

Design and Evaluation of a Topical Rectal Specific Microbicide for HIV prevention

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University of Pittsburgh, 2015

According to the UNAIDS report 2013, there were 2.1 million new HIV infections including adults and children. This indicates that apart from the already existing therapeutic ART (Antiretroviral Therapy), there is a need for development of HIV prevention products which can reduce the risk of HIV transmission from person to person. Such products which are referred to as microbicides could be used as a means of pre exposure prophylaxis (PrEP). A vaginal gel containing 1% tenofovir, a nucleotide reverse transcriptase inhibitor (NtRTI), has been evaluated in clinical trials to be used prior to sexual intercourse for prevention of HIV infection. This vaginal gel was also evaluated for rectal use, where it showed negative side effects. However the same gel with reduced amount of glycerin did not show any side effects rectally. Physiologically, the rectum and vagina are very different. Hence, development of rectal specific microbicide products is needed. In addition to gel formulations, such products may also be in the form of a suppository, enemas, foams etc. [1]. The design of rectal specific gel and suppository products for tenofovir and its combination is the focus of this thesis work.

In addition to tenofovir, other classes of antiretroviral (ARV) drugs such as reverse transcriptase inhibitors, integrase inhibitors (IIs), entry inhibitors, and fusion inhibitors have been evaluated for the development of HIV prevention products [2, 3]. Raltegravir, an II, has shown potential to be a drug candidate for HIV preventative topical product given its anti-HIV activity when challenged post product treatment using a macaque model [4]. Elvitegravir is a potent analog of

raltegravir, which is currently available on the market in the form of a combination ART. Therefore a combination of the NtRTI (tenofovir) and the II (elvitegravir) in the form of a rectal specific gel and a rectal suppository was evaluated in this body of work.

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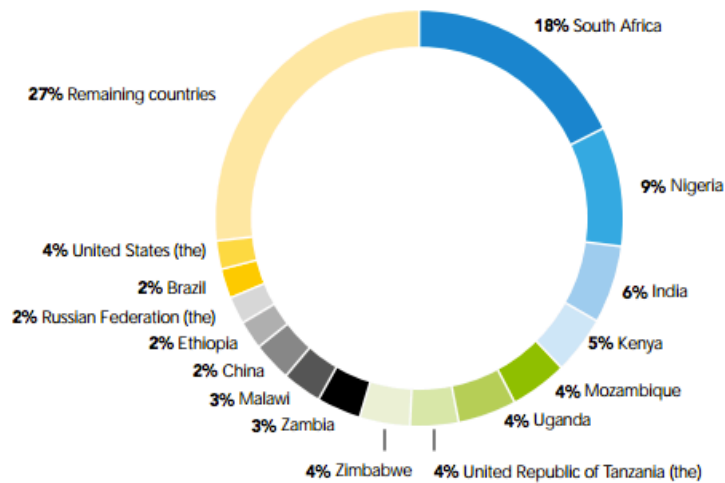
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1. INTRODUCTION

1.1. HIV Prevalence

The United Nations Program on HIV/AIDS (UNAIDS) report in 2013 estimates 35 million people infected with HIV globally [5]. It has been found that, one out of every seventh person in the US is not aware that they are infected with HIV [5]. Figure 1 represents the percentage of people living with HIV in different countries.

People living with HIV by country, 2013



Source: UNAIDS 2013 estimates.

Figure 1: Percentage of population living with HIV in different countries [6]

According to the HIV surveillance report 2014 from Centre for Disease Control's (CDC), the number of new HIV infections has remained stable over the past decade. But in contrast, the number of people living with HIV has increased. There have been approximately 2.1 million new HIV infections worldwide in the year 2013 [7]. Amongst these, majority of new infections are in men who have sex with men (MSM). In 2010, there was a 12% increase in the number of new HIV infections for MSM when compared to the year 2008 [8]. Also 63% of the new HIV infections in 2010 were accounted for MSM [8]. Considering the method of HIV transmission amongst the new infections, 31% of the new infections are due to sexual activity compared to 12% which is due to injection users [9]. Figure 2 shows the ratio of new infections among MSM based on race/ethnicity. These statistics clearly indicate that newer HIV prevention strategies are required for the population that might be at higher risk for HIV transmission through rectal route.

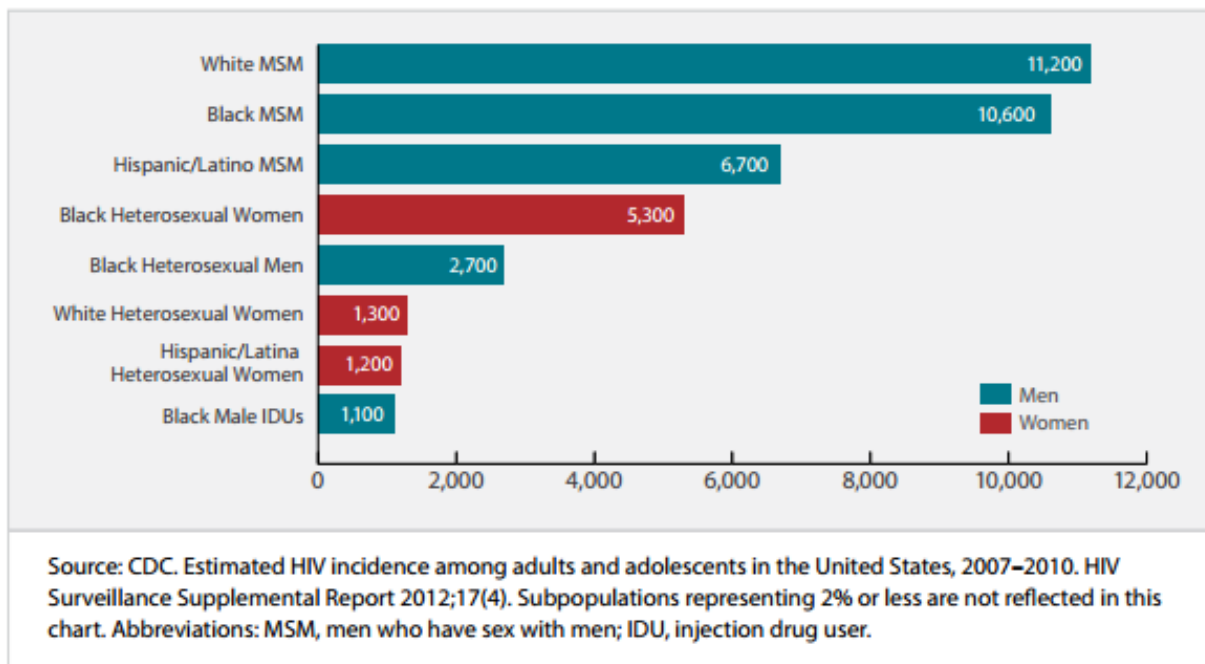


Figure 2: Subset of MSM population based on race/ ethnicity

1.2. Microbicides

The World Health Organization (WHO) defines microbicides as compounds that can be applied inside the vagina or rectum to protect against sexually transmitted infections (STI's) including HIV [10]. They can be formulated as gels, creams, rings, films, tablets, soft gel capsules and suppositories [11]. A few exploratory and acceptability studies are currently under progress to develop rectal microbicides in the form of an enema [12, 13]. Although there have been significant advancements in this field, there are presently no topical microbicides products available on the market. Approximately 23 microbicides are currently at different stages of development in clinical trials [10]. In this work, the development of rectal gels and suppositories as microbicides, their *in-vitro* characterization, preliminary efficacy & toxicity studies and potential drug combinations are presented.

Gels

Gels can be defined as homogenous semi-solid preparations consisting of a liquid phase within a three-dimensional polymeric matrix. They can be broadly categorized based on the nature of gelling agent's utilized i.e. hydrophobic and hydrophilic gels. Polymer based gels are conventional dosage forms which have been widely used in the pharmaceutical industry. For topical applications, the advantages of gels include higher drug loading capacity, less greasy etc. compared to suppositories. Specifically, gels can be delivered in large volumes to the rectal compartment. Due to their physical aspects especially spreadability, as well as familiarity and suitability to the patient, gels are more acceptable compared to other dosage forms.

Suppositories

Apart from gels, suppositories are also a promising rectal drug delivery method, which are widely used for treatment of various diseases [14, 15]. Suppositories are solid dosage forms used for rectal application and in few cases they are used for vaginal applications. Suppositories can utilize two base types i.e. lipophilic or hydrophilic bases. They can be formulated with one or multiple active pharmaceutical ingredients (API). Suppositories are preferred in some diseased conditions as they can be used either for local or systemic action. Drugs which are difficult to administer orally or lead to nausea and vomiting when given orally can be formulated as a suppository. Additionally, studies suggest that greater systemic levels of drugs were reached using rectal suppository compared to oral dosage form [16]. This can be beneficial to our approach as the suppositories can produce a quicker and better effect than oral route.

1.3. Purpose

A vaginal gel microbicide product containing 1% tenofovir (TFV), a nucleotide reverse transcriptase inhibitor (NtRTI), has been evaluated in clinical trials for vaginal use prior to sexual intercourse for prevention of HIV infection [17]. The results from this study indicated 39% overall reduction in HIV acquisition rate among the tested population. According to the GAP report 2014, MSM are 19 times more likely to acquire HIV [6]. This is probably because of the physiological differences between the rectal and vaginal epithelium. Rectal epithelium is thinner and fragile when compared to vaginal lining [13]. The CDC published a report in 2013 which states that about 81% of 37,887 new infections in the United States were gay and bisexual men [18]. This alarming number suggests that there is an urgent need for microbicide products for this specific population. To further investigate and design a rectal-specific formulation, the already existing vaginal TFV gel, which is in clinical trials, was evaluated in the rectal

compartment. However, this vaginal formulation showed poor acceptability and safety concerns *in-vivo* in the rectal compartment such as bloating, pain, urgency and diarrhea [19]. Moreover, the vaginal TFV gel was associated with side effects like colorectal epithelial disruption and sloughing in an *ex-vivo* explant model [20]. Further investigations led to the findings that the vaginal gel was hyperosmolar (3111 mOsmol/kg). Such hyperosmolar products could lead to colonic damage [19]. A recent clinical trial compared a reduced glycerin TFV gel, placebo gel and 2% nonoxynal-9 gel. The results from this clinical trial suggest that there is 87% likelihood of future product use for the reduced glycerin TFV gel [19]. These studies highlight the effectiveness of reduced glycerin TFV gel. Further, they highlight the need for rectal specific formulation due to the physiological differences between rectal and vaginal compartments.

In a study conducted by the CDC, a rectal specific gel formulation containing 1% raltegravir and L-81012, an II under development, was evaluated for efficacy using the pigtailed macaque model [4]. This study suggested that 4 out of 6 macaques were protected when challenged with simian/human immunodeficiency virus (SHIV) after application of the gel product [4]. In studies presented in this thesis, a rectal specific gel formulation which was compatible to the rectal epithelium with respect to osmolality, pH and viscosity was evaluated. A highly potent II, elvitegravir (EVG) was combined with TFV in rectal specific dosage forms to generate a potentially effective product for HIV infection upon rectal exposure.

1.4. Combination Products

Compared to single ARV drugs, combination therapy can significantly reduce HIV risk and possibly reduce resistance [21]. Combination drugs can act on different stages of HIV infection and replication (Figure 3). The various preliminary *in-vitro* studies conducted with different combination formulations have suggested that products containing a combination of two or more drugs may be more effective than a single drug regimen [22]. This effect can be explained because of two reasons: (i) HIV is less resistant to multiple drug therapy and (ii) drugs acting through different mechanisms can block different steps of the HIV replication cycle (probable synergism) [22]. Additionally, combination therapy can be effective for both prophylaxis and treatment [22]. A recent study conducted on a population of 2499 men or transgender women who have sex with men compared an oral product containing combination of ARV drugs, emtricitabine and tenofovir disoproxil fumarate (TDF), with placebo. The results from this clinical trial suggested that this oral ARV combination reduced HIV acquisition rate by 44% and detectable blood levels of the drug was found amongst patients suggesting a probable prophylactic effect [23]. Further investigation is warranted to assess if the combination products provide synergistic or additive effects in reducing HIV acquisition in humans. Our primary goal was to utilize a similar strategy and develop a topical product which could reduce HIV incidence. Moreover, topical products can give high local concentrations of drug(s), thus providing an extra barrier to the initial HIV infection. For this reason, a combination rectal specific microbicide that contains TFV and EVG (an II) was developed.

