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Evaluation of degradation kinetics and physicochemical stability of tenofovir

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Abstract

Tenofovir (TFV) has been proven to prevent the transmission of the Human Immunodeficiency Virus (HIV) through the vagina. But, there is little information available about its stability under various storage and stress conditions. Hence, this study aimed to investigate the degradation behavior and physicochemical stability of TFV using liquid chromatography coupled mass spectrometry (LC-MS) and solid state X-ray diffraction (XRD) analyses. The LC-MS analysis was performed on a QTrap mass spectrometer with an enhanced mass spectrum (EMS) scan in positive mode. A reversed phase C₁₈ column was used as the stationary phase. TFV exhibited degradation under acidic and alkaline hydrolytic conditions. The degradation products with m/z 289.2 and 170 amu have been proposed as 6-Hydroxy adenine derivative of TFV, and (2-hydroxypropan-2-yl)oxy methylphosphonic acid, respectively. A pseudo-first-order degradation kinetic allowed for estimating the shelf-life, half-life, and time required for 90% degradation values of 3.84, 25.34, and 84.22 h in acidic conditions, and 58.26, 384.49, and 1277.75 h in alkaline conditions, respectively. No significant degradation was observed at pH 4.5 (normal cervicovaginal pH) and oxidative stress conditions of 3% and 30% v/v hydrogen peroxide solution. The shelf life of TFV powder at room temperature was 23 months as calculated by using an Arrhenius plot. The XRD pattern showed that the drug was stable and maintained its original crystallinity under the accelerated and thermal stress conditions applied. Stability analyses revealed that the TFV was stable in various stress conditions; however, formulation strategies should be implemented to protect it in strong acidic and alkaline environments.

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Keywords

tenofovir; forced degradation; degradation kinetic; Arrhenius plot

Introduction

Topical microbicides are agents applied within the vagina or rectum to prevent the transmission of sexually transmitted diseases including Human Immunodeficiency Virus (HIV) infections.^[1] Currently, microbicides are the major focus among HIV transmission prevention strategies through the cervicovaginal (CV) mucosa. Tenofovir (TFV), a Food and Drug Administration (FDA) approved microbicide (in its pro-drug form), has been proven to prevent HIV transmission through the vagina.^[2] TFV belongs to the class of anti-retroviral drugs under the sub-category of nucleotide reverse-transcriptase inhibitors (Figure 1A).^[3] It is a weak acidic, hydrophilic drug with the molecular weight of 287.213 g/mol.^[4]

The interaction of microbicides with CV fluid could result in its degradation or chemical modification through hydrolytic pathways since the drug will be exposed to the acidic (pH 3.5 to 4.5) and slightly alkaline environment (pH 7 to 8) of the CV fluid in normal conditions and during sexual intercourse (in the presence of seminal fluid), respectively.^[5] The drug could also be degraded through oxidative hydrolysis since the biologically relevant concentration of hydrogen peroxide (H_2O_2) produced by *Lactobacillus* bacteria present in the normal CV flora is 0.002% to 0.08% v/v.^[6,7]

Elucidating the pH dependent stability of microbicides would also be beneficial in rectal applications since the pH of rectal fluid is around 7 to 8.^[8] Thus, it is essential to characterize the physicochemical stability and degradation mechanism of a microbicide under the above mentioned conditions and according to the International Conference on Harmonization (ICH) guidelines.^[9] In this respect, liquid chromatography coupled mass spectrometry (LC-MS)^[10,11] and solid state X-ray diffraction (XRD)^[12] analyses are well-established and the most versatile techniques for the determination of degradation products and crystalline forms of bio-active molecules, respectively.

