*Journal of Chromatography*, 384 (1987) 13–24
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1011

# ZONE FORMATION IN ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY

## IV. OPTIMIZATION OF PEAK RETENTION IN STEP-GRADIENT ELUTION WITH INTRODUCTION OF COMPETING IONS

ANDERS SOKOLOWSKI
*Department of Analytical Pharmaceutical Chemistry, Biomedical Center, University of Uppsala, Box 574, S-751 23 Uppsala (Sweden)*

SUMMARY

Step-gradient elution by introduction of a co-ion into an ion-pair reversed-phase system was studied. The analytes were organic acids and amines and were used as counter ions in eluents also containing phosphate buffer (pH 4 or 6)–methanol (1:1). The magnitude of the retention decrease obtained for the analytes was dependent on the hydrophobicity and concentration of the co-ion and on a temporary decrease in the counter-ion concentration on introduction of the co-ion. When some prerequisites were fulfilled, a simple equation could be used to predict the retention volume, and then the separation time could be optimized. Extremely narrow peaks were obtained. The peak width was dependent on the co-ion concentration in the second eluent and also on the efficiency of the column, which is responsible for the bandbroadening of the breakthrough front of the co-ion.

## INTRODUCTION

Gradient elution is a powerful technique for decreasing the separation time of analytes having large differences in retention times. The most common technique in reversed-phase liquid chromatography (RPLC)[1,2] is based on a continuous increase of the organic solvent content in the eluent, but a stepwise increase is also used[1,3]. Although the mathematical expressions describing the retention times are rather complicated[1–3], the ion-pair step-gradient technique can be handled by rather simple expressions of the retention[4]. When a co-ion is introduced into the column, equilibrated with the counter ion, the existing equilibria are disturbed. As a result, a co-ion front and a zone containing a lower concentration of the counter ion than in the bulk mobile phase are obained[5]. Both the front and the zone migrate along the column and can increase the migration speed of an analyte, *cf.*, refs. 6–8.

In this work the optimization of the ion-pair step-gradient technique for some organic anions was studied with regard to both peak retention and compression. Quaternary ammonium ions are often used as cationic counter ions but they have

0021-9673/87/$03.50 

the drawback that they decrease the column stability[8,9]. Amines are preferable in this respect[8]. In this study, symmetrical amines, differing in their carbon contents, have been used as counter ions.

## THEORETICAL

The main quantitative expressions for ion-pair retention are described elsewhere[5,7,8,10].

In the single-step gradient elution process used in this study the analyte migrates part of the column distance with an eluent containing the counter ion (eluent 1) and the remaining distance with an eluent containing both the counter- and the co-ion (eluent 2). The retention volume of the analyte is high in eluent 1 but low in eluent 2. The total retention volume of the analyte can be described by the expression[4]

$$V_{R,\mathrm{tot}} = V_{R2} + \frac{v_D(V_{R1} - V_{R2})}{(V_{R1} - V_{RA})} \tag{1}$$

where $V_{R1}$ is the retention volume in the eluent containing only the counter ion (eluent 1), $V_{R2}$ that in the eluent containing both the counter- and the co-ion (eluent 2) in isocratic development, $V_{RA}$ is the retention volume of the co-ion front (breakthrough front), *cf.*, ref. 5, and $v_D$ is the volume eluted between the injection of the analyte and the introduction of eluent 2. Positive $v_D$ values mean that the injection was made before the introduction of eluent 2, negative values that the injection was made after the introduction.

If $V_{R2} < V_{RA}$, the analyte will be concentrated in the co-ion front and will migrate with the front through the rest of the column. Then, eqn. 1 can be simplified[4] to

$$V_{R,\mathrm{tot}} = V_{RA} + v_D \tag{2}$$

This expression is valid if eluent 2 affects the analyte before it is eluted from the column.

## EXPERIMENTAL

### *Apparatus and chemicals*

The apparatus and chemicals were the same as in the Part III[4], with the following exceptions. The columns were packed with μBondapak Phenyl, 10 μm, Batch No. 15 (Waters Assoc., Milford, MA, U.S.A.).

