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REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY OF PYRIMIDINE AND PURINE DERIVATIVES

I. UNBUFFERED BINARY AQUEOUS ORGANIC MOBILE PHASES ON OCTADECYLSILICA

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SUMMARY

High-performance liquid chromatographic separation of representative pyrimidine and purine derivatives on LiChrosorb RP-18 packing with plain, salt-free aqueous organic mobile phases has been studied. Methanol, acetonitrile, dioxan and tetrahydrofuran were used as the organic modifiers. Quantitative data are given for the dependence of the retention parameters on the mobile phase composition. The different organic modifiers are shown to produce significant changes in selectivity for particular types of compounds.

INTRODUCTION

Reversed-phase liquid chromatography (RPLC) has become a well established method for the separation of pyrimidine and purine derivatives, particularly of the biochemically important nucleosides and nucleobases. Typically, *n*-alkyl (mostly *n*-octadecyl) chemically bonded siliceous supports are used as the stationary phase, while aqueous buffers (acidic to neutral) containing an organic modifier, such as methanol[1–7] or acetonitrile[8–10], serve as the eluent. Unbuffered, salt-free eluents have only occasionally been used: aqueous methanol[11–13], aqueous acetonitrile[14,15] and pure water[11].

As the pyrimidine and purine compounds of interest may contain a large variety of ionic and polar functional groups, it is desirable to be able to manipulate their ionic states, and, hence, additions of buffering or ion-interaction reagents to the eluent may be necessary to create suitable conditions for a specific chromatographic separation. Nevertheless, a more thorough investigation of unbuffered mobile phase systems seems to be useful for two reasons. First, a knowledge of the retention characteristics of such simple systems should serve as the basis for a better understanding of some features of the more complicated ionic and/or ionogenic eluents; secondly, these simple systems are preferable in preparative application.

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The aim of the present work was to investigate the retention behaviour of typical pyrimidine and purine compounds on octadecylsilica with neutral binary aqueous organic mixtures. Four different organic modifiers have been examined: methanol and acetonitrile, because of their widespread use and general utility in RPLC; and tetrahydrofuran and *p*-dioxan, two solvents potentially capable of specific selective interactions[16,17] but hitherto unexplored in the chromatography of nucleic acid constituents.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment comprised the following: a Milton Roy Model 396-57 minipump with Model 709 pulse dampener (Laboratory Data Control, Riviera Beach, FL, U.S.A.); a home-made septum injector permitting syringe injection onto the top of the column packing; a 250 × 4.2 mm I.D. stainless-steel column (LiChroma tubing; Applied Science Labs., State College, PA, U.S.A.); either a fixed-wavelength UV detector (Model 1203 UV III monitor, Laboratory Data Control) operated at 254 nm or a differential refractometer (Model RIDK 101; LaboratorníPřístroje, Prague, Czechoslovakia) and a 5-mV potentiometric recorder (Model EZ 13, Laboratorní Přístroje). The column was packed with LiChrosorb RP-18, mean particle size, d_p = 10 μm (E. Merck, Darmstadt, G.F.R.), using a home-made slurry packing apparatus with chloroform at pressure of a 50 MPa. The measurements were made at 20°C.

The eluents were prepared from deionized water and methanol p.a., dioxan p.a. (Lachema, Brno, Czechoslovakia) or tetrahydrofuran p.a. and acetonitrile p.a. (VEB Laborchemie, Apolda, G.D.R.). The compositions of the binary mixtures, denoted by C, are given as % (v/v), *i.e.*, numerically, as the volume, in ml, of the organic solvent which was mixed with (100 − C) ml water.

The commercially available pyrimidines and purines were purchased either from Lachema or from Sigma (St. Louis, MO, U.S.A.). Other derivatives were prepared in this Institute. The samples were dissolved in methanol–water (1:1) to yield concentrations of 50–200 μg/ml, and were injected into the column in 0.5–2.0 μl volumes, using SGE Type B syringes (Scientific Glass, Melbourne, Australia).

