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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF QUATERNARY PYRIDINIUM ALDOXIMES ON CATION-EXCHANGE SILICA GEL MATERIALS

Ch. E. KIENTZ, A. VERWEIJ* and H. L. BOTER
Prins Maurits Laboratory TNO, Chemical Research Division, Postbox 45, 2280 AA Rijswijk (The Netherlands)

SUMMARY

A number of structurally related mono- and bis-quaternary pyridinium aldoximes were analysed by high-performance liquid chromatography on cation-exchange silica gel materials with aqueous mobile phases, containing various amounts of organic solvents. Elution characteristics, such as column efficiency and selectivity, were studied as functions of the composition of the mobile phase, column temperature, type and concentration of the counter-ion, type of ion-exchange material, and pH.

The contribution of ion exchange and liquid–liquid partition to the chromatographic process is discussed.

INTRODUCTION

Pyridinium aldoximes are candidates as antidotes in organophosphate poisoning[1–3]. Their therapeutic usefulness depends primarily on their ability to reactivate the phosphorylated enzyme acetylcholinesterase, thereby restoring the normal transmission of nerve impulses.

Ion-pair high-performance liquid chromatography (HPLC) is frequently used to separate pyridinium aldoximes from biological materials and from other, structurally related pyridinium compounds. Brown *et al.*[4,5] applied ion-pair HPLC to determine TMB-4 and other pyridinium ions in a wide variety of samples. Benschop *et al.*[6] described an ion-pair HPLC procedure to determine different pyridinium aldoximes, such as HI-6 and Toxogonin®, in blood samples. It proved to be difficult to obtain sufficient chromatographic separation of a mixture of structurally related pyridinium aldoximes. In these cases, ion-pair HPLC, although simple and sensitive, suffers mainly from the lack of selectivity. The efficiency of ion-pair methods is rather low when compared with the performances of normal and reversed-phase HPLC. Up to now, compounds which are structurally closely related, such as HI-6 and HS-6, could not be separated by that technique.

Silica gel particles to which a suitable functional (*e.g.*, an ion-exchange) group is bound offer a considerable contact surface to the mobile phase because of their porosity[7] and result in non-swelling and pressure-stable materials. The interest in

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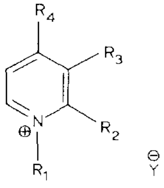
TABLE I

STRUCTURES OF PYRIDINIUM ALDOXIMES AND P_2-AMIDE



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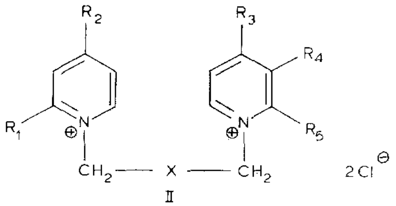

II

Code	*Compound*	*I (mono-quaternary)*					
		R_1	R_2	R_3	R_4	Y	
1	P_2S	CH_3	CH=NOH	H	H	$CH_3SO_3^-$	
2	Ethyl-P_2A	C_2H_5	CH=NOH	H	H	Cl^-	
3	*n*-Propyl-P_2A	C_3H_7	CH=NOH	H	H	Cl^-	
4	*n*-Pentyl-P_2A	C_5H_{11}	CH=NOH	H	H	Cl^-	
5	Benzyl-P_2A	$C_6H_5CH_2$	CH=NOH	H	H	$CH_3SO_3^-$	
6	*n*-Heptyl-P_2A	C_7H_{15}	CH=NOH	H	H	Cl^-	
7	P_2-Amide	CH_3	$C(O)NH_2$	H	H	Cl^-	
8	P_3AM	CH_3	H	CH=NOH	H	Cl^-	
9	P_4AM	CH_3	H	H	CH=NOH	Cl^-	
		II (bis-quaternary)					
		R_1	R_2	R_3	R_4	R_5	X
10	TMB-4	H	CH=NOH	CH=NOH	H	H	CH_2
11	HI-6	CH=NOH	H	$C(O)NH_2$	H	H	O
12	HS-6	CH=NOH	H	H	$C(O)NH_2$	H	O
13	2,2-LüH-6	CH=NOH	H	H	H	CH=NOH	O
14	Toxogonin	H	CH=NOH	CH=NOH	H	H	O

ion-exchange HPLC has grown steadily since appropriate ion-exchange silica gel materials became commercially available. To our knowledge, no publications have hitherto appeared that deal with HPLC of pyridinium aldoximes on ion-exchange silica gel packing materials. The present paper describes a method for the separation of pyridinium aldoximes and related compounds (Table I) having a selectivity superior to that of reversed-phase ion-pair HPLC and a separating efficiency comparable with that currently observed for normal and reversed-phase chromatography.

