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ZONE FORMATION IN ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY

III. STEP-GRADIENT ELUTION OF OLIGODEOXYRIBONUCLEOTIDES

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SUMMARY

Step-gradient elution by decrease of the counter-ion concentration or increase of the co-ion concentration was studied in an ion-pair reversed-phase system. As analytes, polyvalently negatively charged oligodeoxyribonucleotides were used. In the co-ion step-gradient technique, the retention volumes can be predicted by use of a simple equation. This technique gives a drastic decrease in peak width, which makes it possible to decrease the separation time by using an optimized composition of the two eluents used. Furthermore, the detectability of organic ions is considerably improved. The time for the introduction of the second eluent, containing the co-ion, into the column after the sample injection is critical.

INTRODUCTION

During the last decade, ion-pair reversed-phase chromatography has become a common technique for the separation of nucleotides and oligonucleotides[1–7]. When the nucleotides to be separated have different charges and/or hydrophobicities, the separation factors can be very high[1]. However, the last peaks eluted will be very broad and difficult to detect. To solve this so-called general elution problem[8], gradient elution is often used. Usually, the concentration of the organic modifier in the eluent is increased, either continuously[9,10] or stepwise[9,11]. In both cases, mathematical expressions describing the retention have been evaluated[9–11]. In the case of nucleotides, the continuous increase in organic modifier has chiefly been used[2,4–7].

In ion-pair chromatography the retention volume can be regulated by changing the concentration and/or the hydrophobicity of the counter ion or the co-ion, an ion with the same charge as the analyte[1,12–14]. It is possible to obtain a gradient effect by changing the concentration of the organic ionic species in the eluent during the chromatographic analysis, keeping the concentration of the organic modifier constant. It has been shown that the retention volume can be decreased by increasing the co-ion concentration in a single step[14].

In this work, the effect of gradient elution on oligodeoxyribonucleotides has

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been studied by a stepwise change of one organic ionic species in the eluent, either by decreasing the counter-ion concentration or by increasing the co-ion concentration.

THEORETICAL

Retention model

The main quantitative expressions for ion-pair adsorption and retention are described elsewhere[12,13,15,16]. The same approach was used for the oligonucleotides, which are polyvalent anions in the eluents used. They are denoted as ON^{-p}.

If the counter ion, Q^+, and the buffer anion, Z^-, are present in the mobile phase, the net retention volume of ON^{-p} is given, *cf.*, refs. 1 and 17, by

$$V_{N,ON} = \frac{w_s K_0 K_{ONQp} [Q^+]_m^p}{1 + K_{QZ}[Q^+]_m[Z^-]_m} \quad (1)$$

where K_0 is the limited adsorption capacity of the surface, K_{ONQp} is the equilibrium constant for the adsorption of the ion pair ONQp, w_s is the amount of solid phase in the column (g), $[Q^+]_m$ and $[Z^-]_m$ are the concentrations (mol/l) of Q^+ and Z^-, respectively, in the mobile phase. Eqn. 1 is valid if the sample amounts are small and the peaks are symmetrical. It shows that a decrease in the counter-ion concentration results in a decrease in the net retention volume. The higher the charge of the oligonucleotide, the greater will be the decrease in retention volume[1].

If an organic anion, S^-, is also present in the mobile phase, it will compete with ON^{-p} for the limited adsorption sites on the surface of the solid phase[13–15]. The net retention volume will then be

$$V_{N,ON} = \frac{w_s K_0 K_{ONQp} [Q^+]_m^p}{1 + K_{QZ}[Q^+]_m[Z^-]_m + K_{QS}[Q^+]_m[S^-]_m} \quad (2)$$

If $[Q^+]$ and $[Z^-]$ are kept constant and $[S^-]$ is increased the net retention volume of the oligonucleotide will decrease.