Tripentylamine (TrPeA), trihexylamine (TrHexA) and the sodium salts of hexanesulphonic acid and 2-naphthalenesulphonic acid were from Eastman-Kodak (Rochester, NY, U.S.A.). Tributylamine (TrBA), 1-naphthoic acid, 1-naphthylacetic acid and sodium decanesulphonate were from Fluka (Buchs, Switzerland). Sodium 2-anthracenesulphonate was kindly supplied by D. Westerlund. It had been synthesized from sodium 2-anthraquinonesulphonate[11]. TrBA, TrPeA and TrHexA were distilled prior to use. All other substances were of analytical or reagent grade used without further purification.

*Preparation of the eluents*

The eluents were prepared by mixing equal volumes of methanol and phosphate buffer [pH 4.0 or 6.0, ionic strength, $I = 0.032$ (mol/l)]. The pH 4.0 phosphate buffer has a low buffer capacity, but because only the sodium salts of the sulphonates were used as samples, no change of pH occurred. The phosphate buffers were prepared by mixing 1 *M* phosphoric acid and 1 *M* sodium hydroxide. When the eluent contained an amine, an equivalent amount of sodium hydroxide was replaced by the amine to keep the pH and ionic strength constant. When sulphonate was present in the eluent the ionic strength was increased by the corresponding equivalents of sodium sulphonate.

The amines were distilled prior to use in the eluents. They were stored under nitrogen in a refrigerator since it was observed that on storage at ambient temperature they became pale yellow. For TPeA this happened after a few days storage. Even when stored cold, the amines turned the top of the columns yellowish brown, and the column pressure increased when amine-containing eluents were used for several days.

To remove the coloured impurities in the amine-containing eluents, they were pumped through a column packed with μBondapak Phenyl, 10 μm (100 mm × 4.6 mm). The first 50 ml of eluate were discarded, then 200 ml were collected prior to use in the analytical system. If sulphonate were also to be present in the eluent, it was added after this treatment. Before the next 200 ml of eluent were purified, 2 mm of the solid phase at the top of this column were replaced with fresh sorbent, and then 50 ml of methanol were pumped through the column.

TPeA-containing eluents could be used for only 2 days after purification. If they were used later, the column pressure increased and the yellow-brown colour developed. TrBA- and TrHexA-containing eluents did not exhibit these problems after purification.

The concentration of the amines in the eluents was measured by the picrate method[12], using 1- or 5-cm cuvettes, *cf.*, ref. 4.

*Chromatographic technique*

The breakthrough and the step-gradient elution studies were performed according to the breakthrough technique described in ref. 5, using the same equipment. The chromatograph was maintained at 25.00 ± 0.01°C and the flow-rate of the mobile phase was in the range 0.5–0.8 ml/min. The eluent was not recirculated. The analytes were dissolved in the eluent.

## RESULTS AND DISCUSSION

*Amines as counter ions*

The retention volumes of organic anions depend on the concentration and hydrophobicity of the amines used as counter ions (Fig. 1) according to general ion-pair retention principles[7]. The highest concentration of amine in the eluent that could be used without increasing the ionic strength was $1.5 \cdot 10^{-2}$ *M* at pH 6. However, TrHexA could not be used at concentrations higher than $1.0 \cdot 10^{-3}$ *M*, due to its limited solubility.

