STABILIZATION OF A TENOFOVIR ALAFENAMIDE FUMARATE FORMULATION FOR USE IN A SUBCUTANEOUS IMPLANT

by

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Scientific advances in the last three decades have helped transform HIV from a fatal disease into a chronic condition for many people. There has been an increase in interest to explore strategies for the prevention of HIV, commonly known as Pre-exposure prophylaxis (PrEP). Currently Truvada®, a once-a-day oral pill, is the only FDA approved marketed regimen for HIV PrEP. Long-acting (LA) systems requiring less frequent dosing remain a major unmet need. A LA system currently under development is a polycaprolactone (PCL)-based biodegradable subcutaneous implant of Tenofovir alafenamide fumarate salt (TAF) in a castor oil paste. This implant showed sustained zero-order release of TAF over 3 months but failed to release drug in zero-order kinetics post 90 days due to poor drug stability. The present work involves the optimization of this implant to achieve stabilization of the TAF formulation inside the PCL device. First, a stability-indicating HPLC method was developed and validated. Then, a suite of pre-formulation studies were performed to better elucidate the mechanisms of TAF degradation in the device. Additionally, a novel stability model was developed that accelerated TAF degradation inside the implant over 8-folds for rapid formulation screening. This model was utilized to study a wide range of excipients belonging to the class of pH and HLB modifiers. Oil substitutes for castor oil were also explored. Controlling the intra-device pH between 5 – 5.5 was found to be the key determinant of TAF stability. pH modifiers showed most promise in stabilizing TAF, with TAF percent recovery between 90 – 110 % vs. control (<5%). Viable pH modifiers were further

optimized for processability in scale-up efforts. Final optimized formulations containing either dibasic sodium phosphate or sodium citrate were found to stabilize TAF in the accelerated model for over 9 weeks. A percent recovery of 90 – 110 % vs. control (0 %) was obtained. In conclusion, two formulations were identified that increased the stability of TAF in the implant. These formulations can be potentially used as bi-annual, long acting subcutaneous systems for HIV PrEP.

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Preface

The work described in this thesis is funded through a collaboration with Research Triangle Institute (RTI) International, NC, USA and funded by the Bill and Melinda Gates Foundation (OPP1149227).

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1.0 Introduction

According to the WHO statistics, more than 70 million people have been infected with the Human Immunodeficiency Virus (HIV). The epidemic has led to the death of about 50% of those infected. The WHO estimates that by the end of 2017, about 36.9 million individuals were living with HIV/ Acquired Immune Deficiency Syndrome (AIDS). Over 940,000 people died due to HIV-related illnesses in the same year. The African region remained the most severely affected, with nearly 1 in every 25 adults (4.1%) living with HIV and accounting for nearly two-thirds of the people living with HIV worldwide (Figure 1) [1]. The HIV epidemic continues to be a major global public health issue [2]. There have been efforts focused on the development of treatment and preventive strategies for HIV, some of which are highlighted in the upcoming sections.

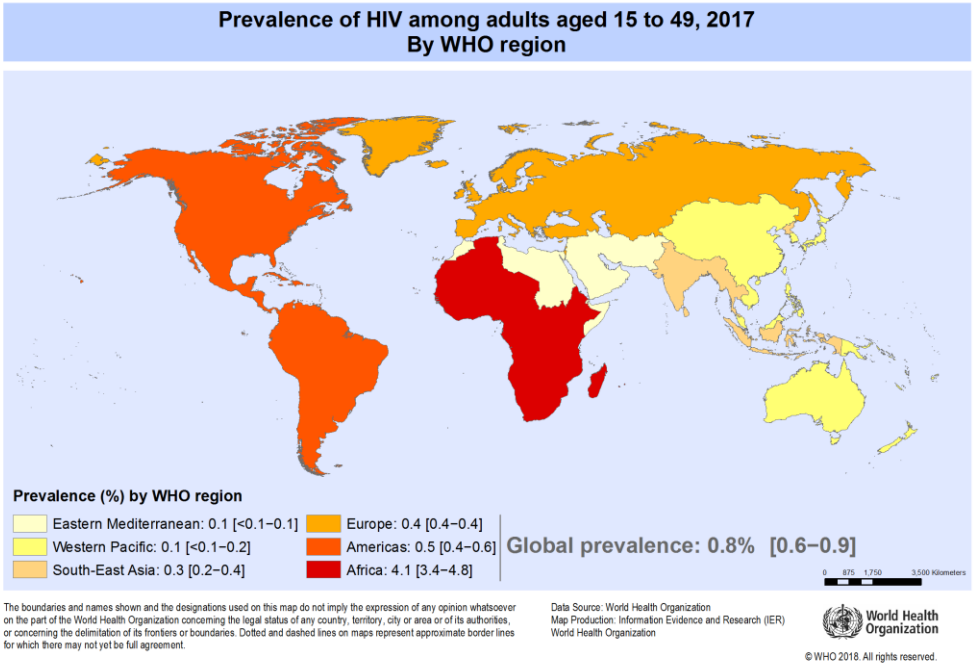

Figure 1 | Prevalence of HIV among adults aged 15 to 49, 2017 (Reprinted with permission from WHO)[1].

1.1 Strategies for tackling HIV infection

For several years, the primary strategy for tackling HIV infection was the treatment of infected individuals. In 1994, a clinical trial in women tested the efficacy of zidovudine to prevent HIV transmission from the mother to the infant [3]. The trial showed that zidovudine is effective in reducing the risk of HIV transmission from mother-infant by approximately two-thirds. This trial marked the milestone which encouraged research in development of preventive strategies for HIV [2]. The two main strategies of tackling HIV, namely treatment and prevention are discussed below.

1.1.1 Treatment of HIV

The primary aim of treating HIV was to prevent clinical progression of the disease while maintaining low drug toxicity and reducing risk of resistance. Identification and characterization of the HIV-1 virus enabled the introduction of azidothymidine or zidovudine (AZT) for HIV treatment in 1987 [4,5]. AZT considerably decreased mortality and opportunistic infections in AIDS patients [6]. Since the introduction of AZT, several antiretroviral (ARV) drugs that target different stages in HIV-1 replication cycle (Figure 2) have been introduced to manage the disease (Table 1). The field was further revolutionized by combination antiretroviral therapy or highly active antiretroviral therapy (HAART) which uses multiple drugs acting on more than one HIV-1 virus life-cycle targets, thereby reducing viral load in the patient. An example of HAART therapy is the introduction of a protease inhibitor and two nucleoside reverse transcriptase inhibitors (NRTIs) which markedly reduced morbidity and mortality [7]. HAART therapy inhibited viral replication, resulting in a long-term suppression of plasma viral load and restoration of the immune function

[8]. Continued therapy of ARVs results in viral load of <50 copies/mL, often to levels considerably lower than the detection levels of currently available clinical assays [9].

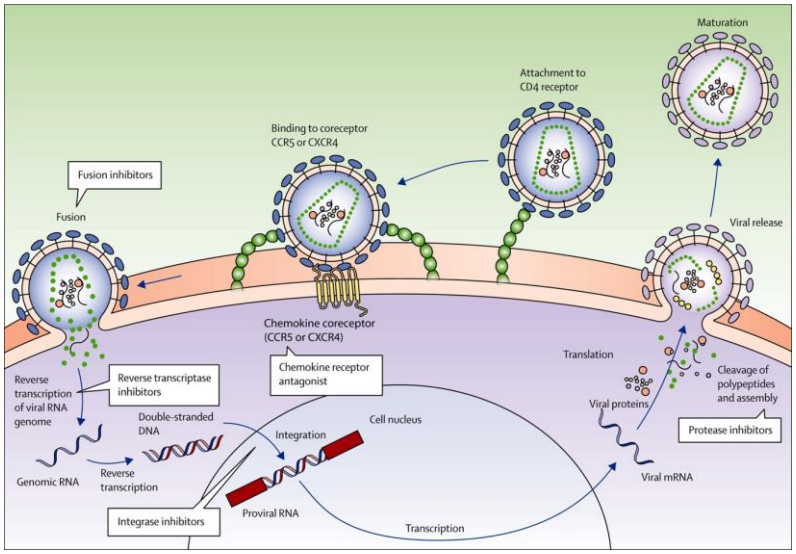

Figure 2 | HIV-1 life cycle illustrating site of action of different classes of ARV drugs. HIV virus fuses to the host site (Fusion inhibitors used) using specific receptors on the host cell and releases its contents inside the cell. Viral DNA is formed by reverse transcriptase enzyme (NRTIs/NNRTIs used). Viral DNA then translocates into the nucleus and integrates with the host DNA (integrase strand transfer inhibitors-InSTI or allosteric integrase Inhibitors-ALLINIs used). Viral RNA copies are used to make viral proteins and genomic unspliced viral RNA assemble to form immature virion buds. Post proteolytic processing, mature virions are formed that can infect other cells (Protease, Capsid or Maturation inhibitors used) [10] Reproduced with permission from Elsevier.

Table 1 | Non-exhaustive list of ARVs used against HIV-1 target sites [11,12].

HIV replication step		ARVs FDA approved	ARV in pipeline
Entry inhibitors		Maraviroc, Enfuvirtide	Fostemsavir, Combinectin, Albuvirtide
Entry inhibitors (monoclonal antibodies)		None	UB-421, Ibalizumab, PRO-140
Reverse transcriptase inhibitors	NRTIs	Emtricitabine, Lamivudine, Zidovudine, Tenofovir alafenamide fumarate, Tenofovir disoproxil fumarate	EFdA, GS-9131

HIV replication step		ARVs FDA approved	ARV in pipeline
	NNRTIs	Efavirenz, Etravirine, Nevirapine, Rilpivirine	Doravirine, Elsufavirine, Rilpivirine long-acting (LA)
Integrase inhibitors		Dolutegravir, Raltegravir	Bictegravir, Cabotegravir, Cabotegravir LA
Protease inhibitors		Atazanavir, Darunavir, Cobicistat, Lopinavir	GS-PS1
Capsid inhibitors		None	GS-CA1
Maturation inhibitors		None	GSK3640254, PA-1050040

1.1.2 Prevention of HIV

Although the progress in HIV treatment has been transformative, HIV is still an incurable disease. It continues to remain a very significant burden on patients, affected communities, healthcare systems and economies around the world [13]. In the absence of an affordable, globally available curative therapy, control of the HIV/AIDS epidemic requires broad implementation of effective and sustainable prevention measures [14].

1.2 Pre-Exposure Prophylaxis

Simply put, pre-exposure prophylaxis or PrEP is the means with which individuals who are at a high risk of contracting HIV, prevent infection. From a clinical perspective, initially, use of condoms coupled with awareness, education and behavioral changes were the only means to effectively prevent HIV infection [15-17]. Male circumcision was found to have compelling epidemiological evidence in preventing transmission of HIV and some other sexually transmitted diseases (STDs) by 50-60%. This was identified over three randomized controlled trails (RCTs)

and over 40 studies [5,18-21]. Development of sensitive and reliable screening methods has almost eliminated the transmission of HIV-1 through blood transfusions [22].

The first proof that ARVs could be effective in PrEP was seen in a clinical trial that studied the reduction in risk of HIV-1 mother-to-infant transmission. It showed that ante- and intra-partum (i.e. time period before and during childbirth respectively) AZT treatment significantly reduced the risk of HIV acquisition by the infant [3,23]. The use of ARVs offered a promising approach to prevent this growing pandemic and change the face of HIV prevention [24].

1.2.1 Oral PrEP

In July 2012, the United States Food and Drug Administration (USFDA) approved Truvada®, a fixed dose combination (FDC) of two NRTIs, Emtricitabine (FTC) and Tenofovir disoproxil fumarate (TDF) in a once-daily pill. There have been numerous clinical trials conducted to evaluate the efficacy of TDF alone or a combination of FTC/TDF as a strategy for oral HIV PrEP. The iPrEx study was a placebo-controlled, randomized clinical trial (RCT) that enrolled 2499 HIV-seronegative transgender women and men who have sex with men (MSM) to study the efficacy of FTC/TDF combination for HIV prophylaxis. A statistically significant ($p=0.005$) reduction in HIV transmission of 44% (95% confidence interval 15, 63) was observed [25]. Another study, the Partners PrEP study, enrolled 4747 HIV-1 serodiscordant couples and randomized the couples to either of the three groups: TDF alone, FTC/TDF combination or placebo. The study found that TDF alone reduces HIV incidence by 67% (95% confidence interval 44, 81, $p<0.001$) and the combination of FTC/TDF reduced HIV incidence by about 75% (95% confidence interval 55, 87, $p<0.001$). The study found no statistically significant difference between the TDF alone and FTC/TDF combination sub-groups [26]. The TDF2 study found a 62% reduction (95%

confidence interval 22, 83, p=0.01) in HIV incidence among men and women uninfected with HIV-1. However, the study was terminated early due to high rates of loss of follow-up [27]. The Bangkok Tenofovir Study studied the efficacy of TDF alone in preventing HIV in people who injected drugs. The study found that TDF alone led to a 48.9% reduction in HIV incidence (95% confidence interval 9.6, 72.2, p=0.01) [28]. To date, daily Truvada® remains the only FDA approved HIV PrEP strategy.

1.2.2 Topical systems for PrEP

Locally acting topical delivery systems offer various advantages over systemically acting conventional solid oral products. Some advantages of topically administered systems include lower risk of systemic toxicity, use as a female-controlled product (where applicable), discretion, self-administration and potential for sustained local delivery. Some topical systems that are being studied for HIV PrEP include vaginal gels, intravaginal rings, vaginal films, vaginal tablets or inserts, and vaginal capsules.

Early research in topical PrEP systems focused on developing over the counter products that incorporate microbicides, offering protection against a broad range of sexually transmitted diseases. The first-generation microbicides that were studied as topical PrEP systems belonged to the class of surfactants. These microbicides were hypothesized to act against a broad range of STIs, including HIV [29]. The use of surfactants was quickly discouraged due to studies showing that this class can potentially increase the rate of infection [30]. Tenofovir (TFV) gel (1%) was the first topical microbicide regimen used in a clinical trial that provided some protection against HIV. The CAPRISA004 study tested the efficacy of 1% TFV gel for HIV prevention when used before and

after sex (Figure 3). It was found that the gel provided an overall 39% reduction in HIV incidence, with increase in adherence leading to increased protection of up to 54% [31].

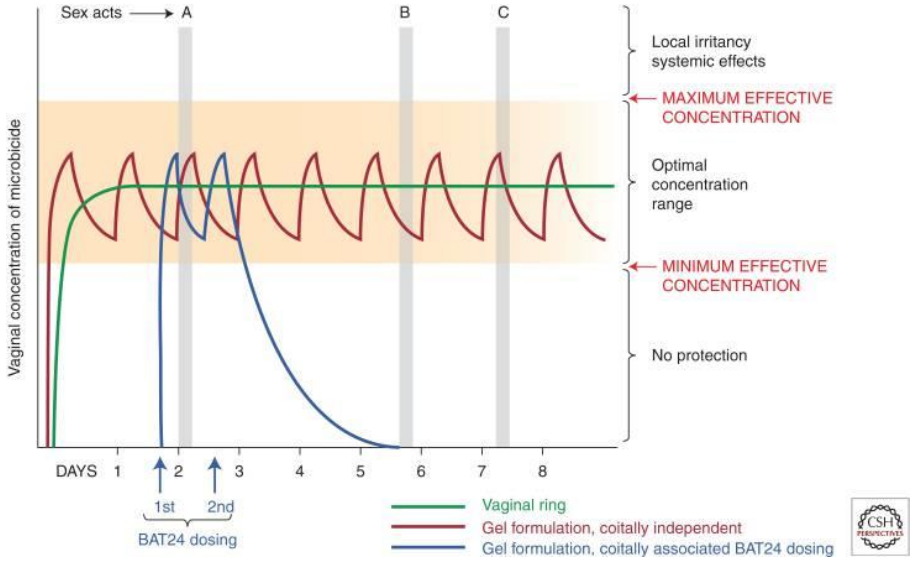

Figure 3 | Early microbicide dosing schedules [29]. Reproduced with permission from Cold Spring Harbor Laboratory Press.

To improve user adherence, intra-vaginal rings (IVR) loaded with microbicides were studied for use in topical PrEP. Torus-shaped IVRs are made with deformable polymers and are filled with the desired drug [30]. Nel, A. and colleagues studied the safety and pharmacokinetics of a dapivirine-loaded IVR in 24 women over a period of 28 days [32]. Dapivirine concentrations of >1000 times the *in vitro* effective concentration values in the vaginal compartment were reported, which further encouraged the development of IVRs in topical PrEP. Drug concentrations of > 4000 times the *in vitro* IC_{99} (concentration at which there is 99% inhibition) in vaginal fluid over a 12-week period have been reported by the same group [30]. The ASPIRE study evaluated the efficacy of a Dapivirine-loaded IVR [33]. This study enrolled 2629 HIV-1 seronegative women. The incidence of HIV infection was found to be reduced by 27% (95% confidence interval 1, 46, $p<0.046$) in the dapivirine ring group vs. the placebo group. Another study (the RING study) was a Phase III study that evaluated the safety and efficacy of the dapivirine ring in women. A 31%

reduction in HIV incidence was found in the dapivirine group when compared to placebo group [34]. Johnson, T. J. and colleagues studied the safety and pharmacokinetics of various TFV-loaded IVRs in an *in vivo* sheep model over a period of 90 days. No significant toxicological effects were observed, demonstrating the potential utility of the IVR for topical PrEP [35]. This high acceptability of current IVRs along with the potential ability to dose every three months, suggest that IVRs may have substantial contribution in HIV PrEP in the near future [36].

Another drug delivery system being studied for topical PrEP is vaginal films. Films are polymeric systems encapsulating the drug in a matrix. Vaginal films can release drug(s) either immediately or over a prolonged period of time. Vaginal films provide advantages of being discrete, reducing product volume and leakage and providing dosing accuracy [37].

It has been recognized that use of PrEP is highly dependent on user acceptability. Thus, it may be essential to prepare multiple dosage forms of the same API in order to provide end-users with choices to select their desired PrEP system [38]. Besides oral and topical systems for PrEP, long-acting injectable systems are being considered for use in HIV PrEP.

1.3 Long-Acting Systems for HIV PrEP

As discussed previously, numerous randomized clinical trials (RCTs) have evaluated the efficacy of employing oral TDF and/or FTC/TDF regimen (iPrEX, Partners PrEP, TDF2, Bangkok Tenofovir Study, PROUD) [39] [25] [26] [27] [28]. Two studies, the FEM-PrEP [40] and VOICE [41] failed to show efficacy of oral PrEP in high risk women. Measurable drug levels could be detected in only 32% females in the FEM-PrEP study and only 29% in the VOICE trial [42]. The safety and efficacy of topical vaginal gel containing 1% TFV has been studied in CAPRISA 004 [31] and VOICE [42]

trials. The range of efficacy of PrEP was found to be between 0 and 75% in the above trials, with a positive link between adherence and efficacy (Figure 4) [43,44]. There is thus a need for long-acting systems that can potentially improve adherence to PrEP regimen.

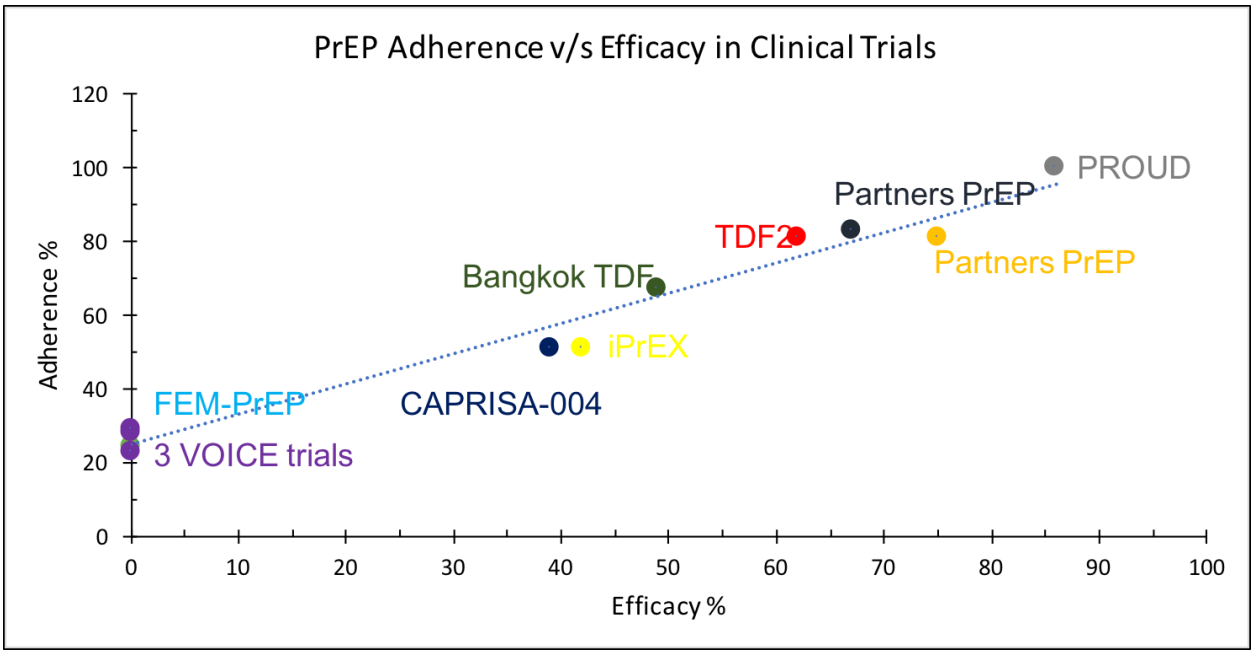

Figure 4 | PrEP Efficacy levels as a function of Adherence levels. As observed in various Clinical Trials conducted on Oral and Topical (approved or under research) PrEP options [45-47].

1.3.1 Need for long-acting PrEP formulations

A crucial advantage of long-acting PrEP formulations is reducing the need of daily dosing in an effort to increase adherence [48]. In the past decade, there is growing evidence and a surge in the research conducted in the area of long acting HIV PrEP. An internet survey of 512 US MSM with median age of 22 years was conducted by Greene et al. The participants were presented with PrEP options of condoms, biomedical interventions of a daily pill or a yearly administered, non-visible, long-acting implant. Over a third of participants preferred condoms as their HIV prevention option, followed by implants and then the pill [49]. A computational modeling study

predicted that long-acting interventions (administered every 8 weeks) will lead to a 44% reduction in newer HIV infections compared to 33% of the daily oral pill [48]. The next section briefly describes some long-acting systems that are being investigated for HIV PrEP.

