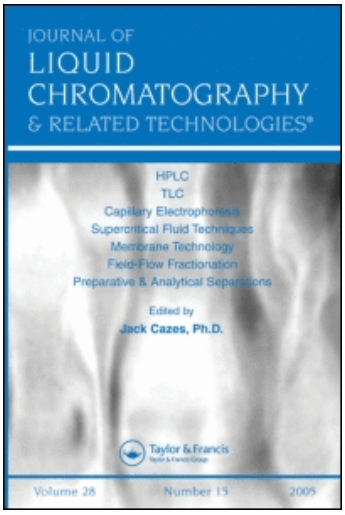

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John P. Caronia[a]; Jonathan B. Crowther[a]; Richard A. Hartwick[a]
[a] Department of Chemistry, Rutgers University, New Brunswick, N. J.

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REVERSED-PHASE SEPARATION OF THE MAJOR DEOXYRIBONUCLEOSIDES AND THEIR MONONUCLEOTIDES USING TETRABUTYLAMMONIUM HETAERONS

John P. Caronia, Jonathan B. Crowther

and Richard A. Hartwick

Department of Chemistry

Rutgers University

New Brunswick, N.J. 08854

ABSTRACT

Simultaneous separation of the four major deoxyribonucleosides and their monophosphate nucleotides was achieved using tetrabutyl ammonium phosphate hetaerons with a reversed-phase (C8) packing material. Baseline resolution for all eight solutes was achieved within 48 minutes, using a 7.5% methanol mobile phase, 2.0 mM in TBA, buffered with 50 mM phosphate at pH 4.8. The effect of methanol and TBA concentrations upon the retention of neutral and anionic solutes was studied in detail. It was determined that changes in solute k' with increasing methanol could be explained by essentially independent phenomena. These are: 1) a decrease in the partition coefficient of the TBA cation with increasing organic concentration, resulting in lower surface charge densities, and 2) a decrease in the hydrophobic interactions of the solutes with the reversed-phase HPLC. The overall effect was a log-linear decrease in k' with increasing methanol concentration. An empirical equation was derived for the above model which was found to be helpful in determining the optimal separation conditions for the nucleosides and nucleotides.

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INTRODUCTION

The simultaneous separation of the nucleosides and their mononucleotides is of significance in many areas of nucleic acid research. Reversed-phase HPLC has become the method of choice for the separation of the nucleosides and their bases (1-2), while ion-exchange is most effective for nucleotide analyses (3). Some success has been achieved in the simultaneous separation of nucleosides and nucleotides using polystyrene based exchangers (4). Such resins however exhibit inherently poor mass transfer properties, resulting in slow analyses and poor efficiencies.

Ion-pairing, or hetaeric HPLC as coined by Horvath (5-7), is uniquely suited for the simultaneous separation of both neutral and ionic solutes (8-11). The apparent separation mechanism accounting for this unique selectivity is the adsorption of hetaerons to the stationary phase (12-15), resulting in a mixed-mode phase with significant ionic and hydrophobic properties. Such dynamic ion-exchange resins produce unique selectivities for separations such as the catecholamines and their metabolites (16,17) and would seem to be equally suited for the simultaneous analysis of the nucleosides and their mononucleotides.

Theory

Relatively few systematic studies into the effect of organic modifier concentration in ion-pair separations have been presented. Therefore, a systematic investigation into

the changes in k' occuring with changes in methanol and tetrabutylammonium phosphate concentrations on a reversed-phase (C8) system for the the deoxyribonucleosides and their nucleotide monophosphates was undertaken. It has been found that the surface concentration of the hetaeron decreases with increasing methanol (18-20). This results in a decreasing retention for ionic solutes (14). At the same time, a neutral molecule normally separated by hydrophobic mechanisms will also decrease retention, usually in a log-linear form (18-20). The net result is a decrease in retention of both neutral and ionic solutes with increasing methanol but for different mechanistic reasons.

