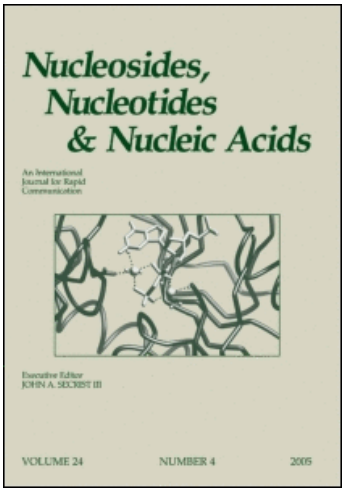

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3'-Azido-3'-deoxy-5'-O-isonicotinoylthymidine: A Novel Antiretroviral Analog of Zidovudine. II. Stability in Aqueous Media and Experimental and Theoretical Ionization Constants

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3′-Azido-3′-deoxy-5′-O-isonicotinoylthymidine: A Novel Antiretroviral Analog of Zidovudine. II. Stability in Aqueous Media and Experimental and Theoretical Ionization Constants

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ABSTRACT

Degradation of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso), an antiretroviral derivative of zidovudine, was investigated in buffer pH 7.4, $\mu = 300$ mOsm at 37, 50 and 60°C, and in water (pH 6.6, 37°C), giving zidovudine (AZT) and isonicotinic acid (INA) as products. The rate constants were determined by reversed-phase HPLC showing pseudo-first-order kinetics related to the residual amount of AZT-Iso. In this way, the studied compound was demonstrated to be 153 times more stable in water than in buffer solution at 37°C. The analytical method was conveniently validated demonstrating to be a rapid and accurate stability-indicating technique. In addition, experimental and theoretical values of pKa were determined.

Key Words: Zidovudine analogs; Antiretroviral; Stability; pKa.

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The first 2′,3′-dideoxynucleoside derivative approved for treatment of HIV-1 infections was 3′-azido-3′-deoxythymidine (AZT, zidovudine).[1,2] This drug needs to be converted to its 5′-*O*-triphosphate analog by cellular enzymes to exhibit inhibitory activity against the reverse transcriptase (RT) of HIV-1, inducing immunologic, virologic, and neurologic improvements in HIV-1 infected patients.[3] Some of the major chemotherapy problems associated with AZT include bone marrow toxicity resulting in anemia and leukopenia, low therapeutic index owing to inhibition of cellular polymerases, low localization in brain, and a short half-life in blood which requires frequent AZT administration to maintain a therapeutic drug concentration.[4,5] In spite of these undesirable effects, this nucleoside reverse transcriptase inhibitor (NRTI) continues to play an important role in the therapy of AIDS extending the life expectancy of individuals with AIDS, particularly in combination with other NRTI, non-nucleoside transcriptase reverse and protease inhibitors.[6–9] In attempts to overcome the problems of rapid elimination of AZT and to increase its therapeutic efficacy, numerous AZT prodrugs have been reported in the literature.[10,11]

In this way, we have been involved in a research program aimed at exploring more convenient novel anti HIV-1 analogs of AZT. Thus, 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso, Fig. 1), a new prodrug of AZT,[12] has presented interesting anti-HIV and cytotoxicity activity as well as lipophilicity and albumin binding properties,[12–15] and therefore is an excellent candidate for preclinical studies.

It is very important to point out that before formulating a new drug, it is essential to determine its chemical stability[16,17] as well as some fundamental physicochemical properties such as lipophilicity and pKa.[18] For this reason and taking into account that AZT-Iso is an ester, in this paper we report stability studies of this compound under the same conditions as those for which biological activity is observed. In addition, the pKa values of AZT-Iso were determined since this

