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REVERSED-PHASE LIQUID CHROMATOGRAPHIC RETENTION AND SELECTIVITY SURFACES

II. DEOXYRIBONUCLEOSIDES

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SUMMARY

By plotting capacity factors and selectivity factors as functions of temperature and pH or methanol concentration of the mobile phase, retention and selectivity surfaces of four deoxyribonucleosides were generated. Although changes in the methanol content had little effect on selectivity, changes in pH and temperature could be used to improve selectivity. However, changes in pH or temperature can be coupled with changes in methanol concentration to optimize the analysis time. In addition, by using lines of constant analysis time (isochrons), it was found that several sets of conditions will result in nearly identical retention times. These isochrons can be used to optimize the resolution of the solutes.

INTRODUCTION

Some analogues of nucleosides are known to be powerful antibacterial and antineoplastic drugs[1,2]. Recently, dideoxy analogues have shown potent antiviral properties against the human immunodeficiency virus (HIV); hence these drugs have potential in the treatment of AIDS[3–8]. The dideoxynucleoside analogue 3-azathymidine (AZT) is now one of the few drugs approved for clinical use. However, this drug has toxic side-effects[9] and other dideoxynucleoside derivatives are being synthesized[10]. High-performance liquid chromatographic (HPLC) methods are being evaluated for the isolation and purification of these new chemotherapeutic agents for clinical trials. Methods are needed to determine rapidly the best conditions for the separation on a large scale and for the determination of these drugs and their metabolites for pharmacological studies.

Frequently, optimization of resolution in HPLC requires maximization of the selectivity. The selectivity of a chromatographic system reflects the total of all interactions between solutes, mobile phase components and the stationary phase in the chromatographic system. These interactions can be manipulated by varying experi-

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mental conditions such as temperature or nature of the mobile phase. The selectivity, as it is related to the partition coefficients of the solutes, is a function of the capacity factors (k') of the solutes. Therefore, a good understanding of the retention behavior of the solutes and their dependence on the experimental conditions is essential for the optimization of the selectivity and subsequently of the resolution.

As a first phase of this study, we recently reported on the retention and selectivity surfaces of deoxyribonucleotides in reversed-phase liquid chromatography (RPLC)[11]. In this paper, we report on the use of these surfaces to optimize RP-LC separations of the naturally occurring deoxyribonucleosides that were used as model compounds prior to studies on the dideoxy analogues.

Among the experimental parameters that control the retention behavior of solutes are temperature and mobile phase composition. In our study we examined the RPLC retention behavior of four deoxyribonucleosides at five column temperatures, five methanol concentrations and five pH values. The 125 experiments, which span a four-dimensional space (including the capacity factor dimension), allow us to characterize any interdependences between the experimental parameters.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Waters 6000A pump (Millipore–Waters, Milford, MA, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector and a Waters M440 absorbance detector at 254 nm. Retention times were recorded with an HP 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) and an Omniscribe (Houston, TX, U.S.A.) recorder. The column was a 110 × 4.70 mm I.D. cartridge packed with 5-μm C_{18} packing material (Whatman, Clifton, NJ, U.S.A.). A guard column packed with Whatman pellicular C_{18} material was installed between the injector and the column.

Materials

Deoxyribonucleosides of the highest purity available were purchased from Sigma (St. Louis, MO, U.S.A.). The following deoxyribonucleosides were used as model compounds: 2′-deoxyadenosine (2-dAdo), 2′-deoxycytidine (2′-dCyd), 2′-deoxyguanosine (2′-dGuo) and thymidine (Thd). Stock solutions (20 mM) were prepared and diluted to $2 \cdot 10^{-5}$ M with doubly distilled, deionized water before injection into the chromatograph. The pH of the solutions was adjusted to 6.0 with potassium hydroxide. All stock solutions were stored at −20°C and the working solutions were kept at 4°C. For the mobile phase, HPLC-grade KH_2PO_4 was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Procedures

The aqueous portion of the mobile phase was 0.01 M KH_2PO_4. The pH of the mobile phase was adjusted with phosphoric acid or potassium hydroxide after methanol and the KH_2PO_4 solution had been mixed. In the first set of experiments the pH and the methanol concentration in the mobile phase were held constant and the temperature was systematically changed, in the second set the pH and temperature were kept constant and the methanol concentration was varied and in the third set the

methanol concentration and temperature were kept constant and the pH was changed, giving a total of 125 chromatographic runs. The temperatures were 26, 30, 35, 40 and 45°C, the pH values were 4.0, 4.5, 5.0, 5.5 and 6.0, the concentrations of methanol were 5.0, 6.0, 7.0, 8.0 and 9.0% (v/v), the flow-rate was 1 ml/min and the chart speed was 0.5 cm/min. The detector was operated at 0.01 absorbance units full scale.

