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Rapid stability-indicating UHPLC method for determination of lamivudine and tenofovir disoproxil fumarate in fixed-dose combination tablets

Danielle Evangelista Rabelo de Souza[a], Nicoly Kaliny Magela Gomes[b], Gerson Antônio Pianetti[b], Bruno Gonçalves Pereira[c], Christian Fernandes[b],*

[a]*Divisão de Controle de Qualidade, Diretoria Industrial, Fundação Ezequiel Dias, Belo Horizonte, Minas Gerais, Brazil*
[b]*Laboratório de Controle de Qualidade de Medicamentos e Cosméticos, Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[c]*Divisão de Desenvolvimento de Medicamentos, Diretoria Industrial, Fundação Ezequiel Dias, Belo Horizonte, Minas Gerais, Brazil*

Lamivudine (3TC) and tenofovir disoproxil fumarate (TDF) are antiretroviral drugs widely used for AIDS treatment. Since safety, efficacy and quality are essential for drug products, stability studies must be performed. Therefore, degradation studies must be carried out to evaluate the degradation products formed. The active pharmaceutical ingredients 3TC and TDF, as well as the tablets containing the combination of these drugs were subjected to a comprehensive forced degradation. 3TC, TDF and the degradation products were analyzed by a stability-indicating ultra-high performance liquid chromatography (UHPLC) method developed and validated. A C8 (100 x 2.0 mm, 2.2 µm) column and a mobile phase composed of 0.1 M ammonium acetate buffer pH 4.0, acetonitrile and methanol in gradient elution, at 0.5 mL/min, were used. The injection volume was 4 µL and detection was at 260 nm. The method was selective, precise, accurate and linear in the range 0.1-0.6 mg mL-1 for the two drugs. 3TC was degraded in acidic, alkaline and oxidative environment and in the presence of metal ions. Two degradation products were observed. TDF was degraded in the same conditions than those 3TC was labile. In addition, TDF was degraded in neutral condition. Again, two degradation products were formed. Chemical structures were proposed for the degradation products using UHPLC-QTOF/MS. The stability-indicating method developed showed to be useful in stability studies and in the quality control of fixed-dose combination tablets containing 3TC and TDF.

Keywords: AIDS, antiviral drug, stability-indicating method, forced degradation, ultra high-performance liquid chromatography.

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Introduction

HIV is a major global public health issue. According to estimates by WHO and UNAIDS there were approximately 37.7 million people living with HIV at the end of 2020, with 1.5 million people becoming newly infected in 2020 globally. Moreover, in 2020, 680,000 people died from HIV-related causes (1).

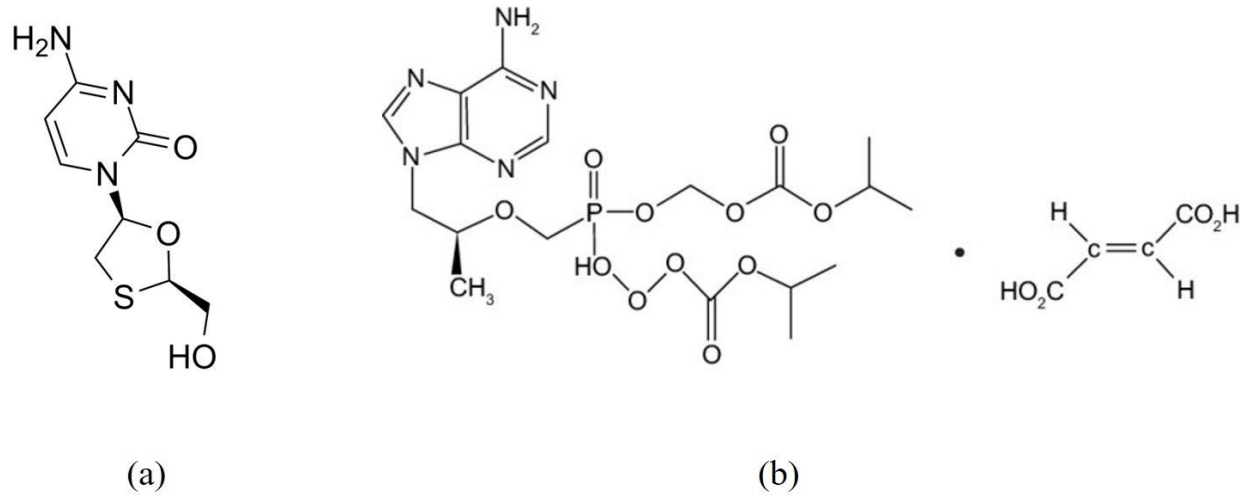
Lamivudine (3TC) (Fig. 1a) and tenofovir disoproxil fumarate (TDF) (Fig. 1b) are nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs), respectively (2, 3). When these two drugs are used in combination the anti-retroviral therapy adherence increases, contributing to reduce mortality, opportunistic infections or severe AIDS-related symptoms (4).

