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RESEARCH ARTICLE

Preformulation studies of novel 5′-*O*-carbonates of lamivudine with biological activity: solubility and stability assays

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Abstract

As a part of preformulation studies, the aim of this work was to examine the solubility and stability of a series of 5′-*O*-carbonates of lamivudine with proven antihuman immunodeficiency virus activity. Solubility studies were carried out using pure solvents (water, ethanol and polyethylene glycol 400 [PEG 400]), as well as cosolvents in binary mixture systems (water–ethanol and water–PEG 400). These ionizable compounds showed that their aqueous solubility is decreasing as the carbon length of the substituent moiety increases, but being enhanced as the pH was reduced from 7.4 to 1.2. Thus, 3TC-Metha an active compound of the series, with an intrinsic solubility at 25 °C of 17 mg/mL, was about 70 times more soluble than 3TC-Octa (0.24 mg/mL), and at pHs of 1.2, 5.8 and 7.4 had intrinsic solubilities of 36.48, 19.20 and 15.40 mg/mL, respectively. In addition, the solubility was enhanced significantly by using ethanol and PEG 400 as cosolvents. A stability study was conducted in buffer solutions at pH 1.2, 5.8, 7.4 and 13.0 and in human plasma at 37 °C. Stability-indicating high-performance liquid chromatography procedure was found to be selective, sensitive and accurate for these compounds and good recovery, linearity and precision were also observed.

Keywords

Aqueous stability, cosolvency, human plasma stability, pH-solubility profiles, prodrugs, solubility

History

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Introduction

The acquired immunodeficiency syndrome (AIDS) has become a serious public health problem around the world and generates much concern in both medical and non-medical circles. Its causative agent is the human T-cell lymphotropic virus, human immunodeficiency virus (HIV), which destroys the immune system cells and causes mortality by allowing opportunistic infections and malignancies to propagate[1]. However, antiretroviral therapy of HIV infection has changed this syndrome from being uniformly fatal into a potentially chronic disease. Today, more than 30 anti-HIV compounds have been formally approved for clinical use in the treatment of AIDS[2,3]. Among them, reverse transcriptase inhibitors (RTIs) are used in drug combination regimens known as Highly Active Anti-Retroviral Treatment (HAART) to achieve the highest possible tolerability and compliance and to diminish the risk of resistance development[1,2].

Lamivudine (3TC, **1**, Figure 1) a RTIs, has a good safety, tolerability and efficacy, but its therapeutic value is limited by a time-dependent development of drug-resistant HIV mutants, which occurs after a few weeks of treatment[1–4]. In an attempt to improve its performance, our investigation group has synthesized 3TC derivatives (**2**–**8**, Figure 1).

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These compounds have been assayed not only against HIV[5] but also against hepatitis B virus (HBV)[6], because 3TC has also been licensed for the treatment of chronic HBV infection[7]. Taking into account not only their improved antiviral properties but also the better lipophilicity achieved compared to the parent compound[8] (Table 1), these derivatives turn into promising candidates to treat affections caused by these viral agents. Because their mechanisms of action are based on hydrolysis and/or enzymatic cleavage of their 5′-OH bonds between 3TC and their attached moieties, stability plays a critical role to explain the activity of these prodrugs. In addition, it is relevant to know the solubility of these drug molecules due to the importance of this parameter in the process of drug discovery and the development of pharmaceutical formulations and biopharmacy.

Considering the potential of these novel 5′-*O*-carbonates of 3TC as antiviral agents, and in an attempt to develop efficient preformulation studies, the purpose of this work was to determine the solubility of **2**–**8** in pure water, ethanol and PEG 400 due the known use of these solvents in pharmaceutical formulations[9]. We also described the study of water binary mixtures of ethanol and PEG 400 as cosolvents, the influence of temperature and pH on solubility in all the used solvent systems. Taking into account essential stability studies for prodrug screening, we also described the aqueous and enzymatic hydrolysis of these carbonates (**2**–**8**), as well as the development and validation of a sensitive, specific, rapid and simple High Performance Liquid Chromatography (HPLC) bioanalytical method, to quantify 5′-*O*-carbonates of lamivudine in different media/matrixes in the presence of 3TC (its only degradation product).

Table 1. Solubility data of **1–8** compounds[a].

Compound	Water[b] 25 °C	Water[b] 37 °C	Ethanol 25 °C	Ethanol 37 °C	PEG 400 25 °C	PEG 400 37 °C	Log P[c]	MW
1, 3TC	65.90 (±0.10)	119.00 (±0.09)	164.00 (±0.09)	256.00 (±0.08)	84.52 (±0.10)	170.00 (±0.07)	−0.91	229.3
2, 3TC-Metha	17.00 (±0.20)	24.90 (±0.08)	41.00 (±0.07)	64.00 (±0.08)	21.13 (±0.09)	42.50 (±0.06)	−0.81	287.3
3, 3TC-Etha	8.56 (±0.10)	14.30 (±0.05)	37.00 (±0.05)	58.00 (±0.06)	19.19 (±0.09)	38.60 (±0.08)	−0.31	301.3
4, 3TC-Pro	4.34 (±0.20)	8.70 (±0.06)	32.00 (±0.10)	50.00 (±0.09)	16.52 (±0.09)	33.23 (±0.06)	0.20	315.3
5, 3TC-Buta	1.13 (±0.07)	6.60 (±0.10)	28.00 (±0.08)	44.00 (±0.07)	14.62 (±0.10)	29.41 (±0.08)	0.86	329.3
6, 3TC-Penta	0.74 (±0.10)	4.50 (±0.10)	25.00 (±0.09)	39.00 (±0.08)	13.05 (±0.07)	26.26 (±0.10)	1.23	343.3
7, 3TC-Hexa	0.52 (±0.05)	2.70 (±0.20)	23.00 (±0.08)	36.00 (±0.05)	12.00 (±0.10)	24.32 (±0.10)	1.57	357.3
8, 3TC-Octa	0.24 (±0.07)	1.18 (±0.06)	16.00 (±0.06)	25.00 (±0.06)	8.45 (±0.05)	17.02 (±0.10)	1.72	385.3

