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Short Communication

Separation of the anomers and isomers of 2′-deoxyuridine and thymidine by capillary zone electrophoresis

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ABSTRACT

Anomerisation and isomerisation take place during acidic degradation of 2′-deoxyuridine (dUrd) and thymidine (Thd) due to the opening and reclosure of the furanose ring. A capillary electrophoretic method was developed which was able to separate dUrd and Thd from their respective anomers and pentopyranosyl isomers. Using a fused-silica capillary (70 cm × 75 μm I.D.) the influence of the type, pH and concentration of the buffer was systematically investigated as well as the effect of voltage and temperature. With a 20 m*M* sodium tetraborate buffer of pH 9.5 and 10 for dUrd and Thd, respectively, good resolution between anomers and isomers was obtained at 20 kV and 50°C.

INTRODUCTION

Degradation studies on 2′-deoxyuridine (dUrd) and thymidine (Thd) necessitated an analytical system that was able to separate all the compounds formed. It has been shown that the hydrolysis of dUrd and Thd is accompanied by the formation of the anomeric and isomeric forms 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)-uracil (αFdUrd), 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)uracil (αPdUrd), 1-(2-deoxy-β-D-*erythro*-pentopyranosyl)uracil (βPdUrd) and 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)thymine (αFThd), 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)-thymine (αPThd), 1-(2-deoxy-β-D-*erythro*-pentopyranosyl)thymine (βPThd) respectively [1]. See Fig. 1 for structures of these compounds. In that paper it was demonstrated that anomerisation and isomerisation of dUrd and Thd take place during degradation, due to the opening and reclosure of the furanose ring. The degradation compounds were at that time separated by two-dimensional thin layer chromatography and quantitated by ^{14}C liquid scintillation counting. To investigate the kinetics of hydrolysis of dUrd and Thd, a liquid chromatographic (LC) method for the analysis of the cold substance was developed previously [2]. Since an LC analysis takes approximately 1/2 hour, the potential of

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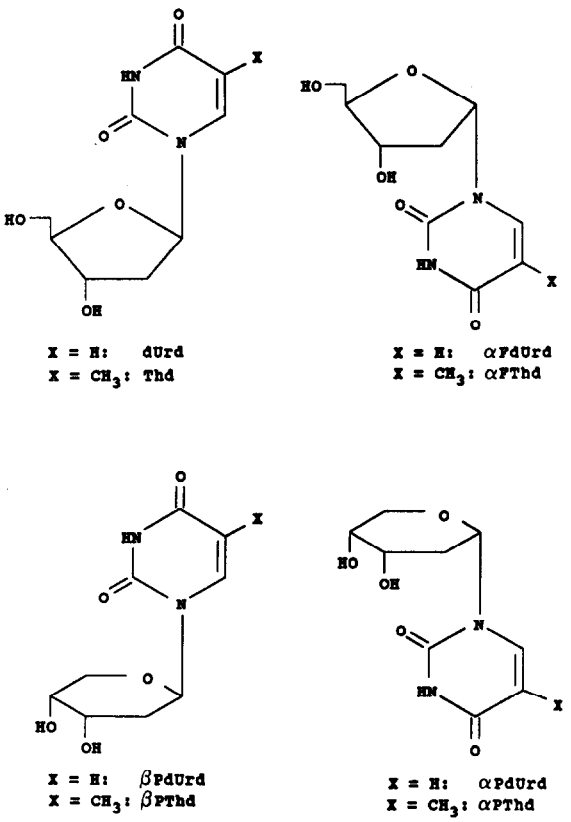

Fig. 1. Structures of dUrd and Thd, and their anomers and isomers.

capillary electrophoresis (CE) was investigated, to speed up the assay. Previous work on CE of nucleosides includes polyacrylamide gel electrophoresis [3], micellar electrokinetic chromatography (MECC) [4–9] and capillary zone electrophoresis (CZE) at pH values ≤ 7 [10–14].