Calculations

The void volume was determined using sodium nitrate as a void volume marker. The capacity factors (k') were averages of three measurements. The selectivity factors (α) were determined for adjacent peaks: 2'dAdo–Thd, Thd–2'-dGuo and dGuo–dCyd.

RESULTS AND DISCUSSION

Retention behavior

Before discussing in detail the effects of each parameter on the retention, some general trends were observed. Increasing the temperature of the column or the methanol concentration of the mobile phase decreased the capacity factors of all the deoxyribonucleosides. The pH of the mobile phase had little influence on the retention of Thd, 2'-dGuo and 2'-dAdo; however, the capacity factor of 2'-dCyd decreased as the pH was increased from 4 to 6.

Effects of temperature. The dependence of the capacity factor on the temperature can be written as

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \varphi \qquad (1)$$

where ΔH and ΔS are the enthalpy and entropy of transfer of the solute between the phases, T is the absolute temperature, φ is the column phase ratio and R is the gas constant. Eqn. 1 indicates that, provided ΔH is negative, the capacity factor will

TABLE I

SLOPES AND INTERCEPTS, AS OBTAINED FROM EQN. 1, FOR 2'-dGuo AT VARIOUS pH VALUES AND PERCENTAGES OF METHANOL IN THE MOBILE PHASE

Parameter	*Methanol (%)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	5	−4712	−4425	−4440	−4238	−4666
	6	−5142	−4518	−4796	−4417	−4377
	7	−4129	−4399	−4462	−4149	−4318
	8	−4536	−4295	−4567	−4380	−4372
	9	−4597	−4382	−4446	−4533	−4301
Intercept	5	−14.17	−13.32	−13.34	−12.68	−14.05
	6	−15.83	−13.80	−14.64	−13.43	−13.36
	7	−13.00	−13.54	−13.78	−12.74	−13.35
	8	−14.16	−13.38	−14.24	−13.63	−13.69
	9	−14.51	−13.78	−14.00	−14.29	−13.57

TABLE II

SLOPES AND INTERCEPTS, AS OBTAINED FROM EQN. 1, FOR Thd AT VARIOUS pH VALUES AND PERCENTAGES OF METHANOL IN THE MOBILE PHASE

Parameter	*Methanol (%)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	5	−3412	−3192	−3161	−3180	−3488
	6	−3243	−3190	−3405	−3252	−3137
	7	−3174	−3068	−3165	−2935	−3035
	8	−3212	−3049	−3202	−3058	−3094
	9	−3207	−3099	−3120	−3185	−2990
Intercept	5	−9.79	−9.15	−9.01	−9.07	−9.53
	6	−9.49	−9.31	−9.96	−9.50	−9.16
	7	−9.43	−9.04	−9.38	−8.60	−9.00
	8	−9.69	−9.13	−9.61	−9.15	−9.34
	9	−9.79	−9.41	−9.48	−9.70	−9.07

decrease with increase in the column temperature. All four solutes studied exhibited this behavior in all the mobile phases, independent of the methanol concentration and pH. Tables I–IV show the slopes ($\Delta H/R$) and intercepts ($\Delta S/R + \ln \varphi$) obtained by regression analysis from eqn. 1 for the four solutes in all mobile phases. The correlation coefficients of the regression analysis were typically >0.99 with the smallest value >0.97.

The data for the four deoxyribonucleosides do not show any clear pattern for the slope and intercept as a function of the methanol concentration. This apparent lack of dependence of the slope and intercept on the methanol concentration is surprising in view of the experimentally observed effect of the mobile phase composition. It appears that the temperature dependence of the capacity factor is not large enough (at least not in the present set of experiments) to be able to relate several factors that affect the

TABLE III

SLOPES AND INTERCEPTS, AS OBTAINED FROM EQN. 1, FOR 2'-dAdo AT VARIOUS pH VALUES AND PERCENTAGES OF METHANOL IN THE MOBILE PHASE