EXPERIMENTAL

Materials

Compounds 1–13 (Table I) were synthesized in this laboratory. Toxogonin, methanol, potassium hydroxide and sodium formate were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.). The following strong cation-exchangers (exchange capacity *ca.* 1 mequiv./g) were used: Partisil-SCX, particle size 10 μm, from Whatman (Maidstone, U.K.), Nucleosil-SA, particle size 5 μm, from

Machery-Nagel (Düren, F.R.G.) and RSILCAT, particle size 5 μm, from RSL-Alltech (Eke, Belgium). All other chemicals and solvents were of analytical grade.

Apparatus

The chromatographic system consisted of a Waters 6000 A solvent delivery system, a Valco 7000 p.s.i. injection valve, a Tracor 970 A variable-wavelength UV detector and a Waters R 401 refractive-index (RI) detector. Columns (25 cm × 5 mm I.D.) were packed according to a modification of the procedure of Linder *et al.*[8] by using a Haskel-100 air-driven constant-pressure pump. They were thermostatted in a laboratory-made water jacket. The columns showed greater stability when used with mobile phases having a high organic solvent content than with mobile phases having a high water content.

RESULTS

Elution characteristics, such as column efficiency and selectivity of the separation of pyridinium aldoximes, were studied as a function of the composition of the mobile phase, column temperature, type and concentration of the counter-ion, type of column packing material, and pH.

The mobile phase

Initial experiments carried out in 0.5 *M* aqueous sodium formate solution as the mobile phase revealed that P_2S, ethyl-P_2A and *n*-propyl-P_2A adhered very strongly to the ion exchanger Partisil-SCX. The elution volumes decreased and the column efficiency (or number of plates) improved on addition of increasing amounts of meth-

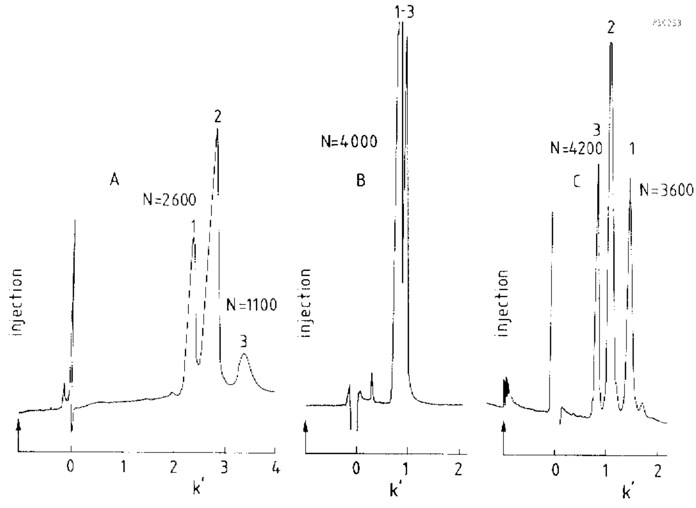

Fig. 1. Chromatograms showing the influence of various amounts of methanol in the mobile phase on elution volume, elution order, and peak shape. Mobile phase: (A) 0.5 *M* sodium formate in methanol–water (10:90); (B) 0.5 *M* sodium formate in methanol–water (50:50); (C) 0.5 *M* sodium formate in methanol–water (80:20). Column: Partisil-SCX, 10 μm; temperature, 20°C; flow-rate, 1 ml/min; detector, RI; *N*, number of plates. Peaks: 1 = P_2S; 2 = ethyl-P_2A; 3 = *n*-propyl-P_2A.

