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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE PHOTOPRODUCTS OF NUCLEIC ACID COMPONENTS

II*. PHOTOHYDRATES OF PYRIMIDINES**

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SUMMARY

The potential of reversed-phase high-performance liquid chromatography for the separation of various ribo and deoxyribo components of nucleic acids containing photohydrates of pyrimidines has been investigated. The chromatographic properties of the modified nucleobases, nucleosides and nucleotides of uracil and cytosine as well as of the dinucleoside monophosphate, ApU, have been studied. Monomers were separated isocratically in pure water and various water–buffer eluents containing methanol at different pH values. Modified and unmodified dinucleoside monophosphates were separated in acetate buffer with a methanol concentration gradient. The modified compounds have shorter retention times and are readily separated from the original ones. Satisfactory resolution has also been obtained for most diastereoisomeric derivatives of the photohydrates.

INTRODUCTION

When studying the effect of light on nucleic acids one needs efficient methods of separation and analysis of the photoproducts formed. High-performance liquid chromatography, particularly its very popular reversed-phase version (RP-HPLC), is the most powerful analytical method of separation of modified components of nucleic acids (for a review, see, *e.g.*, ref. 1). A number of authors have recently reported the RP-HPLC separation of various monomeric[2,3] and oligomeric[4,5] components of nucleic acids containing pyrimidine photodimers of various types. Besides dimers, the photohydrates of pyrimidines[6] produced in nucleic acids are another

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important type of photoproducts[7,8]. Pyrimidine hydrates are also formed in high yield by γ-irradiation of nucleic acids under de-aerated conditions[9] and in acid- and base-catalysed reactions as intermediates, particularly in the acid-catalysed hydrolysis of glycosylated pyrimidine derivatives[10,11].

Until now, mainly thin-layer chromatography[12–18], column chromatography[19], and rapid high-voltage electrophoresis[20,21] have been used for separating and analysing the pyrimidine photohydrates. The disadvantage of these methods, except for electrophoresis, is their inability to separate modified cytosine derivatives because of their instability. The rapid electrophoresis technique has been used only for separating the phosphorylated derivatives of cytosine (mono- and oligonucleotides). Its application to the separation of non-phosphorylated derivatives (nucleobases and nucleosides) has not been demonstrated.

The possible uses of RP-HPLC for separation of photohydrates have not been extensively studied. The separation of some thymine hydrates, including the *cis*-photohydrate, from other derivatives[22] as well as of a mixture of photohydrate isomers from some unmodified derivatives of uracil has been described[10].

In this paper the use of RP-HPLC for the separation of nucleic acid components irradiated with UV light under conditions appropriate for formation of pyrimidine photohydrates has been systematically studied.

MATERIALS AND METHODS

Equipment

The chromatographic separation was performed in isocratic mode on Models 830 and 850, and in gradient mode on a Model 850, high-performance liquid chromatograph (Du Pont, Wilmington, DE, U.S.A.) equipped with a fixed-wavelength (254 nm) detector and variable-wavelength spectrophotometer. A Zorbax ODS column (250 × 4.6 mm) with a Permaphase ODS precolumn (50 × 4.6 mm) were used. Samples were introduced into the column by means of a Rheodyne 7125 injector with a 50-μl loop. A set of three germicidal resonance low-pressure mercury lamps (254 nm, 15 W) was used as the source of the high intensity UV iradiation.

Chemicals and chromatographic standards

Nucleobases, nucleosides and nucleotides were from Reanal (Hungary) and Lachema (Czechoslovakia). If necessary they were purified by HPLC. Dinucleoside monophosphate, ApU*, was obtained from the laboratory of Dr. S. M. Zhenodarova (Institute of Biophysics, Puschino). Methanol, tetrahydrofuran, potassium phosphate and ammonium acetate were obtained from Reachim (U.S.S.R). Deionized distillled water was used.

Sample preparation

Photohydrates of pyrimidines were obtained by UV-irradiation (254 nm) without deaeration of corresponding non-buffered neutral aqueous solutions of the nucleic acid components. To obtain mainly photohydrates the following optimum irradiation conditions were chosen: low concentrations (10^{-4} *M*) and irradiation tem-

* Abbreviations for the bases, nucleosides and nucleotides are as recommended by IUPAC-IUB.

perature (0°C); short irradiation time (less than 10 min) and low degree of photolysis. Under these conditions photohydrates are known to be the major photoproducts of pyrimidines[23,24].

