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Kinetics of the hydrolysis of 2′,3′-dideoxyguanosine: a potent anti-HIV agent

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Summary

2′,3′-Dideoxyguanosine (ddGuo) is a potent inhibitor of the human immunodeficiency virus (HIV) in vitro. Because of the potential interest of this compound for the treatment of AIDS, its chemical stability was studied. The degradation of ddGuo in buffers of different pH and ionic strength was followed using LC. A correlation was found between the logarithm of the rate constant of degradation of ddGuo and the ionic strength of the medium, at constant pH. A pH-rate profile was constructed in the acid region using buffers of the same ionic strength. Activation parameters were determined at pH 7.0.

Introduction

2′,3′-Dideoxyguanosine (ddGuo) (Fig. 1) belongs to the family of 2′,3′-dideoxynucleoside analogues, which contains several potent inhibitors of the human immunodeficiency virus HIV. The following compounds are among those being tested clinically for the treatment of AIDS: 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC) (De Clercq, 1990). ddGuo itself has in vitro activity against HIV comparable to that of ddI (Mitsuya and Broder, 1986), but has not yet been tested in vivo. Because of the potential clinical interest of ddGuo, knowledge of its chemical stability is important for evaluation of its oral resorption, as well as the lifetime of pharmaceutical formulations.

Previous work on the hydrolysis of nucleosides, related to ddGuo, includes spectrophotometric studies on guanosine (Guo) and 2′-deoxyguanosine (dGuo) in dilute acid (Zoltewicz et al., 1970; Hevesi et al., 1972) and of Guo in perchloric acid

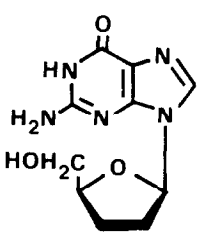

Fig. 1. Structure of 2′,3′-dideoxyguanosine (ddGuo).

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(Zoltewicz and Clark, 1972), polarimetric studies on 7-(β-D-ribofuranosyl)guanine vs the natural 9-isomer (Panzica et al., 1972) and a liquid chromatographic study of the degradation of dGuo (Oivanen et al., 1990). The mechanism of hydrolysis of purine nucleosides in acid media, including those derived from guanine, was established by several groups (Zoltewicz et al., 1970; Garrett and Mehta, 1972a; Hevesi et al., 1972; Panzica et al., 1972; Zoltewicz and Clark, 1972; Romero et al., 1978) and consists of the rate-limiting heterolysis of the protonated nucleoside to a glycosyl oxocarbenium ion and the free purine base. Reaction of the former with water then yields the sugar.

Materials and Methods

2′,3′-Dideoxyguanosine was synthesized according to a previously published procedure (Prisbe and Martin, 1985). Guanine was purchased from Janssen Chimica (Beerse, Belgium). Reagents were of pro analysi quality (Merck, Darmstadt, Germany) and double-distilled water was used throughout. Buffers for the calibration of pH measurements were prepared following the instructions of the European Pharmacopoeia (1980). pH was measured at room temperature with a Consort P 514 pH meter (Turnhout, Belgium) using a Schott pH electrode (Mainz, Germany). Liquid chromatography (LC) was performed with an SP 8700 solvent delivery system (Spectra-Physics, San Jose, CA, U.S.A.), a Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with a 20 μl loop, an L-4000 UV detector (Merck-Hitachi, Darmstadt, Germany) set at 254 nm and an integrator model 3396 A (Hewlett-Packard, Avondale, PA, U.S.A.). A Waters model 990 photodiode-array detector (Milford, MA, U.S.A.) was used to record the on-line UV spectra. Analyses were performed on a Spherisorb ODS 1 10 μm column (250 × 4.6 mm i.d.) thermostated at 25°C, with methanol-0.2 M potassium phosphate buffer pH 5.0-water (10 : 5 : 85 v/v) as the mobile phase. The flow rate was 1.0 ml min^{-1}.

For the kinetic studies, a solution of ddGuo (approx. 1×10^{-4} M) in the buffer of appropriate pH and ionic strength, was stored in a water bath at 26°C (GFL, Hannover, Germany) or in a Memmert oven (Schwabach, Germany) at the other temperatures used. The ionic strength of the buffers was adjusted with KCl. Aliquots were taken at appropriate intervals, neutralized with an equal volume of aqueous KOH or HCl of suitable concentration and frozen at −19°C. Rate constants were calculated from first-order plots of disappearance of ddGuo.

