Journal of Chromatography, 79 (1973) 353–356
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CHROM. 6602

Note

Separation of anomeric pyrimidine nucleosides by thin-layer chromatography

All general procedures for the synthesis of pyrimidine 2′-deoxynucleosides, based on the condensation of an acylated halogeno-2′-deoxyribose with the appropriate pyrimidine derivative, give a mixture of the blocked α- and β-anomers[1–4]. The separation of such anomers usually involves considerable difficulties. The β-anomer is the biologically active form, but it has been shown that yeast RNA contains minor proportions of the α-anomers of cytidine-2′-phosphate and cytidine-3′-phosphate[5], the biological significance of which remains to be clarified. α-Anomers have been reported to be produced by the UV irradiation of nucleosides, nucleotides and calf thymus DNA[6].

Paper chromatography[7] cannot resolve nucleoside anomers, and thin-layer chromatographic (TLC) techniques[8] hitherto used for the separation of anomeric pairs of pyrimidine deoxynucleosides are applicable only to the protected nucleosides[2,9–11] and are therefore of no value for the analysis of materials of biological origin. In the case of one anomeric pair of unblocked purine deoxynucleosides, small differences in R_F values (on Silica Gel G with an ethyl acetate–methanol solvent system) were observed between the purified anomers previously separated by means of ion-exchange column chromatography[12]. More pronounced differences have been reported between the purified anomers of 5-methyluridine[13], by using benzeneboronic acid complexes with the *cis*-glycol system of these ribonucleosides, a method that was initially applied to the separation of ribofuranose from deoxyribofuranose and arabinofuranose[14].

During attempts to separate 5-ethylcytosine from a mixture of the α- and β-anomers of 5-ethyldeoxycytidine by TLC, it was noted that an ether–dimethylformamide solvent system which achieved this separation simultaneously provided a distinct separation of the two deoxynucleoside anomers. This, in turn, prompted a search for other solvent systems that are capable of resolving anomeric keto ribo- and deoxyribonucleosides. Ethyl acetate–methanol and chloroform–methanol were found to be suitable for this resolution and are adequate for analytical and small-scale preparative purposes. They provide simple tools for the analysis of pyrimidine nucleosides of biological origin, for following the purification of anomers by large-scale fractional crystallization, etc.

Materials and methods

Pyrimidines. Uracil (VIII) was obtained from Schuchardt (Munich, G.F.R.) and 5-fluorouracil (VII) was a gift from Hoffman-La Roche (Nutley, N.J., U.S.A.). Both 5-ethyluracil (VI) and 5-ethylcytosine (V) were prepared as described elsewhere[15].

Nucleosides. The preparation of α- and β-5-ethyl-2′-deoxycytidine (Ia and Ib), by condensation of O^2,N^4-bis(trimethylsilyl)-5-ethylcytosine with acylated halogenodeoxyribose, will be described in detail in a subsequent paper. The synthesis of the two anomers of 5-ethyl-2′-deoxyuridine (IIa and IIb) has been reported previously[11]. The

α- and β-anomers of 5-fluoro-2'-deoxyuridine (IIIa and IIIb) were gifts from Dr. J. J. Fox and Hoffman-La Roche, respectively. Uridine (IVb) was a Reanal (Budapest) product, while α-uridine was kindly provided by Dr. J. J. Fox.

Analytical chromatography. R_F values of anomeric pairs of nucleosides, and the corresponding aglycones, were determined on commercial cellulose sheets containing indicator (Chromagram Sheet No. 6065, Eastman, Rochester, N.Y., U.S.A. and DC-Fertigplatten No. 5718/0025 Cellulose F, Merck, Darmstadt, G.F.R.), on silica gel sheets containing indicator (Chromagram Sheet No. 6060, Eastman), and on Silufol UV_{254} silica gel foils (Kavalier, Czechoslovakia) with starch as a binder. Samples of 0.2–0.5 μl (2–5 μg of substance) were deposited as 2-mm diameter spots.

Small-scale preparative chromatography. This was carried out on 20 × 20-cm plates covered with a 1-mm layer of Silica Gel HF_{254} (Merck, Darmstadt) with the aid of a VEB Glasswerke (Ilmenau, G.D.R.) instrument. The plates were dried overnight at room temperature and then activated for 2 h at 120°, following which 2-cm strips were scraped free from the top and sides of each plate. Samples of 20 μl (*ca.* 0.2–0.5 mg) were deposited at a height of 2 cm from the bottom of the plate in the form of a band 2.5 mm wide and 10 mm in length. The plates were developed in a 21 × 12 × 21-cm tank fitted with a ground-glass top, silicone vacuum grease being used to ensure satisfactory sealing of the tank. The tank was lined with filter-paper to a height of 18 cm, and the atmosphere was stabilized for 2 h with 100 ml of the appropriate solvent prior to use. Development was allowed to proceed to the end of the plate. The plate was then dried in a stream of warm air (50°) and, when necessary, developed once again. When this process was repeated n times, the resultant R_F value is given as ${}^nR_F$.