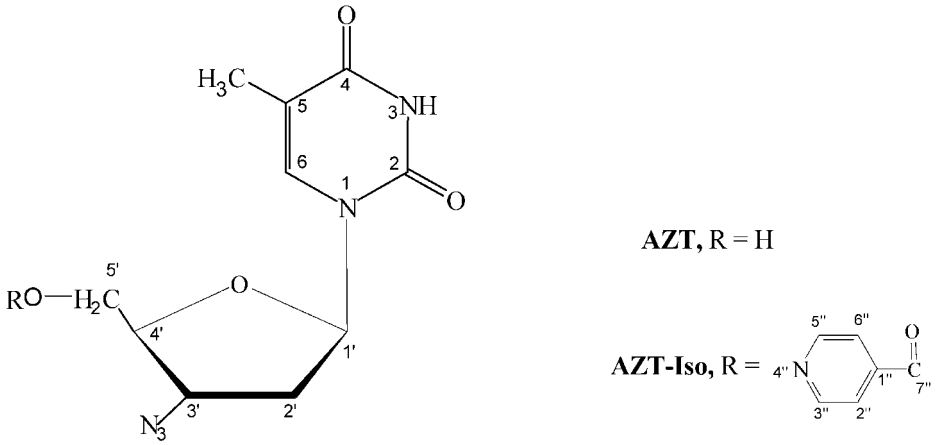

Figure 1. Structure and numeration of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso).

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parameter affects the bioavailability of a drug. Then, it could be possible to calculate the percentage of a soluble drug molecule in the aqueous and the lipophilic media of the organism.[19,20]

RESULTS AND DISCUSSION

Stability Studies

Identification of Degradation Products

The stress kinetics for the degradation of 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso) were followed at 70°C in water. Identification of AZT-Iso and their degradation products, zidovudine (AZT) and isonicotinic acid (INA), was performed by TLC and by comparing their HPLC retention times with authentic samples. A typical chromatogram is shown in Fig. 2. The peak designated as "3" is the parent compound (AZT-Iso, $t_r = 4.25$ min) and those designated "2" and "1"

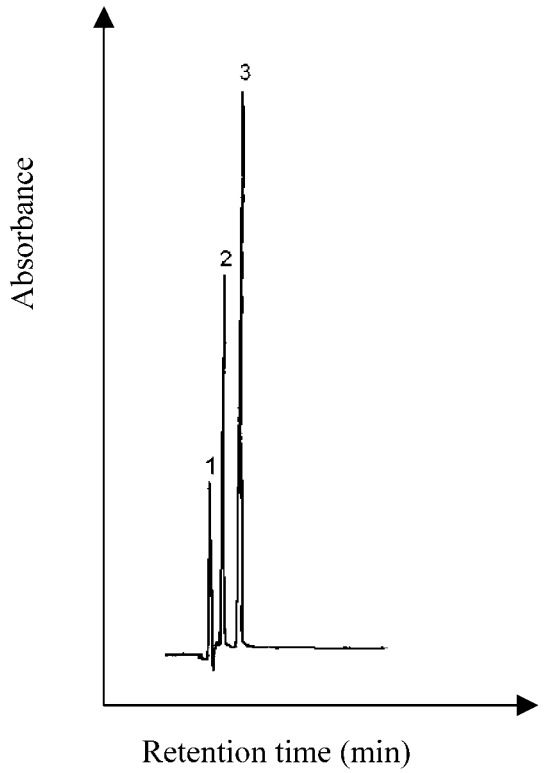

Figure 2. High-pressure liquid chromatograms of AZT-Iso degradation in water at 60°C. Key = (1) isonicotinic acid (INA, $t_R = 2.18$ min); (2) zidovudine (AZT, $t_R = 3.25$ min); (3) 3′-azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso, $t_R = 4.25$ min).

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are the hydrolysis degradation products AZT ($t_r = 3.25$ min) and INA ($t_r = 2.18$ min), respectively. Figure 2 shows an adequate separation among all compounds.

Calibration Graphs and Statistical Analysis

HPLC methods have been used extensively for the quantification of AZT.[21–26] Calibration graphs and statistical analysis of the results are typically performed to validate the analytical method.[27]

Calibration graphs were performed for each compound (AZT-Iso, AZT and INA) showing in lock HPLC chromatogram, the absence of the other compounds. Each AZT-Iso, AZT and INA HPLC peak areas were plotted vs. concentration to verify the linearity of the calibration graph. The analyses were performed in triplicate, showing that the relative standard deviation (RSD) for each experimental point was less than 2%, demonstrating that there is negligible scatter of the experimental points from the regression lines.

