVENTION

3.1 THE RATIONAL OF DESIGNING CMX157 ENEMAS

Since CMX157 has shown promise as a potent TFV prodrug, incorporation into rectal enemas was investigated. After initial solubility studies revealed CMX157 possesses 3mg/ml solubility in 1X PBS, a commonly used buffer that is safe for rectal use, preformulation development for CMX157 enemas began. The target concentration of CMX157 in a water-based enema is 1.76mg/ml based on TFV enemas. As opposed to TAF, CMX157 has organophosphate (esters of phosphoric acid) groups that can be hydrolyzed only under extreme conditions[40]. Likewise, ether is considered relatively unreactive and often used to protect alcohol from oxidation[63]. However, ether can be hydrolyzed by hydrogen bromide, a commonly used acid slightly stronger than HCl. Reactive 1º- alcohol group is subject to oxidation[65]. In conclusion, CMX157 was considered for incorporation into rectal enemas due to its anti-retroviral activities, solubility in PBS, and low chance of hydrolysis under normal conditions.

3.2 MATERIALS & METHODS

3.2.1 HPLC Development

CMX157 was analyzed on a BDS Hypersil (Thermo Fisher Scientific, Waltham, MA) C8 column (3µm, 150 ×4.6 mm) using the Waters HPLC system connected to a diode array detector (Waters 2996 photodiode Array). A gradient elution was utilized by mobile phase A (25mM phosphate buffer and 5mM *t*-butyl ammoniumbromide, pH=3) and B (100% Methanol) for 24 min at a flow rate of 1mL/min at

ambient temperature in auto-sampler. Sample injection volume was 10μL. Table 9 shows the gradient elution.

Table 9 CMX157 gradient elution compositions

Time (min)	Mobile phase A	Mobile phase B
Initial	50%	50%
2	50%	50%
10	10%	90%
20	10%	90%
22	50%	50%
24	50%	50%

3.2.2 CMX157 Enemas Formulas and Preparations

Table 10 shows formulas for isotonic and hypotonic CMX157 enemas in PBS buffered systems. 1XPBS and 0.5XPBS were diluted from purchased 10XPBS. After PBS preparation, CMX157 was added and stirred for 5mins. The resulting solution was sonicated until all components were dissolved. After sonication, pH and osmolality were immediately recorded.

Table 10 Preliminary formulations of CMX157 isotonic and hypotonic enemas

Component (Isotonic)	Amount Per Batch	Component (Hypotonic)	Amount Per Batch
1XPBS	20mL	**0.5XPBS**	20mL
CMX157	35.2mg	**CMX157**	35.2mg

3.3 RESULTS

3.3.1 HPLC Method

An HPLC analytical method was developed and validated. The retention time of CMX157 was observed at 17.9 minutes. The LOD was estimated to be 5µg/mL, and the LOQ was 0.17µg/mL. A linear standard curve was obtained over a range of 0.5~120µg/mL. No CMX157 degradants were detected during the analysis. The perfect overlay among the 10 CMX157 chromatograms represents the repeatability of samples at the low QC level (5ug/mL) (Figure 16). This developed HPLC method was applied to quantify solubility of CMX157 in different solvents and drug concentrations in samples collected throughout the stability studies. The R square of the validation curves were all greater than 0.99. The CV reported as RSD were shown in Table 11.