If the k' contributions from ionic and hydrophobic forces are essentially independent, then the total k' can arise from two contributions;

$$k'_{OBS} = k'_{HYD} + k'_{IONIC} \qquad \text{Eq. 1}$$

It is well documented that the first part of equation 1 can be expressed as;

$$k'_{HYD} = k'_{o} e^{A \cdot C} \qquad \text{Eq. 2}$$

where A is the slope of the decrease in k' with increasing organic, and k' is the intercept.

The latter half of equation 1 represents the ionic contribution of a solute/hetaeron pair. This can be described in terms of hetaeron surface concentration, or

$$k'_{IONIC} = \Theta(C_s) \qquad \text{Eq. 3}$$

where θ is some interaction index, and Cs is the stationary phase concentration of adsorbed hetaeron. This adsorbed surface concentration of hetaeron (or TBA in this experiment) can be accurately predicted by the Freundlich isotherm;

$$C_s = \alpha \cdot C_m^{\beta} \qquad \text{Eq. 4}$$

The basic Freundlich equation concerns the increase in Cs with Cm, the mobile phase concentration of TBA, at constant methanol. It was found experimentally in this study that the intercept of a ln-ln plot of the Freundlich isotherm decreased exponentially with methanol, but that the slope was constant within experimental error. Thus;

$$\alpha = \alpha_o \cdot e^{a \cdot C} \qquad \text{Eq. 5}$$

where a and α_o are the slope and intercept of a plot of the ln α versus the percent methanol.

The above equations are straightforward and are based upon normal adsorption behavior for small molecules on a solid surface. In sum they should describe the decrease in surface TBA concentration as a function of methanol concentration. A further empirical refinement was necessary. The slope of the k' versus Cs plot for each solute was not constant with increasing methanol, but rather decreased exponentially. Thus,

$$\theta = \theta_o e^{\nu \cdot C} \qquad \text{Eq. 6}$$

where Θ_o is the intercept of $\ln \Theta$ versus methanol percent, and ν is the slope. With this final refinement, the overall equation for k' as a function of methanol and TBA mobile phase concentration becomes;

$$k'_{OBS} = k'_o e^{A \cdot C} + \Theta_o e^{\nu \cdot C} (\alpha_o \cdot e^{a \cdot C} \cdot C_m^{\beta}) \qquad \text{Eq. 7}$$

Equation 7 describes the retention of a solute in terms of seven system constants. Three of these, α , β and a, are basic thermodynamic constants for a given pairing ion/stationary/mobile phase combination and need be determined only once. Of the remaining four, k_o' and A are constants for a given solute on a simple reversed-phase system and have been discussed in the literature previously (18-20). The remaining two, Θ_o and ν , are solute specific for a given pairing ion system.

Equation 7 was found to fit quite accurately the retention of the deoxyribonucleosides and their monophosphate nucleotides from 0 to 15% methanol concentrations. While equation 7 is empirical, it is nevertheless useful in optimizing and predicting the qualitative and quantitative effects of the two primary variables in the ion-pair system.

EXPERIMENTAL

Instrumentation

Chromatographic instrumentation consisted of a Waters M 6000 pump, (Waters Associates, Milford, MA) and either a

Waters U6K injector or a Rheodyne 7125 injection valve (Rheodyne Inc., Cotati, CA). The detectors used were either a Waters M 440 detector at 254 nm or a Kratos FS 770 variable wavelength detector (Kratos Analytical Instruments, Ramsey, NJ). Detection of TBA breakthrough curves was accomplished using a detection wavelength of 222 nm., while 254 nm was used for detection of the nucleosides and nucleotides. Data were recorded using either strip chart recorders, or a Hewlitt-Packard 3390A electronic integrator (Hewlitt-Packard, Avondale, PA). A Haskel DSTV 300 pneumatic intensifier pump was used for the packing of all columns.

