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SEPARATION OF 5-ALKYLURACILS AND PURINE BASES IN HYDROLYSATES OF ENZYMATICALLY SYNTHESIZED NUCLEIC ACIDS BY HIGH-PERFORMANCE ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase and reversed-phase ion-pair chromatographic methods were used to determine 5-alkyluracils in the presence of purine bases (mainly adenine) in the hydrolysates of enzymatically synthesized DNA. The effect of ionic strength, pH, concentration of the ion-pairing agent and methanol on the selectivity between alkyluracils and purine bases was examined in order to simplify the routine work and to reduce the time necessary for analysis. Optimal conditions could be developed for the isocratic separation of the various mixtures obtained by hydrolysis of the products of enzymatic synthesis.

INTRODUCTION

Studies of the incorporation of 5-alkyluracils into polydeoxynucleotides produced during *E. coli* DNA polymerase I catalysed synthesis from dATP and 5-alkyl-dUTP in our Institute[1–4] made it necessary to develop a rapid, routine method for the determination of alkyluracils in the presence of adenine (Ade) and other purine bases in hydrolysates. The hydrolysates always contain a purine base, usually Ade and two alkyluracils. The 5-alkyl substituents on the uracils investigated were hydrogen, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl and *n*-hexyl. The results obtained in the separation of the α- and β-anomers of 5-alkyl-2′-deoxyuridines, the phosphate of which we used in the synthesis, were published in a previous paper[5]. In earlier work ion exchange was used in the separation by high-performance liquid chromatography (HPLC) of naturally occurring[6–8] and modified[9,10] bases by various workers. In our hands the ion-exchange method was only moderately successful, because an impurity peak could not be separated from thymine (Thy) and the time necessary for gradient separation and re-equilibration was unacceptably long.

The development of reversed-phase HPLC opened up new possibilities for the analysis of nucleic acid constituents[11–13]. Ion-pair chromatography has also been used for the separation of the naturally occurring bases[14,15].

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EXPERIMENTAL

Reversed-phase and ion-pair chromatographic work was performed on a Perkin-Elmer Series 3 system with an LC 55 detector at 260 nm. A Sigma 10 Data System was used for peak integration and for calculations of response factors. Separations were achieved with a Hypersil ODS (5 μm) column (25 cm × 4 mm I.D.), and a pre-injector guard column (5 cm × 4 mm I.D.) packed with LiChroprep RP-18 was used. Injections were made with a Rheodyne Model 7120 injector with a 20-μl loop. The mobile phase was degassed ultrasonically. The compounds were dissolved in 0.1 *M* hydrochloric acid.

For the reversed-phase separations the mobile phase was a mixture of 0.01 *M* potassium dihydrogen orthophosphate (pH 5.5) and methanol–water (80:20) solution at a flow-rate of 1.0 ml/min. Gradient elution was necessary. The slope of the gradient was chosen according to the composition of the samples. The analyses were performed at ambient temperature.

In ion-pair chromatography the mobile phase was a potassium dihydrogen orthophosphate buffer–methanol mixture with octyl sulphate as counter ion; pH values were measured and adjusted after methanol had been added. The flow-rate was 1.0 ml/min. The solvent composition and pH were varied (see Results and Discussion). The columns and solvent were thermostated at 25°C with a Knauer water-jacket and an MLW ultrathermostat.

Generally, the samples contained Ade and one or more alkyluracils. All of the 5-alkyluracils tested were prepared and identified in our laboratory.

RESULTS AND DISCUSSION

Reversed-phase chromatography

Chromatography on a Hypersil ODS column was successful (Fig. 1). Satisfactory separations were achieved, using a two-step gradient, as shown in Fig. 1. This gradient caused a slight drift in the baseline and the peak shapes were not fully symmetrical, and Ade showed pronounced tailing. Re-equilibration after the gradient was time consuming (15–20 min). With the usual three-component mixtures steeper gradients were tried but, as the baseline drift became larger, the results became less accurate.

pH values between 2.5 and 6.5 were examined and the peak shape of Ade and its separation from Thy were found to be optimal at pH 5.5. The effect of methanol concentration on the k' values is shown in Table I.

Ion-pair chromatography

In this study guanine (Gua) and cytosine (Cyt) were also investigated, as enzymatic experimentx with Gua and Cyt as bases are also planned.

Effect of concentration of ion-pairing agent on capacity factor. Varying the concentration of the ion-pairing agent from 0.001 to 0.01 *M* and the methanol concentration from 10% to 60% (v/v) gave the results for Gua, Ade and Cyt shown in Fig. 2. The capacity factors of the alkyluracils are not influenced by the ion-pairing agent.