Safety, quality and efficacy of pharmaceuticals are essential in drug therapy. Instability of pharmaceuticals can cause changes in physical, chemical, pharmacological or toxicological properties of the drug product (5).

Therefore, degradation studies under heating, humidity, hydrolysis, oxidation, and photolysis conditions and in the presence of metal ions must be carried out to evaluate the degradation products formed (6,7). In this context, a method of analysis selective for the analyte of interest, in the presence of its degradation products formed during degradation studies, must be developed (7). Also, critical analysis of drug degradation profile should be performed.

Due to the possibility of formation of large amount of degradation products and the need for rapid separation, especially in the drug development context, the most suitable approach is UHPLC. This technique employs mobile phases at high linear velocities and high pressures, taking to dramatic increases in resolution, sensitivity and speed (8).

Some studies dealing with 3TC (9-12) or TDF (13-15) stability, have already been published in the literature. Bedse et al. conducted a 3TC degradation study in different conditions. The drug showed instability in acidic, alkaline and oxidative environment. On the other hand, it remained stable in neutral hydrolysis and to light and thermal stress (9).



(a) (b)

Figure 1. Chemical structures of (a) 3TC (CAS 134678-17-4) and (b) TDF (CAS 201341-05-1).

In another study a HPLC stability-indicating method was developed to separate 3TC from its carbonate derivatives with activity against human immunodeficiency and hepatitis B viruses (10). Navaneethan et al. developed HPLC method for determination of 3TC in the presence of degradation products and other antiviral drugs (11). TDF was submitted to hydrolysis and oxidative conditions and a HPLC method was validated for the analysis of TDF and its related substances (13). Only one study has been found in the peer-review literature where 3TC and TDF were simultaneously submitted to degradation and a stability-indicating method by UHPLC was developed for both drugs (16). However, the identification of drug products was not proposed, what makes the value of this study limited. In addition, the forced degradation study must be carried out with the drug product, the placebo and the isolated and associated active pharmaceutical ingredients, in the case of a fixed dose combination. In that study, the degradation study was performed with the tablets and placebo only.

With all this in mind, the aim of this study was to submit 3TC and TDF to forced degradation (neutral, acidic and basic hydrolysis, presence of metal ion, oxidation, high humidity, thermolysis and photolysis); develop and validate a selective UHPLC stability-indicating method for the two drugs; determine the degradation kinetics in tablets under alkaline conditions for determination of reaction rate and half-life; and propose chemical structures and degradation routes for the products formed using UHPLC-QTOF/MS.

Experimental

Chemical, reagents and materials

3TC, TDF, related compound B (chemically named (1-methylethyl) (5RS,8R)-9-(6-amino-9H-purin-9-yl)-5-methoxy-8-methyl-5-oxo-2,4,7-trioxa-5-λ5-phosphanonanoate), tenofovir monohydrated, and fumaric acid reference standards were obtained from U.S. Pharmacopeial Convention (Rockville, USA), all with purity of 100%. 3TC and TDF API were produced by Nortec Química (Duque de Caxias, Brazil). Ammonium acetate and methanol HPLC grade were purchased from Merck (Darmstadt, Germany). Acetic acid glacial, hydrochloric acid, sodium hydroxide, hydrogen peroxide and copper sulphate pentahydrate were purchased from Vetec (Duque de Caxias, Brazil). Acetonitrile HPLC grade was purchased from J. T. Baker (Xalostoc, Mexico). Tablets containing 300 mg of 3TC and 300 mg of TDF were obtained from Farmanguinhos (Rio de Janeiro, Brazil). Placebo was prepared mixing the excipients used in these tablets (croscarmellose sodium and cellulose by Mingtai Chemical, China; pregelatinized starch and hydroxypropylmethylcellulose, polyethyleneglycol 8000 and 400 by Colorcon, USA; lactose monohydrate by DFE Pharma, Germany; sodium glycollate by Amishi Drugs & Chemicals, India; colloidal silicon dioxide by Confarma, USA; magnesium stearate by Peter Greven, Netherlands; and titanium dioxide by Kronos International, USA). Ultrapure water was obtained from a Millipore Integral System (Bedford, USA).

