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Comparative stability study of thymidine and (dideoxy-D-*erythro*-hexopyranosyl)thymine analogues monitored by capillary electrophoresis

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Abstract

A capillary zone electrophoretic method was developed for monitoring the stability of hexopyranosyl analogues of thymidine. The final analytical conditions adopted were as follows: capillary, fused silica, 70 cm (62 cm to detector) × 75 μm I.D.; background electrolyte, 20 m*M* sodium tetraborate (pH 10.5); voltage, 30 kV; detection, UV at 262 nm; and temperature, 15°C. The performance of this analytical system is discussed. The stability study at three pH values allows to compare the stability of the analogues with that of thymidine and to investigate the influence of the configuration of the N-glycosidic bond on the rate constant of degradation.

1. Introduction

The compounds studied are depicted in Fig. 1: **1** = 1 - (2,3 - dideoxy - β - D - *erythro* - hexopyranosyl)thymine; **2** = 1 - (2,3 - dideoxy - α - D - *erythro* - hexopyranosyl)thymine; **3** = 1 - (2,4 - dideoxy - β - D - *erythro* - hexopyranosyl)thymine; **4** = 1 - (2,4 - dideoxy - α - D - *erythro* - hexopyranosyl) - thymine; **5** = 1 - (3,4 - dideoxy - β - D - *erythro* - hexopyranosyl)thymine. They are all modified nucleosides containing a thymine base but carrying a hexopyranosyl instead of a pentofuranosyl sugar. The sugar is deoxygenated in either positions 2 and 3, 2 and 4 or 3 and 4. The synthesis, the incorporation into oligonucleotides, the enzymatic stability of these oligonucleotides and their base-pairing properties are described elsewhere [1–3]. As knowledge of the stability of the N-glycosidic bond in nucleosides is important with respect to their possible pharmaceutical use and to the stability of modified nucleosides in oligonucleotides (depyrimidination can lead to mutations), we undertook a comparative stability study of compounds **1–5** and thymidine (Thd).

2. Experimental

2.1. Reagents

Thymidine and thymine were purchased from Acros Chimica (Beerse, Belgium). The synthesis of **1–5** is described elsewhere [1–3]. All reagents

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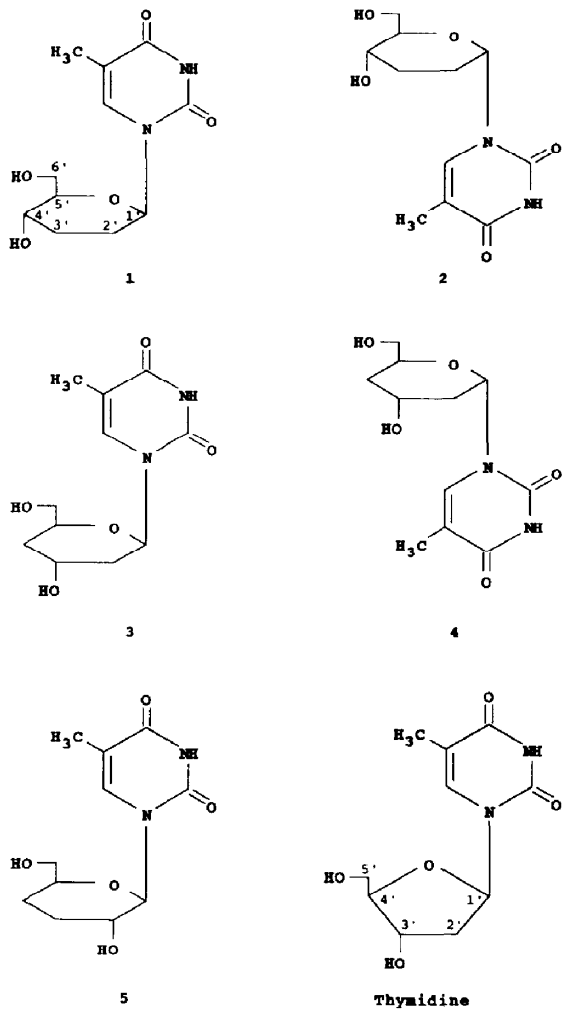

Fig. 1. Structures of thymidine and hexopyranosyl analogues.

were of analytical-reagent grade. Buffers for capillary electrophoresis (CE) were prepared using water obtained from a Milli-Q^{50} system (Millipore, Milford, MA, USA).