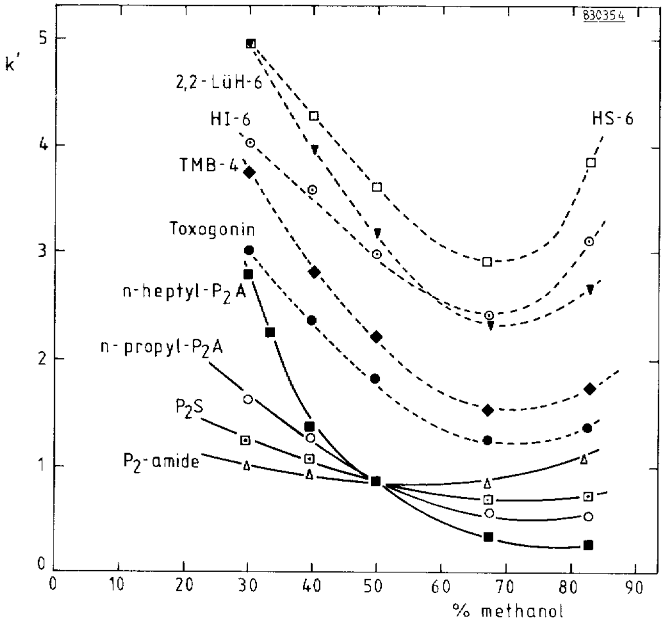

Fig. 2. The retention volume of a number of pyridinium compounds plotted *versus* the methanol content of the mobile phase. Mobile phase: 0.5 *M* potassium formate in methanol–water (pH 6). Column: RSIL-CAT, 5 μm; temperature, 50°C. Curves: ----, bis-quaternary pyridinium compounds; ——, mono-quaternary pyridinium compounds.

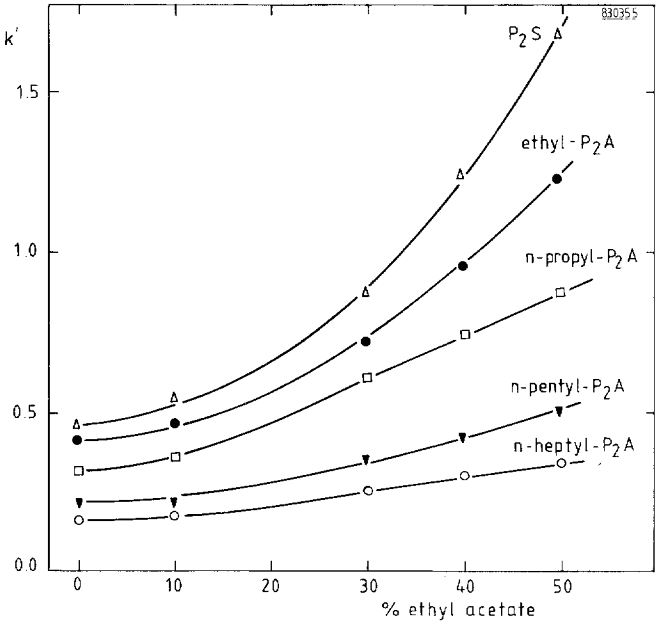

Fig. 3. Retention volume of a number of mono-quaternary pyridinium aldoximes plotted *versus* the ethyl acetate content of the mobile phase. Mobile phase: 0.5 *M* potassium formate in methanol–ethyl acetate [(80 − *x*):*x*:20] (pH 6.3). Column: RSILCAT, 5 μm; temperature, 80°C.

anol. A loss of selectivity was noted at 50% methanol and a reversed elution order at 80% methanol. These effects are presented in Fig. 1.

Subsequently, the retention behaviour of a number of mono- and bis-quaternary pyridinium aldoximes and of the metabolite P_2-amide was studied by varying the percentage of methanol in the mobile phase at constant potassium formate concentration (0.5 *M*), pH 6.3, and a column temperature of 50°C. In Fig. 2, the retention volume of the pyridinium compounds is plotted as a function of the methanol content in the eluent. It can be seen that when the mobile phase contained 50% methanol, the mono-quaternary pyridinium compounds were eluted at the same retention volume. On increasing the methanol content, a minimum retention of both the mono- and bis-quaternary pyridinium compounds was found at 70–75% methanol. The effect of the substitution of methanol in the mobile phase by the more lipophilic solvents ethyl acetate, acetonitrile, or ethanol was also studied. The results are presented in Figs. 3, 4 and 5. Generally, the more lipophilic eluents gave rise to an increase in selectivity and, thus, in the separation of the oximes.