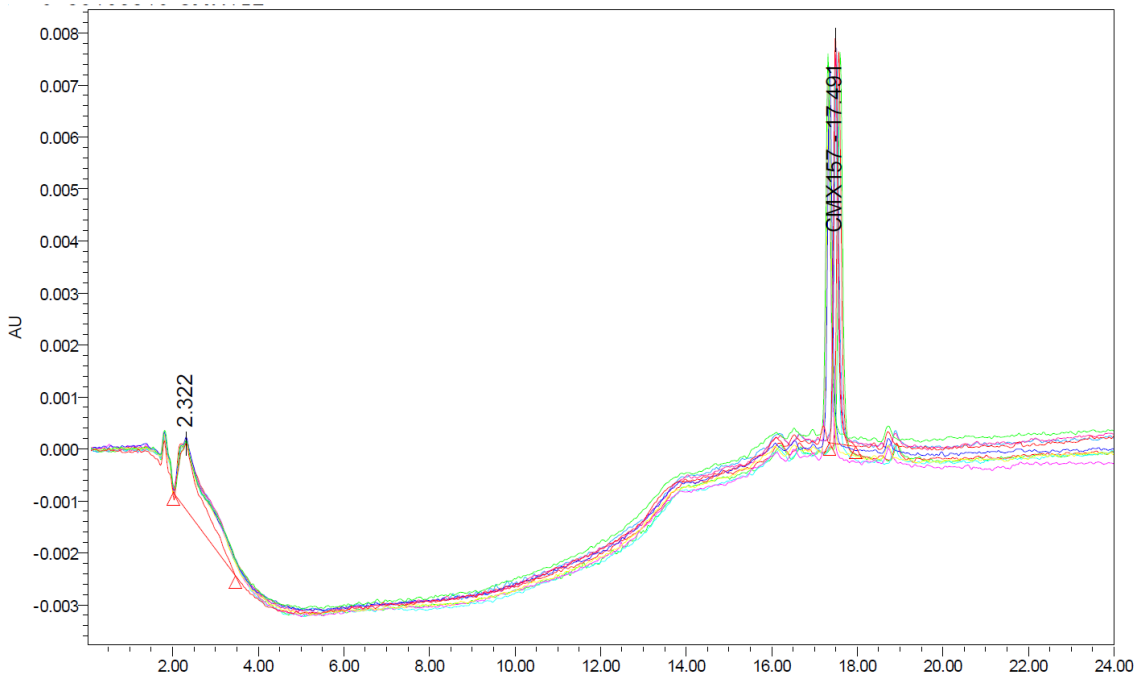

Figure 16 HPLC chromatograms overlay of 10 injections of low QC level of CMX157 (5µg/mL)

10 injections of each QC level were made on each day of three days. RSD was 0.981% for injections shown above. TAF peaks were consistent on each day.

Table 11 Precision results of CMX157 validation for three days

RSD (%)	Low Conc.	Middle Conc.	High Conc.
Precision	7.25%	4.43%	25.4%

3.3.2 CMX157 Enemas Characterizations

The pH and osmolality of CMX157 enemas are shown below.

Table 12 pH of CMX157 enema

	pH
1XPBS CMX157 enemas	6.83 ± 0.01
0.5XPBS CMX157 enemas	6.58 ± 0.02

Data is reported as Mean ± SD, n=3

Table 13 Osmolality of CMX157 enema

	Osmolarity (mosm/kg)
1XPBS CMX157 enemas	306 ± 2
0.5XPBS CMX157 enemas	142 ± 2

Data is reported as Mean ± SD, n=3

4.0 MAJOR SIGNIFICANCE, LIMITATIONS AND FUTURE DIRECTIONS

A safe, effective HIV PrEP strategy with high levels of adherence is urgently needed to protect men and women at highest risk of HIV infection from anal sex, where risk of transmission far exceeds that of penile-vaginal sex [71]. The Development of a Rectal Enema As Microbicide (DREAM) program addresses this critical need to develop a highly effective, safe, and behaviorally-congruent alternative for the prevention of rectal HIV infection [71]. The overall goal of the program is to develop a single dose pericoital enema to deliver a TFV prodrug capable of providing one week of HIV protection[71]. This strategy builds upon proven high levels of efficacy of TFV-based PrEP in adherent persons and directly targets adherence as the greatest weakness of PrEP regimens [71]. Given the common practice of rectal douching with an enema prior to receptive anal sex, using an enema as HIV prevention would require little behavioral change [71]. TAF and CMX157 are potent prodrugs of TFV that have better cellular uptake than TFV, which increases concentration of TFV in PBMCs and are beneficial in reducing dosage and prolonging dosing regimens [26]. As little information is available in the literature regarding the physiochemical characteristics of these two prodrugs, it was necessary to develop crucial preformulation studies to better understand development of microbicide formulations.