2.2. Capillary electrophoresis (CE)

CE was performed using a Spectraphoresis 500 instrument (Thermoseparation Products, Fremont, CA, USA) coupled to a 3396 Series II integrator (Hewlett-Packard, Avondale, PA, USA). Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). The pH of the sodium tetraborate buffer was adjusted with 1 *M* sodium hydroxide solution. Peak areas were corrected.

2.3. Stability study

All samples were incubated at 101°C in a Memmert (Schwabach, Germany) oven using buffers of different pH and with the ionic strength adjusted to 0.06 with KCl. A 0.01 *M* sodium dihydrogencitrate solution was brought to pH 1.2 with 1*M* HCl for the acid buffer. A 0.01 *M* neutral buffer of pH 6.8 and a 0.01 *M* alkaline buffer of pH 12.0 were prepared using the appropriate sodium phosphate species. Solutions (10^{-3} *M*) of the samples in the buffer were incubated for appropriate times and frozen. Just before CE analysis, acid samples were neutralized with 0.09 *M* KOH and alkaline samples with 0.01 *M* HCl.

3. Results and discussion

3.1. Analysis

Although a previous comparative stability study had been performed with liquid chromatography as the analytical technique, it suffered from some problems such as a decrease in retention of the analytes [4], so CE was preferred, also because of its proven speed in the determination of thymidine [5]. There are many papers on the CE of nucleosides, covering both capillary zone electrophoresis [6–11] and micellar electrokinetic capillary chromatography [12–18]. For this study the method used for thymidine [5] was slightly modified to improve the symmetry of the peaks and the stability of the compounds in the electrophoretic system. To achieve these respective aims, the pH was raised to 10.5 and the temperature of analysis was lowered to 15°C. The final analytical conditions were as follows: capillary, fused silica, 70 cm (62 cm to the detector) × 75 μm I.D.; background electrolyte, 20 m*M* sodium tetraborate (pH 10.5); voltage, 30 kV; detection, UV at 262 nm; temperature, 15°C; and current, limited to 150

μA. Samples were injected hydrodynamically for 3 s. Symmetry factors, resolution, migration times, mobilities, R.S.D.s of peak areas and migration times and numbers of theoretical plates are given in Table 1 for each of the compounds investigated. Table 2 collects calibration data accumulated in the concentration range 10^{-5}–10^{-3} *M*, using four calibration points (12 analyses in total). The correlation coefficient was 0.9999 in all instances. No reference substance was available for the unknown, so its mass was expressed as hexopyranosyl nucleoside in mass balance calculations. The detection limit (signal-to-noise ratio = 3) for Thd with this method was found to be 10^{-6} *M*. As six injections of this solution yielded an R.S.D. of 11%, we also regard this as the limit of quantification (LOQ). The equipment injects 4 nl s^{-1} of hydrodynamic injection on a capillary of 75 μm I.D. which gives an LOQ of 3 pg. The intra-day repeatability ($n = 10$) for Thd was 0.9% (migration time) and 1.4% (peak area). The inter-day repeatability ($n = 9$; 9 days) was 8.6% (migration time) and 3.1% (peak area).

Table 2
Calibration data for thymine, thymidine and hexopyranosyl analogues

Compound	*a*	*b*	$S_{y,x}$
Thymine	509	−222	205
Thymidine	372	279	631
1	390	101	226
2	389	263	316
3	359	426	333
4	394	217	193
5	377	128	556

a = Slope; *b* = intercept; *y* = corrected peak area; *x* = concentration (μg · ml^{-1}).

3.2. Stability study

Fig. 2 shows representative electropherograms of samples degraded at pH 1.2. As described before [19], the degradation of thymidine yields not only thymine but also its anomer and pyranosyl isomers. This indicates that in the case of thymidine, degradation does not take place only by destabilization of the N-glycosidic bond after protonation of the heterocyclic base. Part of the mechanistic pathway must also involve sugar protonation and ring opening, which allows the sugar to reorganize into the anomer or pyranosyl isomer.