Stepwise gradient elution

The migration distance of an analyte zone in a column equilibrated with the eluent can be calculated by the expression

$$s = vL/V_R \quad (3)$$

where L is the length of the column, s is the migration distance of the analyte zone when v ml of eluent have been eluted and V_R is the retention volume (ml) of the analyte, *i.e.*, the volume of eluate that is needed to obtain a migration distance equal to L.

In a single-step gradient process, an analyte migrates part of the distance, s_1, along the column with an elution volume, v_1, of eluent 1 and migrates the remaining distance, s_2, with an elution volume, v_2, of eluent 2. The retention volumes of the analyte in the two eluents are V_{R1} and V_{R2}, respectively. The following expressions are valid

$$L = s_1 + s_2 \quad (4)$$

$$V_{R,tot} = v_1 + v_2 \quad (5)$$

where $V_{R,tot}$ is the total retention volume of the analyte. Expressions for v_1 and v_2 can be obtained by combining eqns. 3–5:

$$V_{R,tot} = V_{R2} + \frac{s_1(V_{R1} - V_{R2})}{L} \quad (6)$$

It is assumed that, after introduction, eluent 2, migrates with a constant velocity along the column with a distinct front and reaches the end of the column with a breakthrough volume, V_{RA}. Eluent 2 will affect the retention of the analyte only if its front migrates faster than the analyte zone. The effect will depend not only on the composition of the eluents, but also on how far the analyte has migrated after the introduction of eluent 2.

The front has migrated the distance s_1 and overtakes the sample zone when the elution volume is v_A. The following relationship is valid (*cf.*, eqn. 3):

$$s_1 = \frac{v_A L}{V_{RA}} = \frac{v_1 L}{V_{R1}} \quad (7)$$

The volume, v_D, eluted between the injection of the sample and the introduction of eluent 2 is

$$v_D = v_1 - v_A \quad (8)$$

Combination of eqns. 6–8 then gives

$$V_{R,tot} = V_{R2} + \frac{v_D(V_{R1} - V_{R2})}{(V_{R1} - V_{RA})} \quad (9)$$

This expression is based on the assumption that the concentration changes at the front of eluent 2 are insignificant.

Eqn. 9 shows that $V_{R,tot}$ can be calculated for different values of v_D if V_{R1}, V_{R2} and V_{RA} are known and if the values are constant. If $v_D \leqslant 0$, which means that the sample is injected at the same time or after the introduction of eluent 2, $V_{R,tot}$ will be equal to V_{R2}. This is only valid if $V_{R2} \geqslant V_{RA}$, *i.e.*, the analyte zone migrates with a lower velocity than that of the front; thus, the column behaves as if equilibrated with eluent 2. If $v_D \geqslant (V_{R1} - V_{RA})$ and the analyte is injected before eluent 2 is introduced, the column behaves as if equilibrated with eluent 1, since the front does not reach the sample zone before it emerges from the column. Then $V_{R,tot}$ will be equal to V_{R1}.

EXPERIMENTAL

Apparatus

The pumps were an LDC Model 711-42 solvent-delivery system (Milton-Roy Minipump; Laboratory Data Control, Riviera Beach, FL, U.S.A.) and an Altex Model 100 (Berkeley, CA, U.S.A.). The detectors were an LDC SpectroMonitor III and a Beckman 156 refractive index detector (Berkeley, CA, U.S.A.). A Rheodyne

7125 sample injector (Berkeley, CA, U.S.A.) with a 20-μl loop was used for the sample injections and a Valco (Houston, TX, U.S.A.) CV-6-HPax injector, was used for switching eluent from one pump to the other in the breakthrough experiments. A Labotron eight-way selector valve SS (Kontron AB, Bromma, Sweden) was used at the low-pressure side of the pump in the gradient-elution studies. It is denoted as the "eluent selector".