Tests of the significance of the regression line intercepts were performed to verify whether the intercepts "*a*" differed significantly from zero. Thus, the quantities $t = a/Sa$ were determined (where *Sa* is the standard deviation of the intercept), and then compared to the corresponding tabular data for *t* distribution.[28–31] The calculated values for AZT-Iso ($t = 0.08$), AZT ($t = 1.22$) and INA ($t = 1.11$) were smaller than those tabulated at a 95% significance level with Student's *t* (AZT-Iso, $t = 2.31$; AZT, $t = 2.36$ and INA, $t = 2.57$). This means that the intercepts of the regression lines were not significantly different from zero. In addition, the confidence intervals for the true intercepts and slopes values were calculated from the expressions $a \pm t\ Sa$ and $b \pm t\ Sb$, respectively,[27,32] where *Sa* and Student's *t* are the same as which the previous case and *Sb* is the standard deviation for the slopes. These statistics lead us to infer that the analytical method, determining the concentrations of AZT, AZT-Iso and INA, is free of errors coming from other compounds.

The accuracy, precision and sensitivity values of the proposed method[32–35] were summarized in Table 1.

Based on the validation analyses, HPLC has been selected as a precise and accurate analytical method to follow degradation kinetics of AZT-Iso.

Reaction Order and Rate Constants

Stability studies of AZT-Iso were carried out in a first step in buffer pH 7.4 plasma isotonic, $\mu = 300$ mOsm at 37°C, 50°C and 60°C. The linear relationship ($r > 0.99$) between the natural logarithm (Ln) concentration of the intact molecule drug vs time for all studied temperatures indicates pseudo-first order degradation kinetics for AZT-Iso at constant pH, temperature and ionic strength (Fig. 3). The same hydrolysis kinetics was observed when the study was performed at 37°C in water (pH 6.6).

The degradation rate constants of AZT-Iso, computed by the least-squares linear regression method together with those obtained by extrapolation from the Arrhenius equation,[16] as well as $t_{1/2}$ and t_{90} values, are listed in Table 2.

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Table 1. Statistical data of the analytical method.

Statistical parameters	n	AZT	AZT-Iso	INA
Accuracy (%)	3	100.5 ± 0.4	88.57 ± 8.58	97.04 ± 2.12
Precision				
Repeatability[a] (RSD) Sol. A	5	18.11	0.76	29.32
Sol. B	5	1.36	17.54	8.18
10 μg/mL	2	0.25	0.45	–
0.1 μg/mL	2	0.78	4.66	–
50.2 μg/mL	2	–	–	0.40
1 μg/mL	2	–	–	1.91
Reproducibility[b] (RSD) (5 days)	1	9.44	1.81	36.15
Sensitivity (10^{-4}); μg/mL		7.12 (n = 9)	5.42 (n = 10)	4.01 (n = 7)
LOD; μg/mL		0.1 (n = 9)	0.2 (n = 10)	0.6 (n = 7)
LOQ; μg/mL		0.4 (n = 9)	0.7 (n = 10)	2.0 (n = 7)

AZT-Iso = 3′-Azido-3′-deoxy-5′-O-isonicotinoylthymidine; AZT = 3′-Azido-3′-deoxythymidine; INA = Isonicotinic acid; n = number of dates; Sol. A = Solution A is composed by a mixture of high concentration of AZT-Iso, and low concentration of AZT and INA; Sol. B = Solution B is composed by a mixture of low concentration of AZT-Iso, and high concentration of AZT and INA; RSD = Relative Standard Deviation; LOD = Detection limit; LOQ = Quantification limit;
[a]Precision intraday.
[b]Precision interday.

This table shows that AZT-Iso has a $t_{90} = 1.14$ h and $t_{1/2} = 7.55$ h at 37°C, under conditions that model plasma, with an activation energy (Ea) calculated from Arrhenius plots[16] of 21.76 kcal/mol. As it can be seen, this drug molecule is 153 times more stable in water ($t_{90} = 7.23$ days and $t_{1/2} = 48.04$ days) than in buffer, and therefore AZT-Iso solutions of pH 7.4 should be stored at 0°C ($t_{90} \cong 6$ days and $t_{1/2} \cong 37$ days) to minimize its water hydrolysis process.