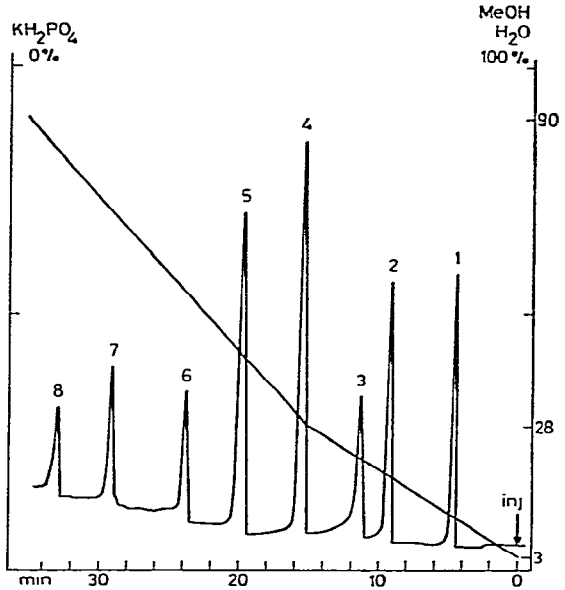

Fig. 1. Gradient separation of adenine and 5-alkyluracils. Peaks: 1 = uracil; 2 = thymine; 3 = adenine; 4 = 5-ethyluracil; 5 = 5-propyluracil; 6 = 5-butyluracil; 7 = 5-pentyluracil; 8 = 5-hexyluracil. The gradient curve is shown. Components: A, 0.01 *M* KH_2PO_4 in water (pH = 5.5); B, methanol–water (80:20). Flow-rate: 1 ml/min.

TABLE I

EFFECT OF METHANOL CONCENTRATION ON CAPACITY FACTORS WITH AND WITHOUT ION-PAIRING AGENT

Mobile phase: 0.01 *M* KH_2PO_4 solution. Concentration of ion-pairing agent: 0 or $5 \cdot 10^{-3}$ *M*.

Compound	*k'*					
	20% Methanol		*40% Methanol*		*60% Methanol*	
	*0**	*$5 \cdot 10^{-3}$**	*0**	*$5 \cdot 10^{-3}$**	*0**	*$5 \cdot 10^{-3}$**
Uracil	0.21	0.27	0.11	0.13	0.09	0.11
Thymine	0.50	0.55	0.20	0.21	0.12	0.14
5-Ethyluracil	1.21	1.27	0.41	0.39	0.20	0.21
5-Propyluracil	3.15	3.29	0.84	0.79	0.31	0.34
5-Butyluracil	9.43	9.36	1.96	1.88	0.60	0.63
5-Pentyluracil	32.96	31.85	4.71	4.57	1.08	1.12
5-Hexyluracil	—	—	12.11	11.44	2.46	2.67
Adenine	0.81	6.75	0.19	1.19	0.22	0.41

* Concentration of ion-pairing agent (*M*).

As alkyluracils show no basic dissociation, whereas the purine bases and Cyt have pK_a values of 3.2–4.6, it is probable that alkyluracils do not form ion pairs in the pH range studied. The capacity factors of Ade, Gua and Cyt increase with increasing concentration of ion-pairing agent, except in solutions containing 60% of methanol. The retention order of Gua and Cyt is reversed if the methanol concen-

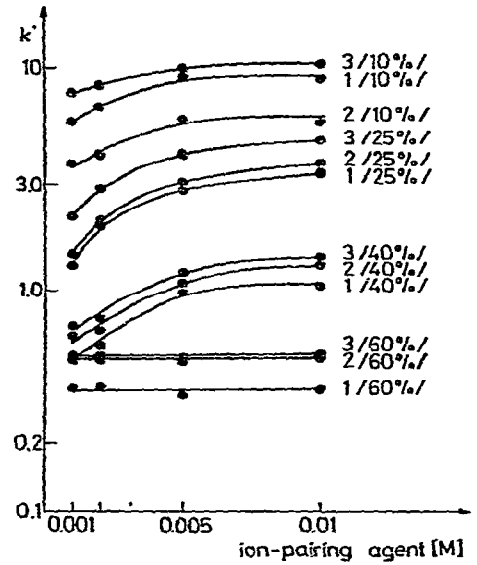

Fig. 2. Influence of concentration of ion-pairing agent at different methanol concentration (in parentheses). Mobile phase: 0.01 *M* KH_2PO_4 (pH = 2.5). Compounds: 1 = guanine; 2 = cytosine; 3 = adenine.