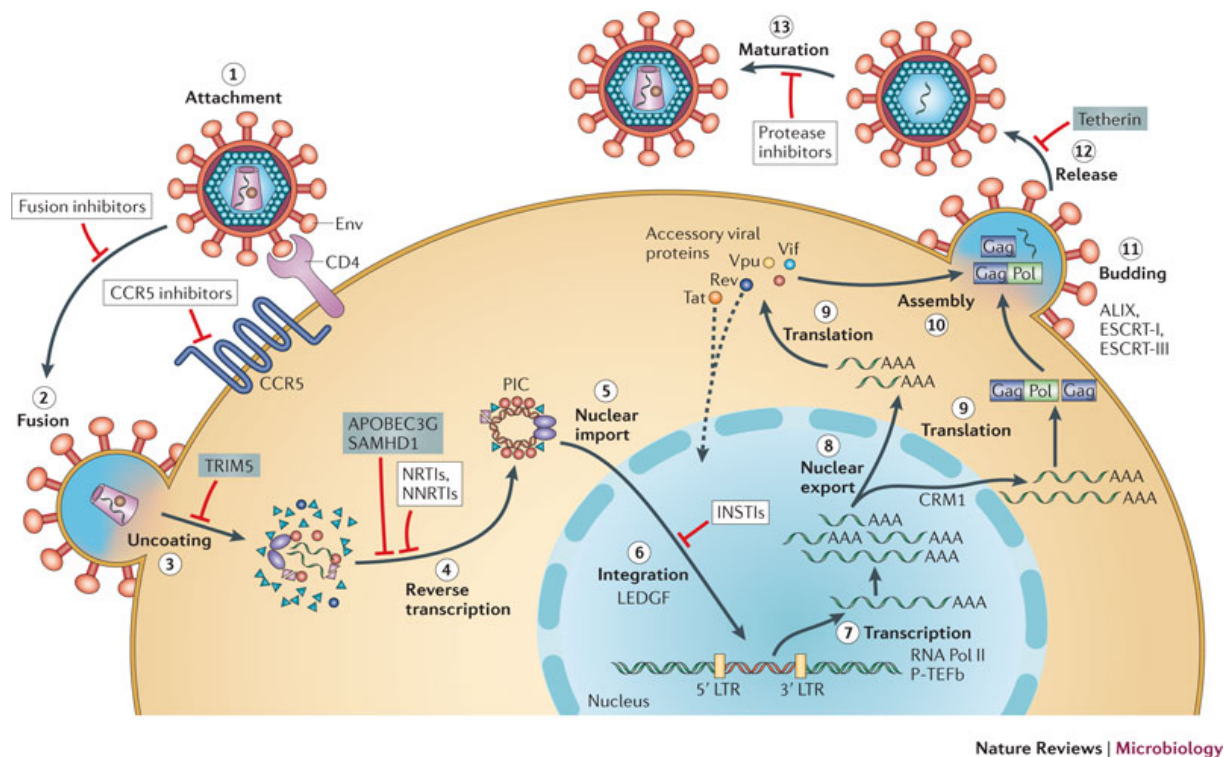


Figure 3: HIV replication life-cycle along mechanism of action of different anti-HIV drug categories [24]

1.4.1. Tenofovir

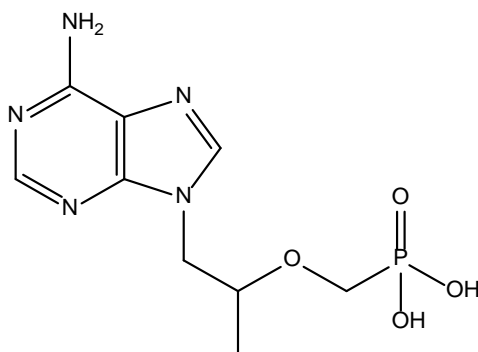


Figure 4: Tenofovir Structure

TFV is an ARV drug, which belongs to NtRTI category. It is currently available in the market as a fumarate salt called tenofovir disoproxil fumarate (TDF) marketed under the brand name

Viread® in the form of an oral tablet. It is also available in combination with other ARV drugs. When the HIV enters the host cell, along with the virus it brings along three enzymes that help in viral replication. TFV blocks HIV reverse transcriptase, thus interrupting the HIV replication cycle (Figure 3). Due to its high water solubility, TFV can be formulated as a hydrogel. Numerous studies and clinical trials have been conducted with TFV in different formulations. It is shown to be safe and effective in animal models and has been proven safe and acceptable in human clinical trials [25].

1.4.2. Elvitegravir

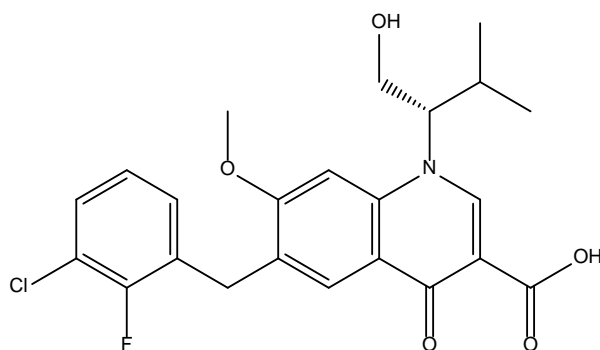


Figure 5: Elvitegravir Structure

EVG belongs to the category, integrase inhibitors (II). Integrase is one of the three enzymes that enter the host cell with HIV. Integrase is responsible for incorporating the viral DNA into the host DNA (Figure 3). EVG blocks the binding site of this enzyme, thus inhibiting the HIV replication cycle. EVG was first approved for use in 2013 by US FDA, in combination with other ARV drugs. Currently it is available in the market only as an oral tablet called Stribild®. This tablet contains a combination of four drugs; elvitegravir, emtricitabine, TDF and cobicistat (EVG/FTC/TDF/COBI). A recent phase III, double blinded clinical trial funded by Gilead Sciences compared this combination with a standard efavirenz, emtricitabine and TDF

(EVG/FTC/TDF) in treatment-naïve patients [26]. The results indicated that the combination that included EVG/FTC/TDF/COBI, would be preferred for initial HIV treatment if approved [26]. Efforts have also been taken to evaluate the possibility of using II by themselves for anti-HIV activity [4].

2. SCIENTIFIC APPROACH

Rectal specific gels have been previously developed by Dr. Rohan's lab [13]. As mentioned before, the focus of this research is to develop a rectal specific formulation. Due to the physiological differences between the vaginal and rectal compartment, a rectal specific formulation is needed. Recently, a clinical trial (CHARM-01) was conducted to test the safety, acceptability and PK/PD of 1% TFV vaginal formulations [27]. Specifically, a vaginal gel, a reduced glycerin vaginal gel, and a rectal specific formulation were administered in the rectal compartment. The results indicated that there was no significant difference in acceptability among the three arms of tested population [27]. Our experimental design is based on developing a product, which is acceptable and compatible with the rectal compartment/epithelium. In addition to gels, suppositories could also be used for rectal application of drugs. Suppositories are advantageous due to the ease of administration without the need for an applicator. Compared to gels, optimum and uniform dosing can be delivered using suppositories. Limitations associated with gels such as leakiness and messiness can be reduced or avoided with suppositories.

In contrast, a study comparing a rectal gel versus a suppository in 77 MSM who were involved in receptive anal intercourse (RAI) suggested that gel was preferred compared to suppositories [28]. However, the authors mentioned that limitations such as the suppository weight used in the study

and study population size could have impacted the outcome [28]. In this study, the weight of suppositories used was 8g. Large and heavy suppositories could cause discomfort, which could have led to preference for gels compared to suppositories. Therefore, small and lightweight suppositories could potentially reduce discomfort and lead to greater acceptability [28]. Following this study, Pines & group conducted a randomized crossover trial comparing rectal drug delivery systems as potential rectal microbicides [12]. Their study design compared an enema, a rectal applicator pre-filled with gel and a rectal suppository. 117 HIV-uninfected males and females were given these products every two weeks for a total of 6 weeks. Their sexual behavior and experience was recorded by conducting fact-to-face (FTF) interviews, computer-assisted self-interviews (CASI) and telephone-assisted self-interviews (T-CASI). The results were evaluated based on different parameters such as product use, components of acceptability, anorectal symptoms and adverse effects (AE's), etc. In this study, 13% of enema users, 15% of gel users and 22% of suppository users reported anorectal symptoms. With respect to AE's, participants reported 12% with enema, 8% with gel and 16% with suppository [12]. Although the results indicate that suppositories were least acceptable compared to others, there was no significant difference between the gel applicator and suppositories. The AE reported were either Grade 1 or Grade 2, and there was no Grade 2 AE reported associated with the suppositories, suggesting better acceptability than enemas. The suppositories used in this study were 1.4g, which is close to the suppository weight we used in our study (1.2-1.5g), indicating lighter suppositories could potentially be more acceptable. Furthermore, various studies have been conducted comparing oral drug delivery with rectal drug delivery methods [14, 16]. These studies suggest that they observed higher plasma levels of drugs given in the form of a rectal suppository when compared to oral dosage form. This could lead to low or equal doses of the

API in suppository giving a similar effect compared to oral route, which will reduce toxicity or side effects associated with the drug.

3. RECTAL SPECIFIC GEL

3.1. Materials

The API TFV used for these formulations was procured from CONRAD, Arlington, VA and EVG was procured from the Centre for Disease Control (CDC), Atlanta, GA. The excipients used in the formulation, sodium carboxymethyl cellulose (CMC/high viscosity), carbomer 974P, disodium EDTA, glycerin, propyl paraben, methyl paraben and 18% sodium hydroxide solution were purchased either from spectrum chemicals or fisher scientific.

The materials required for cell culture, 96 well plates, T75 flasks for cell culture, cell scrapper were either purchased from corning or fisher. The TZM bl cells were stored and maintained in a humidified atmosphere at 5% CO₂ and 37°C. Dulbecco's modification of Eagle medium (DMEM) with L-glutamine, glucose and sodium pyruvate, Dulbecco's Phosphate buffer salt solution without calcium and magnesium (DPBS), Trypsin EDTA (0.25%) in HBSS without calcium, magnesium and sodium bicarbonate used to maintain and culture cells and purchased from Fisher. The lab generated pseudo virus (HIV) was obtained from Dr. Dezzutti's laboratory at Magee Women's Research Institute.

3.2. Formulation

The formula describing the ingredients and their concentrations in the formulation are shown in Table 1.

3.2.1. 1% Elvitegravir Gel

A 1% EVG gel was prepared based on the earlier developed method in Dr. Rohan's laboratory. EVG is very hydrophobic ($\text{Log } p = 4.29$), practically insoluble in water [29]. So the final gel formulation was in the form of a suspension, where glycerin was used to suspend EVG. 90% water required to make the batch was weighed and heated at 65°C to dissolve methyl paraben and propyl paraben in water. An overhead stirrer with a U-shaped propeller blade was attached to this beaker at a constant speed of 50 rpm. The rotation speed was kept at minimum to avoid entrapment of air bubbles in the final gel product. Required amount of disodium EDTA was weighed and added to this beaker with continuous stirring. Once this was completely dissolved, carbomer 974P was added slowly followed by sodium CMC (high viscosity), in small amounts, avoiding clump formation. After overnight stirring, appropriate amount of EVG and glycerin was weighed and transferred to a beaker to make a paste. This paste was then slowly incorporated in the gel formulation with continuous stirring. The mixture was thoroughly mixed to make a paste using a glass rod such that the drug completely gets wet. During the development stage of this gel product, large particles and clumps were observed in the final gel product. Therefore this paste was sonicated and remaining 10% water was used to rinse the beaker and added to the gel to recover any residues. It was allowed to stir for some more time for the drug to get dispersed uniformly. In the final step, 18% sodium hydroxide solution was added in intervals of $100\mu\text{l}$, because carbomer 974P's viscosity increases at higher pH (7-10). Followed by addition of

sodium hydroxide solution, it was left to stir for a 6-8 hours to ensure complete mixing and uniform distribution of the final product.