Results and Discussion

Acid pH-rate profile

The hydrolysis of ddGuo was investigated in acid and alkaline media, using buffers of different

TABLE 1

Observed rate constants (h^{-1}) for the hydrolysis of ddGuo at 26°C in buffers of different pH, type and ionic strength

pH	Buffer type [a]	Ionic strength	k_{obs} (h^{-1}) [b]
1.23	glycine · HCl	0.076	10.05 ± 0.23 ($n = 19$, $x = 5$)
1.23		0.3	12.97 ± 0.11 ($n = 14$, $x = 5$)
1.24		0.4	14.44 ± 0.16 ($n = 15$, $x = 5$)
1.91	phosphate	0.4	3.24 ± 0.02 ($n = 22$, $x = 9$)
2.79	citrate	0.4	0.51 ± 0.004 ($n = 15$, $x = 5$)
2.81	phosphate	0.4	0.47 ± 0.005 ($n = 17$, $x = 6$)
			0.47 ± 0.004 ($n = 16$, $x = 5$)
2.86	glycine · HCl	0.4	0.41 ± 0.005 ($n = 15$, $x = 5$)
4.82	phosphate	0.4	$5.67 \times 10^{-3} \pm 1.2 \times 10^{-4}$ ($n = 26$, $x = 8$)

[a] All buffers were 0.1 M in concentration.

[b] n, total number of chromatographic observations; x, number of points on the time axis.

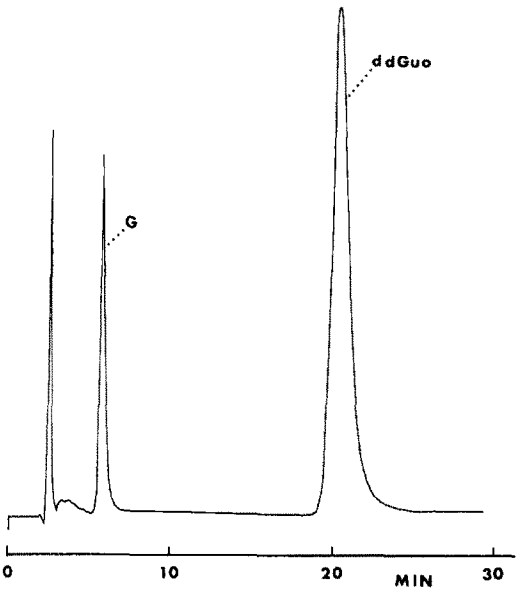

Fig. 2. Chromatogram of a sample of ddGuo, degraded at pH 3.0 (phosphate buffer) and 26°C for 30 min. Column: Spherisorb ODS 1 10 μm (250×4.6 mm). Mobile phase: methanol-0.2 M potassium phosphate buffer pH 5.0-water (10:5:85 v/v). Detection, 254 nm. G, guanine.

ionic strength, and at various temperatures. Table 1 lists the observed rate constants (h^{-1}) for the hydrolysis at 26°C in acid media. One series of experiments was performed per condition, except in the case of pH 2.81 (phosphate buffer). The degradation was followed chromatographically (see Fig. 2 for a typical chromatogram). A calibration experiment demonstrated a linear relationship between the concentration of ddGuo and the detector response in the range $(0.035–4.5) \times 10^{-4}$ M (amount injected ranged from 0.2 to 23 μg).

The degradation of ddGuo in acid media exhibited first-order behaviour, and consisted solely of the hydrolysis of the *N*-glycosidic bond of ddGuo. Indeed, mass balance calculations on the guanine and ddGuo contents in partially degraded samples indicated that guanine is the only UV-absorbing reaction product which is formed upon hydrolysis of ddGuo. The identity and homogeneity of the peak corresponding to the degradation product were further investigated and confirmed using a diode array detector.

