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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOBASES, NUCLEOSIDES AND NUCLEOTIDES

II. MOBILE PHASE COMPOSITION FOR THE SEPARATION OF CHARGED SOLUTES BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The polarity, pH, ion concentration and polarity of the buffer ions of the mobile phase were modified systematically in order to find optimal conditions for the separation of nucleobases, nucleosides and nucleotides by ion-exchange chromatography. The effects of these mobile phase parameters on the retardation of 26 nucleobases, nucleosides and nucleotides on the cation exchanger Partisil-10 SCX and the anion exchanger Partisil-10 SAX were examined and resulted in the formulation of the following simple rules:

(1) These ion exchangers also have reversed-phase and normal-phase properties; their occurrence is determined by the polarity of the mobile phase.

(2) The ion concentration has an effect only on ion-exchange properties.

(3) The pH of the mobile phase determines the degree of protonation of the solutes, and therefore the charge and polarity. Many solutes have different pK values, thus providing a tool for the variation of selectivity.

(4) The polarity of the buffer ions has an indirect effect on ion-exchange chromatography through differential distribution of the buffer ions over the two phases. This differential distribution is caused by reversed-phase or normal-phase characteristics.

The integration of these three types of chromatography has been demonstrated by some examples.

INTRODUCTION

Charged solutes have commonly been separated by ion-exchange chromatography[1–9]. Nucleosides, nucleobases and nucleotides have been separated by ion-ex-

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change chromatography and reversed-phase chromatography[5–31]. Reversed-phase chromatography is increasingly replacing ion-exchange chromatography, especially after the introduction of hydrophobic ions as a component of the mobile phase[32–37]. A disadvantage of ion-exchange chromatography is the unpredictability of some separations; the solutes are not always eluted in the sequence of their charges. Often these complexities can be diminished by the addition of non-polar solvents to the mobile phase[38–41].

Samuelson[38] studied the distribution of ethanol over the two phases of an ion exchanger used in low-pressure liquid chromatography. He found that with up to 30% ethanol in the mobile phase the concentration of ethanol in the stationary phase is always higher than that in the mobile phase. If the concentration of ethanol in the mobile phase is above 35%, then its concentration in the stationary phase is always lower than that in the mobile phase[38]. Eksteen *et al.*[21] used these results to explain the retardation of nucleobases and nucleosides on an anion exchanger in the presence of varying ethanol concentrations. These experiments suggest that reversed-phase and normal-phase interactions may take place in ion exchangers. In reversed-phase chromatography the ion concentration has little effect on retardation[42], but in ion-exchange chromatography it has a pronounced effect[1,2].

The simultaneous occurrence of reversed-phase and ion-exchange interactions may result in unpredictable separations. Therefore, the effects of the polarity, pH, buffer ion concentration and polarity of the buffer ions in the mobile phase on the retardation of 26 nucleobases, nucleosides and nucleotides was studied systematically on anion and cation exchangers. The results led to rules for practical ion-exchange chromatography and a few applications are shown.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment consisted of an Altex Model 100A pump, a Rheodyne 7105 injector and a Perkin-Elmer LC-55 variable-wavelength detector set at 260 nm. The columns were packed with the anion exchanger Partisil-10 SAX and the cation exchanger Partisil-10 SCX (Whatman; 250 × 4.6 mm I.D.). The concentrations of the mobile phase components are always given for the total mixture. The ion concentration is given for the cations.

The pH was measured with an E-516 Titriskop Metrohm Herisau pH meter. Retention times were recorded with a stop-watch and were reproducible with a standard deviation of 0.5%. The solutes were dissolved in water. The flow-rate was 2 ml/min with a pressure between 35 and 70 bar, depending on the column and the composition of the mobile phase. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

In addition to the solutes listed in Table I* and shown in Fig. 1, the following

* Abbreviations used: cAMP = adenosine-3′,5′-monophosphate; 5′-AMP = adenosine-5′-monophosphate; cPMP = purine-riboside-3′,5′-monophosphate; B = Boehringer (Mannheim, G.F.R.); S = Sigma (St. Louis, MO, U.S.A.); P = Pharma-Waldhof (Mannheim, G.F.R.); M = Merck (Darmstadt, G.F.R.); k' = column capacity ratio; α^{cAMP} = selectivity to cAMP.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF SOLUTES