EXPERIMENTAL

Chemicals

dUrd and Thd were purchased from Janssen Chimica (Beerse, Belgium). αFdUrd and αFThd were synthesized according to a previously published procedure [15]. The synthesis of the other compounds is described elsewhere [16]. Reagents were of analytical grade (Merck, Darmstadt, Germany) and Milli-Q water (Millipore, Milford, MA, U.S.A.) was used throughout. The concentration of each of the compounds in the test mixture was approximately 10^{-5} *M* in Milli-Q water.

Capillary electrophoresis

CE was performed on a Spectraphoresis 500 (Spectra Physics, San Jose, CA, USA) coupled to a Model 3396 seriesII integrator (Hewlett-Packard, Avondale, PA, USA). Fused-silica capillaries (70 cm × 75 μm I.D.) were obtained from Spectra Physics. pH adjustments of sodium tetraborate buffer were performed with a 0.1 *M* boric acid solution or a 0.5 *M* sodium hydroxide solution.

RESULTS AND DISCUSSION

To develop a suitable CE method for the resolution of dUrd and its anomer and isomers, the influence of the pH and concentration of the buffer, applied voltage and temperature was successively investigated. Preliminary studies using sodium glycinate buffer of pH 10.8, sodium phosphate buffer of pH 9.3 and sodium tetraborate buffer of pH 9.5 and 20 m*M* each had shown that the latter gave the best separation of the four compounds. The influence of the pH was thus studied using sodium tetraborate buffers and the results of the pH study are summarized in Table I. The separation parameters were calculated following instructions for liquid chromatography in the European Pharmacopoeia [17], for example: resolution = 1.18 × (difference in migration times of the peaks)/(sum of peak widths at half of the peak height). It can be seen that the resolution of αFdUrd and dUrd changes drastically in the neighbourhood of the pK_a of dUrd ($pK_a = 9.3$). Mobility diminishes with a pH increase, because the ionization of the compounds and thus the electrophoretic repulsion from the cathode, increases. The increase in electroosmotic flow velocity is apparently less than the increase in electrophoretic velocity. For subsequent studies a pH of 9.5 was chosen because it yielded a fairly good separation combined with symmetrical peaks. This pH is furthermore obtained when making a 20 m*M* solution of sodium tetraborate in Milli-Q water without any further pH adjustment.

Table II shows the results of experiments in

TABLE I

INFLUENCE OF THE BUFFER pH ON THE SEPARATION CHARACTERISTICS OF dUrd AND ITS ANOMER AND ISOMERS

Electrolyte, 20 m*M* sodium tetraborate adjusted to pH with 0.5 *M* sodium hydroxide or 0.1 *M* boric acid; capillary, fused silica, 70 cm × 75 μm I.D.; temperature, 30°C; voltage, 15 kV; injection, hydrodynamic, 3 s; detection, UV at 262 nm.

pH	Mobility ($cm^2 kV^{-1} min^{-1}$)				Peak symmetry				Resolution		
	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd-dUrd	dUrd-αPdUrd	αPdUrd-βPdUrd
8.6	36.5	36.5	35.9	33.7	[a]	[a]	1.0	0.9	0	2.2	6.8
9.1	37.1	36.9	34.2	32.1	[a]	1.1	0.9	1.0	1.2	9.6	5.8
9.5	36.9	36.1	32.4	30.6	1.1	1.0	1.0	1.1	2.7	11.1	4.9
10.0	29.8	29.1	25.2	23.5	1.0	1.0	1.0	1.0	2.9	13.2	5.4
10.5	25.1	24.6	20.9	19.0	1.0	0.9	1.0	1.1	2.1	12.6	5.4
11.0	22.8	22.5	19.0	17.1	[a]	[a]	1.0	1.0	1.1	10.6	4.8

[a] No separation of peaks at one twentieth of the peak height.

which the concentration of the buffer was modified. Since a good overall resolution combined with good symmetry factors and short analysis times was obtained with a 20 m*M* buffer, this concentration was chosen for further work. The analysis is faster with less concentrated buffer. This is consistent with an increase of the electroosmotic flow when ionic strength diminishes, which was also confirmed by the current monitoring method [18].