As can be seen in Table 1, a correlation exists between the rate constant k of degradation of ddGuo at pH 1.2 and the ionic strength μ of the medium. Regression calculations on these data gave the following relationship between log k and μ:

$$\log k = 0.965 + 0.488\mu \quad r = 0.9999$$

Because of the dependence of the hydrolysis rate of ddGuo on the ionic strength of the medium, μ was maintained at 0.4 throughout all further experiments by the addition of KCl. An increase in hydrolysis rate with increasing ionic strength was also observed by York (1981) for adenine nucleosides with varying sugar entities. On the other hand, other investigators (Anderson et al., 1988) did not find a correlation between the rate constant of degradation of 2′,3′-dideoxyadenosine (ddA) and the ionic strength of the medium, when comparing $\mu = 0.01$ with $\mu = 0.15$. For guanosine, a change in μ from 0.10 to 1.00 at pH 2.30 resulted in a 32% increase in the rate constant (Zoltewicz et al., 1970). The increase observed here for ddGuo at pH 1.2 going from $\mu = 0.076$ to 0.4 was about 44%. The dependence of the rate constant k on the ionic strength of the medium can be explained by a primary salt effect on the activity coefficients of the reactants involved (Frost and Pearson, 1961). Table 1 further shows the rate constants k, obtained with various buffers of constant ionic strength. The small differences between the rate constants obtained for different buffers at pH about 2.8 can be attributed to the small differences in pH. In previous studies on very similar nucleosides such as Guo and dGuo (Zoltewicz et al., 1970), as well as other purine nucleosides such as ddA (Anderson et al., 1988) and adenine derived nucleosides (Garrett and Mehta, 1972a) no general acid-base catalysis by the buffers used was reported. All the results were therefore pooled to establish the acid pH-rate profile of hydrolysis of ddGuo. Regression analysis on these data yielded the following relationship between log k (h^{-1}) and pH:

$$\log k = 2.34 - 0.95\text{pH} \quad r = 0.9999$$

The slope being close to −1 confirms first-order kinetics with respect to the hydrogen ion for the degradation of ddGuo. Comparison of our results for ddGuo with those obtained for ddA leads to the conclusion that ddGuo is less stable than ddA in acid media. Indeed, Anderson et al. (1988) obtained rate constants (h^{-1}) for the degradation of ddA in dilute HCl media, which yield the following pH-rate profile:

$$\log k = 2.32 - 1.08\text{pH} \quad r = 0.9989$$

We also investigated the stability of ddA in the same medium (dilute HCl) using our chromatographic method, and obtained a comparable pH-rate profile, namely:

$$\log k = 2.45 - 1.12\text{pH} \quad r = 0.9999$$

A comparison can be made between the stability of ddGuo in acid media, and that of Guo and dGuo, so as to evaluate the influence of hydroxylation of the sugar on the stability of the *N*-glycosidic bond. When comparing values from different sources, however, it should be borne in mind that absolute statements are difficult to make, simply because of the influences of different ionic strengths. It is nevertheless possible to observe a certain tendency in the series Guo, dGuo and ddGuo. Indeed, the hydrolysis of ddGuo is approx. 44 times faster than that of dGuo at pH 1 and 30°C (data for dGuo given by Zoltewicz et al., 1970) while dGuo is 143 times more reactive than Guo at 30°C (Zoltewicz et al., 1970). It can be concluded that the influence of 2′-deoxygenation on the hydrolysis rate at 30°C is greater than that of a subsequent 3′-deoxygenation. This was also noted for the analogous adenosine series (York, 1981). This trend is consistent with the previously established mechanism of degradation via a glycosyl oxocarbenium ion, which is destabilized through the inductive effect of hydroxyl groups (Zoltewicz et al., 1970; Garrett and Mehta, 1972a; Hevesi et al., 1972; Anderson et al., 1988).

In comparison with some other 2′,3′-dideoxynucleosides, active against HIV, the following order of stability exists: 3′-azido-3′-deoxythymidine (AZT) > ddC > ddA > ddGuo > ddI (data for ddC and AZT taken from Oivanen et al., 1990, and for ddI from Anderson et al., 1988).

TABLE 2

Observed rate constants (h^{-1}) for the hydrolysis of ddGuo at pH 7.0, as a function of temperature and using 0.1 M potassium phosphate buffer with $\mu = 0.4$

Temperature (°C)	k_{obs} (h^{-1}) [a]
60	$1.44\times10^{-3} \pm 1.2\times10^{-4}$ ($n = 24$, $x = 9$)
81	$2.75\times10^{-2} \pm 3.8\times10^{-4}$ ($n = 16$, $x = 7$)
95	$1.23\times10^{-1} \pm 1.7\times10^{-3}$ ($n = 24$, $x = 8$)
101	$3.01\times10^{-1} \pm 3.2\times10^{-3}$ ($n = 19$, $x = 7$)

[a] n, total number of chromatographic observations; x, number of points on the time axis.