3.2.2. Elvitegravir: Tenofovir Gel (1%:1%)

This gel was also formulated in a very similar manner as described above with minor modifications. Unlike EVG, TFV was easily dissolved in water without glycerin. In this formulation, the same procedure was followed until the addition of disodium EDTA. Glycerin was added which was followed by addition of required amount of TFV in small amounts. TFV and carbomer 974P's solubility is pH dependent, hence 18% NaOH solution was used [30]. One fourth of the entire amount of 18% NaOH solution is used initially followed by enough to obtain required pH. The remaining procedure is same as described earlier for 1% EVG gel. The final combination gel product has TFV dissolved in the medium and EVG suspended in the matrix with the help of glycerin.

Ingredients	1% Elvitegravir (% w/w)	1% Elvitegravir + 1% Tenofovir (%w/w)	Role
Elvitegravir	1	1	API
Tenofovir	-	1	API
Carbomer 974P	0.5	0.5	Polymer
Sodium CMC (high viscosity)	1	1	Polymer
Glycerin	2.5	2.5	Humectant
Methylparaben	0.18	0.18	Preservative
Propylparaben	0.02	0.02	Preservative
Disodium EDTA	0.01	0.01	Chelating Agent
Sodium Hydroxide Solution 18% (w/v)	1.15	1.96	pH modifier
Purified Water	93.65	91.83	Vehicle

1N HCl or 1N NaOH qs	pH 7.0 ± 0.2	pH 7.0 ± 0.2	
Total	100	100	

Table 1: Formula for 1% Elvitegravir and Combination gel product

3.3. Characterization

3.3.1. HPLC Methods

A previously developed ultra-HPLC assay was used to quantify TFV in the gel formulation [30]. A Waters Acquity UPLC (Ultra Performance Liquid Chromatography) equipped with a TUV detector and EMPOWER data-processing software was used. Separations were achieved on an ACQUITY UPLC BEH C18 column (1.7µm, 2.1 X 50mm; Waters) fitted with a guard column (1.7µm, 2.1 X 50mm; Vanguard) at ambient temperature. The mobile phase was a mixture of 90% 10mM K₂HPO₄ + 5mM t-Butylammonium bisulfate (tBAHS) and 10% methanol adjusted to pH 5.7 using 10% phosphoric acid. The observed retention time was 2.8 ± 0.3 minutes. A calibration curve was prepared in the range 1-200µg/ml and unknown concentrations were calculated using the instrument software.

For EVG analysis, analytical method and column were obtained from CDC for characterization and formulation purpose. This method was modified to suit our HPLC systems, product matrix and other conditions. The HPLC system used to develop the assay for EVG was an Ultimate 3000 (Dionex) which is equipped with a photodiode array detector (275 nm). Chromeleon version 6.70 (Chromatography Management System) was used as the data management and analysis software. Separations were achieved on a Synergi 4µ Polar column (150 X 2.00 mm, RP

80A; phenomenex) at 37°C with a flow rate of 0.5ml/ min. The mobile phase was a mixture of 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile in the ratio of 30:70. The observed elution time was 7.1 ± 0.2 mins. A calibration curve was made in the range 1-100 μ g/ml and unknown concentrations were calculated using the software.

3.3.2. Viscosity

The viscosity for these gel products was determined using Brookfield DVIII+ cone/plate viscometer. Since the gels were viscous, we used high viscosity viscometer (HA) and spindle number CP 52. The method set used to conduct this test covered shear rates from 0-30 rpm. The tests were conducted at two different temperature conditions i.e. 25°C and 37°C. The latter temperature was preferred in order to assess the gel's behavior at normal human body temperature.

3.3.3. Osmolality & pH

Osmolality for the gel formulations was measured using Freezing point Micro-Osmometer (Model 3320) by Advanced Instruments Inc. 20 μ l of the sample was withdrawn from the batch using a specially designed syringe. This was then inserted into the sampling chamber and results were displayed on the screen as mOsm/kg. pH was measured using an Orion ROSS ultra-flat surface probe specially designed for high viscosity liquids calibrated at pH 4, 7 and 10.

3.3.4. Stability

As per ICH guidelines Q1A (R2), we made a stability protocol for the two drug products i.e. 1% EVG gel and the combination gel containing 1% EVG and 1% TFV. The ICH guidelines suggest three different temperature conditions, long-term (25°C/ 60% Relative Humidity) (RH), intermediate (30°C/ 65% RH) and accelerated (40°C/ 75% RH). But due to limited availability of the active pharmaceutical ingredient (API), we included only accelerated and long-term conditions in our stability design. Both the products were tested for stability for 6 months at accelerated conditions and 12 months at long-term conditions. At every time point the formulations were tested for appearance, pH, viscosity, osmolality and drug content uniformity. The samples were analyzed in triplicate for each characterization method except viscosity, where duplicates were utilized.

To test drug content uniformity, samples of product was analyzed from top, middle and bottom part of the gel container for HPLC assay. Briefly, the samples were diluted in a 20ml volumetric flask, with 0.2% formic acid-in-water and Acetonitrile (70:30). This was followed by vortexing and sonication for ten minutes to ensure that the gel is completely dissolved. Approximately 1 ml of the sample was withdrawn and filtered into a HPLC vial using a syringe and 0.22µm PTFE filtration device. Each sample was injected twice. Data was reported as mean \pm standard deviation (SD) and % relative SD.

3.4. *In-vitro* dissolution

This test was carried out using Franz Cell system manufactured by Hanson Research Corporation. This system consists of a Franz diffusion cell which is connected to a vacuum pump assembly, autoplus multifill sample collector and a temperature regulated water bath.

The test is carried out in multiple steps. The cell drive system consists of six individual cells. Each cell has an outer water jacket which is connected to the speed and temperature control regulators. Every cell consists of a donor compartment, receptor compartment and a membrane compartment. Regenerated cellulose (RC) membrane discs (Spectra/Por[®]) with molecular weight cut off (MWCO) of 6000-8,000 Da and 33 mm diameter were utilized. Previous clinical trials and studies conducted with potential microbicides have employed 3.5-4 ml of the gel product as the dosing volume [19, 31]. Due to instrument limitations, only 0.4 mL of the gel samples was loaded into the donor compartment. The receptor compartment was initially primed to maintain 4ml of the dissolution medium at all times. Each receptor chamber has a sampling port, from where the AutoPlus Multifill Collector would collect specified amounts of sample at regular time intervals and replaces that amount with same amount of fresh dissolution medium which was placed in a water jacketed vessel to maintain the temperature. Samples were collected at 0, 15, 30, 45, 60, 120, 180, 240, 300, 360, 480 minutes (11 sampling points) and analyzed using HPLC. The drug concentrations observed in these samples were within the range of HPLC analysis. Preliminary studies showed that no dilution was required to quantify the drug concentration in the samples. Therefore samples obtained from the auto sampler were directly analyzed using HPLC. The HPLC method used for analysis was the same as described in section 3.3.1. The concentration of the samples analyzed using HPLC were in the range of detection and calibration curve. The dissolution medium used for this experiment was 5% sodium dodecyl sulfate (SDS) dissolved in milliQ water.

3.5. Anti-HIV activity studies

The anti-HIV activity was determined by conducting efficacy and toxicity studies with the TZM bl cells. We compared 1% TFV gel, 1% EVG gel, combination gel (TFV: EVG), placebo gel and free drugs as controls. A clear bottom see through 96 well plate was seeded with TZM bl cells at the concentration of 1×10^4 cells per well. The cells were incubated at 37°C for 24 hours. On day 2, all the plates were exposed to different concentrations of the product dissolved in 0.1% DMSO/DMEM. To test for toxicity, cells were treated with serial dilutions of test solution. Each well received 100 µl of test solutions. To calculate cell viability and determine toxicity 48 hours after initial seeding, 100 µl of medium was replaced with CellTiter Glo and recorded luminescence using SoftMax Pro 6.2.2. on SpectraMax M3 plate reader.

For efficacy studies, along with serial dilutions of the test products 100 µl of medium containing HIV-1_{Bal} (~3000 TCID₅₀) and DEAE dextran solution was added. To determine efficacy and anti-HIV activity, 72 hours after initial seeding 100 µl of media was replaced with Bright Glo and luminescence was recorded. The results were calculated by comparing the test products with positive (cells with HIV only) and negative controls (cells only).

3.6. Results

The studies reported in the literature with gels suggested that specific products are required for rectum and vagina due to their physiological differences [19, 32]. Factors such as pH and osmolality are crucial and they can be modified to obtain rectal specific formulation from vaginal gels. For example, the side effects associated with rectal application of vaginal products were

attributed to hyper osmolality of the formulations [32]. The rectum has minimum amount of fluid and very low buffer capacity [33]. The WHO guidelines specified that osmolality and pH range should be less than 1200 mOsm/kg and 5.5-7 respectively [34]. Results from our studies (Table 2 to 5) meet these requirements and therefore are expected to cause minimal or no side effects to the rectal cavity.

Stability

The results from stability studies of gel products with single and combination drugs conducted according to ICH guidelines are shown in figures 6 to 9 and tables 2 to 5. All the rectal gels showed opaque/white appearance based on visual observation. pH of the gels was between 6.2 and 6.5 indicating their suitability for rectal applications. Osmolality of the gels prepared with single drug was between 370-410 mOsm/kg. However, the gels containing two drugs showed an increased osmolality in the range of 440-475 mOsm/kg. For viscosity, as mentioned earlier in section 3.3.2, shear rates were obtained from 0-30 rpm. Product viscosity values are reported at 10 rpm. The viscosity observed for EVG gels was between 40,000-50,000 cPs at 25°C and between 30,000-45,000 cPs at 37°C. The viscosity at 10 rpm was much lower (~30,000 cPs) for combination gels compared to gels prepared with single drugs.

Based on our in-house criteria and previous studies, the specification for content uniformity was set at less than 10% RSD. The results reported in Figures 6 to 9 for drug content is an average of six samples. Results show that the drug content was between 90-110% for 1% EVG gels and 90-120% for combination gel products and RSD less than 10%.

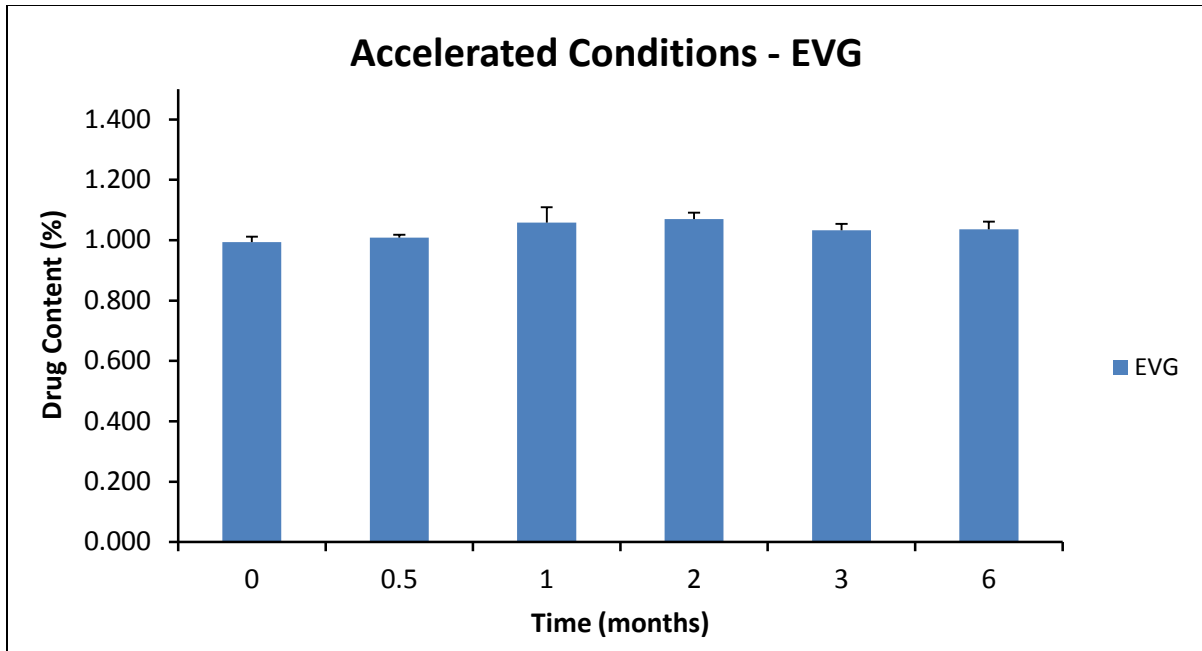


Figure 6: % Drug Content of Elvitegravir Gel under Accelerated Stability Conditions