Solvents. The solvents used were *sec.*-butanol (Riedel, Hanover, G.F.R.); isopropanol (for chromatography, UCB, Brussels, Belgium); chloroform and ethyl acetate (*pro analysi*, POCH, Gliwice, Poland), both redistilled at least once weekly; methanol (*pro analysi*, POCH); diethyl ether (*pro narcosi* P.H.P., Pronit, Pionki, Poland, containing 2 % of ethanol); dimethylformamide (VEB Laborchemie, Apolda, G.D.R.); and concentrated ammonia solution (25 %, d = 1.18).

The solvent systems used were as follows (v/v): (A) water-saturated *sec.*-butanol (20°), upper phase; (B) isopropanol–conc. ammonia solution–water (7:1:2); (C) diethyl ether–dimethylformamide (7:3); (D) diethyl ether–dimethylformamide (8:2); (E) chloroform–methanol (85:15); (F) chloroform–methanol (9:1); (G) chloroform–methanol (8:2); and (H) ethyl acetate–methanol (94:6).

Results and discussion

The TLC of microgram amounts of the nucleoside anomer pairs I–IV on commercial cellulose sheets, and the widely applied solvent systems A and B, gave virtually no separation of anomers. Eastman silica gel and $Silufol_{254}$ sheets gave small, but reproducible, differences in R_F values between the α- and β-anomers (Table I). These differences were accentuated appreciably with diethyl ether–dimethylformamide solvents (C,D), giving satisfactory separations for aminodeoxynucleoside anomers (Ia and Ib); and with ethyl acetate–methanol for keto ribo- and deoxyribonucleosides (IVa and IVb). Chloroform–methanol solvents (E,F,G,) gave a reasonable separation of the ketodeoxynucleoside pairs IIa and IIb, and IVa and IVb. It will be noted from Table I that the β-deoxynucleosides migrate faster than the α, whereas the opposite occurs with ribonucleosides.

TABLE I

CHROMATOGRAPHY ON EASTMAN (E) AND SILUFOL$_{254}$ (S) SILICA GEL SHEETS OF α- AND β-ANOMERS OF VARIOUS NUCLEOSIDES AND THEIR FREE BASES

Compound	*R_F value*				
	Solvent system (and type of sheet)				
	C(E)	*D(E)*	*E(E)*	*H(E)*	*H(S)*
α-5-Ethyl-2′-deoxycytidine (Ia)	0.26	0.15	0.14		
β-5-Ethyl-2′-deoxycytidine (Ib)	0.30	0.18	0.15		
5-Ethylcytosine (V)	0.12	0.08	0.17		
α-5-Ethyl-2′-deoxyuridine (IIa)	0.86	0.85	0.40	0.40	0.15
β-5-Ethyl-2′-deoxyuridine (IIb)	0.86	0.85	0.43	0.45	0.19
5-Ethyluracil (VI)	0.89	0.89	0.51	0.59	0.29
α-5-Fluoro-2′-deoxyuridine (IIIa)	0.78	0.76	0.32	0.41	0.18
β-5-Fluoro-2′-deoxyuridine (IIIb)	0.80	0.79	0.35	0.45	0.24
5-Fluorouracil (VII)	0.80	0.82	0.38	0.53	0.33
α-Uridine (IVa)	0.71	0.64	0.25	0.21	0.08
β-Uridine (IVb)	0.71	0.59	0.23	0.19	0.09
Uracil (VIII)	0.77	0.74	0.41	0.41	0.17

Attempts to separate the enantiomers L- and D-thymidine by using the above solvent systems were unsuccessful. Slight differences between the two enantiomers could be observed after 10 or more developments in the same direction, but these differences were insufficient to distinguish the two in a mixture.

The preparative TLC of mixtures of anomeric pairs on activated Silica Gel HF$_{254}$ (Table II), following a number of developments in the same direction, made it possible to separate 0.2–0.5 mg/cm with a layer thickness of 1–2 mm. The best

TABLE II

SEPARATION OF 1:1 MIXTURES OF ANOMERIC PYRIMIDINE NUCLEOSIDES BY MULTIPLE DEVELOPMENT

(a) On an analytical scale, 2–5 μg in the form of a 2-mm round spot, on Silufol$_{254}$ sheets (denoted by S); (b) on a semi-preparative scale, 0.2 – 0.5 mg/cm in the form of a band, on 1–2 mm layers of Merck Silica Gel HF$_{254}$ (denoted by M).