[a]Solubility data are expressed as mg/mL ± (SD). $n = 2$; [b]Water pH 6.5; [c]Ref. Ravetti et al. (2008).

Compound	R
1, 3TC	H
2, 3TC-Metha	$C(O)OCH_3$
3, 3TC-Etha	$C(O)OCH_2CH_3$
4, 3TC-Pro	$C(O)O(CH_2)_2CH_3$
5, 3TC-Buta	$C(O)O(CH_2)_3CH_3$
6, 3TC-Penta	$C(O)O(CH_2)_4CH_3$
7, 3TC-Hexa	$C(O)O(CH_2)_5CH_3$
8, 3TC-Octa	$C(O)O(CH_2)_7CH_3$

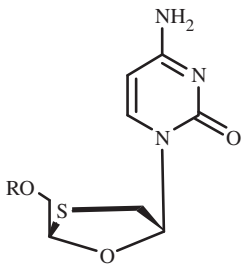

Figure 1. Chemical structure of lamivudine (**1**) and its derivatives (**2–8**).

Methods

Materials and equipment

Lamivudine, a generous gift from Filaxis (Buenos Aires, Argentina), was used without purification. The corresponding prodrugs, obtained by association of 3TC with different aliphatic alcohols, were prepared as previously reported[5]. Acetonitrile and methanol, both of HPLC grade, and PEG 400 and ethanol, both of analytical grade, were purchased from Sintorgan (Buenos Aires, Argentina), with sodium dihydrogen phosphate monohydrate analytical grade being provided by Merck (Darmstadt, Germany). The water was purified using a Milli-Q water-purification system (Millipore®), and all solutions used for HPLC were filtered through a Millipore® Type FH filter (0.45 µm pore size) (Millipore S.A.S., Molsheim, France) before being vacuum degassed.

All other chemicals and solvents were of analytical grade and used as delivered. Human plasma was supplied by the Blood Bank, Universidad Nacional de Córdoba, Argentina, and collected from healthy volunteer blood donors. It was then fractioned in aliquots (10 mL) under sterile conditions, and immediately frozen until used. Human plasma samples were cleaned up by off-line Solid Phase Extraction (SPE), using Sep Pak Plus C18 extraction cartridges (Water Corp). Buffer solutions of 25 mM were prepared as follows: pH 1.2, 42.5 mL of 0.2 M hydrochloric acid and 0.12 w/v potassium chloride; pH 5.8, potassium phosphate monoacid (0.08 w/v) and sodium phosphate monobasic dihydrate (0.34 w/v); pH 7.4, potassium phosphate monoacid (0.26 w/v) and sodium phosphate monobasic dihydrate (0.15 w/v); and pH 13.0, 0.10 w/v potassium chloride and 0.05 w/v sodium hydroxide. The pH values were measured by a Crison GLP-21 pH meter (Modena, Italy). A Haake-DS thermostatic bath (37 ± 0.1 °C) (Karlsrthhe, Germany) was used for assays requiring temperature control. A Rolco centrifuge (Buenos Aires, Argentina) was employed for ultrafiltration procedures and a TestLab Ultrasonic Cleaner (Dongguan, China) utilized for sample preparation. Ultraviolet spectrophotometric (UV) studies were carried out with a Shimadzu Model UV-160A spectrophotometer, using 1 cm quartz cells, and HPLC assays were carried out with an Agilent Series 1100 chromatograph using an UV detector at $\lambda = 272$ nm, equipped with a Phenomenex® column, Hypersil ODS 10 µ particle diameter of 250 mm in length and 4.6 mm internal diameter. Data were produced by means of a Peak Simple Chromatography Data System (version 2.86®). Thin layer chromatography was performed on plates from Merck (Silica Gel 60 F254) (Darmstadt, Germany).

Solubility studies

Drug calibration curves

To determinate the solubility of prodrugs **2–8**, as well as the parent compound **1,** standard curves were constructed in each medium. Stock solutions of **1–8** (1×10^{-6} mol/mL) were prepared in appropriate solvents and the concentration of the standard solutions ranged between 2×10^{-8} and 1×10^{-7} mol/mL. The analytical method used was UV at $\lambda = 272$ nm.

Equilibrium assays

An excess amount of drug was added to 1 mL solvent (aqueous buffer, water, ethanol or PEG 400) in glass vials and placed into a shaking water bath at 25 ± 0.1 °C and 37 ± 0.1 °C at 60 rpm for 24 h. Then, these solutions were centrifuged at 12 500 rpm for 10 min, and the supernatant filtered through 0.45 µm pore diameter membranes. The first portion was discarded to ensure saturation of the filter and an aliquot of the remaining filtrate was diluted with the corresponding solvent and then analyzed. All solubility studies were carried out in duplicate.