Columns

Whatman Partisil 5-C8 packing material (Whatman Inc., Clifton, N.J.) was used throughout the ion-pair studies. Particle size for all experiments was 5 micron. The columns were packed in the laboratory using the slurry method. For the 10 cm columns, 1.2g of material was slurried in 10 ml of isopropyl alcohol (IPA), and was packed upwards at 5000 psi pressure for 10 minutes using acetone as the packing solvent. Without releasing the pressure, the columns were inverted, and packed downwards for another 5 minutes. The pressure was shut off with a high-pressure valve, and the apparatus allowed to equilibrate for 5-10 minutes before removal of the column. After assembly, columns were washed and equilibrated using 60% methanol/water mixtures and were tested for reproducibility of k' values and for efficiencies

using test solutes of interest. Commercially prepacked columns were also used, in which cases the accuracy of k' values were again confirmed.

Preparation of Reagents and Chemical Standards

The eluent used consisted of HPLC grade methanol (Burdick & Jackson,Muskegon MI), double distilled water, with a 0.05M buffer prepared from ACS grade KH_2PO_4 (Fisher Scientific Co., Fairlawn NJ) and NaOH (Fisher) at a pH of 4.80. The concentration of buffer remained constant for all the eluents. The pairing ion used was tetrabutyl ammonium phosphate (TBAP) (Eastman Kodak Co, Rochester NY). All eluents were filtered through 0.45 micron membrane filters, then degassed with helium before use. Small changes in total ionic strength occurred as the concentration of TBA was varied. Such changes were minimized through the use of relatively high ionic strengths. Working standards of the solutes were prepared at 0.025mg/ml.

Determination of Adsorption Isotherms

Frontal elution breakthrough curves were used in order to determine directly the adsorption of the pairing ion TBA on the stationary phase surface. Absorbance measurements at 222nm proved to be sufficiently sensitive to allow for the monitoring of eluting TBA concentration profiles. The identity of the breakthrough fronts were confirmed by using a simple water-methylene chloride extraction system. An anionic dye (in this case a water soluble blue ink)

partitioned into the organic phase in the presence of TBA. When determining the breakthrough volumes, a solvent system was first run with a given percent methanol and buffer but no TBA ion. The ion-pair containing eluent made from the same batch of methanol-water buffer was then flushed through the system up to the column union. Initial time was taken as the start-up of the carefully calibrated pump.

RESULTS

Effect of Pairing Ion Concentration on Retention

The k' values of the nucleosides and their monophosphate nucleotides were found to be a linear function when plotted against the surface concentration of adsorbed TBA, as shown in Figure 1 for the nucleoside dGuo, and the nucleotide dAMP. This direct relationship between adsorbed hetaeron and k' has been reported previously (14). A maximum in k' is observed for the charged solutes, followed by a decrease in retention. It is likely that this maximum results from mobile phase pairing, fitting the general model presented by Horvath et al (5-7). However, in the region prior to this maximum, i.e., up to about 0.18 umole m for dAMP, or 4-5 mM TBA in the mobile phase, it seems likely that the dynamic ion-exchange model most accurately describes the data (12-15). The slope of this curve is labelled as θ . It is interesting to note that the k' values of the nucleosides decrease linearly, while the k' of the charged nucleotides increase linearly with surface TBA concentration.