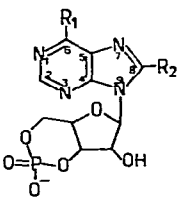
No.	*Compound*	*Source*	*Hydrophobicity* at pH 3.0*	*Electrophoretic mobility** at pH 3.0*
1	cAMP	P	0.8	− 3.1
2	N^6-Monobutyryl-cAMP	B	3.9	− 8.4
3	6-Chloro-cPMP	B	2.0	−10.8
4	8-Amino-cAMP	B	0.6	+ 0.5
5	8-Dimethylamino-cAMP	B	3.5	+ 0.1
6	8-Benzylamino-cAMP	B	11.2	+ 0.7
7	5′-AMP	B	0.3	− 2.9
8	Adenosine	M	1.1	+11.1
9	Adenine	B	0.8	+19.5
10	8-Bromo-cAMP	B	2.0	− 5.6
11	8-Methoxy-cAMP	B	1.0	− 2.9

* Hydrophobicity is expressed as the column capacity ratios (k') of the solutes on a reversed-phase column (RP-8, Riedel-de Haën, Hannover, G.F.R.) in 10% methanol–10 m*M* ammonium formate (pH 3.0)[42].

** The electrophoretic mobility is expressed as the rate of movement in cm/h of the solutes to the negative pole (positive sign) or positive pole (negative sign) of a Pherograph high-voltage electrophoretic apparatus. The electrophoretic movement is divided by the R_F value of the solute in ascending paper chromatography, and thus corrected for adsorption of the solutes to the paper.

compounds were investigated the same way (data not shown, except in Figs. 6 and 7): 8-hydroxy-cAMP (B), xanthosine-3′,5′-monophosphate (B), 5-aminoimidazole-4-carboxamide-1-ribose-3′,5′-monophosphate (AICAR) (B), adenosine-N^1-oxide (S), 2′-deoxyadenosine (M), 5′-tosyladenosine (P), 2′,3′-isopropylideneadenosine (S), 2′,3′-O-*p*-methoxybenzylideneadenosine (S), guanosine (P), 2′-deoxyguanosine (M), cytidine (P), 2′-deoxycytidine (P), uridine (P), thymidine (B) and purine riboside (S).

The electrophoretic mobility of the solutes was determined in 0.04 *M* citrate–hydrochloric acid (pH 3.0) on Whatman 3MM paper using Pherograph high-voltage electrophoresis at 2000 V. Adsorption of the solutes to this paper was determined in this buffer by ascending paper chromatography. As an indication of the charge of the solutes at pH 3.0 the electrophoretic mobility was divided by the R_F value for paper chromatography.

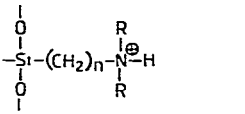


No	R_1	R_2
1	$-NH_2$	-H
2	$-NH-CO-CH_2-CH_2-CH_3$	-H
3	-Cl	-H
4	$-NH_2$	$-NH_2$
5	$-NH_2$	$-N(CH_3)_2$
6	$-NH_2$	$-NH-CH_2-C_6H_5$
10	$-NH_2$	-Br
11	$-NH_2$	$-O-CH_3$

Fig. 1. Structures of the cyclic nucleotide derivatives.

Structure of the ion exchangers

The information on the structure of Partisil-10 SAX and SCX was kindly provided by Dr. F. Rabel, Whatman Inc. (personal communication). Partisil-10 SAX and SCX are chemically modified silica gel resins, which still contain silanol groups. The functional groups are shown in Fig. 2.



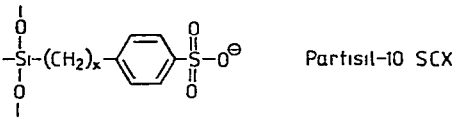


Fig. 2. Structures of the functional groups of Partisil-10 SAX and Partisil-10 SCX.

In the anion exchanger Partisil-10 SAX, the tertiary ammonium moiety for ion-exchange properties is connected to the silica gel matrix via an aliphatic spacer. In Partisil-10 SCX, cation-exchange properties are derived from the sulphonyl moiety, which is connected to the silica gel matrix via a spacer composed of aliphatic and aromatic moieties.