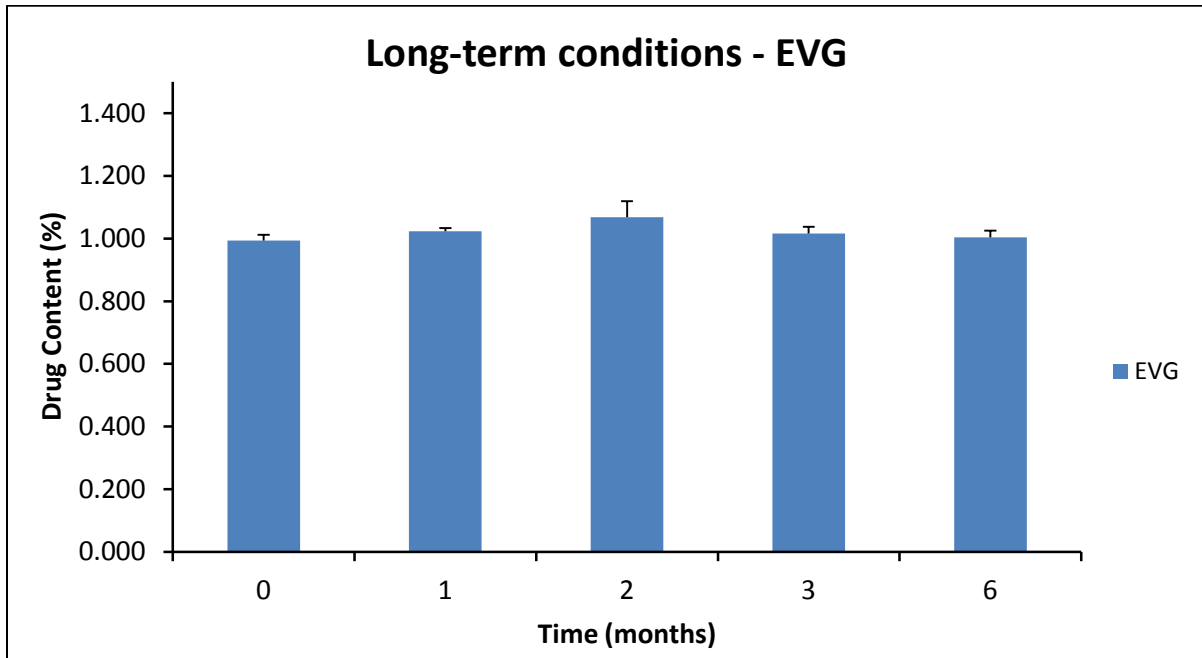


Figure 7: % Drug Content of Elvitegravir Gel under Long-term Stability Conditions

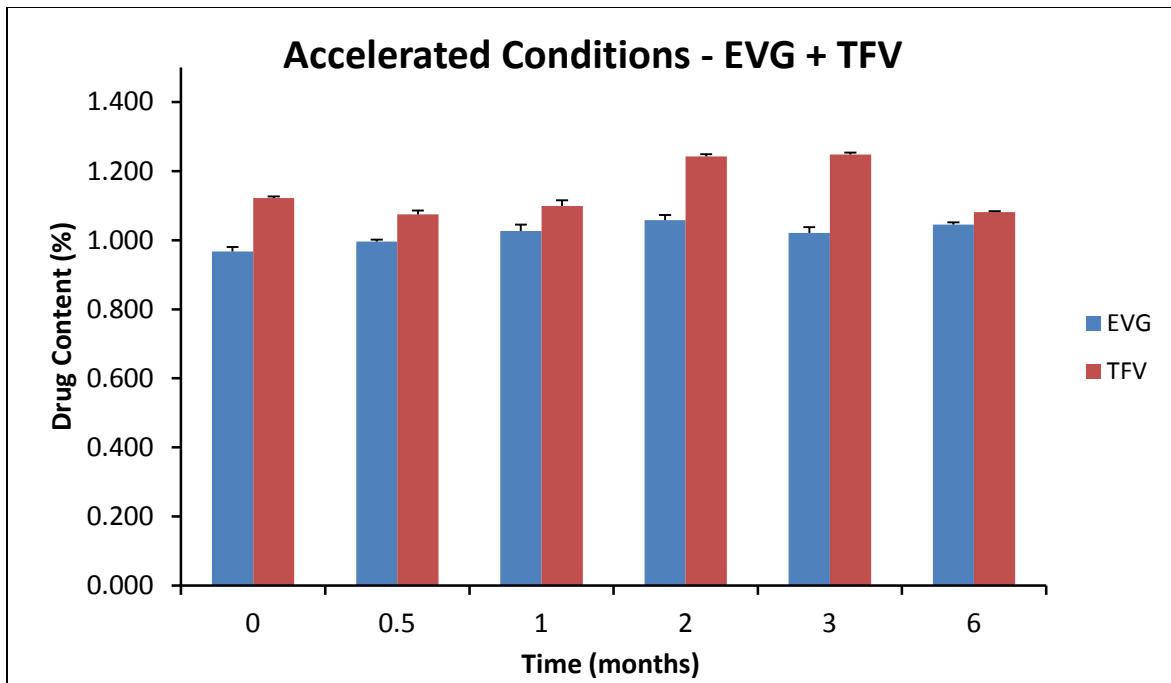


Figure 8: % Drug Content of Combination Gel under Accelerated Stability Conditions

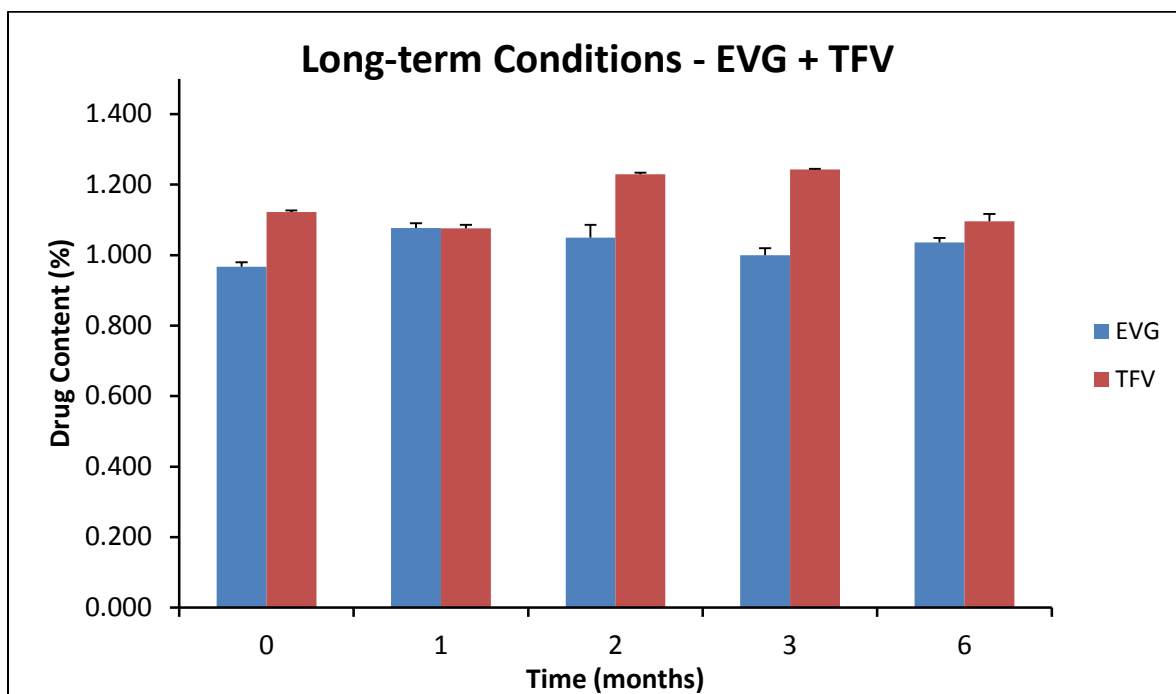


Figure 9: % Drug Content of Combination Gel under Long-term Stability Conditions

		Accelerated Studies (\pm SD, n=3)					
Tests		Time 0	T 2 weeks	T 1 Month	T 2 Month	T 3 Month	T 6 Month
pH		6.29 \pm 0.020	6.33 \pm 0.020	6.29 \pm 0.032	6.47 \pm 0.011	6.33 \pm 0.020	6.35 \pm 0.010
Osmolality (mOsm/kg)		372.67 \pm 0.57	398 \pm 4.72	384.66 \pm 2.08	399.66 \pm 19.55	384.33 \pm 5.68	409.667 \pm 5.13
Viscosity (cPs)	At 25°C	47724 \pm 2104	49113 \pm 2385	49312 \pm 140	48121 \pm 1262	47129 \pm 701	40084 \pm 280
	At 37°C	41473 \pm 841	42863 \pm 1403	42763 \pm 420	42168 \pm 420	39191 \pm 420	32742 \pm 280

Table 2: 1% Elvitegravir Accelerated Stability Study results

		Long-term Studies (\pm SD, n=3)				
Tests		Time 0	T 1 Month	T 2 Month	T 3 Month	T 6 Month
pH		6.29 \pm 0.020	6.26 \pm 0.015	6.53 \pm 0.030	6.35 \pm 0.01	6.34 \pm 0.023
Osmolality (mOsm/kg)		372.67 \pm 0.57	375.66 \pm 1.15	381.33 \pm 6.80	375.33 \pm 2.08	397.33 \pm 5.13
Viscosity (cPs)	At 25°C	47724 \pm 2104	51495 \pm 420	52586 \pm 561	51693 \pm 420	50106 \pm 1543
	At 37°C	41473 \pm 841	43954 \pm 140	43756 \pm 420	43458 \pm 841	43359 \pm 140

Table 3: 1% Elvitegravir Long-term Stability Study results

		Accelerated Studies (\pm SD, n=3)					
Tests		Time 0	T 2 weeks	T 1 Month	T 2 Month	T 3 Month	T 6 Month
pH		6.40 \pm 0.01	6.43 \pm 0.005	6.44 \pm 0.050	6.49 \pm 0.017	6.43 \pm 0.026	6.39 \pm 0.0
Osmolality (mOsm/kg)		441.67 \pm 7.63	453 \pm 11.53	451 \pm 4.35	458.33 \pm 4.93	452 \pm 1.0	475.33 \pm 10.78
Viscosity (cPs)	At 25°C	35651.18 \pm 420	51197.52 \pm 841	36413 \pm 982	34826.22 \pm 420	35520.6 \pm 280	31750.4 \pm 0.0
	At 37°C	27483.94 \pm 982	30460.54 \pm 701	30758.20 \pm 1683	30496.32 \pm 190	27682.38 \pm 420	26094.86 \pm 421

Table 4: Elvitegravir + Tenofovir (1:1) Accelerated Stability Study results

		Long-term Studies (\pm SD, n=3)				
Tests		Time 0	T 1 Month	T 2 Month	T 3 Month	T 6 Month
pH		6.40 \pm 0.01	6.39 \pm 0.005	6.52 \pm 0.052	6.473 \pm 0.005	6.427 \pm 0.020
Osmolality (mOsm/kg)		441.67 \pm 7.63	450.33 \pm 7.02	445.33 \pm 9.29	442.33 \pm 6.11	463.33 \pm 9.86
Viscosity (cPs)	At 25°C	35651.18 \pm 420	37604.38 \pm 140	37802.82 \pm 420	38993.46 \pm 140	36612.18 \pm 701
	At 37°C	27483.94 \pm 982	31055.86 \pm 140	30857.42 \pm 420	29865.22 \pm 1543	31090.86 \pm 1817

Table 5: Elvitegravir + Tenofovir (1:1) Long-term Stability Study results

***In vitro* dissolution**

Given the hydrophobicity of EVG, it was necessary to incorporate a surfactant in the dissolution medium used. A 5% SDS aqueous solution was used as the dissolution medium. The dissolution samples were analyzed separately for EVG and TFV. The dissolution study was conducted for 8 hours. Approximately 20% of TFV in the gel product was released in the tested time. However, no release was observed for EVG. Preliminary solubility studies were performed for EVG using different concentration of surfactant such as Cremophor and SDS in water. EVG was insoluble in water, but showed solubility in SDS, which was dependent on the concentration of SDS. Further optimization needs to be done to develop a better dissolution technique and overcoming the solubility barrier for EVG.