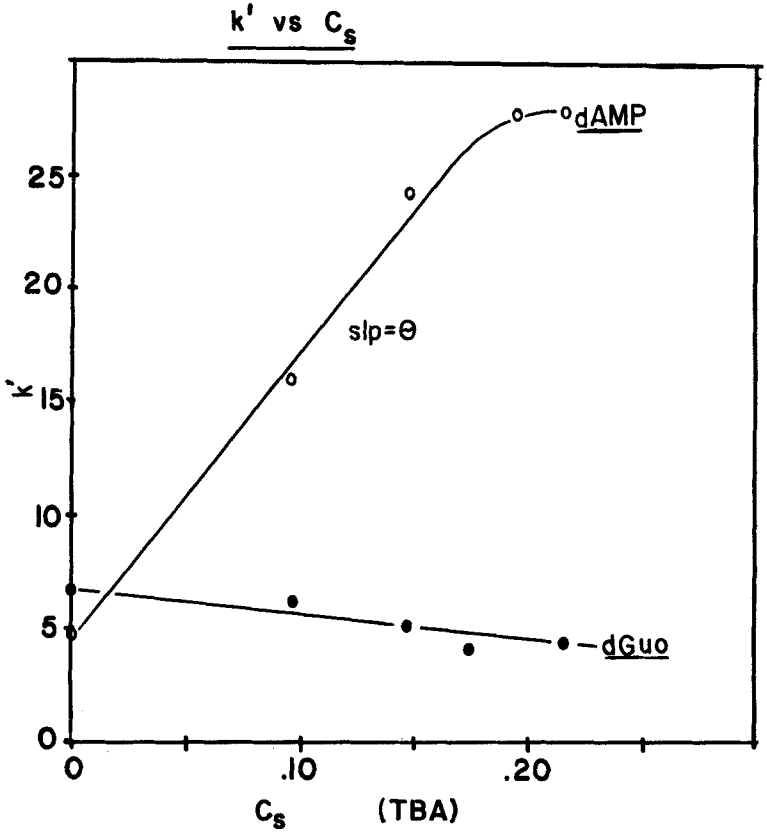


Figure 1

Plot of k' vs. concentration of pairing ion Cs, on the stationary phase, for the negatively charged nucleotide dAMP, and the nucleoside dGuo (for conditions see experimental).

Effect of Methanol Concentration

The k' values for all eight of the nucleosides and nucleotides were found to decrease logarithmically with increasing volume percent of methanol in the mobile phase. Figure 2 shows this log-linear relationship for the dAMP solute over the range of 0-10% (v/v) methanol/water, using a

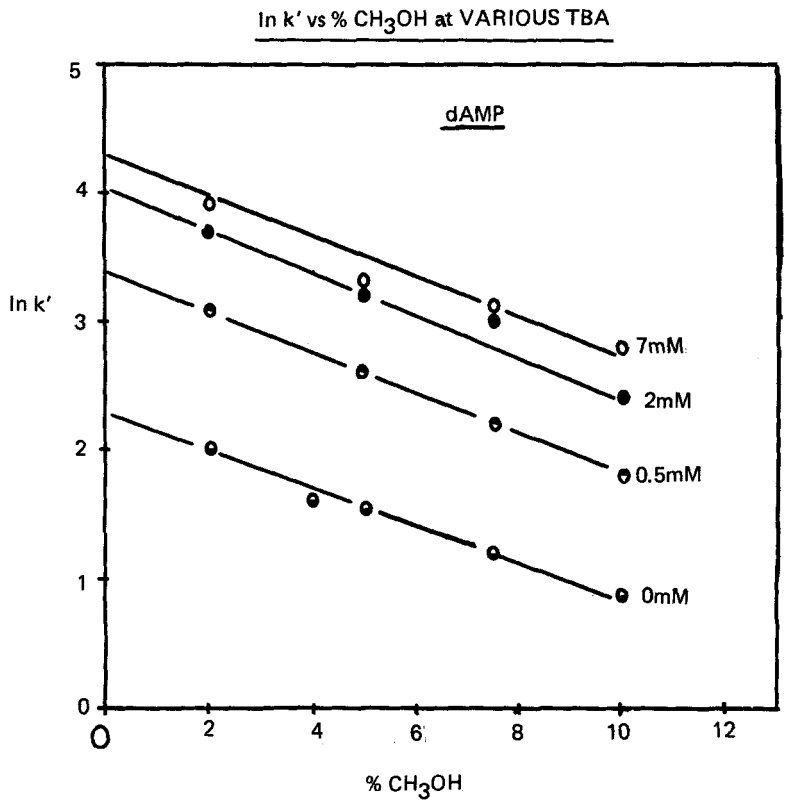


Figure 2

Plot of ln k' vs. % methanol for a negatively charged nucleotide, dAMP, at several TBA concentrations.