Chromatographic conditions

The separation was performed at room temperature or at 35°C at an eluent flow-rate of 1 ml/min. The following eluents were used for isocratic separation of monomer components of nucleic acids: deionized water, in some cases with addition of several tenths per cent of tetrahydrofuran; 1% methanol in 50 m*M* KH_2PO_4 (pH 4.5); 1% methanol in 100 m*M* ammonium acetate (pH 6.8). For the separation of modified and unmodified ApU, a methanol concentration gradient in 100 m*M* ammonium acetate (pH 6.8) was used.

Identification of photohydrates

To identify photohydrates we made use of their characteristic property, *i.e.*, reversion to the original substances upon acid or heat treatment[6]. In the case of the uracil derivatives, both photohydrates and possible photodimers are converted by heat, but only photohydrates are converted by acid[24]. So we compared the chromatograms of irradiated samples heated for 1 h at 80°C or unheated and those treated with acid or untreated under similar conditions. For the cytosine derivatives, irradiation of aqueous samples gives rise only to photohydrates, but not to photodimers[24]. In this case only heated and unheated irradiated samples were chromatographed and compared. Upon rechromatography the peaks of the assumed photohydrates disappeared and a peak at the position of the unmodified component increased in intensity. Another independent method of identification of photohydrates is the determination of their spectral properties. Photohydrates of the uracil derivatives are known to show insignificant absorption compared with the original substances, at about 254 nm, and show comparable absorption at about 230 nm[6]. Photohydrates of the cytosine derivatives have analogous spectral properties at acidic pH[6]. At neutral pH, photohydrates of the cytosine derivatives, unlike the unmodified substances, show little absorption at higher than 270 nm, but have characteristic maxima at about 250 nm[6]. Hence, after the separation, the photohydrates were detected at two wavelengths (254 and 230 nm for uracil derivatives, 254 and 230 nm for cytosine derivatives at acidic pH, 270 and 254 nm for cytosine derivatives at neutral pH).

In most cases the repeated separations with detection at 254 nm and at other wavelengths (220–270 nm, every 10 nm) yielded the general pattern of the adsorption spectra of individual peaks. Comparison with the spectra of authentic compounds[6] confirmed the assignments. The photohydrates identified were in fact the major photoproducts (major peaks), their yields being in good agreement with the extent of photolysis of the original components.

RESULTS AND DISCUSSION

The use of pure water as an eluent gives a good resolution of the photomodified from the original unmodified non-phosphorylated derivatives of Ura. Fig. 1 shows the chromatogram of a sample containing dUrd (peak 3) and its photoproducts. The irradiation conditions were chosen to produce photohydrates (too low concentration

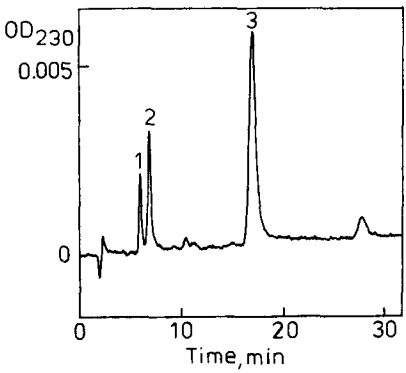

Fig. 1. Chromatogram of dUrd and its photohydrates. Column: Zorbax ODS (250 × 4.6 mm). Eluent: water. Temperature: 35°C. Flow-rate: 1 ml/min. Peaks: 1 and 2 = diastereomers of photohydrates; 3 = dUrd.

for dimer formation). The assumed hydrates have similar retention times, they revert to dUrd upon acid treatment, have similar spectral properties and in accordance with published data[25] have similar yields. So we propose that peaks 1 and 2 correspond to the hydrate diastereoisomers of dUrd differing in orientation of the hydroxyl group photoadded at the 6 position of the base attached to the deoxyribose ring. The isomer in which the hydroxyl group is directed towards the oxygen atom of the deoxyribose ring should in principle be more hydrophobic, since the two polar groups have a partially common hydration shell, *i.e.*, the hydrophobic groups come into contact to a lesser extent with the solvent (so-called "proximity effect"[26]). On the basis of this we suppose that peak 2 corresponds to the isomer of deoxyuridine photohydrate in which the hydroxyl group is oriented towards the ring oxygen atom of deoxyribose. Unfortunately, because of the small quantities of substance available, we cannot experimentally identify the specific type of isomer. Therefore the isomers are indicated conventionally according to their order of elution.