There are several high performance liquid chromatography (HPLC),^[4,13,14] LC-MS,^[15–18] matrix-assisted laser desorption/ionization (MALDI),^[19] and liquid chromatography-tandem mass spectrometry (LC-MS/MS)^[20–25] based assays that have been reported in the literature for the analysis of TFV alone or in combination with other bio-active molecules. Fragmentation behavior of TFV under varying collision energy (5–55 V) using nitrogen as the collision activation dissociation gas has also been reported.^[26] A critical feature of an effective microbicide is that it should be stable at various climate/storage environments.^[27] However, there is little information available about TFV stability under the various storage and stress conditions. Hence, the integral aim of the present study is to investigate the physicochemical stability and degradation behavior of TFV under the selected conditions of temperature, pH, percent relative humidity (% RH), and H_2O_2 in according to the ICH guidelines.^[9]

Materials and methods

Chemicals and reagents

TFV was purchased from Zhongshuo Pharmaceutical Co. Ltd (Beijing, China). Acetonitrile (HPLC grade), sodium hydroxide (NaOH), and hydrogen peroxide (H₂O₂) were from Sigma-Aldrich (St Louis, MO, USA). Hydrochloric acid (HCl) was from Fisher Scientific (Pittsburgh, PA, USA). Deionized water for all experiments was obtained through a Millipore Milli Q water purification system (Millipore Corp., Danvers, MA, USA). All other chemicals were of analytical grade and used as obtained from suppliers.

Liquid chromatography (LC) and mass spectrometry (MS) conditions

LC analysis was carried out on a UFLC Shimadzu prominence system (Shimadzu USA manufacturing Inc., Torrance, CA, USA) consisting of an LC-20 AD low pressure gradient pump, SPD-M20A photodiode array (PDA) detector, SIL-20AST auto sampler, and DGU-20As degasser. A reversed phase Waters Symmetry® C₁₈ column (150 mm × 4.6 mm, 5 µm) was used as a stationary phase. The detection was carried out at 259 nm. The LC elution conditions were set as follows (all solvent percentages were volume fractions): mobile phase-A, 0.1% v/v formic acid in water; mobile phase-B, 0.1% v/v formic acid in acetonitrile; mobile phase-C, 30% A + 70% B. The time program was: 0.01 min, 100% A; 10 min, 100% C; 13 min, 100% B; 14 min, 100% A; and 18 min, 100% A. The flow rate was 0.6 mL min⁻¹ at ambient temperature conditions. A sample injection volume of 10 µL was used in each analysis.

MS studies were performed on a 3200 QTrap mass spectrometer (Applied Biosystems Sciex, Framingham, MA, USA). Spectra were recorded using Enhanced mass spectrum (EMS) scan in positive mode. Analyst® software version 1.4.2 (Applied Biosystems Sciex, Framingham, MA, USA) was used for data acquisition. MS operational parameters were set as follows: Collision activated dissociation (CAD): high; Ion source Gas1 (GS1): 50 psi; Gas2 (GS2): 50 psi; Turbo ion spray voltage (IS): 5500 V; Source temperature (TEM): 350°C; Collision energy (CE): 5 V; Declustering potential (DP): 2 V, and Entrance potential (EP): 2 V. Nitrogen gas was used as the nebulizer, and the scan rate was 4000 amu/sec. The spectra were obtained by scanning between 100 and 700 amu.

Forced degradation analysis

The drug at the concentration of 1 mg mL⁻¹ was subjected to stress degradation under acidic (0.1 M HCl, pH 1), alkaline (0.1 M NaOH, pH 13), and neutral (H₂O) hydrolytic conditions by refluxing up to 5 days.^[9,28] The stability of TFV under the normal pH condition of CV flora (4.5)^[5] was determined at 25°C and 40°C (analyzed up to 10 days) and under refluxing conditions up to 5 days at the concentration of 1 mg mL⁻¹. The oxidative degradation studies were carried out by exposing TFV (1 mg mL⁻¹) to 3% and 30% v/v aqueous solutions of H₂O₂ for up to 7 days at room temperature. The high H₂O₂ concentration compared to that present in normal vaginal flora (0.002% to 0.08% v/v)^[6] was selected for stress degradation according to the ICH guidelines.^[9]

To determine the long-term storage stability of TFV, a 12-month study was performed at -20°C , 5°C , $25^{\circ}\text{C}/60\% \text{ RH}$, and under the accelerated condition of $40^{\circ}\text{C}/75\% \text{ RH}$ (drug powder was spread in a glass dish to have a thin homogeneous layer). Thermal (dry heat) accelerated stress stability analysis was performed up to 12 months at 40°C , 50°C , and 60°C and for one month at 80°C . TFV was also subjected to thermal stress at 25°C and 40°C under acidic (0.1 M HCl: pH 1; 0.01 M HCl: pH 2; HCl: pH 4.5) and alkaline (0.1 M NaOH: pH 13; 0.01 M NaOH: pH 12) conditions up to 10 days. The pH of all the solutions was measured using a SevenEasy pH meter (Mettler Toledo, Schwerzenbach, Switzerland) at room temperature.