TAF is a promising microbicide candidate for incorporation into enemas as it has sufficient solubility in water and other commonly used solubility media such as water, saline and PBS. In this study, TAF aqueous solubility is sufficient to achieve the 1.76mg/ml target concentration without aide of solubilizers. Our clinical collaborator set this target concentration for the TFV enema. However, TAF was prone to degradation under stressed conditions, an acidic environment (pH≤4), and high temperature environment (≥25°C). These characteristics have been anticipated from the chemical structure of TAF. TAF has a carboxyl ester bond labile to hydrolysis. Amines and phenols present in TAF's structure are

susceptible to electron transfer oxidation to yield N-oxides, hydroxylamine, and sulfones [63, 64]. Benzylic carbons and tertiary carbons, present in TAF benzene ring and carbon chain, which contain labile hydrogen, are also susceptible to oxidation to form hydro peroxides or ketone s[64, 65]. There are many strategies available to stabilize drug products against various environments. For oxidative environments, antioxidant reagents such as antioxidant vitamins or EDTA were proposed to solve instability in oxidative environment. For stressed acidic and thermal conditions, micelles made by cationic surfactants, such as CTAB and DTAB, were proposed to suppress hydrolysis [70]. More research is needed for stabilization of TAF in water-based media, in order to progress TAF enemas to animal studies.

Little data is obtained in this thesis work about the chemical characteristics of CMX157. As predicted from the chemical structure of CMX157, it may have less solubility compared with TAF due to its nineteen carbon chain and be subject to oxidation attributed to that 1º-alcohol group that links to organophosphate is a reactive group, which is subject to oxidation [65]. The HPLC method developed for CMX157 needs further optimization. Preliminary solubility data showed that CMX157 was soluble in 1XPBS (3mg/ml). Preliminary CMX157 formulations in PBS buffered system have reached the desired pH and osmolality range without further adjustments. Drug content evaluation, stability testing, and some necessary pre-formulation work are needed to optimize this formulation. A once-weekly dosing regimen of CMX157 was recommended because PBMC levels of TFV-DP remained detectable for six days after the single 400 mg dose due to high intracellular uptake of CMX157 [26]. Solubilizing CMX157 might be needed to achieve this one-week dosing regimen, which should enhance patient compliance. Refrigerator storage has been recommended for CMX157. We observed degradation peaks when temperature was not well controlled. Therefore, extra caution regarding temperature control should be used when developing analytical tools and determining storage conditions for CMX157.

The goal of the work written in this thesis was to develop a rectally applied, HIV-preventive, TFV prodrug enema. Dosing recommendations for future work with TAF and CMX157 for non-human primate and human studies based on both multi-compartment pharmacokinetics and antiretroviral pharmacodynamics is not currently available [71]. Formulations developed in this study sought to promote colonic absorption without compromising epithelial integrity in the colorectum by osmolality and pH control [32, 50]. The pH range (pH 6-8) of TFV prodrug enemas was selected based on studies conducted by Lin Wang (Magee Women's Research Institute, Pittsburgh, PA) and World Health Organization (WHO) recommendations [32, 39]. The aim was to relieve potential discomfort resulting from extreme pH. However, due to the varying physiochemical characteristics between different compounds, the pH range should be chosen based on achieving stable formulations of the specific drug substance.

In conclusion, these studies demonstrated the feasibility for formulation of 1.76 mg/ml of TAF in an enema dosage form. However, further work to enhance stability of TAF in this aqueous based enema dosage form is needed to prevent against oxidative, hydrolytic, and thermal conditions.