The influence of the applied voltage is summarized in Table III. A voltage of 20 kV was chosen for further work, because it allowed a fast separation with symmetric peaks and very good resolution. It is apparent that the electroosmotic velocity increases more with increasing voltage than the electrophoretic velocity of the negatively charged compounds, since the overall mobility rises with rising voltage.

Table IV indicates that temperature does not significantly change the separation pattern. Due to a lowering of the viscosity, the separation was speeded up with a temperature increase. A temperature of 50°C was selected for subsequent studies.

Fig. 2 depicts the separation of dUrd from its

TABLE II

ELECTROPHORETIC PARAMETERS FOR THE SEPARATION OF dUrd FROM ITS ANOMER AND ISOMERS USING DIFFERENT CONCENTRATIONS OF SODIUM TETRABORATE BUFFER OF pH 9.5

Other conditions were as described for Table I.

Buffer concentr.	Mobility ($cm^2 kV^{-1} min^{-1}$)				Peak symmetry				Resolution		
	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd-dUrd	dUrd-αPdUrd	αPdUrd-βPdUrd
10 m*M*	44.1	43.3	40.5	39.0	0.9	0.9	1.0	0.9	1.9	6.3	2.8
15 m*M*	39.1	38.3	35.0	33.2	0.9	0.9	0.9	0.9	2.4	9.0	4.0
20 m*M*	36.9	36.1	32.4	30.6	1.1	1.0	1.0	1.1	2.7	11.1	4.9
25 m*M*	31.9	31.2	27.3	25.5	1.0	1.0	1.0	1.0	2.9	13.8	5.9
30 m*M*	30.0	29.5	25.4	23.5	1.0	0.8	1.0	0.9	2.0	15.1	6.4

TABLE III

ELECTROPHORETIC PARAMETERS FOR THE SEPARATION OF dUrd FROM ITS ANOMER AND ISOMERS WHEN APPLYING DIFFERENT VOLTAGES

Electrolyte, 20 m*M* sodium tetraborate pH 9.5. Other conditions were as described for Table I.

Voltage	Mobility ($cm^2 kV^{-1} min^{-1}$)				Peak symmetry				Resolution		
	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd-dUrd	dUrd-αPdUrd	αPdUrd-βPdUrd
5 kV	34.7	34.0	30.3	28.4	1.0	1.0	1.0	1.0	1.9	9.0	4.5
10 kV	36.3	35.6	31.9	30.1	1.0	1.0	1.1	0.9	2.5	10.8	4.8
15 kV	36.9	36.1	32.4	30.6	1.1	1.0	1.0	1.1	2.7	11.1	4.9
20 kV	37.7	36.9	33.1	31.1	1.0	1.1	1.0	1.0	2.7	10.5	4.6
25 kV	39.4	38.6	34.8	32.9	0.9	0.9	0.8	0.9	2.3	9.3	4.1
30 kV	41.7	40.8	37.0	35.0	1.0	1.0	0.9	1.1	2.0	7.7	3.5

anomer and isomers under the finally chosen conditions. The linearity of the detector was tested in a range of 10^{-3} *M* dUrd (228 μg/ml dUrd) to $3.9 \cdot 10^{-6}$ *M* dUrd (0.89 μg/ml dUrd) using 9 calibration points (27 data points). The following regression curve was obtained: peak area = 1601 + 4481 × concentration (μg/ml); r = 0.9999; standard error of y estimate = 2039; standard deviation of slope = 10.