Anion exchanger

In ion-exchange chromatography the buffer ion concentration and the pH are the important mobile phase parameters that influence the retardation of charged

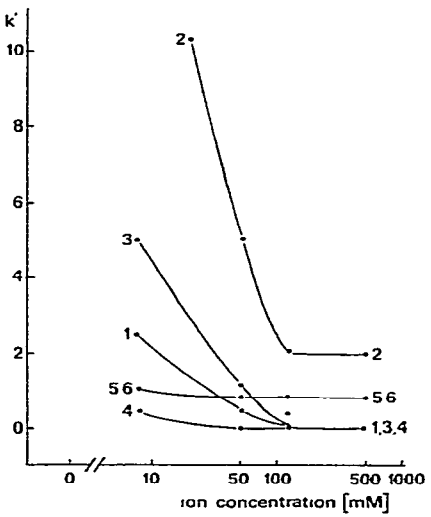


Fig. 3. Influence of ion concentration on column capacity ratios. Stationary phase: Partisil-10 SAX anion exchanger. Mobile phase: sodium phosphate buffer (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 3 = 6-chloro-cPMP; 4 = 8-amino-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP.

solutes. The ion concentration mainly affects column capacity ratios and pH affects column capacity ratios and selectivity. In Fig. 3 the buffer ion concentration increased from 6 to 500 m*M*. Above about 100 m*M* the ion concentration no longer influences the retention times. Although ion-exchange chromatography is virtually absent, there is still retention, especially of the nucleotides with hydrophobic substituents (Fig. 3, Table I). Reversed-phase interactions can be diminished by the addition of an apolar solvent to the mobile phase[42]. The addition of small amounts of acetonitrile to the mobile phase (Fig. 4) results in a reduction of the retention times of the nucleotides with hydrophobic substituents (Fig. 4, Table I). This may indicate that acetonitrile removes reversed-phase interactions. At high acetonitrile concentrations, the retention of most nucleotides increases. This suggests the introduction of normal-phase chromatography, probably due to activation of silanol groups at high acetonitrile concentrations.

The effects of the mobile phase components on the retardation of charged solutes by ion-exchange chromatography can be shown more clearly for a cation-exchange column.

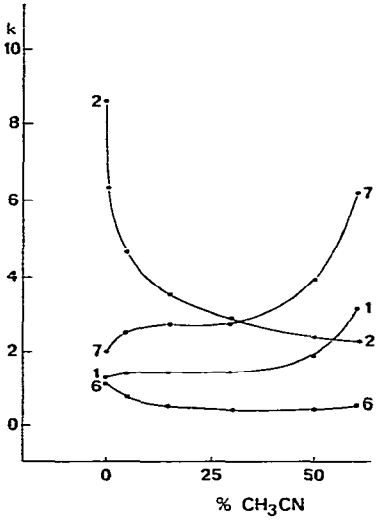


Fig. 4. Influence of polarity of the mobile phase on column capacity ratios. Stationary phase: Partisil-10 SAX anion exchanger. Mobile phase: 20 m*M* triethylammonium formate (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 6 = 8-benzylamino-cAMP; 7 = 5'-AMP.

Cation exchanger

Partisil-10 SCX consists of a silica gel matrix to which sulphonyl groups are coupled via a spacer composed of aliphatic and aromatic moieties (Fig. 2); silanol groups are present (Dr. F. Rabel, Whatman Inc., personal communication). Due to these components, the following types of chromatography can take place on this cation exchanger: cation exchange on the sulphonyl groups; reversed-phase chromatography on the aromatic and aliphatic spacer; and normal-phase chromatography on the unprotected silanol groups of the matrix. Each of these types of chromatography may be effective.

Polarity of the mobile phase

If reversed-phase, cation-exchange and normal-phase interactions are involved in the retardation of a solute on a cation exchanger, the polarity of the mobile phase will determine the types of interactions that are predominant. Reversed-phase and cation-exchange properties may be present if the mobile phase does not contain an apolar solvent. Addition of apolar solvent to the mobile phase will remove the reversed-phase properties. Silanol groups may become activated at a high concentration of an apolar solvent in the mobile phase.

Cation-exchange properties will be present at all polarities. An explanation for the effects of the polarity of the mobile phase on retardation of the nucleobases and nucleosides will be given later.

In buffer alone several nucleotides are retarded on a cation exchanger (Fig. 5). The retention times of the nucleobases, nucleosides and nucleotides are not very well correlated with their charges (Fig. 6a). The addition of acetonitrile to the mobile phase results in a reduction of the retention times of the nucleotides, especially of the hydrophobic ones, such as N^6-monobutyryl-cAMP (2), and 8-dimethylamino-cAMP (5) (Fig. 5). Between approximately 15% and 40% acetonitrile, the retention times of the nucleobases, nucleosides and nucleotides change only slightly (Fig. 5).