There are several limitations in the presented studies that may be addressed in future work. For solubility studies, solubility results obtained in this work require further investigation. The current developed HPLC method for TAF is not fully stability-indicating as the oxidative degradant peak could not be detected. This might result in decreased solubility results if there was any oxidation occurring during the solubility study. The current HPLC method for TAF should be adjusted to obtain more information on TAF degradants. In the hydrolysis test by different buffers with varying pH, acidic and basic buffers were used, but neutral buffers were not tested. In the formulations we developed in this project, pH 6-8 was selected as the target range, with pH=6 being more acidic and pH=8 being more basic. According to the current pH range (pH=1,2,4 and 11) used in the hydrolysis test of TAF, little information is obtained that predicts whether TAF is stable in this range. Hydrolysis studies of TAF at

pH=6 to pH=8 buffers are needed to justify the hypothesized pH range, with respect to formulation stability. In TAF hydrolysis and thermal studies, the UV detector detected hydrolysis peaks eluted at consistent time points that all had the same absorption, suggesting that one degradant could be detected by the developed HPLC method. However, the identity of this degradant is unclear, which should be explored to rule out any toxic degradants.

In this thesis work, not only has the chemical characteristics of TAF been explored by preformulation studies, but the formulations of TAF and CMX157 enema were also developed. These studies represent the first time that TAF and CMX157, as TFV prodrugs, have been developed as an enema product intended to be used as a rectal microbicide. Meanwhile, the preformulation studies on solubility and stability of TAF under various conditions provide insight into the possibility of developing other liquid based formulations. Enemas, incorporating TFV prodrugs, could offer a means of protection for people who engage in AI. The understandings and findings in this thesis work serve as a basis for future development of rectal microbicide enema products, as well as provide important information required for formulation of TFV prodrugs.

5.0 ACKONWLEDGEMENT

Research reported in this thesis was supported by NIAID of the National Institutes of Health under award number U19AI113127. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

6.0 APPENDIX A

ABBREVIATION

1. Men who have sex with men—MSM
2. Anal intercourse—AI
3. Dendritic cells—DCs
4. Sexually Transmitted Disease—STDs
5. Centers for Disease Control and Prevention—CDC
6. Pre-exposure prophylaxis—PrEP
7. Tenofovir disoproxil fumarate—TDF
8. Emtricitabine—FTC
9. Antiretrovirals—ARVs
10. Nucleotide/nucleoside reverse transcriptase inhibitors—NRTIs
11. Reverse transcriptase—RT
12. Tenofovir—TFV
13. Reduced glycerin TFV gel—RG-TFV gel
14. Tenofovir alafenamide fumarate—TAF
15. Tenofovir-diphosphate—TFV-DP
16. Darunavir/cobicistat/emtricitabine/tenofovir alafenamide—D/C/F/TAF
17. Darunavir/cobicistat/emtricitabine/tenofovir disoproxil fumarate—D/C/F/TDF

18. Hexadecyloxypropyl-Tenofovir—CMX157
19. hexadecyloxypropyl—HDP
20. Unprotected receptive anal intercourse—URAI
21. Simulated colon solution—SCS
22. Active pharmaceutical ingredients—APIs
23. tris buffered saline—TBS
24. High performance liquid chromatography—HPLC
25. Phosphate buffer solution—PBS
26. Hydrochloric acid—HCl
27. Sodium hydroxide—NaOH
28. Hydrogen peroxide—H_2O_2
29. U.S. Food and Drug Administration—FDA
30. The International Council for Harmonisation—ICH
31. Limit of detection—LOD
32. Limit of quantification—LOQ
33. Ethylenediaminetetraacetic acid—EDTA
34. High Performance Liquid Chromatography—HPLC
35. Quality control-QC
36. Coefficient of variation-CV
37. Relative standard deviation-RSD