Stability studies

Chromatographic conditions

For the HPLC analyses, the mobile phase consisted of a mixture of methanol-buffer phosphate (pH 7.4; 12.5 mM) (70:30, v/v). A volume of 20 µL was injected in triplicate and the analyses were conducted at 25 °C with a flow-rate of 1 mL/min. Thin layer chromatography was performed at room temperature using dichloromethane–methanol (90:10 v/v) as the mobile phase.

Validation of HPLC method

Specificity. The specificity of the analytical method was demonstrated by extracting and analyzing spiked 3TC and **2–8** derivatives from three different lots of normal human plasma and buffer solutions at different pHs. Identification of **2–8** and its degradation product was performed by HPLC and their retention times revealed no interference of the analytes with the corresponding matrix by the observation of an adequate separation among all compounds.

Linearity. The calibration curves for 3TC and its carbonates were obtained at ten different concentrations equally distributed in the range from 5.0×10^{-8} to 8.0×10^{-7} mol/mL, and five injections of each concentration were performed.

Limit of detection (LOD) and limit of quantification (LOQ). The samples were spiked with decreasing analyte concentrations before being analyzed. The LOD was determined as the lowest amount of analyte in each sample which could be detected with a between-run coefficient of variation (RSD) < 20%, but not necessarily could be quantified as an exact value. In addition, the LOQ was calculated as the lowest amount of analyte in a sample which could be quantitatively determined with an adequate precision and accuracy[10].

Precision. The precision data was obtained by analyzing two concentrations of **1–8** (8×10^{-8} and 8×10^{-7} mol/mL), with the precision being calculated as the relative standard deviation (RSD).

Recovery. Recovery of 3TC and its derivatives in buffer and human plasma matrices was assayed at three different concentrations: 8×10^{-8}, 3×10^{-7} and 8×10^{-7} mol/mL (low, medium and high), respectively.

Stability measurements in aqueous buffers

Stability studies of **2–8** prodrugs were performed at pH 1.2, pH 5.8, pH 7.4 and pH 13.0 at 37 °C. One milliliter of buffer was equilibrated at 37 °C for 10 min prior to the introduction of each solution of **1–8** in dimethylsulfoxide (DMSO) to yield an initial concentration of 5×10^{-7} mol/mL, which was then mixed using a vortex for 30 s. Samples of 100 µL were withdrawn at suitable time intervals, and 400 µL of methanol (MeOH) were added to each one. These mixtures were immediately cooled in an ice bath and stored in a −20 °C freezer. Upon removal of the last samples, the stored solutions were allowed to warm up to room temperature, and then the disappearance of **2–8** was monitored by HPLC by measuring the peak area of each compound. The pseudo-first-order rate constant for the disappearance of compounds under investigation was determined by using linear regression of the natural logarithm of concentration versus time. Samples for determining the chemical stability were assayed as three replicates.

Stability measurements in human plasma

A 50 µL aliquot of **1–8** stock solution (5×10^{-5} mol/mL) was added to 5 mL of human plasma pre-incubated at 37 °C. Aliquots (200 µL) were taken from the incubation media at regular intervals for 1.3 h and were immediately placed into an ice-cold tube. The cartridges were sequentially preconditioned with pro-analysis grade methanol (5 mL) and water Milli-Q (5 mL). To prepare the samples for the SPE, 1.8 mL of water Milli-Q was added to a 200 µL matrix sample containing the drug under analysis and then the solution was appropriately homogenized. The resulting solution was applied to the preconditioned cartridge by means of a 5 mL syringe at a flow rate of one drop every 2 s. The loaded cartridge was washed with 1 mL of Milli-Q water, and an air stream was then forced through it to dry the cartridge by applying positive pressure with a syringe. Finally, the drug was eluted from the cartridge with 1 mL of HPLC-grade methanol and quantified by HPLC before being assayed with three replicates.

Results

Water, ethanol and PEG 400 solubility

The traditional equilibrium method for solubility determination of **1–8** in water (S_w), ethanol (S_{eth}) and PEG 400 (S_{PEG400}) was employed, with an excess of drug molecule being added to the solvent system under stirring at constant temperature[11,12]. Because pharmaceutical products may be subject to wide temperature variations in the stored conditions, the water solubility for **1–8** was analyzed at both 25 ± 0.1 °C and 37 ± 0.1 °C (Table 1), which showed its values increasing with temperature. The average of the duplicate determinations was also reported.

The solubility of **1–8** increased with the polarity of the organic solvents (dielectric constant (D) for ethanol: 24.30; PEG 400: 12.17). Thus, 3TC-Metha, the most soluble compound, was at 25 ± 0.1 °C 1.94 times more soluble in ethanol than in PEG 400, and 2.4 times more soluble than in water. At 37 ± 0.1 °C, this compound showed the same ratio of solubility, being 1.5 times and 2.6 times more soluble in ethanol than in PEG 400 or water, respectively. The greater solubility of these derivatives in ethanol than in PEG 400 suggests that the solubility was principally governed by the intermolecular interactions among the solvent molecules.