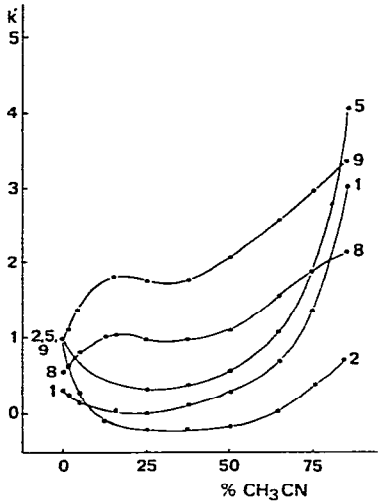


Fig. 5. Influence of polarity of the mobile phase on column capacity ratios. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* triethylammonium formate (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 8 = adenosine; 9 = adenine.

The order of elution from the column follows the order of electrophoretic mobility only with 25% acetonitrile in the mobile phase (Fig. 6b).

The decrease in the retention times of the nucleotides on the cation exchanger between 0% and 25% acetonitrile ($\Delta k'$ SCX) is proportional to the hydrophobicity of the solutes (k' RP-8) (Fig. 7). This clearly shows that reversed-phase interactions occur in the absence of acetonitrile, and that acetonitrile removes these reversed-phase interactions.

Above 50% acetonitrile in the mobile phase, nucleotides become even more

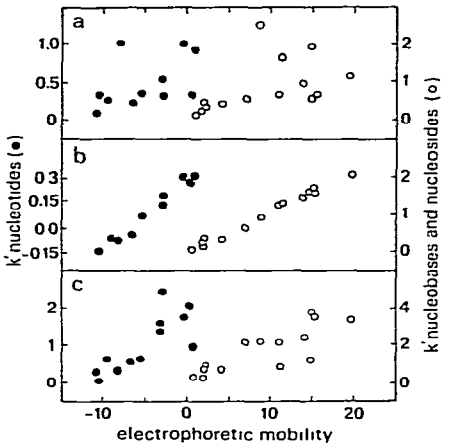


Fig. 6. Correlation between electrophoretic mobility of the solutes and their column capacity ratios at different polarities of the mobile phase. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* triethylammonium formate (pH 3.0). Solutes: 26 nucleotides, nucleosides and nucleobases (see Experimental). (a) no acetonitrile; (b) 25% acetonitrile; (c) 75% acetonitrile; ●, column capacity ratios of nucleotides; ○, column capacity ratios of nucleobases and nucleosides. For the expression of electrophoretic mobility, see Table I.

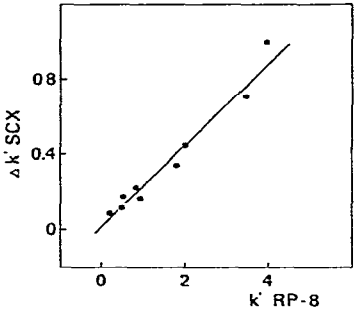


Fig. 7. Correlation between hydrophobicity of the nucleotides and the difference in column capacity ratios on an ion exchanger in the absence of acetonitrile and with 25% acetonitrile. Abscissa: hydrophobicity of the nucleotides, expressed as column capacity ratios on a reversed-phase column (k' RP-8[42]). Ordinate: decrease in column capacity ratios on Partisil-10 SCX cation exchanger if the mobile phase changed from 5 m*M* triethylammonium formate (pH 3.0) to 5 m*M* triethylammonium formate–25% acetonitrile (pH 3.0) ($\Delta k'$ SCX).

retarded (Fig. 5) and, as in the absence of acetonitrile, the order of elution does not correspond closely to the order of the electrophoretic mobilities of the solutes (Fig. 6c). The selectivity of the nucleotides in relation to cAMP changes minimally above 65% acetonitrile (Fig. 8). Only 5′-AMP is retarded more strongly than cAMP at increasing acetonitrile concentrations. This suggests retardation by normal-phase interactions of the phosphate moieties with the silanol groups. This phosphate moiety is identical in all cyclic nucleotides, is more polar in 5′-AMP and is not present in the nucleobases and nucleosides.