Column temperature

The column temperature was varied from 40 to 80°C to study the influence on both the retention volume and the column efficiency, expressed as the number of plates. The results obtained with the mobile phase–0.5 *M* potassium formate in methanol–water (80:20) are presented in Table II. As can be seen, the retention volume decreased regularly with increasing temperature. For all compounds, the column efficiency ultimately reached *ca.* 20,000 plates/25 cm.

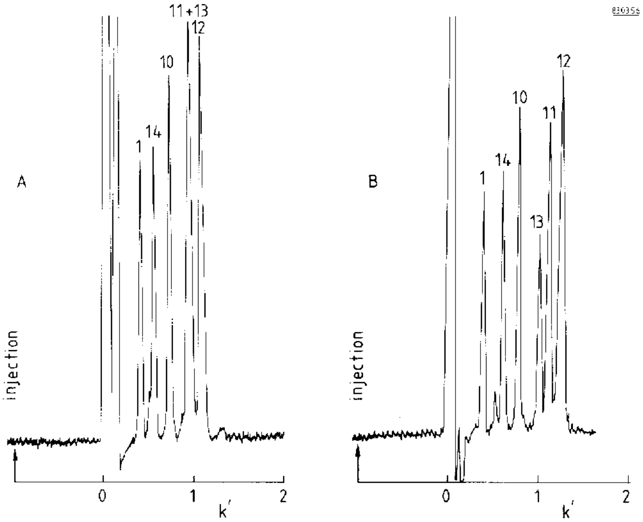

Fig. 4. Chromatograms of a number of pyridinium aldoximes with and without acetonitrile as a mobile phase modifier. Mobile phase: (A) 0.5 *M* potassium formate in methanol–water (80:20) (pH 6.3); (B) 0.5 *M* potassium formate in methanol–acetonitrile–water (68:12:20) (pH 6.3). Column: Nucleosil-SA, 5 μm; temperature, 80°C; flow-rate, 1 ml/min; detector, RI.

TABLE II

INFLUENCE OF COLUMN TEMPERATURE ON THE RETENTION VOLUME AND NUMBER OF PLATES OF SOME QUATERNARY PYRIDINIUM ALDOXIMES

Mobile phase, 0.5 *M* potassium formate in methanol–water (80:20).

Compound	*Temperature (°C)*									
	43		*55*		*63*		*74*		*83*	
	V (ml)	*N (25 cm^{-1})*	*V (ml)*	*N (25 cm^{-1})*	*V (ml)*	*N (25 cm^{-1})*	*V (ml)*	*N (25 cm^{-1})*	*V (ml)*	*N (25 cm^{-1})*
P_2S	4.06	19,500	4.06	19,000	3.96	20,000	3.92	19,300	3.78	19,900
Toxogonin	8.82	16,600	8.61	21,300	8.40	22,000	8.26	22,100	7.91	22,000
2,2-LüH-6	14.28	13,100	13.65	17,200	12.88	20,800	12.46	19,500	11.90	20,400
HI-6	18.48	12,300	17.64	14,600	16.52	17,500	15.96	18,200	15.12	19,900
HS-6	23.66	9400	22.12	13,300	20.58	14,400	19.46	15,800	18.06	19,000