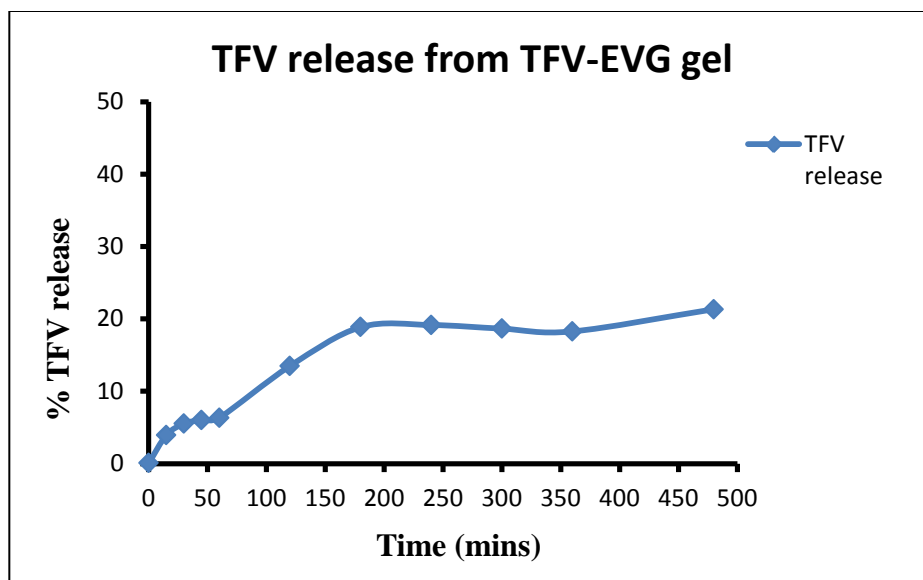


Figure 10: Tenofovir release from TFV-EVG combination gel

Anti-HIV activity and cytotoxicity of developed product

The primary goal to conduct this assay was to determine the toxicity and efficacy of the new products. For this purpose, TZM bl cells were used. These are HeLa cells engineered to express the C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) receptors. These receptors are used by viruses such as HIV to infect the host immune system.

In order to assess the effect of product concentrations on cell viability, TZM bl cells were incubated with different dilutions of free drugs and gel products. To compare activities, free EVG, free TFV, both the gel products and placebo gel were tested. As shown in figures 11 and 12, all the products and free drugs tested did not show any reduction in cell viability. Cell viability was obtained based on the effect of treatments of cell ATP production. The percent cell viability was observed to be greater than or equal to 100%.

Cell toxicity with Tenofovir

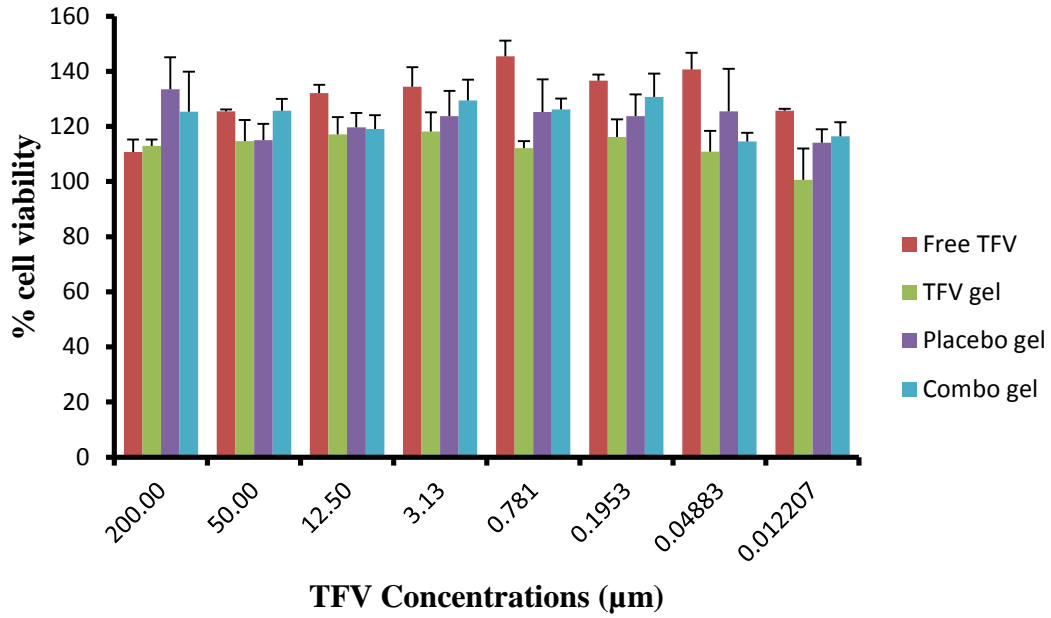


Figure 11: Cell toxicity with free TFV, TFV gel, placebo gel and combination gel

Cell toxicity with Elvitegravir

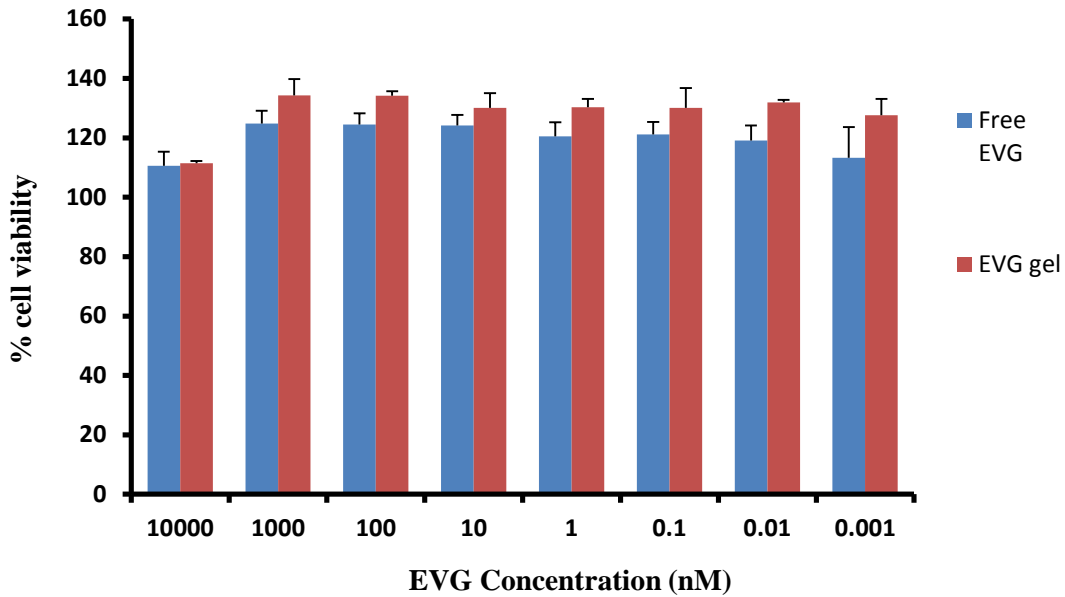


Figure 12: Cell toxicity with free EVG, EVG gel

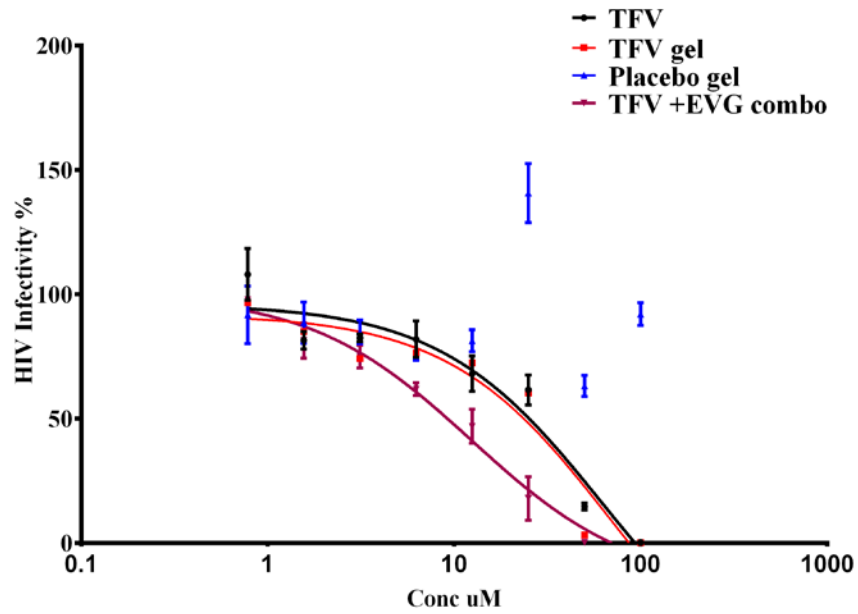


Figure 13: Efficacy Results for free TFV, TFV gel, placebo gel, TFV+EVG combo gel

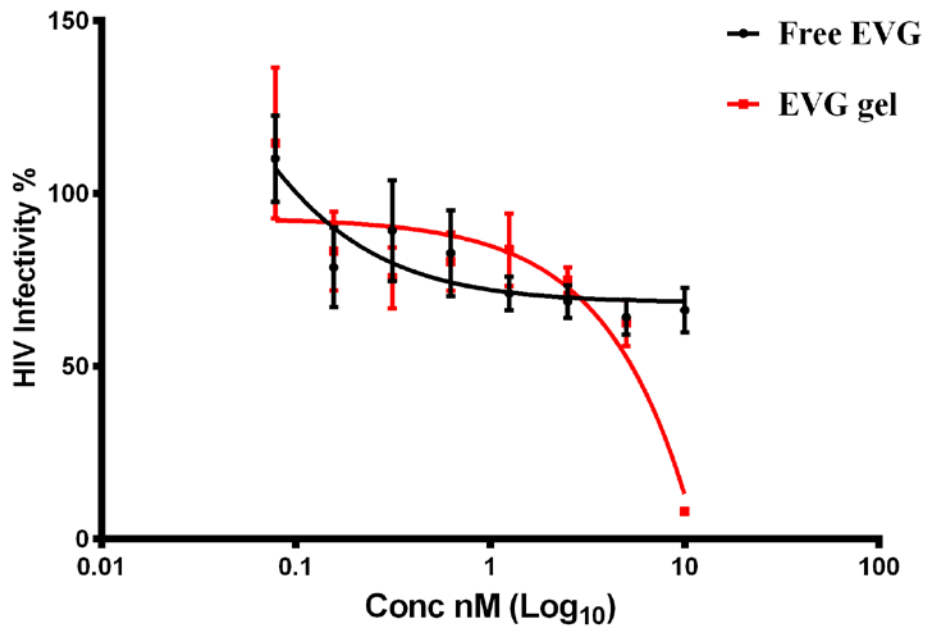


Figure 14: Efficacy Results for free EVG and EVG gel

In comparison with gel products, 1% TFV and free TFV, a leftward shift was observed in the HIV infectivity % vs concentration for the combination gel. This result indicates a lower IC_{50} for the combination gel compared to free TFV and 1% free TFV gel. The IC_{50} values for free TFV, TFV gel and combination gel were 62.41, 69.46 and 12.39 μM respectively. Further investigation needs to be done to evaluate whether it shows a synergistic or additive effect.

For EVG, the predicted IC_{50} value for EVG gel is ~15 nM. The solubility of EVG in the DMEM/DMSO could be a limiting factor. Further optimization might be needed to correctly obtain the IC_{50} values and efficacy results for free EVG and EVG gels.

4. RECTAL SUPPOSITORY

As mentioned in section 2, studies have been conducted comparing gels versus suppositories as potential rectal microbicides. This could be achieved by optimizing the formulation and reducing the weight of the suppository. In our case, we made suppository formulations with various fat soluble bases which are available in the market and also different combination of Polyethylene Glycol (PEG's) with various molecular weights.

4.1 Materials

The suppository bases, Polyethylene Glycols (PEG) of different molecular weights were obtained from spectrum. Other fat soluble bases such as Suppocire A/AP/BS2 were from Gattefosse, Witepsol H5/ H15/ E75/ W35 were purchased from Cremer Health. The disposable plastic suppositories molds were purchased from PCCA.