Parameter	*Methanol (%)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	5	−4573	−4447	−4661	−4143	−4525
	6	−4291	−4472	−4749	−4762	−4637
	7	−3944	−4362	−4492	−4174	−4482
	8	−4324	−4265	−4593	−4416	−4512
	9	−4265	−4344	−4440	−4541	−4163
Intercept	5	−12.67	−12.26	−12.94	−11.24	−12.51
	6	−11.88	−12.51	−13.36	−13.39	−13.06
	7	−10.97	−12.30	−12.75	−11.73	−12.75
	8	−12.35	−12.16	−13.24	−12.66	−13.01
	9	−12.38	−12.58	−12.89	−13.24	−12.05

TABLE IV

SLOPES AND INTERCEPTS, AS OBTAINED FROM EQN. 1, FOR 2'-dCyd AT VARIOUS pH VALUES AND PERCENTAGES OF METHANOL IN THE MOBILE PHASE

Parameter	*Methanol (%)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	5	−4088	−3676	−3673	−3463	−3765
	6	−3931	−3761	−3933	−3712	−3581
	7	−3601	−3741	−3763	−3367	−3545
	8	−3900	−3665	−3904	−3635	−3579
	9	−3928	−3797	−3886	−3768	−3441
Intercept	5	−12.84	−11.77	−11.94	−11.38	−12.20
	6	−12.47	−12.18	−12.89	−12.33	−12.01
	7	−11.57	−12.20	−12.60	−11.37	−12.06
	8	−12.62	−12.11	−13.11	−12.37	−12.31
	9	−12.85	−12.62	−13.18	−12.96	−11.96

retention. With a wider range of experimental parameters, the interrelationships between these parameters may be established rigorously.

Tables I–III also show that there are no apparent relationships between the value of the slope or intercept for 2'-dAdo, 2'-dGuo and Thd and the pH of the mobile phase. This lack of relationship is not surprising as the capacity factors of these three solutes appear to be independent of pH. With 2'-dCyd the situation is different as its capacity factor is a strong function of the pH of the mobile phase; however, even with 2'-dCyd, as is shown in Table IV, there are no clear patterns in the behavior of the slope and intercept of eqn. 1.

Although there was no clear correlation between temperature effects and mobile phase effects, the slopes and intercepts of the ln k' *versus* $1/T$ plots show distinct differences in the solutes. Table V gives the average slopes and intercepts and the standard deviations. For all solutes the coefficient of variation of the slope and the intercept is 5% or less. The retention order is not related to any order in the slopes or intercepts. The reason for this lack of correlation is the different chemical natures of the solutes. However, the different slopes and intercepts mean that temperature changes can be utilized in the optimization of the selectivity. In Fig. 1, ln k' of 2'-dAdo, 2'-dGuo and Thd are plotted *versus* $1/T$ at a given pH and methanol concentration. Fig. 1 clearly demonstrates that a temperature change, say an increase, can cause either

TABLE V

AVERAGE SLOPES AND INTERCEPTS OF ALL SOLUTES ACCORDING TO EQN. 1

Solute	*Slope ± S.D.*	*Intercept ± S.D.*
2'-dGuo	−4467 ± 208	−13.75 ± 0.61
2'-dAdo	−4423 ± 198	−12.52 ± 0.62
2'-dCyd	−3724 ± 176	−12.32 ± 0.50
Thd	−3176 ± 129	−9.35 ± 0.32

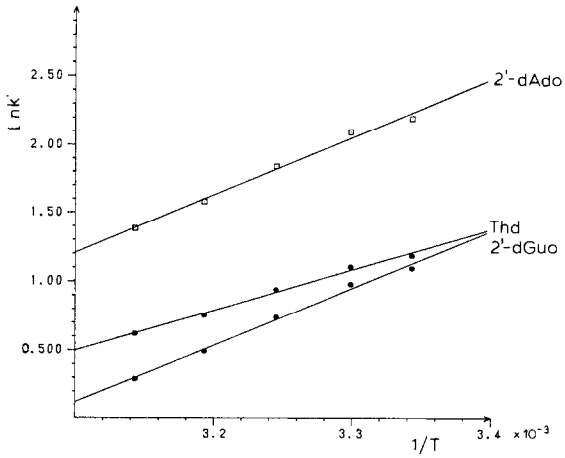

Fig. 1. Plots of ln k' values of 2'-dAdo, Thd and 2'-dGuo as a function of the reciprocal of temperature.

a decrease (between 2'-dAdo and Thd) or an increase (between Thd and 2'-dGuo) in the selectivity. We shall elaborate further on this point in the section dealing with selectivity.

