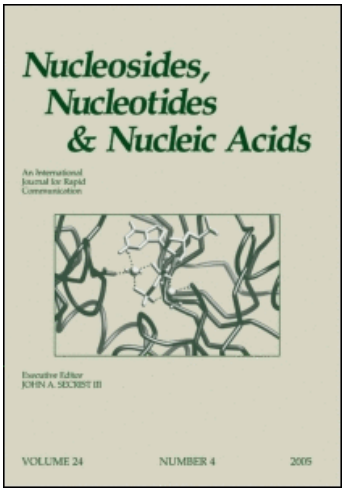

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Comparative Liquid Chromatographic Stability Study of Thymidine and 1-(2-Deoxy-α-D-Erythro-Pentofuranosyl) Thymine

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To cite this Article Van Schepdael, A. , Heerinckx, F. , Van Aerschot, A. , Herdewijn, P. , Roets, E. and Hoogmartens, J.(1994) 'Comparative Liquid Chromatographic Stability Study of Thymidine and 1- (2-Deoxy-α-D-Erythro-Pentofuranosyl) Thymine', Nucleosides, Nucleotides and Nucleic Acids, 13: 5, 1113 — 1123
To link to this Article: DOI: 10.1080/15257779408011882
URL: http://dx.doi.org/10.1080/15257779408011882

NUCLEOSIDES & NUCLEOTIDES, 13(5), 1113-1123 (1994)

COMPARATIVE LIQUID CHROMATOGRAPHIC STABILITY STUDY OF THYMIDINE AND 1-(2-DEOXY-α-D-*ERYTHRO*-PENTOFURANOSYL)THYMINE

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Abstract. A liquid chromatographic method was developed for the separation of thymidine from its anomer and isomers. The method was implemented during a comparative stability study of thymidine and 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)thymine.

Introduction

The stability of thymidine (Thd) in acidic media has been thoroughly investigated in the past, using UV-spectrophotometry as the analytical method. The influence of hydroxylation of the sugar on the rate of degradation was studied in 1N HCl[1]. Specific acid catalysis was shown to take place[2] in the region of pH 0 to 0.6. Degradation rate constants were determined at pH 1[3], at pH 1.5 to 3.0[4,5] and at pH 4.0 to 6.5[6]. A study performed in 2M $HClO_4$ proved that anomerization and isomerization take place during acidic degradation and thus showed that the sugar ring opens to form an intermediary acyclic Schiff base[7] and likewise these isomerizations were shown to occur during treatment of thymidine with hydrobromic acid[8]. Structures of the compounds formed apart from thymine, namely 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)thymine (αThd), 1-(2-deoxy-ß-D-*erythro*-pentopyranosyl)thymine (ßPT) and 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)thymine (αPT) are given in Figure 1.

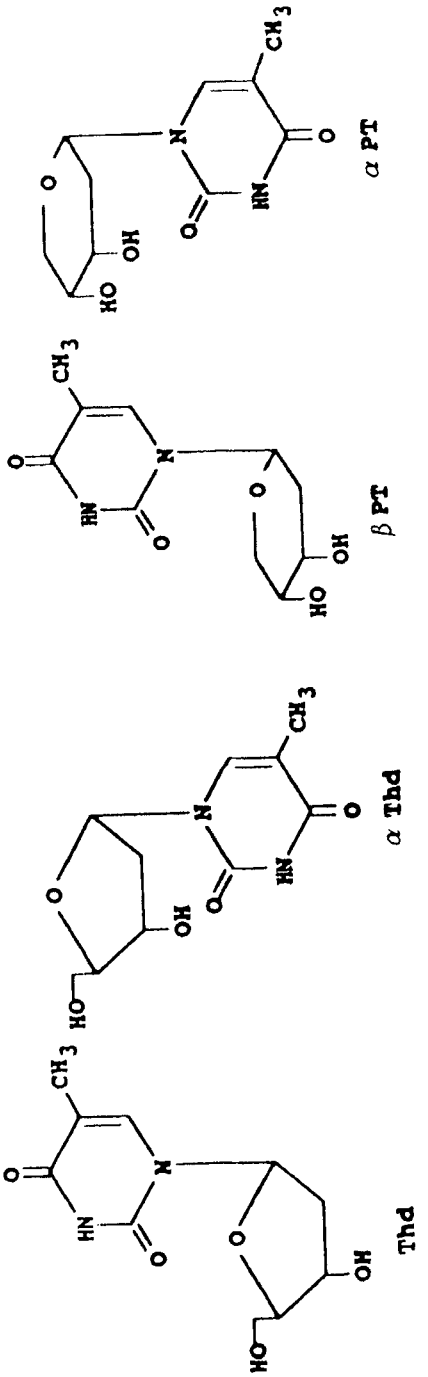

FIG. 1. Structure of thymidine and its anomer and isomers.