Anomeric mixture	*Components*	*^nR_F value*[a]						
		Solvent system (and type of plate)						
		C(S)	*D(M)*	*E(M)*	*F(M)*	*G(S)*	*H(S)*	*H(M)*
I	α-5-Ethyl-2′-deoxycytidine (Ia)	^{7}0.46	^{9}0.72					
	β-5-Ethyl-2′-deoxycytidine (Ib)	^{7}0.57	^{9}0.80					
II	α-5-Ethyl-2′-deoxyuridine (IIa)				^{10}0.49	^{4}0.80	^{9}0.66	^{3}0.68
	β-5-Ethyl-2′-deoxyuridine (IIb)				^{10}0.56	^{4}0.84	^{9}0.74	^{3}0.76
III	α-5-Fluoro-2′-deoxyuridine (IIIa)			^{14}0.66		^{4}0.67	^{9}0.75	^{3}0.66
	β-5-Fluoro-2′-deoxyuridine (IIIb)			^{14}0.73		^{4}0.71	^{9}0.83	^{3}0.72
IV	α-Uridine						^{11}0.55	
	β-Uridine						^{11}0.63	

[a] The superscript before each R_F value represents the number of developments (n).

separations were obtained for the anomers Ia and Ib with solvent D, for IIa and IIb with solvent H, and IIIa and IIIb with solvent H, using 0.5 mg/cm with a gel layer thickness of 2 mm. Development times at room temperature with these plates were 75–90 min. It is possible that satisfactory preparative separations of the ribonucleoside anomers could be attained with the aid of benzeneboronic acid complexes (see above).

Attempts were made to use the Silufol sheets for small-scale preparative separations. These sheets have the advantage that the time for a single development for a 15 × 15 cm plate with solvents E–G is about 15 min, and with solvents C and D about 30 min. Furthermore, these plates include starch as a binder, which is a good adsorbent for nucleoside chromatography. The TLC of I–IV on these sheets was similar to that with Eastman silica gel, except that the spots exhibited lower mobilities. Because of tailing, these sheets could not be used with solvent systems C and D. In contrast, they gave good separations of the anomers of IV with solvent H.

In general, it will be noted that the separation of anomeric nucleoside pairs proceeds in solvent systems that include a component of low polarity (ethyl acetate, chloroform, diethyl ether) supplemented with a small proportion of a more polar solvent (methanol, dimethylformamide). The samples to be separated must be strongly adsorbed, hence the utility of silica gel. On the other hand, alumina proved to be too strong an adsorbent and was unsuitable for the above purposes. For separations by multiple development, the ${}^{1}R_F$ value should not exceed 0.2 so that, in accordance with the equation for multiple development[16,17], 10–15 developments may be used to achieve good separations.

We acknowledge the skilled technical assistance of Mrs. MARIA ŻYLONIS and Miss MAGDALENA PIWNICKA. This investigation was supported by the Polish Academy of Sciences, and, in part, by The Wellcome Trust, the World Health Organization and the Agricultural Research Service of the U.S. Department of Agriculture.

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1 G. E. HILBERT AND T. B. JOHNSON, *J. Amer. Chem. Soc.*, 52 (1930) 4489.
2 M. PRYSTAŠ, I. FARKAŠ AND F. ŠORM, *Collect. Czech. Chem. Commun.*, 30 (1965) 3123.
3 M. W. WINKLEY AND R. K. ROBINS, *J. Org. Chem.*, 33 (1968) 2822.
4 J. J. FOX, N. YUNG, I. WEMPEN AND M. HOFFER, *J. Amer. Chem. Soc.*, 83 (1961) 4066.
5 H. G. GASSEN AND H. WITZEL, *Biochim. Biophys. Acta*, 95 (1965) 244.
6 R. A. SANCHEZ AND L. E. ORGEL, *J. Mol. Biol.*, 47 (1970) 531.
7 E. VISCHER AND E. CHARGAFF, *J. Biol. Chem.*, 1968 (1947) 781.
8 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, New York, 1963, pp. 185–199.
9 M. PRYSTAŠ AND F. ŠORM, *Collect. Czech. Chem. Commun.*, 31 (1966) 1035.
10 M. PRYSTAŠ AND F. ŠORM, *Collect. Czech. Chem. Commun.*, 31 (1966) 1053.
11 M. ŚWIERKOWSKI AND D. SHUGAR, *J. Med. Chem.*, 12 (1969) 533.
12 M. L. WOLFROM, H. W. WINKLEY AND P. MCWAIN, in W. W. ZORBACH AND R. S. TIPSON (Editors), *Synthetic Procedures in Nucleic Acid Chemistry*, Interscience, New York, 1968, p. 168.
13 J. FARKAŠ, L. KAPLAN AND J. J. FOX, *J. Org. Chem.*, 29 (1964) 1469.
14 E. J. BOURNE, E. M. LEES AND H. WEIGEL, *J. Chromatogr.*, 11 (1963) 253.
15 T. D. KULIKOWSKI AND D. SHUGAR, *Acta Biochim. Pol.*, 18 (1971) 209.
16 A. JEANES, C. S. WISE AND R. J. DIMBER, *Anal. Chem.*, 23 (1951) 415.
17 H. P. LENK, *Z. Anal. Chem.*, 184 (1961) 107.

Received December 19th, 1972