The retention times, t_R, were measured as the distance between the injection point and the peak maximum on the chromatogram. Retention (capacity) factors, k, were evaluated from the relationship:

$$k = (t_R - t_M)/t_M$$

Each k value was obtained from at least four measurements. The mobile phase hold-up time, t_M, was determined from injections of ^2H_2O, as was originally proposed by Karch *et al.*[18]. Table I lists values of V_M, the hold-up volume of the column (dead volume), calculated from t_M and the appropriate volumetric flow-rate, together with the total column porosities, $\varepsilon = V_M/V_c$, where V_c is the volume of the empty column (3.46 ml in this case).

As is seen, differing V_M values were obtained for different mobile phase compositions. This is in general agreement with recent findings of McCormick and Karger[19], who also have given explanations for such phenomena. The marked decrease in

TABLE I

DEAD VOLUME, V_M, AND POROSITY OF THE LICHROSORB RP-18 COLUMN

Eluent	V_M *(ml)*	ε
Methanol–water (20:80, v/v)	2.32	0.67
Methanol–water (10:90, v/v)	2.34	0.68
Methanol–water (2:98, v/v)	2.34	0.68
Acetonitrile–water (8:92, v/v)	2.08	0.60
Acetonitrile–water (2:98, v/v)	2.12	0.61
Dioxan–water (2:98, v/v)	2.05	0.59
Tetrahydrofuran–water (2:98, v/v)	2.08	0.60
Water	1.68	0.49

V_M in pure water is most striking; again, however, similar observations have been made previously[19,20].

RESULTS AND DISCUSSION

When chromatographed on LiChrosorb RP-18 with aqueous methanol, acetonitrile, tetrahydrofuran or dioxane, the various pyrimidine and purine derivatives fell into three groups.

(1) Compounds such as orotic acid, orotidine, barbituric acid, uric acid and 4,6-dihydroxypyrimidine emerged as sharp, narrow peaks with elution times shorter than the mobile phase hold-up time, t_M, *i.e.*, they were excluded from at least part of the intraparticle void volume. These compounds possess pK_a values between 5.7 (uric acid) and 2.1 (orotic acid) (*cf.*, refs. 21 and 22) and, hence, must be present, wholly or predominantly, as anions in the neutral mobile phase. Exclusion of ionized species from *n*-alkyl bonded siliceous packings is known[23–25] and could be eventually exploited as a means for group separations.

(2) Some amino derivatives displayed a distinct tendency to tailing. This is attributed, in accordance with the literature[17,26], to heterogeneous interactions involving both the alkyl chains and the free silanol groups of the packing. Typical values of the asymmetry factor, A_s (defined as the rear to front bandwidth ratio at 10% peak height), were 4–5 for adenine (the worst case) and 2.5–3 for cytosine. More symmetrical peak shapes were observed with the corresponding ribosides and deoxyribosides, and reasonable chromatograms were obtained for such substances. Nevertheless, the retention data were influenced by the concentrations of the solutes (even in the nanogram to microgram range investigated) and for this reason cytosine and adenine derivatives will not be included in the present evaluation.

(3) The other compounds, mostly derivatives of uracil and various oxopurines, gave symmetrical or nearly symmetrical peaks with A_s values of 1.1–1.2. They are listed (together with the abbreviations used) in Table II. It is noted that several substances with amino functionalities belong to this group, such as guanine, 5-aminouracil and 4-amino-6-hydroxypyrimidine. There is no obvious explanation for the different behaviour of these amino compounds compared to that of the adenine and cytosine group mentioned above. Their pK_a values[21,22] do not differ greatly