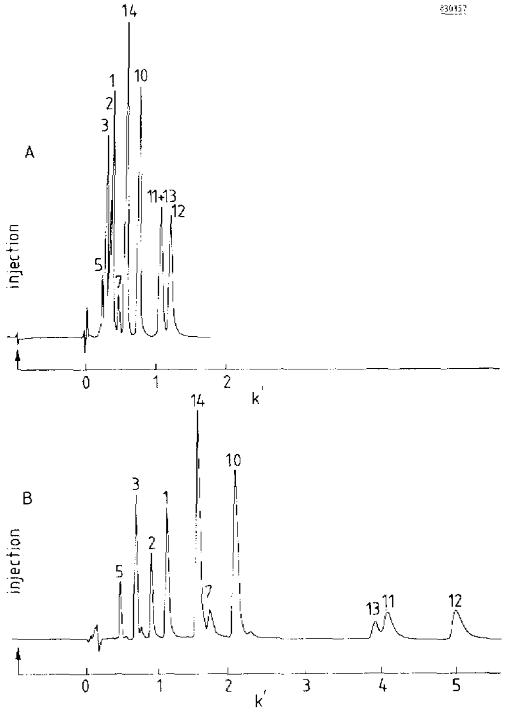

Fig. 5. Chromatograms of a number of pyridinium compounds with methanol or a mixture of ethanol and ethyl acetate, added to the mobile phase. Mobile phase: (A) 0.5 *M* potassium formate in methanol–water (83:17) (pH 6.3); (B) 0.5 *M* potassium formate in ethanol–ethyl acetate–water (67:17:16) (pH 6.6). Column: Nucleosil-SA, 5 μm; temperature, 80°C; flow-rate, 1 ml/min; detector, UV (290 nm).

Type and concentration of the counter-ion

The influence of the sodium ion concentration on the retention volume (expressed as k') of a number of mono-quaternary pyridinium compounds with methanol–water (80:20) as the mobile phase and the ion-exchanger Nucleosil-5-SA is presented in Fig. 6. A linear correlation between k' and the reciprocal value of the sodium ion concentration was found when the latter was below 0.1 *M*. The diverging lines suggest an increasing resolution with more diluted mobile phases, but a considerable peak broadening of the respective compounds appeared simultaneously.

When potassium was substituted for sodium in the mobile phase, a linear correlation was also found between k' and the reciprocal value of the potassium concentration at higher counter-ion concentrations (Fig. 7).

Column packing material

Cation exchangers of three different brands showed different selectivity patterns. The better separation capability of RSILCAT 5 μm in comparison with Nucleosil-SA 5 μm and Partisil-SCX 10 μm is shown in Fig. 8, where the chromatograms represent the elution of a mixture of quaternary pyridinium compounds.

Another difference was found when the sodium ion concentration was reduced from 0.5 to 0.1 *M*. This caused a large drop in the number of plates of the Nucleosil and RSILCAT columns from 15,000 to 1500.

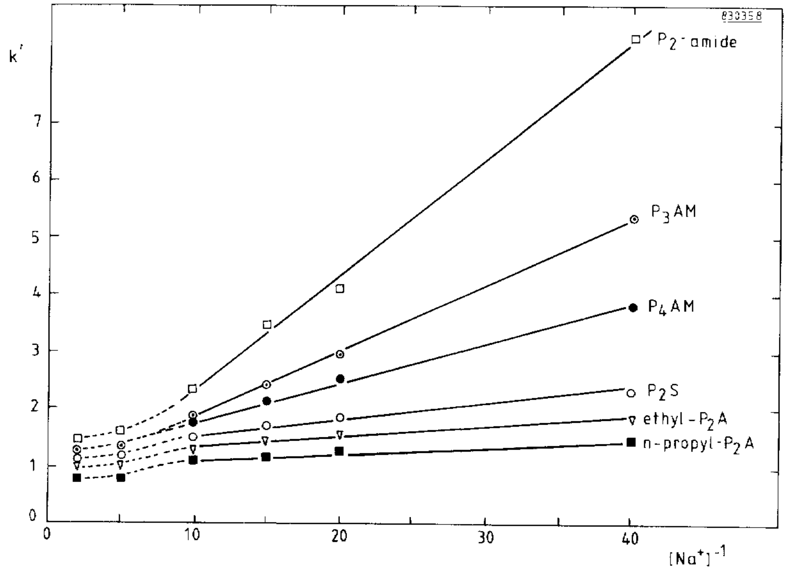

Fig. 6. The influence of the inverse of the molar sodium ion concentration on k'. Mobile phase: methanol–water (80:20), containing different amounts of sodium formate. Column: Nucleosil-SA, 5 μm; temperature, 80°C.