Determinations of pKa Values

Experimental Values

The importance of using drug pKa during preformulation is well known. Potentiometric analysis is a convenient method for dissociation constant determination, with accuracy and reproducible results. In this way, the experimental pyrimidinic base pKa values for AZT-Iso (pKa = 8.89) and for the standards thymidine and AZT compounds were pKa = 9.43 and pKa = 9.85, respectively. The measured pKa value for thymidine was similar to that of literature (pKa = 9.79),[36] showing the usefulness of this methodology for this family of compounds.

Conversely, it was found that the potentiometric method does not have sufficient sensitivity to detect the pKa values of either the pyridine ring nitrogen of AZT-Iso or OH aliphatic sugar moiety of thymidine and AZT compounds. This fact is due to the very weak base characteristic of those groups.

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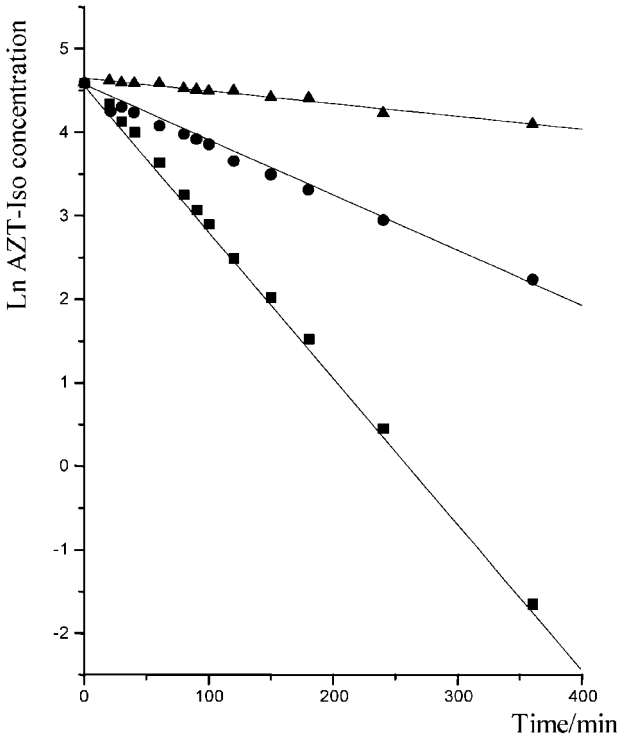

Figure 3. Plots of the observed pseudo-first order kinetic degradation of AZT-Iso in buffer solution pH 7.4 at different temperatures. Key (▲) 37°C; (●) 50°C; (■) 60°C.

It is important to point out that AZT-Iso at physiological pH (pH 7.4) is 96.87% non-ionized, and taking into account that AZT-Iso is 16 times more lipophilic than AZT,[13] this drug could display better absorption properties than AZT.

Table 2. Hydrolysis rate constants, $t_{1/2}$ values and t_{90} values of AZT-Iso in buffer (pH 7.4, $\mu = 300$ mOsm).

Temp. (°C)	$k_{obs} \times 10^4$ (min^{-1})	r	t_{90} (h)	$t_{1/2}$ (h)
0*	0.129 (±0.004)	–	134.4	892.5
25*	3.746 (±0.026)	–	4.643	30.84
37	15.30 (±0.09)	−0.99236	1.137	7.550
50	64.40 (±0.13)	−0.99722	0.2701	1.794
60	178.3 (±0.1)	−0.99914	0.09757	0.648
37[a]	0.1002 (±0.0055)	−0.9876	173.6	1153

k_{obs} = rate constants of the pseudo first-order kinetics of hydrolysis reaction; r = linear correlation coefficient; Ea = 21.76 kcal/mol.
*Extrapolated value.
[a]Stability in water.