Table I lists the chromatographic parameters of Ura, Urd and dUrd and the corresponding hydrates when water was used as an eluent. The resolution parameter, R_s, which characterizes the resolution of neighbouring peaks, in a few cases cannot be determined because of the significant superimposition of photohydrate peaks. For this reason it is not included in Table I. Unlike the diastereoisomers of hydrates of

TABLE I

CAPACITY, k', AND SELECTIVITY, α, FACTORS FOR THE IRRADIATED MONOMERIC URACIL DERIVATIVES

Eluent: water. Temperature: 35°C. Other conditions as in Materials and Methods. Indices 1 and 2 denote photohydrates, 3 denotes unmodified substances.

	k'_1	k'_2	k'_3	$\alpha_{2,1}$	$\alpha_{3,2}$
Ura	–	0.64	1.43	–	2.23
Urd	0.69	1.05	3.17	1.52	3.02
dUrd	1.33	1.70	5.21	1.28	3.06

glycosylated derivatives of pyrimidines, for Ura (as for Cyt, see below) the enantiomers of the base hydrates are not resolved. The resolution of enantiomers is generally a special problem for which ligand-exchange chromatography on chiral stationary phases is usually employed[27].

Use of water as eluent without the addition of salts and organic solvents makes it possible to obtain a good resolution of the peaks that are of interest to us for Ura and its derivatives not containing ionogenic phosphate groups. Addition of several tenths per cent of tetrahydrofuran, which sometimes improves the separation[3], in our case decreased the resolution of photohydrate isomers and lowered the value of $\alpha_{2,1}$ (not shown).

Use of chromatography with pure water or water–organic eluents is inadequate for cytosine derivatives due to the broadening and tailing of the peaks[28]. For this reason, we used buffered eluents containing methanol for separating the photomodified derivatives of Cyt. The various uracil derivatives were studied under analogous conditions. Separation was performed at different pH values, since as the pH changes the charge on the components also changes due to protonation (deprotonation) of nitrogen bases and phosphate groups, which may affect the peak resolution. However, the stability of hydrates also changes with pH[6,23]. This must be taken into consideration when choosing the optimum separation conditions.

Tables II and III give the chromatographic parameters for pyrimidine derivatives, including nucleobases, nucleosides and some nucleotides, and their photohydrates at pH 4.5 and 6.8. In all cases, good separation of modified components from the unmodified ones is observed; addition of water molecules to nucleobases results in a reduction in the capacity factors of the substances on the reversed phase. Some of the listed pyrimidines were also well separated in the phosphate buffer at pH 5.5 (data not shown). The tables indicate that conditions are available for all the compounds investigated under which a good resolution of photohydrate diastereoisomers is observed.

When considering the changes in chromatographic properties upon hydration of pyrimidines one should take into account at least four factors: saturation of the C5–C6 bond, addition of proton or of hydroxyl and change in conformation (for the

TABLE II

CAPACITY AND SELECTIVITY FACTORS FOR IRRADIATED MONOMERIC DERIVATIVES OF URACIL AND CYTOSINE

Solvent: 1% methanol in 0.05 *M* KH_2PO_4 (pH 4.5). Other conditions as in Materials and Methods. Indices 1 and 2 denote photohydrates, 3 denotes unmodified substances.

		k'_1	k'_2	k'_3	$\alpha_{2,1}$	$\alpha_{3,2}$
Ura	(35°C)	–	0.31	0.95	–	3.06
Urd	(35°C)	0.37	0.64	2.32	1.73	3.63
dUrd	(35°C)	1.00	1.28	4.25	1.28	3.32
pU	(35°C)	0.28	0.37	0.64	1.32	1.73
Cyt	(35°C)	–	0.13	0.54	–	4.33
Cyd	(23°C)	0.16	0.16	2.23	1.00	13.95
dCyd	(23°C)	0.34	0.39	2.50	1.18	6.40
pC	(35°C)	0.10	0.14	0.48	1.40	3.43

TABLE III

CAPACITY AND SELECTIVITY FACTORS FOR IRRADIATED MONOMERIC DERIVATIVES OF URACIL AND CYTOSINE

Solvent: 1% methanol in 0.1 *M* ammonium acetate (pH 6.8). Other conditions as in Materials and Methods. Indices 1 and 2 denote photohydrates, 3 denotes unmodified substances.