Effect of pH. The only deoxyribonucleoside to be affected by pH changes in the range studied (4–6) is 2'-dCyd. The capacity ratio of this solute decreases with increasing pH at all temperatures and methanol concentrations studied. This decrease is surprising since the N-3 site in its pyrimidine base has a pK_a value of 4.3 and a pH increase above this value makes the molecules less charged. Previously we found that charge formation decreases the retention in the RP-LC of ribonucleosides[12]. Therefore, an increase in k' values would be predicted at pH values greater than 4.3.

All the other solutes investigated were relatively unaffected by pH changes. As Thd only has a basic pK of 9.8, we did not except pH changes to influence the k' values. Also, as the pK of 2'-dAdo is 3.8 and that of 2'-dGuo is 2.5, it was not surprising that the pH did not affect the retention behavior as the molecules are not charged in the pH range 4–6.

Effect of methanol concentration. Over the narrow range of methanol concentrations studied here (5 9%), the general equation that describes the relationships between k' and methanol concentration is

$$\ln k' = b - aX \tag{2}$$

where a and b are solute-related constants and X is the percentage of methanol. The capacity factors of all four solutes decreased as the methanol concentration was increased, irrespective of the temperature and pH.

Tables VI–IX give the values of a and b for the four solutes at all pH values and temperatures. Unlike the results with temperature changes, there are some clear trends in the behavior of the slopes and intercepts. First, at any given temperature and mobile phase composition, the order of the intercepts is 2'-dAdo > Thd ≅ 2'-dGuo > 2'-dCyd, which is the order of the capacity ratios of the solutes. Second, the intercepts decrease as the temperature increases. This behavior is identical with that of the

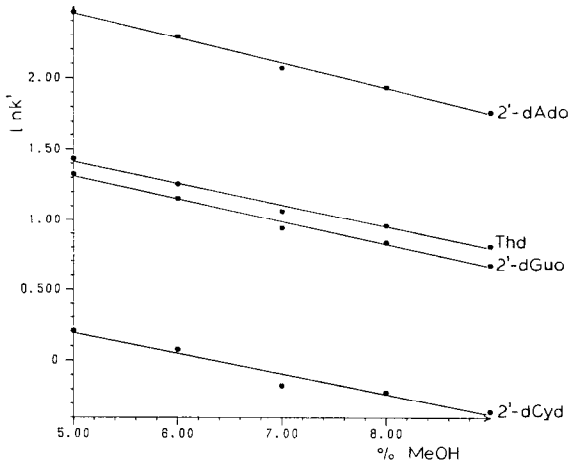

Fig. 2. Plots of ln k' values of 2'-dAdo, Thd, 2'-dGuo and 2'-dCyd as a function of percentage of methanol (MeOH) in the mobile phase. Temperature, 30°C; pH 4.

capacity ratios. Third, for 2'-dCyd, the slopes increase and the intercepts decrease with increasing pH. All other solutes do not show any particular trends in the slope and intercept as a function of pH.

Although the average slopes for 2'-dAdo, Thd and 2'-dGuo (averaged over all temperatures and pHs) are 0.1738 (S.D. 0.0093) 0.1646 (S.D. 0.0109) and 0.1533 (S.D. 0.0098), respectively, the difference is not sufficient to indicate different rates of change of the capacity ratios over the narrow range of methanol concentrations studied. A plot of ln k' *versus* methanol concentration would give nearly parallel lines for the solutes (Fig. 2). Thus the retention of all the solutes is affected similarly by increasing the methanol concentration up to 9%. However, over a large change in methanol concentration, changes in the selectivity are expected to occur. This behavior is

TABLE VI

SLOPES AND INTERCEPTS FOR THE RELATIONSHIP BETWEEN LN k' AND THE METHANOL CONTENT (EQN. 2) FOR 2'-dAdo AT VARIOUS TEMPERATURES AND pH VALUES IN THE MOBILE PHASE

Parameter	*Temperature (°C)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	26	0.181	0.173	0.179	0.169	0.177
	30	0.177	0.158	0.178	0.181	0.191
	35	0.186	0.158	0.164	0.185	0.184
	40	0.171	0.169	0.175	0.182	0.171
	45	0.169	0.157	0.165	0.179	0.166
Intercept	26	3.53	3.50	3.56	3.46	4.50
	30	3.31	3.17	3.34	3.36	3.38
	35	3.12	2.92	2.98	3.14	3.07
	40	2.82	2.80	2.85	2.91	2.78
	45	2.59	2.50	2.55	2.65	2.53

TABLE VII

SLOPES AND INTERCEPTS FOR THE RELATIONSHIP BETWEEN LN k' AND THE METHANOL CONTENT (EQN. 2) FOR Thd AT VARIOUS TEMPERATURES AND pH VALUES IN THE MOBILE PHASE