The objective of this work was to study the stability of Thd and αThd. A comparative stability study of Thd and αThd is relevant because of recent interest in oligonucleotides containing α- and ß-thymidine[9,10,11]. These data will also complement analogous comparisons between the α- and ß-anomeric forms of adenosine[12] and deoxyuridine[13].

Experimental section

Thd was purchased from Janssen Chimica (Beerse, Belgium) as well as thymine and all other reagents, which were of analytical grade. The synthesis of αPT and ßPT was published elsewhere[14] and αThd was prepared following a previously described procedure[15]. Double distilled water was used throughout and tetrahydrofuran was distilled in the presence of ferrous sulfate and kept in the dark at 5 °C. Stability studies were performed at pH 1.0 using a 0.1 M glycine.HCl buffer which was adjusted to an ionic strength of 0.4 with KCl. A 10^{-4} M solution of Thd or αThd was incubated at 103 °C in a Memmert oven (Schwabach, Germany) and quenched to pH 4 at appropriate intervals with a solution of potassium hydroxide, after which the samples were frozen. They were analyzed as a series using following liquid chromatographic equipment : a Milton Roy mini pump (Laboratory Data Control,Riviera Beach, FL, USA) at a flow rate of 1 ml/min, a Marathon injector (Spark Holland, Emmen, The Netherlands) with a 20 µl loop, a Spectro Monitor 3100 detector (Milton Roy, Riviera Beach, FL, USA) at 262 nm and an integrator model 3396 A (Hewlett-Packard, Avondale, PA, USA). Column temperature was maintained at the desired value with a jacket connected to a thermostating bath (Julabo, Seelbach, Germany). The stationary phase was Hypersil C_8 5 µm (Shandon, Astmoor, Cheshire, U.K.), packed in a 250 x 4.6 mm ID column and the mobile phase consisted of methanol, 0.2 M potassium phosphate buffer pH 5.0 and water in a ratio of 0.1:5:94.9, v/v. The test mixture for development work contained

approximately 10^{-5}M of each of the compounds. Other stationary phases used were PLRP-S 1000 Å (Polymer Labs, Church Stretton, Shropshire, U.K.), RSil C_{18} LL 5 μm (Biorad, Eke, Belgium), LiChrosorb C_8 5 μm (Merck, Darmstadt, Germany), Nucleosil 100-5 C_8 5 μm (Macherey-Nagel, Düren, Germany), Partisil C_8 5 μm (Whatman, Clifton, NJ, U.S.A.) and Chromspher C_8 5 μm (Chrompack, Middelburg, The Netherlands).

Results and discussion

Analytical method development. For the stability study it was necessary to resolve both Thd and αThd. In literature a bidimensional thin-layer chromatographic system[16] with scintillation detection of radioactively labeled compounds was used before[7]. This was however not thought to be convenient for the purpose of this work. Other workers reported on liquid chromatographic (LC) methods to analyze thymidine or its main acidic degradation compound thymine using reversed phase columns with mobile phases containing a low amount of organic modifier[17-21]. However, none of these studies reported the separation of anomers or isomers. It was therefore necessary to develop a new LC method. It was first attempted to form an ion-pair in basic media (pK_a of Thd = 9.8) and analyze this on polymeric stationary phases, which are known to be stable at extreme pH values. Since other rather lipophilic compounds such as sugar degradation compounds are generated during kinetic studies, such an ion-pair would allow to use a higher percentage of organic modifier and thus keep the lipohilic compounds from accumulating on the column. The conditions in which Thd was retained most (column : PLRP-S 1000 Å, mobile phase : 0.2 M cetyltrimethylammonium bromide pH 12.0 - 0.2 M potassium phosphate buffer pH 12.0 - water (0.5:5:94.5, v/v), temperature : 60 °C, detection : UV at 262 nm) unfortunately did not allow to separate Thd and

TABLE 1. Initial choice of a stationary phase for the separation of αPT, Thd, αThd and ßPT. Mobile phase : tetrahydrofuran - 0.2 M potassium phosphate buffer pH 4.0 - water (0.1:5:94.9, v/v). Flow rate : 1.0 ml/min. Detection : UV at 262 nm. Temperature : 20±1 °C.