The presence of co-ions in the amine-containing eluents resulted in decreased

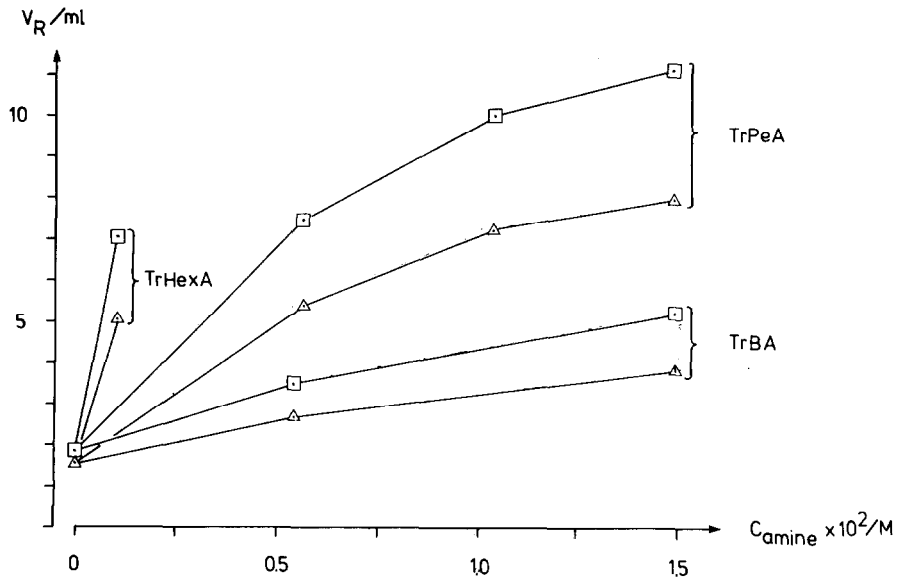


Fig. 1. Retention volumes of carboxylic acids as ion pairs with different amines. Eluents: phosphate buffer (pH 6.0)–methanol (1:1) containing different amines. Analytes: □ = 1-naphthylacetic acid; △ = 1-naphthoic acid.

retention volumes for the analytes, *cf.*, refs. 6 and 8. Hexane-, octane- and decanesulphonate were tested with TrPeA or TrHexA as counter ions. Decanesulphonate, the most hydrophobic sulphonate, decreased the retention volumes most effectively. Fig. 2 shows the effect on some carboxylic acids when TrPeA was used as the counter ion and octanesulphonate as the co-ion.

Since TrPeA showed a rapid degradation when used as the counter ion in the eluents (see Experimental). TrHexA was preferred. When the pH of the phosphate buffer was decreased, the retention volume of anionic analytes increased for the same concentrations of TrHexA, *cf.*, ref. 7. This is shown in Table I. Although the reason for this is not known, the ratio $[H_2PO_4^-]/[HPO_4^{2-}]$ increases and could increase the ion-pair distribution. Furthermore, the protolysis of free silanol groups is suppressed, and this may change the character of the solid phase. Since the highest concentration of TrHexA that could be used was $1.0 \cdot 10^{-3}$ *M*, a pH of 4 was used to obtain sufficiently high retention volumes of the most retained analyte in the step-gradient elution studies.

*Breakthrough retention volume of the co-ion*

When an eluent containing $1.0 \cdot 10^{-3}$ *M* TrHexA was introduced into a column equilibrated with phosphate buffer (pH 4.0)–methanol (1:1) a breakthrough curve was obtained, which was detected by a refractive index detector. The retention volume was 11.5 ml[5]. If a second eluent, also containing decanesulphonate, was then introduced, two breakthrough curves were obtained. Increased concentrations of decanesulphonate resulted in decreased retention volumes of the first breakthrough,

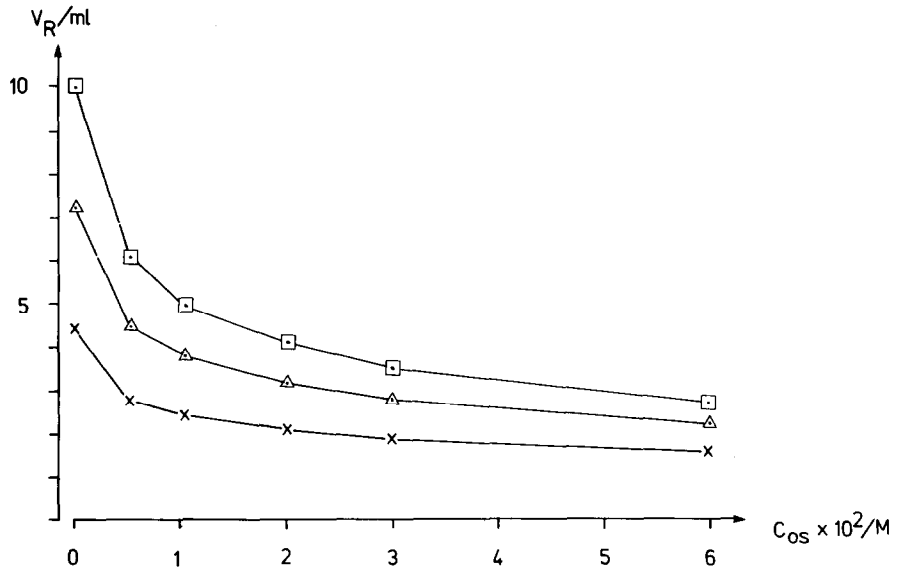


Fig. 2. Retention volumes of carboxylic acids in the presence of TrPeA and octanesulphonate (OS). Eluent: $1.04 \cdot 10^{-2}$ *M* TrPeA and OS in phosphate buffer (pH 6.0)–methanol (1:1). Analytes: □ = 1-naphthylacetic acid; △ = 1-naphthoic acid; × = 2,4-dinitrobenzoic acid.