BIBLIOGRAPHY

1. *About Microbicides Fact Sheet*. 2014 25-March-2014 [cited 2016 May 19th]; Available from: http://www.mtnstopshiv.org/node/706.
2. *HIV in the United States: At A Glance*. 2015 September 29 [cited 2016 May 19th]; Available from: http://www.cdc.gov/hiv/statistics/overview/ataglance.html.
3. Nunes, R., B. Sarmento, and J. das Neves, *Formulation and delivery of anti-HIV rectal microbicides: advances and challenges.* Journal of Controlled Release, 2014. **194**: p. 278-294.
4. Andrea. *Types of immune cells present in human PBMC*. 2012 Nov 12 Available from: http://technical.sanguinebio.com/types-of-immune-cells-present-in-human-pbmc/.
5. *HIV Overview-The HIV Life Cycle*. 2015 September 22 [cited 2016 May 11th]; Available from: https://aidsinfo.nih.gov/education-materials/fact-sheets/19/73/the-hiv-life-cycle.
6. Wilen, C.B., J.C. Tilton, and R.W. Doms, *HIV: cell binding and entry.* Cold Spring Harb Perspect Med, 2012. **2**(8).
7. Shattock, R.J. and J.P. Moore, *Inhibiting sexual transmission of HIV-1 infection.* Nat Rev Microbiol, 2003. **1**(1): p. 25-34.
8. Wilton, J. *From exposure to infection: The biology of HIV transmission*. 2011; Available from: http://www.catie.ca/en/pif/fall-2011/exposure-infection-biology-hiv-transmission.
9. McElrath, M.J., et al., *Comprehensive assessment of HIV target cells in the distal human gut suggests increasing HIV susceptibility toward the anus.* Journal of acquired immune deficiency syndromes (1999), 2013. **63**(3): p. 263.
10. Baggaley, R.F., R.G. White, and M.-C. Boily, *HIV transmission risk through anal intercourse: systematic review, meta-analysis and implications for HIV prevention.* International journal of epidemiology, 2010: p. dyq057.
11. *PrEP*. 2016 [cited 2016 July 12]; Available from: http://www.cdc.gov/hiv/basics/prep.html.
12. Roth, G. *Second Injectable PrEP Shows Promise*. 2016 [cited 2016 April 13]; Available from: http://www.hivplusmag.com/prevention/2016/4/13/second-injectable-prep-shows-promise.
13. Markowitz, M. *Long-acting PrEP injection tolerable and acceptable for male volunteers, but dose adjustment needed*. [cited 2016 25 February]; Available from: http://www.aidsmap.com/Long-acting-PrEP-injection-tolerable-and-acceptable-for-male-volunteers-but-dose-adjustment-needed/page/3039277/.
14. Smith, D.K., et al., *Condom effectiveness for HIV prevention by consistency of use among men who have sex with men in the United States.* JAIDS Journal of Acquired Immune Deficiency Syndromes, 2015. **68**(3): p. 337-344.
15. effrey Laurence, M.D., Rowena Johnston, Ph.D. *Heterosexual Anal Sex: Implications for HIV Prevention*. 2010 July 14 [cited 2016 May 11]; Available from: http://www.amfar.org/articles/in-the-lab/2010/heterosexual-anal-sex--implications-for-hiv-prevention-(july-2010)/.