The columns, 100 mm × 4.6 mm I.D., were equipped with modified Swagelok connectors and Altex 250-21 filters and were packed with LiChrosorb RP-18, 5 μm (Merck, Darmstadt, F.R.G.). A water-bath, HETO Type 02 PT 923 (Birkerød, Denmark), was used to thermostat the chromatograph. The pH was measured with a Model 801 A/digital pH meter (Orion Research, MA, U.S.A.), equipped with a Type 401 combined electrode (W. Ingold, Urdorf, Switzerland). The spectrophotometric measurements were made with a Zeiss PMQ II Spektralphotometer (Carl Zeiss, F.R.G.).

Chemicals

Methanol was of Merck p.a. quality. Tetrapentylammonium (TPeA) iodide (Eastman-Kodak, Rochester, NY, U.S.A.) was converted into the hydroxide by shaking the aqueous solution with silver oxide[18] before use in the eluents. Sodium octanesulphonate was also from Eastman-Kodak. All other substances and solvents were of analytical or reagent grade used without further purification.

The oligodeoxyribonucleotides were kindly supplied by J. B. Chattopadhyaya. They had been synthesized by a phosphotriester method[19]. The abbreviations used are as follows: A = 2'-deoxyadenosine and T = thymidine. In ApTp, A is the 5'-end and Tp the 3'-end of the oligonucleotide. An internucleotidyl phosphodiester linkage is denoted as p ($pK_a \approx 3$) and 3'-terminal p denotes a phosphomonoester ($pK_1 \approx 3.8$ and $pK_2 \approx 6$–7)[20,21].

Preparation of the eluent

The eluent was prepared by mixing equal volumes of methanol and phosphate buffer (pH 7.0) of ionic strength $I = 0.032$ (mol/l) and a total phosphate concentration of $1.51 \cdot 10^{-2}$ *M*. The phosphate buffer was prepared by mixing 1 *M* phosphoric acid and 1 *M* sodium hydroxide. When the eluent contained TPeA, an equivalent amount of sodium hydroxide was replaced by TPeA hydroxide to keep the ionic strength constant. Because octanesulphonate was added as the sodium salt, an increase of the ionic strength was expected.

The concentration of TPeA in the eluent was measured by the picrate method[22], using 1- or 5-cm cuvettes. The eluents were diluted at least 20 times in phosphate buffer (pH 6.5, $I = 0.1$) before the picrate extraction, in order to minimize phase-volume changes due to extraction of methanol.

Chromatographic technique

The chromatograph was thermostatted by circulating water from a water-bath kept at 25.00 ± 0.01°C. The eluent reservoir was kept in the thermostatted water-bath, which also thermostatted the analytical column by pumping water through a glass jacket, mounted on the column.

The flow-rate of the mobile phase was in the range of 0.5–0.8 ml/min. The

eluent was not recirculated. The oligonucleotides injected were dissolved in the eluent but their exact concentration in the injected sample was not known.

The hold-up volume of the column, V_m, was determined from the peak obtained when sodium nitrate was injected. When TPeA was present in the eluent the nitrate ion was retained. In those cases, the value of V_m used was that from the preceding or succeeding experiment without TPeA present in the eluent.

In the gradient-elution studies the eluent selector was used in combination with the LDC pump. The volume from the eluent selector to the top of the analytical column was measured as 1.74 ml, including the volume of the pump and of the injector loop. This volume was included in all calculations of volumes.

The adsorption and desorption studies were performed according to the breakthrough technique described in ref. 15, using the same equipment.

Peak efficiency and resolution determination

The number of theoretical plates was measured by use of the retention time, t_R, of the peak maximum and the width of the peak, w_t, measured at the base between the tangents to the inflexion points, in time units.

In gradient elution it is not correct to use the parameters N and H. In this study, the parameters of the gradient-elution peaks were measured in the ordinary way, but are indicated as *"N"* or *"H"* to indicate the peak-compression effect. The resolution, R_S, of two analytes was measured as the difference between the peak maxima, divided by the mean peak width. In gradient elution the resolution was also measured in this way.