$V_1$, while those of the second, $V_2$, increased (Table II). The results indicate that the first breakthrough represents the retention of decanesulphonate and the second the retention of TrHexA[5].

The refractive index (RI) detector only registers the total change in the refractive index of the eluate. It is assumed that the breakthrough of decanesulphonate creates a negative TrHexA zone[5], and the sum of the decanesulphonate and TrHexA signals results in an elution profile containing two breakthrough curves (Fig. 3). This means that there are three different regions of mobile phase composition migrating along the column. The first region contains the bulk concentration of TrHexA, the second contains the bulk concentration of decanesulphonate, but at a smaller concentration than the bulk of TrHexA, and the third contains decanesulphonate and TrHexA at the bulk concentrations of both. The phase composition of these three regions forms the basis for the understanding and utilization of the ion-pair step-gradient elution technique.

TABLE I

DEPENDENCE OF RETENTION VOLUME ON pH

Eluent: $1.03 \cdot 10^{-3}$ *M* TrHexA in phosphate buffer–methanol (1:1).

| *Analyte* | $V_R$ *(ml)* | |
|---|---|---|
| | *pH 4.0* | *pH 6.0* |
| 2,4-Dinitrobenzoic acid | 3.85 | 3.21 |
| 2-Naphthalenesulphonate | 5.93 | 4.90 |

TABLE II

RETENTION VOLUMES OF THE BREAKTHROUGH CURVES ON INTRODUCTION OF DECANESULFONATE (DS) AND TrHexA INTO A COLUMN EQUILIBRATED WITH $1.00 \cdot 10^{-3}$ *M* TrHexA

| $C_{DS}$ (*M*) | $V_1$ (*ml*) | $V_2$ (*ml*) |
|---|---|---|
| $2.40 \cdot 10^{-3}$ | 6.8 | 33 |
| $5.00 \cdot 10^{-3}$ | 5.1 | 49 |
| $1.00 \cdot 10^{-2}$ | 4.0 | 60 |

*Step-gradient elution*

When a mixture of 2,4-dinitrobenzoic acid (DNBA) and 2-anthracenesulphonate (AS) was injected into a column equilibrated with TrHexA (eluent 1) and then an eluent, also containing decanesulphonate (eluent 2), was introduced into the column (positive $v_D$ values), only the retention volume of AS was affected (Fig. 4). The separation was complete ($R_S > 1.5$) for all $v_D$ values tested. A plot of $V_{R,tot}$ *versus* $v_D$ gives a straight line with slope +0.98 and intercept 4.1, and indicating that the retention of AS is in accord with eqn. 2. The intercept is in good agreement with the retention volume of the decanesulphonate breakthrough front, $V_{RA}$ (Table II). This means, further, that AS is concentrated in the decanesulphonate front ($V_{R2} < V_{RA}$).

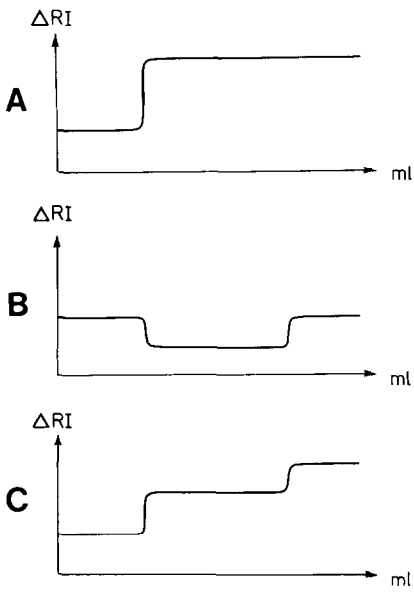


Fig. 3. Hypothetical and observed elution profiles after introduction of decanesulphonate into a column equilibrated with TrHexA. For compositions of the eluents see Table II. (A) Hypothetical breakthrough of decanesulphonate; (B) hypothetical negative TrHexA zone; (C) observed elution profile.