Instrumentation and analytical conditions

The stability-indicating method was developed and validated using an UHPLC system (Shimadzu Nexera) coupled with a diode-array detector. The software LabSolutions was used for data acquisition and processing. The column used was a Shim-pack XR-C8 (100 x 2.0 mm; 2.2 µm) from Shimadzu and was kept at 35 ºC. The mobile phase was composed of 0.1 M ammonium acetate buffer in acetic acid 0.1% (v/v) pH 4.0, methanol and acetonitrile, in gradient elution, in a flow-rate of 0.5 mL min^{-1}. Detection was at 260 nm, injection volume was 4 µL and auto injector temperature was 15 ºC.

The identification of the degradation products was conducted in an UHPLC system (Agilent 1290 Infinity) coupled with an UV (260 nm) and a Q-TOF mass spectrometer (6540 UHD Accurate-Mass Agilent)

equipped with an electrospray ion source. The software Agilent Mass Hunter was used for data acquisition and processing. The source temperature was 120 ºC, the capillary voltage was 4000 Kv and the electrospray ionization was at positive mode. Collision gas was nitrogen with a voltage of 15, 20 and 25 eV. Separation was performed in a ZORBAX Extend C18 (50 x 2.1 mm, 1.8 µm) column and was kept at 35 ºC. The mobile phase was composed of water and acetonitrile in gradient elution at 0.5 mL min^{-1}. Injection volume was 4 µL.

Preparation of the standard solutions

Standard solutions of 3TC and TDF, separated and associated, were prepared at 1 mg mL^{-1} in water and were submitted to ultrasound during 45 minutes. A dilution was performed to 0.5 mg mL^{-1} in 0.1 M ammonium acetate buffer in 0.1% (v/v) acetic acid pH 4.0. Then, one portion of each standard was immediately filtered into vial maintained at 15 ^{o}C and injected in the UHPLC.

Forced degradation and preparation of sample solutions

Each sample (3TC and TDF, separated and associated, tablets and placebo) were prepared in volumetric flask at 1 mg mL^{-1}. For tablets, the average weight of twenty fixed-dose combination tablets (lamivudine 300 mg and tenofovir desoproxil 300 mg) was determined and the tablets were crushed. The amount of powder equivalent to 25 mg of 3TC and 25 mg of TDF were transferred to a 25 mL volumetric flask. For the preparation of the placebo, approximately 33.72 mg of the mixture of the excipients, equivalent to 0.08 of the average weight, were transferred to a 25 mL volumetric flask. After, the degradation solutions (acid, alkaline, neutral, oxidative and metal ion) were added to the volumetric flasks and submitted to ultrasound for 45 minutes at 80 Hz. Afterwards, solutions were vortexed with controlled temperature. Then, one portion of each sample was diluted again to 0.5 mg mL^{-1} with 0.1 M ammonium acetate buffer in 0.1% (v/v) acetic acid pH 4.0, filtered to vial and injected in the UHPLC. For neutral (ultrapure water), acid (0.1 M HCl), alkaline (0.01 M NaOH) and metal ion (0.05 M $CuSO_4$), solutions were kept at 70 ºC; for alkaline (0.0001 M NaOH) and oxidative (0.3% (v/v) H_2O_2), solutions were kept at 25 ºC. Therefore, two different concentrations of NaOH were required for stress testing experiments. All these conditions were established in order to obtain at least 10% of degradation. Under photolysis (6 lux.h and 1000 watt.h/m^2), moisture (30 °C and 75% UR) and thermolysis (90 °C) conditions, sufficient amount of the samples was placed in petri plates for degradation. Then, each sample was aliquoted and diluted to 1 mg mL^{-1} in water. Finally, one portion of each sample was diluted again to 0.5 mg mL^{-1} with 0.1 M ammonium acetate buffer in 0.1% (v/v) acetic acid pH 4.0, filtered to vial and injected in the UHPLC. The degradation solutions, diluent solution (0.1 M ammonium acetate buffer in 0.1% (v/v) acetic acid pH 4.0), ultrapure water, mobile phase and placebo were injected into the UHPLC to evaluate the selectivity of the chromatographic method.

Method validation

The stability-indicating method was validated according to Brazilian validation guideline and a procedure to assess linearity by ordinary least squares method (17-19). All calculations were performed using Microsoft Excel 2010. The parameters evaluated were: specificity, linearity, range, precision (repeatability and intermediate precision), accuracy, limits of quantification and detection and robustness.

Degradation kinetic

Degradation kinetic for all stress conditions studied was assessed. At each sampling, the remaining 3TC and TDF content was determined. Then, graphs of remaining concentration versus time were constructed for each condition. Linear regression was used to evaluate the best fit of the line and to calculate the straight-line equation of the graph. In order to determine the reaction order, the y-axis was established as concentration (zero order), logarithm of the concentration (first order) and inverse of the concentration (second order). R^2, k (relation between concentration and time variation) and RSD were evaluated to determine the reaction order. From k value, half-life ($t½$) and shelf life ($t90$) were calculated (20).