It is known that the intrinsic solubility of a drug is determined by its polarity and crystallinity[13], with the polarity of a drug molecule being commonly evaluated by the logarithm of its octanol-water partition coefficient (log P), while the crystallinity of a drug is usually evaluated by its melting point (MP). Although this latter property could not be determined, because these compounds decomposed with temperature, the solubility of **1–8** compounds was inversely related to log P[8] and molecular weight (MW)[5] (Table 1). In general, the aqueous solubility of small molecules depends on their lipophilicity (log P)[14,15], and evidently the addition of an alcohol moiety to the 5′-position of lamivudine drastically reduced its polarity as the number of carbon atoms increased, which was confirmed by their log P values (Table 1).

In all cases, the solubility showed a certain parallelism with the solubility in water of the alcohols acting as substituent moieties of the 3TC molecule, which depended on the strength of the attraction of the corresponding OH group. As the alkyl chain length in alcohols increased, their ability to form hydrogen bonds with the drug molecules decreased[9]. Hence, the solubility also decreased. Thus, the first three alcohols (methanol, ethanol and propanol) are completely miscible, whereas from the fourth one (butanol) the solubility of the alcohols started to decrease, and then heptanol all alcohols are considered to be immiscible[16].

Cosolvency

Considering that **2–8** have lower solubilities than 3TC, a cosolvency study was carried out to try to improve this situation, because solutions having quite different properties are obtained when cosolvents are added to water. The cosolvency phenomenon involves using the empiric-analytical model proposed by Yalkowsky et al.[13,17], which allows the cosolvent effect on the solubility of active principles that are slightly soluble in aqueous media to be expressed by the following equation:

$$\log Sm = \log Sw + \sigma f \quad (1)$$

where Sm and Sw are the solubility of the solute in water–cosolvent binary mixture system and in pure water, respectively; f is the volume fraction of cosolvent in the solute-free solvent mixture; and σ is the cosolvent solubilization power for a particular solute–cosolvent system, whose value is experimentally