The presence of reversed-phase, cation-exchange and normal-phase chromatography on one column can be very confusing when optimizing separations, as the effects of the mobile phase parameters cannot be easily predicted. Retardation on an

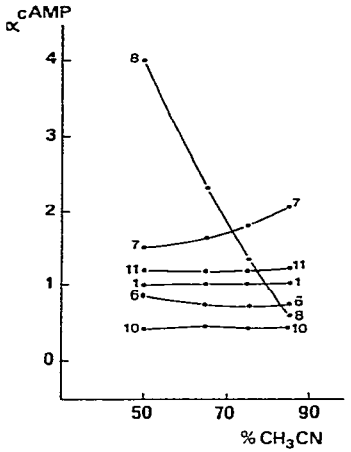


Fig. 8. Influence of polarity of the mobile phase on the selectivity of the solutes to cAMP (α^{cAMP}) at high acetonitrile concentrations in the mobile phase. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* triethylammonium formate (pH 3.0). Solutes: 1 = cAMP; 6 = 8-benzylamino-cAMP; 7 = 5'-AMP; 8 = adenosine; 10 = 8-bromo-cAMP; 11 = 8-methoxy-cAMP.

ion-exchange column is caused solely by ion-exchange properties, if a moderate amount of apolar solvent is added to the mobile phase.

Concentration of buffer ions

The buffer ion concentration is that mobile phase component which is always used to change column capacity ratios in ion-exchange chromatography[1,2]. An increase in concentration decreases retention times. The ion concentration has minor

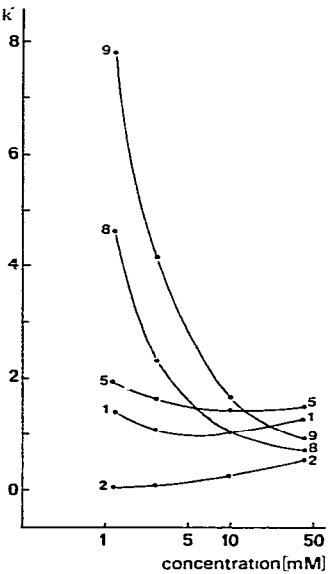


Fig. 9. Influence of concentration of buffer ions on column capacity ratios. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: triethylammonium formate–75% acetonitrile (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 8 = adenosine; 9 = adenine.

effects on reversed-phase chromatography[42]. To show the effect on normal-phase chromatography, this parameter was varied in the presence of 75% acetonitrile. Fig. 9 clearly shows that the ion concentration has minor effects on the nucleotides, which were assumed to be mainly retarded by normal-phase chromatography. The ion concentration strongly influences the retention times of the nucleobases and nucleosides, which is as expected for cation-exchange interactions.

pH of the mobile phase

The pH of the mobile phase influences the charge of the solutes, and consequently their polarity. Protonation of a basic atom group results in stronger cation-exchange interactions, and in an increase in polarity. Both phenomena are shown in Fig. 10A. At 65% acetonitrile, a decrease in pH results in stronger cation-exchange interactions of the nucleobases and nucleosides, and in stronger normal-phase interactions of the nucleotides. Most solutes will have different pK values; therefore, this parameter should also have a strong effect on selectivity. This is shown for some nucleotides in Fig. 10B.

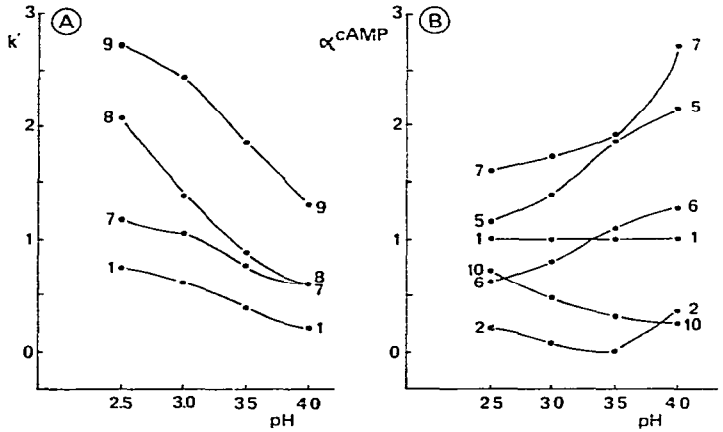


Fig. 10. Influence of pH on column capacity ratios (A) and selectivity to cAMP (B). Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 mM triethylammonium formate–65% acetonitrile. Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP; 7 = 5'-AMP; 8 = adenosine; 9 = adenine; 10 = 8-bromo-cAMP.

Polarity of the buffer ions

The polarity of the buffer ions has a strong effect on retardation on a reversed-phase column, partly due to differential distribution between the mobile phase and the stationary phase[37,42]. On this cation exchanger not only do cation-exchange properties occur, but normal-phase and reversed-phase interactions can also take place. Owing to these normal-phase and reversed-phase properties, the polarity of the buffer ions may have an effect on the concentrations of these ions in the stationary phase and, consequently, on the retardation of a solute by ion-exchange interactions. To study the effect of this parameter on retardation, the polarity of the cations was varied for all compounds at different acetonitrile concentrations. The concentration of cations in the mobile phase is kept constant. The results for one nucleoside are shown in Fig. 11.