RESULTS AND DISCUSSION

In the adsorption and desorption as well as in the gradient-elution studies two different eluents were used. The column was initially equilibrated with the first eluent, which may contain adsorbable organic ions, *e.g.*, the counter ion. A second eluent, which differed from the first eluent in the content of one organic ion, was then in-

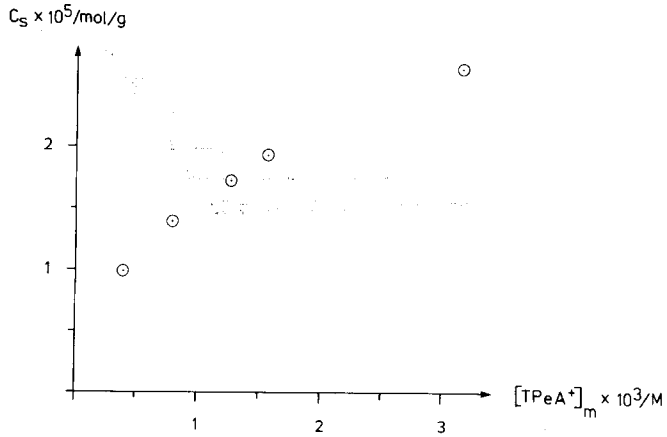


Fig. 1. Adsorption isotherm of TPeA as a phosphate ion pair.

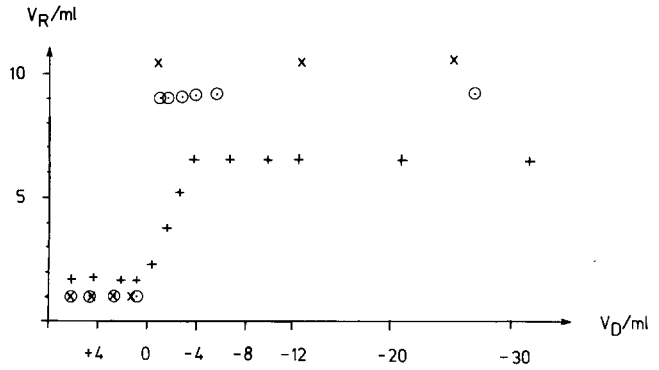


Fig. 2. Dependence of the retention volumes of nucleotides, injected at different times in relation to the introduction of the TPeA-containing eluent. See text for definition of v_D. Analytes: Ap (+); TpTp (×); ApAp (○). Eluents: 1, phosphate buffer (pH 7.0, I = 0.032)–methanol (1:1); 2, $1.57 \cdot 10^{-3}$ M TPeA in the eluent 1.

troduced into the column. The volume eluted between the sample injection and the introduction of eluent 2 is denoted as v_D (ml). Positive values of v_D mean that the injection was made before the introduction of eluent 2, negative values that the injection was made after the introduction.

Adsorption of TPeA as a phosphate ion pair

The adsorption of TPeA on the solid phase as a phosphate ion pair was studied by use of the breakthrough technique (Fig. 1). No fitting of the adsorbed amounts by the adsorption model was attempted, *cf.*, ref. 15, because there were too few data. A plot of $1/[QPA]_s$ *versus* $1/[Q^+]_m$ (ref. 12) gave a curved line for TPeA, indicating a heterogeneous surface.

To investigate the effect on the retention volume when TPeA was introduced into the column, some oligonucleotides were injected at different v_D values (Fig. 2). TpTp and ApAp were not separated and had very low retention volumes in the TPeA-lacking eluent. In the second eluent, TpTp and ApAp were strongly retained and were separated. For Ap, a gradual increase in the retention volume was observed when $v_D < 0$, until a constant value of 6.5 ml was obtained at $v_D \leqslant -3.7$ ml. The reason for this is that Ap will migrate through only part of the column in the TPeA-containing eluent. With the actual TPeA concentration and column used, the retention volume of the front was 9.9 ml and, consequently, Ap, when injected with $v_D > -3.7$ ml, will be eluted from a column only partly equilibrated with the TPeA-containing eluent. TpTp and ApAp have about the same retention as the TPeA front; thus, they will migrate in the column as if it were equilibrated with TPeA (for negative v_D values).