Sample preparation and stability analysis

A stock solution of TFV (10 mM) in Milli Q water was diluted with mobile phase - A to yield the solutions in the concentration range of 7.81–500 μM for the calibration curve. The stressed samples were collected after each time period and diluted with the same mobile phase to the concentration of 250 μM and to make them suitable for LC analysis.^[28] For comparison, a freshly prepared solution of TFV (250 μM) and the blank samples (without TFV, processed in the similar way as of stressed samples) were also analyzed in triplicate ($n = 3$). The drug was considered stable if there was $< 10\%$ degradation of the initial amount was observed.^[29,30]

Solid state X-ray diffraction (XRD) analysis

The effect of temperature and % RH conditions on the crystal structure of TFV powder (samples stored at -20°C , 5°C , $25^{\circ}\text{C}/60\% \text{ RH}$, $40^{\circ}\text{C}/75\% \text{ RH}$, 50°C , and 80°C) was determined using solid state powder XRD analysis. The XRD patterns were obtained using a Rigaku MiniFlex automated X-ray diffractometer (Rigaku, The Woodland, TX, USA). The samples were mounted on single-crystal Si zero-background plates for analysis. The analysis was performed at room temperature using Cu K α radiation produced at 35 kV and 15 mA, with a Ni filter. The scan angle (2θ) was from 5° to 40° with a step size of $0.05^{\circ} 2\theta$ and 3 sec per step. The diffraction patterns were processed using Jade 8+ software (Materials Data, Inc., Livermore, CA, USA).

Stability analysis using Arrhenius equation plot

The influence of temperature on the degradation kinetics of TFV was determined using accelerated stability testing and Arrhenius equation^[31–33] (Eqn (1)).

$$\ln(k) = \ln(A) - \frac{E_a}{RT} \quad (1)$$

Where, k is the degradation rate constant, A is the frequency factor, E_a is the activation energy, R is the gas constant and T is the absolute temperature in degrees Kelvin. The k value depends on the E_a and is characteristic of a specific compound.^[34]

Based on the pseudo-first-order reaction kinetics,^[31] Eqn (2) was generated into its logarithmic (base 10) form.

$$\log \frac{C}{C_0} = - \frac{k' t}{2.303} \quad (2)$$

In this Eqn (2), k' , C_0 , C and C/C_0 are the pseudo-first-order rate constant, initial concentration and concentration of drug remaining after time t , and fraction of drug remaining after time t , respectively. The values of k' at each temperature can be determined using Eqn (2), from the slope of the regression equation generated from the plot between $\log$ % drug remaining and time (t) in months.

The value of $1000/T$ (in Kelvin) was calculated for each temperature and the Arrhenius plot between $\ln(k')$ vs. $1000/T$ was constructed. The slope and intercept values of this plot were equal to $-E_a/R$ and $\ln(A)$, respectively, according to Eqn (1). The E_a was calculated by multiplying the slope value by R ($8.314 \text{ J.mol}^{-1} \cdot \text{Kelvin}^{-1}$). The significance of the E_a value is to determine the temperature dependency of a chemical reaction. The higher the value of E_a for a chemical reaction the greater the acceleration with increase in temperature and the more the stability of a drug is temperature dependent.^[34,35] Generally, drugs with lower E_a values have significantly longer shelf-lives.^[34] The rate constant (k'_{25}) that corresponds to room temperature (25°C) was calculated from the regression equation.

The k'_{25} value was used for the calculation of shelf life (t_{90}), half-life (t_{50}), and the time required for the drug to decrease its initial amount by 90 % (t_{10}). The determination of the t_{90} , t_{50} , and t_{10} values was based on Eqn (2).