		k'_1	k'_2	k'_3	$\alpha_{2,1}$	$\alpha_{3,2}$
Ura	(35°C)	–	0.37	0.77	–	2.08
Urd	(35°C)	0.40	0.60	2.21	1.50	3.68
dUrd	(35°C)	0.86	1.11	3.73	1.29	3.36
pU	(35°C)	*	*	0.12	–	–
Cyt	(35°C)	–	0.16	0.67	–	4.19
Cyd	(23°C)	0.17	0.22	1.74	1.26	7.90
Cyd	(35°C)	0.20	0.24	1.68	1.20	7.00
dCyd	(23°C)	0.38	0.76	3.46	2.00	4.55
dCyd	(35°C)	0.26	0.59	2.66	2.27	4.51
pC	(35°C)	*	*	0.08	–	–

* Retention time close to elution time of an unretained substance, hence an accurate determination is impossible.

derivatives of Ura) due to disappearance of conjugation. The addition of an hydrophilic electronegative hydroxyl group appears to dominate as regards the change in hydrophobicity, leading to its reduction.

The major chromatographic features of the investigated hydrate-containing samples (considerable changes in capacity factors upon hydration, and elution of the hydrated components before the unmodified ones) are in general agreement with the results of Prior *et al.*[10]. They also separated photohydrates of Ura derivatives from the unmodified compounds by RP-HPLC with water as eluent. However, the problem of the separation of diastereoisomers was not studied, attention being focused on the dehydration processes with mixtures of photohydrate isomers.

The existence of diastereoisomers had earlier been shown for hydrates of Urd, Cyd, dThd, pU and Cp[15,25,29,30]. However, as far as we know, the separation of diastereoisomers has been performed only for Urd[19] on the column, and for dThd[15] by thin-layer chromatography. In the present work a satisfactory resolution of diastereoisomers was obtained for all the compounds investigated, including those for which the existence of isomers had not been demonstrated by other techniques (see Tables I–III, Fig. 1 and Fig. 2 showing the chromatograms for dUrd and dCyd and their photohydrates).

As mentioned above, the isomer in which the hydroxyl-group is closer to the oxygen atom of the sugar ring should be more hydrophobic because of the "proximity effect"[26]. For this reason we suppose that the second peak in each chromatogram of glycosylated derivatives corresponds to the photohydrate isomer in which the hydroxyl group is oriented toward the oxygen atom of the sugar ring. Unfortunately we could not identify unambiguously the particular type of isomer and thus verify this assumption. The photohydrates of the uracil derivatives are stable under all conditions investigated[6] (half-life, $t_{1/2}$, not less then 10^4 min). In the course of separation (about 10 min), not more than one tenth per cent of the available photoproducts of the uracil derivatives can decay. The resolution depends only slightly on

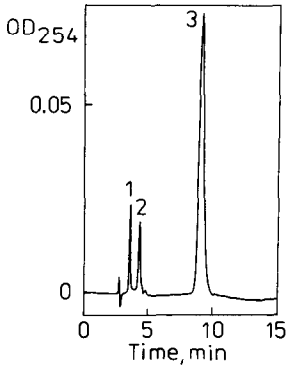

Fig. 2. Chromatogram of dCyd and its photohydrates. Eluent: 1% methanol in 0.1 *M* ammonium acetate (pH 6.8). Column and other conditions as in Fig. 1. Peaks: 1 and 2 = diastereomers of photohydrates; 3 = dCyd.

pH for the bases and nucleosides, and so all the conditions investigated are adequate for separating irradiated non-phosphorylated uracil derivatives. As for irradiated pU, the best resolution occurs at pH 4.5 (all the three expected peaks are resolved). An increase in pH results in a double ionization of the phosphate group, which in turn leads to a reduction in retention time and to worsening peak resolution. The peak of the unmodified component has $k' = 0.12$, and unresolved peaks of photohydrate isomers are eluted practically in the void volume.

For photohydrates of the cytosine derivatives instability becomes noticeable during the separation. Hence this should be taken into account when choosing the optimum conditions for separation, though estimations made on the basis of literature data[6] show that hydrate decomposition will not be great within the elution time of the corresponding peaks (not more than several per cent under all conditions investigated). The best resolution is observed at pH 6.8 for non-phosphorylated cytosine derivatives (see, for example, Fig. 2). This pH corresponds to the maximum stability of photohydrates, where the rate of conversion into the parent substances does not depend on the type of buffer anion; $t_{1/2}$ is about 10^3 min, which means there will not be more than 1% photohydrate decomposition during the separation. The resolution at pH 4.5 is higher for the phosphorylated cytosine derivative (pC); the decay of its hydrate does not depend on the buffer type at all. Although a rise in pH increases the stability of photohydrates of pC, it is seen from Table III that corresponding peaks are less resolved. Therefore we believe that pH 4.5 is more suitable for pC, despite the fact that during the separation several per cent of its photohydrates decay. An increase in stability of the photohydrates under these separation conditions can be attained by cooling the column, the more so as the variation in temperature apparently affects the resolution very slightly (compare lines 6 and 7, 8 and 9 in Table III).