Desorption of TPeA

When an eluent lacking TPeA was introduced into the column equilibrated with TPeA, the quaternary ammonium compound was desorbed from the surface of the solid phase (Fig. 3). The retention volume to the point P, V_{RP}, where the concentration of TPeA starts to decrease, was dependent on the initial TPeA concentra-

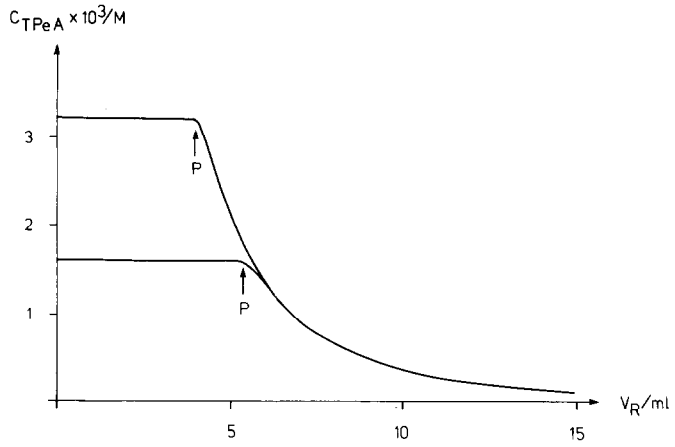


Fig. 3. Desorption profiles of TPeA. The column was equilibrated with $1.60 \cdot 10^{-3}$ and $3.21 \cdot 10^{-3}$ M TPeA, respectively, prior to introduction of the TPeA-lacking eluent. P denotes the point where the TPeA concentration starts to decrease.

tion in the column; the higher its concentration, the smaller was V_{RP} (*cf.*,ref. 14). This behaviour is similar to that discussed by Helfferich[23] concerning the equilibrium changes in the system. It is of interest that when about 6 ml of the TPeA-lacking eluent had been pumped into the column the two different initial concentrations of TPeA gave the same desorption profile.

The desorption profile was similar to that for the desorption of N,N-dimethylprotryptiline (DMPT) from μBondapak Phenyl[14], but showed more tailing. The DMPT adsorption studies indicated an homogeneous surface of μBondapak Phenyl[15], while the TPeA adsorption indicated an heterogeneous surface of LiChrosorb RP-18.

The tailing of the TPeA desorption profile was very extensive, as demonstrated by continuously collecting 100-μl fractions of the eluate, which were then measured by the picrate method (see Experimental). Still, after 36 ml there was a detectable amount of TPeA, $4 \cdot 10^{-5}$ M. However, after collection and measurement of 100 ml of the eluate, it was demonstrated that the amounts adsorbed and desorbed were equal.

During the desorption studies, nucleotides were injected after the introduction of the TPeA-lacking eluent. When injected just after the introduction, there was a drastic decrease in the retention volumes, but when injected later the effect levelled off. As a further reflection of the slow desorption, after 50 ml the retention volumes had still not reached the values obtained with the TPeA-lacking eluent.

Step-gradient elution

Stepwise concentration changes of the counter ion (decrease) or the co-ion (increase) will decrease the analyte retention volumes, *cf.*, eqns. 1 and 2, and in favourable cases improve the apparent chromatographic efficiency.

Counter-ion desorption. Fig. 4 shows the effect of the TPeA desorption on the retention volumes and the resolution of TpTp and ApAp for different v_D values. The retention volumes and the resolution decreased with decreasing v_D.