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Prediction Values

Since the pKa of N-pyridine and OH groups of AZT-Iso and pyrimidinic nucleosides, respectively are too weak, to be experimentally determined, linear free energy relationships (LFER) using the Hammett equation, were employed to predict the pKa's (Eq. (1)),

$$\mathrm{pKa} = \mathrm{pKa}^{\circ} - \rho \sum \sigma \tag{1}$$

where pKa° is the pKa of the unsubstituted acid or base, σ is a constant for the particular substituent, and ρ is the constant for a particular equilibrium reaction.[37]

pKa for Imide Group. Although Hammett-type equations are available for many families of compounds, no relations were found for pyrimidinic bases. For this reason, as a first step to calculate AZT-Iso pKa values, 2-pyridone (Fig. 4a) was used (Eq. (2)) as a model for pyrimidinic nucleosides.

$$\mathrm{pKa} = 11.65 - 4.28 \sum \sigma \tag{2}$$

From Eq. (2), the pKa of 2-pyrimidone (Fig. 4b) could be calculated considering the structural similarity of this compound with pyrimidinic nucleosides. In this way, Eq. (3) was constructed, using $\sigma = 0.71$ for heterocyclic nitrogen according to Hammett approximations, leading to a pKa = 8.6 for 2-pyrimidone [pKa (exp) = 8.59].[37]

$$\mathrm{pKa}_{\text{2-pyrimidone}} = 11.65 - 4.28\,\sigma_{\mathrm{N}} = 8.6 \text{ (calc.)} \tag{3}$$

Assuming this pKa for 2-pyrimidone, the σ value for the second carbonyl group of pyrimidine base, $\sigma_{\text{2-CO,}}$ was calculated applying to Eq. (2) uracyl compounds (pKa = 9.46),[36] using a value of $\sigma_{\text{1-N}} = 0.71$ for the nitrogen at position 1, which leads to Eq. (4). Then, a $\sigma_{\text{2-CO}} = -0.20$ was obtained.

$$9.46 = 11.65 - 4.28\ (\sigma_{\text{1-N}} + \sigma_{\text{2-CO}}) \tag{4}$$

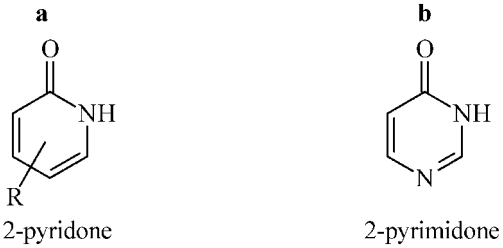

Figure 4. a) Chemical structure of 2-pyridone. b) Chemical structure of 2-pyrimidone.

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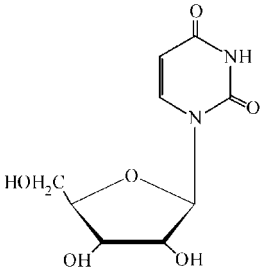
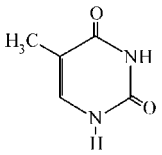
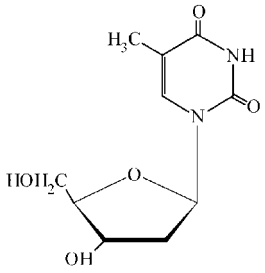
Table 3. Theoretical ionization constant calculations of the imide group of pyrimidinic nucleosides.


Uridine


Thymine


Thymidine

Comp.	Equation $pKa = pKa^{\circ} - \rho^{a}\sum\sigma^{b}$	pKa Calc.	pKa Exp.	ΔpKa
Thymine	$pKa = pKa_{2\text{-Pyridone}} - \rho(\sigma_{1\text{-N}} + \sigma_{2\text{-CO}} + \sigma_{5\text{-Me}})$	9.77	9.90	0.13
Thymidine	$pKa = pKa_{2\text{-Pyridone}} - \rho(\sigma_{1\text{-N}} + \sigma_{2\text{-CO}} + \sigma_{5\text{-Me}} + \sigma_{Sug}{}^{c})$	10.05	9.79	0.26
Uridine	$pKa = pKa_{2\text{-Pyridone}} - \rho(\sigma_{1\text{-N}} + \sigma_{2\text{-CO}} + \sigma_{Sug}{}^{c})$	9.77	9.30	0.47

[a] $\rho = 4.28$.
[b] $\sigma_{1\text{-N}} = 0.71$; $\sigma_{2\text{-CO}} = -0.20$; $\sigma_{5\text{-Me}} = -0.07$; $\sigma_{Sug} \cong -0.07$; $pKa_{2\text{-Pyridone}} = 11.65$.
[c] The $\sigma_{Sug} \cong -0.07$ is an approximated value, that adjust well to pKa prediction of thymidine and uridine.