4.1.1 Formulation

Suppositories are usually prepared using either fat-soluble bases or water-soluble bases. Commonly used bases were chosen for both the categories, which are readily available in the

market and compatible with the active pharmaceutical ingredient (API) i.e. TFV and EVG. Due to the limited availability of API, suppositories containing single drugs were not manufactured. However, the suppositories consisting of both the drugs i.e. a combination suppository was formulated.

The dosing levels which were determined based on historical studies conducted with TFV. Several studies and clinical trials previously conducted with TFV in the field have used 4ml as the administered gel dosing amount for vaginal as well as rectal administration [35]. These gels were either placebo or 1% drug loaded. 4ml of a 1% TFV gel product would deliver 40 mg of the drug. This previously used dosing level for TFV was used as the dosing level for both APIs in the combination products developed in this study.

For fat-soluble base containing suppositories were prepared using bases such as cocoa butter, Witepsol H15, Suppocire A/AP/ BS2. These bases were a combination of mono, bi or tri glycerides (C₁₀-C₁₂). Cocoa butter is one of the most commonly used suppository bases. The remaining bases were chosen based on the decision tree obtained from respective manufacturers. This decision tree aided in selecting the required base which was based on the properties of API.

For water soluble base-containing suppositories, polyethylene glycol (PEG) of different molecular weights, namely, 3350, 1000, 400 and 8000 were used.

4.2 Characterization

4.2.1 Appearance

The appearances of the suppositories were recorded based on color, texture size and shape. Each suppository was visually examined for cracks or pits formed due to air entrapment while pouring into molds.

4.2.2 HPLC methods

TFV and EVG analysis was performed on the same instrument. The HPLC system we used to develop the assay for EVG was an Ultimate 3000 (Dionex) which used a photodiode array detector (275 nm). Chromeleon version 6.70 (Chromatography Management System) was used for data management software and analysis purpose. Separations were achieved on a Synergi 4 μ Polar column (150 X 2.00 mm, RP 80A; phenomenex) at 37°C with a flow rate of 0.5ml/ min. The mobile phase was a mixture of 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile in the ratio of 30:70. The observed elution time was 7.1 ± 0.3 minutes. For TFV the separations were achieved on a Gemini 5 μ C18 110 (150 \times 4.60mm; phenomenex) at 37°C.

4.2.3 Weight variation

Based on the batch size, each suppository was individually weighed as well as collectively. The collective weight was divided by the number of suppositories for the respective batch and average weight was found. The variation of each individual suppository from the average weight was calculated. According to the USP guidelines, in a batch of 10 units, none should lie beyond the range of 85% to 115% and the relative standard deviation should be less than or equal to 6%. The USP has criterias and specifications if one or more than one unit falls outside the specified range.

4.2.4 Hardness

Hardness testing was evaluated using Texture Analyzer TA.XT.plus. The instrument has a fixed solid bases and a height adjustable L-shaped arm. This arm has a probe holder with a slot, to fit a probe based on the test requirement. We used the probe TA-58 which is a flat surfaced probe. The instrument was operated using “Exponent” software. The software has predefined methods already established for different dosage forms and tests. The parameters for these methods were modified to suit the suppository testing. The instrument’s height and force was calibrated before beginning of every sample set.

The suppository was trimmed from both the ends and flattened so that it stands vertically. Each suppository was trimmed to $2.2 \text{ mm} \pm 0.2\text{mm}$. The height was calibrated to 10 mm and the length the probe would after hitting the suppository surface was increased to 5mm from 2mm to ensure complete breakage of the suppository. We were able to test the hardness only for placebo suppositories due to unavailability of API.

4.2.5 Melting point

The Differential Scanning Calorimeter (DSC) was used to measure the melting point of the suppositories. The difference between placebos and drug loaded suppositories were noted. A small amount (2-8 mg) of sample was weighed in an aluminum crucible and sealed using the mettler Toledo sealer. The heat flowing through this crucible was compared to an empty crucible as a reference and change in heat flow was measured. The computer software displayed results in

the form of a graph with time on the X axis and temperature as the Y axis. The peak of this graph was considered as the melting point of the sample.

4.2.6 Disintegration time (DT)

The DT for suppositories was determined using Electrolab Disintegration tester apparatus, Model no: ED 2L, Version 1.1. 700ml of 1X PBS at pH 7.4 was used as the medium. This apparatus primarily consists of an electric water bath which programmed to be heated at $37 \pm 0.5^\circ \text{C}$. This water bath has slots for 2 beakers; each of the beakers has a basket assembly which consists of 6 tubes which are open from both ends with mesh at the bottom and a basket arm. The basket arm is connected to the hook arms of the equipment. The hook arms of the equipment have a fixed motion which allows the basket to go in and out of the beaker in an up and down fashion. When filled with water, this motion will cause the basket assembly containing the required dosage form unit to dip in and out of water at the rate of 30 ± 1 strokes / minute. These strokes in and out of water cause the suppository to start disintegrating. The point at which the PEG based suppositories completely melts and the time at which the fat soluble bases disintegrate is determined.

4.3 *In-vitro* drug release

The dissolution for the suppositories was conducted on Distek dissolution apparatus. We used the USP Apparatus Type 1 basket for this experiment. The clear glass dissolution vessels with 1 liter capacity were used. The distance between the inner surface of the vessel and the basket was

manually adjusted to 25 ± 2 mm. Sink conditions were made and 500ml of 5% SDS was used as the dissolution medium.

Each suppository was weighed individually and placed inside the basket. The dissolution medium was added to the vessel and allowed to equilibrate with the water bath temperature i.e. 37°C. Two ml aliquots were collected at 15, 30, 45, 60, 75, 90, 105, 120, 150 and 180 minutes and replaced with fresh medium. These samples were filtered using a syringe and 0.22 μ m PTFE filtration device, diluted and analyzed using HPLC. The raw data from HPLC was analyzed using Microsoft excel and a release profile was obtained. The same procedure was followed for both types of suppositories.

4.4 Results

Weight Variation

As described in section 4.2.3, the USP specifications for dosage weight variation were met and results were within permissible limits. The results displayed in table 6 are the respective weights for individual drug loaded suppository for Witepsol H15 and PEG 3350/1000/400 in the ratio 60:30:10.

Sr. No.	PEG 3350/1000/400		Witepsol H15	
	Weight (g)	Variation	Weight (g)	Variation
1	1.5052	0.0068	1.2480	0.0097
2	1.4955	-0.0029	1.2272	-0.0111
3	1.5056	0.0072	1.2368	-0.0015
4	1.4849	-0.0135	1.2374	-0.0009
5	1.5175	0.0191	1.2122	-0.0261
6	1.5085	0.0101	1.2516	0.0133
7	1.4977	-0.0007	1.2190	-0.0193
8	1.4958	-0.0026	1.2387	0.0004
9	1.5081	0.0097	1.2337	-0.0046
10	1.5077	0.0093	1.2393	0.0010
11	1.5046	0.0062	1.2470	0.0087
12	1.5157	0.0173	1.2338	-0.0045
13	1.4648	-0.0336	1.2560	0.0177
14	1.466	-0.0324	1.2685	0.0302
15	-	-	1.2260	-0.0123
Average	1.4984		1.2383	
SD	0.0157		0.0136	

Table 6: Weight variation for combination (EVG:TFV) suppositories

Hardness

A method was developed using texture analyzer to test the hardness of the suppositories. Placebo suppositories were tested for every formulation and results were obtained in the form of a graph with time on the X-axis and force on the Y axis. The graph showed a lot of peaks based on the varying pressure. The first peak was usually the highest one which represented the breaking point of the suppository. Fat soluble suppositories like Suppocire A and Witepsol H15 were tough and brittle in nature, so they broke as soon as the probe hit the surface. But the PEG bases suppositories were soft in nature and did not break apart at once. As the probe kept going through the suppository, it kept losing its shape and formed shreds. The results for hardness of every formulation are reported in table 7.

Formulation (Suppository base)	Hardness (kgs; Mean \pm SD, n=4)
Suppocire A	2.830 \pm 0.277
Witepsol H15	5.076 \pm 0.981
Cocoa butter	0.385 \pm 0.293
PEG 8000/400 (60:40)	2.575 \pm 0.210
PEG 3350/1000 (25:75)	2.046 \pm 0.205
PEG 3350/1000/400 (60:30:10)	1.314 \pm 0.140

Table 7: Placebo suppository Hardness

Melting Point

The melting point was calculated by measuring the peak of the graph obtained from the DSC. The melting points of different formulation are displayed in table 8. There was minimal or no difference between drug loaded and placebo suppositories. The PEG based suppositories usually tend to dissolve at body temperature and not melt [36]. This explains the high melting points observed for two PEG based formulations.

Formulation (Suppository base)	Melting point (n=2)	
	Placebo	Drug Loaded
Suppocire A	37.79	37.15
Witepsol H15	34.77	34.73
Cocoa butter	32.97	33.09
PEG 8000/400 (60:40)	56.39	56.33
PEG 3350/1000 (25:75)	37.86	36.34
PEG 3350/1000/400 (60:30:10)	55.08	56.4

Table 8: Melting Point for fat soluble and water soluble suppositories

Disintegration

The disintegration results were based on visual observations. 1X PBS at pH 7.4 was used as the disintegration medium. This medium was primed with the water bath temperature before the test. Sinkers were used to keep the suppositories dipped inside the liquid surface. The presence of oil and glycerides in the fat soluble suppositories makes them insoluble in aqueous medium. They disintegrate forming minute oil droplets and also free insoluble API, which makes the medium turbid. The results were recorded when the suppositories completely lost their shape and integrity. Hence, the results are reported as a range and not a specific time.

Fat soluble suppositories

Suppocire A > Witepsol H15 > Cocoa Butter

PEG based suppositories

PEG 8000/400 > PEG 3350/1000 > PEG 3350/1000/400

Formulation	Disintegration time range (mins)
Suppocire A	7-8
Witepsol H15	6-8
Cocoa butter	3-4
PEG 8000/400 (60:40)	12-15
PEG 3350/1000 (25:75)	8-9
PEG 3350/1000/400 (60:30:10)	7-8

Table 9: Disintegration Time of Fat soluble and PEG based suppositories

***In-vitro* dissolution**

Triplicates samples were analyzed for every formulation. Two ml aliquots were withdrawn and replaced with fresh medium at each time point. 40mg was used as the theoretical weight for both the drugs and percentage drug release was calculated. For fat soluble bases, a sustained release profile was observed over a period of three hours. Witepsol H15 showed the maximum drug release, followed by Suppocire A and then cocoa butter (Figures 15 to 17). In all the formulations, greater TFV levels were observed in comparison to EVG. This was expected considering the hydrophobicity of the drugs.

For PEG based suppositories, all the formulation showed an initial burst release followed by a plateau phase. The combination of PEG 3350/1000/400 in the ratio 60:30:10 showed maximum drug release compared to other two formulations (Figures 18 - 19). Most of the drug was released within the thirty minutes. There was minimal or no difference of drug release for all the formulations.