16. *Effectiveness of Prevention Strategies to Reduce the Risk of Acquiring or Transmitting HIV*. 2016 Jan 7 2016; Available from: http://www.cdc.gov/hiv/risk/estimates/preventionstrategies.html.
17. Newman, E. *Injectable PrEP: The Next Order of the Day*. 2015 May 4 Available from: http://betablog.org/injectable-prep-the-next-order-of-the-day/.
18. Newman, E. *Injectable PrEP: The Next Order of the Day*. 2015 [cited 2015 May 4]; Available from: http://betablog.org/injectable-prep-the-next-order-of-the-day/.
19. McGowan, I., *Rectal microbicides: can we make them and will people use them?* AIDS and Behavior, 2011. **15**(1): p. 66-71.
20. Garg, A.B., J. Nuttall, and J. Romano, *The future of HIV microbicides: challenges and opportunities.* Antiviral Chemistry and Chemotherapy, 2009. **19**(4): p. 143-150.
21. Shattock, R.J. and Z. Rosenberg, *Microbicides: topical prevention against HIV.* Cold Spring Harb Perspect Med, 2012. **2**(2): p. a007385.
22. *AIDSinfo Drug Database-Tenofovir (microbicide)*. April 14, 2016; Available from: https://aidsinfo.nih.gov/drugs/272/tenofovir--microbicide-/0/patient.
23. Karim, Q.A., et al., *Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women.* science, 2010. **329**(5996): p. 1168-1174.
24. Sarafianos, S.G., et al., *Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition.* Journal of molecular biology, 2009. **385**(3): p. 693-713.
25. Karim, Q.A., *The global HIV epidemic: current status and challenges.* Current HIV/AIDS Reports, 2013. **10**(2): p. 111-112.
26. Herman, B.D. and N. Sluis-Cremer, *Molecular Pharmacology of Nucleoside and Nucleotide HIV-1 Reverse Transcriptase Inhibitors*. 2012: INTECH Open Access Publisher.
27. Birkus, G., et al., *Cathepsin A is the major hydrolase catalyzing the intracellular hydrolysis of the antiretroviral nucleotide phosphonoamidate prodrugs GS-7340 and GS-9131.* Antimicrob Agents Chemother, 2007. **51**(2): p. 543-50.
28. Adams, J.L. and A.D. Kashuba, *Formulation, pharmacokinetics and pharmacodynamics of topical microbicides.* Best Practice & Research Clinical Obstetrics & Gynaecology, 2012. **26**(4): p. 451-462.
29. Dezzutti, C.S., et al., *Reformulated tenofovir gel for use as a dual compartment microbicide.* J Antimicrob Chemother, 2012. **67**(9): p. 2139-42.
30. Fuchs, E.J., et al., *Hyperosmolar sexual lubricant causes epithelial damage in the distal colon: potential implication for HIV transmission.* J Infect Dis, 2007. **195**(5): p. 703-10.
31. Anton, P.A., et al., *RMP-02/MTN-006: A phase 1 rectal safety, acceptability, pharmacokinetic, and pharmacodynamic study of tenofovir 1% gel compared with oral tenofovir disoproxil fumarate.* AIDS research and human retroviruses, 2012. **28**(11): p. 1412-1421.
32. Dezzutti, C.S., et al., *Development of HIV-1 rectal-specific microbicides and colonic tissue evaluation.* PloS one, 2014. **9**(7): p. e102585.
33. Lee, W.A., et al., *Selective intracellular activation of a novel prodrug of the human immunodeficiency virus reverse transcriptase inhibitor tenofovir leads to preferential distribution and accumulation in lymphatic tissue.* Antimicrob Agents Chemother, 2005. **49**(5): p. 1898-906.
34. Painter, G.R., et al., *Evaluation of hexadecyloxypropyl-9-R-[2-(Phosphonomethoxy)propyl]-adenine, CMX157, as a potential treatment for human immunodeficiency virus type 1 and hepatitis B virus infections.* Antimicrob Agents Chemother, 2007. **51**(10): p. 3505-9.
35. A. Mills, R.O., G. Crofoot, Jr, C. McDonald, P. Shalit, J. Flamm, D. Shamblaw, M. Saag, H. Cao, H. Martin, M. Das, C. Liu, L. Zhong, C. Callebaut, J. Custodio, A. Cheng, S. McCallister. *48 Week Study of the First PI-based Single Tablet-Regimen (STR) Darunavir/Cobicistat/Emtricitabine/ Tenofovir Alafenamide (D/C/F/TAF) vs. Darunavir (DRV)*

boosted by Cobicistat (COBI) and Emtricitabine/Tenofovir Disoproxil Fumarate (TVD) in HIV-Infected Treatment-Naïve Adults. 2014 September 5-9 [cited 2016 May 11]; 54th Interscience Conference on Antimicrobial Agents and Chemotherapy]. Available from: http://www.natap.org/2014/ICAAC/ICAAC_07.htm.