Table 3 shows the calculated and experimental pKa values for thymine, thymidine and uridine, demonstrating that $\sigma_{2\text{-CO}} = -0.20$ is a reliable value to predict the pKa of pyrimidinic nucleosides, since no literature data were found for this moiety.

pKa for Aromatic Nitrogen. AZT-Iso has an additional basic pKa corresponding to the isonicotinoyl aromatic nitrogen, which was calculated applying the Hammett equation for pyridine compounds (Eq. 5) and employing $\sigma_{CO2Et} = 0.72$ as a representative *p*-ester group. As can be seen, the calculated pKa of 0.932 corresponds to an extremely weak base moiety.

$$pKa_{N\text{-}Ar} = pKa^{\circ}_{Pyr^{-}} - \rho\sum\sigma$$
$$pKa = 5.18 - 5.90\sigma_{p\text{-}COOEt} = 5.18 - 5.90 \times 0.72 = 0.932 \quad (5)$$

pKa for Hydroxyl Group. Taking into consideration the aliphatic character of deoxyribose ring, the Taft equation was applied to calculate the ionization constant of 5′-OH group of AZT and thymidine molecules. Then, the equation corresponding to primary alcohols (RCH_2OH) (Eq. (6)) was used.[37]

$$pKa = 15.9 - 1.42\sum\sigma^{*} \quad (6)$$

The validity of Eq. (6) was corroborated calculating the pKa of 5′-OH group of the thymidine (Eq. (7)) and AZT (Eq. (8)), taking into account that the effect of a group as expressed in the σ^{*} constant is halved for each saturated carbon atom that

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separates the OH center from the substituent.[37] Thus, for thymidine, the σ^* values of Eq. (7) are, $\sigma^*_{oBu} = 1.68$, $\sigma^*_{OH} = 1.34/4$ and $\sigma^*_{2\text{-pyrimidinyl}} = 1.67/16$.

$$\begin{aligned} pKa_{Thym} &= 15.9 - 1.42(\sigma^*_{oBu} + \sigma^*_{OH} + \sigma^*_{2\text{-Pyrimidinyl}}) \\ pKa_{Thym} &= 15.9 - 1.42(1.68 + 0.335 + 0.104) = 12.89(calc) \\ pKa_{Thym} &= 12.85(exp) \\ \Delta pKa &= 0.04 \end{aligned} \tag{7}$$

As it can be seen from Eq. (7), a ΔpKa of 0.04 was observed between experimental (pKa = 12.85) and calculated (pKa = 12.89) values. Applying this equation, the 5′-OH pKa of AZT was calculated using Eq. (8), with a $\sigma^*_{N3} = 2.62/4$, obtaining a pKa value of 12.44.

$$\begin{aligned} pKa_{AZT} &= 15.9 - 1.42(\sigma^*_{oBu} + \sigma^*_{N3} + \sigma^*_{2\text{-Pyrimidinyl}}) \\ pKa_{AZT} &= 15.9 - 1.42\,(1.68 + 0.655 + 0.104) = 12.44(calc) \end{aligned} \tag{8}$$

Table 4 presents a summary with experimental and calculated pKa values for AZT-Iso and the studied pyrimidinic nucleosides, showing a good relationship between both methods.

The pKa for the NH imide group of AZT-Iso was calculated using the equation for thymidine of Table 3 and the sigma Hammett constant $\sigma_{2\text{-CO}} = -0.20$. This value was pKa = 10.05 (exp. pKa = 8.89), showing a ΔpKa = 1.16 since the ionization constant was determined in a DMF/water medium, due to its sparing water solubility. For this reason, the calculation of pKa value of AZT-Iso was very important.

Moreover, the similar pKa values for AZT, thymidine and AZT-Iso, corresponding to the imide group (NH) of the pyrimidinic base, can be attributed to the fact that structural differences are sufficiently separated from each other to have only minor effects.