The t_{50} is the time required for 50% degradation. This was calculated by replacing C and t with $C_0/2$ and t_{50} , respectively, in Eqn (2). This gives Eqn (3) after logarithmic calculations.

$$t_{50} = \frac{0.693}{k'} \quad (3)$$

The shelf life (t_{90} :time required to decrease initial drug amount by 10%) was calculated by replacing C and t with $0.9 C_0$ and t_{90} ; whereas, t_{10} was calculated by replacing C and t with $0.1 C_0$ and t_{10} in Eqn (2), which are given by Eqns (4), and (5), respectively.

$$t_{90} = \frac{0.105}{k'} \quad (4)$$

$$t_{10} = \frac{2.303}{k'} \quad (5)$$

Method validation

The developed method was validated with respect to the ICH guidelines Q2:R1^[36] as explained below.

Linearity—The calibration curve was plotted between the LC peak area of the extracted wavelength chromatogram (XWC) in the mAU.min (y axis) and the corresponding drug concentrations in the range of 7.81–500 μM (x axis). The intercept, slope, and square of the correlation coefficient (r^2) values were determined by linear regression analysis of the data.

Limit of quantification (LOQ) and limit of detection (LOD)—The LOQ is defined as the lowest concentration of an analyte that can be quantified; whereas, LOD is the concentration of an analyte that can only be detected with acceptable precision and accuracy under the stated operational conditions.^[36] The LOQ and LOD values were calculated using the signal-to-noise (S/N) ratios of 10:1 and 3:1, respectively.

Precision and accuracy—The intra-(repeatability), inter-day, and intermediate (analyst-to-analyst) precision was analyzed using three quality control (QC) samples (62.5, 250, and 500 μM) in replicate ($n = 6$). The analysis was performed on the same day for intra- and on three different days within a week for inter-day precision. To analyze the intermediate precision, the whole experiment was conducted by a different analyst in replicate ($n = 6$). Accuracy was determined by spiking the QC samples of TFV with a mixture of stressed samples. The percentage mean recovery was calculated from the calibration curve.

Robustness—The robustness of a method is its ability to remain unaffected by small deliberate variations in the method parameters.^[36] The following changes in the method parameters were examined: the flow rate of the mobile phase (± 0.2 unit); ratio (% v/v) of mobile phase-A: mobile phase-B at 10 min (± 5 units); and the detection wavelength (± 1 unit). The variation in the peak area of the LC chromatogram of TFV was calculated and the acceptance criterion of %RSD $< 2\%$ was considered for each parameter.

Specificity and selectivity—Specificity analysis was based on the resolution of the LC chromatogram of TFV in the presence of its degradation products.^[36] The selectivity was assessed by comparing LC chromatograms obtained by injecting blank samples corresponding to media used in each stress condition with the standard TFV to determine any interfering peaks at the retention time of TFV.

Results and discussion

Validation of the LC method

The retention time of TFV was found to be 6.00 ± 0.01 min ($n = 6$) as shown in the representative XWC spectrum (Figure 1A). A good linearity was observed within the concentration range of 7.81–500 μM with an r^2 value of 0.998 ± 0.001 ($n = 3$). The LOQ and LOD values were found to be 3.90 μM and 1.90 μM , respectively. The method was found to be precise as the %RSD values for inter-, intra-day, and intermediate precision was $< 2\%$ (Table 1). The percent mean recovery of TFV was within the acceptable range of 90–110% (Table 1). The impact of minor variations in the method parameters was within the acceptable limits, which ensured robustness of the method. No interfering peaks of stress conditions media and products were observed at the retention time of TFV.

Proposed degradation behavior of TFV

The native TFV showed its molecular ion ($M + H$) peak with m/z 288.2 amu (Figure 1B). The degradation behavior of TFV under various stress conditions was explained below.

Hydrolytic and oxidative degradation—The TFV exhibited extensive degradation under acidic (0.1 M HCl) condition, as about 27% degradation occurred after day one (Figure 1C). The extent of degradation of TFV after 24 h was sufficient to be considered an acid labile drug.^[28] However, the study was continued up to 5 days under refluxing conditions, and it was observed that there was > 95% degradation after 5 days of reflux as shown in the XWC spectrum (inset Figure 1C). The acidic condition resulted in degradation products namely A_1 and A_2 with m/z of 170 and 289.2 amu, respectively, as shown in Figure 1D. The drug was rapidly degraded in acidic conditions; however, the absorbance (height) of the TFV peak decreased, without a corresponding rise in peaks of the degradation products. This indicated that the drug was further hydrolyzed in strong acidic conditions, perhaps to non-chromophoric low molecular weight compounds caused by an alteration of the bonds in the degradation products. It has been previously observed and reported by various authors that degradation products with low molecular weight and weak or non-chromophoric groups cannot be retained on the LC column and hence no or very low intensity peaks were observed in the LC analysis.^[37–39]