Long-acting (LA) systems for PrEP have the potential to be administered weekly, monthly, bi-annually or with even lesser frequency. Some of the currently studied LA systems for HIV PrEP include biodegradable or non-biodegradable implants and long-acting injectables. These LA-systems answer numerous challenges that are unmet by oral pills and topical dosage forms. Their attributes include discretion, user-independence, long dosing intervals, reduction in daily pill fatigue and better long-term adherence [30,50]. The following sub-sections highlight few systemic long acting systems for HIV PrEP.

1.3.2 Long-acting Injectables

A survey of over 400 patients that are currently taking daily HIV treatment showed that over 80% of patients would favor a monthly injectable formulation over daily regimens [51]. Currently, there are two LA injectable systems that have advanced to Phase III clinical trials. One contains the HIV integrase strand inhibitor Cabotegravir (CAB). The second system under trial is a combination of crystalline nanoparticulate CAB and Rilpivirine (RPV), an NNRTI. Both these poorly water-soluble drugs are administered every 4 or 8 weeks, as an intra-muscular (IM) depot injection [52-56]. A Phase I study evaluating the pharmacokinetics of RPV as an IM depot was conducted recently [57]. The study showed that RPV suppressed HIV replication in the rectal tissue for up to 4 months.

While the first generation of LA injectable systems that are mentioned above show considerable promise, LA injectables have many hurdles to overcome before reaching the market.

Challenges include irreversibility in case of toxicity in patients, possibility of dose dumping, release rate control and seroconversion of the HIV virus to its resistant strains after discontinuation [30,58-60]. The patients can show signs of toxicity to ARVs used in LA systems. Since controlling adverse reactions after administration of injections becomes difficult, a month-long oral lead-in period prior to the administration of the LA preparation was performed in the aforementioned trials [61]. The release rate is also a critical criterion as 'dose dumping' should be prevented. While stopping the LA PrEP regimen, there is a dangling sword of a pharmacokinetic tail i.e. below protective concentrations of ARV in plasma for a long period of time. Exposure to the virus during this phase may result in seroconversion to a resistant strain of HIV. For example, one case of seroconversion with NNRTI-resistant virus strain was observed 80 days after stopping a 300 mg IM dose of RPV LA injectable therapy [62]. Close monitoring after discontinuation is thus critical when using a long-acting injectable system.

1.3.3 Implants

There are various implants that are currently under development for long-acting HIV PrEP. Chua, C. Y. X. and coworkers are currently investigating a refillable transcutaneous nanofluidic implant for FTC and Tenofovir Alafenamide fumarate (TAF), an NRTI. [63]. This implant has a medical grade titanium drug reservoir with two silicone resealable ports (venting port and drug loading port) for transcutaneous refilling. The titanium reservoir can be loaded with either 3g of FTC powder or 200mg of TAF powder. The drug-loaded device was studied in an *in vivo* macaque model. The device was implanted subcutaneously in dorsal scapular region. The device was refilled by attaching needles to the venting and drug loading ports. The needle attached to the venting port was used to withdraw and remove any remaining drug present in the device. This

created a negative pressure inside the titanium drug reservoir, drawing fluid from the drug loading syringe, thereby refilling the device. This implant demonstrated sustained release of both the drugs over 83 days with clinically relevant preventive TFV-diphosphate (TFV-DP) concentrations (the active drug metabolite of TAF) achieved within 3 days after implantation. However, the 'refillable' attribute can have unfavorable consequences as it opens up avenues for complications during the refilling step and the overall feasibility needs to be proven.

Merck & Co. is currently developing a drug-eluting polymeric implant of an investigational drug MK-8591 (4'-ethynyl-2-fluoro-deoxyadenosine; EFdA), a NNRTI [64]. EFdA is one of the most potent ARVs with an EC_{50} value in lower nM to pM range [65]. Its extraordinary potency coupled with the long intracellular half-life of its active metabolite, EFdA-triphosphate makes it an excellent ARV choice for implants. EFdA was formulated into hot melt extruded, monolithic, bioerodible (either acid terminated poly (lactic acid) (PLA) or ester terminated poly(caprolactone) (PCL) polymeric) and non-bioerodible (Poly (ethylene vinyl acetate) (EVA) polymeric) implants. In pre-clinical studies of EFdA PCL implant in rodents, drug release was maintained at 10µg/day and cumulative drug release was approximately 45% at 17.6 months. In the non-human primate model, administration of EFdA implant achieved clinically-relevant preventive and sustained drug levels over 6 months. Being an investigational drug, although the initial results are very encouraging, its suitability, efficacy and toxicity in larger clinical trial settings still needs to be answered.

Researchers at the Oak Crest Institute, CA have developed a match-stick size (40 x 1.9 mm) subdermal implant that delivers TAF in an *in vitro* sustained, pseudo zero-order release between 4 and 30 days at 0.92mg/day [66]. A proof of principle study in beagle dogs demonstrated good release over 40 days and no adverse events related to the treatment. This implant is a medical-

grade platinum cured silicone scaffold tube with 14 delivery channels that are mechanically punched into it [67]. The team aims to optimize the implant for sustained release to provide one year of drug delivery.

Intarcia is a Boston-based company that licensed the Duros® technology from Alza as the Medici Drug Delivery System™. This titanium implant consists of a mini-osmotic pump that delivers a steady rate of drug housed in its reservoir cavity. The Medici platform is currently under a second USFDA review after successfully completing Phase III trials for year-long delivery of exenatide, a peptide indicated for Type 2 diabetes. The company is currently in the pre-clinical stage of evaluating the Medici platform to administer TAF subcutaneously once or twice a year [68].

1.4 Long-acting Thin Film Polymeric Device for HIV PrEP

Another biodegradable implant under development is by researchers at Research Triangle Institute (RTI) International, NC. The initial prototype developed was the Thin Film Polymeric Device (TFPD) containing TAF as the ARV [69]. The TFPD is a reservoir type implant containing TAF slurry or paste with castor oil, that relies on the surrounding biological fluid to penetrate the drug paste, where it solubilizes and creates a constant diffusion flux out of the implant. Initial prototypes of the device were fabricated using PCL films of various thicknesses. This 'thin film' was wrapped around a rod-shaped mold of desired diameter to form a tube. The cylindrical tube was sealed at one end and filled with a slurry of TAF in PEG 300. The drug filled tube was finally sealed at the other end to form the TFPD implant. The release rates varied between 0.4 – 2.5 mg/day, depending on the thickness of PCL used and length of the implant. A linear release rate was observed throughout the life of the implant, but non-linear kinetics were observed when TAF

was almost entirely depleted in the implant. TAF stability remained unchanged within the device up to 49 days, after which a 19% reduction in TAF stability was observed until 89 days (n=1). This decrease was most likely due to hydrolysis of TAF into its degradants [70].

The TFPD prototype has since then been modified to improve processability and facilitate scale-up. The modified device is fabricated by hot melt extrusion process. PCL polymer (80kDa) at different thicknesses of 70, 100, and 200µm are extruded. These PCL tubes are then filled with the TAF slurry and crimp sealed using an impulse heat-sealer to form the final implant, now called the extruded polymeric device (EXPD) (Figure 5).

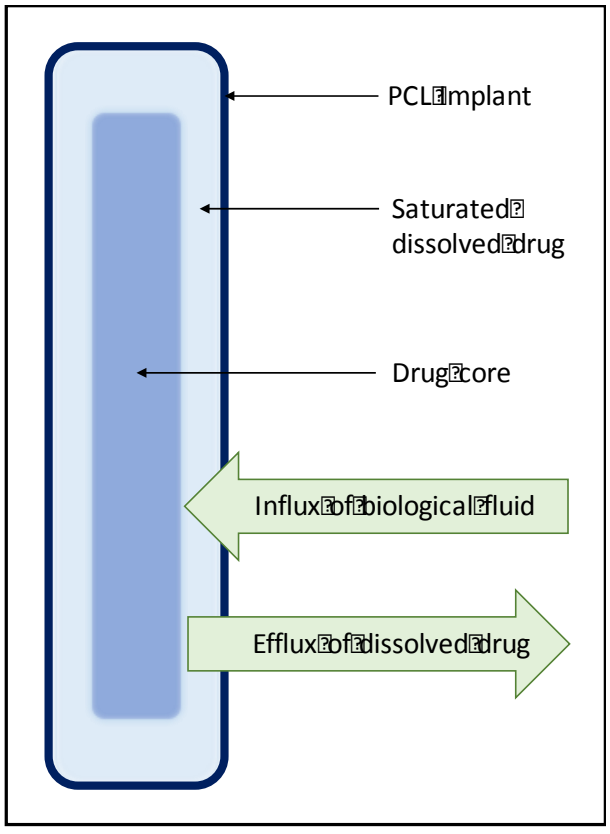

Figure 5 | Reservoir based PCL EXPD implant (adapted from [69]).

EXPD is a biodegradable, subcutaneous PCL device that can achieve sustained zero-order release of TAF. The device is compatible with existing trocar applicators like those used with

Implanon® and Jadelle®. A trocar is used to deliver many contraceptive implants [71]. Use of a commercially available applicator to deliver these implants can reduce cost and improve its accessibility, which is particularly important in developing nations. The following sections describe the properties of PCL and TAF, which are the two main components of the implant. Studies performed using the EXPD implant will be discussed in section 1.4.3.

1.4.1 PCL Polymer for drug delivery

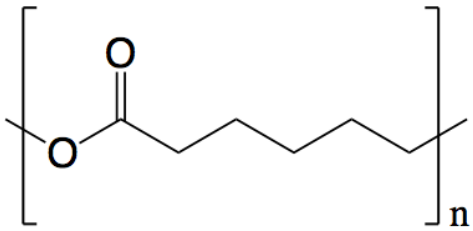

Figure 6 | Poly (ε-caprolactone).

Poly(ε-caprolactone) (PCL) (Figure 6) was one of the earliest biodegradable polymers first synthesized in the 1930's [72,73]. PCL is semi-crystalline in nature. The amorphous regions have a glass transition temperature of -60°C and the crystalline parts melt between 59 – 64°C. PCL polymers are graded by molecular weight, which can vary anywhere from 3 – 80 kDa [74]. PCL is being increasingly recognized as a suitable polymer not only for tissue engineering applications but also for long-acting drug-eluting systems [75].

PCL has good flexibility, can be formulated into a variety of shapes, is non-toxic and highly bio-compatible [76]. The homopolymer of PCL has a long biodegradation rate of 2-4 years. Non-enzymatic cleavage of PCL begins in the polymer's amorphous regions, leading to formation of carboxyl end-groups, which cause further polymer degradation due to auto-catalysis [77]. The initial

cleavage of bonds in amorphous regions is dependent on the permeability of water into the formulation. Increase in polymer molecular weight increases the hydrophobicity, reducing the rate of polymer hydrolysis [76]. However, PCL can be chemically modified by co-polymerization with more hydrophilic polymers, thereby increasing its degradation rate [78]. The homopolymer of PCL is most suitable for long-acting systems which require the drug to be released over long periods of time, usually over 6 months. PCL also has a higher permeability to small molecules and its long degradation time leads to slow formation of acidic monomers. Thus, PCL does not create an acidic environment *in vivo*, unlike polymers composed of lactic acid and glycolic acid [78]. This is especially advantageous for drugs that are sensitive to hydrolytic cleavage in the presence of acids. Once degraded, the polymer gets fully resorbed and excreted from the body.

The most important advantage of PCL is that it is approved by the FDA and there are several PCL-based products, such as Capronor®, that are currently in the market. This makes PCL an attractive polymer not only for academic research but also for clinical use. Much research has been conducted for use of PCL in drug-delivery systems such as microspheres, nanospheres, sutures, wound dressings and dental fixtures [75]. PCL-based microspheres and implants have been used for injectable systems intended to deliver contraceptives [79,80]. Recently, thin film devices of PCL have also been reported to be studied for ocular drug delivery and for HIV PrEP [81].

1.4.2 Use of TAF as an anti-retroviral in EXPD Implant

Although a number of ARVs are available for treatment and prevention of HIV, many of them are not suitable for long-acting PrEP systems. ARVs with high potency are most suitable to provide sustained drug levels, practical dose loading in the system and longer durations of action [52]. Other properties of the ARV that play a role in drug selection for LA systems include its

physicochemical properties like solubility, log P (partition coefficient), molecular size and pKa, and pharmacokinetic parameters like $t_{½}$, volume of distribution, t_{max} and C_{max}. These properties also play a role during formulation development, assessing drug stability, rate of drug release and duration of action from the implant [82].

The use of TAF as a potential ARV for LA systems has been recognized recently. This second-generation prodrug was developed to overcome the drawbacks presented by its parent drug Tenofovir and Tenofovir disoproxil fumarate (a first-generation prodrug of tenofovir). Some of the challenges exhibited by these two drugs are summarized below.

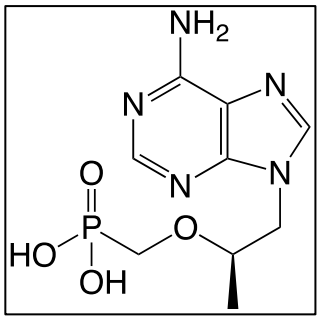

Figure 7 | Tenofovir.

Tenofovir (TFV), an NRTI, is a nucleotide analog of adenosine monophosphate. Chemically, it is (9-[(R)-2(phosphonomethoxy)propyl] adenine) and is also known as PMPA (Figure 7). It is a hydrophilic compound with a low oral bioavailability (around 17.1%) as seen animal models, as it exists in its anionic form at physiological pH and does not undergo diffusion across gastrointestinal tract membranes [83,84].

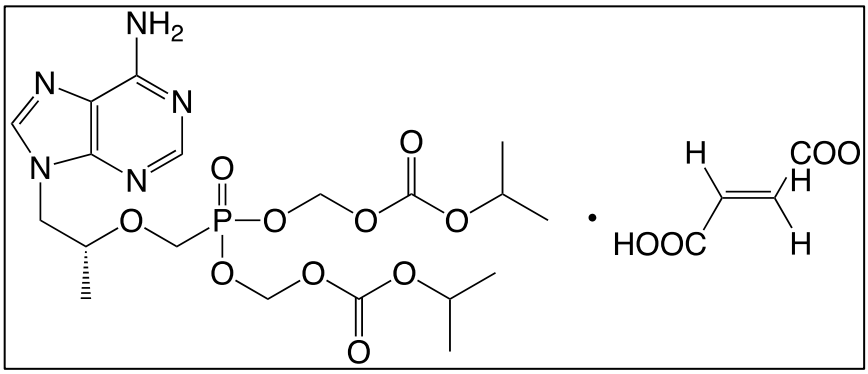

Figure 8 | Tenofovir Disoproxil Fumarate.

TDF (Figure 8), the first-generation bis-phosphonate ester-prodrug of TFV was approved by FDA in 2001 and was marketed as Viread®, a 300mg once-a-day oral pill. It is a potent drug with a long plasma half-life of 17h. TDF is absorbed from the GI tract and gets hydrolyzed to TFV. TFV undergoes intracellular uptake where it is phosphorylated twice to its active form, TFV-DP. TFV-DP competes with indigenous nucleotide deoxyadenosine 5'-triphosphate and inhibits activity of HIV RT by incorporating into viral DNA and causing chain termination [85]. However, it was observed that TFV eliminated mainly by the kidneys, in its unchanged form in urine (~50%) by both glomerular filtration and active tubular secretion [86,87]. Prolonged residence of TFV in plasma showed signs of progressive tubular nephrotoxicity causing moderate-to-severe renal impairment, along with potential risks of bone fracture and osteoporosis [88,89]. TDF is marketed as prescription fixed dose combinations (FDCs) with a number of other ARVs. FDCs include Atripla® (TDF/FTC/efavirenz), Complera® (TDF/FTC/RPV), Stribild (TDF/FTC/elvitegravir (EVG)/cobicistat (COBI)), Cimduo (Lamivudine, TDF), Delstrigo (Doravirine, Lamivudine, TDF), Truvada (TDF/FTC), etc.

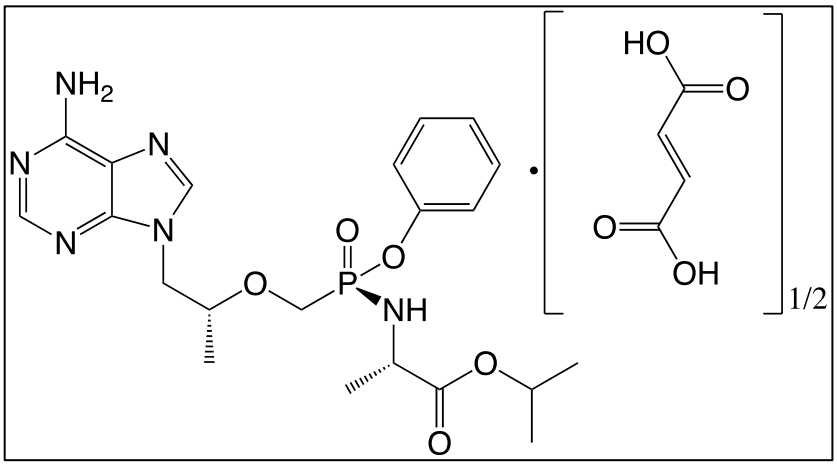

Figure 9 | Tenofovir Alafenamide Fumarate.

Tenofovir Alafenamide Fumarate (TAF or GS-7340) (Figure 9), a second-generation phosphonoamidate prodrug of TFV was developed as a more potent, pharmacologically safer prodrug of TFV [90] (Table 2). It was approved by the FDA in 2015 as Genvoya®, a FDC of COBI, EVG, FTC and TAF (10mg oral dose of TAF). TAF is isopropyl (2S)-2-(1R)-2-(6-aminopurine-9-yl)-1-methyl-ethoxy] methyl-phenoxy-phosphoryl amino propanoate, present as a hemifumarate salt. It is a purine analog retaining the backbone scaffold of TDF but having difference side chains. It is marketed as FDCs Biktarvy (Bictegravir sodium, FTC, TAF), Descovy (FTC, TAF), Genvoya (COBI, EVG, FTC, TAF) and Odefsey (FTC, RPV, TAF), among others.

Clinical trials have demonstrated that TAF significantly reduces renal and bone toxicities when compared to TDF [89,91]. TAF has started replacing TDF in many of the fixed dose combinations filed with the FDA. It has not yet received approval to be used for HIV PrEP. Table 2 compares the various physicochemical and pharmacokinetic properties of TFV and its prodrugs.

Table 2 | Comparison of physicochemical properties of TFV and its prodrugs.

Parameters	TFV	TDF	TAF	References
Solubility in water	~5mg/mL	13.4mg/mL	~4.7mg/mL	[92,93]
Log p	1.25		1.6	[94]
pKa	3.75		3.96	[94]

Parameters	TFV	TDF	TAF	References
Clinical dose	30mg TDF		10mg	[94]
FDA approval for treatment (Year)	✓ (2001)		✓ (2015)	[95]
FDA approval for PrEP (Year)	✓ (2012)		✗	
Pharmacokinetic profiles				
Bioavailability (oral)	25% fasting 39% high-fat meal		35%	[96]
$t_{½}$ (hr)	17hr as TFV		0.51hr	[94]
EC_{50} HIV-1 (PMBCs)	1.2µM	0.015µM	0.003µM	[97,98]
Intracellular half-life	150-180 hr as TFV-DP			[93]
Major elimination pathway	Renal (~50%) unchanged		Metabolism (>80%)	[95]
C_{max}	300 ng/mL		160 ng/mL	[93]
T_{max}	2.3 hr		1 hr	[93]
AUC	2290 ng.hr/mL		210 ng.hr/mL	[93]

1.4.2.1 Mechanism of Action

TAF is marketed as a safer and more potent prodrug of TFV. TAF requires Cathepsin A for conversion to TFV. The lysosomal carboxypeptidase Cathepsin A is a ubiquitously expressed hydrolase enzyme with particularly high expression in lymphoid cells [99,100]. Cathepsin A hydrolyzes the carboxyester bond of TAF to produce TFV. Dependence of TAF on Cathepsin A for activation reduces is conversion to TFV in the gut or plasma. Thus, it is very efficient in delivering TFV directly to the peripheral blood mononuclear cells (PBMCs), such as lymphocytes and other HIV targets (over 1000-fold increase compared to TFV and 30-fold increase over TDF). Inside the cells, TAF gets converted to TFV, which is eventually phosphorylated [101] (Figure 10). As mentioned previously, the diphosphate form of TFV or TFV-DP acts as a competitive RT inhibitor, preventing HIV proliferation. TAF produces metabolites that are impermeable and hence get effectively trapped into the cells, reducing their plasma exposures. The superior ability of TAF

to deliver and retain TFV primarily in the cells makes it safer as high plasma levels of TFV are known to be associated with nephrotoxicity. This ability of TAF to concentrate TFV intracellularly also makes it very potent, allowing use of a very low dose to provide clinical levels of protection against HIV infection [101].