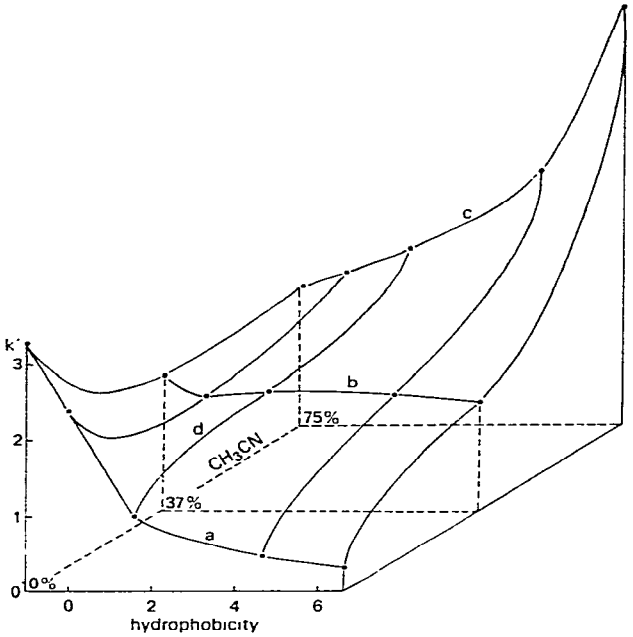


Fig. 11. Influence of hydrophobicity of the buffer ions and polarity of the mobile phase on column capacity ratios of adenosine. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* cations (pH 3.0). Cations used: ammonium, ethylammonium, triethylammonium, tributylammonium and tetrabutylammonium. Their hydrophobicities were calculated with the hydrophobic fragmental constant[44]. For (a), (b), (c) and (d), see text.

In the absence of acetonitrile (Fig. 11, curve a), reversed-phase properties are present in this cation exchanger, which results in attraction of hydrophobic components of the mobile phase by the stationary phase. An increase in the hydrophobicity of the cations results in an increase in their concentration in the stationary phase, and therefore in reduced cation-exchange interactions (Fig. 11, curve a). At 37.5% acetonitrile (Fig. 11, curve b), the polarities of the two phases are the same, so that the hydrophobicity of the cations has no effect on the distribution of the cations over the two phases, and also has no effect on the retardation of adenosine (Fig. 11, curve b). At 75% acetonitrile (Fig. 11, curve c) the stationary phase is more polar than the mobile phase. An increase in the hydrophobicity of the cations results in a decrease in their concentration in the stationary phase, and therefore in longer retention times of solutes retarded by cation-exchange interactions (Fig. 11, curve c).

The influence of different acetonitrile concentrations on the retardation of nucleobases and nucleosides on this cation exchanger in the presence of triethylammonium formate can now be more easily explained (Fig. 5; Fig. 11, curve d). Triethylammonium is a hydrophobic cation and is attracted to the stationary phase by reversed-phase interactions that occur in the absence of acetonitrile. Addition of acetonitrile to the mobile phase removes the reversed-phase characteristics, and the concentration of triethylammonium ions in the stationary phase decreases and retention times increase. Between 15% and 40% the polarities of the two phases are identical, and no changes in retention times are observed. A further increase in the

acetonitrile concentration makes the stationary phase more polar than the mobile phase, resulting in a further decrease in the concentration of triethylammonium ions in the stationary phase. The retention times of the nucleosides and the nucleobases will increase still further (Fig. 5; Fig. 11, curve d).

Practical aspects of ion-exchange chromatography

The increasing replacement of ion-exchange columns for the separation of charged solutes is based mainly on the ease of handling mobile phase parameters in reversed-phase chromatography. Although this replacement is justified by excellent separations of almost all classes of compounds, ion-exchange columns can be even more valuable if their properties are understood better. The following rules may help to optimize mobile phase compositions for ion-exchange chromatography:

(1) Ion-exchange columns based on a silica gel matrix also have reversed-phase and normal-phase properties. The polarity of the mobile phase determines the types of interactions that can be involved in retardation.

(2) The pH of the mobile phase is a general parameter affecting column capacity ratios and especially selectivity. This parameter is important if the solutes have different pK values.

(3) The ion concentration has negligible effects on reversed-phase and normal-phase interactions, but has strong effects on ion-exchange interactions. Higher ion concentrations reduce the retention times of solutes retarded by ion-exchange interactions.