Structure proposals of the degradation products

Samples containing a mixture of all stress conditions used for 3TC and TDF were injected using UHPLC-QTOF/MS system. The peaks with the most intense abundance were selected and fragmented. Chemical structures for the degradation products were proposed.

Results and Discussion

Stability-indicating method development

A stability-indicating method was developed by UHPLC in order to selectively separate 3TC and TDF from their degradation products formed in the forced degradation studies. Initially, an exploratory gradient with methanol and water, from 5 to 95% of methanol, was tested for 60 minutes to know the behavior of the analytes. Afterwards, ammonium acetate buffer and methanol were chosen as mobile phase. Acetic acid was able to improve the peak shape, improving resolution, since it minimized the existence of ionized silanol groups (21). However, the adequate resolution among some peaks was obtained only with the addition of acetonitrile and gradient elution, as follows: 90% buffer, 0% methanol and 10% acetonitrile (0-5 min isocratic), 0% buffer, 90% methanol and 10% acetonitrile (5-7 min gradient), 90% buffer, 0% methanol and 10% acetonitrile (7-10 min isocratic), 100% buffer, 0% methanol and 0% acetonitrile (10-15 min gradient). The wavelength of maximum absorption for 3TC and

TDF were at 270 and 261 nm, respectively. Since TDF response (peak height) was lower than that for 3TC, the wavelength chosen was 260 nm, the wavelength of maximum absorption for TDF.

Forced degradation

After five days, no degradation was observed in neutral condition (70 ºC) for 3TC isolated, associated and in tablets; on the other hand, TDF degraded after five hours. Two degradation products, named TD, were formed ($DP1_{TD}$ and $DP2_{TD}$ with retention times of 2.8 min and 5.8 min, respectively). At acid condition (0.1 M HCl at 70 ˚C), TDF degraded faster; in two hours of exposure, and the same two degradation products were formed. In contrast, 3TC degraded in three days, and two degradation products were observed ($DP1_{3TC}$ 1.8 min and $DP2_{3TC}$ 3.2 min). Due to TDF lability, the alkaline degradation had to be done separately for each API. 3TC degradation was made in 0.01 M NaOH at 70 °C in one day with only one degradation product ($DP2_{3TC}$ 3.2 min). On the other hand, TDF degradation was made in 0.0001 M NaOH at 25 °C in nine days, forming two degradation products ($DP1_{TD}$ 2.8 min and $DP2_{TD}$ 5.8 min). H_2O_2 0.3% (v/v) at 25 °C was used for 3TC and TDF oxidation. In this case, 3TC degradation occurred faster than that for TDF, forming only one degradation product ($DP1_{3TC}$ 1.8 min); for TDF, one degradation product ($DP2_{TD}$ 5.8 min) was formed. When exposed to 0.05 M $CuSO_4$ at 70 °C, TDF and 3TC degraded, each one, in two products, the same already described. 3TC and TDF did not degrade when subjected to exposure in a high humidity chamber (30 ˚C and 75% RH) for 39 days. Thus, both 3TC and TDF were considered stable to moisture. The samples were also exposed at 90 °C for 21 days and 3TC and TDF were found not to degrade. For photodegradation studies, samples were exposed to 1.2 million lux.h (visible) and 200 watt.h/m^2 (ultraviolet). Under the conditions studied, 3TC and TDF did not degrade. Therefore, it can be concluded that both 3TC and TDF were stable to light. Fig. 2 shows the chromatogram of a mixture of all samples obtained in the forced degradation studies. As can be seen, all peaks were appropriately separated in less than 10 minutes.

In a general way, TDF is much more labile than 3TC, except in oxidative condition. In the presence of H_2O_2 0.3% (v/v), 3TC isolated degraded more than when associated with TDF. On the other hand, TDF isolated degraded less than when associated or in the tablets. In these cases, the presence of 3TC and/or excipients (in the case of tablets) seems to favor TDF degradation. Table 1 summarizes the results of degradation for 3TC and TDF.