Nucleosides **1** and **3** only show the formation of thymine. Formation of their α-anomers **2** and **4**, respectively, might have taken place but the latter had probably already degraded because of their much higher degradation rate (a factor of 14 and 20 respectively; see Table 3). Nucleosides **2** and **4** show the formation of their (more stable) β-anomers **1** and **3** respectively. During degradation of **2**, small amounts of two unknown compounds were observed. These could be sugar isomers because their degree of formation is comparable to that of the anomer and this pattern of degradation is known for thymidine.

Table 1
Electrophoretic parameters for thymidine and hexopyranosyl analogues

Compound	Symmetry factor	Resolution with thymine	Migration time (min)	Mobility (cm^2 kV^{-1} min^{-1})	RSD (%)		No. of theoretical plates
					Peak area ($n = 3$)	Migration time ($n = 3$)	
Thymidine	1.0	15.8	7.0	23.3	1.2	0.3	139 000
1	1.1	4.7	6.8	24.0	0.3	0.8	81 500
2	1.0	11.5	6.3	25.9	1.0	0.2	165 500
3	1.0	17.7	7.5	21.8	1.1	1.6	178 600
4	1.0	15.8	6.7	24.4	0.6	0.4	186 000
5	0.9	13.5	7.3	22.4	0.6	0.1	176 400

For conditions, see text. Repeatability experiments were performed on 10^{-4} *M* solutions.