50 mM phosphate buffer eluent. The lower curve at 0 mM TBA represents a simple reversed-phase (C8) system. The linear correlation coefficients at 0 mM TBA was found to average 0.9293 for all solutes, with no coefficient being less than 0.9000.

When TBA is present in the mobile phase, this same log-linear decrease in retention is observed, but at higher or lower k' values. Thus, the ln k' values for dAMP

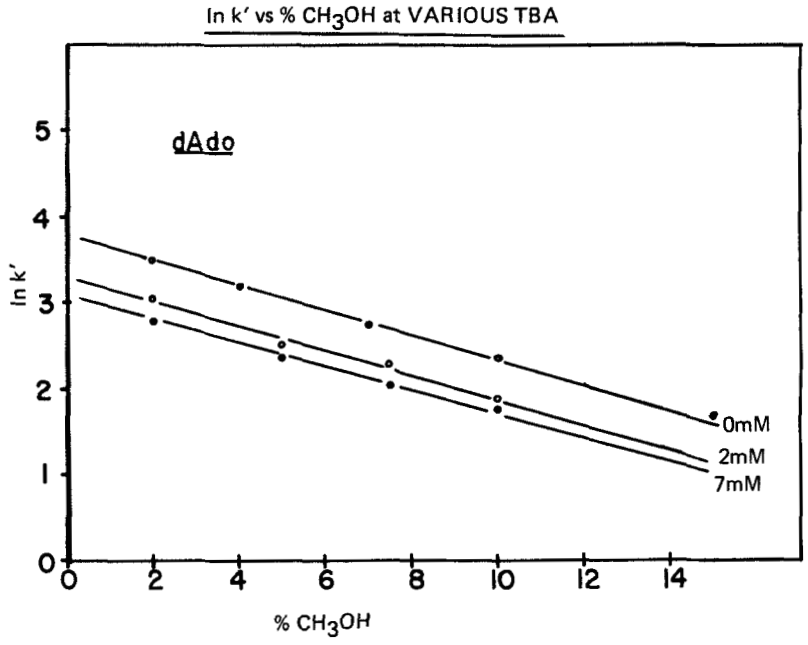


Figure 3

Plot of lnk' vs. % methanol for a nucleoside, deoxyadenosine, at various TBA concentrations

increase steadily with increasing TBA, with equal slopes but greater intercepts.

For the nucleoside dAdo, as shown in Figure 3, the same parallel ln k' lines are observed. The intercepts however decrease with increasing TBA. This is presumably due to repulsion of the solutes from the increasingly cationic stationary phase.

The TBA adsorption to the stationary phase was found to fit a Freundlich isotherm, Eq. 4 . Stationary phase concentrations were determined using frontal elution techniques. Within experimental error, the Freundlich