TABLE II

VALUES OF THE INTERCEPTS, ln k_0, AND SLOPES, A, OF EQN. 1 FOR PYRIMIDINES AND PURINES ON LICHROSORB RP-18

Compound	*Methanol–water**		*Acetonitrile–water***		*Dioxan–water****		*Tetrahydrofuran–water****	
	ln k_0	*A*	*ln* k_0	*A*	*ln* k_0	*A*	*ln* k_0	*A*
2-Hydroxypyrimidine	−0.48	0.079	−0.49	0.230	−0.36	0.240	−0.66	0.240
2-Hydroxy-5-methylpyrimidine	0.86	0.094	0.82	0.258	0.71	0.310	0.10	0.280
Uracil (Ura)	−0.29	0.074	−0.29	0.228	−0.14	0.251	−0.41	0.341
Uridine (Urd)	0.77	0.122	0.85	0.326	0.76	0.381	0.34	0.479
2′-Deoxyuridine (dUrd)	1.35	0.127	1.37	0.346	1.13	0.387	0.72	0.490
1-β-D-Arabinofuranosyluracil	1.30	0.132	1.31	0.372	1.05	0.373	9.65	0.481
2,2′-Anhydro-1-β-D-arabinofuranosyluracil	−0.56	0.105	−0.45	0.244	−0.61	0.261	−0.84	0.401
Thymine (Thy)	1.07	0.090	1.08	0.255	1.08	0.313	0.71	0.410
5-Methyluridine	1.84	0.131	1.85	0.356	1.71	0.427	1.24	0.511
Thymidine (dThd)	2.52	0.151	2.54	0.395	2.12	0.410	1.72	0.590
1-Methylthymine (m^1Thy)	2.25	0.124	2.22	0.308	1.86	0.360	1.22	0.515
5-Hydroxymethyluracil	−0.20	0.097	−0.24	0.264	−0.15	0.297	−0.47	0.390
4-Amino-6-hydroxypyrimidine	−0.41	0.075	−0.36	0.240	−0.21	0.313	−0.46	0.360
4-Amino-6-hydroxy-1-β-D-Rbf-pyrimidine§	1.09	0.137	1.09	0.362	0.82	0.367	0.52	0.519
5-Aminouracil (n^5Ura)	−1.08	0.058	−1.01	0.162	−0.10	0.027	§§	
2-Hydroxypurine	−0.05	0.106	−0.09	0.304	−0.17	0.320	−0.65	0.295
Hypoxanthine (Hyp)	0.52	0.099	0.47	0.294	0.38	0.327	0.06	0.381
Inosine (Ino)	1.88	0.158	1.96	0.446	1.39	0.457	0.95	0.640
1-Methylinosine (m^1Ino)	2.79	0.181	2.83	0.464	2.13	0.520	1.56	0.770
Xanthine (Xan)	0.78	0.108	0.70	0.308	0.65	0.303	0.40	0.439
Xanthosine (Xao)	—	—	—	—	1.90	0.450	1.31	0.620
Guanine (Gua)	0.65	0.107	0.62	0.303	0.92	0.153	0.80	0.060
Guanosine (Guo)	1.98	0.151	2.02	0.422	1.60	0.450	1.12	0.519
2′-Deoxyguanosine (dGuo)	2.24	0.150	2.30	0.434	1.86	0.470	1.49	0.560
1-Methylguanosine (m^1Guo)	2.76	0.161	2.86	0.456	2.46	0.533	1.82	0.653
Isoguanosine	1.72	0.153	1.77	0.402	2.45	0.403	2.03	0.360

* 2–20% (v/v) methanol.
** 1–6% (v/v) acetonitrile.
*** 1–4% (v/v) dioxan or tetrahydrofuran
§ Rbf = Ribofuranosyl.
§§ Non-linear.

(cytosine, 4.4; adenine, 4.2; guanine, 3.2; 4-amino-6-hydroxypyrimidine, 1.4) and all should exist almost wholly as neutral molecules at pH 7. Tentatively, it is suggested that the heterogenous interactions become operative only with molecules of a certain functionality and a certain rigid molecular shape —the "template effect" as discussed by Knox and Pryde[27].

Methanol–water

Experimental ln k values are plotted against C in Fig. 1 for several representative solutes. Although the ln k *versus* C relationship is generally non-linear[28], the present data for the methanol–water system obey a linear relationship over the whole