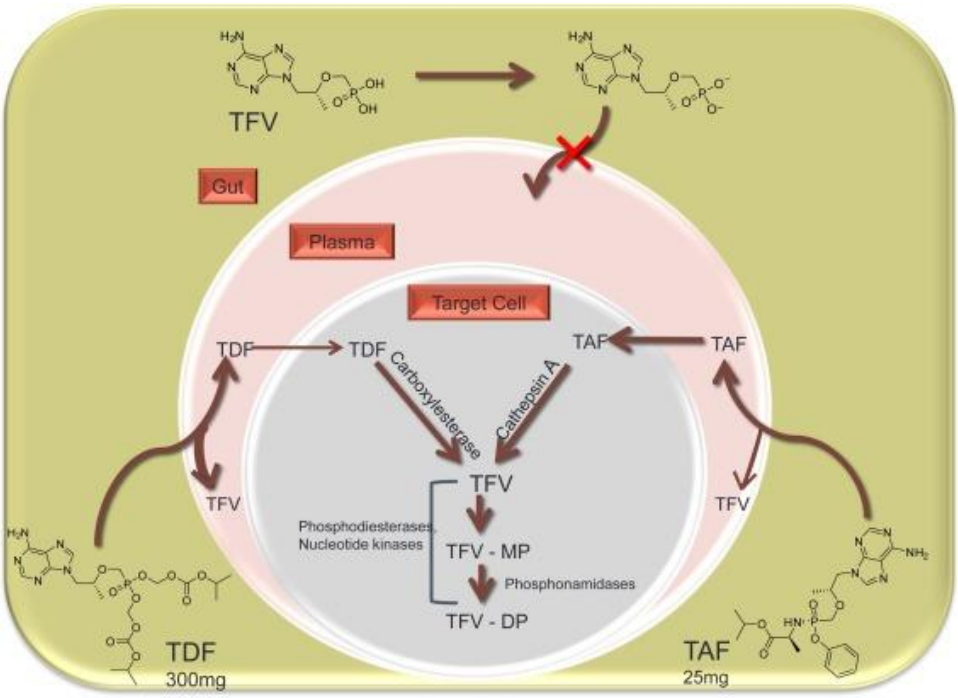

Figure 10 | Pharmacokinetic mechanism of action of TFV, TDF and TAF [102]. Reproduced with permission from Elsevier.

1.4.3 Previous studies on RTI's TAF EXPD Implant

An exploratory *in vivo* study was conducted for the EXPD implant (RTI International, NC, USA) in rabbits for three dose levels of TAF (low dose releasing 0.2mg/d; medium dose releasing 0.4mg/d; high dose releasing 0.8mg/d) for a period of 63 days [103]. *In vivo* PBMC TFV-DP levels were between 443±529 and 1293±905 fmol/10^6 cells for low and high dose respectively. Median vaginal and cervical tissue levels of TFV-DP achieved in the high dose group were 139 fmol/mg

and 74 fmol/mg. A *post hoc* analysis of cryopreserved PBMCs from the iPrEx study determined that a concentration of 16fmol/10^6 cells of TFV-DP was associated with 90% protection [104]. However, cryopreservation can lead to a 33 – 67% loss of TFV-DP. Thus, TFV-DP concentrations more than 16-48 fmol/10^6 cells have been predicted to be required for HIV PrEP [66]. The levels of TFV-DP in the *in vivo* study for EXPD implant far exceeded the predicted preventive levels for HIV PrEP.

Another pharmacokinetic study was conducted in rabbits over 3 months using the TAF EXPD implant. Overall, PBMC, TFV-DP and plasma TFV were sustained throughout the 91-day study, with plasma TAF concentrations being detected as early as 6 hours post implantation. Individual data from rabbits at day 49 and day 91 had similar profiles for the aforementioned analytes, demonstrating that the EXPD implant had similar profiles sustained over 3 months. A daily release rate of 0.75±0.06 mg/d was observed in an *in vitro* release experiment performed in parallel with the rabbit study. Although a zero-order profile was observed initially, by 80 days some of the EXPDs started showing erratic release profiles. Also, significant degradation of TAF was observed in the implants. At day 91, TAF stability was found to be between 5.3-10%. This warranted additional insight into stability issues of TAF as an API and optimization of the EXPD implant formulation to achieve sustained release for 6 months [105].

The EXPD implant developed by RTI is made from PCL of 80kDa molecular weight. The implant has an outer diameter (OD) of 2.5mm and length of 40mm. It can be made of variable thicknesses, including 45, 70, 100 and 200µm [106]. Implants with thickness ≥100µm, demonstrated sustained *in vitro* release profiles for over four months. The group has also established shelf life stability (≥ 95% stability of TAF) of the implant at room and accelerated storage conditions over 6 months [106]. When this implant was studied in female rabbits, it was observed that the implants

were easy to administer and retrieve. These implants were also well tolerated and showed minimal local tissue reactivity [107].

End-user feedback studies were conducted for the EXPD implant by surveying 105 young men and women in South Africa to offer insight on their preferred attributes. Discreetness, flexibility of the implant, long duration (>6 months), biodegradable nature and comfort were the major desired attributes identified by the study population. Possibility of any side effects and 'plastic' appearance of the implant could potentially disincentivize users [108]. On-going efforts focus on developing a PCL implant containing TAF showing sustained zero-order release of around six months.

1.4.3.1 Stability issues of TAF

Stress studies are usually performed during the pre-formulation stage of drug product development. They are vital to mechanistically understand the degradation profile and kinetics of the parent Active Pharmaceutical Ingredient (API). This helps us to identify potential causes of degradation and instability of the molecule which can be mitigated using various strategies including formulation composition. Stress studies performed on TAF revealed that the API was highly sensitive to hydrolytic degradation, particularly acid mediated degradation [102]. Acidic pH causes cleavage of P-N bond of the phosphoramidate group in TAF [109], while alkaline pH causes cleavage of P-O bond [110] (Figure 11).

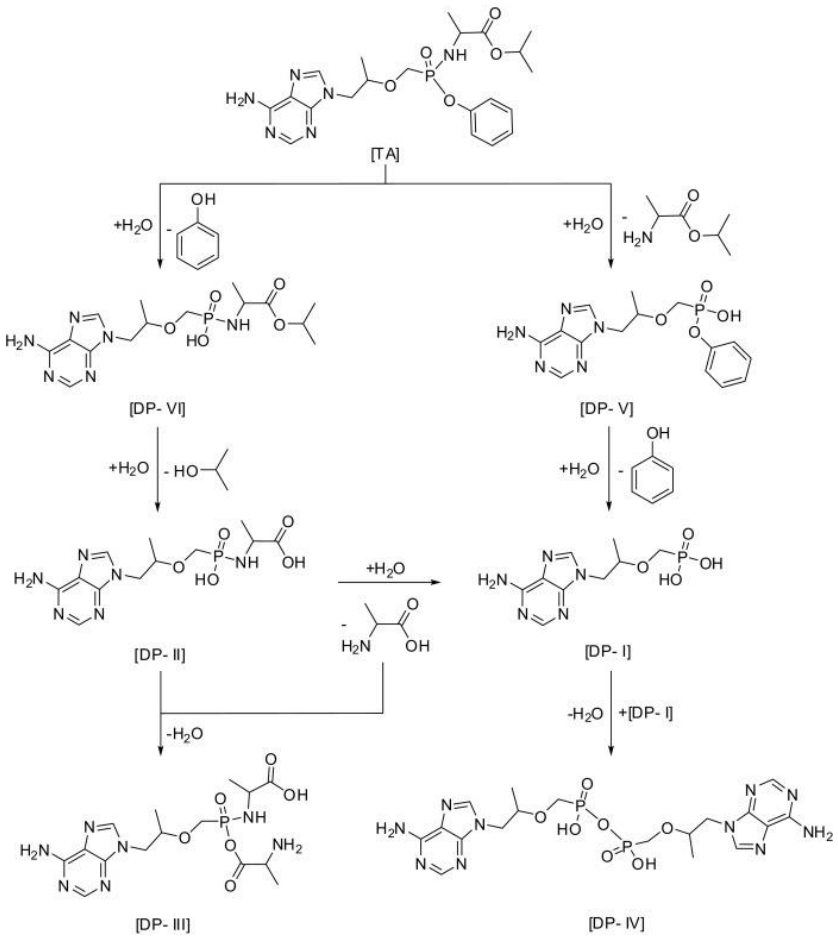

Figure 11 | Potential degradation pathway of TAF under hydrolytic conditions [102]. Reproduced with permission from Elsevier.

1.4.4 Optimization of RTI EXPD Formulation – An Unmet Need

Studies on the EXPD implant have highlighted need to optimize the formulation and device characteristics (thickness of PCL, molecular number and molecular weight) in order to maintain stability of TAF for the lifetime of the device [105,111,112]. The device is a PCL shell with an inner core of TAF paste. Optimization of the core formulation for stabilization of TAF was required to achieve a suitable device that could provide sustained drug release for TAF over a period of 6 months while restricting levels of TAF degradants in the core of the device to more acceptable

levels. Instability of the device as it approaches depletion may be attributed to the hydrolytic instability of the API by itself and the inability of the excipients to prevent degradation.

1.4.4.1 Lack of model to predict degradation

The studies mentioned above indicate a reduction in the stability of TAF in the device towards the end of device study. Real time *in vitro* stability and release studies for a 6-month implant are extremely time consuming. In order to optimize the core formulation, it was first required to develop an accelerated degradation model. Employing accelerated models that predict potential real-time drug release or stability has been widely utilized in formulation development [113,114]. Forced degradation studies on drug substance and product are a routinely followed protocol for any regulatory submission. Although accelerated models can be applied to quickly screen formulations, it is to be noted that unless they employ approved compendial method for analysis, real-time release and stability have to be demonstrated [115].

The **scope of work** for this thesis is derived from the unmet needs discussed above and revolves around the optimization of TAF core suspension formulation that is filled inside the EXPD implant.

2.0 Project Hypothesis and Goal

The overarching **project goal** was to optimize the TAF formulation in a long-acting PCL subcutaneous implant for HIV PrEP. This implant was previously developed by our collaborators, RTI International, NC and is intended for bi-annual administration. It consists of a 3:1 ratio of TAF dispersed in castor oil, filled in a PCL polymer tube to form a monophasic reservoir-based implant system. As illustrated in Figure 5, once the device is implanted, it draws in the surrounding physiological fluid, thereby assuming a biphasic system of an oily phase (castor oil) and an aqueous phase (physiological fluid). TAF migrates to the aqueous phase where it dissolves and eventually diffuses out of the implant following zero-order release kinetics. In an *in vitro* release study conducted on this implant by our collaborators, TAF stability reduced by over 90% at the end of 3 months. Erratic drug release profiles were observed around 90 days in the *in vitro* study [105].

The erratic drug release and poor drug stability that were observed previously can be attributed to two potential causes. Firstly, prior literature suggests that TAF degrades in acidic as well as basic pH environments [102]. This can be mitigated by altering and maintaining the intra-device pH to a range where TAF displays maximum stability. Maintaining intra-device pH can potentially reduce the degradation of TAF and increase the duration of action of the implant. Secondly, TAF is susceptible to hydrolytic degradation when exposed to an aqueous environment. Thus, if the migration of TAF from the oily phase of castor oil to the aqueous phase inside the implant is reduced by modulating the partitioning behavior of TAF, it would reduce the possibility of TAF degradation. Hence the project **hypotheses** derived are two-fold:

A. Modifying the partitioning behavior of TAF from the oil phase to the aqueous phase inside the polymeric implant will alleviate hydrolytic degradation of TAF
B. Modifying the pH environment inside the polymeric implant will assist in stabilization of TAF by preventing hydrolytic degradation

In order to achieve the project hypotheses, the specific aims identified are:

Specific Aim 1: To perform pre-formulation mechanistic studies to understand how TAF destabilizes in the current formulation in PCL device

Specific Aim 2: To develop a screening method to accelerate TAF degradation in device for excipient screening

Specific Aim 3: To perform formulation modification and optimization for improving the stability of TAF in implant.

Chapter 4 describes the development and validation of a stability-indicating HPLC method that was used to quantify TAF and some of its degradants in the rest of the work. Chapters 5, 6 and 7 delineate the methods undertaken and results obtained to address specific aims 1, 2 and 3 respectively.

3.0 Materials

TAF API (Gilead, CA, USA), Fumaric acid (FA) (TCI, OR, USA, >99% purity) Tenofovir-phenyl (TFV-P) (BOC Sciences, NY, USA, 98+% purity) and Tenofovir (TFV) (Gilead, CA, USA) were kindly provided by RTI International, Durham, NC. PCL EXPD (Sigma grade (Sigma Aldrich, USA) and PuraSorb PC12 medical grade (Corbion Purac, Netherlands) 80kDa, 100µm thickness, 2.5mm outer diameter, ~6cm length) tubes were kindly provided by RTI International, Durham, NC. Acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC Grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Buffer components – monobasic potassium phosphate (KH_2PO_4, ACS grade) and dibasic potassium phosphate (K_2HPO_4, USP grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA); tetrabutyl ammonium bromide (t-BAB, 99+% purity) was obtained from ACROS Organics (NJ, USA). Ultrapure water was obtained from an in-house Milli-Q® water purification system (Millipore Sigma Advantage A10). Dibasic sodium phosphate (Fisher Scientific, US, USP grade), Sodium citrate dehydrate granular (Spectrum, US, USP grade), Glycine (Spectrum, US, USP grade), Meglumine (Spectrum, US, USP grade), Tromethamine (Spectrum, US, USP grade) were used. Castor oil (Spectrum, USA, USP grade), Anise oil (Spectrum, US, FCC grade), Corn oil (Spectrum, US, NF grade), Cottonseed oil (Spectrum, US, NF grade) and Soybean oil (Spectrum, US, NF grade) were used. Super-refined castor oil (Croda, USA, USP/NF grade) and Super-refined sesame (Croda, USA, USP/NF grade) samples were kindly provided by Croda, USA. Labrafac Lipophile WL 1349 (Gatteefosse, France, USP/NF Grade), Labrafil M 2130 CS (Gatteefosse, France, USP/NF Grade), Gelot 64 (Gatteefosse, France) and Transcutol HP (Gatteefosse, France, EP/USP/NF Grade) samples were kindly provided by Gatteefosse, France.

4.0 Stability indicating HPLC Analytical Method Development and Validation

HPLC analysis is an essential tool utilized during the entire life-cycle of pharmaceutical product development [116]. It is used to separate and quantify the drug substance and its degradants. After developing an analytical method, it is crucial to establish the reliability of the output data. This is performed by validating the method as per set guidelines like the International Conference on Harmonization (ICH) guidelines. Validation proves that the developed analytical method can 'demonstrate that it is suitable for its intended purpose' [117]. In this chapter, a stability indicating HPLC method was developed and validated for TAF and its degradants as per ICH guidelines.

4.1 Methods

4.1.1 Preparation of standard solutions

Standard solutions of TAF, FA and degradants TFV and TFV-P were prepared. TAF standard was prepared by dissolving TAF API in ACN: MeOH: Water 22:10:68 to obtain a concentration of 500µg/mL. This stock was used for further dilutions for the calibration curve from 10µg/mL to 200µg/mL. TFV stock solution was prepared by dissolving TFV in ACN: MeOH: Water 22:10:68 to obtain a concentration of 500µg/mL. TFV-P stock solution was prepared by dissolving TFV-P in ACN: MeOH: Water 22:10:68 to obtain a concentration of 500µg/mL. FA stock solution was prepared by dissolving FA in ACN: MeOH: Water 22:10:68 to obtain a concentration of 500µg/mL. This stock was used for specificity studies.

4.1.2 Preparation of mobile phase

The mobile phase was composed of (25mM phosphate buffer+ 5mM tetrabutylammonium bromide (t-BAB) pH 6.0) (Buffer pH 6.0) and Methanol in a ratio of 80:20. Phosphate buffer comprised of dibasic potassium phosphate and monobasic potassium phosphate adjusted to pH 6.0 using a 30% H_3PO_4 solution.

4.1.3 HPLC System and Chromatographic conditions

The HPLC system (Thermo Scientific, Dionex UltiMate 3000 UHPLC+ focused) consisting of an auto-sampler injector, a quaternary pump, column oven and a diode array detector was used. Chromeleon™ Chromatography Data System 7.0 software was used for HPLC equipment control, data acquisition and integration. Chromatographic separation was achieved using a C18 reverse phase column with tetramethylsilyl (TMS) endcapping (Phenomenex, Gemini® 5 µm C18 110 Å, LC Column 150mm x 4.6 mm) protected by a Guard Cartridge (Phenomenex, SecurityGuard™ cartridges with 2.0 internal diameter) for separating TAF and degradants. The chromatographic conditions are summarized in Table 3.

Table 3 | Chromatographic conditions.

Column	Phenomenex Gemini C18 150x4.6 mm, 110Å, 5µm
Mobile phase	MPh A: Buffer pH 6.0: Methanol 80:20 MPh B: Acetonitrile 100%
UV	260 nm
Flow	1 mL/min
Solvent	Acetonitrile: Methanol: H_2O 22:10:68 (for standards) Methanol 100% & Acetonitrile: H_2O 25: 75 (for samples)

Injection volume	20 uL
Column temperature	**25°C**
Auto-sampler temperature	**5°C**
Run time	17 min

The analysis was performed with a gradient elution (Table 4) of mobile phase A (MPh A) composed of (25mM phosphate buffer+ 5mM tetrabutylammonium bromide (t-BAB) pH 6.0) (Buffer pH 6.0) and Methanol in a ratio of 80:20, and mobile phase B (MPh B) composed of 100% Acetonitrile (ACN). The flow rate was 1.0 mL/min, with 20 µL injection volume at a run time of 17 minutes. Auto-sampler was set at 5°C and column temperature at 25°C. TAF, TFV, TFV-P and FA were determined by ultra-violet (UV) detection at 260nm.

Table 4 | Gradient for HPLC analytical method.

Time (min)	MPh A (%)	MPh B (%)	Curve
0	95	5	-
6	95	5	5
8	75	25	1
12	75	25	5
14	95	5	5
17	95	5	5

4.1.4 Drug content

Drug content was determined using the developed and validated HPLC method described above. The current device consists of a PCL device containing TAF in a castor oil based slurry. To analyze TAF drug content, it is essential to extract TAF from the dosage form. PCL device containing TAF paste was cut from one end using sharp clean scissors and was placed in a scintillation vial containing 10mL 100% methanol and stirred for 1 hour to ensure complete dissolution of paste. This was further diluted 10 times using 25% ACN in water and injected in the HPLC system. If turbidity was observed after dilution, they were filtered using 13mm 0.22µm

PTFE syringe filters (Restek™) and then injected. Working concentration for TAF samples was set to be around 100µg/mL in the matrix.

4.1.5 Analytical method validation

For this project a stability indicating HPLC analytical method was developed and validated according to International Conference on Harmonization (ICH) guidelines [117].

Specificity of the analytical method is the ability of the method to quantify the analyte of interest in the presence of degradants, formulation excipients, matrix, buffer and degradation products. It was determined by injecting standard of TAF API, sample of TAF API in matrix, blank, mobile phase, individual degradants and combined degradants using the developed method.

Linearity of the analytical method was determined from triplicate analytical curves obtained by HPLC analysis of TAF standard solution on three different days.

The accuracy was determined at three levels of TAF in castor oil matrix: 20% of working concentration at 20µg/mL, 100% of working concentration at 100µg/mL and 120% of working concentration at 120µg/mL. This was achieved by adding 2mg, 10mg and 12mg of TAF API to 3.33mg of castor oil and processing it as per drug content procedure for TAF recovery. The recovery experiments were performed in triplicate for each level.

Precision was determined by repeatability (intra-day precision) and reproducibility (by different analyst on a different day) by triplicate injections of six TAF samples in castor oil. It was calculated as a relative standard deviation (RSD) of results from three standard solution injections (injected at 100µg/mL).

Lower limit of quantification (LOQ) and lower limit of detection (LOD) were identified based on signal-to-noise (S/N) ratio obtained of concentration levels injected in triplicates over three days. LOD was set at S/N of at least 3.3 and LOQ was set at S/N of at least 10.

4.1.6 Stress studies of TAF in water and PBS matrices

All stress studies were performed at an initial drug concentration of 1mg/mL in water and PBS matrices. For both groups, samples were studied up to 72h with sampling done at 0 time, 4h, 24h, 48, and 72h for PBS group and 0 time, 4h, 24h and 72h for water group. For all matrices, acid hydrolysis was performed in 0.01N HCl at room temperature in the dark, basic hydrolysis was performed in 0.01N NaOH at room temperature in dark and unstressed sample was also placed at room temperature in dark. Oxidative degradation studies were carried out at room temperature in the dark at two concentration levels of 0.3% and 3% hydrogen peroxide. Photolytic degradation studies were performed at room temperature by placing samples in clear glass vials exposed to light. Lastly, samples were placed in dark, at 45°C to study effect of heat on TAF degradation. Samples were withdrawn at pre-defined time points and subjected to HPLC analysis after suitable dilution.

4.2 Results

In this chapter, a rapid, simple and sensitive stability indicating HPLC analytical method was developed for determination of TAF and its degradants. This method was validated according to the International Conference on Harmonization (ICH) guidelines [117] by testing for specificity,

linearity, range, limit of detection and quantification, accuracy, precision, stability of solution and robustness.

4.2.1 Specificity

Specificity of the analytical method, or its ability to resolve TAF and its degradants, was established by spiking a 100 µg/mL standard of TAF with FA and the known degradants Tenofovir and Tenofovir-phenyl. The retention time of TAF was observed at 14.545± 0.076 min. The degradant retention times were observed to be 4.205 ± 0.017 min for TFV and 10.312 ± 0.009 min for TFV-P. Retention time for Fumaric acid was 5.666 ± 0.084 min. The peaks were well separated from parent API peak (Figure 12) which elutes at 14.545± 0.076 min with resolution between the closest peaks of FA and TFV >2. This result confirms the specificity of the analytical method.