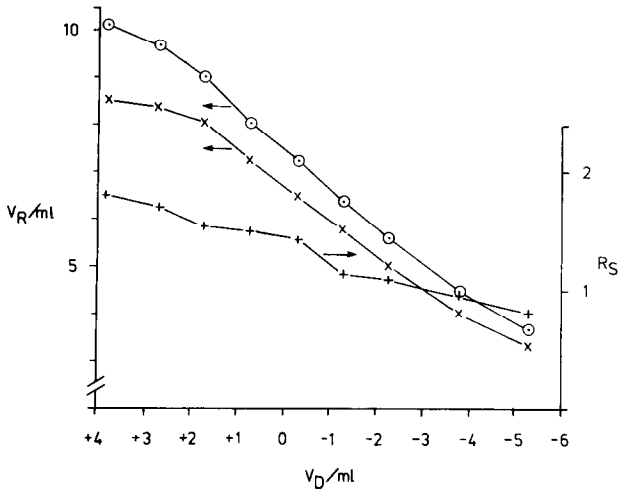


Fig. 4. Effect on the retention volumes and resolution, R_S (+), of using step-gradient elution after introduction of a TPeA-lacking eluent. Analytes: TpTp (○); ApAp (×). Eluents: 1, $1.57 \cdot 10^{-3}$ *M* TPeA in phosphate buffer (pH 7.0, $I = 0.032$)–methanol (1:1); 2, eluent 1 lacking TPeA.

On introduction of the second eluent, lacking TPeA (positive v_D value), the analyte initially migrates with a constant retention volume, which is dependent on the TPeA concentration in the first eluent. If the retention volume of P, V_{RP} (see Fig. 3), is smaller than for the analyte, P can reach and pass the analyte zone before it is eluted from the column. Behind P, the TPeA concentration continuously decreases, giving a continuous counter-ion gradient. The value of the total retention volume of the analyte will depend on the equilibration concentration of TPeA, and on the v_D value.

Since TpTp had the largest retention volume in the TPeA-containing eluent, it was affected by the point P earlier than was ApAp. This gives the resolution decrease shown in Fig. 4. Under isocratic conditions, R_S is 1.83 in the TPeA-containing eluent and 0.1 in the TPeA-lacking eluent.

Between $v_D = +3.8$ and -3.8 ml, peak compression was obtained. The effect

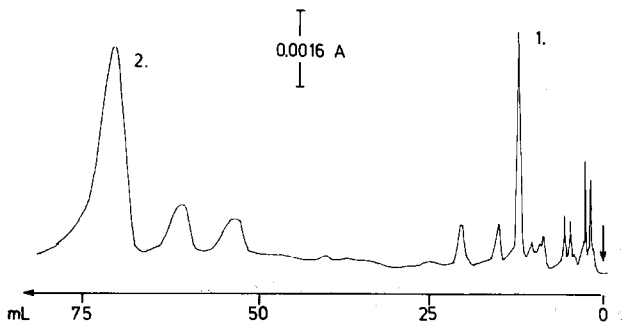


Fig. 5. Isocratic separation of oligonucleotides. Eluent: $1.63 \cdot 10^{-3}$ *M* TPeA in phosphate buffer (pH 7.0, $I = 0.032$)–methanol (1:1), flow-rate, 0.49 ml/min. Peaks: 1 = TpTp; 2 = TpTpTpTp.

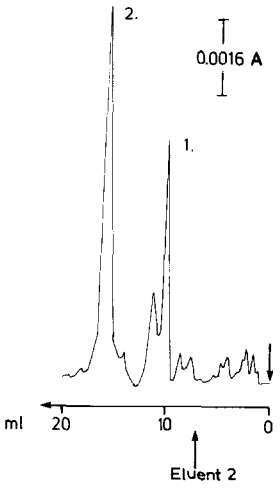
was greatest in the range $v_D = +1.7$ to -0.4 ml. The value of "H" for ApAp was 0.05 and for TpTp it was 0.03, which is about three times lower than in the isocratic experiment with the TPeA-containing eluent. The peak compression is a consequence of the continuous decrease of the TPeA concentration in the mobile phase. The concentration is lower at the rear of the peak than at the front; thus, the rear migrates under conditions which increase the elution velocity compared to that of the front, *cf.*, eqn. 1.