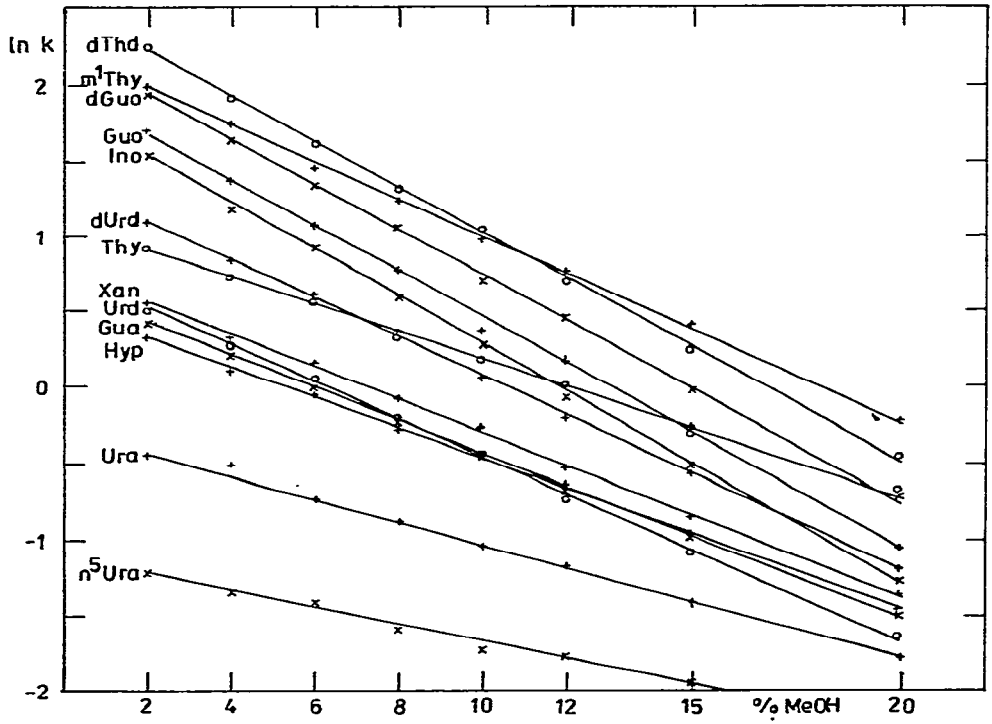


Fig. 1. Plot of ln k values in the methanol–water system against volume percent of methanol (MeOH).

composition range from 20 to 2% methanol, with a high correlation coefficient (typically, $r = 0.98$)

$$\ln k = \ln k_0 - A\,C \tag{1}$$

where A denotes the slope and $\ln k_0$ the intercept of the lines. Values of $\ln k_0$ and A are summarized in Table II.

The dependence of k on the mobile phase composition displays characteristic features for certain compound types. Thus, values of A are very similar for compounds differing only in one functional group (*cf.*, *e.g.*, 2-hydroxypurine, Xan, Hyp and Gua) or in the sugar moiety (Urd, dUrd, arabinosyluracil). On the other hand, nucleosides have systematically higher A values than their parent bases (Urd *versus* Ura, Ino *versus* Hyp, etc.) and this can lead to significant changes in selectivity or even to reversals of the elution order (Thy and dUrd, m¹Thy and dThd, etc.).

It is interesting to compare these results with the comprehensive set of data, of Brown and coworkers[1,2,29]. In spite of the fact that the latter authors used mixtures of acidic phosphate buffers with methanol (and, moreover, different types of octadecylsilica), the overall trends are very similar for solutes common to both sets of data, *i.e.*, mainly uracils and oxopurines. As an example, Fig. 1 of the present study cann be directly compared with Fig. 1 of ref. 29: the relative positions of the lines for Ura, Urd, Hyp, Xan, Ino and Guo are almost the same. It appears that chromato-

graphy of such compounds is scarcely affected by the presence of the buffering salts.

The chromatographic measurements were commenced with higher concentrations of methanol and then proceeded gradually to eluents richer in water; the retention times naturally rose in accordance. On changing from 2% methanol to pure water as eluent the retentions initially increased further and tended to values roughly corresponding to the linear extrapolation of the ln k *versus* C relationship, as established with different contents of methanol. However, as more water passed through the column, the retention times began to fall again and stabilized only after long column equilibration, requiring at least 150–200 column volumes. The steady-state retention times in pure water were even lower than those obtained in 10% methanol and the corresponding k values [calculated with the appropriate (low) value of V_M, *cf.*, Table I] bore no relation to the extrapolated k_0 values. It must be emphasized with regard to Table II that ln k_0 represents merely the intercept of the ln k *versus* C relationship for binary mixtures in the indicated range of compositions and is by no means a measure of the retention in pure water.