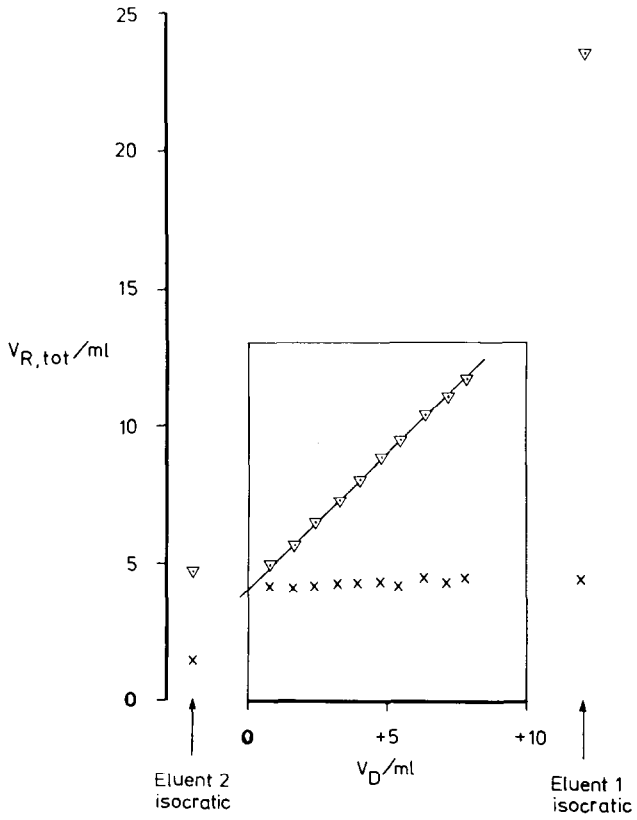


Fig. 4. Dependence of the total retention volume, $V_{R,tot}$, on time for step-gradient elution. Positive $v_D$ values; see text for definition of $v_D$. Eluents: 1, $1.00 \cdot 10^{-3}$ *M* TrHexA in phosphate buffer (pH 4.0)–methanol (1:1); 2, $9.99 \cdot 10^{-3}$ *M* sodium decanesulphonate in eluent 1. Analytes: ▽, 2-anthracenesulphonate; ×, 2,4-dinitrobenzoic acid.

A drastic decrease in peak width, $4\sigma$, was obtained when AS was affected by eluent 2. When the AS zone is concentrated in the front, the peak will be very narrow, since the rear of the AS zone migrates under conditions creating a greater velocity than that of the front. The difference in co-ion concentration between the front and rear depends on the steepness of the front, which is dependent on the column efficiency[13]. By using a very efficient column very narrow peaks can be obtained. The peak width of AS was 0.13 ml for all $v_D$ values, while in isocratic elution with eluent 1 the peak width was 2.4 ml and with eluent 2 it was 0.53 ml. As a consequence, the higher $V_{R,tot}$ is for AS (high $v_D$), the higher will be the measured efficiency, "*N*"[4]. For $v_D = +7.79$ ml, "*N*" was calculated to be $1.3 \cdot 10^6$ plates per m.

The measured efficiency cannot be compared with those of isocratic systems, since the peak width was unchanged independent of $V_{R,tot}$. It is of greater interest that the peak was 16 times higher than in an isocratic elution with eluent 1 and 4 times higher than with eluent 2.