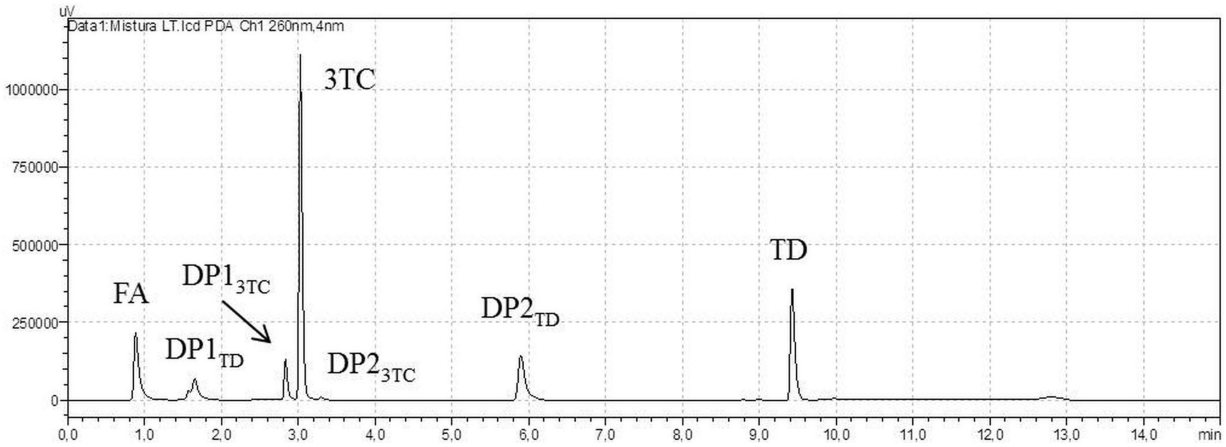


Figure 2. Chromatogram of a mixture of all degradation conditions obtained in the developed stability indicating UHPLC method. FA: fumaric acid; $DP1_{TD}$: degradation product 1 from TD; $DP2_{TD}$: degradation product 2 from TD; 3TC: lamivudine; $DP1_{3TC}$: degradation product 1 from 3TC; $DP2_{3TC}$: degradation product 2 from 3TC TD: tenofovir desoproxil.

Table 1. Degradation percentage obtained for 3TC and TDF in different conditions and times of exposition.

Sample	H_2O 70 ˚C		HCl 0.1 *M* 70 ˚C		NaOH 0.01 *M* 70 ˚C		NaOH 0.0001 *M* 25 ˚C		H_2O_2 0.3% (v/v) 25 ˚C		$CuSO_4$ 0.05 *M* 70 ˚C	
	%	Time	%	Time	%	Time	%	Time	%	Time	%	Time
3TC isolated	0	5 days	16	3 days	25	1 day	-	-	45	15 hours	13	1 day
3TC associated	0	5 days	19	3 days	29	1 day	-	-	31	15 hours	18	1 day
3TC tablets	0	5 days	13	3 days	23	1 day	-	-	31	15 hours	17	1 day
TDF isolated	23	5 days	24	2 hours	-	-	13	9 days	10	3 days	13	1 hour
TDF associated	18	5 days	24	2 hours	-	-	26	9 days	19	3 days	25	1 hour
TDF tablets	24	5 days	24	2 hours	-	-	28	9 days	23	3 days	22	1 hour

Method validation

Selectivity

To prove method selectivity for 3TC and TDF, the mixture of the degraded samples, in all conditions, was injected. Resolution was higher than 2.0 for all pair of peaks. Also, peak purities for 3TC and TDF were higher

than 97% (Table 2). The degradation solutions, placebo and the diluent (0.1 M ammonium acetate buffer prepared in 0.1% (v/v) acetic acid pH 4.0) were injected and no peaks coeluting with 3TC, TDF, $DP1_{3TC}$, $DP2_{3TC}$, $DP1_{TD}$ and $DP2_{TD}$ were observed.

Table 2. Retention time, resolution and peak purity obtained in the evaluation of selectivity.

Peak	RT (min)	Resolution	Purity (%)
FA	0.886	---	---
$DP1_{3TC}$	1.657	5.116	---
$DP1_{TD}$	2.851	9.166	---
3TC	3.044	2.201	98.3145
$DP2_{3TC}$	3.317	2.907	---
$DP2_{TD}$	5.907	18.991	---
TD	9.425	23.981	97.7936

Linearity

Six solutions were prepared with concentrations equally spaced between 20% and 120% (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg mL^{-1}) of the theoretical concentration of analysis, 0.5 mg mL^{-1}. The presence of outliers, normality, independence and homoscedasticity of the residuals were evaluated and showed to be in agreement with all the least squares method assumptions. Also, the regression was statistically significant and lack of adjustment to the linear model was not observed. The graphs were plotted for 3TC and TDF and equations of the calibration curves and determination coefficients were: $y = 8x10^6 x + 6231.8$ (R^2 0.9983) and $y = 5x10^6 x + 135053$ (R^2 0.9989), respectively. So, the stability-indicating method presented a linear behavior for both 3TC and TDF (17-19).