Parameter	*Temperature (°C)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	26	0.168	0.147	0.154	0.149	0.160
	30	0.165	0.136	0.156	0.157	0.173
	35	0.165	0.137	0.150	0.155	0.161
	40	0.162	0.138	0.153	0.151	0.154
	45	0.158	0.138	0.148	0.147	0.152
Intercept	26	2.41	2.26	2.33	2.27	2.33
	30	2.23	2.02	2.19	2.21	2.27
	35	2.04	1.86	1.96	2.01	2.00
	40	1.87	1.72	1.84	1.82	1.81
	45	1.69	1.57	1.65	1.65	1.65

different from that of changes in temperature, where plots of ln k' *versus* $1/T$ for Thd had substantially different slopes from those of 2'-dAdo and 2'-dGuo.

Combined effects of two experimental parameters. The data acquired allow us to examine the effect of combining two experimental parameters. For example, Fig. 3 shows a retention surface obtained by plotting the experimental capacity factors of 2'-dAdo, at a given pH, *versus* changes in the temperature and in the methanol concentration. The surface was plotted as k' *versus* methanol concentration and T rather than ln k' *versus* $1/T$ and methanol concentration in order to emphasize the curvature of the actual retention surface. This surface is typical of the retention

TABLE VIII

SLOPES AND INTERCEPTS FOR THE RELATIONSHIP BETWEEN LN k' AND THE METHANOL CONTENT (EQN. 2) FOR 2'-dGuo AT VARIOUS TEMPERATURES AND pH VALUES IN THE MOBILE PHASE

Parameter	*Temperature (°C)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	26	0.177	0.158	0.167	0.151	0.170
	30	0.176	0.147	0.164	0.167	0.191
	35	0.177	0.149	0.156	0.168	0.179
	40	0.173	0.150	0.165	0.166	0.167
	45	0.157	0.148	0.161	0.166	0.165
Intercept	26	2.42	2.28	2.36	2.21	2.34
	30	2.21	1.98	2.13	2.16	2.27
	35	1.94	1.75	1.81	1.90	1.92
	40	1.72	1.55	1.66	1.67	1.64
	45	1.35	1.53	1.41	1.46	1.41

TABLE IX

SLOPES AND INTERCEPTS FOR THE RELATIONSHIP BETWEEN LN k' AND THE METHANOL CONTENT (EQN. 2) FOR 2'-dCyd AT VARIOUS TEMPERATURES AND pH VALUES IN THE MOBILE PHASE

Parameter	*Temperature (°C)*	*pH*				
		4	*4.5*	*5*	*5.5*	*6*
Slope	26	0.130	0.122	0.133	0.136	0.154
	30	0.132	0.116	0.143	0.148	0.164
	35	0.142	0.119	0.135	0.154	0.159
	40	0.173	0.119	0.144	0.151	0.151
	45	0.125	0.124	0.143	0.148	0.147
Intercept	26	1.44	1.15	0.99	0.86	0.91
	30	1.29	0.93	0.91	0.80	0.82
	35	1.13	0.74	0.62	0.64	0.56
	40	0.83	0.57	0.50	0.43	0.36
	45	0.63	0.41	0.30	0.24	0.15

behavior of all the solutes at all pHs. The surface has the shape of a tilted sheet with a minimum at the highest percentage of methanol and highest temperature corner and a maximum at the lowest percentage of methanol and lowest temperature corner. As a result of the shape of the surface, isochrons (lines of constant analysis time) can be found if so desired. Stepping along an isochron is accomplished by changing two experimental parameters, which in this example are methanol concentration and temperature. In the surface shown in Fig. 3, the conditions of 5% methanol and 40°C, 7% methanol and 35°C and 9% methanol and 26°C will all result in nearly identical retention times. Such isochrons can be used in the optimization of the resolution of different combinations of solutes[13–15].

Although the general shape of the k'–%methanol–temperature surface is similar for all the solutes studied, the slopes of the surfaces differ between solutes.

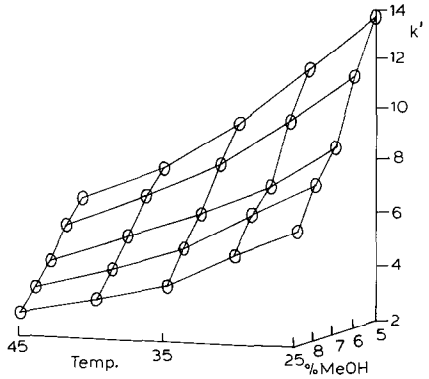

Fig. 3. Retention surface generated by plotting k' values of 2'-dAdo as a function of temperature (°C) and percentage of methanol in the mobile phase. Each circle on an intersection of two or more lines on the surface represents an experimental point.