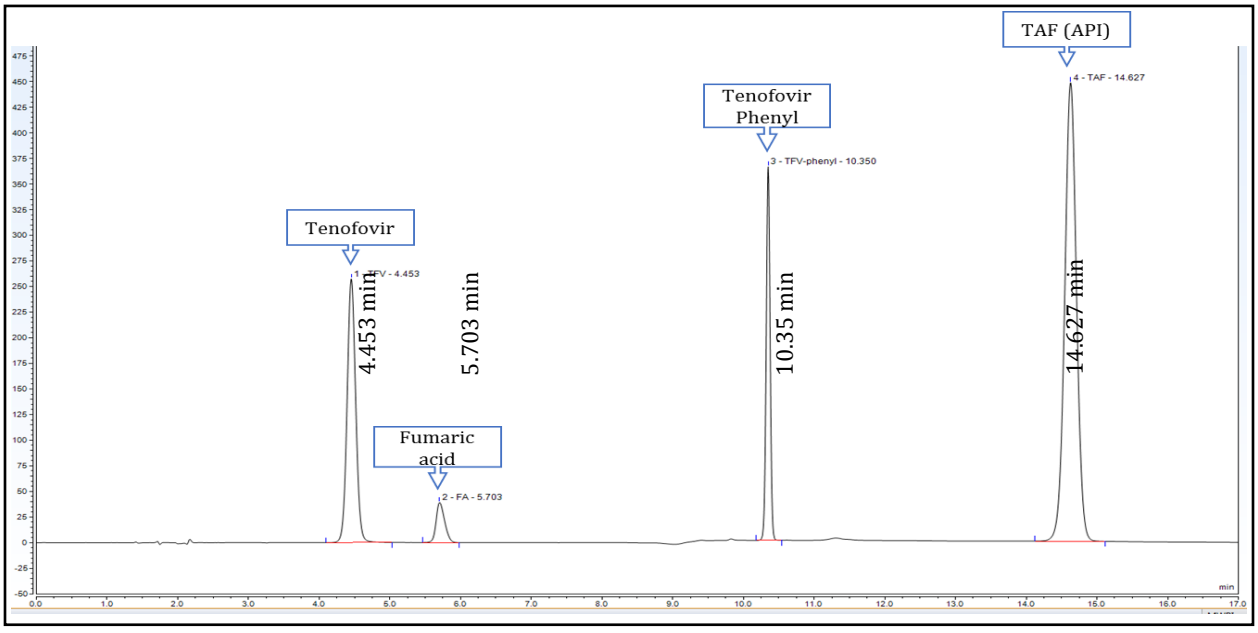

Figure 12 | Representative chromatogram showing the known degradant peaks, FA and parent API peak. Chromatogram obtained by injecting a sample of 100µg/mL TAF standard spiked with FA, TFV and TFV-P. The

retention time of TAF was observed at 14.545± 0.076 min, FA was 5.666 ± 0.084 min, TFV was 4.205 ± 0.017 min and TFV-P was 10.312 ± 0.009 min.

4.2.2 Linearity and Range

A linearity range of calibration curve was run from 10 µg/mL to 200 µg/mL with a co-efficient of determination (R^2) of the regression line >0.999 over 3 days. This indicates a linear relationship between TAF concentration and the area under analyte peak (Figure 13). Figure 14 illustrates the overlay of the three individual linearities.

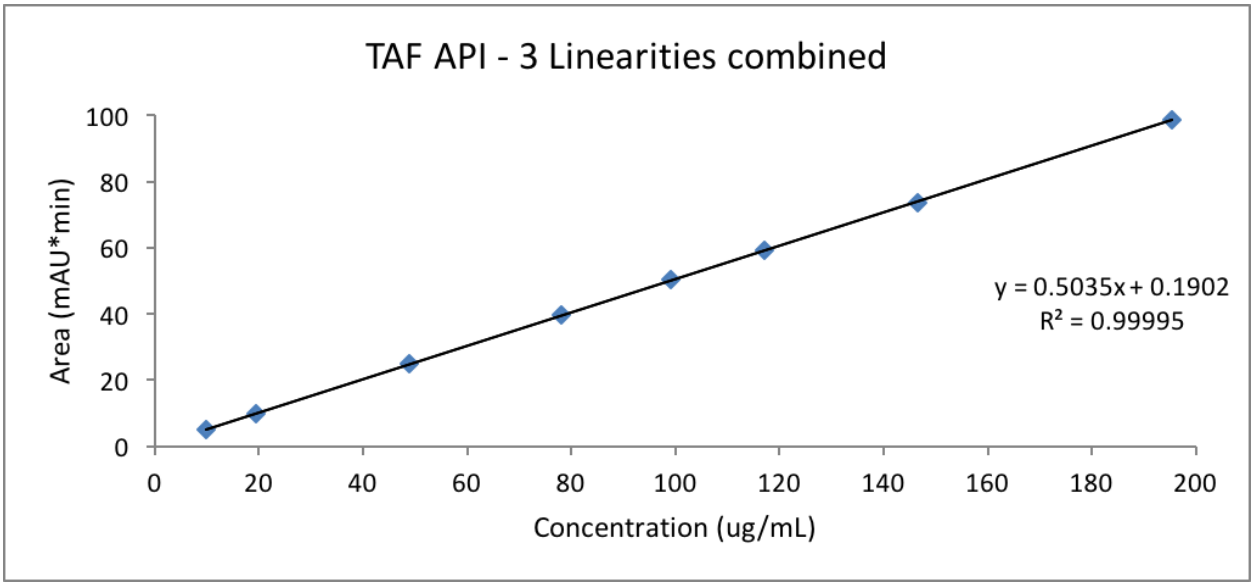

Figure 13 | Three linearities of TAF drug substance performed on three different days - combined. Three calibration curves, from 10µg/mL to 200µg/mL of TAF standard were run with each level injected in triplicates, on three different days. Average area (mAU*min) v/s TAF concentration (µg/mL) was combined for each linearity and was plot.

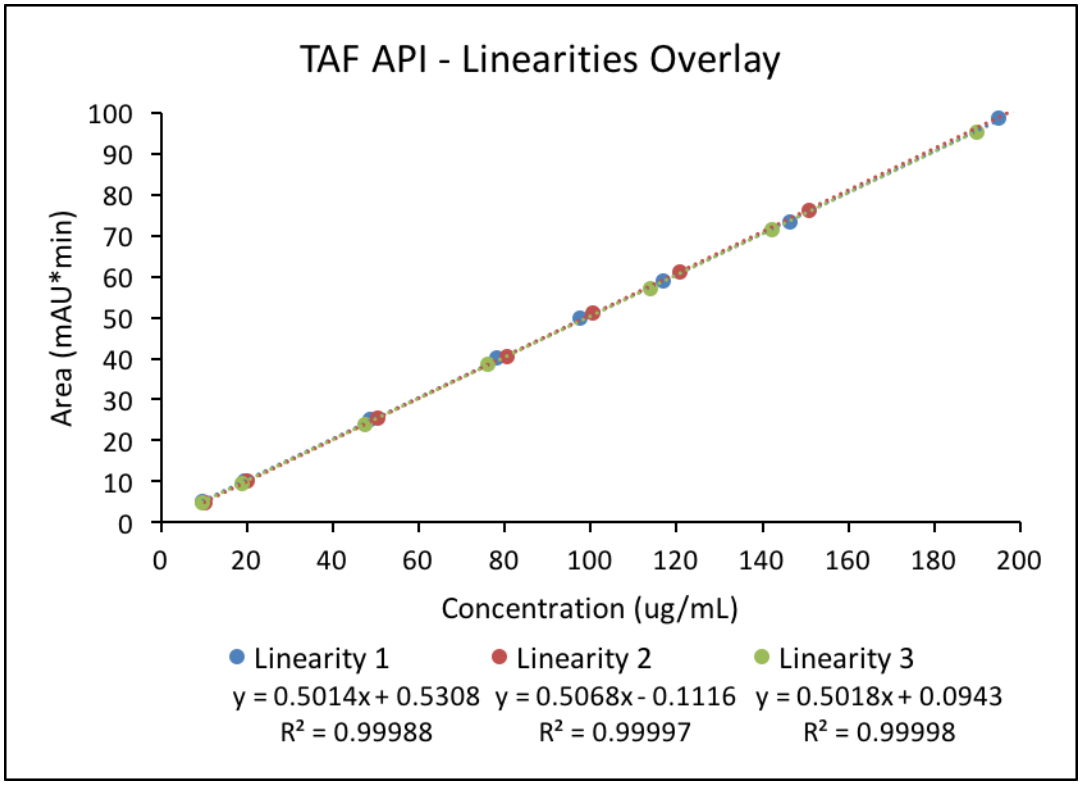

Figure 14 | Three linearities of TAF drug substance performed on three different days - overlay. Three calibration curves, from 10µg/mL to 200µg/mL of TAF standard were run with each level injected in triplicates, on three different days. An overlay of Average area (mAU*min) v/s TAF concentration (µg/mL) was plot for individual linearity.

Linearities of FA and individual degradant standards TFV and TFV-P were analyzed from 2µg/mL to 200µg/mL over three days. The regression equation obtained from the compiled linearity standard curves was used for calculating the degradants during formulation development and optimization throughout the document (Table 5).

Table 5 | Regression equation of TFV, TFV-P and FA. Three calibration curves, from 2µg/mL to 200µg/mL of FA and degradation standards TFV and TFV-P were run with each level injected in triplicates, on three different days. Regression equation calculated from graph of average area (mAU*min) v/s concentration (µg/mL) (data not shown) of three linearities for each analyte is listed below.

Analytes	R^2	Regression equation
TFV	0.9999	y = 0.9045x + 0.1359

TFV-P	0.99849	y = 0.7007x + 1.5432
FA	0.99985	y = 0.171x + 0.0.0836

4.2.3 Limit of quantification (LOQ) and limit of detection (LOD)

The limit of quantification (LOQ) and limit of detection (LOD) was estimated to be 1.25 µg/mL (S/N ratio = 12.43 ± 1.06) and 0.6 µg/mL (S/N ratio = 3.82 ± 0.35) respectively over n=3 injections over 3 days.

4.2.4 Accuracy

The accuracy of the method was tested across three levels (n=3 per level), 20% of the working concentration (20 µg/mL), 100% of working concentration (100 µg/mL) and 120% of working concentration (120 µg/mL). The acceptance criteria were set at 90-110% for each level and was met (Table 6).

Table 6 | Accuracy determination of analytical method. Accuracy was tested at three levels (n=3 per level, each level injected in triplicates): 20% of the working concentration (20 µg/mL), 100% of working concentration (100 µg/mL) and 120% of working concentration (120 µg/mL). The acceptance criteria were set at 90-110% for each level.

Level	%TAF Recovery ± SD	% RSD
20%	107.42 ± 0.51	0.48%
100%	107.17 ± 0.8	0.75%
120%	106.22 ± 1.36	1.28%

4.2.5 Precision

Repeatability (intra-day) and reproducibility (different day and different analyst on same instrument) was performed to analyze the precision of the analytical method. 6 determinations at 100% level of working concentration (TAF concentration around 100µg/mL) in sample matrix of castor oil and one determination of TAF standard (100µg/mL concentration) were analyzed in triplicate. The % RSD of TAF was found to be below 2%, confirming the repeatability and reproducibility of the method (Table 7).

Table 7 | Precision of Analytical Method. Repeatability (intra-day) and reproducibility (different day and analyst on the same HPLC instrument) was performed. 6 TAF samples at 100% level of working concentration (100µg/mL) in matrix of castor oil; injected in triplicates and one 100µg/mL TAF standard, injected in triplicates was analyzed. %RSD of %TAF recovery for each sample or standard was calculated, with acceptance criteria set at %RSD <2%.

Analyst	%RSD TAF Standard	%RSD TAF Sample
Analyst 1 (n=6 for TAF sample)	0.06	1.32
Analyst 2 (n=6 for TAF sample)	0.07	0.99
Analyst 1 and 2 (n=12 for TAF sample)	0.60	1.60

4.2.6 Stability of solution

TAF standard (n=1) and TAF sample (n=1) solutions of 100µg/mL were prepared and kept in the auto-sampler at a temperature of 5 °C and tested for % assay up to 172h. Acceptance criteria was set at difference in % assay of TAF at 172h with respect to 0h should be < 8%. Percent difference between 0 and 42h for TAF standard was found to be 1.62% and TAF sample was

0.65%. Percent difference between 0 and 172h (long-term solution stability) for TAF standard was found to be 6.94% and TAF sample was 5.61%.

4.2.6.1 Robustness

The method was found to be robust to flow rate changes from 1.0mL/min (working condition) to 1.2mL/min, changes in initial gradient composition MPh A (PO_4 Buffer pH 6.0: MeOH 80:20): B (ACN) from 95:5 to 90:10 and 100:0. The robustness of the system was also tested by modifying the column temperature at 20 °C and 30 °C. The acceptance criteria were set at number of theoretical plates (NTP) not below 30000, resolution between FA and TFV in specificity injections (RF) >2 and symmetry factor (T) of TAF between 0.9-1.1. The lowest resolution between FA and TFV was observed to be 2.88 (data not shown) and the lowest NTP was observed to be ~36000 (data not shown). As seen in Table 8, all criteria were met, indicating that the developed analytical method is robust.

Table 8 | Robustness of the analytical method. Conditions tested: working conditions, flow rate changes from 1.0mL/min (working condition) to 1.2mL/min, changes in initial gradient composition MPh A (PO4 Buffer pH 6.0: MeOH 80:20): B (ACN) from 95:5 to 90:10 and 100:0 and changing the column temperature from 25°C to 20 °C and 30 °C. One sample of TAF standard and sample each, and a specificity injection of TAF spiked with FA, TFV and TFV-P was injected in triplicates for each condition. The acceptance criteria were set at NTP not below 30000, resolution between FA and TFV in specificity injections (RF) >2 and symmetry factor (T) of TAF between 0.9-1.1.

Condition	Type	Avg. TAF RT	NTP	T	RF (TFV and FA)
Working condition	Standard	14.430	42009	0.99	5.41
	Sample	14.427	42389	1.00	
Flow 1.2 mL/min	Standard	13.080	47199	0.98	5.01
	Sample	13.077	47291	0.98	
Mobile Phase A:B 90:10	Standard	13.937	42423	0.98	8.10
	Sample	13.937	42432	0.98	
Mobile Phase A:B 100:0	Standard	14.573	36564	1.02	2.88
	Sample	14.603	36023	1.03	
	Standard	14.527	37343	1.03	4.70

Condition	Type	Avg. TAF RT	NTP	T	RF (TFV and FA)
Column temp 20°C	Sample	14.520	37691	1.02	
Column temp 30°C	Standard	14.293	45494	0.96	5.95
	Sample	14.293	45468	0.95	

4.2.7 Stress studies

TAF was subjected to forced degradation studies (or stress studies) to determine if the analytical method could separate the different degradants produced. Stress conditions such as acidic (0.01N HCl) and basic (0.01N NaOH) pH, oxidation (0.3% and 3% hydrogen peroxide (H_2O_2)) and thermal and photolytic exposure were studied. Since the sample matrix for stress studies should be relevant for the parent drug, TAF was stressed in water and PBS matrices (to simulate physiological fluids) for a period of 72 hours.

4.2.7.1 TAF in Water matrix

An aqueous solution of TAF (1 mg/mL) was tested over 72 hours in unstressed condition (Figure 15-A). The % TAF recovery was within the limits of 90-110%. 0.3% of TFV was formed and unknown degradant with relative retention time (RRT) 0.70 was the major degradant at 4.5% after 72 hours. TAF was found to be labile to hydrolysis when exposed to mildly acidic (0.01N HCl, theoretical pH 2) and basic conditions (0.01N NaOH, theoretical pH 12) (Figure 15-B and C). As seen in Figure 15-B, in acidic conditions, the degradation of TAF (41.4% after 72hr) was followed by consequent formation of the major unknown degradant with RRT 0.70 (63.1% after 72hr). In basic conditions, TAF degraded after immediate exposure to 0.01N NaOH, with 21.8% TAF recovery at 0 time and could not be detected after 4 hours (Figure 15-C). TFV-P was the major degradant at 0 time at 50.5% detected. Over time, the concentration of TFV-P steadily

decreased with 39.8% observed at 4 hours and 4.81% at 24 hours. An increase of an unknown degradant with RRT 0.64 was observed with 46.4% at 4 hours, increasing to over 100% at 72 hours. As seen in Figure 15-D, degradation of TAF was observed when exposed to 0.3% H_2O_2 (around 10% degraded over 72 hr). Less than 5% of an unknown degradant with RRT 0.70 was observed at the final time point. At higher concentration of H_2O_2 (Figure 15-E), almost 30% TAF degraded at 72 hours, showing that TAF is prone to oxidative stress. When exposed to 45°C, a 25.6% reduction in TAF recovery was observed over 3 days (Figure 15-F). The major degradant at the final time-point was an unknown degradant with RRT 0.70 at 27.6%. As observed in Figure 15-G, when exposed to photolytic degradation, TAF was found to undergo degradation with 89.2% TAF recovered at 72 hours. The major degradant was an unknown degradant with RRT 0.70 (10.5% at 72 hr).

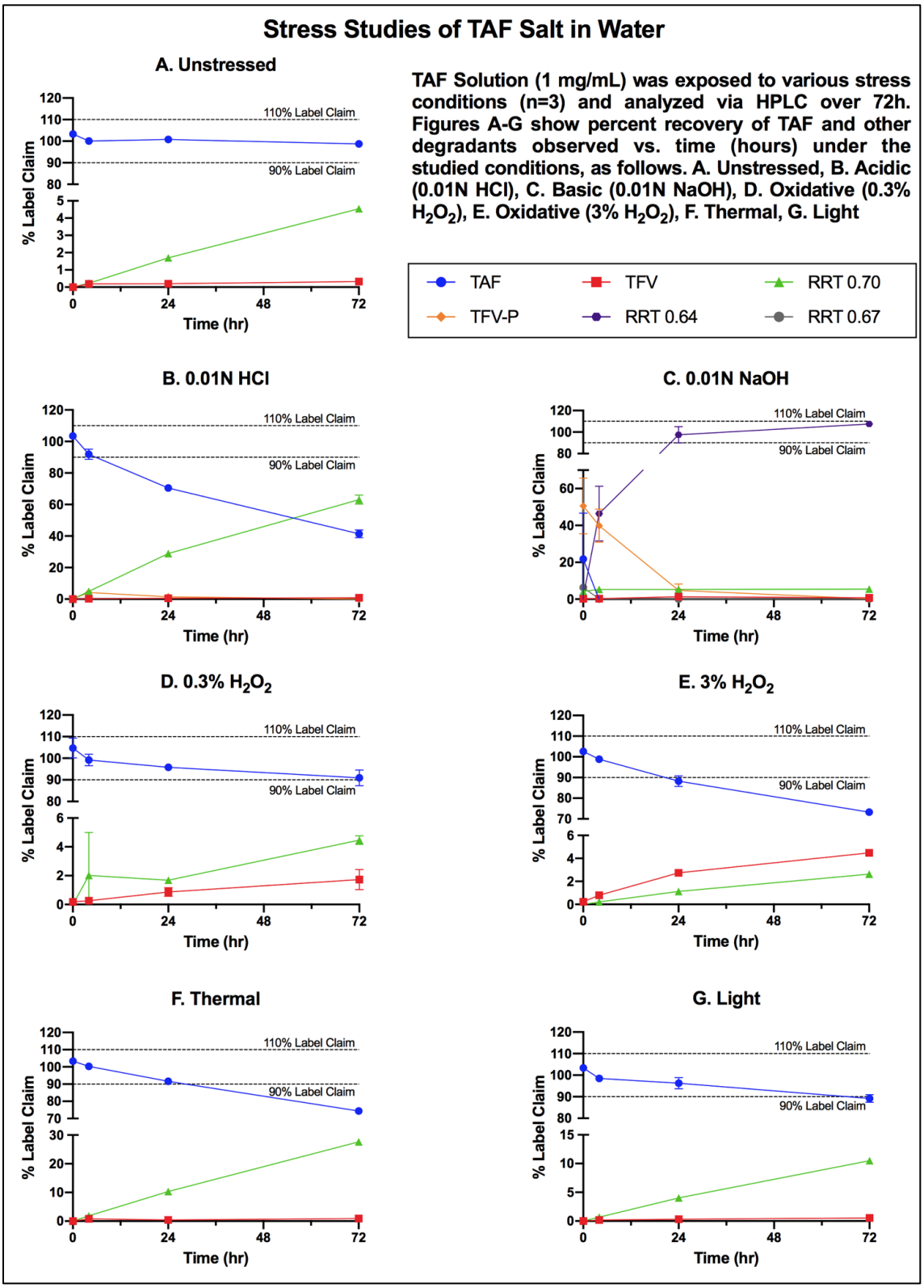

Figure 15 | Stress studies of TAF in water matrix. Data represented as mean ± SD for all groups (n=3 per condition per time-point).

4.2.7.2 TAF in PBS matrix

TAF in PBS was tested up to 72 hours in unstressed condition at room temperature (Figure 16-A). The % TAF recovery was within the limits of 90-110%. 0.3% of TFV was formed and TFV-P was the major degradant at 4.1% after 72 hours. TAF stability was pH dependent with greater degradation at a higher pH level compared to a lower pH level (Figure 16-B and 16-C). In acidic conditions (Figure 16-B), the degradation of TAF was followed by consequent formation of the major degradant TFV-P (major degradant at 4.5% after 72hr exposure). In basic conditions (Figure 16-C), degradation of TAF also began after immediate exposure to 0.01N NaOH, with 82.8% TAF recovery. TAF could not be detected after 4 hours and the major degradant at this time-point was TFV-P (62.7%). A trend of degradant formation was observed with a sharp increase in TFV-P formation at 4 hours followed by its subsequent decline and gradual formation of an unknown degradant with RRT 0.64. At 72 hours, the major degradants were RRT 0.64 at 66.4% and TFV-P at 28.7%. A small amount of TFV was also observed at this time-point. Around 5% of an unknown degradant with RRT 0.70 was formed at 4 hours which remained constant over 3 days. TAF in PBS underwent oxidative degradation when exposed to 0.3% (Figure 16-D) and 3% H_2O_2 (Figure 16-E) with 53.8% and 7.6% recovery at 72 hours respectively. TFV-P was the major degradant for both concentrations of hydrogen peroxide, at 19.5% and 35% for 0.3% and 3% H_2O_2 respectively at 72 hours. Other degradants were TFV and an unknown degradant with RRT 0.68. TFV-P degradant formation began to plateau after 48 hours for both groups. For 3% H_2O_2 group, TFV-P concentration was highest at 48 hours as well (36.3%) and was stable over the next 24 hours. It was followed by increase in formation of TFV, 5.9% at 48 hours to 9.4% at 72 hours. TAF in PBS degraded to 47.3% after 72 hours when exposed to 45 °C (Figure 16-F). TFV-P was the major degradant at 29.3%, 9.4% TFV and 2.9% of an unknown degradant with RRT 0.70 was

formed at 72 hours. TAF in PBS underwent photolytic degradation with TAF recovery around 70.8% and TFV-P major degradant at 18.8% after 72 hours (Figure 16-G). This validated stability indicating analytical method was applied on pre-formulation studies and drug content calculations of stability experiments throughout the course of this project.