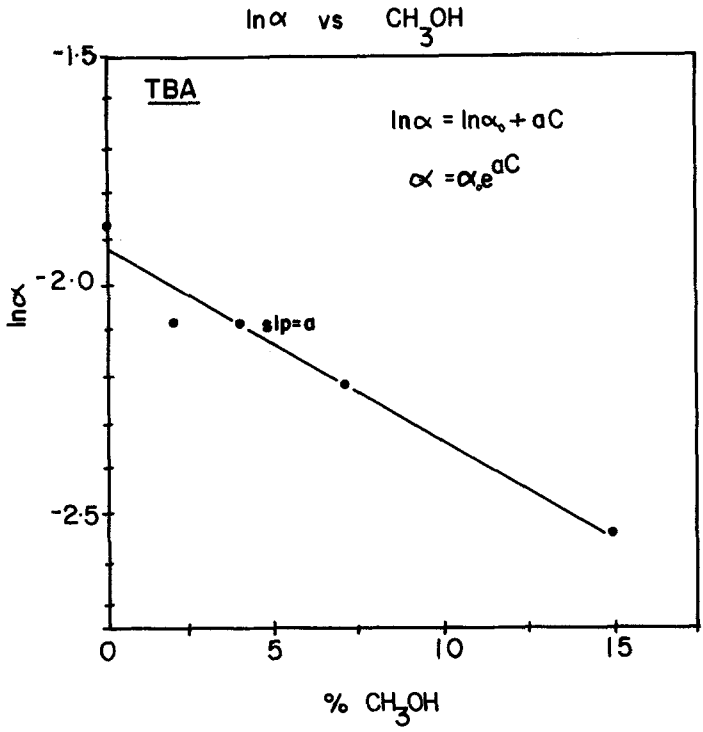


Figure 4

Plot of ln α vs. % methanol in the mobile phase. Alpha is the intercept of the Freundlich isotherm for the adsorption of TBA onto the stationary phase. The slope of the plot is a, and the intercept, α_o. All other conditions the same as figure 1.

slopes for TBA on the C8 stationary phase were found to be constant with increasing methanol, while the intercepts decreased exponentially, as shown in Figure 4.

The slopes of the k' versus Cs curves or θ, decreased exponentially with increasing methanol, as shown in Figure 5 for the deoxynucleotides. The same log-linear relationship was also observed for the deoxynucleosides.

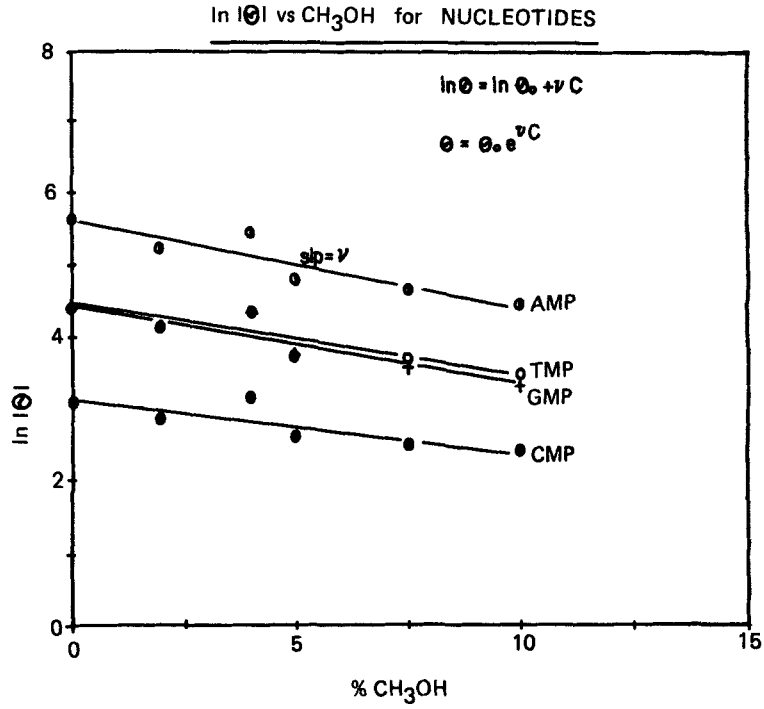


Figure 5

Plot of k' of dNMP, vs. Cs at various methanol concentrations. The slope $_\theta$ varies systematically with increasing organic modifier concentrations.

Net Effect of Methanol and TBA Concentration

The overall effect of TBA and methanol upon the k' of the deoxynucleosides and their mononucleotides can be described by equation 7 for mobile phase concentrations of up to 15% methanol and at TBA concentrations of up to 7 mM TBA. The net effect of increasing methanol at constant TBA is to decrease retention for all solutes. At constant methanol, the deoxynucleosides will decrease in retention, while the deoxymononucleotides will increase. The rate of increase or decrease can be predicted with reasonable