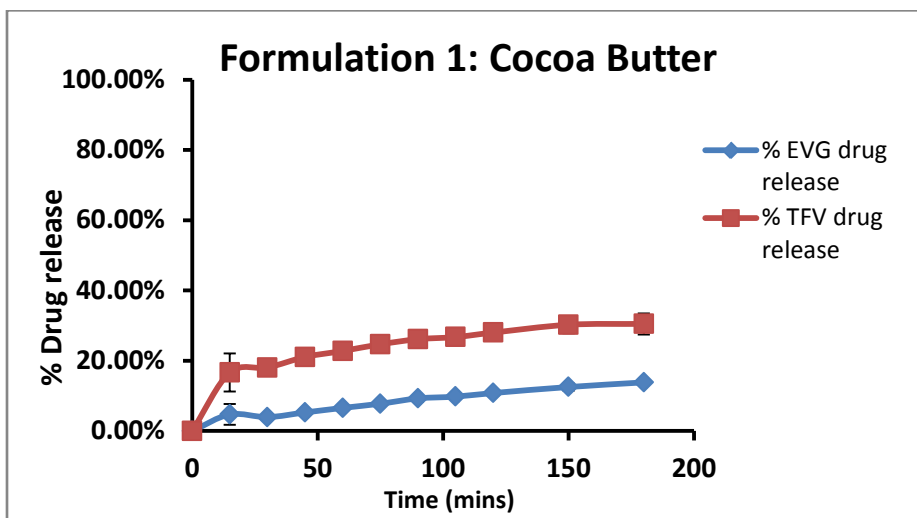


Figure 15: TFV & EVG release from cocoa butter suppository

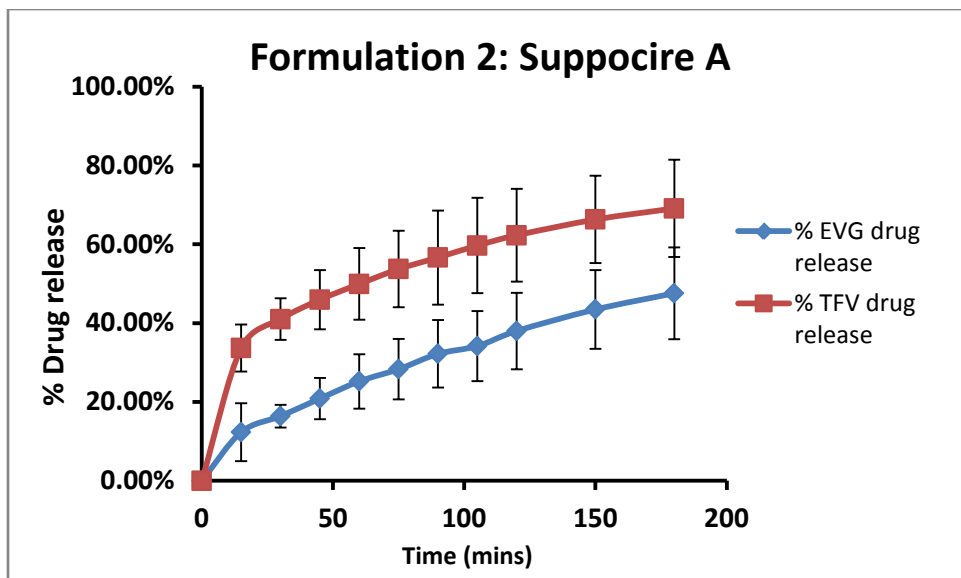


Figure 16: TFV & EVG release from Suppocire A suppository

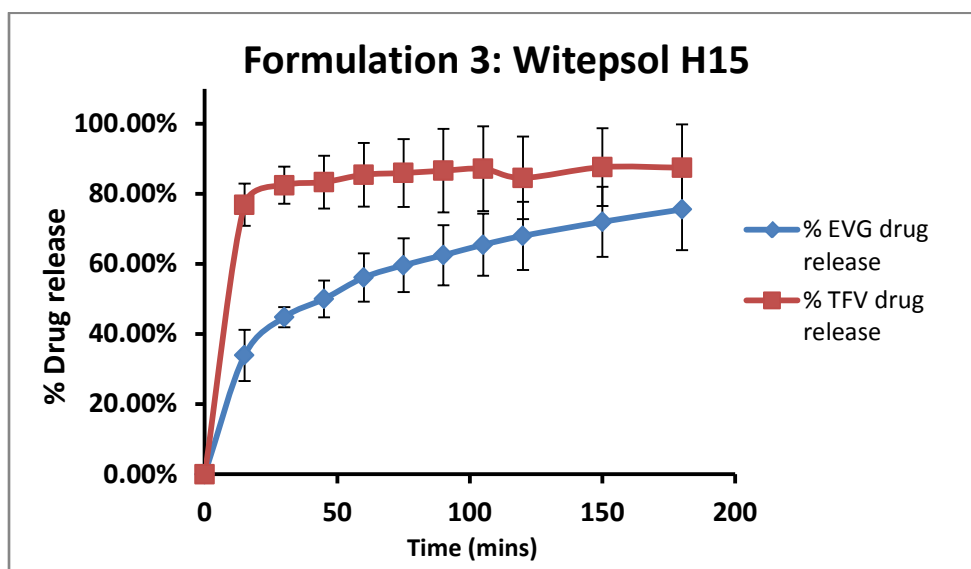


Figure 17: TFV & EVG release from Witepsol H15 suppository

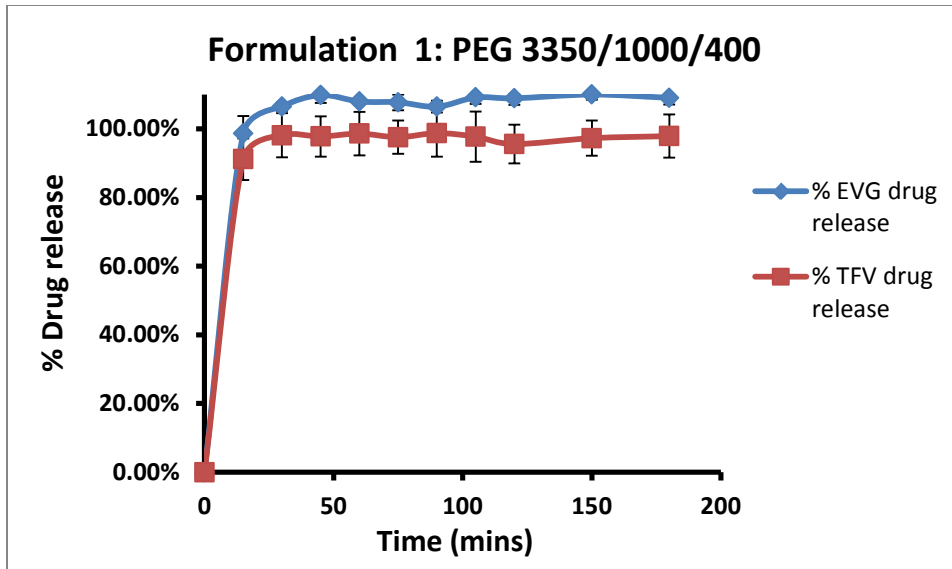


Figure 18: TFV & EVG release from PEG 3350/1000/400 (60:30:10) suppository

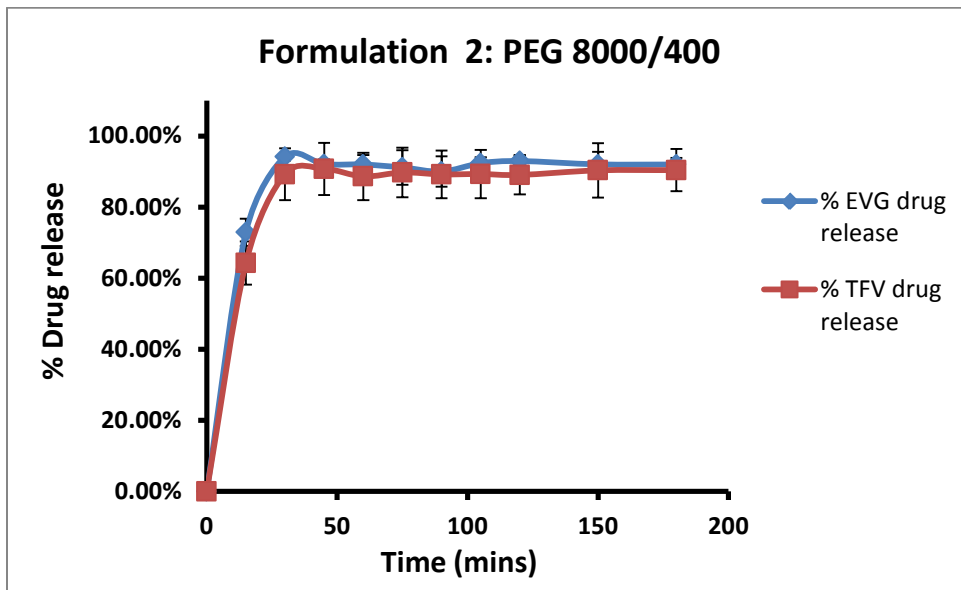


Figure 19: TFV & EVG release from PEG 8000/400 (60:40) suppository

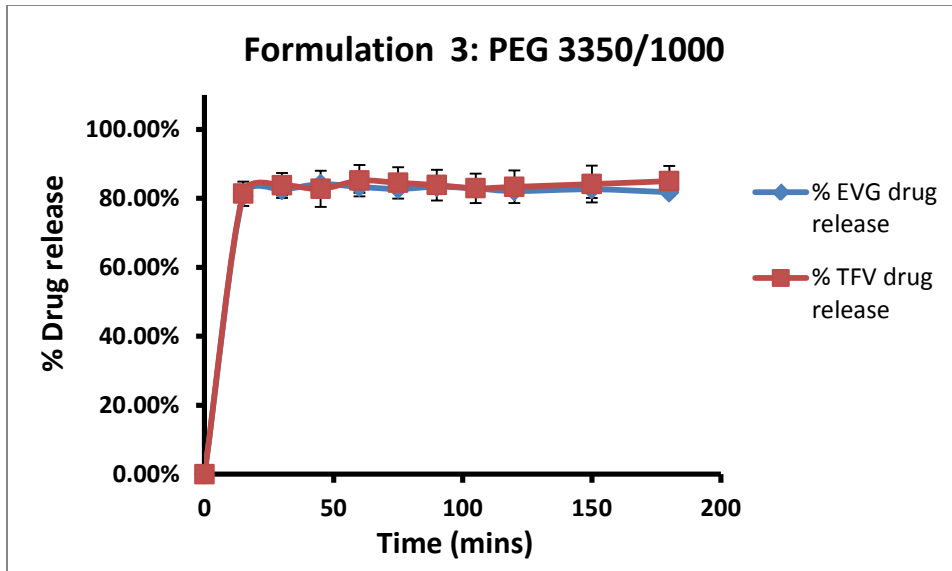


Figure 20: TFV & EVG release from PEG 3350/1000 (25:75) suppository

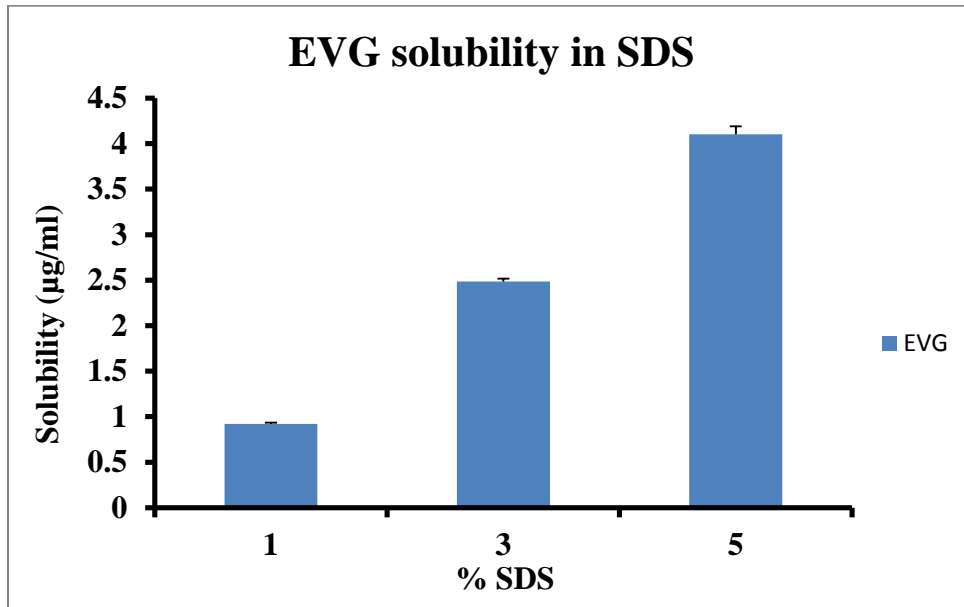


Figure 21: EVG solubility in increasing concentrations of SDS

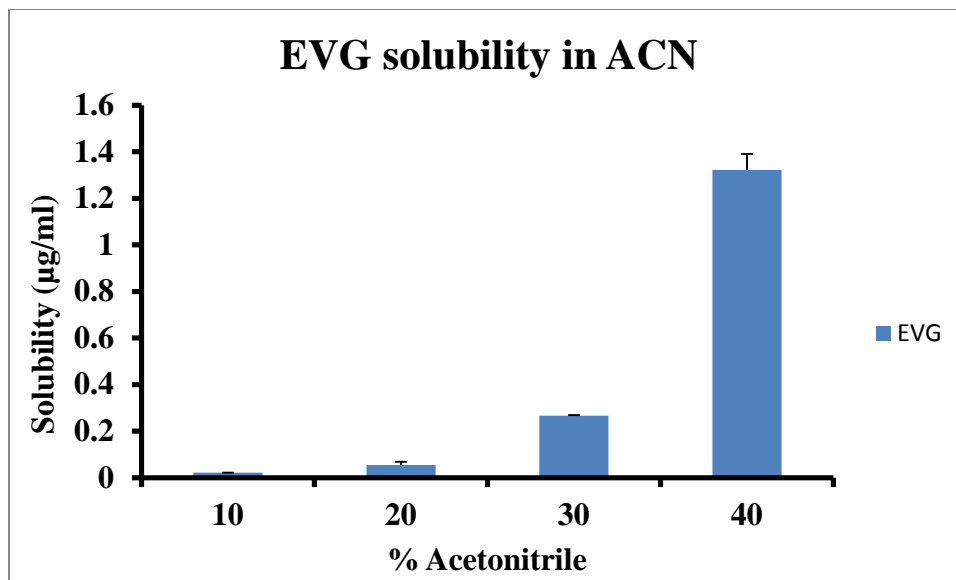


Figure 22: EVG solubility in increasing concentrations of Acetonitrile

5. DISCUSSION

The ultimate goal of this project was to develop a safe, effective and acceptable rectal dosage form for HIV prophylaxis. Tremendous research and efforts have been taken over the past decade to develop vaginal and rectal products for HIV prevention. For this purpose various dosage forms have been studied to deliver a range of ARV's. The most commonly used topical dosage forms are creams, lotions, foams, gels, suppositories and ointments. Gels and suppositories have been widely utilized for rectal applications. From a microbicide product development point of view and considering the factors affecting patient compliance and acceptability, within this body of work gel and suppositories were studied for their potential as rectal microbicide dosage forms.