Table 4. pKa values of pyrimidinic nucleosides.

	Calculated pKa			Experimental pKa		
Comp.	NH-base	OH-sugar	N-Ar	Found	Lit.	ΔpKa
Thymine	9.77	–	–	–	9.90[a]	–
Uridine	9.75	–	–	–	9.30[a]	–
Thymidine	10.05	12.85	–	9.43[a]	9.79[a], 12.89[b]	0.62[c], −0.04[d]
AZT	10.05	12.64	–	9.85[a]	–	0.20[c]
AZT-Iso	10.05	–	0.932	8.89[a]	–	1.16[c]

[a]NH-base.
[b]OH-sugar.
[c]$\Delta pKa\ (NH\text{-}base) = pKa_{calc.} - pKa_{exp.}$
[d]$\Delta pKa\ (OH\text{-}sugar) = pKa_{calc.} - pKa_{lit.}$

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CONCLUSIONS

HPLC has demonstrated to be simple, precise, specific and a good stability-indicating technique due to the ability of the method to simultaneously and accurately measure the analyte (AZT-Iso) response in the presence of its degradation products (AZT and INA), which follows pseudo first-order degradation kinetics. Though in plasma conditions, AZT-Iso hydrolyzes at 37°C faster than in water, its chemical stability could give enough time to AZT-Iso to reach lipophilic tissues, before this prodrug converts into AZT by intracellular enzymes. It is important to point out that the covalent bond between AZT and the isonicotinoyl moiety is labile enough to easily regenerate the anti HIV parent compound.

In addition, because of the very weak acid character exhibited by the pyrimidinic base of AZT-Iso, it will be a non-ionized form at physiological and gastric pHs (96.87% and 100% respectively). On the other hand, as we can see from the calculated pKa value of the aromatic moiety (pKa 0.932, Eq. (5)) it has a base behavior with 100% and 78.72% of non-ionized forms at physiological and gastric pHs, respectively.

Previously, we pointed out that AZT-Iso (Log P = 0.82) was 16.4 times more lipophilic than AZT (Log P = 0.05), which achieves a higher intracellular concentration by crossing biological membranes by passive diffusion.[12]

EXPERIMENTAL SECTION

Materials

3′-Azido-3′-deoxythymidine (AZT), a generous gift from Filaxis (Buenos Aires, Argentina), was used without purification. Isonicotinoyl chloride hydrochloride and isonicotinic acid (INA) were purchased from ALDRICH and SIGMA Companies, respectively. 3′-Azido-3′-deoxy-5′-O-isonicotinoylthymidine (AZT-Iso) was prepared as previously reported.[12] All chemicals and reagents were of analytical grade and all solvents were purified by distillation prior to use. Standard buffer solutions were purchased from Carlo Erba (Argentina). Water used for buffers and for mobile phase in high-performance liquid chromatography (HPLC) was double distilled and deionized through a Milli-Q water purification system (Millipore). All the solvents used for HPLC were filtered through a 0.45 μm filter prior to use. Silica gel type H, size 10–40 μm, without binder (Sigma), was used for flash chromatography and precoated 60 F 254 silica gel plates (Merck) for thin layer chromatography (TLC).

Buffer Solution

A phosphate buffer solution (81 mL of Na_2HPO_4 0.2 M and 19 mL of NaH_2PO_4 0.2 M), pH 7.40 plasma isotonic (μ = 300 mOsm) was used in these investigations. The solution was freshly prepared and the pH value was measured at the experimental temperature using a research pH meter with a SC-glass electrode.

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Apparatus

Ultraviolet spectrophotometric (UV) studies were carried out with a Shimadzu Model UV-160A spectrophotometer, using 1 cm quartz cuvettes. An Orion Model 5A 520 pH meter was used to measure the pH of the buffer solutions with a glass-reference electrode. For kinetic measurements the constant-temperature bath was regulated by a Haake D8 thermostat with a precision of $\pm 0.1^{\circ}C$.