Accuracy

Accuracy was evaluated by spiking the placebo with known amounts of 3TC and TDF and calculating the percentage recovery at 20, 100 and 120% (0.1, 0.5 and 0.6 mg mL^{-1}) of the working concentration. Recoveries were within the range of 98 to 102%, showing the stability-indicating method accuracy (Table 3) (17, 22).

Precision

Precision was evaluated at three levels (0.1, 0.5 and 0.6 mg mL^{-1}), with three independent replicates. The intra-day precision was assessed by quantification of 3TC and TDF in the same day by the same analyst. The inter-days precision was performed by repeating the same procedure on another day. The relative standard deviations (RSD) obtained in the intra-day precision were from 0.10 to 1.76% for 3TC and from 0.28 to 0.83% for TDF. In the evaluation of inter-days precision, the RSD were in the range 0.09– 0.84% and 1.00– 1.22% for 3TC and TDF, respectively. All results were lower than 2.0%, showing the method precision (17).

Table 3. Accuracy for the developed UHPLC stability-indicating method.

Theoretical concentration (mg mL^{-1})	Replicate	Recovery 3TC (%)	Mean ± sd 3TC (%)	Recovery TDF (%)	Mean ± sd TDF (%)
0.1	1	99.66	99.43 ± 0.70	101.11	101.36 ± 0.33
	2	99.99		101.73	
	3	98.64		101.25	
0.5	1	100.43	100.24 ± 0.67	97.99	98.26 ± 0.38
	2	100.79		98.70	
	3	99.49		98.10	
0.6	1	99.68	99.15 ± 0.96	100.35	100.40 ± 0.26
	2	99.74		100.69	
	3	98.04		100.18	

LOD and LOQ

The detection (LOD) and quantification (LOQ) limits were evaluated based on the standard deviation of the intercept (s_b) and the slope of the calibration curve (a) according to the formulas: LOD = $3(s_b/a)$ and LOQ = $10(s_b/a)$. These are theoretical values since s_b and a were obtained from the calibration curves, according to the legislation used to validate the method (17). The detection limits for 3TC and TDF were 0.0168 and 0.0276 mg mL^{-1}, respectively; and the quantification limits were 0.0554 and 0.0911 mg mL^{-1}, respectively.

Robustness

The following parameters were evaluated: oven temperature (25 and 45 °C), mobile phase flow-rate (0.4 and 0.6 mL/min), detection wavelength (254 and 271 nm), injection volume (3 and 5 µL) and pH of 0.1 M ammonium acetate buffer (3.0 and 5.4). These parameters were varied according to the statistical method of Youden and Steiner (23). The developed method was robust for the parameters: oven temperature, detection wavelength and pH of 0.1 M ammonium acetate buffer. Greater rigor should be employed when using the injection volume and mobile phase flow-rate.

Degradation kinetic in alkaline condition

Degradation kinetic for all studied conditions was evaluated. However, alkaline medium was the only condition where the results were appropriate in terms of coefficient of determination and relative standard deviation (20).

Degradation kinetic for 3TC in tablets, submitted to 0.01 M NaOH at 70 °C, was established through determination of remaining drug content on days 0, 1, 2, 3, 4 and 5. The best fit to the model was found for second order kinetic (Fig. 3), which means that degradation depends on the concentration of two reagents, NaOH and excipients or TDF. The following equations were used to calculate the degradation constant (k_2), half-life ($t_{1/2}$) and shelf life (t_{90}), respectively, for 3TC in tablets exposed to alkaline condition,

$$k_2 = \frac{\frac{1}{C} - \frac{1}{C_0}}{t} \quad \text{Equation 1}$$

$$t_{1/2} = \frac{1}{k_2 . C_0} \quad \text{Equation 2}$$

$$t_{90} = \frac{1}{9k_2 . C_0} \quad \text{Equation 3}$$

Where C is the concentration of 3TC remaining in a time; C_0 is the initial concentration of 3TC; k_2 is the second order degradation constant; and t is time. The degradation constant k_2 was 0.0014% day^{-1} and $t_{1/2}$ was 194 years. Also, t_{90} was established as 21.6 years, which means 3TC would take 21 years to degrade 10% in the conditions employed.

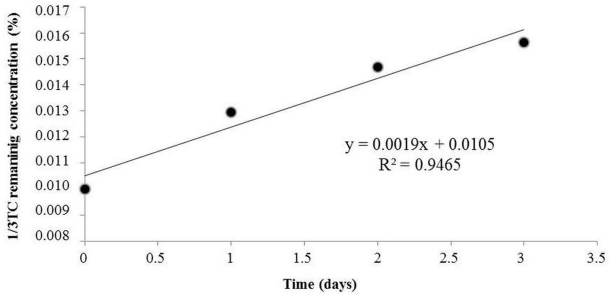


Figure 3. Second order degradation kinetic for 3TC in alkaline environment (0.01 M NaOH) at 70 °C.