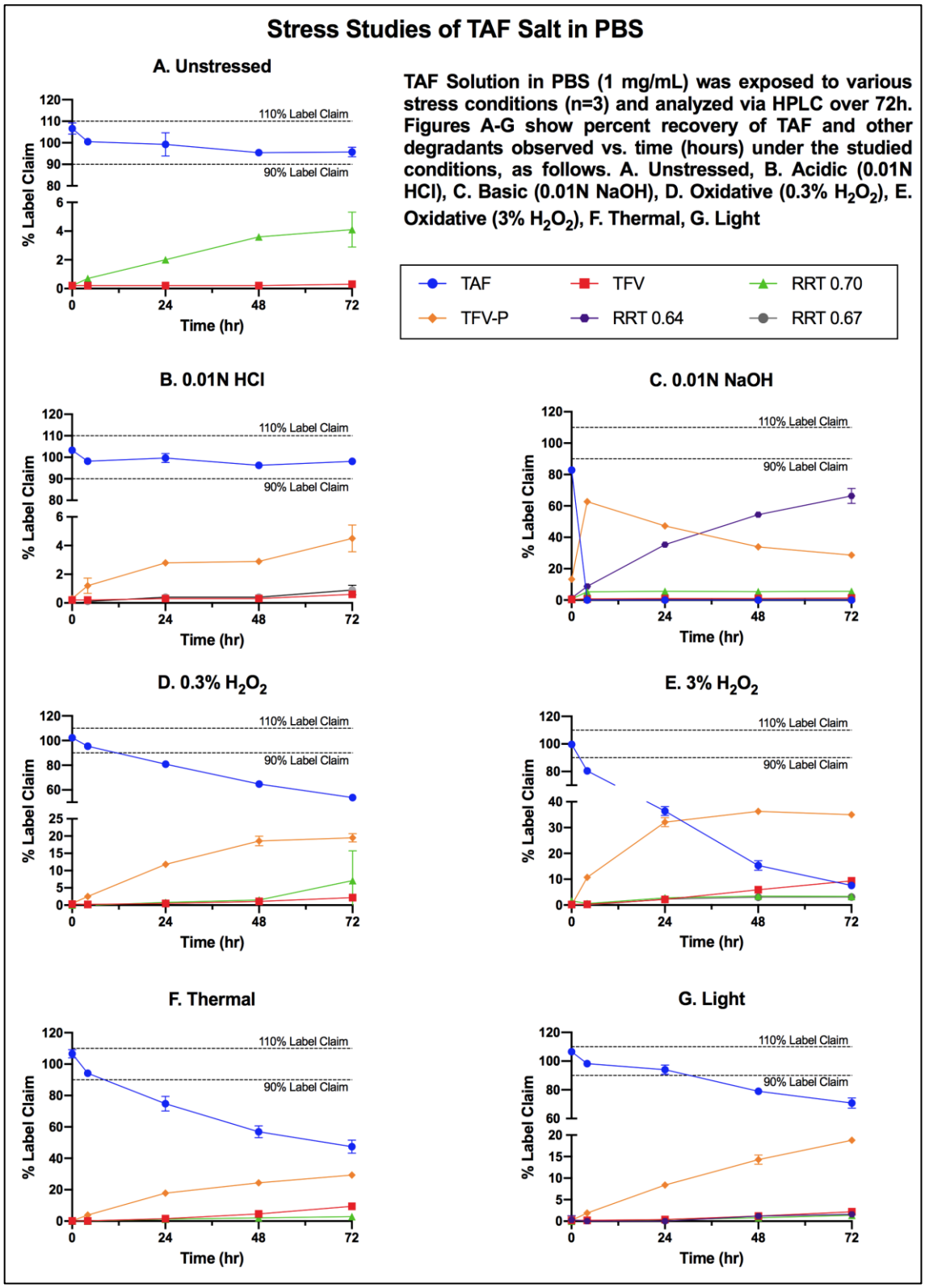

Figure 16 | Stress studies of TAF in PBS matrix. Data represented as mean ± SD for all groups (n=3 per condition per time-point).

4.3 Discussion

A stability indicating analytical method was developed and validated in section 4.2. The representative chromatogram shows that the parent API peak (TAF) and the known degradant peaks are well resolved without any interference from extraneous components, demonstrating the specificity of the developed method (Figure 12). The method was found to be linear for TAF within the concentrations of 10 to 200 µg/mL as identified over three days (Figure 14). Further, linearity experiments of the known degradants TFV, TFV-P as well as FA were performed, and their regression equations were obtained (Table 5). This would help in quantifying the TFV, TFV-P and FA analytes in the HPLC assay performed during the course of the project. An LOD of 0.6µg/mL and an LOQ of 1.25µg/mL was observed over three days which shows that the developed method was very sensitive.

For accuracy experiment, level of 20%, 100% (working concentration) and 120% were chosen (Table 6). 20% level was selected as it is important to ensure good recovery of TAF even at lower concentrations that may be typically observed while performing accelerated stability experiments. The acceptance criteria of 90-110% was met at all three levels showing that the analytical method was accurate.

Both repeatability (multiple samples processed by the same analyst) and reproducibility (multiple samples processed by different analyst on a different day) studies demonstrated %RSD values less than 2% (Table 7). This established the precision of the developed analytical method.

It is important to monitor the stability of solutions (both TAF standards and samples) in the auto-sampler to make sure that the solutions tested will remain stable during the duration of analysis. The TAF recovery up to 172 hours was within the set specifications of 90-110% which proved the stability of TAF solutions in the auto-sampler.

ICH guidance defines robustness or ruggedness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters [117]. Variations in our robustness analysis included varying the column temperatures (from 25°C to 20°C and 30°C), increasing the flow to 1.2mL/min and changing the initial gradient compositions from 95:5 MPh A: MPh B to 90:10 and 100:0 MPh A: MPh B. Specifications of system suitability (performed to determine the applicability of an analytical system and its fit, prior to its use [118]) like symmetry factor (between 0.9-1.1) of the main analyte of interest – TAF, number of theoretical plates (>30,000), as well as the resolution between the closest eluting peaks of TFV and FA (at 4.205 ± 0.017 min and 5.698 ± 0.084 min respectively). The accuracy of peak integration/quantitation and peak height of an analyte is reduced if there is tailing or fronting (asymmetry) observed in the eluted peak. Hence a symmetry factor of 0.9-1.1 ensures accurate integration and quantitation of the analytical data [119]. Another 'system suitability' parameter is the number of theoretical plates or NTP. NTP is an indicator of column efficiency with a simple assumption that larger the NTP, better the quality of analyte separation [120]. NTP of over 30000 was set and the lowest resolution between the closes pair of degradants TFV and TFV-P was observed to be 2.88 (data not shown), well above the set specification of 2 (Table 8). Hence, the developed analytical method was found to be robust.

Stress studies are performed by forced degradation of the analyte of interest after exposing it to conditions like hydrolytic stress (acidic and basic pH), oxidative, thermal and photolytic stress and comparing the trend of degradation with respect to an unstressed control. The degradants obtained through this exposure did not co-elute with the TAF main peak, further demonstrating that this analytical method is stability indicating. It also highlighted the degradation trend of TAF when exposed to the aforementioned conditions. Published [102] studies as well as the stress study

we performed identified that TAF was liable to hydrolytic degradation. We performed our stress studies in water and PBS as the matrix should closely recapitulate the environment that the API would be exposed to. We chose these two matrices, as the drug will be exposed to these elements during the process of formulation development and when it will be implanted (PBS or physiological fluid). For both matrices, acidic as well as basic pH conditions led to increased degradation of TAF (Figures 15-B, C and 16-B, C). As observed in literature, cleavage of P-N bond of the phosphoramidate group at lower pH and cleavage of P-O bond at higher pH may cause this instability (degradation products as seen in Figure 11). Thermal and oxidative stress had lower impact on TAF stability compared to acidic and basic pH conditions (Figure 15-D-G and 16-D-G). Certain limitations were observed with the current analytical method which are discussed below.

4.3.1 Limitations and future directions

Although the developed HPLC analytical method was successful in characterizing TFV and TFV-P degradants of TAF drug substance, a few unknown impurities (RRT 0.64, 0.67 and 0.70) were also detected. There is need for additional identification of these unknown impurities using mass spectroscopic analysis or matching with respect to known degradant standards. The degradation pathway identified in Figure 11 can also be used as a reference to obtain degradant standards and evaluate the specificity of the currently developed method. Additional robustness studies can be performed that evaluate the effect of change of buffer pH (from 6.0 to 5.5 or 6.5), reducing the flow to 0.8mL/min from 1.0mL/min and changing in column type. Lastly, stress

studies can be performed in castor oil to better understand the degradation profile (if any) of the drug substance in its formulation matrix.

4.3.2 Impact of current work

The developed and validated analytical method is one of the few TAF analytical assays that are stability-indicating, robust, simple, fast and reliable. Extensive characterization of the drug substance, fumaric acid and two of its known degradants will be beneficial to the field. This analytical method can be employed for the quantitation of TAF not only in the current formulation development effort, but also in the quantification and analysis of other formulations (enemas and other implants under research) and routine quality control as well.

5.0 Pre-formulation mechanistic studies to understand TAF degradation (Specific aim 1)

This section addresses the first specific aim of the work, which is to mechanistically understand the destabilization behavior of TAF in the current castor oil formulation. Pre-formulation studies are carried out to assess the physico-chemical characteristics of the drug substance in order to identify properties that may impact drug product stability and formulation development [121]. The overall objective of the pre-formulation panel of experiments is to obtain information that is utilized in the development and optimization stages of a pharmaceutical product as well as for the scale-up and commercialization efforts [122]. Solubility and hygroscopicity studies are part of a general battery of pre-formulation investigations to understand the API under study. Since TAF degrades due to pH dependent hydrolysis, it was vital to understand the pH range where TAF exhibits maximum stability. Hence a study to identify the optimal intra-device pH range was performed. Finally, an experiment that quantified the partitioning of TAF from castor oil to an aqueous layer was performed to better understand the migration behavior of the API.

5.1 Methods

5.1.1 Solubility study

Solubility of TAF was measured in both water and 1x PBS pH 7.4 over three replicate samples at ambient temperature. 1x PBS was prepared by diluting 10x PBS (Corning, Fisher Scientific, USA) 10 times using MilliQ water. Excess quantity of TAF was well suspended in both

water and 1x PBS in 4mL amber color vials. After 48 hours, 200µL sample was aliquoted, centrifuged at 10,000 rpm for 10 min using accuSpin Micro 17 Micro-centrifuge (Fisher Scientific, USA). Supernatant solution was diluted 100 times using ACN: MeOH: Water 22:10:68 diluent and analyzed for drug content using HPLC.

5.1.2 Hygroscopicity study

Hygroscopicity of TAF was measured in triplicate in glass petri-dish. Each petri-dish was weighed prior use (m1). Approximately 50mg of TAF was added in each of the vessels and weighed (m2). This assembly was placed in a desiccator at ambient temperature containing a saturated solution of ammonium sulfate (80% relative humidity) for 24 hours (monitored using Fisherbrand™ Traceable™ Thermometer/Clock/Humidity Monitor, Fisher Scientific, USA). After 24 hours, the vessel is reweighed (m3). Percent increase in mass is calculated using the formula:

$$\%\ increase\ in\ mass = \frac{\mathrm{m3} - \mathrm{m2}}{\mathrm{m2} - \mathrm{m1}} \times 100$$

The results were interpreted as per follows:

Deliquescent: sufficient water is absorbed to form a liquid,

Very hygroscopic: % increase in mass ≥ 15 %,

Hygroscopic: 15 % > % increase in mass ≤ 2 %

Slightly hygroscopic: 2% > % increase in mass ≤ 0.2 % [123]

5.1.3 pH range for maximum TAF stability inside the PCL device

Sodium phosphate buffers were prepared. pH was adjusted by mixing NaH_2PO_4 (Sigma Aldrich, USA) and Na_2HPO_4 (Sigma Aldrich, USA) in varying concentrations to get three buffers of final pH 5.0, 5.5 and 6.0. TAF was dissolved in each buffer at the concentration of 1mg/mL by stirring for one hour. Each buffer sample was further divided into two vials. One vial was placed at ambient temperature and other at elevated temperature (40°C). Samples were drawn at 0 time, at 4 and 10 days and analyzed for drug and degradant content using HPLC.

5.1.4 Partitioning behavior study

TAF was dissolved in castor oil (Spectrum, US USP grade) (500µg/mL) by stirring for 7 days. 9.025g of this TAF solution in castor oil was weighed (corresponding to 9.5mL of castor oil, density = 0.95g/cm^3) in 15mL centrifuge tubes for 5:95 Water: Castor oil group. Similarly, 8.075g castor oil was weighed (corresponding to 8.5mL of castor oil) in 15mL centrifuge tubes for 15:85 Water: Castor oil group. 0.5mL and 1.5mL of MilliQ water was added to each tube for 5:95 and 15:85 group respectively. These tubes were stirred on an orbital rotator at 50 rpm. TAF drug content in oil phase was analyzed using HPLC for 0-time analysis before mixing with water. Samples in duplicates were drawn at 5 min, 1 hour and 24 hour time points for both groups from both the aqueous and oil phase. Additionally, a sample point was drawn at 5 hours for 15:85 group to better monitor the partitioning of TAF in presence of higher concentrations of water. At each time point, the centrifuge tubes were centrifuged for 30 min at 5000 rpm (Sorvall Legend RT Centrifuge, Thermo Scientific, USA) prior to sampling. The recovery of TAF and its degradants

(if any) from both the oil and water phase were studied to better understand partitioning behavior. Hence samples were collected from both the aqueous and oil phase using the procedure below.

Oil phase sampling: Sample was drawn from the oil phase using a transfer pipette. 0.95g of castor oil sample was weighed in a 20mL scintillation vial and 10mL methanol was added. Sample was stirred for 1 hour in the dark and further diluted 10 times using 25% acetonitrile in water for drug and degradant content using HPLC analysis.

Water phase sampling: A plastic 10mL slip tube syringe with a 25G x 1 (0.5mm x 25mm) removable needle (BD Precision Glide Needle, NJ, USA) was used to sample the water phase. The syringe was initially filled with air. Air was gently expelled while inserting the needle through the oil phase. Once sampled from the aqueous phase, the syringe was quickly removed from the tube and the needle was detached. This method was used to prevent oil traces contamination while sampling the higher density aqueous phase in centrifuge tubes. [124]. Sample was diluted two times using ACN: MeOH: Water 22:10:68 diluent for drug and degradant content using HPLC analysis.

5.2 Results

Pre-formulation experiments were performed before formulation stabilization and optimization efforts were carried out. A pH range where TAF was most stable was first identified. Further, the partitioning behavior of the drug substance in presence of two concentrations of water was studied. Lastly, the solubility and hygroscopicity of TAF was determined.

5.2.1 Solubility studies for TAF

Solubility of TAF was measured in both water and 1x PBS pH 7.4 over three replicate samples at room temperature. TAF solubility after 48 hours was found to be 24.7±1.3 mg/mL in water and 28.3±0.2 mg/mL in 1x PBS pH 7.4 analyzed by HPLC (Table 9).

Table 9 | Solubility of TAF in water and 1x PBS at ambient temperature. Excess quantity of TAF was suspended in MilliQ water and 1x PBS in 4mL amber color vials. After 48 hours, 200µL was aliquoted and centrifuged at 10,000 rpm for 10 min. The supernatant solution was diluted 100 times using ACN: MeOH: Water 22:10:68 diluent and analyzed for drug content by HPLC. Data represented as mean ± SD; n=3 per group

Matrix	TAF (mg/mL) (n=3)
Water	24.7 ± 1.3
PBS (1x)	28.3 ± 0.2

5.2.2 Hygroscopicity studies for TAF

The average % mass increase (n=3) was found to be 0.28% over 24 hours at room temperature. As per the criteria listed in the European Pharmacopoeia [123], TAF was found to be slightly hygroscopic.

5.2.3 Identification of optimal pH for maximum stability of TAF inside the device

Following the stress studies, it was established that TAF stability is pH dependent. In order to stabilize TAF in the castor oil formulation, it was essential to identify an optimum pH inside the PCL device. Previous studies on TAF in Dr. Rohan's laboratory had identified pH around 5.5 as the ideal pH for maximum stability of TAF in solution from a tested range of 5.0 to 7.0

(unpublished data). Hence, we chose to narrow the range further by testing the TAF stability at pH 5.0, 5.5 and 6.0. Experiments were performed at room temperature and elevated temperature (40°C) and followed for 10 days. As seen in Figure 17-A and C, TAF showed better stability between a pH range of 5.0 to 5.5 at room temperature and at 40°C. At room temperature, over two-fold reduction in the concentration of the major impurity TFV was observed at pH 5.0-5.5 compared to pH 6.0 (Figure 17-B). At elevated temperature, least degradation of TAF was observed at pH 5.0 and 5.5 (Figure 17-D). Thus, subsequent formulation development experiments were aimed at maintaining the intra-device pH between 5.0 – 5.5.

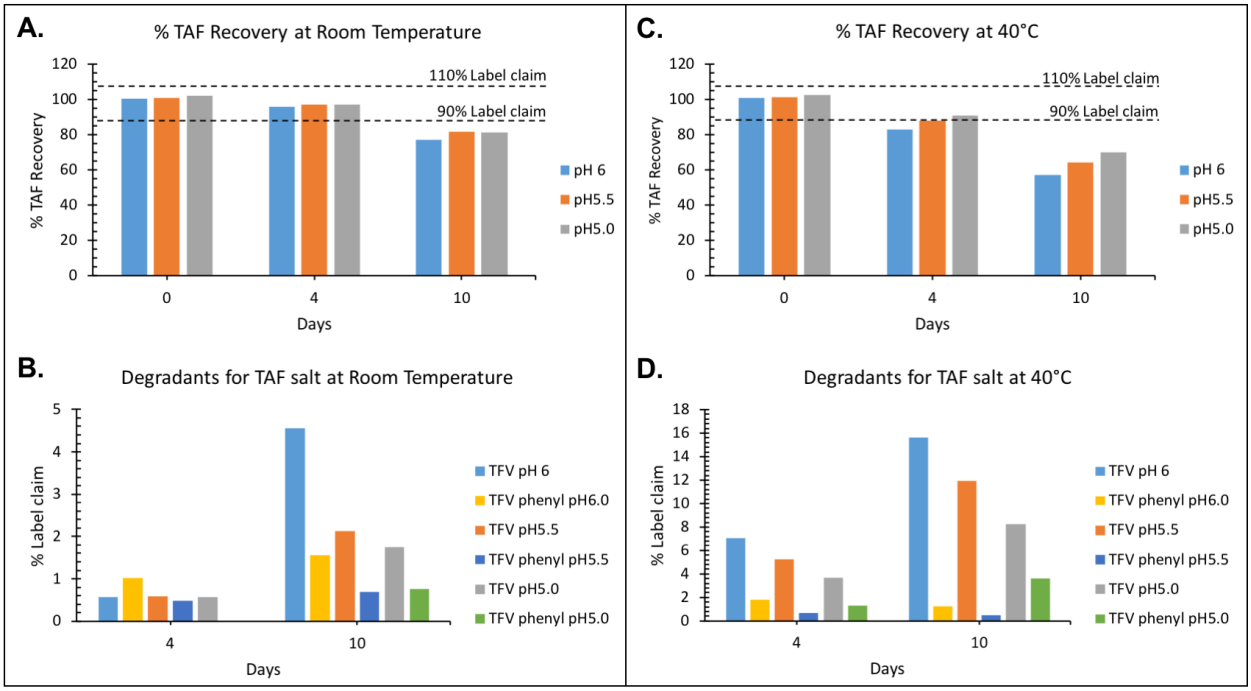

Figure 17 | Determination of optimum pH range for TAF stability. A. Percent TAF Recovery at room temperature, **B**. Degradants of TAF at room temperature, **C**. Percent TAF Recovery at 40°C and **D**. Degradants of TAF at 40°C. Three Na_2HPO_4 buffers of pH 5.0, 5.5 and 6.0 were prepared. TAF was dissolved in each buffer at the concentration of 1mg/mL by stirring for one hour. Each buffer sample was divided into two vials. One vial was placed at ambient temperature and other at 40°C. Samples were taken at 0 time, at 4 and 10 days and analyzed for drug and degradant content by HPLC. (Data represented as mean label claim; n=2 per time-point per group).

5.2.4 Partitioning studies of TAF in castor oil

Prior data from our collaborator, RTI International, NC, USA, suggests that the TAF implant begins to demonstrate erratic *in vitro* release behavior at around 90 days. The ingressed water content (determined by loss on drying) at 90 days was observed to be around 5%, which increased to ~12% at 120 days (Alice Li, Ph.D., RTI International, NC, personal communication, April 12, 2018). In our studies, TAF dissolved in castor oil (500 µg/mL) was exposed to 5% and 15% of water over a period of 24 hours to determine the partitioning behavior of TAF. TAF recovery was analyzed in both phases and greater partitioning into aqueous phase was observed with time. Over 20% of TAF partitioned into the water phase for 85:15 (castor oil: water) group (Figure 18-B) while only around 8% partitioned for 95:5 (castor oil: water) group (Figure 18-A). Subsequently, TAF did not degrade in the oil phase whereas it underwent hydrolysis in the aqueous phase to form TFV, TFV-P. After 24 hours, 0.02% of TFV and 0.08% of TFV-P was formed in the 95:5 (castor oil: water) group and 0.2% and 0.3% of TFV and TFV-P was formed respectively in the 85:15 (castor oil: water) group. An unknown degradant of RRT 0.26 was formed and was the major degradant at 1.2% and 3.7% in both 5% and 15% water samples respectively after 24 hours.