High-Performance Liquid Chromatography Analysis (HPLC)

The HPLC measurements were assayed on a Konik 500 G chromatograph, using an UV detector at λ 265 nm and a Waters column, Spherisorb 250×4 mm, 5 μm particle size, packed with a C18 (octadecyl silane) chemically bonded non-polar stationary phase. The mobile phase was methanol:water (70:30) and the flow rate was 0.85 mL/min. The samples were injected into the column with a Rheodyne (Model 7125) injector of a 20 μL loop. The experiments were performed in triplicate at room temperature.

Analytical Method Validation

The stock solutions were prepared each day from 10, 10 and 50 μg/mL of AZT, AZT-Iso and INA respectively, using water as solvent. Corresponding aliquots were diluted with water in order to obtain final concentrations in the range to 0.1–10 μg/mL for AZT and AZT-Iso, and 0.5–50 μg/mL for INA. The solutions were analyzed by HPLC and the area of the peak vs. the concentration was plotted, finding that the response to the concentration was linear over the range of studied concentrations, in agreement with Beer′s law. The column was flushed at the end of each day with water followed by methanol.

The accuracy of the proposed method was determined by analyzing three known concentrations of synthetic mixtures of the assayed compounds, and it is expressed as the recovery percentage ± standard deviation (σ_{n-1}) between the obtained and expected values. The concentrations of the standard mixtures were chosen to give similar conditions to the stability studies. The precision was evaluated through their RSD parameters. For intraday precision (or repeatability), the study was performed in quintuplets from two different "real" solutions.[33] In this way, two solutions of AZT-Iso (10 μg/mL) were stored under stress conditions at 68°C, 10 min (sol. A) and 5 h (sol. B). Thus, the sol. A has high AZT-Iso concentration and low AZT and INA concentrations, and the sol. B has low concentration of AZT-Iso and high concentrations of AZT and INA. To determine the interday precision (or reproducibility), the same sample solution was analyzed during five consecutive days, and injected three consecutive times each day. The sensitivity of the method was analyzed from the calibration graph intercepts of AZT-Iso, AZT and INA. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the confidence intervals of the intercepts plus three and ten values of $S_{x/y}$,

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respectively.[32,34,35] Also, the precision of the method was analyzed by the measurement of high and low concentration solutions of each compound, and each sample was injected five consecutive times.

Kinetic Studies

Identification of Degradation Products

40 μg (1.07×10^{-7} mol) of AZT-Iso were dissolved in 10 mL of water. The solution was stored at 70°C, during 10 h. Then, the flask was removed from the bath, immediately cooled in an ice bath, and assayed at room temperature in duplicate by HPLC (methanol:water, 70:30 as the mobile phase at a flow-rate of 0.85 mL/min) and by TLC (ethyl acetate-petroleum ether (80:20)) techniques. The reaction mixture was assayed against standard solutions of AZT, AZT-Iso and INA.

Stability Assays

A stock solution of AZT-Iso (50 μg/mL) was prepared in water. Aliquots were taken from the stock solution and diluted with buffer phosphate solutions of pH 7.4 (Na_2HPO_4–NaH_2PO_4, $\mu = 300$ mOsm) and water (pH 6.6) up to final concentration of 5.37×10^{-6} M. All sample solutions were filled into 7 mL flasks and stored at the appropriate temperature (37°C, 50°C and 60°C for buffer solutions and 37°C for water solutions) in a constant temperature bath, while the "zero time" sample was maintained at −20°C. The flasks were withdrawn at suitable time intervals, immediately cooled in an ice-bath and stored in a freezer until analyzed. All kinetic studies were done in duplicate. Upon removal of the last sample, the stored solutions were equilibrated to room temperature and analyzed by HPLC (methanol:water, 70:30 as the mobile phase at a flow-rate of 0.85 mL/min).

Potentiometric Determination of pKa

The pKa value of AZT-Iso was determined by a potentiometric method[38] using a glass electrode and an automatic dossier (0.1 mL each 20 s). A solution of 15 mL of sulfuric acid (0.0672 N) containing 0.3 mM (112 mg) of AZT-Iso in 5 mL of water:DMF (10:7.65) was titrated with sodium hydroxide solution (0.0590 N). As a first step, the thymidine pKa was determined as standard compound (Lit. pKa = 9.9)[36] in order to validate this methodology to pyrimidinic nucleoside systems, which involve a weak acid.

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