The phenomena associated with changing from a mobile phases containing an organic modifier to pure water (and *vice versa*) were quite reporducible provided that the long equilibration times necessary were taken into account. Very similar observations have recently been reported by Scott and Simpson[30], who also noticed the anomalously low retentive characteristics of LiChrosorb RP-18 in water and presented a plausible explanation; they classify LiChrosorb RP-18 as a "brush"-type reversed-phase packing, having free hydrocarbon chains which probably interact between themselves when no organic additive is present in the aqueous mobile phase.

Acetonitrile–water

The ln k values obtained were plotted against the acetonitrile content of the mobile phase and some typical relationships are depicted in Fig. 2. As acetonitrile is a stronger eluent than methanol in RPLC, the useful concentration range is narrower than in the case of the methanol modifier, but even in this range the dependences show a definite curvature. Nevertheless, linear interpolation is fully justified between 6 and 1% acetonitrile, and, hence, the parameters of eqn. 1 have been calculated and are given in Table II for this region. As in the previous case, the ln k_0 values can be regarded only as a mathematical aid in interpreting the retention data, but it may be noted that, for most solutes, they are closely similar for methanol–water and for acetonitrile–water. A full coincidence, of course, would be expected if the underlying relationship, as derived from measurements in the binary mixtures, were valid also when the common component of the two binaries became the single constituent of the mobile phase.

Compared to methanol–water, the slopes (A values) are generally greater for acetonitrile–water, but the differences between individual solutes are similar, the rate of change of retention with the volume fraction of the modifier being more pronounced for nucleosides than for the bases; again, both separation factors and elution orders are affected in this way.

p-Dioxan–water and tetrahydrofuran–water

The results for these two systems will be treated together, as they have common features. For selected solutes, the ln k *versus* C relationships are plotted in Figs. 3 and

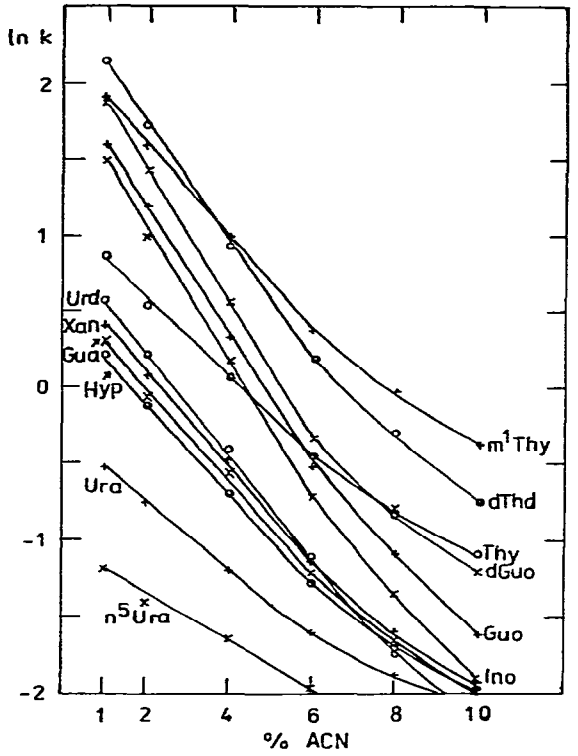
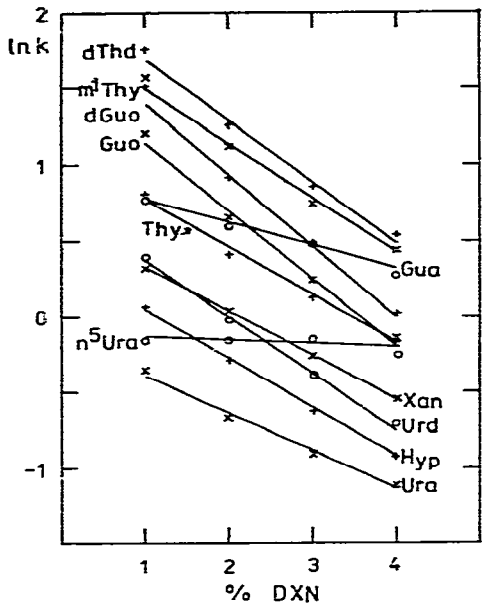


Fig. 2. Plot of ln k values in the acetonitrile–water system against volume percent of acetonitrile (ACN).