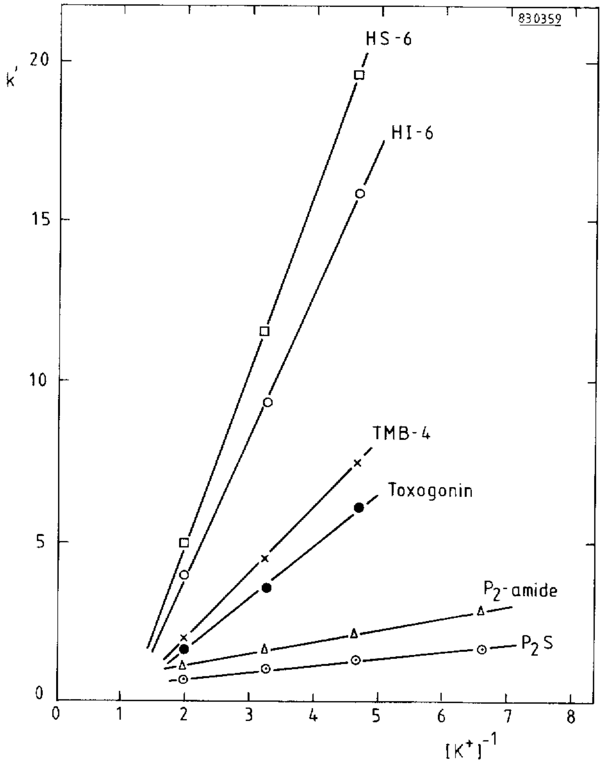

Fig. 7. The influence of the inverse of the molar potassium ion concentration on k'. Mobile phase: methanol–water (80:20), containing different amounts of potassium formate. Column: RSILCAT, 5 μm; temperature, 80°C.

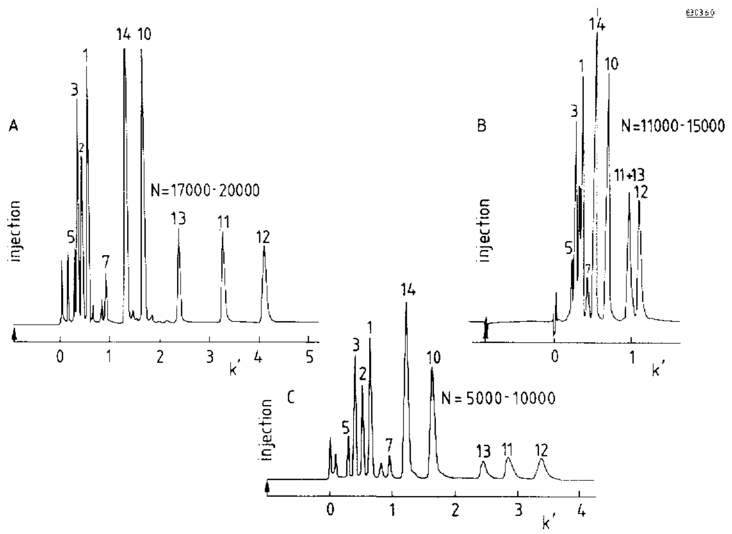

Fig. 8. Chromatograms showing the difference in separation capability of RSILCAT, 5 μm (A), Nucleosil-SA, 5 μm (B), and Partisil-SCX, 10 μm (C) for a mixture of pyridinium compounds. Mobile phase: 0.5 *M* potassium formate in methanol–water (80:20) (pH 6.3); temperature, 80°C; detector, UV (290 nm).

When the concentration of the potassium ion was lowered from 0.5 to 0.1 *M*, the plate number of RSILCAT decreased only a small extent from 20,000 to 15,000, whereas the plate number on Nucleosil again showed a big drop from 15,000 to 2500 plates.

The number of plates on Partisil-SCX remained constant on decreasing the sodium as well as the potassium ion concentration, but in the case of potassium as counter-ion the number of plates obtained for mono-quaternary and bis-quaternary pyridinium compounds differed considerably and amounted to 10,000 and 5000, respectively.

pH

The pH of the mobile phase 0.5 *M* potassium formate in methanol–water (80:20) was varied by adding different amounts of potassium hydroxide or formic acid. In Fig. 9 the *k'* values of some quaternary pyridinium compounds are presented as a function of the pH of the eluent. Although the results suggest that the application of basic eluents is to be preferred because they give better resolution, some disadvantages were observed. At pH values above 7 the number of plates obtained for the different compounds proved to be poorly reproducible, some oximes (*e.g.* HS-6 and HI-6) decomposed, and the column conditioning, which generally was completed within 15 min, took several hours.