(4) The polarity of the buffer ions has an indirect effect on ion-exchange interactions owing to a differential distribution of the buffer ions between the two phases. This partition is caused by the presence of reversed-phase and normal-phase properties.

During the last 2 years these rules have been applied to different separation problems on a variety of ion-exchange columns. Combining them with the rules for the separation of charged solutes by reversed-phase chromatography[42], 2′-(2,4-dinitrophenoxy)-cAMP and 2′-(2,4-dinitrophenoxy)-5′-AMP can be separated in several chromatographic systems (Fig. 12). On a reversed-phase column a high pH is necessary in order to obtain full expression of the charge differences between phosphate diesters and phosphate monoesters. Neither the polarity of the buffer ions nor their concentration is decisive. The methanol concentration was varied in order to achieve sufficient resolution and minimal analysis times (Fig. 12A). On an anion exchanger the reversed-phase properties should be removed by the addition of a moderate amount of acetonitrile, because both compounds have the very lipophilic dinitrophenoxy moiety in common. Neither pH nor the polarity of the buffer ions is decisive. The ion concentration was varied in order to obtain an optimal separation (Fig. 12B). On a cation exchanger a high concentration of acetonitrile is required in order to introduce normal-phase chromatography. Neither pH, the polarity of the buffer ions, nor ion concentration is decisive. The acetonitrile concentration was varied in order to achieve separation (Fig. 11C). On this cation exchanger there is still another range of parameters with which separation of these negative solutes is possible. Both solutes have a very hydrophobic dinitrophenoxy moiety, but they have a different polarity at the phosphorus moiety. Reversed-phase properties in a cation exchanger can produce selectivity for these solutes. Only acetonitrile is an important

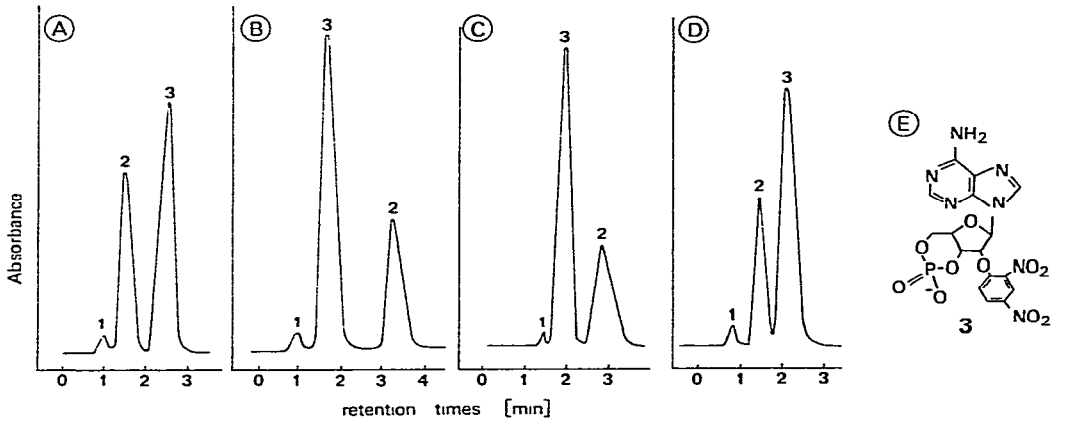


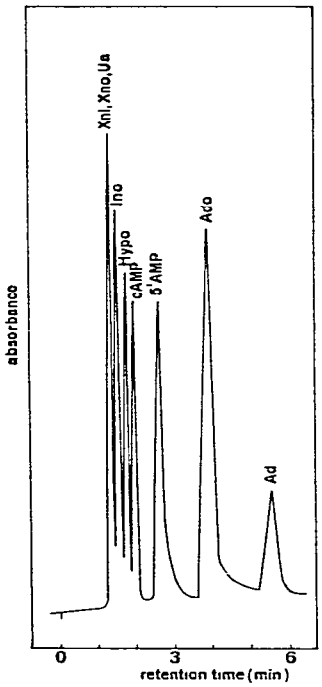
Fig. 12. Separation of 2′-(2,4-dinitrophenoxy)-cAMP and 2′-(2,4-dinitrophenoxy)-5′-AMP in different chromatographic systems. (A) Reversed-phase column (RP-8, Riedel-de Haën, 300 × 3 mm I.D., self-packed) in 15 m*M* Na_2HPO_4/H_3PO_4–46% methanol (pH 6.6); flow-rate, 2 ml/min. (B) Anion exchanger (Partisil-10 SAX) in 25 m*M* triethylammonium formate–20% acetonitrile (pH 3.0); flow-rate, 3 ml/min. (C) Cation exchanger (Partisil-10 SCX) in 2 m*M* triethylammonium formate–73% acetonitrile (pH 3.0); flow-rate, 3 ml/min. (D) Cation exchanger (Partisil-10 SCX) in 2 m*M* triethylammonium formate–1.5% acetonitrile (pH 3.0); flow-rate, 3 ml/min. (E) Structure of 2′-(2,4-dinitrophenoxy)-cAMP. Solutes: 1 = impurity, probably 2,4-dinitrophenol; 2 = 2′-(2,4-dinitrophenoxy)-5′-AMP; 3 = 2′-(2,4-dinitrophenoxy)-cAMP.