The drug was relatively stable under alkaline (0.1 M NaOH) hydrolytic conditions. Since, there was < 5% degradation after day one, the study was continued up to 5 days and < 20% degradation was observed after day 5. One major degradation product (B_1) of m/z 289.2 amu was observed as shown in the XWC (Figure 1E) and EMS spectrum (Figure 1F). The intensity of the TFV peak in the LC chromatogram decreased with a corresponding increase in the intensity of B_1 . The drug was considered practically stable under neutral hydrolytic conditions since insufficient (< 10%) degradation was observed after 5 days (Figure 1G). However, a very small peak of degradation product (W_1) with m/z 289.2 amu was observed after day 5 as shown in the XWC (Figure 1G) and EMS spectrum (Figure 1H). The slow rate of formation of W_1 was due to the fact that reactions at neutral pHs are non-catalytic and a very long study time may be required under these conditions to obtain significant degradation of a bio-active molecule.^[40,41]

Although, the drug was relatively stable at pH 4.5 (< 10% degradation), the degradation product A_2 (m/z 289.2 amu) was observed under refluxing conditions for 5 days as shown in its XWC (Figure 2A) and EMS spectrum (Figure 2B). However, no significant degradation (< 5%) and degradation products were observed at pH 4.5 whether the temperature was 25°C (Figure 2C and 2D) or 40°C (Figure 2E and 2F) as analyzed up to 10 days. TFV was also found stable under oxidative stress conditions in 3% and 30% v/v H_2O_2 solutions up to 7 days at room temperature (data not shown). On exposure to acidic (0.1 and 0.01 M HCl) and alkaline (0.1 and 0.01 M NaOH) conditions at 25°C and 40°C, the drug was found stable (< 5% degradation) (data not shown).

Proposed structures of degradation products and degradation pathways of TFV under hydrolytic conditions—The structural instability of TFV could be due to

the deamination of its adenine nucleus and degradation of the ({[propan-2-yl]oxy} methyl) phosphonic acid side chain under hydrolytic conditions. It was observed that TFV was primarily degraded through hydrolytic mechanism under acidic and alkaline conditions. The degradation products have been observed and shown by pathways-A and, B in Figure 3. The degradation products (A_2 , B_1 , W_1) with m/z 289.2 amu were proposed as 6-Hydroxy derivative of TFV (pathway-A). This could be formed due to the proton mediated hydrolytic deamination ($-NH_2$) of the adenine nucleus of TFV. It has been previously explained that deamination of adenine is easier under acidic and alkaline conditions owing to the lower energy barriers compared to the neutral conditions.^[40,41] This could be the reason why a very small peak of 6-Hydroxy derivative of TFV was observed under a neutral environment compared to acidic and alkaline conditions even after 5 days of reflux.

The degradation product (A_1) of m/z 170 amu was proposed as (2-hydroxypropan-2-yloxy) methylphosphonic acid as shown by pathway-B in Figure 3. This could be formed due to the cleavage of N-C bond between the adenine nucleus and ({[propan-2-yl] oxy} methyl) phosphonic acid side chain of TFV. A very low intensity peak of A_1 was observed in LC chromatogram, which could be due to its non-chromophoric nature. Overall, the stability analysis revealed that the degradation of TFV was to the combined effects of pH, and temperature since the major degradation products were observed under hydrolytic conditions only.

Degradation kinetics of TFV under hydrolytic conditions—The kinetic plots (Figure 4) were constructed between log (% drug remaining) versus time in hours using Eqn (2) under acidic and alkaline hydrolytic conditions. The amount of drug remaining after each time point was calculated from the linear regression equation and represented in terms of mean $\pm$ standard deviation (SD) after triplicate analysis ($n = 3$). The kinetic plots were found to be linear in both acidic (r^2 : 0.986 ± 0.001) and alkaline (r^2 : 0.991 ± 0.004) conditions ($n = 3$), and the degradation of TFV followed pseudo-first-order kinetic behavior. The k 'values determined from the slope of kinetic plots using Eqn (2) were calculated as 2.73×10^{-2} (± 0.01) h^{-1} and 0.18×10^{-2} (± 0.01) h^{-1} in acidic and alkaline conditions, respectively, ($n = 3$).