Therefore, the selectivity between the solutes can be manipulated by changing the methanol concentration and the temperature of the column. The dependence of the selectivity on these parameters will be examined in a later section.

Plotting the effects of the pH and either the temperature or the methanol concentration *versus* k' gives a surface with the shape of an inclined sheet for 2'-dGuo, dAdo and Thd. The incline is in the direction of decreasing methanol concentration. Because of the small effect of the pH on the retention, the isochrons on this surface occur at a relatively constant pH; the surface for 2'-dCyd has the shape of a twisted sheet similar to that in Fig. 3.

Selectivity behavior

Changes in temperature and pH should have a much greater effect on the selectivity than changes in the methanol concentration. We shall first discuss the individual effects of the experimental parameters on the selectivity, then we shall analyze the combined effects of these parameters.

Effect of temperature. From eqn. 1, we can write an expression for the selectivity as a function of changes in the temperature:

$$\ln \alpha = -\frac{\Delta H_2 - \Delta H_1}{RT} + \frac{\Delta S_2 - \Delta S_1)}{R} \tag{3}$$

where α is the selectivity (defined as the ratio k_2/k_1), subscript 2 identifies the more retained compound and subscript 1 the less retained compound.

Eqn. 3 allows us to develop an implicit equation relating the rate of change in the selectivity with changing temperature:

$$\frac{\mathrm{d}\ln \alpha}{\mathrm{d}T} = \frac{1}{RT^2}(\Delta H_1 - \Delta H_2) \tag{4}$$

As ΔH is usually negative, then if $\Delta H_2 > \Delta H_1$, the selectivity improves as the temperature is increased. If $\Delta H_2 < \Delta H_1$ then $\mathrm{d}\ln \alpha/\mathrm{d}T$ is negative and the selectivity deteriorates as the temperature is increased. An example is the selectivity between Thd and 2'-dGuo. Under the conditions in Fig. 1, ΔH for Thd is -5.82 kcal/mol whereas that for 2'-dGuo is -8.22 kcal/mol. According to eqn. 4, the selectivity between these two solutes should increase with increasing temperatures. Fig. 1 shows that the selectivity indeed improves with increasing temperature. The rate of selectivity change with change in temperature is a function of the selectivity itself and, inversely, of the square of the temperature. Therefore, the relative importance of the difference in the enthalpies and $1/T^2$ will determine whether the rate at which α increases with T will increase, decrease or remain constant. In the example discussed above, the improvement in the selectivity "accelerates" with increasing temperature. For these two solutes, it is much better to operate at high temperatures. The selectivity will improve at an increasing rate and the retention times will decrease. However, the resolution will not necessarily increase with increasing temperature, as k' can decrease with increasing T and the efficiency of the column may be affected adversely.

From eqn. 3, we can extrapolate the temperature at which the column is no longer selective ($\alpha = 1$) towards the two solutes Thd and 2'-dCyd. In the example here, this temperature is about 20°C.

Fig. 1 shows that for 2′-dAdo and Thd the selectivity decreases with increasing temperature. For 2′-dAdo the enthalpy is −8.27 kcal/mol. Therefore, $\Delta H_2 < \Delta H_1$ and, according to eqn. 4, the selectivity should indeed decrease with increasing temperature. The temperature at which the selectivity of these two solutes is lost is extrapolated to be about 123°C.

Therefore, any optimization scheme should take into account the possibility that α of neighboring solutes can increase or decrease with changes in the experimental parameters.

Effect of methanol concentration. Using eqn. 2, it can be shown that the dependence of selectivity on the mobile phase composition is

$$\ln \alpha = X(a_1 - a_2) + (b_2 - b_1) \tag{5}$$

where a and b are the constants from eqn. 2 and the subscripts identify the solutes. The selectivity can increase, decrease or remain constant as the mobile phase composition changes:

$$\frac{\mathrm{d}\ln \alpha}{\mathrm{d}X} = a_1 - a_2 \tag{6}$$

If $a_1 > a_2$, then the selectivity will increase with increasing amount of the modifier, whereas the trend will be the opposite for $a_1 < a_2$. For example, for the solutes 2′-dAdo and Thd, at 35°C and pH 5 the slopes a_1 and a_2 are 0.164 and 0.149 respectively and the intercepts $(b_2 - b_1)$ are 2.98 and 1.96, respectively. According to eqn. 6, α decreases with increase in methanol concentration. From eqn. 5, we can extrapolate that at 68% methanol, α will be 1 and at higher methanol contents the retention order will be reversed. This extrapolation assumes that eqn. 2 is correct over a wide range of methanol concentrations. For the methanol concentration range studied in this work, the selectivity does not vary much for any of the solute pairs.