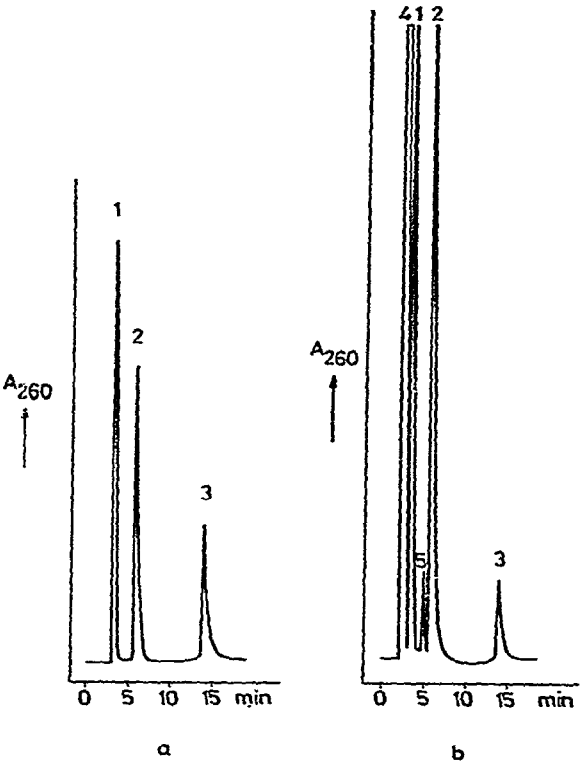

Fig. 3. Chromatogram of standard mixture (a) and hydrolysate (b). Peaks: 1 = thymine; 2 = adenine; 3 = pentyluracil; 4 and 5 = impurities in hydrolysis mixture. Solvent: 0.01 *M* KH_2PO_4 buffer–methanol (60:40) containing 0.005 *M* octyl sulphate (pH = 2.5); flow-rate, 1 ml/min.

tration is changed from 10% to 60%. This difference in solvent selectivity between Gua and Cyt is obviously due to the difference in the two ring systems.

Influence of methanol concentration. As expected from the results of the reversed-phase experiments, the methanol concentration in the mobile phase has a significant effect on the capacity factors, which increase with the number of methylene groups in the aliphatic chains (Table I). If no ion-pairing agent is present, the effect of methanol concentration is significantly different on Ade, Cyt, Gua and alkyluracils. When no ion-pairing agent is added, the variation of k' values of alkyluracils with methanol concentration is the same as in the presence of an ion-pairing agent, whereas the retention of Ade, Cyt and Gua is influenced to a lesser extent than in the presence of ion-pairing agent and the k' values show minima at a methanol concentration of 40%.

Effect of pH. Variation of the pH from 2.5 to 5.0 affects the k' values only of purine bases and Cyt (Table II). Alkyluracils have constant k' values in the pH range studied.

TABLE II

EFFECT OF pH ON CAPACITY FACTORS

Mobile phase: 0.01 *M* KH_2PO_4 solution. Methanol concentration: 25%. Concentration of ion pairing agent 0.001 *M*.

Compound	*k'*			
	pH 2.5	*pH 3.3*	*pH 4.1*	*pH 5.0*
Thymine	0.52	0.55	0.52	0.57
5-Ethyluracil	1.06	1.09	1.09	1.13
Adenine	2.92	3.19	1.34	0.94
Cytosine	2.30	2.90	0.96	0.41
Guanine	1.44	1.60	1.51	0.43

Effects of ionic strength. Variation of the concentration of the buffer from 0.01 to 0.2 *M* has a small effect of the capacity factors. The k' values are slightly lowered in the case of Ade, Cyt and Gua (*e.g.*, for Ade from 1.4 to 0.4) and there is almost no effect with alkyluracils.

The complete selectivity of the ion-pairing agent towards the purine bases and Cyt indicated the possibility of influencing the k' values of these compounds. We could shift their peaks to appropriate positions on the chromatogram relative to the other bases occurring in the hydrolysis mixture. The use of different methanol concentrations for the various mixtures gave the possibility of optimizing the separation times. (Compare Fig. 1 with Fig. 3).

We consider that similar selective ion pairing can also be used in other analytical problems.

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REFERENCES

1 J. Sági, A. Szabolcs, A. Szemzõ and L. Ötvös, *Nucleic Acids Res.*, 4 (1967) 2767.
2 J. Sági, S. Brahms, J. Brahms and L. Ötvös, *Nucleic Acids Res.*, 6 (1979) 2839.
3 J. Sági and L. Ötvös, *Nucleic Acids Res.*, 7 (1979) 1593.
4 J. Sági, R. Nowak, B. Zmundzka, A. Szemzõ and L. Ötvös, *Biochim. Biophys. Acta*, 606 (1980) 196.
5 Á. H. Csárnyi, A. Szabolcs, M. Vajda and L. Ötvös, *J. Chromatogr.*, 169 (1979) 426.
6 P. R. Brown, S. Bobic and F. L. Hanley, *J. Chromatogr.*, 99 (1974) 587.
7 A. Floridi, C. A. Palmerini and C. Fini, *J. Chromatogr.*, 138 (1977) 203.
8 E. H. Edelson, J. G. Lawless, C. T. Wehr and S. R. Abbot, *J. Chromatogr.*, 174 (1979) 409.
9 D. B. Lakings, T. P. Waalkes and J. E. Mrochek, *J. Chromatogr.*, 116 (1976) 83.
10 J. M. Essigmann, W. F. Busby, Jr., and G. N. Wogan, *Anal. Biochem.*, 81 (1977) 384.
11 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 126 (1976) 679.
12 A. Wakizaka, K. Kurosaka and E. Okuhara, *J. Chromatogr.*, 162 (1979) 319.
13 R. A. Hartwick, S. P. Assenza and P. R. Brown, *J. Chromatogr.*, 186 (1979) 647.
14 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
15 E. Melanie and E. Kenneth, *J. Chromatogr. Sci.*, 17 (1979) 531.