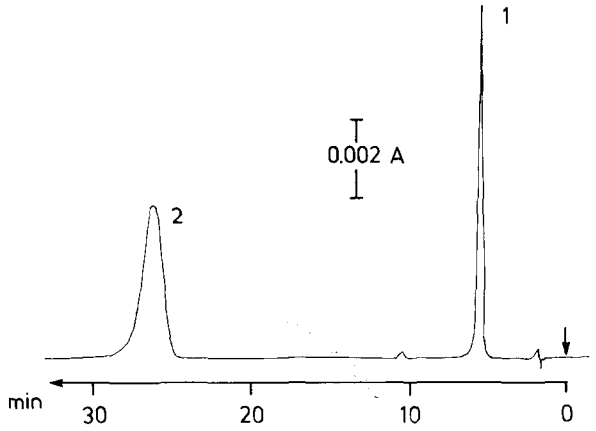


Fig. 5. Isocratic separation of anions. Eluent: $1.00 \cdot 10^{-3}$ *M* TrHexA in phosphate buffer (pH 4.0)–methanol (1:1). Peaks: 1 = 2,4-dinitrobenzoic acid ($C^0 = 2.0 \cdot 10^{-4}$ *M*), 2 = 2-anthracenesulphonate ($C^0 = 5.0 \cdot 10^{-5}$ *M*). Flow-rate: 0.71 ml/min.

The peak width of DNBA was unchanged for all $v_D$ values and had the same value as in isocratic elution with eluent 1, 0.46 ml, while with eluent 2 it was half as wide, 0.22 ml.

The features of the step-gradient technique are most easily demonstrated by a comparison of isocratic elution (Fig. 5) with step-gradient elution (Fig. 6), where $v_D = 3.7$ ml. In gradient elution the retention volume of the peak eluted was reduced 2.5 times and the detectability considerably improved.

When decanesulphonate was replaced by hexanesulphonate, keeping the concentration at $1.0 \cdot 10^{-2}$ *M*, the retention (Fig. 7) and "*N*" of both DNBA and AS were affected. On increasing $v_D$ (positive) the total retention increased. For DNBA a straight line with a slope of +1 was obtained when $V_{R,tot}$ was plotted against $v_D$.

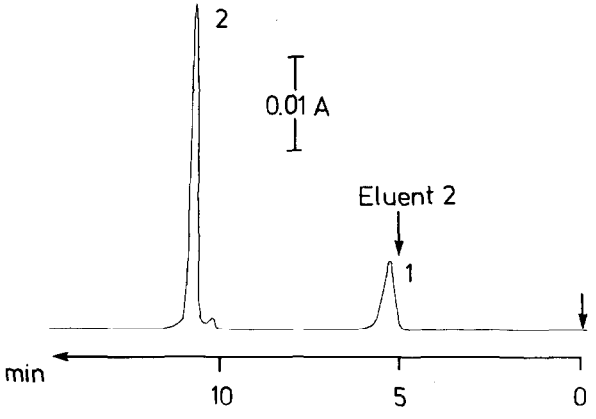


Fig. 6. Step-gradient elution by introduction of a co-ion; $v_D = +3.73$ ml. For eluents 1 and 2 see Fig. 4; peaks, see Fig. 5; Flow-rate: 0.74 ml/min.

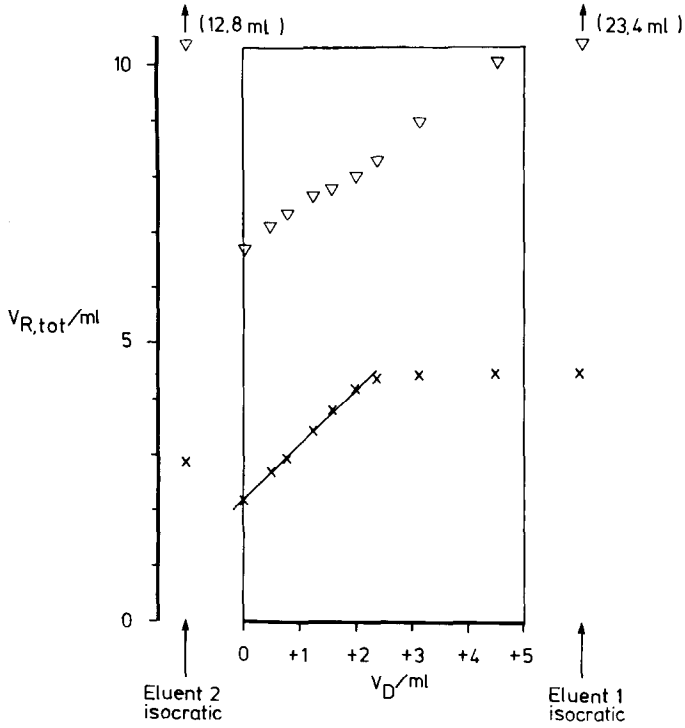


Fig. 7. Dependence of the total retention volume, $V_{R,tot}$, on time for step-gradient elution. Positive $v_D$ values. Details as in Fig. 4 except eluent 2, $1.00 \cdot 10^{-3}$ *M* sodium hexanesulphonate in eluent 1.