The same procedure was established for TDF in tablets, with the exception of the medium, temperature and times of analysis, which were 0.0001 M NaOH, 25 °C, and 0, 2, 4, 7, 8, 9 and 10 days. The degradation followed zero order kinetic (Fig. 4); so, degradation depends on the concentration of the active pharmaceutical ingredient. The following equations were used to calculate the degradation constants (k_0), $t_{1/2}$ and t_{90}, respectively, for TDF in tablets exposed to alkaline condition,

$$k_0 = \frac{C_0 - C}{t} \quad \text{Equation 4}$$

$$t_{1/2} = \frac{C_0}{2k_0} \quad \text{Equation 5}$$

$$t_{90} = \frac{C_0}{2k_0} \quad \text{Equation 6}$$

Where C is the concentration of TDF remaining in a time; C_0 is the initial concentration of TDF; k_0 is zero order degradation constant; and t is time. The calculated constant was 2.62% day^{-1}, $t_{1/2}$ was 19 days and the time required to decrease TDF content in 10% (t_{90}) was 3.8 days.

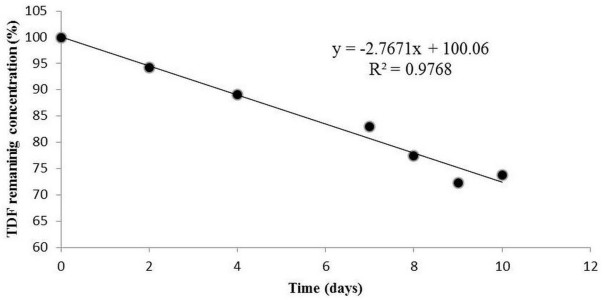


Figure 4. Zero order degradation kinetic for TDF in alkaline environment (0.0001 M NaOH) at 25 °C.

Chemical structure of degradation products

To determine the chemical structures of $DP1_{3TC}$ and $DP2_{3TC}$, the samples from forced degradation studies were analyzed using UHPLC-QTOF/MS. Initially, three ions with significant abundance were obtained, *m/z* 112, 230 and 459 (Fig. 5).

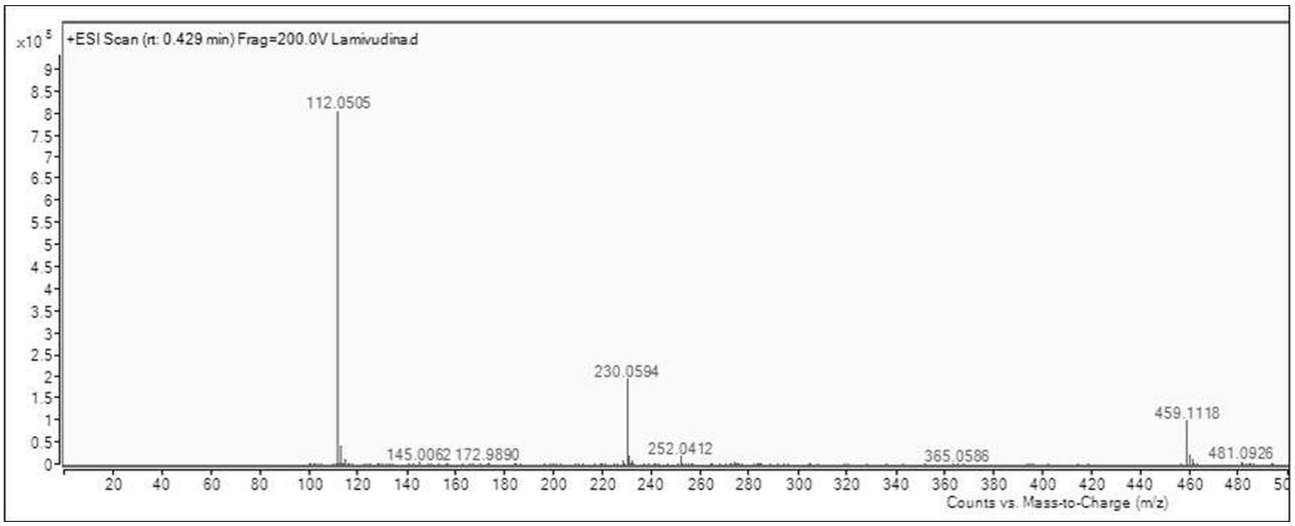


Figure 5. Mass spectrum obtained for the mixture of 3TC, $DP1_{3TC}$ and $DP2_{3TC}$.