Effect of pH. With the exception of selectivities involving 2′-dCyd, pH changes should not greatly affect the α values. It will be shown shortly that this is so.

Combined effects of two experimental parameters. The outcome of changing two experimental parameters can be important in terms of the selectivity. The complexity of the selectivity surface is a function of the retention behavior of the individual solutes. When the experimental parameters concerned are temperature and methanol concentration, the greatest change in the selectivity surface is usually in the temperature direction. A typical surface is the selectivity between Thd and 2′-dGuo (not shown but easily constructed from the data in the tables). The surface is an inclined sheet, with α increasing with increasing temperature. In the temperature direction the selectivity changes from 1.05 at 26°C to 1.35 at 45°C. In the methanol direction the surface is flat. Hence the methanol axis is not a factor in the optimization of the selectivity between these two solutes, although it may be of great importance with regard to the analysis time.

The selectivity surface between 2′-dAdo and Thd (not shown) can be approximated by a curved inclined sheet. In the methanol direction there is a shallow maximum that occurs at around 6% methanol. However, unlike the previous case, α increases with decreasing temperatures.

Similar behavior is illustrated in Fig. 4, which shows the selectivity surface

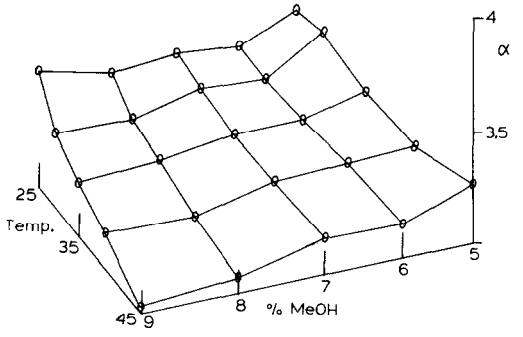

Fig. 4. Selectivity surface generated by plotting α values of 2′-dGuo–2′-dCyd as a function of temperature (°C) and percentage of methanol in the mobile phase at a mobile phase pH of 6.8. Each circle on an intersection of two or more lines on the surface represents an experimentally determined α value.

between 2′-dGuo and 2′-dCyd as a function of changes in temperature and mobile phase composition. The surface has the shape of a twisted sheet, *e.g.*, α increases when the temperature and methanol concentration decrease. The highest selectivity occurs around 26°C and 5% methanol.

These surfaces allow the optimization not only of the resolution but also of the analysis time. From Fig. 4, the lines of constant selectivity can be found. Therefore, once the desired selectivity has been found, the experimental parameters can be changed to retain a constant selectivity at the minimum analysis time.

The surfaces that were obtained by changing the pH of the mobile phase and the temperature can have similar shapes to the temperature–methanol surfaces. For Thd–2′-dGuo the surface (not shown) is an inclined sheet that is flat in the pH direction, with α increasing with increasing temperature. For 2′-dAdo–Thd the surface (not shown) is a curved inclined sheet. In the pH direction there is a shallow maximum around pH 5. In the temperature direction, α increases significantly.

The selectivity surface for 2′-dGuo–2′-dCyd is shown in Fig. 5. The influence of the pH is much more important than for the previous two surfaces. The large change in the retention of 2′-dCyd as a function of pH is manifested by a very large effect on the selectivity. In fact, the changes in the temperature direction are dwarfed by the increase in the pH direction.

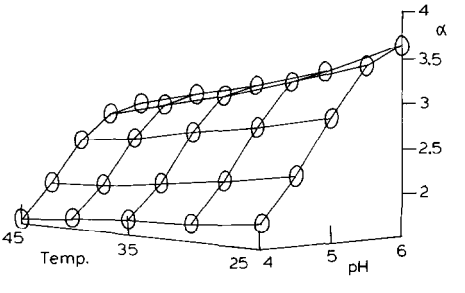

Fig. 5. Selectivity surface generated by plotting α values of 2′-dGuo–2′-dCyd as a function of temperature (°C) and mobile phase pH in a mobile phase that contains 9% methanol. Each circle on an intersection of two or more lines represents an experimentally determined α value.