Since the resolution between TpTp and ApAp was relatively small, there is no great advantage in using the step-gradient technique to improve the separation time for this sample mixture. By using two analytes with a very large resolution in the TPeA-containing eluent, the gradient effect will be more pronounced.

Fig. 5 shows an isocratic separation of a mixture of TpTp and TpTpTpTp in a TPeA-containing eluent. The retention volumes were 12.5 and 74.3 ml respectively. The TpTpTpTp peak showed high asymmetry, and the efficiency was very low (*cf.*, ref. 10). In a TPeA-lacking eluent there was no resolution at all, and the retention volumes were very small (*ca.* 1 ml).

When the step-gradient technique was used for this nucleotide mixture, TpTpTpTp was affected most (Fig. 6). The retention volume was 15.8 ml, and the peak was symmetrical. Due to the narrower peak obtained, the injected TpTpTpTp was diluted ten times, compared to the concentration in Fig. 5. The dilution may also decrease the asymmetry of the peak[13]. TpTp was affected only to a minor degree ($V_R = 10.1$ ml). When the v_D value was decreased, the retention volumes of both nucleotides were decreased and so was the resolution. When v_D was less than $+2.4$ ml, no resolution was obtained.

Co-ion adsorption. If the column is first equilibrated with TPeA (counter ion) and then a second eluent, also containing octanesulphonate (co-ion), is introduced,



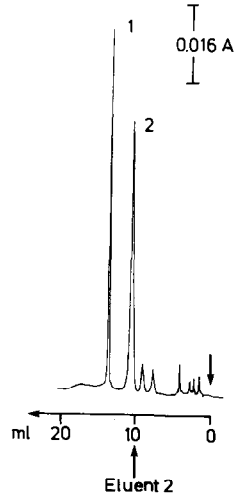


Fig. 6. Step-gradient elution, utilizing the TPeA desorption effect; $v_D = +7.6$ ml. Eluents: 1, as in Fig. 5; 2, eluent 1 lacking TPeA. Peaks as in Fig. 5. Flow-rate: 0.59 ml/min.

Fig. 7. Step-gradient technique with introduction of a co-ion; $v_D = +10.6$ ml. Eluents: 1, as in Fig. 5; 2, eluent 1 plus $1.00 \cdot 10^{-2}$ M octanesulphonate. Peaks as in Fig. 5. Flow-rate: 0.48 ml/min.

the octanesulphonate will give a distinct breakthrough front as it is eluted from the column[15]. When the column was equilibrated with respect to both TPeA and octanesulphonate, the retention volumes of TpTp and TpTpTpTp were smaller (*cf.*, eqn. 2) than when only TPeA was present, and no resolution was obtained between the nucleotides.

When the octanesulphonate eluent was introduced after the injection of the nucleotide sample (Fig. 7), the retention volume of TpTpTpTp was drastically decreased and a very narrow peak was obtained, compared to that obtained by isocratic elution (Fig. 5). TpTp was unaffected, which means that it was eluted from the column before the octanesulphonate front reached it. Due to the extremely narrow peak of TpTpTpTp, the peak width was three times smaller than for TpTp, and the chromatographic resolution was still very high (R_S = 5.2). "*N*" was 50000 plates for TpTpTpTp, while it was only 2700 for TpTp. The injected solution of TpTpTpTp was diluted 100 times, compared to the concentration in Fig. 5, while the TpTp concentration was unchanged. This shows that this gradient technique may have great potential for the analysis of low concentrations of an ionic organic analyte in the sample.

When v_D was less than +6.0 ml, no resolution was obtained, and for values less than +3.0 ml only one peak appeared. Not only the retention volume of the TpTpTpTp peak was affected. When the two eluents from Fig. 7 were used, TpTp was also affected, but to a much smaller degree. The total separation time was decreased by the use of a lower initial concentration of TPeA in the eluent. The analytes were then retained to a smaller extent (eqn. 1) and lower v_D values could be used while resolution was maintained.