36. Markowitz, M., Zolopa, A., Ruane, P., Squires, K., Zhong, L., Kearney, B.P. & Lee, W. . *GS-7340 Demonstrates Greater Declines in HIV-1 RNA than Tenofovir Disoproxil Fumarate During 14 Days of Monotherapy in HIV-1 Infected Subjects* 2011 February 27 - March 2 [cited 2016 May 11]; Conference on Retroviruses and Opportunistic Infections,Paper # 152LB]. Available from: http://www.natap.org/2011/CROI/croi_23.htm.
37. DURHAM, N. *Chimerix's Antiviral CMX157 Demonstrates Positive Phase 1 Results with Favorable Pharmacokinetics, Safety & Toler...- EXHIBITS POTENT IN VITRO ACTIVITY AGAINST XMRV AND HIGHLY RESISTANT HIV*. 2010 [cited 2016 June 16th].
38. Lanier, R. *Hexadecyloxypropyl Tenofovir(CMX157) Has Enhanced Potency in Vitro against NRTI Resistant HIV Relative to Tenofovir and a Favorable Preclinical Profile. CHIMERIX INC*. 2008 [cited 2016 June 16th]; Available from: http://www.natap.org/2008/ResisWksp/ResisWksp_19.htm.
39. Organization, W.H., *Use and procurement of additional lubricants for male and female condoms: WH.* 2012.
40. *Hydrolysis of organic compound*. Available from: http://www.avogadro.co.uk/organic/hydrolysis/hydrolysis.htm.
41. Lautenschlager, C., et al., *Drug delivery strategies in the therapy of inflammatory bowel disease.* Adv Drug Deliv Rev, 2014. **71**: p. 58-76.
42. *Beginner's Guide To Anal Douche and Enema*. [cited 2016 June 29th]; Available from: http://www.herballove.com/solutions/beginners-guide-anal-douche-and-enema.
43. Javanbakht, M., et al., *Prevalence and types of rectal douches used for anal intercourse: results from an international survey.* BMC infectious diseases, 2014. **14**(1): p. 1.
44. Carballo-Dieguez, A., et al., *The use of rectal douches among HIV-uninfected and infected men who have unprotected receptive anal intercourse: implications for rectal microbicides.* AIDS Behav, 2008. **12**(6): p. 860-6.
45. McGowan, I., *Rectal microbicides: can we make them and will people use them?* AIDS Behav, 2011. **15 Suppl 1**: p. S66-71.
46. Louissaint, N.A., et al., *Distribution of cell-free and cell-associated HIV surrogates in the colon following simulated receptive anal intercourse in men who have sex with men.* Journal of acquired immune deficiency syndromes (1999), 2012. **59**(1): p. 10.
47. Hendrix, C.W., et al., *Quantitative imaging and sigmoidoscopy to assess distribution of rectal microbicide surrogates.* Clin Pharmacol Ther, 2008. **83**(1): p. 97-105.
48. Leyva, F.J., et al., *Isoosmolar enemas demonstrate preferential gastrointestinal distribution, safety, and acceptability compared with hyperosmolar and hypoosmolar enemas as a potential delivery vehicle for rectal microbicides.* AIDS Res Hum Retroviruses, 2013. **29**(11): p. 1487-95.
49. Ensign, L.M., et al., *Enhanced vaginal drug delivery through the use of hypotonic formulations that induce fluid uptake.* Biomaterials, 2013. **34**(28): p. 6922-9.
50. Maisel, K., et al., *Enema ion compositions for enhancing colorectal drug delivery.* J Control Release, 2015. **209**: p. 280-7.
51. Sandle, G., *Salt and water absorption in the human colon: a modern appraisal.* Gut, 1998. **43**(2): p. 294-299.
52. Devroede, G.J. and S.F. Phillips, *Conservation of sodium, chloride, and water by the human colon.* Gastroenterology, 1969. **56**: p. 101-109.