Stationary phase	Capacity factor				Symmetry factor				Resolution		
	αPT	Thd	αThd	ßPT	αPT	Thd	αThd	ßPT	αPT-Thd	Thd-αThd	αThd-ßPT
Hypersil C18 5 μm	10.5	16.8	17.6	24.0	1.3	a	a	1.3	12.1	1.3	5.7
RSil C18 LL 5 μm	4.7	6.6	7.0	9.7	1.8	a	a	1.7	b	b	3.3
Hypersil C8 5 μm	5.7	8.9	9.4	13.5	1.0	a	a	1.0	9.5	1.2	8.4

a : no separation of peaks at 1/20 of the peak height.
b : no separation of peaks at 1/2 of the peak height.

αThd, so that it was necessary to turn to silica-based reversed-phases after all.

For the LC development work a test mixture consisting of Thd, αThd, ßPT and αPT was used, because all these compounds are formed during acidic degradation of Thd[7]. At first, three types of stationary phases were tested, namely Hypersil C_8 and C_{18} 5 μm and RSil C_{18} 5 μm. For these initial studies the mobile phase developed previously for 2'-deoxyuridine[22] was used at 20 °C. Looking at the symmetry factors in Table 1 it was preferred to use Hypersil C_8 for further development work. Chromatographic parameters were calculated according to the European Pharmacopoeia[23].

In a second step the pH of the mobile phase was varied, the results of which are summarized in Table 2. No significant differences could be noticed and a pH of 5.0 was chosen for subsequent studies.

The results of experiments with different organic modifiers yielded the data in Table 3. Acetonitrile, dioxane, diethyl ether and tetrahydrofuran gave no baseline separation of Thd and αThd. Out of the remaining possibilities methanol was chosen because it combined an acceptable analysis time with good resolution. Even if the mobile phase contains only a small percentage of methanol,

TABLE 2. Influence of the mobile phase pH on the separation of αPT, Thd, αThd and ßPT. Stationary phase : Hypersil C_8 5 µm. Mobile phase : tetrahydrofuran - 0.2 M potassium phosphate buffer pH x - water (0.1:5:94.9, v/v). Flow rate : 1.0 ml/min. Detection : UV at 262 nm. Temperature : 20±1 °C.

x	Capacity factor				Symmetry factor				Resolution		
	αPT	Thd	αThd	ßPT	αPT	Thd	αThd	ßPT	αPT-Thd	Thd-αThd	αThd-ßPT
1.4[a]	5.0	7.8	8.2	12.2	1.1	b	b	1.1	9.0	1.0	8.1
2.0	5.9	9.2	9.7	13.9	1.1	b	b	1.0	9.4	1.1	7.9
3.0	5.9	9.2	9.7	13.9	1.1	b	b	1.0	9.5	1.1	8.4
4.0	5.7	8.9	9.4	13.5	1.0	b	b	1.0	9.5	1.2	8.4
5.0	5.5	8.6	9.1	13.1	1.0	b	b	1.1	9.2	1.1	8.7
6.0	5.5	8.9	9.4	13.2	1.1	b	b	1.1	10.0	1.3	8.1

a : a pH of 1.4 was obtained by adding 1 N $HClO_4$ instead of potassium phosphate buffer.
b : no separation of peaks at 1/20 of the peak height.

TABLE 3. Influence of type and concentration of the organic modifier on the separation of αPT, Thd, αThd and ßPT. Stationary phase : Hypersil C_8 5 µm. Mobile phase : organic modifier - 0.2 M potassium phosphate buffer pH 5.0 - water (0.1:5:94.9, v/v). Flow rate : 1.0 ml/min. Detection : UV at 262 nm. Temperature : 20±1 °C.