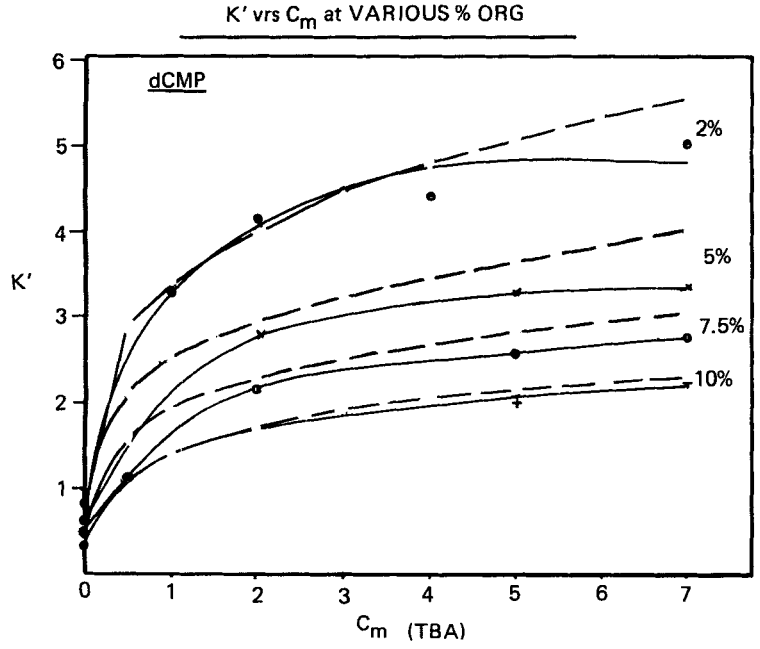


Figure 6

Comparisons of the calculated (broken lines) and observed (solid lines) k' values for the negatively charged mononucleotide dCMP. Experimental conditions the same as in Fig. 1.

Table 1

Summary of retention constants for equation 7 for the eight deoxynucleosides and their mononucleotides. Stationary phase: Whatman C8. Mobile phase: methanol/water, 50 mM in phosphate. Temperature:ambient. Valid range: 0-15% (v/v) methanol, 0-7 mM TBA.

	dCyd	dGuo	dThd	dAdo	dCMP	dGMP	dTMP	dAMP
k'_o	3.42	13.74	15.33	48.42	0.88	3.42	3.74	9.97
A	-.122	-.145	-.137	-.150	-.072	-.188	-.125	-.130
Θ_o	-6.75	-26.58	-40.44	-121.51	22.64	88.23	84.77	267.70
ν	-.145	-.197	-.220	-.221	-.074	-.116	-.101	-.121

for TBA: α_o =0.146 a= -0.0418 β = 0.308

accuracy according to equation 7. Figure 6 shows the predicted and observed values for dCMP, using the system constants presented in Table 1.