53. Savjani, K.T., A.K. Gajjar, and J.K. Savjani, *Drug solubility: importance and enhancement techniques.* ISRN Pharm, 2012. **2012**: p. 195727.
54. Baka, E., J.E. Comer, and K. Takács-Novák, *Study of equilibrium solubility measurement by saturation shake-flask method using hydrochlorothiazide as model compound.* Journal of pharmaceutical and biomedical analysis, 2008. **46**(2): p. 335-341.
55. Brittain, H.G., *Thermodynamic vs. Kinetic Solubility: Knowing Which is Which.* American Pharmaceutical Review, 2014.
56. *Science*. [cited 2016 June 21]; Available from: http://www.sirius-analytical.com/science/solubility/solubility-definitions.
57. Apley, M., et al., *Determination of Thermodynamic Solubility of Active Pharmaceutical Ingredients for Veterinary Species: A New USP General Chapter.*
58. Blessy, M., et al., *Development of forced degradation and stability indicating studies of drugs—A review.* Journal of Pharmaceutical Analysis, 2014. **4**(3): p. 159-165.
59. Ngwa, G., *Forced degradation as an integral part of HPLC stability-indicating method development.* Drug delivery technology, 2010. **10**(5): p. 56-59.
60. Magari, R.T., *Assessing shelf life using real-time and accelerated stability tests: although accelerated tests are needed, real-time tests are the ultimate proof.* Biopharm international, 2003. **16**(11): p. 36-48.
61. *Inactive Ingredients Database Download*. April 30 2016 [cited 2016 June 22]; Available from: http://www.fda.gov/Drugs/InformationOnDrugs/ucm113978.htm.
62. Birkus, G., et al., *Activation of 9-[(R)-2-[[(S)-[[(S)-1-(Isopropoxycarbonyl)ethyl]amino] phenoxyphosphinyl]-methoxy]propyl]adenine (GS-7340) and other tenofovir phosphonoamidate prodrugs by human proteases.* Mol Pharmacol, 2008. **74**(1): p. 92-100.
63. *Functional Groups*. Available from: http://chemed.chem.purdue.edu/genchem/topicreview/bp/2organic/function.html.
64. Młochowski, J. and H. Wójtowicz-Młochowska, *Developments in synthetic application of selenium (IV) oxide and organoselenium compounds as oxygen donors and oxygen-transfer agents.* Molecules, 2015. **20**(6): p. 10205-10243.
65. JAMES. *1°, 2°, 3°, 4°*. Available from: http://www.masterorganicchemistry.com/2010/06/16/1%C2%B0-2%C2%B0-3%C2%B0-4%C2%B0/.
66. By Michelle Duncan, P., and Irene Zaretsky, MS, *Do the Math for Shelf Life Predict stability using data collected at higher temperatures*. 2011. p. 8.
67. Porter, W.R., *Thermally accelerated degradation and storage temperature design space for liquid products.* Journal of Validation Technology, 2012. **18**(3): p. 73.
68. Antonio, M.A., L.K. Rabe, and S.L. Hillier, *Colonization of the rectum by Lactobacillus species and decreased risk of bacterial vaginosis.* Journal of Infectious Diseases, 2005. **192**(3): p. 394-398.
69. Barreiro-Iglesias, R., et al., *Solubilization and stabilization of camptothecin in micellar solutions of pluronic-g-poly (acrylic acid) copolymers.* Journal of Controlled Release, 2004. **97**(3): p. 537-549.
70. Leung, M.H., H. Colangelo, and T.W. Kee, *Encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis.* Langmuir, 2008. **24**(11): p. 5672-5675.
71. Hendrix, C.W. *Development of Rectal Enema As Microbicide (DREAM)* Available from: http://grantome.com/grant/NIH/U19-AI113127-01.