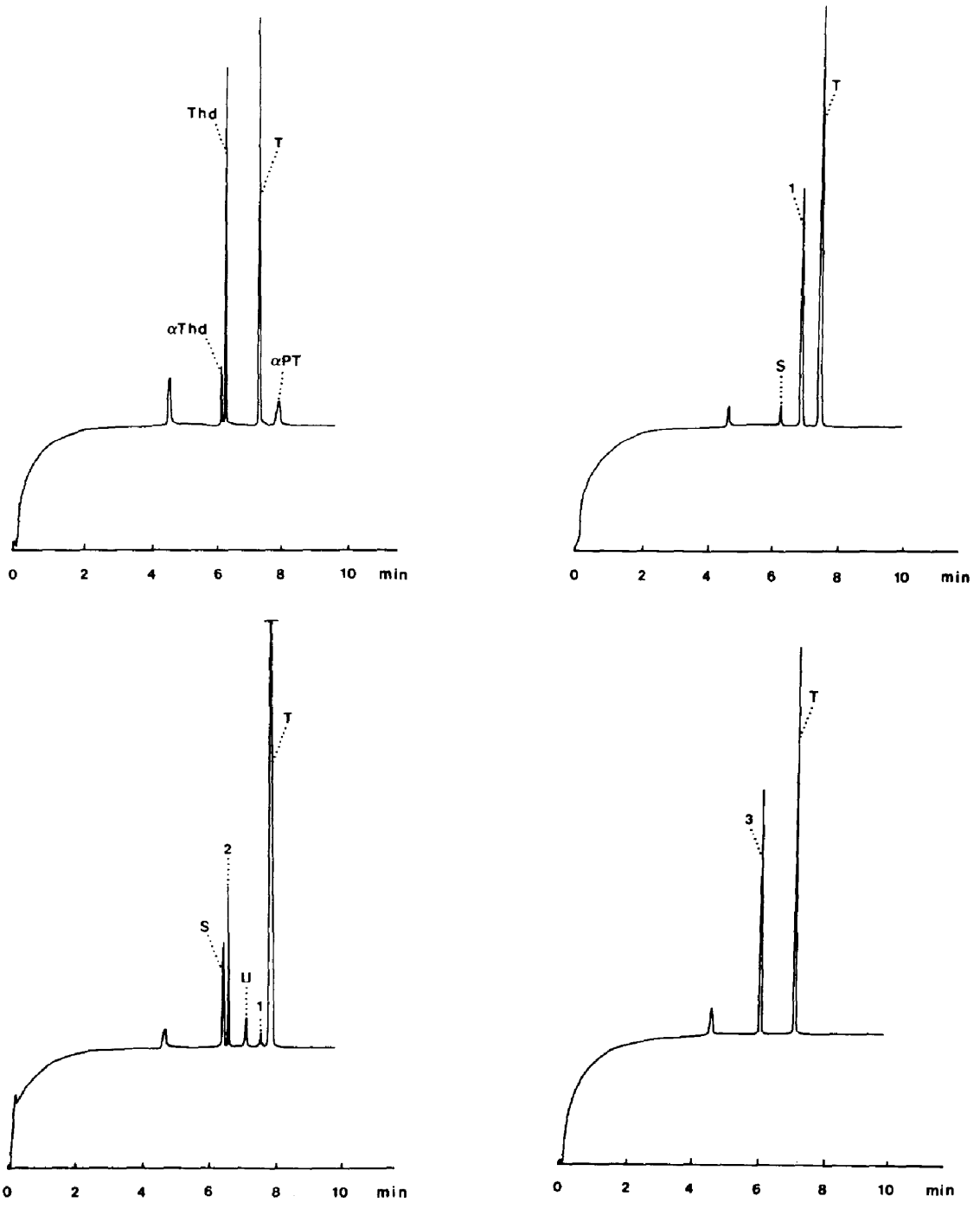

Fig. 2. Electropherograms of thymidine and hexopyranosyl analogues degraded at pH 1.22 and 101°C for the following periods: thymidine, 30 h; **1**, 216 h; **2**, 30 h; **3**, 216 h; **4**, 21 h; **5** 216 h. S = Synthetic impurity; U = unknown. αThd = 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)thymine; Thd = thymidine; T = thymine; αPT = 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)thymine. For analytical conditions, see text.

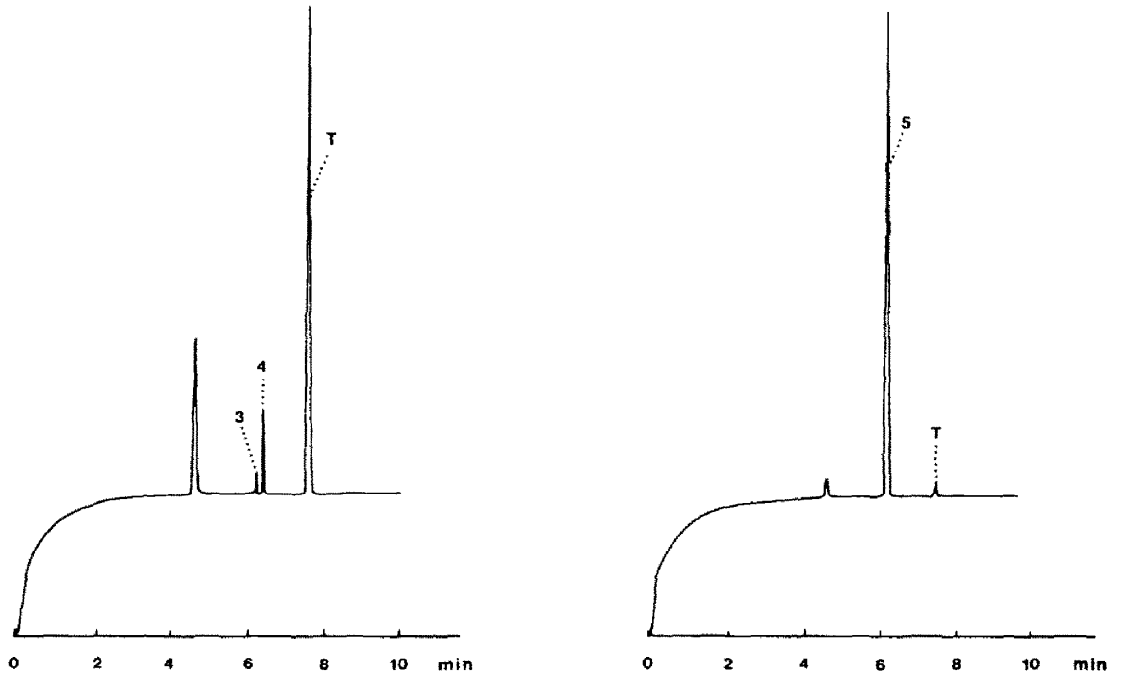

Fig. 2 (*continued*).

The hexopyranosyl sugar possibly isomerizes into a hexofuranosyl sugar. This could explain why nucleosides **3**, **4** and **5** apparently do not form any isomers: they do not possess a 4′-hydroxyl group. On the other hand, for **1** no other degradation products apart from thymine could be distinguished. Nucleoside **5** was only degraded 1.7% after 216 h and is thus much more stable.