4; in Table II, the parameters of the linear interpolation are given for all compounds. The course of the ln k dependence shows a definite curvature even in the restricted composition range available for practical separations; the linear correlation according to eqn. 1 must be regarded only as an approximation. According to Schoenmakers *et al.*[28], the departures from linearity of the ln k *versus* C dependence are always more pronounced for organic modifiers less polar than methanol.

Some similarities between the dioxan–water and tetrahydrofuran–water systems and the two binaries discussed previously are readily apparent from the Figures or can be deduced from Table II, in particular the relative magnitudes of the slopes for simple bases and nucleosides. However, in other respects there are significant differences. Isoguanosine, the retention of which was always lower than that of guanosine (Guo) in methanol–water and acetonitrile–water, becomes one of the most retarded solutes and emerges far behind Guo. The most striking changes apply to Gua and n^5Ura, not only is the retention markedly enhanced over the whole composition range, but, moreover, the character of the composition dependence is altered. For n^5Ura the degree of retention is almost invariable in dioxane–water, whereas with the tetrahydrofuran–water system the retention decreases with increasing water content.

Whereas in methanol–water and acetonitrile–water, guanine always emerged before guanosine, the elution order is reversed with the tetrahydrofuran and dioxan modifiers (although, with the latter, only at $C > 2.5\%$). This is probably the first



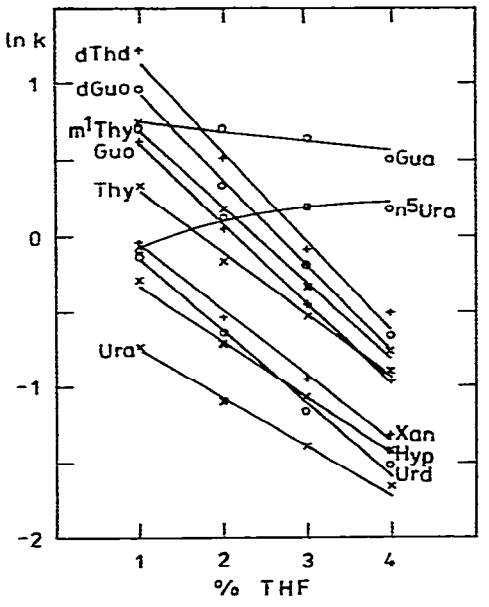


Fig. 3. Plot of ln k values in the dioxane–water system against volume percent of dioxan (DXN).

Fig. 4. Plot of ln k values in the tetrahydrofuran–water system against volume percent of tetrahydrofuran (THF).

known case, in RPLC on octadecylsilica, of a ribonucleoside eluting faster than the parent base. Brown and Grushka[31] have discussed the peculiarity of the typical RPLC retention order of nucleoside/base pairs and attributed the higher retention factors of nucleosides to their ability to form hydrophobic aggregates in the mobile phase. The present result for Gua and Guo seems indirectly to corroborate this; if the "normal" elution order (nucleoside after base) can be altered by a change in the mobile phase composition, then it must be related to mobile-phase interactions and cannot be considered as an inherent property of the alkylsilica stationary phase.

Comparison of the four modifiers

Besides the retention shifts, pointed out in the preceding paragraphs, there are some further differences between the modifiers that affect separation selectivities and are significant enough to have practical importance. In order to compare methanol, acetonitrile, dioxan and tetrahydrofuran it is necessary to normalize the conditions with respect to the water content, as this primarily determines the elution strength. Here, normalization has been done arbitrarily, from the practical point of view. The retention of the thymidine/deoxyuridine pair was taken as the basis for comparison since the separation factor ($\alpha = k_2/k_1$) for these compounds was at least altered by the nature of the organic modifier. In Table III, k values of the pyrimidine and purine derivatives are compared for four different systems, each containing a different modifier and a different water content, but all resulting in $k \approx 3$ for thymidine. (The values were obtained by linear interpolation according to eqn. 1.)