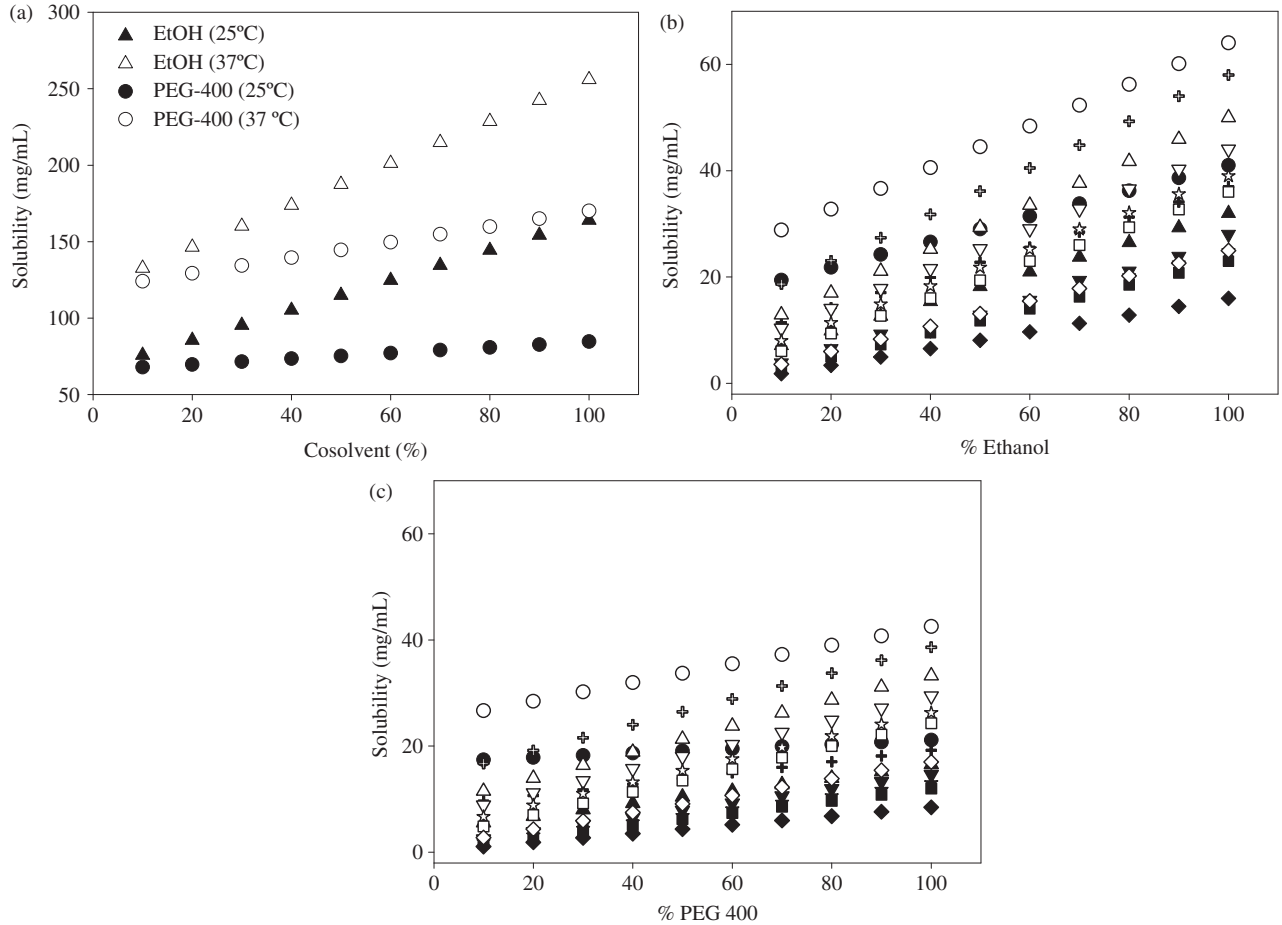

Figure 2. Solubility versus cosolvency. (a) 3TC versus % of ethanol and PEG 400 at 25 and 37 °C. (b) Ethanol (%): ● ○ 3TC-Metha (25 °C, 37 °C); ●○ 3TC-Etha (25 °C, 37 °C); ▲ △ 3TC-Pro (25 °C, 37 °C); ▼▽ 3TC-Buta (25 °C, 37 °C); ⋆⋆ 3TC-Penta (25 °C, 37 °C); ■ □ 3TC-Hexa (25 °C, 37 °C); ◆ ◇ 3TC-Octa (25 °C, 37 °C). (c) PEG 400 (%): ● ○ 3TC-Metha (25 °C, 37 °C); ⋆ 3TC-Etha (25 °C, 37 °C); ▲ △ 3TC-Pro (25 °C, 37 °C); ▼▽ 3TC-Buta (25 °C, 37 °C); ⋆ 3TC-Penta (25 °C, 37 °C); ■ □ 3TC-Hexa (25 °C, 37 °C); ◆ ◇ 3TC-Octa (25 °C, 37 °C).

obtained from the slope of the straight-line plot of log Sm versus f (Equation 1). The importance of Equation 1 is that σ is not related to the crystalline structure of the solute, and therefore this predictive method does not require knowledge of the melting temperature or melting enthalpy energy values.

Figure 2 shows the solubility of **1**–**8** compounds in water–ethanol and water–PEG 400 binary mixed-solvent systems at the two assayed temperatures (See Supporting Information, Tables S1–S4).

Yalkowsky and Roseman proposed a log-linear model (Equation 1) which describes the phenomenon of the exponential increase of the aqueous solubility for non-polar organic compounds as the cosolvent concentration is increased[11,18–20]. When the difference log Sm – log Sw of **1**–**8** was plotted against f, a nonlinear plot was obtained as shown in Figure 3, which could be indicating the non-ideality of the solvent mixture in the **1**–**8** solubilization. It is known that ethanol, when used as a cosolvent increases the cohesion forces of water whereas PEG 400 reduces these. Consequently, the surface tension, dielectric constant and solubility parameters are decreased[21,22]. As can be seen, water–10% ethanol mixture enhanced water solubility more than water–10% PEG 400 mixture, indicating that cosolvency was adequate for preformulation of **2**–**8**.

pH-solubility

One of the major factors responsible for the dissolution of an organic compound is its ability to dissociate into ionic species, which in turn depends on the pH of the medium. Compounds **1**–**8** contained one ionizable group and their behavior resembled weak basic drugs. The solubility data of **1**–**8** in different aqueous buffer solutions are listed in Table 2, where the averages of duplicate determinations are reported.

The total solubility of **1**–**8** (S_T) at a particular pH is the sum of the solubility of unionized species and the solubility of ionized species as described by the following Henderson–Hasselbach equations,

$$S_T = Sw\left[1 + 10^{(pKa-pH)}\right] \quad (2)$$

$$S_T = Sw(1 + Kb[H^+]/[Kw]) = Sw \times [1 + \text{antilog}(pKa - pH)] \quad (3)$$

where S_T is the total buffer aqueous solubility, Sw is the intrinsic aqueous solubility and Kb is the base dissociation constant. From Table 2, the aqueous solubility of **1**–**8** at 25 °C showed significant decreases for increases of the pH value, in agreement with Equation 3 [23]. From Equation 3, the pKa of the amine group of **1**–**8** was calculated (Table 2), which was very useful for pKa determination of the 3TC derivatives, because the potentiometric one did not have enough sensibility.

The pKa values of the NH_2 group of **1**–**8** was found to range between 4.1 and 5.7, with both the percentage of drug ionized and the solubility increasing with decreasing pH values of the medium. The addition of an acidic buffer is an easy and effective