Modifier	Capacity factor				Symmetry factor				Resolution		
	αPT	Thd	αThd	ßPT	αPT	Thd	αThd	ßPT	αPT-Thd	Thd-αThd	αThd-ßPT
a	7.6	11.9	12.7	18.6	1.2	1.2	1.1	1.1	9.1	1.5	8.3
CH_3CN	7.1	11.1	11.9	17.4	1.1	1.1	b	1.1	9.2	1.4	8.1
dioxane	8.5	13.3	14.1	20.5	1.1	b	b	1.1	9.3	1.3	8.1
Et_2O	4.4	7.0	7.3	10.4	1.0	b	b	1.0	9.8	1.2	8.7
MeOAc	6.3	9.7	10.3	15.0	1.1	1.0	1.0	1.1	8.7	1.4	8.5
c	7.5	11.6	12.4	18.3	1.1	1.0	1.0	1.1	12.2	2.0	10.7
THF	5.5	8.6	9.1	13.1	1.0	b	b	1.1	9.2	1.1	8.7
MeOH	7.4	11.6	12.4	18.1	1.0	1.1	1.0	1.1	9.5	1.5	8.9
MeOH (0.5 % v/v)	6.7	10.3	11.0	16.1	1.0	1.1	1.1	1.1	8.7	1.4	7.9

a : mobile phase : 0.2 M potassium phosphate buffer pH 5.0-water (5:95, v/v)
b : no separation of peaks at 1/20 of the peak height.
c : ethylene glycol methyl ether

TABLE 4. Influence of the temperature of the column on the separation of αPT, Thd, αThd and ßPT. Stationary phase : Hypersil C_8 5 µm. Mobile phase : methanol - 0.2 M potassium phosphate buffer pH 5.0 - water (0.1:5:94.9, v/v). Flow rate : 1.0 ml/min. Detection : UV at 262 nm.

Tempera-ture (°C)	Capacity factor				Symmetry factor				Resolution		
	αPT	Thd	αThd	ßPT	αPT	Thd	αThd	ßPT	αPT-Thd	Thd-αThd	αThd-ßPT
20±1	10.4	16.5	18.1	26.7	1.4	1.1	1.0	1.5	11.0	2.4	9.2
35±1	7.4	11.0	12.0	18.5	1.2	1.2	1.2	1.3	6.4	1.5	7.3
40±1	5.2	7.7	8.4	13.3	1.2	1.2	1.1	1.1	5.9	1.4	7.6
45±1	4.3	6.3	6.8	10.9	1.1	a	a	1.2	5.5	1.2	7.4

a : no separation of the peaks at 1/20 of the peak height.

it has some advantages over one containing only buffer, namely better symmetry factors and a faster analysis.

The stationary phase was also used at different temperatures out of which 20 °C was selected for reasons of good resolution (Table 4). It has to be noted that the latter experiments were performed on a new Hypersil stationary phase so that somewhat different chromatographic parameters were obtained.

Indeed, after use of the first Hypersil C_8 stationary phase during a period of 2 months the retention of Thd suddenly dropped to zero. Since the column performed well during an analysis with aromatic substances it was concluded that the reversed phase material was not damaged and that the retention of thymidine must be based on a more complex mechanism than simple distribution interaction only. An attempt was made to circumvent this problem by using another type of stationary phase. LiChrosorb C_8, Nucleosil 100-5 C_8 and Partisil C_8, all 5 µm, could however not separate Thd and αThd. With Chromspher C_8 5 µm as stationary phase and 0.2 M potassium phosphate buffer pH 5.0 - water (5:95, v/v) as mobile phase, the overall separation was comparable to that obtained on Hypersil but after some time the problems of lack of retention occurred too. Finally, it was observed that washing the stationary