Pertinent information can be easily obtained from Table III. For example, the

ABLE III

ALUES OF THE RETENTION FACTORS, *k*, IN MOBILE PHASES OF COMPARABLE STRENGTHS FOR YRIMIDINES AND PURINES ON LICHROSORB RP-18

ompound	*Methanol–water (9.5:90.5, v/v)*	*Acetonitrile–water (3.5:96.5, v/v)*	*Dioxan–water (2.5:97.5, v/v)*	*Tetrahydrofuran–water (1.0:99.0, v/v)*
Hydroxypyrimidine	0.29	0.27	0.38	0.41
Hydroxy-5-methylpyrimidine	0.96	0.91	0.93	0.83
racil (Ura)	0.37	0.34	0.46	0.47
ridine (Urd)	0.68	0.74	0.83	0.87
-Deoxyuridine (dUrd)	1.15	1.17	1.17	1.26
β-D-Arabinofuranosyluracil	1.04	1.00	1.12	1.18
2'-Anhydro-1-β-D-arabinofurano-syluracil	0.21	0.27	0.28	0.29
hymine (Thy)	1.23	1.21	1.35	1.35
Methyluridine	1.79	1.82	1.90	2.08
hymidine (dThd)	2.97	3.16	3.00	3.09
Methylthymine (m^1Thy)	2.89	3.13	2.61	2.03
Hydroxymethyluracil	0.33	0.31	0.40	0.42
-Amino-6-hydroxypyrimidine	0.33	0.30	0.37	0.44
-Amino-6-hydroxy-1-β-D-Rbf-pyrimidine	0.81	0.83	0.90	1.60
-Aminouracil (n^5Ura)	0.20	0.20	0.85	0.98
-Hydroxypurine	0.35	0.31	0.38	0.39
[ypoxanthine (Hyp)	0.66	0.57	0.64	0.73
iosine (Ino)	1.46	1.49	1.28	1.36
-Methylinosine (m^1Ino)	2.92	3.32	2.29	2.20
anthine (Xan)	0.78	0.68	0.89	0.96
anthosine (Xao)	—	—	2.18	1.99
iuanine (Gua)	0.69	0.64	1.72	2.09
iuanosine (Guo)	1.72	1.72	1.60	1.82
'-Deoxyguanosine (dGuo)	2.25	2.16	1.97	2.53
-Methylguanosine (m^1Guo)	3.42	3.52	3.06	3.22
soguanosine	1.31	1.43	4.22	5.31

biochemically important quadruplet Hyp, Xan, Gua and Urd emerge very close together in methanol–water eluents, buffered[1,29] or unbuffered. Their relative retentions have been recalculated in terms of the separation factors (α values) and are given in Table IV. The reference compound ($\alpha = 1$) was chosen individually for each system so as clearly to display the pairs most difficult to resolve. Two such pairs, with $\alpha = 1.03$, occur in the methanol–water system (Hyp/Urd and Urd/Gua), whereas in acetonitrile–water all four solutes are more separated. In dioxan–water and tetrahydrofuran–water, owing to the selective retardation of Gua, the separation can be enhanced further.

The work reported here has been done on a single type of octadecylsilica. Nevertheless, preliminary experience with another type of reversed-phase packing indicates that the selectivity changes in the separation of nucleic acid components through the choice of the organic modifier have more general validity.

TABLE IV

SEPARATION FACTORS, α, FOR HYPOXANTHINE, XANTHINE, GUANINE AND URIDINE ON LICHROSORB RP-18 IN DIFFERENT MOBILE PHASES (SEE TEXT)

Compound	*Methanol–water (9.5:90.5, v/v)*	*Acetonitrile–water (3.5:96.5, v/v)*	*Dioxan–water (2.5:97.5, v/v)*	*Tetrahydrofuran–water (1.0:99.0, v/v)*
Hyp	0.97	0.89	0.77	0.84
Xan	1.14	1.06	1.07	1.10
Gua	1.03	1	2.07	2.40
Urd	1	1.16	1	1

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