It was observed that the initial amount of TFV was dramatically reduced from 249.68 ± 3.71 μM to 11.66 ± 0.42 μM in acidic conditions, and moderately from 257.25 ± 0.60 μM to 208.81 ± 1.27 μM ($n = 3$) in alkaline conditions after 5 days of reflux. The t_{50} , t_{90} , and t_{10} values determined using Eqns (3), (4), and (5)^[31] were 25.34 ± 0.05 , 3.84 ± 0.01 , and 84.22 ± 0.17 h in acidic conditions; whereas, the values were 384.49 ± 10.32 , 58.26 ± 1.56 , 1277.75 ± 34.28 h in alkaline conditions, respectively, ($n = 3$).

Physicochemical, long-term, accelerated and thermal stress stability of TFV

Physicochemical stability (XRD analysis)—The results of the XRD analyses were shown in Figure 5. The characteristic peaks of native TFV (Figure 5A) were observed at the diffraction angle of $2\theta = 7.44^\circ$, 14.89° , 18.11° , 18.59° , 22.41° , 23.45° , 24.75° , 28.90° , and 29.80° . The XRD pattern of the unstressed sample of TFV obtained here was similar to that obtained in a previous study.^[42] Results confirmed that the drug was stable and maintained

its original crystalline diffraction pattern under the variable stress conditions of temperature and % RH (Figure 5B–5G).

Arrhenius equation plot—The degradation kinetic plot of TFV powder between $\ln$ (% drug remaining) and time (t) in months stressed at 40, 50, and 60°C was shown by Figure 6A. The k' values determined from the slope of the regression equation were observed as $0.60 (\pm 0.04)$, $0.70 (\pm 0.06)$, and $0.84 (\pm 0.06) \times 10^{-2} \text{ months}^{-1}$ at 40, 50, and 60°C, respectively, ($n = 3$). A linear relationship between $\ln(k')$ in months^{-1} and $1000/T$ (in Kelvin) at 40°C (313.15 Kelvin), 50°C (323.15 Kelvin), and 60°C (333.15 Kelvin) was observed in the Arrhenius plot (Figure 6B). The slope of this plot and r^2 values were found to be -1.75 ± 0.29 and 0.974 ± 0.032 , respectively, ($n = 3$). The k'_{25} value was calculated as $0.45 \times 10^{-2} (\pm 0.03) \text{ months}^{-1}$ ($n = 3$).

The E_a value derived from the slope of Arrhenius plot was calculated as $14.54 \pm 2.45 \text{ kJ.mol}^{-1}$ ($n = 3$). The low E_a value obtained here reflected that the stability of TFV under accelerated conditions was temperature independent.^[34] The t_{90} of TFV calculated using the obtained k'_{25} value and Eqn (4) was found to be 23.40 ± 1.56 months (1.95 ± 0.13 years) after triplicate analysis ($n = 3$). This was close to the shelf life of TFV (24 months: two years) at room temperature found in the literature under the recommended storage conditions.^[43]

Conclusions

In this study, the physicochemical stability and degradation kinetics of TFV under hydrolytic, oxidation, thermal and accelerated stress conditions was analyzed using a validated LC-MS method. The drug exhibited degradation under acidic (0.1 M HCl) and alkaline (0.1 M NaOH) hydrolytic conditions, following a pseudo-first-order degradation kinetics behavior. Based on LC-MS data, the structure of the degradation products and degradation pathways were proposed. The drug was found to be stable under oxidation, thermal, and accelerated stress conditions. Solid state XRD analysis confirmed that the TFV powder was stable and maintained its original crystalline pattern under the variable stress conditions of temperature and % RH.