Since 3TC molar mass is 229.26 g mol^{-1}, and the molecule undergoes nitrogen protonation in the aromatic ring, it may be suggested that the *m/z* 230 signal refers to the protonated 3TC. The *m/z* 112 seems to be cytosine, chemically named 4-aminopyrimidin-2(1*H*)-one, and showed in Fig. 6a. This impurity was identified in The International Pharmacopoeia (Impurity E) (24). The *m/z* 459 suggests the formation of a dimer (Fig. 6b). To the best of our knowledge, this is the first time this degradation product is described in the literature. So, $DP1_{3TC}$ has *m/z* 112 and $DP2_{3TC}$ has *m/z* 459.

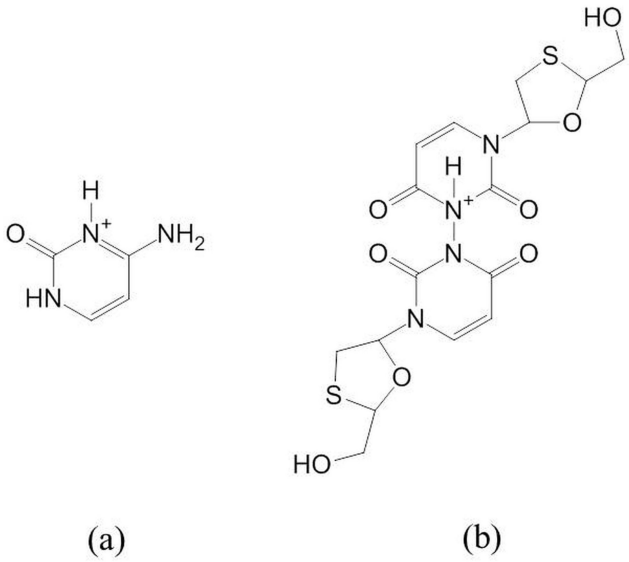


Figure 6. Proposed chemical structure for (a) $DP1_{3TC}$ and (b) $DP2_{3TC}$.

The same analyzes were done to determine $DP1_{TD}$ and $DP2_{TD}$. Several ions with significant abundance were obtained (mass spectrum not showed), among them *m/z* 176 and 288. Since tenofovir molecule undergoes protonation at the amino group, the *m/z* 176 suggests the formation of the degradation product showed in Fig. 7b, already described as Impurity *K* on The International Pharmacopoeia (24). According to Silva (2014), the two ester groups present in TDF structure are subject to hydrolysis to form carboxylic acid and alcohol (25). As the molar mass of tenofovir is 287.23 g mol^{-1}, and as the molecule can undergo protonation at the amino group, it can be suggested that the *m/z* 288 is tenofovir (Fig. 7a). In order to confirm the proposed structures complementary studies are required. Nuclear magnetic resonance and infrared spectroscopy are appropriate techniques for this purpose.

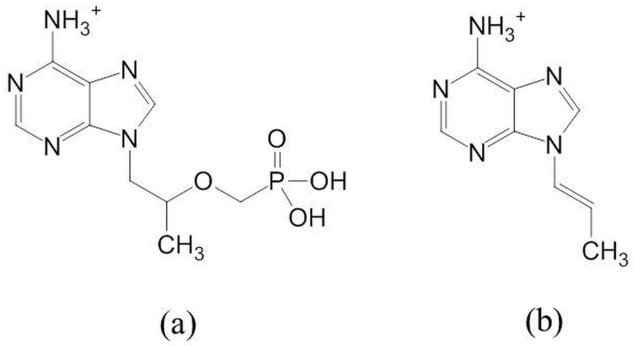


Figure 7. Proposed chemical structure for (a) $DP1_{TD}$ and (b) $DP2_{TD}$.

Conclusions

An UHPLC stability-indicating method for determination of 3TC and TDF in fixed-dose combination tablets was developed. A comprehensive forced degradation study was performed subjecting 3TC and TDF API and tablets to neutral, acidic and alkaline hydrolysis, oxidation, metal ion, heat, moisture and light. The two drugs showed different behavior against the degradation conditions. 3TC was susceptible to acid, basic, oxidative and by metal ions degradation. In neutral condition, there was no degradation. TDF showed degradation faster than 3TC due to the labile characteristic of the molecule itself. It degraded under neutral, acidic, basic, oxidative and by metal ions conditions. Both presented no degradations in high humidity, photolysis and thermolysis. The stability-indicating method was validated, showing to be able to determine 3TC and TDF in the presence of their degradation products. Finally, the chemical structures of $DP1_{3TC}$, $DP2_{3TC}$, $DP1_{TD}$ and $DP2_{TD}$ were proposed.

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Conflict of interest

The authors declare no conflicts of interest.

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