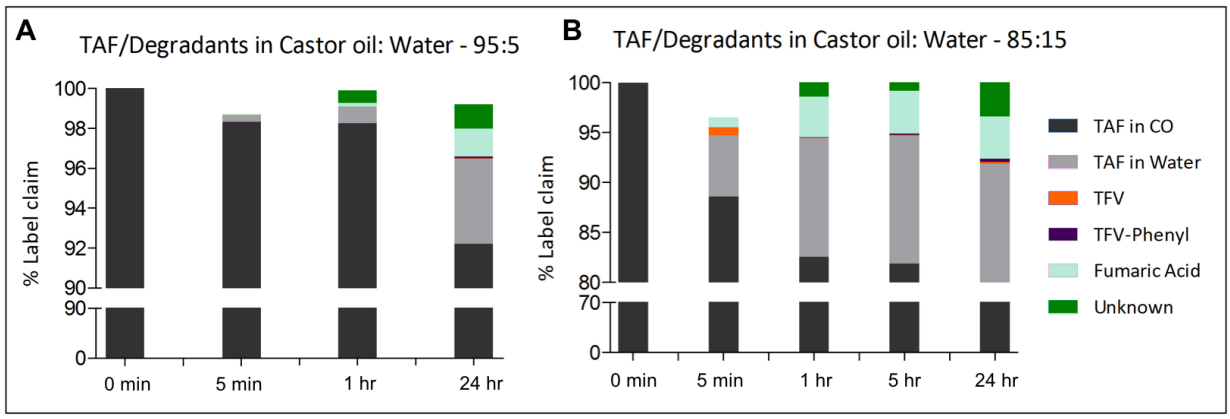

Figure 18 | Partitioning behaviour of TAF; in **A.** 95:5 and **B.** 85:15 castor oil: water samples. TAF was dissolved in castor oil at concentration of 500µg/mL. Weights equivalent to 9.5mL or 8.5mL of castor oil were taken in 15mL centrifuge tubes for 5:95 Water: Castor oil group and 15:85 Water: Castor oil group respectively. 0.5mL and 1.5mL of MilliQ water was added to each tube for 5:95 and 15:85 group respectively. These tubes were stirred on an orbital rotator at 50 rpm for 24 hours. TAF drug content in oil phase was analyzed by HPLC for 0 time, and drug content was analysed in both oil and aqeuous phase for further time points. (Data represented as mean; n=2 per group per time-point).

5.3 Discussion

Pre-formulation studies are sets of experiments performed prior to formulation development and optimization. The information generated through these studies is crucial in identifying key critical properties of the drug that contribute to determining pathways for drug degradation, its instability, compatibility with excipients, suitability for a particular dosage form and impact of manufacturing and storage during scale-up [122]. All of these factors need to be considered before developing the new chemical entity into a safe, stable and efficacious dosage form.

Following stress studies performed in Section 4.2.7, it was identified that both extremes of the pH range (acidic as well as basic) were not viable for TAF stability. Hence, we decided to focus on a narrow range of pH that was mildly acidic, i.e. between 5.0 and 6.0. Two temperatures - ambient and elevated (40°C) were chosen to evaluate the effect of pH while conducting bench-top research experiments as well temperatures that closely mimic physiological temperatures. We observed that a controlled pH of 5.0-5.5 (Figure 17) was optimum in ensuring the highest stability. At elevated temperature (40°C) least degradation of TAF was observed at pH 5.0 and 5.5. This range can be used while making the paste of TAF and castor oil and to study the intra-device pH while performing various *in vitro* studies in the future. The hygroscopic character of the API can influence the behavior of the drug substance during manufacturing, packaging, storage and transport and can also impact the stability of the formulation. Hygroscopicity study revealed that TAF API was slightly hygroscopic.

When the device is fabricated, it consists of TAF in a mono-phasic oily paste. Post-implantation, it draws in physiological fluid from the surrounding and the drug partitions into the aqueous phase. Since the implant consists of two predominant phases, the oil and aqueous phase, it was important to understand the partitioning of the API between these two phases. As mentioned above, TAF degrades in the presence of an aqueous component. Hence, understanding the degree of partitioning or distributing between the two phases can give an idea of the expected degree of degradation of TAF within the implant. While choosing the aqueous to oily phase ratio, we attempted to simulate an *in vitro* release condition observed in the data obtained by our collaborators. Observing TAF partitioning at these conditions of 95:5 and 85:15 oil: water could help us understand the behavior of drug degradation and hence the stability of the implant at those days. As seen in Figure 18, more than two-fold increase in drug partitioning into the aqueous phase

for the 85:15 oil: water group versus the 95:5 oil: water group over 24 hours was observed. The drug that distributed into the water phase degraded while the drug in the oil phase was stable. As mentioned above, the major degradation pathway in the aqueous phase is pH dependent. Based on our studies, controlling the pH of TAF in the aqueous phase to 5.0-5.5 can reduce its degradation. This data supports the hypothesis that controlling the pH environment inside the device can assist in TAF stabilization.

To summarize, in this panel of pre-formulation experiments, a pH of 5.0-5.5 was observed to be ideal for enhanced stability of the system. Also, modifying the drug distribution was crucial to avoid TAF migration and subsequent hydrolysis into the ingressed physiological fluid.

5.3.1 Limitations, impact and future directions

A limitation of the pre-formulation studies conducted in this chapter is that it is difficult to measure the pH inside the device as the outer diameter of the device is just 2.5 mm. The pH range can be thus estimated by measuring pH of TAF in castor oil paste during paste preparation. Whether the same pH range can be extrapolated to ensure maximum TAF stability needs to be evaluated by conducting pH dependent recovery studies of the paste. An impact of this experiment is that the pH range of 5.0-5.5 can be utilized for stabilization efforts in other TAF formulations as well. A second limitation observed is that the hygroscopicity of TAF paste in castor oil needs to evaluated. Lastly, it would be useful to determine the solubility of TAF in aqueous solutions maintained at different pH to demonstrate any pH dependent solubility of the API.

6.0 Development of Accelerated TAF Degradation Models for Rapid Excipient Screening (Specific Aim 2)

As mentioned before (section 1.4.3), in the rabbit PK *in vitro* study conducted by our collaborators [105], an erratic release profile of TAF was observed around 11 weeks. In this study, TAF stability at 91 days (n = 4 devices) was observed to be between the range of 5.3-10%. On the contrary, around 90% stability was observed in two devices evaluated at 49 days. For a system that is aimed at longer durations of action, from a product development perspective, it may not be beneficial to wait for over 3 months to observe degradation/stabilization of TAF in the formulation. Hence, it is pertinent that for quick excipient screening during formulation development and for formulation optimization; accelerated TAF degradation models need to be developed. This section addresses the second specific aim of this project, i.e. to develop a screening method to accelerate TAF degradation in the device for quick excipient screening.

6.1 Methods

Two accelerated models were developed in this chapter. The model developed in section 6.1.1 was used for excipient screening experiments conducted in sections 7.2.1 to 3 and 7.4. Model developed in section 6.1.2 was only used for formulation optimization experiments in section 7.3.2.

6.1.1 Paste preparation to be incorporated inside the PCL device

TAF and oil paste was prepared using a mortar and pestle. Briefly, TAF and castor oil was weighed in a weighing boat (usual ratio of TAF: castor oil was 3:1). Using two spatulas, the entire TAF powder was wetted with the oil, making sure it was mixed until it was well incorporated, and all the powder was visually wetted with oil. This mixture was transferred into a clean mortar and pestle. It was gently grinded 3 times to form a homogenous paste. This paste was back-loaded into a 1mL luer lock syringe. A 14G blunt tip luer-lock stainless steel needle unless mentioned otherwise (Hamilton™ Metal Hub Blunt Point needle, Fisher Scientific, USA) was applied. This 'paste assembly' was used to fill the PCL devices.

6.1.2 Device preparation

The extruded PCL tubes (either Corbion Purasorb PC12 grade, 100µm thickness, 2.5mm outer diameter (OD) or Sigma 80kDa grade, 100µm thickness, 2.5mm OD) were first cut as per the required length and crimped on one side using a heat impulse sealer. The heat impulse sealer used for crimp sealing was lined by a release liner before crimping. Markings were made on the PCL tube using an ultra-fine sharpie at pre-determined lengths. The PCL tubing was then placed on top of the needle (paste assembly) such that the needle tip is just below the sealed end. Pressure was gently applied on the syringe plunger such that the paste was deposited near the sealed end of the PCL tube. Simultaneously, the tube was moved upwards to assist in filling the paste. The tube was filled till the mark on the PCL tube and the other end was crimped to form the final device.

6.1.3 Accelerated TAF degradation models developed in PCL Device

Four models were designed in order to identify a model that can accelerate TAF degradation in the implant (research or Sigma grade of PCL used). They were developed to accelerate the degradation of TAF drug in the paste present inside the device. These four conditions varied either by the void space, surface area of interaction between the paste and PBS or the integrity of the PCL device. All four models were stored in an aluminum pouch (Polyester/Foil laminates RFE-042, Amcor Flexibles, Zurich). They were placed in duplicates for stability monitoring in an incubator maintained at 45°C/ 75% relative humidity using saturated salt solutions of potassium chloride (Ricca Chemicals, TX, USA) (monitored using Fisherbrand™ Traceable™ Thermometer/Clock/Humidity Monitor, Fisher Scientific, USA). TAF paste was analyzed at 0 time for drug content. Sampling was performed at 2, 4 and 6 weeks for model M1, M2 and M3 and at 2 and 5 weeks for M4 model. Device was cut open using clean pair of scissors and placed in a 20mL scintillation vial containing 10mL methanol. It was stirred for one hour in dark and diluted 10 times using 25% acetonitrile in water for HPLC analysis. A schematic of the four models is illustrated in Figure 19.

All models had 4 groups:

1. Group 1: Open packaging + PBS: Device placed in an aluminum pouch that had one end unsealed. Device contained PBS
2. Group 2: Sealed packaging + PBS: Device placed in an aluminum pouch which was sealed. Device contained PBS
3. Group 3: Open packaging + no PBS: Device placed in an aluminum pouch that had one end unsealed. Device did not contain PBS

4. Group 4: Sealed packaging + no PBS: Device placed in an aluminum pouch which was sealed. Device did not contain PBS

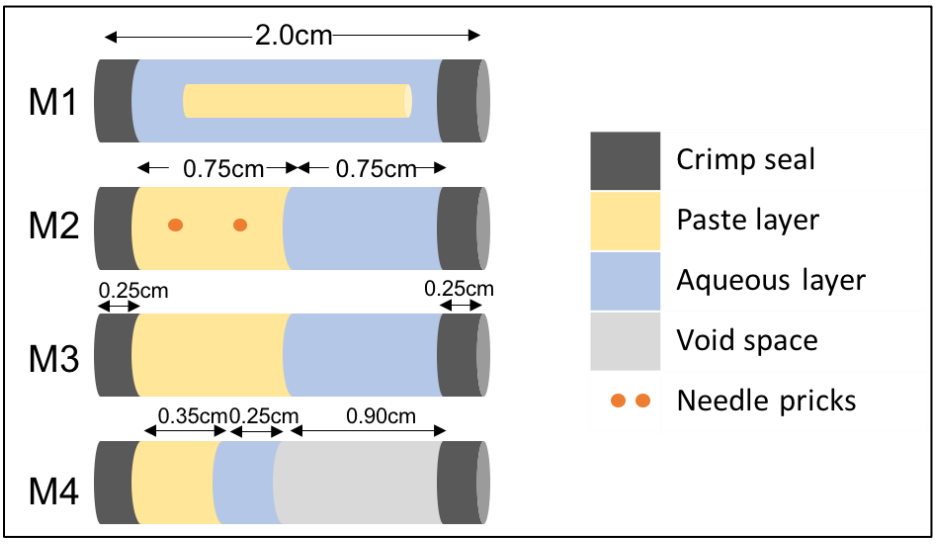

Figure 19 | Schematic of accelerated models (M1 to M4). M1 model: device filled with ~15mg TAF paste and surrounded by 30µL of PBS, M2 model: device filled upto 1cm with TAF paste (~30mg) and remaining half filled with PBS (15µL); PCL implant on the paste side was pricked with a needle to further stress the system, M3 model: device filled upto 1cm with TAF paste (~30mg) and remaining half filled with PBS (15µL); device was not pricked, M4 model: device was filled upto 6mm with TAF paste (~15mg), additional 2.5mm with PBS (10µL) and remaining 9mm was left empty to accommodate for increase in osmotic pressure due to degradant formation. All devices were individually placed in aluminium foil laminates, with (Sealed sub-group 2 and 4) or without (Open sub-group 1 and 3) crimp sealing. Half of the overall devices were filled with PBS (sub-group 1 and 2, 'w/PBS') and the other half were not filled with PBS (sub-group 3 and 4).

6.1.3.2 Proposed accelerated model - M1

Paste was prepared as per procedure described in section 6.1.1. One end of a 2cm PCL tube was crimped. The device was filled initially with 30µL of 1x PBS for groups 1 and 2 and no PBS was added for groups 3 and 4. Approximately 15mg of TAF paste was injected into this device

using a 17G stainless steel blunt tip needle and the other end of the tube was crimped. This model simulated the ingress of PBS from all sides.

6.1.3.3 Proposed accelerated model – M2

Paste was prepared as per procedure described in section 6.1.1. One end of a 2cm PCL tube was crimped. A marking was made at 1 cm from the outer crimped end of the device. It was filled with TAF paste (~30mg) up to this mark using a 14G blunt tip stainless steel needle. 1x PBS (15µL) was added from top for groups 1 and 2 and the other end was crimped. No PBS was added for groups 3 and 4. The PCL implant on paste side of the device was pricked using a sharp-tip 20G needle twice to further stress the device.

6.1.3.4 Proposed accelerated model – M3

Paste was prepared as per procedure described in section 6.1.1. One end of a 2cm PCL tube was crimped. A marking was made at 1 cm from the outer crimped end of the device. It was filled with TAF paste (~30mg) up to this mark using a 14G blunt tip stainless steel needle. 1x PBS (15µL) was added from top for groups 1 and 2 and the other end was crimped. No PBS was added for groups 3 and 4. This model was not pricked.

6.1.3.5 Proposed accelerated model – M4

Paste was prepared as per procedure described in section 6.1.1. One end of a 2cm PCL tube was crimped. A marking was made at 6mm from the outer crimped end of the device. It was filled with TAF paste (~15mg) up to this mark using a 14G blunt tip stainless steel needle. 1x PBS (10µL) was added from top for groups 1 and 2. The remaining 9mm was left empty to

accommodate for increase in osmotic pressure due to degradant formation and the other end was crimped. No PBS was added for groups 3 and 4.

After identifying one accelerated degradation model in the Sigma grade of PCL, it was further verified by testing it in the clinically used (medical) PuraSorb PC12 grade of PCL. The PuraSorb PC 12 grade of PCL was going to be used for excipient screening and formulation optimization and hence validating the effectiveness of the developed accelerated model in this grade was necessary.

6.1.4 Alternate accelerated stability model developed to screen pH modifier concentrations in auto-sampler inserts

The methods and results of this model will be discussed in detail in section 7.3.2.

6.1.5 Statistical Analysis

Statistical analysis was performed while comparing the two grades of PCL – Sigma and PuraSorb PC12. Results were presented as mean % TAF recovery ± standard deviation (SD). Unpaired t test was performed in GraphPad Prism version 8.0.0. p value <0.05 was considered statistically significant and ns = not significant.

6.2 Results

Four accelerated screening models were evaluated to identify one model that can accelerate TAF degradation inside the device for rapid excipient screening. These models were tested for a duration of 6 weeks and the % TAF degraded was analyzed using the stability indicating HPLC method. One selected model was further tested in the clinically used PuraSorb PC12 (medical grade) PCL to validate the applicability of the model for excipient screening and optimization.

6.2.1 PCL device-based screening models

Four models (M1 to M4) were tested using the research grade (Sigma grade) of PCL over a period of either 5 or 6 weeks to identify one optimal model for excipient screening at 45°C/ 75% RH. In M1, M2 and M3 models, study arms of control, open pouches with (Group 1) and without (Group 3) PBS and sealed pouch without PBS (Group 4) had %TAF degradation lower than 20% after 6 weeks. Interestingly, study arm of sealed pouch with PBS (Group 2) showed highest degradation (~100% at 6 weeks) across all three models. M3 model accelerated TAF degradation the fastest, with 92% TAF degraded within 2 weeks (Figure 20-C). In contrast, TAF degradation in M1 and M2 models was found to be 45% (Figure 20-A) and 66% (Figure 20-B) respectively. However, at 4 weeks, devices in M1, M2 and M3 model were observed to have ruptured. The rupture of PCL device may have been caused due to osmotic pressure build-up inside the device and may have contributed to the near 100% degradation of TAF inside the paste.

Thus, a fourth accelerated model (M4 model) was developed that incorporated a void space to account for the increased osmotic pressure inside the device. Percent TAF degradation in the

M4 model was found to be 98% over 5 weeks (Figure 20-D). As observed in the earlier three models, the Group 2 (sealed with PBS) demonstrated highest TAF degradation.

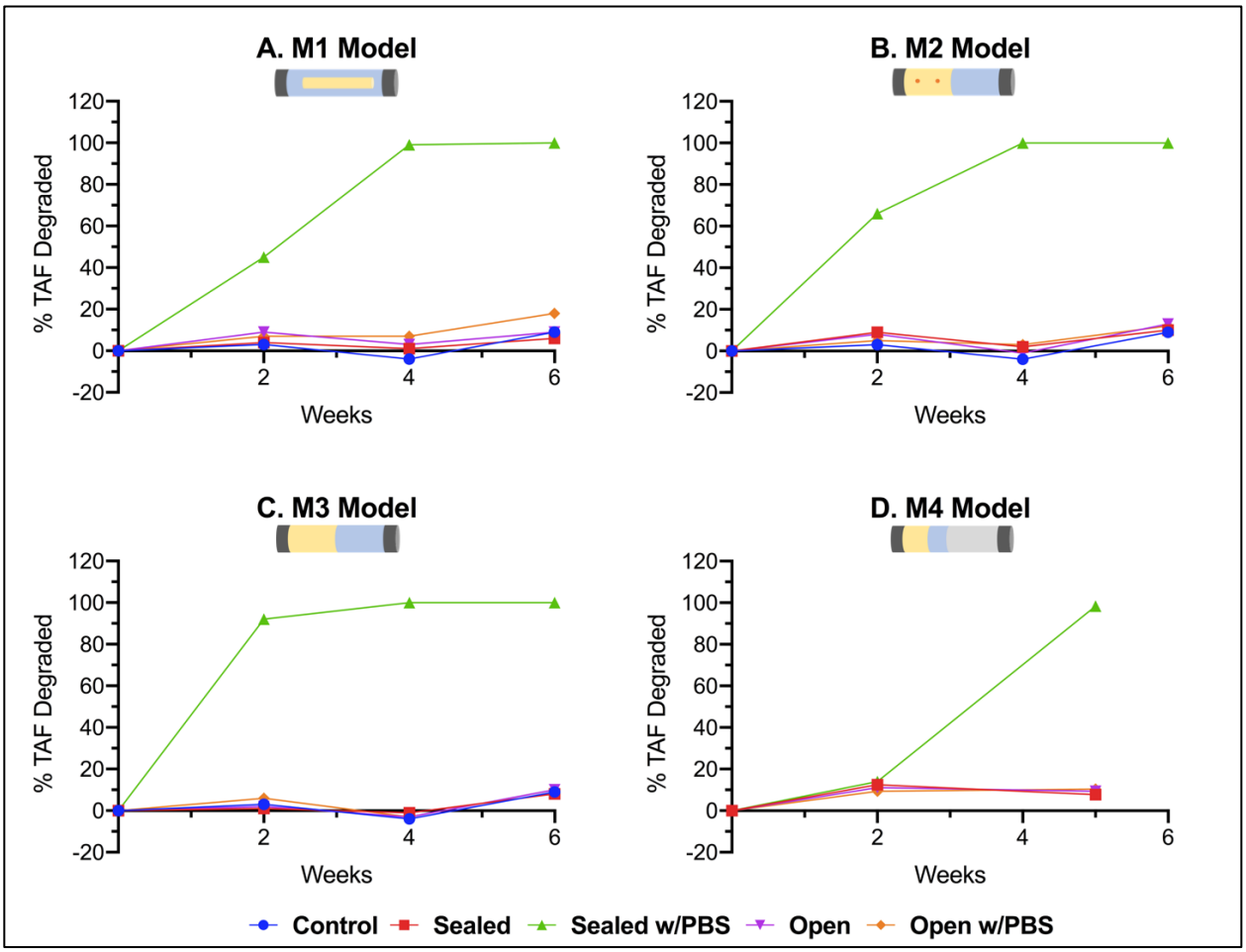

Figure 20 | **Evaluation of accelerated stability models of TAF in implant.** Figures above show %TAF degraded v/s time (weeks) (n=2 per sub-group per time-point) : **A.** M1 model (device filled with ~15mg TAF paste and surrounded by 30µL of PBS), **B.** M2 model (the 2cm device was filled upto 1cm with TAF paste (~30mg) and remaining half was filled with PBS (15µL). The PCL implant on the paste side was pricked with a needle to further stress the system), **C.** M3 model (the 2cm device was filled upto 1cm with TAF paste (~30mg) and remaining half was filled with PBS (15µL). The device was not pricked), **D.** M4 model (the 2cm device was filled upto 6mm with TAF paste (~15mg), additional 2.5mm with PBS (10µL) and remaining 9mm was left empty to accommodate for increase in osmotic pressure due to degradant formation). All devices were individually placed in aluminium foil laminates, with (Sealed sub-group) or without (Open sub-group) crimp sealing. Half of the overall devices were filled

with PBS as described (sub-groups ‘w/PBS’) and the other half were not filled with PBS. Approprate controls were established for each model and all devices were placed in incubators set at 45°C/75 %RH.

The devices did not rupture and there was a considerable amount of degradation in the Sigma grade PCL based M4 model (group 2 – sealed with PBS) and hence, this set-up was further evaluated in the medical grade (PuraSorb PC12) of PCL. Since further experiments were to be conducted in the clinically relevant PuraSorb PC12 grade of PCL, it was essential to validate the degradation profile of the M4 set-up in devices made with this grade of PCL. Thus, a PCL grade comparison study was performed to evaluate the degradation profiles of TAF in devices made of both PCL grades. Also, a large gap in TAF degradation was observed in the sampling points of M4 model, (from ~15% degradation at week 2 to ~98% degradation at week 5 (Figure 20-D)). Hence, we closely monitored the degradation trend by weekly sampling for the PCL grade comparison study. As seen in Figure 21, there was no statistical difference between TAF degradation trend in both the research and medical grade of PCL over 3 weeks.