Besides the separation of photohydrated monomeric components of nucleic acids, we probed the possibility of separation of oligonucleotides containing pyrimidine photohydrates. As an example, dinucleoside monophosphate ApU is given. Adenine is quite stable to UV irradiation[24], and dimerization is obviously impossible

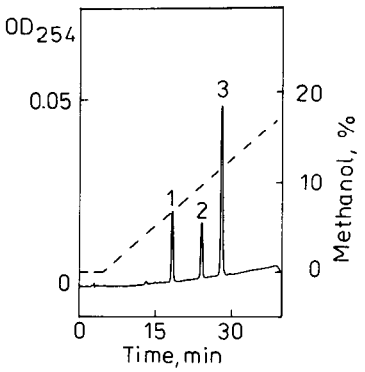

Fig. 3. Chromatogram of ApU after irradiation under conditions appropriate for uridine photohydrates production. Eluent: A, 1% metanol in 0.1 *M* ammonium acetate (pH 6.8); B, 21% methanol in 0.1 *M* ammonium acetate. Gradient: 0–84% B shown with dotted line. Column and other conditions as in Fig. 1. Peaks: 1 and 2 = modified (photohydrated) ApU; 3 = unmodified ApU.

(dilute solution). Thus the major photoproducts of this oligomer are dinucleoside monophosphates containing photohydrates of Ura.

Fig. 3 shows a chromatogram of irradiated ApU. The two major peaks of the modified substance we attribute to oligomers containing diastereoisomers of photohydrates of Urd. The retention time of the modified substance is again reduced. However the value of the resolution parameter, R_s, for the isomers of the photohydrates (about eight) is significantly higher that in the case of monomeric components (*e.g.*, for deoxyuridine photohydrates in Fig. 1, $R_s = 1.48$). Such comparison of R_s for the gradient and isocratic modes of separation seems to be reasonable since the elution times of the substances in question are comparable. The difference in hydrophobicity of the two dinucleoside monophosphates containing the isomers of the hydrates of Urd is explained in part by the same conformational factor as in the case of the hydrates of monomeric compounds, *i.e.*, the orientation of the hydroxyl group toward (or away from) the ring oxygen atom of ribose. In addition, space-filling models indicate that the orientation of this hydroxyl group toward the oxygen atom of the ribose ring means also its interior localization in the structure of the dinucleoside monophosphate. This results in partial shielding of this oxygen from contact with the solvent. The steric factors due to the presence of this oxygen atom lead to a more open conformation of the dinucleoside monophosphate. Either of these factors can increase the hydrophobicity of the substance, which can be classified as more hydrophobic only on the basis of a consideration of the conformations of the uridine hydrates.

The possibility of RP-HPLC separation of hydrates of oligonucleotides is important for model photochemical investigations and for studying the process of photohydration of polynucleotides. Because of the instability of pyrimidine hydrates, the main method of hydrolysis of polynucleotides for quantitative analysis is the enzymatic one yielding short ($n \leqslant 3$) oligonucleotides[31].

The investigations carried out demonstrated that in most the cases one can select the conditions under which good resolution of all peaks expected can be observed (R_s is at least greater than unity, *e.g.*, baseline resolution in Figs. 1–3). The

advantage of the technique described is the rapidity of separation (for monomers, less than 20 min), the peaks of unstable photoproducts being eluted during several minutes. In this respect RP-HPLC is preferred to rapid electrophoresis[20,21] where typical times for photohydrate separation are 20–40 min.

For quantitation of the photoproducts at very low degrees of modification, radioactive labelling is very often used. The observed elution of photomodified compounds before unmodified ones is an advantage in this case. At a reversed order of elution, *e.g.* under normal-phase chromatography conditions, even the usual slight peak tailing of the unmodified component at great excess of its radioactivity would dramatically limit the range of accurate determination of photoproducts[3].

The separation of photohydrates, in particular of their isomers, is indispensable for studying their physical-chemical properties. To study some of these properties one can make use of the data obtained directly from chromatographic separation. Thus, for example, we observed somewhat different stabilities of isomers of photohydrates of dCyd at neutral pH, from the changing ratios of the peak heights in chromatograms of irradiated sample chromatographed in successive periods of time.

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