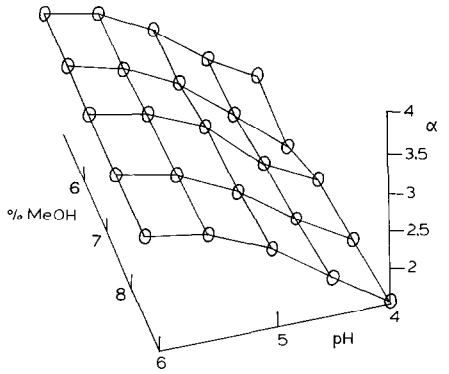

Fig. 6. Selectivity surface generated by plotting α values of 2′-dGuo–2′-dCyd as a function of mobile phase methanol concentration and pH at a temperature of 45°C. Each circle on an intersection of two or more lines represents an experimentally determined α value.

For the pH–methanol content axis, the behavior of the surfaces can be predicted from the previous discussion. Thus, for Thd–2′-dGuo the surface is essentially flat with a very gentle increase in α as the methanol concentration increases. For 2′-dAdo–Thd the surface is also essentially flat with very shallow maxima in both directions (at pH 5 and 6% methanol). Finally, as shown for 2′-dGuo–2′-dCyd in Fig. 6, the selectivity surface increases with increasing pH.

CONCLUSIONS

The optimum conditions as chosen from the retention and selectivity surfaces for the deoxyribonucleosides are temperatures of 35–40°C, pH 5.0–5.5 and a methanol concentration of 7%. A chromatogram of a separation at 40°C, pH 5.5 and 7%

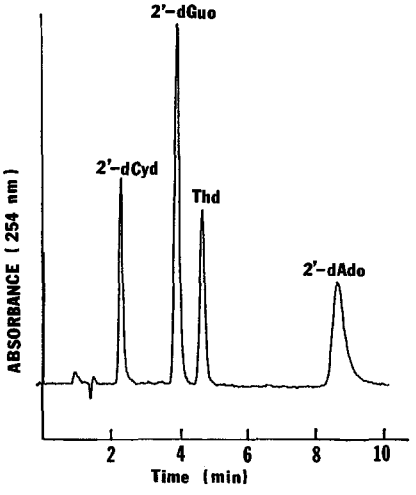

Fig. 7. Chromatogram of the four deoxyribonucleosides in order of retention time: 2′-dCyd, 2′-dGuo, Thd, 2′-dAdo. Conditions: temperature, 40°C; pH, 5.5; methanol concentration, 7%; flow-rate, 1.0 ml/min.

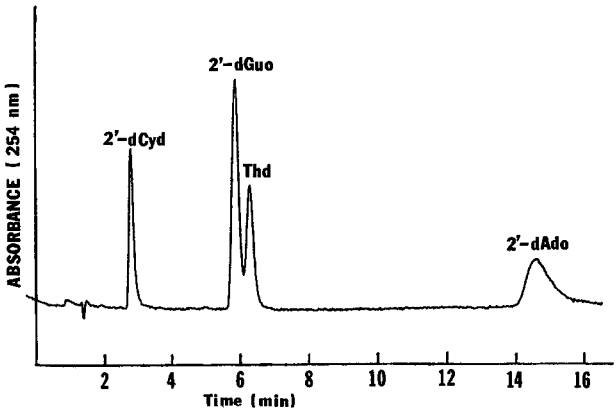

Fig. 8. Chromatogram of the four deoxyribonucleosides in order of retention time as in Fig. 7. Conditions: temperature, 25°C; pH, 5.5; methanol concentration, 7%; flow-rate, 1.0 ml/min.

methanol is shown in Fig. 7. A chromatogram in which the mobile phase is the same but the temperature is 25°C shows that the resolution of 2'-dGuo and Thd is decreased and the retention time of 2'-dAdo is greatly increased (Fig. 8). The selectivity can be changed by appropriate manipulation of the experimental parameters. More important, the experiments described indicate which experimental parameters are important in the optimization of selectivity and resolution of the RP-LC separation of deoxyribonucleosides. In the present instance, it is clear that changes in the methanol concentration have little effect on the selectivity of the system. For improvement in the selectivity, changes in pH and temperature are much more important. It is possible, however, that adjusting only one parameter, such as the pH or the temperature, will be sufficient to achieve an acceptable separation. Changes in the methanol concentration can then be coupled with changes in the pH or in the temperature to manipulate the analysis time. Therefore, lines of constant selectivity on the surfaces may indicate the optimization not only of the resolution but also of the analysis time.

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