The degradation of the nucleosides seems to take place through the same mechanism as for thymidine in view of the degradation compounds formed and in view of relative stabilities of the different analogues. The higher stability of **5** can indeed be due to the presence of its 2′-OH, which destabilizes the positively charged intermediate and/or decreases protonation of the sugar oxygen. Mass balance calculations performed on the acid-degraded series fitted for all compounds except for **3**, even after repeating the experiment (30% loss of total mass after 216 h). The reason for this is not clear. The amount of synthetic impurities present in **1** and **2** did not change during the experiment and these substances were therefore not taken into account during mass balance calculations. Degradation experiments at neutral and alkaline pH revealed the formation of thymine as the sole degradation product.

Table 3 contains the observed pseudo-first-order degradation rate constants at the three pH values tested. The hydrolysis rate increases towards the acidic region.

In contrast to thymidine [4], the difference in the hydrolysis rates of α- versus β-anomers (**1** vs. **2**, **3** vs. **4**) is significant in acidic, neutral and alkaline media. As discussed before [4], thymidine anomers display the same kinetics in acidic media for stereoelectronic and conformational reasons. The N-glycosidic bond bears the same relationship to the lone pair of electrons of the ring oxygen whether it is in the α- or β-position [20]. It was also stated [20] that the puckering of the ring usually adjusts so that the substituent is quasi-axial in either case.

^{1}H NMR measurements at various temperatures [21] and molecular mechanics analysis [22] have further shown the flexibility of the

Table 3
Observed rate constants of degradation of thymidine and hexopyranosyl analogues at 101°C and $\mu = 0.06$

Compound	pH 1.2					pH 6.8					pH 12.0				
	k (h^{-1})	N	x	n	y	k (h^{-1})	N	x	n	y	k (h^{-1})	N	x	n	y
Thymidine	0.04516 ± 0.00088	16	8	1	2	0.0044 ± 0.00016	13	8	1	2	0.0015 ± 0.00013	32	8	2	<1
1	0.00637 ± 0.00021	30	8	2	2	0.0025 ± 0.00017	16	8	1	1	0.00052 ± 0.000047	32	8	2	<1
2	0.09110 ± 0.0020	32	8	2	4	0.0171 ± 0.00053	32	8	2	5	0.0039 ± 0.00014	28	8	2	1
3	0.00766 ± 0.00024	28	8	2	2	0.0032 ± 0.000073	32	8	2	2	0.00071 ± 0.0067	32	8	2	<1
4	0.15426 ± 0.00403	28	7	2	6	0.011 ± 0.00071	28	7	2	4	0.0067 ± 0.00037	14	7	1	2
5	m					s					s				

N = No. of electropherograms; x = No. of points on the time axis; n = No. of experiments; y = duration of experiments in half-lives; m = degradation negligible after 216 h; s = stable after 309 h.

thymidine sugar ring, which can adopt different conformations. Few reports exist on the conformation of glucopyranosyl-containing nucleosides. When 2,3-dideoxy-β-D-glucopyranosyl rings with either adenine or thymine as base were incorporated into an oligonucleotide, they were shown by NMR to be in the most stable chair conformation with all substituents equatorial and with both the adenine and the thymine base in the *anti* conformation [23]. This is consistent with a report [24] that large heterocyclic structures on the anomeric carbon of glucopyranosides, especially when they carry a positive charge, are in an equatorial position. This is due to a reverse anomeric and steric effect. When comparing the hydrolysis of acetals with an α- or β-bond, the α-isomer seems to cleave more easily because its conformation adapts better to a situation in which the ring oxygen lone pair is antiperiplanar with respect to the exocyclic bond [25,26].

Table 3 also allows one to compare the stability of thymidine containing a pentofuranosyl sugar with the anologue carrying a hexopyranosyl sugar (**3**). The latter is more stable than its pentofuranosyl counterpart towards acid hydrolysis. This has also been noted in the field of carbohydrates, where glycosides of 2-deoxyhexoses are much more stable than those of the corresponding 2-deoxypentoses and hexofuranosides of deoxy sugars are much more acid labile than the hexopyranosides [27].

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