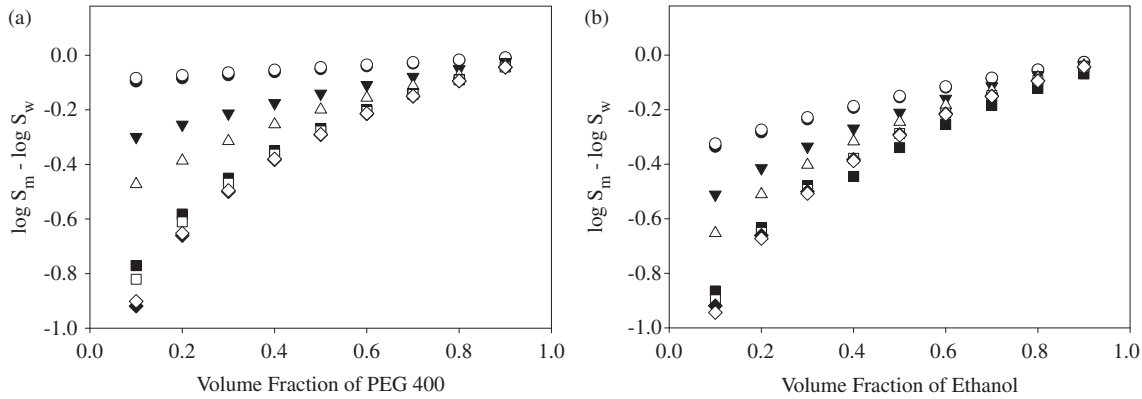

Figure 3. Log Sm – log Sw versus volume fraction of both cosolvents at 25 °C. ● 3TC; ○ 3TC-Metha; ▼ 3TC-Etha; ▲ 3TC-Pro; ■ 3TC-Buta; □ 3TC-Penta; ◆ 3TC-Hexa; ◇ 3TC-Octa.

Table 2. Solubility data of **1–8** compounds versus pH at 25 °C.[a]

Compound	pH 1.2	pH 5.8	pH 6.5[b]	pH 7.4	pKa
1, 3TC	103.00 (±0.06)	67.20 (±0.10)	65.90 (±0.10)	63.60 (±0.10)	4.10
2, 3TC-Metha	36.48 (±0.07)	19.20 (±0.08)	17.00 (±0.20)	15.40 (±0.05)	4.91
3, 3TC-Etha	20.42 (±0.05)	10.25 (±0.05)	8.56 (±0.10)	6.70 (±0.04)	5.10
4, 3TC-Pro	11.00 (±0.02)	5.40 (±0.06)	4.34 (±0.20)	3.30 (±0.10)	5.20
5, 3TC-Buta	5.40 (±0.03)	2.60 (±0.10)	1.13 (±0.07)	0.95 (±0.05)	5.25
6, 3TC-Penta	2.03 (±0.05)	0.95 (±0.10)	0.74 (±0.10)	0.63 (±0.02)	5.40
7, 3TC-Hexa	1.37 (±0.07)	0.73 (±0.05)	0.52 (±0.05)	0.45 (±0.05)	5.65
8, 3TC-Octa	0.82 (±0.08)	0.41 (±0.06)	0.24 (±0.07)	0.15 (±0.03)	5.70

[a]Solubility data are expressed as mg/mL ± (SD), $n = 2$; [b] pH 6.5 = water.

Table 3. Calibration parameters for **1–8** compounds.

Compound	Slope ± (SD)	Intercept ± (SD)	r^2	Recovery (%)[a]	Precision (RSD)[b]	LOD (mol/mL)	LOQ (mol/mL)	t_r (min)
1, 3TC	$1.0 \times 10^7 \pm (0.3 \times 10^6)$	−7.7 ± (134,0)	0.999	99.6, 98.9, 100.2	0.07, 0.03	2.1×10^{-9}	2.3×10^{-9}	2.68
2, 3TC-Metha	$2.4 \times 10^6 \pm (1.1 \times 10^5)$	−10.0 ± (47,5)	0.998	97.5, 100.9, 99.4	0.40, 0.04	6.9×10^{-9}	7.6×10^{-9}	3.68
3, 3TC-Etha	$1.3 \times 10^6 \pm (0.7 \times 10^5)$	16.0 ± (29,3)	0.998	99.2, 99.6, 104.8	0.59, 0.04	6.6×10^{-9}	9.5×10^{-9}	4.15
4, 3TC-Pro	$5.0 \times 10^6 \pm (1.8 \times 10^5)$	18.9 ± (75,4)	0.999	101.7, 99.5, 99.9	0.05, 0.01	6.7×10^{-10}	1.4×10^{-9}	4.79
5, 3TC-Buta	$3.1 \times 10^6 \pm (0.9 \times 10^5)$	12.2 ± (37,6)	0.999	100.4, 100.6, 99.9	0.03, 0.05	1.6×10^{-9}	3.2×10^{-9}	6.97
6, 3TC-Penta	$8.9 \times 10^6 \pm (2.9 \times 10^5)$	14.9 ± (111,0)	0.999	99.5, 99.8, 100.1	0.05, 0.01	8.0×10^{-10}	1.0×10^{-9}	7.69
7, 3TC-Hexa	$4.4 \times 10^6 \pm (1.7 \times 10^5)$	18.3 ± (74,9)	0.999	99.4, 99.5, 99.9	0.01, 0.07	6.3×10^{-10}	7.1×10^{-10}	10.33
8, 3TC-Octa	$2.5 \times 10^6 \pm (0.8 \times 10^5)$	20.0 ± (24,4)	0.999	97.6, 101.0, 100.4	0.12, 0.07	1.9×10^{-9}	3.6×10^{-9}	11.31

[a]$c_1 = 8 \times 10^{-8}$; $c_2 = 3 \times 10^{-7}$; $c_3 = 8 \times 10^{-7}$ mol/mL. [b]$c_1 = 8 \times 10^{-8}$; $c_2 = 8 \times 10^{-7}$ mol/mL.
$n = 3$.

way to increase its solubility. Thereby, the new derivatives were more soluble in pH 1.2 and from pH 5.8–7.4 with the solubility not changing due to the existence of unionized species.