When the TPeA concentration was kept constant and the octanesulphonate concentration in the second eluent was decreased, the retention decrease of TpTpTpTp was smaller (Fig. 8). This is probably due to the increased retention volume of the front[15]. The analyte thus migrated a longer distance through the col-

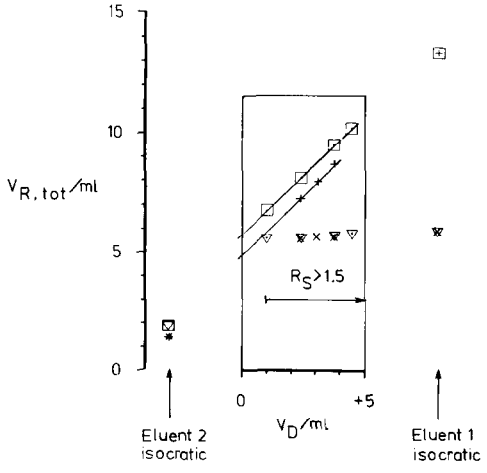


Fig. 8. Plot of $V_{R,tot}$ *versus* v_D. Eluents: 1, 7.76 · 10^{-4} *M* TPeA in phosphate buffer (pH 7.0, I = 0.032)–methanol (1:1); eluent 1 with addition of octanesulphonate [2.51 · 10^{-4} *M* for TpTp (▽), TpTpTpTp (□); 3.80 · 10^{-4} *M* for TpTp (×), TpTpTpTp (+)].

umn (higher s_1) before the front reached the analyte zone. In the v_D range investigated, the TpTp retention volume was unchanged at both concentrations of octanesulphonate.

A plot of $V_{R,tot}$ *versus* v_D should give a straight line with slope $(V_{R1} - V_{R2})/(V_{R1} - V_{RA})$ (eqn. 9), but only if the analyte is affected by the second eluent. TpTpTpTp gives straight lines at both concentrations of octanesulphonate (Fig. 8). The slopes of the lines are very close to +1, indicating that $V_{R2} = V_{RA}$. However, the intercepts are much higher than the V_{R2} values.

If it is assumed that the analyte retention volume is smaller in the second eluent, V_{R2}, then the retention volume of the front, V_{RA}, the analyte will be concentrated in the front. This means that the analyte retention volume is the same as that of the front and migrates through the rest of the column (s_2) with the front. V_{R2} in eqn. 9 can thus be exchanged for V_{RA}, resulting in the simple expression:

$$V_{R,tot} = V_{RA} + v_D \tag{10}$$

A plot of $V_{R,tot}$ *versus* v_D should now give a straight line with the slope +1 and the intercept V_{RA}.

The validity of eqn. 10 was tested by measurement of the retention volumes for the TpTpTpTp peak from the point where eluent 2 was introduced (v_D) in the two cases. Constant values were obtained, 5.7 and 4.8 ml (see intercepts in Fig. 8), at octanesulphonate concentrations of $2.5 \cdot 10^{-3}$ and $3.1 \cdot 10^{-3}$ M, respectively, indicating that V_{R2}, in fact, equals V_{RA} for this compound.

Higher concentrations of TPeA in the eluents also gave straight lines for TpTpTpTp, but the slopes were less than +1, indicating that the retention volume of TpTpTpTp in the second eluent was larger than that of the octanesulphonate front. In this case, eqn. 9 may be valid.

CONCLUSIONS

Two main principles can be used for the step-gradient technique introduced in this paper:

(1) decreasing the counter-ion concentration by desorption, and

(2) increasing the co-ion concentration.

The first principle, resulting in decreased retention by a gradual decrease of the counter-ion concentration, suffers from the drawback of slow desorption. The second principle, based on the competition for adsorption sites by the introduction of a co-ion, seems to be most fruitful for further development. Strong effects on the retention volumes and on the peak efficiencies were obtained, indicating possibilities for considerable improvements in analysis time, selectivity and detectability.

The equations developed permit the total retention volume for the analyte eluted last to be calculated.

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