Attempts have been made previously to develop a topical microbicide for rectal or vaginal administration in different dosage forms [4, 17, 31]. One of the drug candidates used in these studies, raltegravir, belongs to the category of integrase inhibitors, which has been used as a candidate for PrEP-based inhibition of HIV acquisition [4]. Another potent analog of raltegravir is EVG, which is available in the market as an oral tablet. We developed topical dosage forms, namely gels and suppositories containing EVG and TFV. Our study demonstrates the ability to formulate EVG alone and as a combination in a topical dosage form for rectal specific drug delivery for the first time. The combination of EVG and TFV in a single dosage form is

challenging because they are hydrophobic and hydrophilic respectively. These drugs can either be suspended or molecularly dispersed in gels and suppositories. Since EVG is hydrophobic, glycerin was used as a suspending agent for incorporation in the aqueous gel base.

A 1% EVG and TFV/EVG combination gel formulation was developed based on a previous rectal specific formulation designed in Dr. Rohan's laboratory (unpublished results). Typically, hydrogels consist of gelling agents, preservatives, chelating agents, API etc. The role of each excipient chosen in the formulation is shown in Table 1. Sodium CMC and Carbopol formed the backbone of the gel product. Carbopol is an acidic white powder, which partially uncoils when added to water. To increase viscosity, acidic carbopol can be neutralized to its salt form, which further uncoils the polymer and leads to cross-linkages. In addition to this, pH modifiers were included in the formulation to modify the pH suitable to rectal compartment (pH 5-7). TFV is acidic in nature and therefore its solubility is pH dependent. Hence, to stabilize the system and achieve greater viscosity, second polymer sodium CMC was added. Other ingredients such as humectants were included in the formulation to maintain the water content of the gels because lower water levels could cause irritation to the rectal epithelium. Being water based formulation, it is susceptible to microbial growth, and hence preservatives, methyl and propyl parabens, were added. For single and combination-drug containing gels, a uniform opaque and white product was obtained, which was easy to disperse. Hydrophilic and hydrophobic drugs were successfully incorporated in a single formulation. Their presence collectively did not impact the aesthetic appearance and pH of the combination gels. Additionally, the pH and osmolality were within the specifications suggested for rectal specific products.

EVG was shown to be stable under acidic, alkaline and oxidative conditions. A recent study evaluated the stability of EVG under forced degradation conditions such as acidic, basic,

oxidation, heat and photolysis [37]. EVG was shown to be stable under these conditions. As shown in Table 2 to 5, gel products were stable under accelerated and long-term storage conditions with minimal changes in the tested attributes. The drug content monitored for 6 months was within 90-110% compared to time zero for 1% EVG gel and 80-120% for the combination gel product (Figures 6 to 9). According to the ICH guidelines Q1A(R2), 5% change in the assay causes a significant change in the stability and quality of drug product. These guidelines suggest that if there is a significant change for accelerated conditions, the stability can be evaluated based on results from intermediate and long-term studies. Long-term stability for the gel products is ongoing, therefore, the shelf-life of the products cannot be determined at this point. Once all the stability data is obtained product shelf life will be estimated.

In order to understand the release profile from gel products, *in-vitro* dissolution studies were performed using Franz diffusion cell system. Due to EVG's hydrophobicity, pure aqueous solvents could not be used for dissolution studies. Therefore, solubility was determined in aqueous organic mixtures and also water containing surfactants. Our results indicated that EVG was soluble in water containing either acetonitrile or SDS. The solubility of EVG was dependent (Figure 21 and 22) on the concentrations of acetonitrile and SDS. Based on the solubility (4.1 mg/ml) of EVG, 5% w/v SDS in water was used as the dissolution medium to maintain sink conditions. From the dissolution samples, TFV and EVG were analyzed separately using HPLC. In spite of using a dissolution medium that has high solubility for EVG, it could not be detected in the dissolution samples in the tested 8 hours of dissolution time. In order to delineate the effect of EVG adsorption to RC membrane, adsorption study was conducted. Less than 10% drug (~ 5 µg) was shown to be adsorbed during 20 hours incubation with RC membrane in dissolution media containing known concentrations of EVG at 37°C. Adsorption to RC membrane could be

one of the potential reasons for not detecting EVG in the receptor compartment of Franz diffusion cell. Furthermore, attempts were made to improve the in-vitro dissolution method of EVG gel. The withdrawal of sample from receptor compartment might create an air bubble underneath the membrane hampering the diffusion of gel product through the membrane. Detection and quantification of EVG was unsuccessful despite repeated attempts to modify and improve the dissolution technique. This could probably be a limitation of the equipment, as the entire gel product is not exposed to the medium compared to other dissolution systems. Thus, only TFV results were reported from the combination gel product and further investigation is needed to address this problem and obtain drug release profile for EVG gel. As shown in Figure 10, only 20% of the drug release was observed for TFV in 8 hours. The permeability through the RC membrane could be a limiting factor for TFV release. This indicates that further optimization is needed to establish an *in-vitro* dissolution method to characterize the gel products.

To evaluate the safety and efficacy of these gel products, TZM bl reporter assay was conducted. This in-vitro cell based model for efficacy and safety testing has been well established in the literature [20, 38, 39]. The TZM bl cells express CCR5 and CXCR4 co-receptors, which are required for HIV attachment and entry in to the host immune cells. As shown in Figures 11-12, the gel products did not reduce cell viability. For efficacy, IC_{50} values were calculated for free drugs and gel products by measuring the % HIV inhibition. Compared to free TFV and 1% TFV gel, the combination product showed a significantly lower IC_{50} (12.39 μ M). The predicted IC_{50} of EVG gel determined in a separate experiment was 15 nM. These results indicate that both the drugs were active in the combination product and partially or fully contributed to the reduced HIV infectivity. In order to understand whether the combination gel product shows synergistic or additive effect, further investigation is needed.

As mentioned earlier in this discussion and in section 2, suppositories can also be used as potential rectal microbicides. A comparative study between enema, gels and suppositories concluded that suppository and gels are equally acceptable [12, 28]. Therefore to explore and develop rectal suppositories, a small pilot study was carried out to observe preliminary API behavior within the suppository bases. Our results showed that both the APIs were dispersed in the suppository base but not dissolved. Adjuvants such as surfactants, plasticizers can be utilized in the suppository formulations to improve the solubility and alter the drug-base interactions. Previous studies suggest that addition of an appropriate adjuvant to the suppository can achieve desired physicochemical characteristics of suppositories [40]. However, in the current work adjuvants were not included in the formulation.

A single suppository containing TFV and EVG was formulated. The drug loading for these suppositories was calculated based on the gel dosing volume (4ml) used in early pre-clinical and clinical studies [32, 35]. The suppository bases employed in this work are widely used in the industry. Cocoa butter is a naturally occurring fat and Witepsol H15 and Suppocire A are well known semi-synthetic glyceride bases with a hydroxyl value of 5-15 and 25 respectively. Both the drugs did not show any major effect on the suppository formulations. Out of the six formulations prepared, three utilized water soluble PEG bases and three utilized fat soluble bases.

Drug extraction methods from the combination suppositories were developed and both the drugs were quantified separately using HPLC. The PEG based suppositories showed greater than 90% drug recovery, whereas the fat soluble bases showed 20-80% depending upon the type of fatty base. This reduced drug recovery could be attributed to the presence of saturated triglyceride fatty acids. These fatty acids tend to retain drugs in the formulation because of the drug-base

interactions. The weight variation results were within the limits specified by USP. No suppository was outside the range of 90-110% of average weight. A hardness testing method was developed, optimized and tested using placebo suppositories. The results show that the fat soluble suppositories such as Suppocire A and Witepsol H15 showed greater hardness compared to other formulations. For the disintegration test, the results were obtained based on visual observations. For the fat soluble bases the drug loaded suppositories melted earlier compared to the respective placebo suppository. But for PEG based suppositories there was no difference observed between drug loaded and placebo suppositories. The melting point of the suppositories was evaluated using the DSC. Usually suppositories are designed to dissolve at body temperature and release the drug. Although the melting point of two PEG formulations was observed to be around 54°C, the suppository will dissolve and release the drug at body temperature. It has been shown that PEG bases dissolve in the rectal compartment rather than melting, eventually releasing the drug [36].

In-vitro drug release for the combination suppositories was performed using 5% SDS as the dissolution medium. The results indicate that TFV shows a much faster and higher release in the fat soluble bases compared to EVG, whereas no difference was observed in the release profile for PEG bases. This result can be explained considering the hydrophilic nature of TFV. When TFV is exposed to aqueous medium such as SDS in water, it would release faster. On the contrary, EVG would retain in the fatty suppository base given the presence of triglyceride. Hydroxyl value for a base indicates the amount of free hydroxyl groups available. A higher number of free hydroxyl groups in the bases increases the possibility of its interaction with the API. Results indicate that Suppocire A released lower amounts of the drugs when compared to Witepsol H15. As mentioned earlier, the hydroxyl value of Suppocire A is 25, which is higher compared to

Witepsol H15 (5-15). Hence, the API has higher tendency to bind to Suppocire A than Witepsol H15. The API's compatibility with the suppository bases was analyzed on the basis of solubility. Most of the suppository bases are in solid form at room temperature and liquefies only when heated. Visual confirmation of drug solubilization could not be observed due to inability of the bases to be in liquid state at room temperatures. This drug-base interaction could lead to delayed release of the API from the formulation. Further optimization is needed to choose suppository bases that provide better drug recovery and desirable drug release profile.

6. CONCLUSIONS & FUTURE DIRECTIONS

In conclusion, this work led to the formulation of rectal specific 1%EVG gel, a combination gel and suppository containing 1% EVG and 1% TFV. This is the first study where attempts were made to formulate EVG in the form of a topical product. Both gels and suppositories efficiently incorporated either single or combination drugs. Furthermore, toxicity and efficacy results of the gel products showed promising results. Further investigation is needed to compare the combination gel products with both 1% EVG and 1% TFV gel alone. As mentioned earlier, the gel and suppository products were sent to CDC for in-vivo studies in macaques. Results from in vivo studies will give a better understanding of the drug release profile, toxicity, local and systemic drug levels.

Future studies will attempt to develop topical dosage forms for a potent analog of TFV, tenofovir alafenamide fumarate (TAF). Studies have suggested that lower concentrations of TAF can produce similar effects with a much higher concentration of TFV [41]. As mentioned in the discussion earlier, in spite of repeated attempts we were unable to analyze EVG using the existing *in-vitro* dissolution technique. Therefore, there is a need of developing a suitable dissolution method to assess drug release from such gels. Also, since we were unable to determine the solubility of the APIs in the suppositories, it could be achieved using DSC.

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