At $v_D$ values higher than +2.4 ml, the retention was unchanged and equal to that in isocratic elution with eluent 1. Eqn. 2 is valid for DNBA in the region $v_D = 0$ to +2.0 ml. The intercept of the line was in good agreement with the retention volume of the breakthrough front for $1.00 \cdot 10^{-2}$ *M* hexanesulphonate (2.0 ml). The peak width of DNBA was 0.13 ml in the region where it was affected by hexanesulphonate. In this region *"N"* also increased to a maximum of 150000 plates per m. At higher $v_D$ values the peak width continuously increased and *"N"* decreased to the values obtained with eluent 1, 0.47 ml and 16000 plates per m, respectively. With eluent 2 the peak width was 0.28 ml.

The AS peak was also affected, but to a lesser extent, compared to the effect of decanesulphonate. With increasing $v_D$ the retention volume of AS increased, but there was no linear relationship. With eluent 1 the retention volume of AS was very high, and thus, when eluent 2 was introduced, AS was very quickly reached by the hexanesulphonate front. The actual concentration of hexanesulphonate does not have enough competing effect to concentrate AS in the front. This means that the front will pass the AS zone, but the migration velocity of AS will be increased. Fig. 3B shows that the concentration of TrHexA, the counter ion, was decreased on introduction of decanesulphonate; a negative TrHexA zone was obtained. Also, this decrease in the TrHexA concentration will increase the migration velocity of AS.

TABLE III

EFFECT ON RETENTION VOLUME AND EFFICIENCY OF THE INTRODUCTION OF HEXANESULPHONATE BY THE STEP-GRADIENT TECHNIQUE, COMPARED TO AN ISOCRATIC ELUTION

Eluents: 1, $1.00 \cdot 10^{-3}$ *M* TrHexA in phosphate buffer (pH 4.0)–methanol (1:1); 2, $1.00 \cdot 10^{-2}$ *M* sodium hexanesulphonate in eluent 1.

| *Analyte* | *Eluent 2 (isocratic)* | | *Step gradient,* $v_D = +1.6$ *ml* | | *Eluent 1 (isocratic)* | |
|---|---|---|---|---|---|---|
| | $V_R$ *(ml)* | *N (per m)* | $V_{R,tot}$ *(ml)* | *"N" (per m)* | $V_R$ *(ml)* | *N (per m)* |
| DNBA | 2.9 | 15 000 | 3.8 | 133 000 | 4.5 | 16 000 |
| AS | 12.8 | 17 000 | 7.8 | 25 000 | 23.4 | 17 000 |

When TrHexA is returned to its original bulk concentration (end of the negative TrHexA zone) the migration velocity of AS will decrease. The migration velocity of AS will be changed twice in different directions, and thus, no linear relationship between $V_{R,tot}$ and $v_D$ is expected.

With increasing positive $v_D$ values, the peak width of AS first decreased to 0.62 ml at $v_D$ +2.4 ml and then increased. At higher $v_D$ values, *"N"* increased, but the effect was not so pronounced as for DNBA.

For small positive $v_D$ values it was observed that $V_{R,tot}$ for both DNBA and AS was smaller than in isocratic development with eluent 2 (Fig. 7). This indicates the effect on the retention of the negative TrHexA zone (Fig. 3B), in combination with the hexanesulphonate. For $v_D = +1.6$ ml the separation time was smaller and *"N"* was higher for the two analytes than in isocratic elution with eluent 2 (Table III).