parameter for reducing retention times. The other parameters will hardly have any effect (Fig. 11D). Notice the order of elution in these four separations.

For the study of the degradation of cyclic nucleotide derivatives in a liver homogenate[43] a separation system was required for the simultaneous analysis of nucleotides, nucleobases and nucleosides. It would be practical if chromatography is relatively independent of derivatization of the cAMP molecule. The nucleotides can be separated on the basis of the phosphate moiety and the nucleosides and nucleobases according to their charge and polarity. Therefore, a mixture of cation-exchange and normal-phase chromatography was chosen. The pH and polarity of the buffer ions are relatively unimportant. The retention times of the nucleotides were varied with acetonitrile (normal-phase chromatography) and the retention times of the nucleobases and nucleosides were varied with the ion concentration (cation-exchange chromatography). A chromatogram is shown in Fig. 13.

S-Adenosyl-L-methionine (Boehringer; Fig. 14) was chromatographed on another Partisil-10 SCX column that differed from that described above by the necessity for a 100-fold higher ion-concentration in order to obtain the same retention times in cation-exchange interactions. The S-adenosyl-L-methionine peak kept a constant column capacity ratio of 0.8 if the ammonium acetate concentration was above 1.5 *M*. At this high ion concentration the peak could be shifted to the dead volume by adding propanol, again indicating reversed-phase properties. By decreasing the ion concentration in the presence of propanol the S-adenosyl-L-methionine peak was shifted to higher column capacity ratios, indicating cation-exchange interactions.

Only if reversed-phase and cation-exchange properties are present simultaneously do two S-adenosyl-L-methionine peaks appear. The peaks are interconver-



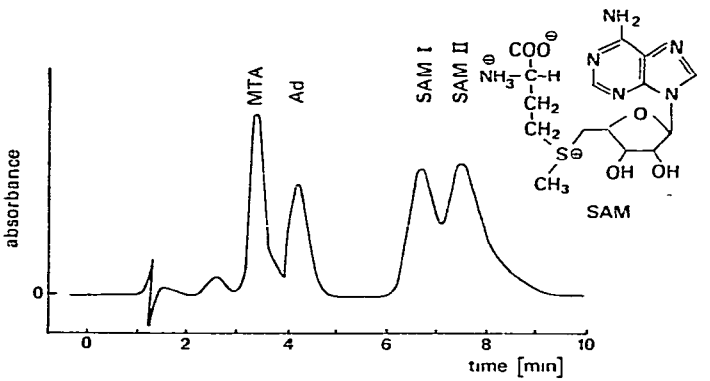


Fig. 13. Separation of cyclic AMP and its enzymatic degradation products by mixed chromatography. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 3 m*M* triethylammonium formate–67% acetonitrile (pH 3.0); flow-rate, 3 ml/min. Solutes: Xni = xanthine; Xno = xanthosine; Ua = uric acid; Ino = inosine; Hypo = hypoxanthine; cAMP = adenosine-3′,5′-monophosphate; Ado = adenosine; Ad = adenine.

Fig. 14. Separation of two stereoisomers of S-adenosyl-L-methionine by mixed chromatography. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 1.1 *M* ammonium acetate (pH 5.6); flow-rate, 2 ml/min. Sample: commercial S-adenosyl-L-methionine was boiled for 5 min at pH 1 in 0.1 *M* HCl, which resulted in racemization and partial degradation. Peaks: MTA = 5′-deoxy-5′-methylthioadenosine; Ad = adenine; SAM I = (−)-S-adenosyl-L-methionine; SAM II = (+)-S-adenosyl-L-methionine.

tible on heating and are equally sensitive to alkaline pH. Most probably these two peaks are the two stereoisomers of S-adenosyl-L-methionine at the sulphonium atom (Fig. 14).

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