Thus, the M4 model incorporating PBS, placed in a sealed aluminum laminate (Group 4 – sealed with PBS) and exposed to 45°C/ 75% RH incubator conditions were used for further excipient screenings and formulation optimization.

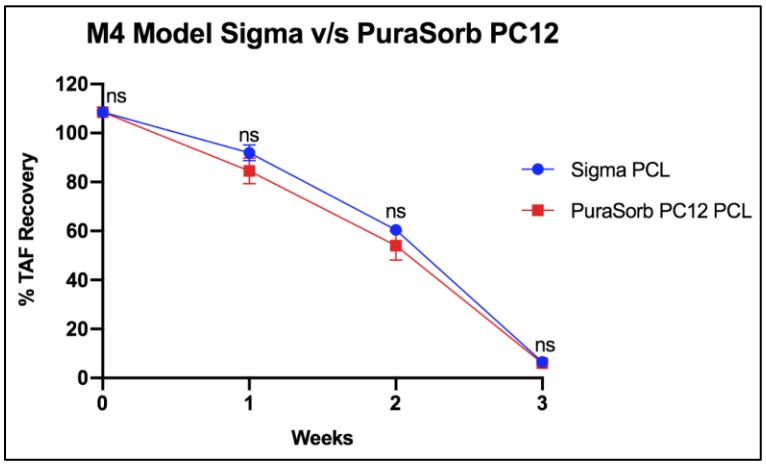

Figure 21 | M4 model comparing the two grades of PCL – research grade (Sigma) or medical grade (PuraSorb PC12). TAF paste in castor oil was filled in tubes of both these polymers as per procedure in section 6.1.3.5. Samples were analyzed for TAF drug recovery every week. Multiple t-test was used to analyze data at each time-point (ns = not significant). (Data represented as mean±SD; n=3 per group per time-point)

6.3 Discussion

In this chapter, an accelerated degradation model of TAF in PCL implant was developed. Four models M1 to M4 were proposed and were evaluated at incubator conditions of 45°C/75%RH. The models (Figure 19) included simulations of PBS ingress from all sides (M1 model), artificial inflictions made on the PCL polymer for greater exposure to incubator conditions (M2 model) and PBS ingress from one side of the paste with the absence (M3 model) and presence (M4 model) of a void space. Void space was accounted for as it would alleviate the effect of increased osmotic pressure due to high solute concentration into the device and prevent its impact on PCL polymer integrity.

All four models were placed in four groups into the incubator – Group 1 (open packaging with PBS), group 2 (sealed packaging with PBS), group 3 (open packaging without PBS) and

group 4 (sealed packaging without PBS). Although unsealed pouches had greater exposure to high humidity of the incubator, it also led to the evaporation of the liquid inside the device. This explained why both groups 1 and 3 (with and without PBS respectively) in the unsealed group (Figure 20) had lower levels of degradation compared to the sealed packaging arm with PBS (group 2). For group 2 (sealed with PBS), M3 model showed highest degradation compared other model set-ups. However, the devices in M1, M2 and M3 model of this group were found broken within 4 weeks due to increased osmotic pressure. Once the device was ruptured, the integrity of PCL was lost, accelerating TAF degradation. Hence greater degradation was observed for these three groups compared to M4 model, where the devices were intact for 5 weeks. It was necessary to validate this model in the medical PuraSorb PC12 grade of PCL as this would be eventually used in the clinic. Figure 21 shows that a similar trend with no statistical differences observed in TAF degradation in devices made of the PuraSorb PC12 grade when compared to the Sigma grade of PCL.

6.3.1 Limitations and future directions

A potential limitation of the accelerated model M4 in the 2 cm PCL device incorporates a void space, which is not a true representation of the final commercial implant. The impact of the variability in length of the implant and the void space on the stability of the full length 4 cm commercial implant is hence unknown. Lastly, the physiological fluid ingress in a subcutaneously implanted device will be from all directions whereas the M4 model demonstrates only an uni-directional effect of PBS on TAF degradation. Whether the accelerated model can closely recapitulate the *in vivo* conditions, is yet to be studied.

6.3.2 Impact

A novel accelerated degradation model was developed for rapid screening of excipients for formulation development. The novel degradation method utilized only 15mg of API, conserving the amount of TAF required for the study. TAF recovery observed at 21 days in this accelerated system was comparable to the TAF recovery observed around 91 days in the real-time *in vitro* release study conducted by our collaborators [105]. This shows that the M4 model accelerated degradation of the drug substance over 4-folds. The developed accelerated model can be further extended for use in routine quality control analysis during implant fabrications. It can also be used for screening additional excipients and ARVs that can be delivered using the PCL platform.

7.0 Formulation Modification and Optimization to Improve TAF Stability in Implant (Specific aim 3)

The objective of this project is to optimize the TAF formulation in a long-acting PCL subcutaneous implant for HIV PrEP. In the earlier sections, specific aims 1 and 2 were addressed by highlighting the mechanism of TAF destabilization and by developing an accelerated model (M4 model) for quick excipient screening. The third and final specific aim was to perform formulation modification and optimization for improving the stability of TAF in the implant. We hypothesized that modulating the partitioning behavior of TAF from the oily to aqueous phase; and maintaining the intra-device pH to a level where TAF is most stable, might alleviate its degradation inside the implant. Hence, in this section, the current 3:1 ratio of TAF: Castor oil formulation was modified by incorporating various classes of pharmaceutical additives to enhance stability – Class I: HLB modifiers, Class II: different oils and Class III: pH modifiers. These formulations were evaluated in the developed accelerated model M4 and the excipients that stabilized TAF over a period of 3 weeks were short-listed. Formulations incorporating these short-listed excipients were further optimized and finally evaluated for a long-term (9 week) study in the M4 accelerated model.

7.1 Methods

7.1.1 Formulation modification – Excipient Class I: HLB modifiers

Four HLB modifiers – labrafac WL 1349 (HLB = 1), transcutol (HLB = 4), labrafil M2130 (HLB = 9) and gelot 64 (HLB = 10) were studied. It has been reported that the overall HLB values of mixtures are additive [125], i.e. it is a fractional ratio of the quantities of the individual components. Paste of TAF and various oil mixtures was fabricated as per the procedure described in section 6.1.1. HLB modifiers were added in pre-defined concentrations to both sesame and castor oil matrices, thus modifying the final HLB of the system (Table 10). The excipients were evaluated in the M4 accelerated model (n=3 per time-point) for 3 weeks with sampling at 0time, 1 week, 2 weeks and 3 weeks.

Table 10 | Concentration of HLB modifiers used and final HLB of mixtures with castor and sesame oil.

HLB Modifier	Excipient HLB	HLB modifier (% w/w)	Oil (% w/w)	Final HLB of system with Sesame oil	Final HLB of system with Castor oil
Labrafac™ lipophile WL 1349	1	20	80	5.8	11.4
Transcutol	4	20	80	6.4	12.0
Labrafil M2130	9	10	90	7.2	13.5
Gelot™ 64	10	5	95	7.15	13.8

7.1.2 Formulation modification – Excipient Class II: Different oils

The second strategy explored to stabilize TAF in the formulation was to replace castor oil with different oils such as cottonseed oil (HLB = 10), corn oil (HLB = 9), anise oil (HLB = 11.7) and soybean oil (HLB = 7). All the four oils selected have been used in FDA approved parenteral formulations [126]. Pastes consisting of TAF in various oils were fabricated as per the procedure described in section 6.1.1. The pastes were then placed into the M4 accelerated model (n=3 per time-point) and stability was monitored for 3 weeks with sampling at time 0, 1 week, 2 weeks and 3 weeks.

7.1.3 Formulation modification – Excipient Class III: pH modifiers

The final class of additives studied were the pH modifiers namely: dibasic sodium phosphate (Na_2HPO_4), sodium citrate, glycine, tromethamine, meglumine and a combination of sodium citrate and glycine (Table 11). These excipients were chosen as they have been already used in FDA approved parenteral formulations [126]. Paste of TAF with excipients and castor oil was fabricated as per the procedure described in section 6.1.1. The excipients were evaluated in the M4 accelerated model (n=3 per time-point) for 3 weeks with sampling at 0time, 1 week, 2 weeks and 3 weeks.

The concentrations of pH modifiers were calculated to be 1.5 times the molar equivalence to the amount of fumaric acid in TAF (TAF contains approximately 10% fumaric acid [93]; also calculated by HPLC (data not shown)). For the combination of sodium citrate and glycine, equimolar concentration of the individual excipient to fumaric acid was selected. It was assumed that 15mg of TAF paste (1.5mg or 0.0129mM of FA) is filled in the device subjected to M4

accelerated model. Thus, pH modifiers amounting to 0.0194mM (1.5× molar equivalent of FA) were added for groups of Na_2HPO_4, sodium citrate, glycine, tromethamine, meglumine and 0.0129mM for the combination of sodium citrate and glycine.

Table 11 | Formulation composition of TAF in castor oil + pH modifer excipients.

pH Modifier Groups	Formulation Ingredients (%)				
	TAF	Excipient 1	Excipient 2	Castor oil	Total
Control	75.0	-	-	25.0	100
Na_2HPO_4	60.4	11.5	-	26.6	100
Sodium citrate	55.3	19.0	-	25.7	100
Citrate (Excipient 1) + Glycine (Excipient 2)	64.7	14.7	4.3	28.6	100
Tromethamine	64.8	10.2	-	25.0	100
Glycine	68.4	6.6	-	25.0	100
Meglumine	59.7	15.0	-	25.3	100

7.1.4 Formulation optimization – Improving TAF paste syringe-ability

A qualitative assessment was performed to identify the optimal ratio for the combinations of TAF and pH modifiers to castor oil. Initially, a 3:1 (TAF + excipient): castor oil paste was formed as per procedure in section 6.1.1. A small accurate quantity of castor oil was sequentially added to this mixture and mixed using a mortar and pestle until a syringe-able paste was formed. It was checked by loading and dispensing of the paste from the syringe as per procedure in section 6.1.1. The final composition of the mixture was calculated.

7.1.5 Formulation optimization – Identification of ideal pH modifier concentration

Three levels of concentrations of pH modifiers in TAF paste were selected for this study. The concentration was calculated in molar equivalence to the amount of fumaric acid in TAF form (TAF contains approximately 10% fumaric acid [93]). pH modifiers in molar equivalents of 0.5 times, 1 time and 1.5 times that of fumaric acid were studied using the auto-sampler HPLC insert screening model over 4 weeks. Briefly, a 40mm PCL tube was crimp sealed on one end and filled entirely with the TAF paste of interest. The other end was crimp sealed. Using a #11 blade, the filled PCL device was sliced to make 3.5mm discs (Figure 22-A). These discs were placed horizontally in an HPLC auto-sampler glass insert such that the PCL tube wall is parallel to the insert wall. 15µL 1x PBS was added from top to submerge the disc (Figure 22-B). This assembly was placed in an amber colored 2mL HPLC vial, capped and placed in 45°C/ 75% RH incubator. Samples were analyzed bi-weekly for 4 weeks for drug content.

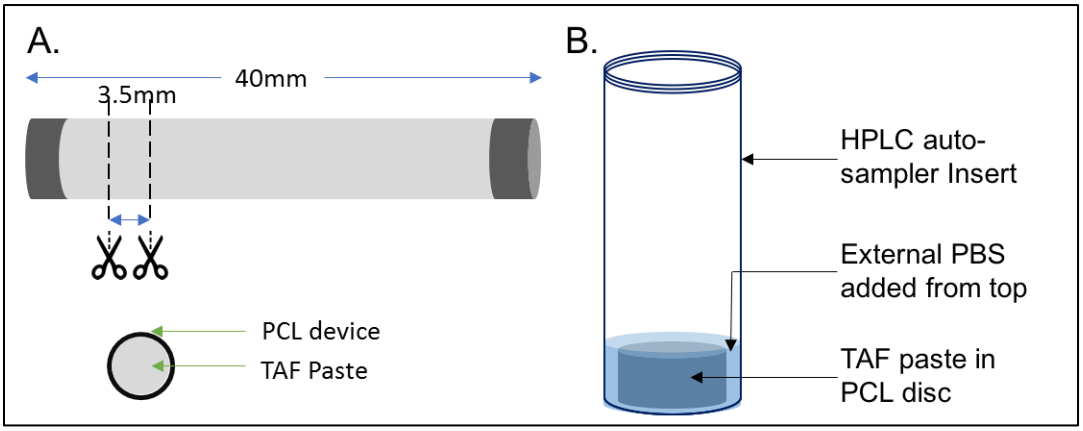

Figure 22 | Schematic of In-insert model. A. Assembly and cross-section of the TAF paste in PCL disc and **B.** Set-up of TAF paste in PCL disc inside the HPLC auto-sampler insert. Paste of TAF and pH modifiers in varying concentrations in castor oil was prepared. A 40mm PCL tube was crimp sealed on one end and filled entirely with the TAF paste of interest. The other end was crimp sealed. Using a #11 blade, the filled PCL device was carefully sliced

to make 3.5mm discs. This disc were placed horizontally in an HPLC auto-sampler glass insert so that the PCL tube wall is parallel to the insert wall. 15µL 1x PBS was added from top to submerge the disc. This assembly was placed in an amber colored 2mL HPLC vial, capped and placed in 45°C/ 75% RH incubator. Samples were analyzed at 0 time, 2 and 4 weeks for drug content.

7.1.6 Formulation optimization - Accelerated stability testing of final formulations

The optimized formulae in Table 12 (section 7.2.4.2) were tested for 9 weeks in M4 accelerated model to evaluate their long-term accelerated stability. Multiple runs were performed for each pH modifier group to evaluate its inter-batch reproducibility. Two separate runs were performed for Na_2HPO_4 (n=3 per time-point) and sodium citrate (n=3 per time-point) whereas three runs were performed for tromethamine (n=3 per time-point).

7.2 Results

The accelerated stability model identified in the earlier chapter was used for rapid screening of excipients in this section. Various classes of pharmaceutical additives were studied to enhance TAF stability in the formulation namely: HLB modifiers added to TAF in castor oil formulation, different oils to replace castor oil in the TAF formulation and pH modifiers added to TAF in castor oil formulation.

7.2.1 Formulation modification – Excipient Class I: HLB modifiers

After 1 week, all HLB modifiers except transcutol in both castor oil (Figure 23-A) and sesame oil (Figure 23-B) groups had a % TAF recovery within 90-110%. However, at 2 weeks the % TAF recovery was <60% for all groups. TAF recovery in all groups was observed to be < 10% at 3 weeks. None of the HLB modifiers were successful in preventing degradation of TAF in both castor and sesame oils. Thus, modification of HLB in the formulation may not be a suitable strategy to stabilize TAF.

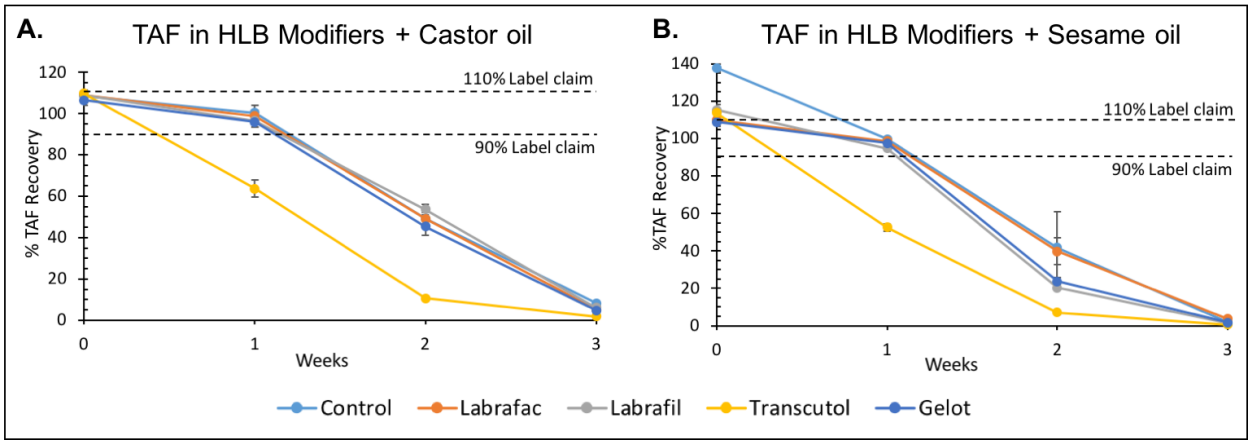

Figure 23 | %TAF Recovery for HLB modifiers in Castor oil over 3 weeks. Four HLB modifiers – labrafac WL 1349 (HLB = 1), transcutol (HLB = 4), labrafil M2130 (HLB = 9) and gelot 64 (HLB = 10) and control (TAF in castor oil) were evaluated. Paste of TAF and various oil mixtures and control was fabricated as per the procedure described in section 6.1.1. HLB modifiers were added in pre-defined concentrations as listed in table 10 to both sesame and castor oil matrices. The excipients were evaluated in the M4 accelerated model for 3 weeks with samples taken at 0 time, 1, 2 and 3 weeks. (Data represented as mean ± SD, n=3 per group per time-point).

7.2.2 Formulation modification – Excipient Class II: Different oils

Although cottonseed, corn and soybean oil improved TAF stability (% TAF recovery between 50-60%) with respect to castor oil control (zero % TAF recovery) (Figure 24), they were insufficient by themselves in stabilizing TAF within 90-110% level throughout the experiment. Additionally, challenges were faced while fabricating the pastes incorporating these oils. Hence additional work for improving processability of the paste is warranted along with combination of other classes of additives to further stabilize the formulation.

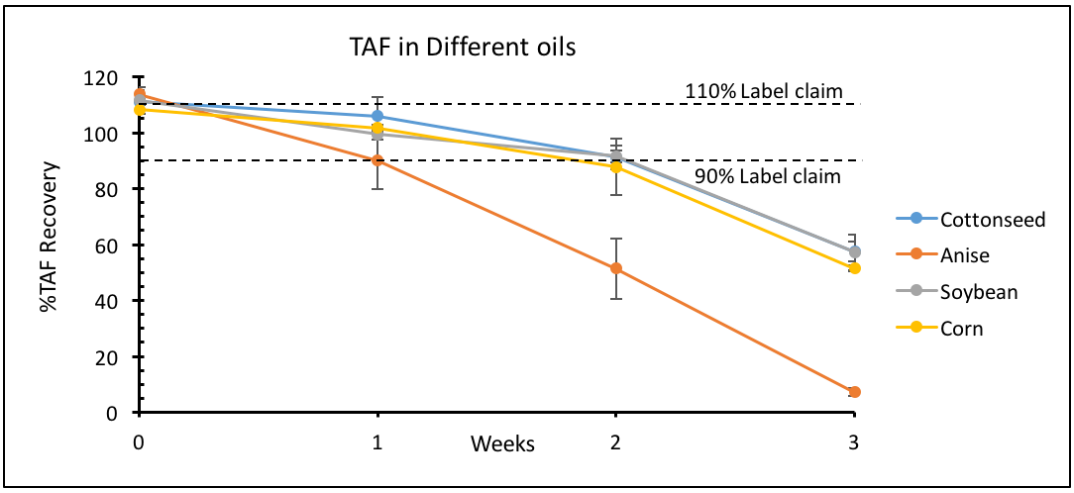

Figure 24 | %TAF Recovery in Different Oils over 3 weeks. Castor oil was replaced with different oils namely cottonseed oil (HLB = 10), corn oil (HLB = 9), anise oil (HLB = 11.7) and soybean oil (HLB = 7). Paste of TAF and various oils was fabricated as per the procedure described in section 6.1.1. The excipients were evaluated in the M4 accelerated model for 3 weeks with samples taken at 0 time, 1, 2 and 3 weeks. (Data represented as mean ± SD, n=3 per group per time-point).

7.2.3 Formulation modification – Excipient Class III: pH modifiers

As seen in (Figure 25), after 3 weeks, all pH modifiers except glycine had a %TAF recovery within 90-110%. On the contrary, <5% of TAF Recovery was observed in the control group. Thus,

controlling the pH environment within the PCL device greatly improved TAF stability. Based on the results observed in Figure 25, pH modifiers Na_2HPO_4, sodium citrate, tromethamine and meglumine were chosen for further optimization.

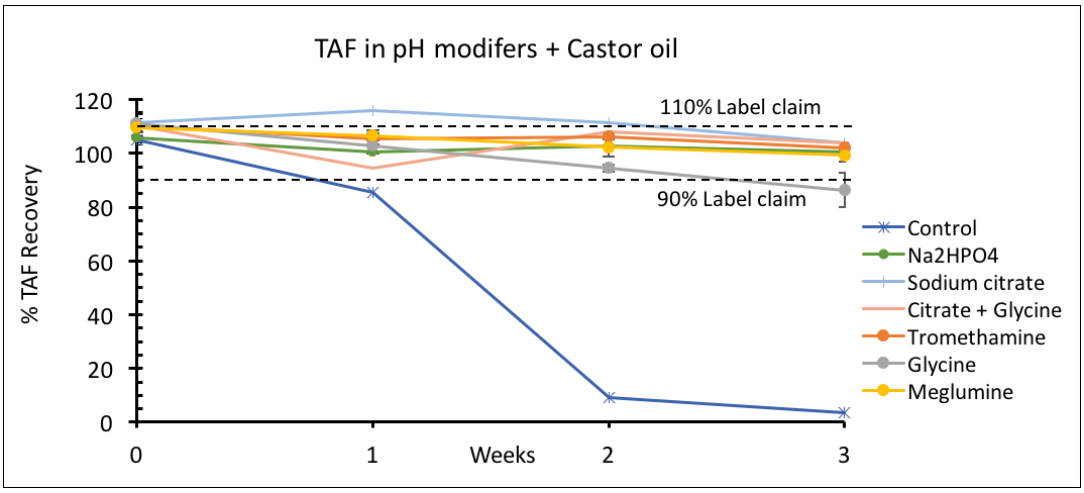

Figure 25| %TAF Recovery for pH modifier class of additives over 3 weeks (n=3 per time-point). 6 groups of pH modifiers were studied: dibasic sodium phosphate, sodium citrate, tromethamine, glycine, meglumine and a combination of sodium citrate and glycine along with a control of TAF in castor oil without any additive. Paste of TAF and various pH modifiers in castor oil and control was fabricated as per the procedure described in section 6.1.1. pH modifiers were added in pre-defined concentrations as listed in table 11 to the castor oil matrices. The excipients were evaluated in the M4 accelerated model for 3 weeks with samples taken at 0 time, 1, 2 and 3 weeks. (Data represented as mean ± SD, n=3 per group per time-point).