The limit of detection for a S/N ratio of 3 was approximately 10^{-6} *M* for dUrd using a 3-s hydrodynamic injection. Six injections of this solution showed a R.S.D. of 18.0% on the peak area, which would correspond to a limit of quantification of approximately 2.7 pg (given the apparatus injects approximately 4 nl per s for a 75-μm capillary).

Table V contains the R.S.D. values for repeated hydrodynamic 3-s and 9-s injections within-day and 3-s injections between-day of the dUrd test mixture. The same table also mentions R.S.D. values for a 1-kV 9-s electrokinetic injection of a $1.2 \cdot 10^{-4}$ *M* solution of dUrd in sodium tetraborate electrolyte, which yielded peak areas for dUrd comparable to those for a 3-s hydrodynamic injection. On inspection of the table, one can conclude that within-day repeatability is far better than between-day repeatability and

TABLE IV

ELECTROPHORETIC PARAMETERS FOR THE SEPARATION OF dUrd FROM ITS ANOMER AND ISOMERS AT DIFFERENT TEMPERATURES

Electrolyte, 20 m*M* sodium tetraborate, pH 9.5; voltage, 20 kV. Other conditions were as described for Table I.

T	Mobility ($cm^2 kV^{-1} min^{-1}$)				Peak symmetry				Resolution		
	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd-dUrd	dUrd-αPdUrd	αPdUrd-βPdUrd
20°C	29.4	28.7	25.2	23.4	1.1	0.9	1.1	1.0	2.6	11.9	5.1
30°C	37.7	36.9	33.1	31.1	1.0	1.1	1.0	1.0	2.7	10.5	4.6
40°C	39.4	38.5	34.4	32.4	1.1	0.9	0.9	0.9	1.4	9.4	4.0
50°C	43.6	42.5	38.4	36.4	0.9	1.0	0.9	1.0	2.3	8.4	3.8
60°C	48.0	46.8	42.6	40.6	0.9	0.9	1.0	1.0	2.1	7.5	3.4

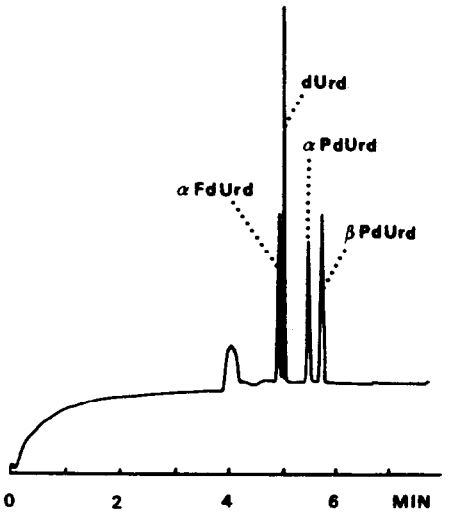

Fig. 2. Capillary zone electrophoresis of a mixture containing dUrd and its anomer and isomers. Electrolyte, 20 m*M* sodium tetraborate, pH 9.5; capillary, fused silica, 70 cm × 75 μm I.D.; temperature, 50°C; voltage, 20 kV; injection, hydrodynamic, 3 s; detection, UV at 262 nm.

hydrodynamic injection is preferable over the electrokinetic mode.

The conditions for dUrd were then applied on a mixture of Thd and its anomer and isomers. Since αFdUrd and dUrd were only poorly resolved and since Thd has a slightly higher pK_a value (9.8) than dUrd, it was decided to raise the pH to 10. This yielded the electropherogram shown in Fig. 3.