Stability studies

Validation of HPLC method

The HPLC method reported here provides a simple procedure to determine concentrations of **1–8** compounds. Table 3 summarizes some of their relevant analytical data obtained with this procedure.

The standard curves for **1–8** were adequately described by least-squares linear regression. The calibration data revealing an excellent linearity over the concentration ranges studied, with correlation coefficients values (r^2) between 0.998 and 0.999. No interference from endogenous substances was observed in any plasma sample of 3TC or in their carbonate derivatives.

The limits of detection (LOD) and quantitation (LOQ) are summarized in Table 3. Precision was calculated as the relative standard deviation (RSD), where the standard deviation (SD) and mean corresponded to six injections.

Recovery was calculated as the percentage of deviation between the nominal and the observed concentration.

Identification of degradation compounds

Accelerated degradation studies for **2–8** were carried out in acid and basic media at 70 °C to identify their degradation products. In all cases, 3TC was detected as the only product in the reaction media (Figure 4a), which is also the parent compound of these prodrugs. Compounds **2–8** and 3TC were identified by comparing their retention times with those of standard samples by the TLC and HPLC techniques.

Using HPLC, no interference of 3TC with **2–8** was observed for any compound, revealing that these prodrugs could be determined in the presence of their degradation product. For example, Figure 4(b) shows the HPLC chromatogram for 3TC-Pro ($t_R = 4.79$ min) in the presence of its degradation product, 3TC ($t_R = 2.68$ min).

Aqueous stability

The chemical stability of 3TC compounds **2–8** was studied under experimental conditions of biological relevance, that is, at pHs 1.2, 5.8 and 7.4, and at a temperature of 37°C. The degradation of all compounds at the corresponding pH, as investigated by

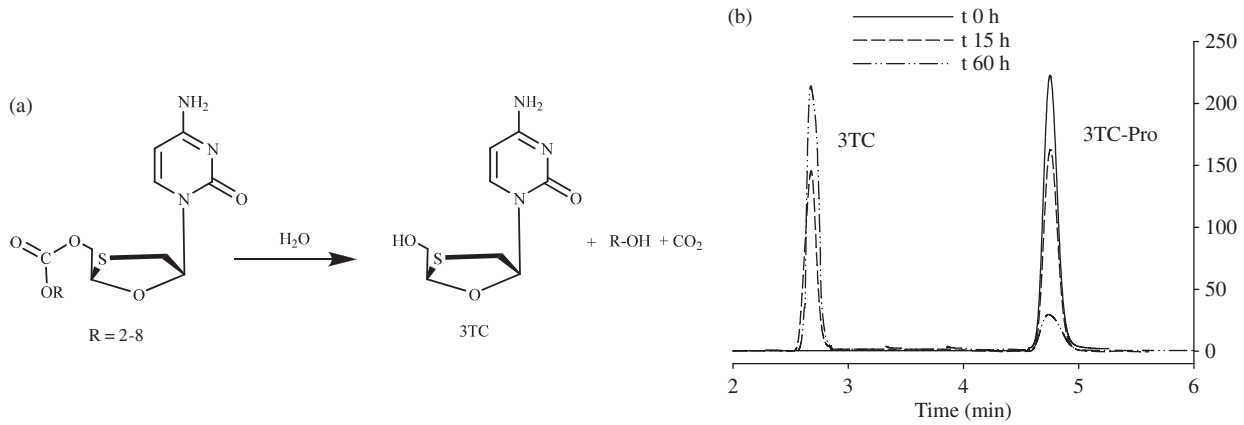

Figure 4. (a) General reaction of hydrolysis of carbonates of 3TC. (b) High Performance Liquid Chromatography (HPLC) of 3TC-Pro ($t_R = 4.79$ min) and its degradation product 3TC ($t_R = 2.68$ min).

Table 4. Rate constant and half-lives ($t_{1/2}$) of the chemical and enzymatic hydrolysis of **1**–**8** compounds at 37.0 ± 0.1 °C.

	Aqueous hydrolysis							
	pH 1.2		pH 5.8		pH 7.4		Enzymatic hydrolysis	
Compound	k (hs^{-1})	$t_{1/2}$ (hs)	k (hs^{-1})	$t_{1/2}$ (hs)	k (hs^{-1})	$t_{1/2}$ (hs)	k (min^{-1})	$t_{1/2}$ (min)
1, 3TC	Stable[a]	–	Stable[a]	–	Stable[a]	–	Stable[a]	–
2, 3TC-Metha	0.022	31.95	0.007	106.61	0.006	114.95	0.042	16.32
3, 3TC-Etha	0.018	39.27	0.006	110.00	0.005	141.46	0.037	18.59
4, 3TC-Pro	0.032	21.59	0.024	28.92	0.020	35.17	0.093	7.48
5, 3TC-Buta	0.025	27.37	0.015	46.63	0.014	49.33	0.066	10.57
6, 3TC-Penta	0.007	99.00	0.002	348.24	0.002	378.77	0.020	35.20
7, 3TC-Hexa	0.010	72.03	0.005	145.59	0.004	160.45	0.021	33.27
8, 3TC-Octa	0.043	16.05	0.031	22.01	0.030	22.85	0.127	5.44

[a]Stable: no degradation products observed by HPLC after 20 d (480 h) of experiment, $n = 3$.

monitoring the concentration of intact prodrug as a function of time, displayed strict pseudo-first-order kinetics for more than two half-lives. The rate constants for regeneration of lamivudine from **2**–**8** obtained from the linear regression of a pseudo-first-order plot using log concentration versus time are shown in Table 4, together with the $t_{1/2}$ values calculated from the hydrolysis rate constants in buffer solutions. However, it should be noted that at pH 13.0 all compounds were highly unstable and their rate constants could not be determined.