To summarize the formulation modification efforts, three classes of additives were studied in the accelerated model to stabilize TAF in the paste formulation. Class of pH modifiers showed most promise with five out of six excipients stabilizing TAF within the 90-110% recovery level over 3 weeks. Additional efforts were carried out in order to optimize these preliminary formulations in the next section.

7.2.4 Optimization of the modified TAF formulation

A drawback of pH modifiers class of excipients was the increased difficulty of paste fabrication. Also, a higher concentration (1.5x molar equivalent to fumaric acid in TAF paste) of pH modifiers was chosen for the preliminary screen performed above. Thus, further experiments were focused on improving syringe-ability (or paste fabrication) of TAF formulation by altering the ratio of solid excipients (TAF+ pH modifier): castor oil. Further, the concentration of pH modifiers in the TAF paste was also optimized.

7.2.4.1 Paste optimization to improve syringe-ability

A qualitative assessment was performed to identify the optimal ratio for the combinations of TAF and pH modifiers to castor oil. Four pH modifiers, dibasic sodium phosphate, sodium citrate, tromethamine and meglumine were initially selected for paste optimization. The pastes were studied for syringe-ability as per procedure described in section 7.3.1. However, combination of TAF and meglumine with castor oil could not form a syringe-able paste. Hence, dibasic sodium phosphate, sodium citrate and tromethamine were further advanced in the optimization process.

7.2.4.2 Paste optimization to identify ideal excipient concentration

For all three pH modifiers, %TAF Recovery for 1.5x molar equivalent of fumaric acid (FA) groups fell within 90-110% range. pH modifiers at 0.5x molar equivalent of FA degraded faster than the other two concentration groups for all 3 excipients (Figure 26-A-C). pH modifiers at equimolar concentrations of FA also demonstrated %TAF recovery within 90-110% range. However, equimolar concentrations of pH modifiers need to be evaluated in the M4 accelerated model before advancing to paste optimization and final screening stages. Thus, for all three pH

modifiers, concentrations at 1.5x molar equivalent of FA were used in the optimized paste compositions (Table 12) and were used for further experiments.

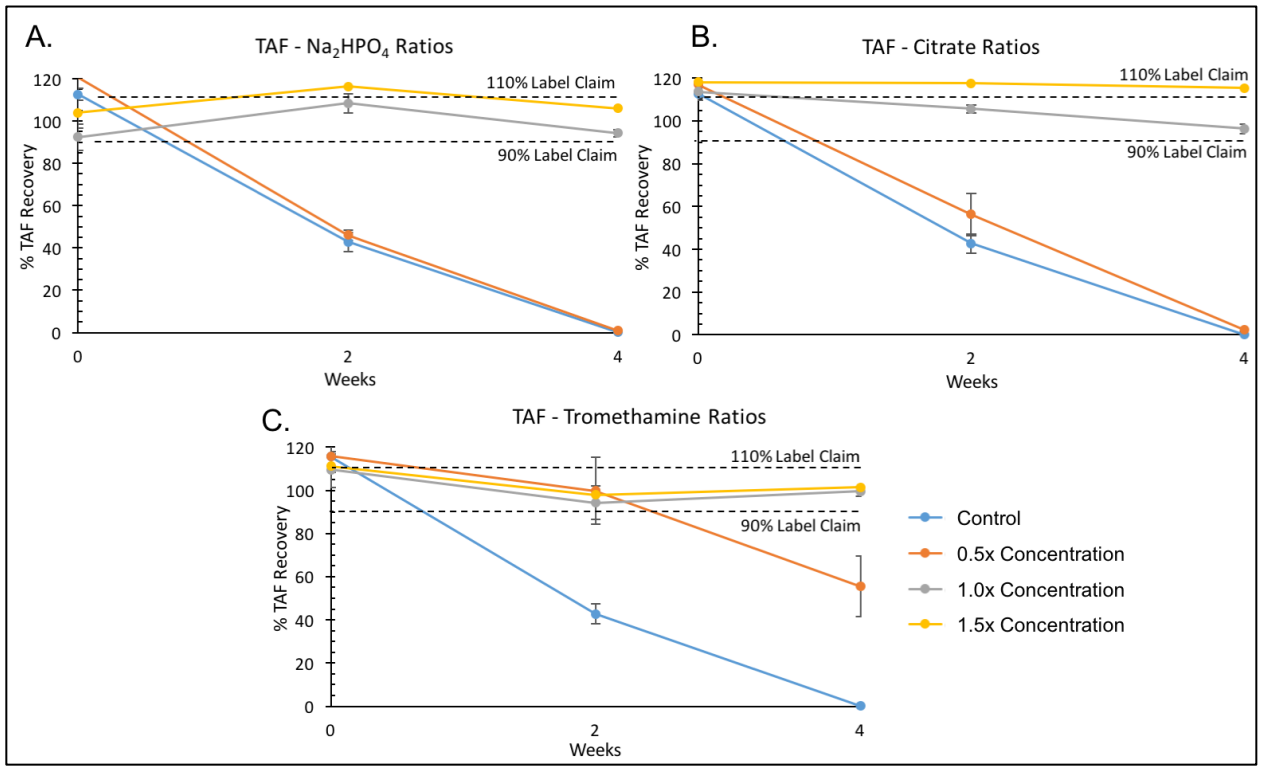

Figure 26 | Optimization of pH modifier concentration using the HPLC insert model. A. TAF and dibasic sodium phosphate in castor oil, **B.** TAF and sodium citrate in castor oil and **C.** TAF and tromethamine in castor oil. (n=3 per time-point). pH modifiers dibasic sodium phosphate, sodium citrate and tromethamine were added to TAF paste in castor oil in molar equivalents of 0.5 times, 1 time and 1.5 times that of fumaric acid. The samples were studied using the auto-sampler HPLC insert screening model over 4 weeks with sampling at 0 time, 2 and 4 weeks. (Data represented as mean ± SD, n=3 per group per time-point).

Table 12 | Final optimized compositions for three pH modifiers.

Ingredients	Dibasic sodium phosphate	Sodium citrate	Tromethamine
TAF (%w/w)	62	56.222	59.421
Castor oil (%w/w)	26.5	25	31.25
pH modifier (%w/w)	11.5	18.778	9.329

7.2.5 Accelerated stability testing of optimized TAF compositions

TAF recovery for all three runs of tromethamine was outside the set specifications of 90-110% (Figure 27-C). % TAF recovery for both Na_2HPO_4 (Figure 27-A) and sodium citrate (Figure 27-B) groups for both runs were between 90-110% range. Zero percent TAF was recovered for all controls at week 9, highlighting the superior stability imparted by the pH modifiers. From results observed in Figure 27, two stable TAF formulations (containing pH modifiers Na_2HPO_4 or sodium citrate) were identified.

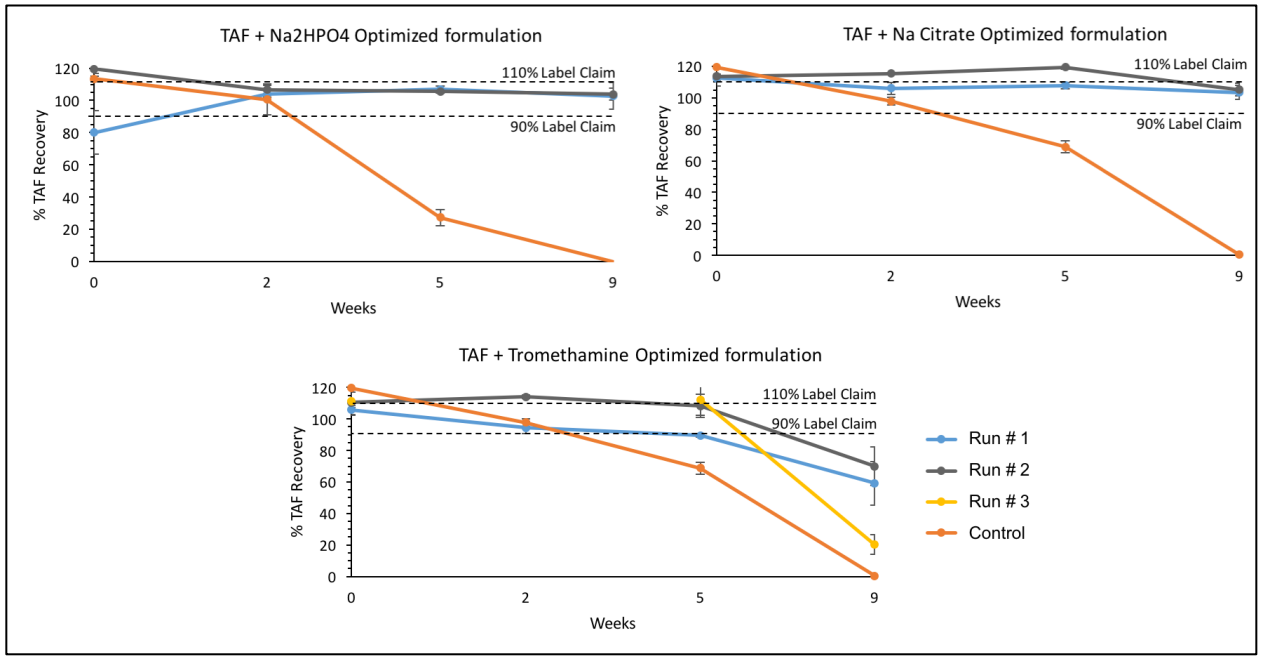

Figure 27 | Final optimized formulations evaluated in long term in accelerated model M4. Three groups of pH modifiers namely dibasic sodium phosphate, tromethamine and sodium citrate were studied. Paste of TAF and various pH modifiers in castor oil and control was fabricated as per the procedure described in section 6.1.1. pH modifiers equivalent to 1.5 times fumaric acid were added in pre-defined concentrations as listed in table 12 to the castor oil matrices. The excipients were evaluated in the M4 accelerated model for 9 weeks with samples taken at 0 time, 2, 5 and 9 weeks. (Data represented as mean ± SD, n=3 per group per time-point).

7.3 Discussion

The overarching project goal of this work was to optimize TAF formulation in a long-acting PCL subcutaneous implant for HIV PrEP. Two hypotheses derived from the literature were articulated to address the goal. The first hypothesis was to modify the partitioning behavior of TAF in order to reduce the migration from the oil phase to aqueous phase. It was assumed that this will alleviate the hydrolytic degradation of TAF. HLB is an empirical expression to determine the hydrophilic and hydrophobic nature of a multi-phasic system. It shows the ease of compatibility of the organic or oil phase with water. Higher the HLB value, greater is the hydrophilicity, and lower the HLB value, greater is the hydrophobicity of the oil and lesser is the ease of mixing with water. HLBs are additive [125]. Two experiments were performed to modify the HLB value of the system. The first strategy employed the use of different HLB modifiers and the second strategy was to substitute castor oil by different oils. Castor oil has an HLB of 14 whereas sesame oil has an HLB of 7. Although we hypothesized that incorporating different excipients with different HLB values would ultimately reduce TAF degradation, it was observed that HLB modifiers did not affect the %TAF recovery for any of the groups compared to control (Figure 23). This can be due to the fact that we added only between 5-20% of HLB modifiers that marginally modified the overall HLB of the oil mixture (Table 10). Higher concentrations of the modifiers could have a greater impact on the HLB of 7 and 14 of sesame and castor oil respectively, but it can potentially have a negative impact on the processability and syringe-ability of the paste. Moreover, impact of the use of HLB modifiers on release kinetics of TAF from the paste is not known.

The second strategy to tackle the migration of TAF from oil to aqueous phase was by substituting castor oil with different oils. We selected four oils (cottonseed, corn, soybean and anise oil) that were previously FDA approved for parenteral use. Cottonseed, soybean and corn oil

moderately improved the stability of TAF (between 50 – 60% TAF recovery, Figure 24) whereas only 7.2% TAF could be recovered from anise oil devices at the end of three weeks. All the groups were out of specifications of 90-110% at the end of the experiment. Also, it was difficult to syringe all four pastes formed using TAF and these oils. Thus, cottonseed, corn, soybean and anise oils cannot be used individually to stabilize TAF. Moreover, additional efforts will have to be taken to improve the processability of the pastes. Although the present work did not evaluate formulation modifications incorporating cottonseed, corn or soybean oils, they can potentially stabilize TAF, especially when supplemented with other additives, such as pH modifiers. Since pastes of TAF in castor oil were easier to formulate and syringe, further efforts were dedicated to stabilizing TAF with different additives in a castor oil matrix.

Based on literature and our pre-formulation studies, the second hypothesis was to modify the intra-device pH in order to prevent TAF degradation. We identified a non-exhaustive list of pH modifiers that are already approved by the FDA for parenteral delivery (Table 11). These pH modifiers were formulated as a TAF+ pH modifier in castor oil paste and tested in the M4 accelerated model over a period of 3 weeks. While the control degraded to almost 0% TAF by three weeks, all pH modifiers studied improved TAF recovery (Figure 25). Five out of the six pH modifiers studied (all except glycine) had a %TAF Recovery within specifications of 90-110% after three weeks. This supported the hypothesis that modifying the intra-device pH assisted in TAF stability. Overall, pH modifiers were better at improving the stability of TAF in the accelerated model compared to the other two classes of excipients. Four excipients (dibasic sodium phosphate, sodium citrate, tromethamine and meglumine) were down-selected for a qualitative assessment of syringe-ability and paste processability. Meglumine group could not form a paste that could easily pass through the syringe and hence was not selected for further optimization.

Next, the concentration of pH modifier in the implant was optimized. Three concentration levels were selected for this experiment – 0.5x, 1x and 1.5x the molar equivalent of fumaric acid. TAF drug substance has around 10% fumaric acid, a dicarboxylic acid. Neutralizing the acidic carboxylic groups of fumaric acid can prevent the reduction of pH by the solubilized drug and help in maintaining the pH within the implant. An alternate accelerated model was developed that utilized HPLC auto-sampler inserts for this experiment (Figure 22). This model was developed and used as it reduced the consumption of the expensive medical grade PCL tubes. 0.5x group degraded faster than 1x and 1.5x with the latter two groups falling within specifications after 4 weeks (Figure 26). 0.5x concentration of all excipients was insufficient in stabilizing TAF within the implant. This can be due to the inadequate neutralization of FA inside the TAF paste. 1.5x concentration had overall least reduction in %TAF recovery and was chosen as the optimal pH modifier concentration for TAF implant. This data suggests that neutralization of FA inside the device might be a viable strategy for TAF stabilization efforts. Although 1.0x concentrations of pH modifiers were also successful in stabilizing TAF, they needed to be evaluated in the M4 accelerated model before their use in the implant can be further explored.

Using the identified optimized formulae of three pH modifiers (dibasic sodium phosphate, sodium citrate and tromethamine, Table 12), a long term (9-week) accelerated stability was set up in the M4 model. At least two individual runs were performed to check the reproducibility of the formulations. Tromethamine containing implants were out of specification after 5 weeks (Figure 27-C). After the final 9-week accelerated study, the formulations containing sodium citrate and dibasic sodium phosphate significantly improved the stability of TAF inside the implant (Figure 27-A and B). To summarize, three formulations of TAF in castor oil were identified and studied

in a long term accelerated stability model. Two of the three formulations studied were found to be stable up to 9 weeks.

7.3.1 Limitations and future directions

Although the work described in this chapter is promising, there are a few potential limitations of the study, as described below. First, the excipient toxicity of the pH modifiers at the studied concentrations is unknown. Although all the selected excipients have been previously used in FDA approved parenteral dosage forms, the concentrations used in the implants have not demonstrated safety and acceptability in animal models. Moreover, the pH modifiers studied are highly water soluble. In an *in vivo* setting, their solubility and distribution kinetics inside the device after ingress of physiological fluids has not been studied. Once dissolved, they can potentially migrate outside the device, which might compromise their neutralizing ability.

Secondly, the effect of PCL polymer on the degradation of TAF is unknown. PCL may not contribute to acidic moieties inside the device, but the impact of low pH caused by FA and acidic degradants of TAF on the integrity of PCL is unknown. Lastly, although the accelerated model establishes superior stability of our selected formulations vs. control, the extrapolation of this model for estimating real-time *in vivo* stability or *in vitro* release is yet unknown. Nevertheless, the developed models are applicable for quick screening of excipients and to identify potential stability issues. Additional work needs to be done to answer the above listed limitations.

In future, the optimized formulations would be studied for *in vitro* release, and real-time and accelerated stability as per ICH guidelines. Additional work needs to be done to assess the scalability of the developed formulations for commercial manufacturing. A suitable equipment needs to be identified that can enable automated paste manufacturing. Various characterization

techniques, such as measuring rheological characteristics of the paste, paste homogeneity and drug content uniformity, may need to be introduced during scale-up. The impact of sterilization technique on paste stability needs to be evaluated. The formulations need to be tested in a long term *in vivo* dog pharmacokinetic model before translating the implant to the clinic. Lastly, there is potential for additional work to be performed to extend the duration of action of the implant to one year.

7.3.2 Impact

This implant is the first implant that is biodegradable in nature. It utilizes PCL which has already been approved by the FDA and is a well-established biodegradable polymer. It has precedent for use in contraceptive implant, with Capronor® being in the market for over a quarter century. Due to PCL polymer's proven track record, there are many commercial vendors available which is important from a large-scale manufacturing perspective and while translating this implant to the clinic. The devices are extruded using HME, a robust technique that has been used for large scale production of formulations for many years. The PCL EXPD platform also has the potential to deliver other potent ARVs for HIV PrEP. The active pharmaceutical ingredient in this implant is TAF, a highly potent anti-retroviral drug. TAF has been already approved by the FDA in 2015 for oral consumption in a tablet dosage form (Genvoya®). This would be beneficial in getting a relatively faster approval for TAF in the implant system in the FDA regulatory pathway compared to other new chemical entities. The two down-selected stabilizers (pH modifiers) have been used in FDA approved parenteral formulations, which can further reduce regulatory burden of the device. Systematic formulation development was carried out to identify two formulations that are stable under heavily accelerated conditions. These modified formulations of TAF in castor oil

incorporated in a long-acting polymeric biodegradable implant can possibly demonstrate sustained zero-order release of TAF over six months. The excipients identified in this study can be potentially used to overcome TAF instability in other formulations, such as enemas, gels and other implants.

8.0 Conclusion

Multiple reports have broadly identified few key attributes that need to be considered for the success of HIV PrEP. It is pertinent to keep in hindsight that PrEP is employed in a 'real-world' setting, where attributes such as socio-economical differences, gender inequalities, user preferences, high-risk groups such as sex-workers, cultural norms and societal stigmas exist. Although the daily 'pill' or Truvada® might be an answer for a select group of population for HIV prevention, availability of user-independent long acting system remains an unmet need.

The aim of the current work was to stabilize TAF formulation in a subcutaneous PCL-based implant intended for use as a LA system for HIV PrEP. First, a stability indicating HPLC method was developed and validated as per ICH guidelines. This analytical method can be extended for quantification and analysis of TAF and its degradants for other formulation platforms as well. Further, pre-formulation studies were performed to better understand the degradation of TAF inside the implant. A novel accelerated degradation model was then developed for rapid screening of excipients for formulation development. This model accelerated the degradation of the drug substance over 4-folds, affording quick screening and optimization of formulation excipients. The accelerated TAF degradation model can be extended for use in stability testing during routine quality control analysis. It can also be employed for screening of additional excipients and ARVs intended to be used in the PCL platform. During formulation development, over fifteen stabilizers belonging to the classes of HLB or pH modifiers were screened using the developed accelerated model. During formulation modification efforts, concentrations of the most promising excipients from this preliminary screen were further optimized. Formulations containing either sodium citrate or dibasic sodium phosphate were identified to be the most

suitable and effective for preventing TAF degradation inside the implant. Two independent batches of the final optimized formulations were found to be stable over 9 weeks in the accelerated model, suggesting the reproducibility of the results.

In the niche area of long-acting HIV PrEP systems, the present work is novel and has the potential to be easily scalable and translatable to the clinic. It is biodegradable, has the potential for bi-annual administration and utilizes established and commonly used methods for implant administration. The user-independent subcutaneous implant described in this thesis can hopefully answer the persistent problem of lack of long-acting systems for HIV PrEP.

Appendix A Abbreviations

AIDS	:	Acquired immune deficiency syndrome
ALLINI	:	Allosteric integrase inhibitors
API	:	Active pharmaceutical ingredient
ARV	:	Anti-retroviral
AZT	:	Azidothymidine or Zidovudine
CAB	:	Cabotegavir
COBI	:	Cobicistat
CRL	:	Complete response letter
EFdA	:	4’-Ethynyl-2-fluorodeoxyadenosine
EVA	:	Ethylene vinyl acetate
EVG	:	Elvitegravir
EXPD	:	Extruded polymeric device
FTC	:	Emtricitabine
GS-7340	:	Tenofovir alafenamide fumarate, or TAF
HAART	:	Highly affective anti-retroviral therapy
HIV	:	Human immunodeficiency virus
InSTI	:	Integrase strand transfer inhibitors
IVR	:	Intra-vaginal rings
PCL	:	Poly(caprolactone)

PLA	:	Poly (lactic acid)
PrEP	:	Pre-exposure prophylaxis
LA	:	Long acting
MSM	:	Men who have sex with men
NRTI	:	Nucleoside reverse transcriptase inhibitors
OD	:	Outer diameter
PBMC	:	Peripheral blood mononuclear cell
PMPA	:	9-[(R)-2(phosphonomethoxy)propyl] adenine, or TFV
RCT	:	Randomized controlled trial
RPV	:	Rilpivirine
RTI	:	Research Triangle Institute
STD	:	Sexually transmitted diseases
TAF	:	Tenofovir alafenamide fumarate, or GS-7340
TDF	:	Tenofovir disoproxil fumarate
TFPD	:	Thin film polymeric device
TFV	:	Tenofovir, or PMPA
TFV-DP	:	Tenofovir diphosphate
USFDA	:	United States Food and Drug Administration
WHO	:	World Health Organization

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