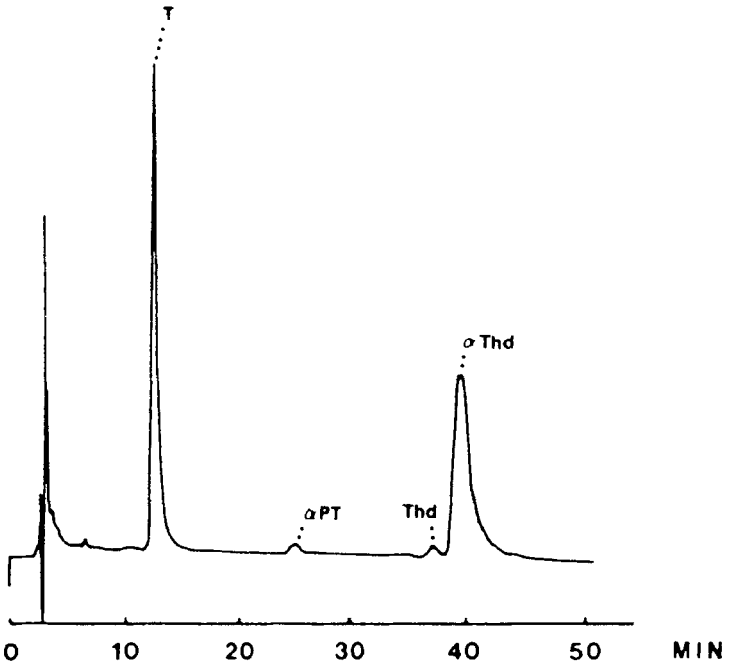

FIG. 2. Chromatogram of αThd degraded at pH 1.0. Stationary phase : Hypersil C_8 5 μm. Mobile phase : methanol - 0.2 M potassium phosphate buffer pH 5.0 - water (0.1:5:94.9, v/v). Flow rate : 1.0 ml/min. Temperature : 20±1 °C. Detection : UV at 262 nm.

phase with a strongly eluting mobile phase such as acetone - perchloric acid 1 N - water (50:5:45, v/v) restored it performance. This washing was performed weekly and did not affect the column during the span of this study.

Figure 2 shows a chromatogram of an acid degraded sample of αThd under the conditions finally selected. A calibration experiment performed for Thd, αThd and thymine proved a linear relationship between the peak area and the concentration. In later mass balance calculations the obtained regression equations were used, while αPT and βPT were expressed as Thd, because they were not available in large enough quantities. Detection limits were 1.0 ng and 0.5 ng for Thd and αThd respectively (signal to noise ratio = 3).

TABLE 5. Observed degradation rate constants of Thd and αThd at pH 0.99, T = 103 °C and μ=0.4.

	k (h^{-1})	N	x	n	$t_{1/2}$
Thd	0.082±0.0076	15	7	2	1.5
αThd	0.067±0.0048	17	7	2	1.2

N= total number of analyses
x= number of points on the time axis
n= number of independent experiments
$t_{½}$=number of half-lives during which tested

Stability study. Samples of Thd and αThd were then subjected to a pH of 1.0 at 103 °C. Mass balance calculations showed that, apart from thymine and the anomer and isomers, no other compounds were formed. The anomer and isomers were only side-products of the degradation since their molar percentage never exceeded 6%. The observed degradation rate constants are displayed in Table 5. There was no significant difference at the 1% level. For the corresponding adenosine[12] and deoxyuridine[13] analogues the difference in stability was also very small (factors 1.4 and 1.3 respectively) with the latter being statistically significant at the 1% level. It is believed that these differences are not really relevant and that the reactivities of the α- and ß-nucleosides discussed here, are comparable. This would be consistent with conformational information available. Indeed, it was stated that the $C_{1'}$-N_1 bond stands in the same relationship to the lone pair electrons on the ring oxygen whether it is in the α- or ß-position[24]. In α- as well as ß-nucleosides the pyrimidine ring (either uracil or thymine) furthermore prefers the *anti* position, so that on that level no difference in preferences exists[25]. It is thus presumed that the large similarity in conformation about the N-glycosidic bond is consistent with very similar stabilities in acidic medium.

Acknowledgements

A. Van Aerschot (Senior Research Associate) and A. Van Schepdael (Senior Research Assistant) thank the Belgian National Fund for Scientific Research for financial support. The authors thank D. Everaert (Laboratory for Analytical Chemistry and Medicinal Physicochemistry, K.U.Leuven) for helpful discussions. The secretarial help from A. Decoux and I. Quintens is gratefully acknowledged.

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Received 9/7/93
Accepted 1/11/94