Hydrolysis in neutral and alkaline media

The degradation at neutral and alkaline pH was too slow to be monitored at room temperature, and experiments at pH 7.0, 9.0 and 12.0 were therefore carried out at higher temperatures. Table 2 shows the observed pseudo-first-order rate constants for the hydrolysis of ddGuo as a function of temperature at pH 7.0. Regression analysis, performed on these data, yielded the following Arrhenius relationship:

$$\log k = 18 - 6940 \times 1/T \quad r = 0.9991$$

Using this relationship, the k value found at pH 7.0 and 26°C is $(6.31 \pm 2.22) \times 10^{-6}$ h^{-1}, which corresponds to a shelf-life (90%) of 1.9 ± 0.7 years for the conservation of a ddGuo solution at pH 7.0, $\mu = 0.4$ and 26°C. The half-life of ddGuo in the human stomach is less than 2 min, as can be deduced from the data in Table 1 (pH 1.2 and 26°C).

The data in Table 2 also allow us to calculate the activation enthalpy and entropy at pH 7.0 and 26°C. The following values were found: $E_a = 31.8 \pm 0.9$ kcal mol^{-1}, $\Delta H^{\neq} = 31.2 \pm 0.9$ kcal mol^{-1}, $\Delta S^{\neq} = 13.7 \pm 0.4$ e.u.

The activation enthalpy increases with increasing deoxygenation of the sugar. Indeed, values of $\Delta H^{\neq}$ for Guo and dGuo are 20.9 and 22.5 kcal mol^{-1} respectively (Hevesi et al., 1972). The activation entropy displays a parallel behaviour with

TABLE 3

Observed rate constants (h^{-1}) for the hydrolysis of ddGuo in the alkaline region, using 0.1 M potassium phosphate buffer with $\mu = 0.4$

pH	Temperature (°C)	k_{obs} (h^{-1}) [a]
9.0	95	$6.71\times10^{-3}\pm1.2\times10^{-4}$ ($n=24$, $x=8$)
	101	$2.47\times10^{-2}\pm6.5\times10^{-4}$ ($n=15$, $x=5$)
12.0	95	$2.43\times10^{-3}\pm9.9\times10^{-5}$ ($n=10$, $x=4$)
	101	$7.41\times10^{-3}\pm3.6\times10^{-4}$ ($n=24$, $x=8$)

[a] n, total number of chromatographic observations; x, number of points on the time axis.

$\Delta S^{\neq}$ for Guo and dGuo being -11.3 and $+4.3$ e.u., respectively (Hevesi et al., 1972). This is consistent with observations for ddA (Anderson et al., 1988) where $\Delta S^{\neq}$ values became significantly more positive with removal of both 2'- and 3'-hydroxyl groups. We note, however, that Zoltewicz found a $\Delta S^{\neq}$ of $+12.7$ e.u. for dGuo, which differs considerably from the value of $+4.3$ e.u. obtained by Hevesi et al. (Zoltewicz et al., 1970). The positive value of $\Delta S^{\neq}$ is further consistent with the prevalence of an A_1 or unimolecular mechanism of degradation for ddGuo (Schaleger and Long, 1963).

Some experiments were carried out at pH 9.0 and 12.0, and at 95 and 101°C. The results are summarized in Table 3, and show that ddGuo is remarkably stable in the alkaline region. Calculation of a pH-rate profile for both temperatures 95 and 101°C, using the data for pH 7.0, 9.0 and 12.0, yields comparable slopes of approx. -0.3 with rather poor correlation ($r = 0.9252$ and 0.9503, respectively). This is completely different from the situation for ddA, the hydrolysis of which increases when the pH rises above 9 (Anderson et al., 1988). The behaviour of ddGuo in alkaline media seems more closely comparable to that of ddI (Anderson et al., 1988) which also showed a flattening of the log k vs pH plot in the alkaline region. A tentative explanation for this behaviour can be found in the ionization of both ddI and ddGuo in position N_1H-C_6O (*enol* form 6-hydroxyl) with a reported pK_a of 9.12 ± 0.02 for ddI (Anderson et al., 1988) and 9.25 ± 0.01 for Guo (Christensen et al., 1970). At higher pH the negative charge present in the lactam function repels the negative hydroxyl ions, which have been shown to attack nucleophilically adenine nucleosides in the base moiety (Garrett and Mehta, 1972b; Lehikoinen et al., 1986 and references cited therein).

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