The shelf life of TFV at room temperature was found to be about 23 months, calculated by using the Arrhenius equation. Stability analysis revealed that formulation strategies, such as encapsulation of TFV in nanoparticles, should be implemented to protect it from strong acidic and alkaline conditions. However, stability of TFV at pH 4.5 (normal CV pH), oxidative, accelerated, and thermal stress conditions makes it suitable for long-term storage and vaginal controlled delivery applications as a topical microbicide. However, the degradation products of TFV needs to be further separated using semi-preparative HPLC method and characterized using nuclear magnetic resonance (NMR) and Infra-red (IR) spectroscopy in the future work. Semi-preparative HPLC analysis would be helpful in order to facilitate the structural studies on the degradation products that are present at very low concentration.

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Figure 1.

Extracted wavelength chromatogram (XWC) and Enhanced mass spectrum (EMS) of Tenofovir (TFV): Unstressed sample (1 mg mL⁻¹) (A and B), and stressed samples under Acidic (0.1 M HCl), analyzed after day one (C and D); Alkaline (0.1 M NaOH), analyzed after day 5 (E and F); and Neutral (water) hydrolytic conditions, analyzed after day 5 (G and H), respectively. The inset figures C and D showed the XWC and EMS spectra of stressed samples of TFV under acidic (0.1 M HCl) conditions, analyzed after 5 days of reflux, respectively. The left and right panels of the Figure showed XWC and EMS spectrum, respectively. A₁, A₂, B₁, and W₁ are the degradation products of TFV as discussed in Figure 3.

Figure 2.

Extracted wavelength chromatogram (XWC) and Enhanced mass spectrum (EMS) of stressed sample of Tenofovir (TFV) (1 mg mL^{-1}) under acidic pH 4.5, analyzed after 5 days of reflux (A and B); TFV samples stressed under acidic pH 4.5 at 25°C , analyzed after 10 days (C and D); TFV samples stressed under acidic pH 4.5 at 40°C , analyzed after 10 days (E and F), respectively. The left and right panels of the Figure showed XWC and EMS spectrum, respectively. A₂ is the degradation product of TFV under acidic condition as discussed in Figure 3.

Figure 3.
Proposed degradation pathways and degradation products structure of Tenofovir (TFV)
under hydrolytic stress conditions.

Figure 4. Pseudo-first-order degradation kinetic plots of Tenofovir (TFV) under acidic (0.1 M HCl; ■) and alkaline (0.1 M NaOH; ◆) hydrolytic conditions.

Figure 5.

Solid state X-ray diffraction (XRD) of Tenofovir (TFV) in different environmental conditions. From bottom to top, (A) TFV-unstressed sample, (B) TFV at -20°C , (C) TFV at 5°C (D) TFV at $25^{\circ}\text{C}/60\% \text{ RH}$, (E) TFV at $40^{\circ}\text{C}/75\% \text{ RH}$, (F) TFV at 50°C and, (G) TFV at 80°C . Each sample was analyzed after 12 months of stressed conditions applied except the sample at 80°C which was analyzed after one month of exposure.

Figure 6.

Degradation kinetic plot of Tenofovir (TFV) (A), constructed between $\ln (\% \text{ drug remaining})$ and time in months at 40°C (◆), 50°C (■), and 60°C (▲). (B) Arrhenius plot of TFV between $\ln (k')$ in month^{-1} and $1000/T$ (in Kelvin) at 40°C, 50°C, and 60°C.

Table 1

Precision and accuracy of the method

Actual amount (μM)	Intra-day: within- a day, (n = 6)		Inter-day: three days within- a week, (n = 6)	
	Precision: recovered amount $\pm$ SD (% RSD)	Accuracy: percent mean recovery $\pm$ SD	Precision: recovered amount $\pm$ SD (% RSD)	Accuracy: percent mean recovery $\pm$ SD
62.50	59.54 $\pm$ 0.18 μM (0.31)	95.27 $\pm$ 0.29	61.39 $\pm$ 0.40 μM (0.65)	98.71 $\pm$ 0.64
250	250.26 $\pm$ 0.18 μM (0.07)	100.11 $\pm$ 0.07	250.53 $\pm$ 0.24 μM (0.10)	100.21 $\pm$ 0.10
500	472.22 $\pm$ 0.50 μM (0.11)	94.44 $\pm$ 0.10	478.65 $\pm$ 1.56 μM (0.33)	95.73 $\pm$ 0.31