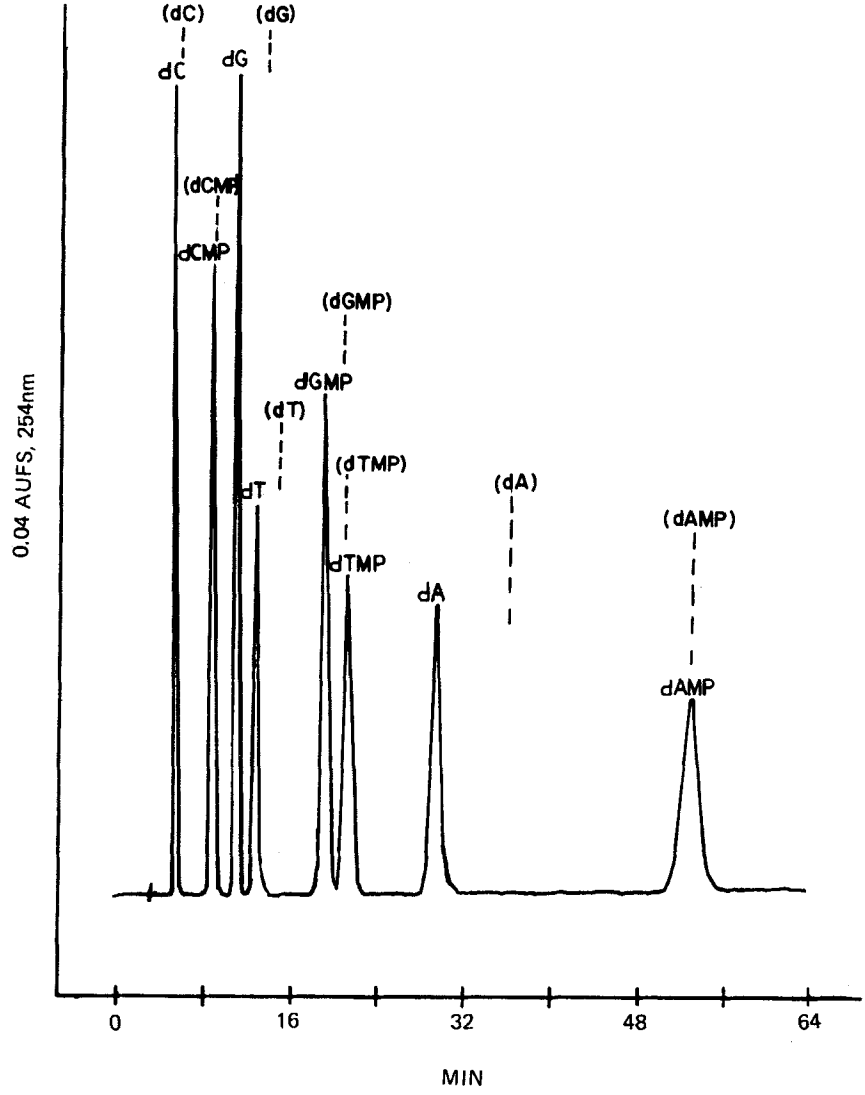
Excellent qualitative accuracy, and reasonable quantitative fits were observed for all eight solutes using equation 7. Over the range of conditions studied, equation 7 proved to be accurate to within 5-10% for all eight of the deoxynucleosides and their nucleotide monophosphates.

Final Separation Conditions

Figure 7 presents the simultaneous separation of the eight deoxyribonucleosides and their monophosphate nucleotides. Baseline separation was achieved for all solutes, with a total analysis time of 50 minutes. The retention times as predicted by equation 7 are presented as broken lines. This separation offers considerable flexibility in conditions. Changing the methanol or TBA concentration will drastically alter the elution pattern, according to EQ. 7. Thus, if only several of the solutes studied need be separated, new conditions can be readily calculated and form the basis for at least the initial starting conditions. These conditions can then be fined tuned empirically at the instrument.

CONCLUSIONS

An ion-pair separation of the major deoxyribonucleosides and their monophosphate nucleotides has been presented. Complete separation of all solutes was achieved in under 1 hour, with excellent efficiencies. An



Figures 7

Chromatogram of the eight test solutes comparing the observed and predicted retention times according to Eq. 9. Mobile phase: 0.050 M phosphate buffer, 2.0 mM TBA, 7% (v/v) methanol in water. Temperature, ambient. Column: Whatman Partisil 5 C8 (4.6 mm id X 250 mm).

empirical function was derived to aid in the optimization of separation conditions. It appears that ion-pair HPLC is uniquely suited for the separation of the nucleic acid constituents, since in many cases both charged and non-charged solutes are involved. Ion-pairing offers the flexibility of changing the retention of the charged solutes at a much greater rate than the neutral solutes, resulting in excellent control over the selectivity of the system.

The mechanism of separation appears to fit a dynamic ion-exchange model most accurately. If this is indeed the case, then it ought to be feasible to synthesize stationary phases with properties similar to those observed in ion-pairing systems, but without the problems inherent in ion-pairing. These shortcomings include the addition of foreign ions to the mobile phase, long equilibration times and diminished column lifetimes. The synthesis of such phases are being studied in our laboratory and will be reported in the literature.

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