DISCUSSION

Figs. 1 and 2 demonstrate that, depending on the methanol content of the mobile phase, different separation mechanisms determine the elution volume as well as the elution order. The order P_2S < ethyl-P_2A < *n*-propyl-P_2A < *n*-heptyl-P_2A,

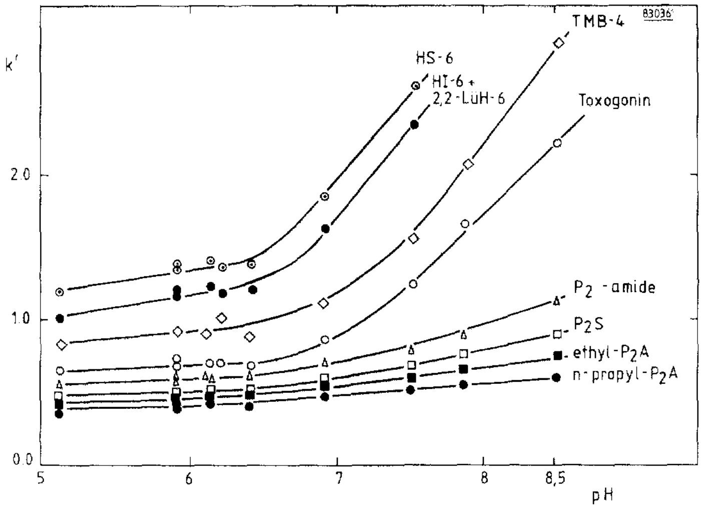

Fig. 9. Retention behaviour (k') of a number of pyridinium aldoximes and P_2-amide plotted *versus* the pH of the mobile phase. Mobile phase: 0.5 M potassium formate in methanol water (80:20). Column: Nucleosil-SA, 5 μm; temperature, 80°C.

found with a mobile phase containing low percentages of methanol, points to the action of an ion-exchange mechanism and Van der Waals forces. The pK_a value of the oxime group of these pyridinium aldoximes[9] is 7.8. As a consequence, the compounds will present themselves at pH 6 as positively charged solute particles, and an exchange will take place with the cation-exchange material. The interaction of the organic part of the solute ions with the organic site of the stationary phase will then determine the elution sequence.

The reversed elution order is found when an eluent with a high percentage of methanol is used. In that case, a normal-phase separation, in which a partition takes place between the mobile phase and the hydrophilic (aqueous) stationary phase, will probably play an important role. This assumption is sustained by the corresponding sequence of distribution coefficients (log p) of P_2S, ethyl-P_2A, n-propyl-P_2A and n-heptyl-P_2A being -3.2, -2.7, -2.2 and -0.2, respectively, as obtained in a comparable liquid–liquid partition system composed of n-octanol and water[10]. From Fig. 3 it can be seen that the addition of ethyl acetate to the mobile phase influences the retention of a number of pyridinium aldoximes to a varying extent. The increase of the retention volume decreases in the order P_2S > ethyl-P_2A > n-propyl P_2A > n-pentyl-P_2A > n-heptyl-P_2A. This may be explained on the basis of an increasing solubility of the homologues with higher alkyl chains in the more lipophilic mobile phases. Alterations in retention behaviour of the different oximes, observed on addition of ethyl acetate or other organic solvents, may thus be used to improve the resolution. This beneficial effect is demonstrated in the case of the addition of acetonitrile (Fig. 4) and of a mixture of ethyl acetate and ethanol (Fig. 5) to the mobile phase, composed of methanol (80%), water (20%) and potassium formate.

At pH values above 7 the retention volumes increase considerably (Fig. 9). It can be seen that the retention behaviour of the aldoxime- and amide-containing

TABLE III

SOME CHEMICAL CHARACTERISTICS OF THE CATION-EXCHANGE MATERIALS, TOGETHER WITH THE RETENTION OF P_2-AMIDE AND HS-6

Packing material	*k'*		*Weight percent.**		*Functional group***
	P_2-amide	*HS-6*	*SO_3^-*	*Organic carbon*	
Nucleosil-SA	0.5	1.2	1.7	8.0	Propylphenylsulphonic acid
Partisil-SCX	1.0	3.4	1.3	2.1	Phenylsulphonic acid
RSILCAT	1.0	4.3	3.5	4.6	Phenylsulphonic acid

* Derived from elemental sulphur and carbon analysis and corrected for packing densities.