The chemical stability measurements revealed that the **2**–**8** prodrugs of lamivudine were relatively unstable at pH 1.2 and their observed t ranged from 16.05 h (compound **8)** to 99.00 h (compound **6**). At pH 7.4, a similar behavior was observed, but with t values higher than those of pHs 1.2 and 5.8. However, carbonates **4**, **5** and **8** still manifested a lower stability at pH 7.4 than the rest of compounds.

These results are consistent with literature reports indicating that carbonate esters are relatively stable at pHs 5.8 and 7.4 [24–26].

Human plasma studies

It is generally known that proteolytic enzymes hydrolyze esters rapidly and that carbonates and carbamates are types of esters which, when hydrolyzed, give CO_2 and the corresponding alcohols or amines[26–29].

A product analysis revealed that at 37 °C the plasma-catalyzed hydrolysis of these 5′-*O*-carbonates quantitatively afforded the corresponding parent lamivudine compound. In human plasma, as well as in aqueous media, hydrolysis of **2**–**8** also followed pseudo-first-order degradation kinetics. Table 4 shows the different rate constants under the above conditions, together with the $t_{1/2}$ values calculated from the hydrolysis rates in this matrix.

The susceptibility of compounds **2**–**8** to undergo enzymatic hydrolysis was shown by comparing their half-lives with that of buffer pH 7.4. It is clear that human plasma enzymes markedly accelerated the hydrolysis rate of these 5′-*O*-carbonates. For example, compound **6**, the most stable compound in human plasma, presented a $t_{1/2}$ value of 35 min and a $t_{1/2}$ of 378 h at pH 7.4.

Despite this fast enzymatic hydrolysis, all these compounds were clearly demonstrated to have remained in the human plasma sufficient time to be able to act as prodrugs before hydrolysis took place. These prodrugs with 4 to 6 carbon atoms in their alkyl chains fitted the active sites of these esterolytic enzymes better than carbonates with 2, 3 or 8 carbon atoms in their alkyl chain. These results suggest that prodrugs **2**–**8** are good substrates for the esterase and can efficiently generate the parent drug. Although the structural features influenced the chemical and enzymatic stability, no clear correlation between anti-HIV activity[5] and stability was observed in this series.

Discussion

From the results of this study, it is clear that the equilibrium solubility method is appropriate for determining the solubility of compounds **1**–**8**. Aqueous solubility decreased as the carbon length of the substituent increased, but as expected, this parameter increased with a rise in temperature. It was noted that the ratio of solubility for each compound was maintained constant at the two assayed temperatures. In addition, **1**–**8** presented an enhanced solubility as the pH was reduced, due to free amino group ionization (having a weak basic character of **1**–**8**, with pKa values between 4.1 and 5.7). In the range of pH 5.8–7.4, a slight variation in solubility was observed, with the maximum solubility being observed at pH 1.2 for all compounds.

In summary, the following conclusions can be stated: (a) All prodrugs had greater solubility in the presence of cosolvents. (b) Compounds **1**–**8** were more soluble in ethanol than in PEG 400, due to the polarity of the prodrugs and the dielectric constants of the employed solvents. (c) The solubility was higher with ethanol and PEG 400 cosolvents than with aqueous solution alone. (d) With ethanol and PEG 400 as cosolvents, a linear relationship between solubility versus percentage of each cosolvent was established.

Stability studies are important requirements of prodrug screening, which usually need to have acceptable aqueous stability, but are able to cleave to active drugs under certain enzymatic conditions.

Therefore, a simple and efficient reverse-phase HPLC method was developed for the determination and quantification of lamivudine and their prodrug derivatives in different buffers and human plasma, and found to be specific, linear, accurate and precise across the experimental analytical range. Most of the 5′-*O*-carbonate prodrugs exhibited a good chemical stability, revealing that their structures influence the rate of hydrolysis (Table 4). It is important to note that 3TC remained unchanged in chemicals and plasma media, with no degradation products being observed by HPLC after 20 d (480 h) of experiments. Thus, these studies have shown the effectiveness of carbonate linkage to create a prodrug from a well-known drug.

For these aliphatic carbonates, the rates of chemical and enzymatic hydrolysis were found to be independent of the length of the aliphatic chains under the conditions stated, as previously observed for other types of compounds[26]. It is clear, however, that more detailed studies of the kinetics of the enzyme–substrate interaction are still needed to reveal the true nature of the hydrolysis mechanism of these carbonate derivatives.

These new prodrugs could be successful pharmacological agents not only in the treatment of patients with AIDS and Hepatitis B but in also co-infected ones. In view of their solubilities shown using 10% ethanol as the cosolvent and their acceptable stabilities at physiological pHs and in human plasma as preformulation parameters, which are essential to achieve good oral bioavailability, further assays such as biopharmaceutics and pharmacokinetics (biodistribution, permeability, etc.) should now to be conducted to assess the full potential of these prodrugs.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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