Moreover, it is possible to obtain a good separation of dUrd and Thd from their main degradation compounds uracil and thymine, respectively. The same conditions can be used as described above, except for temperature which has to be lowered to 30°C. Electrophoretic parameters are given in Table VI, which shows

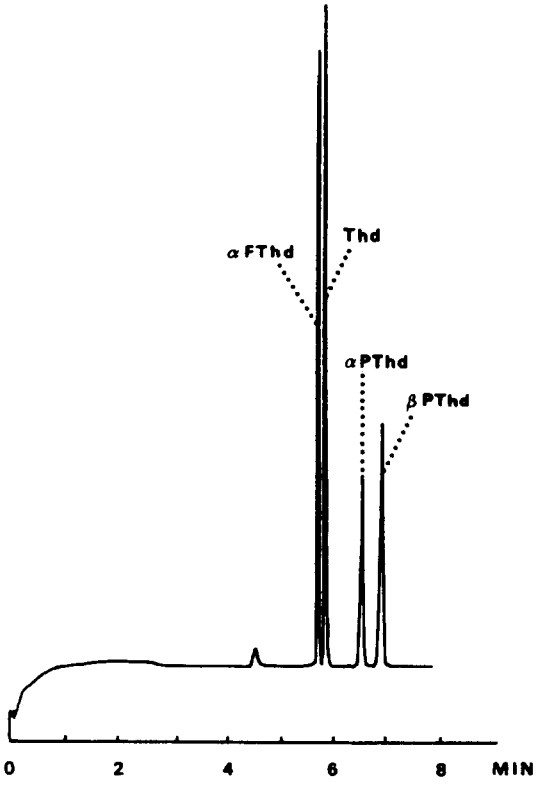

Fig. 3. Capillary zone electrophoresis of a mixture containing Thd and its anomer and isomers. Electrolyte, 20 m*M* sodium tetraborate, pH 10.0; capillary, fused silica, 70 cm × 75 μm I.D.; temperature, 50°C; voltage, 20 kV; injection, hydrodynamic, 3 s; detection, UV at 262 nm.

TABLE V

R.S.D. VALUES OF MIGRATION TIMES AND PEAK AREAS FOR REPEATED INJECTIONS ($n = 9$)

	R.S.D. (migration times) (%)				R.S.D. (peak areas) (%)			
	αFdUrd	dUrd	αPdUrd	βPdUrd	αFdUrd	dUrd	αPdUrd	βPdUrd
Hydrodynamic injection								
within-day, 3 s	0.2	0.2	0.2	0.2	3.0	1.1	1.8	1.0
within-day, 9 s	0.3	0.3	0.4	0.4	1.0	0.2	1.4	1.1
between-day, 3 s	2.1	2.1	2.3	2.4	2.1	1.5	2.8	2.2
Electrokinetic injection								
within-day, 1 kV, 9 s		3.0				3.9		

TABLE VI

ELECTROPHORETIC PARAMETERS FOR THE SEPARATION OF TWO ARTIFICIAL DEGRADATION MIXTURES OF dUrd AND Thd

U = uracil. T = thymine. Electrolyte, 20 m*M* sodium tetraborate pH 9.5 (dUrd) and 10.0 (Thd); capillary, fused silica, 70 cm × 75 μm I.D.; temperature, 30°C (dUrd) and 50°C (Thd); voltage, 20 kV; injection, hydrodynamic, 3 s; detection, UV at 262 nm.

Mobility ($cm^2 kV^{-1} min^{-1}$)					Peak symmetry					Resolution			
αFdUrd	dUrd	U	αPdUrd	βPdUrd	αFdUrd	dUrd	U	αPdUrd	βPdUrd	αFdUrd-dUrd	dUrd-U	U-αPdUrd	αPdUrd-βPdUrd
37.7	36.9	33.8	33.2	31.4	1.0	1.0	[a]	1.0	1.0	2.6	9.1	1.3	4.3
αFThd	Thd	T	αPThd	βPThd	αFThd	Thd	T	αPThd	βPThd	αFThd-Thd	Thd-T	T-αPThd	αPThd-βPThd
35.7	35.0	32.0	30.9	29.1	1.0	1.1	1.0	1.0	1.0	2.2	7.0	2.4	4.1

[a] No separation of peaks at one twentieth of the peak height.

that uracil and thymine migrate between dUrd or Thd and their α-pyranosyl counterparts.

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