** As mentioned by the respective suppliers.

compounds show the same tendency. However, a difference would be expected, as in aqueous solutions above pH 8–9 the mono-quaternary aldoxime molecules will mainly exist as zwitter-ions, which will not be adsorbed on the negatively charged groups of the stationary phase according to classical ion-exchange experiments of P_2S[11]. Zwitter-ions seem to be absent as a result of a decreased dissociation of the oxime group due to the relatively high dielectric constant of the methanol–water (80:20) eluent.

The ion-exchange mechanism seems to be still operative in mobile phases with a lipophilic character. It is well known that, if this mechanism occurs, k' will be inversely proportional to the concentration of the counter-ion in the eluent[12]. In Figs. 6 and 7 this relationship is clearly demonstrated for the sodium and potassium ions, respectively. Moreover, as potassium ions will have a greater affinity for the negatively charged parts (SO_3^-) of the stationary phase, their addition to the mobile phase causes a steeper decline of the retention volume of the pyridinium compounds when compared with the addition of sodium ions.

The retention behaviour was greatly dependent on the column packing material used (Fig. 8), but it was impossible to find a correlation between the respective retention values and the weight percentages of SO_3^- or organic carbon of the cation exchanger, as shown in Table III. From this it may be concluded that neither the binding of the positively charged pyridinium compounds to the ionic part (SO_3^-) of the stationary phase nor the interaction with the organic part of this material predominates in the elution process. Probably a normal-phase partition between an aqueous phase and an organic mobile phase plays a predominant role, accompanied by an ion-exchange process of minor importance.

A preliminary experiment, carried out with bare silica (Fig. 10), which behaves mainly as an aqueous phase and to a small extent as a weak cation exchanger, with 0.5 *M* potassium formate in methanol–water (80:20) as the mobile phase, supports the preponderant role of normal-phase liquid–liquid partition, as demonstrated in the case of pyridinium compounds by a retention sequence similar to those obtained on the ion-exchange materials (Fig. 8). Contrary to the classical or fixed-site ion-exchange systems in normal-phase liquid–liquid (*e.g.*, ion-pair) chromatography, the equilibria are dynamic, giving rapid mass transfer of the solutes[12]. This fact, in addition to the elevated (80°C) column temperatures used, will result in high chromatographic efficiencies (20,000 plates/25 cm, Table II), which are generally difficult to obtain with fixed-site ion-exchange columns.

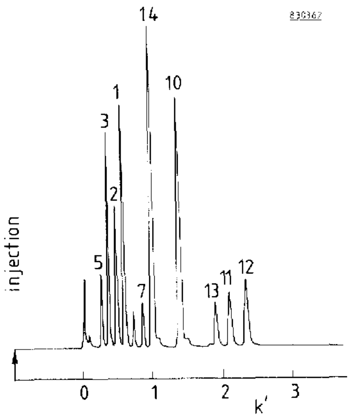

Fig. 10. Separation of pyridinium aldoximes and P_2-amide on bare silica (LiChrosorb, 5 μm). Conditions as in Fig. 8.

CONCLUSIONS

From the HPLC analysis of a number of mono- and bis-quaternary pyridinium compounds on three different cation-exchange materials, with sodium or potassium formate in methanol–water (80:20) as the mobile phase, it may be concluded that as well as a distinct contribution from an ion-exchange mechanism a predominant role must be ascribed to a normal-phase or liquid–liquid partition.

The elution characteristics, such as column efficiency and selectivity, depend on the column temperature, type and concentration of the counter-ion, pH, type of ion-exchange material and on the lipophilicity of the mobile phase.

Generally, it is preferable to use a column temperature of 80°C, potassium as the counter-ion, a pH between 5 and 7, and RSILCAT as column material. The selection of the eluent will depend on the composition of the sample.

The strong cation-exchange silica gel materials, in combination with highly lipophilic eluents and potassium as a counter-ion showed a selectivity superior to that of reversed-phase ion-pair HPLC as well as a separation efficiency comparable with that currently observed for normal- and reversed-phase chromatography.

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