*Step-gradient elution with negative* $v_D$ *values*

When AS was injected after the introduction of decanesulphonate (negative $v_D$ values), $V_{R,tot}$ was larger the later the injection was made (Fig. 8), but at larger

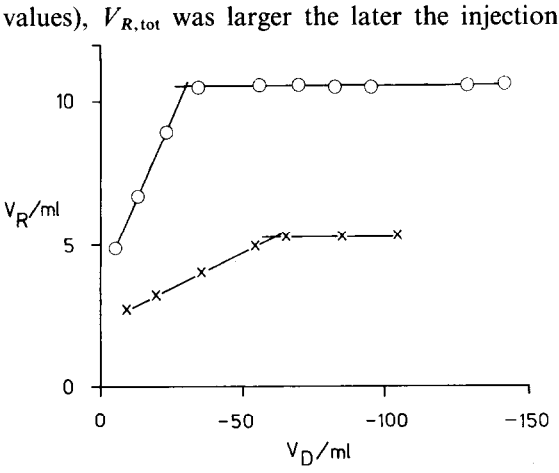


Fig. 8. Dependence of the total retention volume, $V_{R,tot}$, on time for step-gradient elution. Negative $v_D$ values. Eluents: 1, $1.00 \cdot 10^{-3}$ *M* TrHexA in phosphate buffer (pH 4.0)–methanol (1:1); 2, $2.42 \cdot 10^{-3}$ (○) or $1.01 \cdot 10^{-2}$ *M* (×) decanesulphonate in eluent 1. Analyte: 2-anthracenesulphonate.

negative $v_D$ values the retention volume was constant. The latter value is assumed to be the retention volume in eluent 2 when the column is equilibrated.

The increase in the retention volume shows the effect from the end of the negative TrHexA zone, *i.e.*, when the TrHexA concentration returns to the bulk concentration (Fig. 3B). AS migrates partly in the zone where the TrHexA concentration is lower and partly with the bulk concentration. The later AS was injected after the introduction of decanesulphonate the longer was the distance it migrated with the bulk concentration of TrHexA, and thus, the retention volume was increased. The position ($v_D$ values) where the retention volume of AS starts to be constant is in good agreement with the second breakthrough retention volumes given earlier (Table II).

The effect of the negative TrHexA zone on the retention of AS demonstrates further that eqn. 1 can be used only if the retention in the negative zone is known and if the analyte is not affected by the end of this zone (the TrHexA concentration increases).

*Reequilibration of the system*

When the column was equilibrated with an eluent containing both TrHexA and decanesulphonate (eluent 2 in the step-gradient elution) the decanesulphonate had to be desorbed prior to the next experiment. This could be performed by introduction of an eluent containing only TrHexA (eluent 1). However, the desorption of the anion and the reequilibration would then have been a lengthy process. To speed up the reequilibration, a large volume of methanol was injected, just after the TrHexA-containing eluent was introduced into the column. When 1.0 ml of methanol was injected, both TrHexA and decanesulphonate were completely desorbed. This was confirmed by use of the refractive index detector and continuous injections of DNBA. TrHexA must then be adsorbed before the column is equilibrated. This was achieved when 12 ml (the breakthrough retention volume) of the TrHexA-containing eluent were pumped through the column. When only 0.5 ml of methanol were injected the desorption was incomplete, and a larger volume (25 ml) of eluent 1 had to be pumped through the column before it was equilibrated. The injection of methanol, in combination with the introduction of the TrHexA-containing eluent, seems to be the most rapid way of reequilibrating the column. At the actual concentrations of TrHexA and decanesulphonate, this technique is three times as fast as the introduction of eluent 1 alone.

## CONCLUSIONS

Ion-pair step-gradient elution provides excellent possibilities for decreasing the analysis times and the detection limits of ionic analytes. By using suitable conditions, the separation times are decreased, while complete resolution is maintained. The total retention volume can in most cases be calculated by use of a simple equation. The peak width of late eluted peaks can be reduced to a large extent by increasing the hydrophobicity and/or the concentration of the co-ion in the second eluent. Early eluted peaks can also be affected by using more hydrophilic co-ions. In this case, late eluted peaks will also be affected but to a lesser extent. The system can be reequilibrated quickly by injection of a large volume of methanol in combination with the introduction of an eluent containing only the counter ion.

ACKNOWLEDGEMENTS

I am very grateful to G. Schill for his interest in this work and for fruitful discussions, to D. Westerlund for critical reading of the manuscript and to M. Edlund and B.-O. Häglund for technical assistance. A grant from the IF Foundation of Pharmaceutical Research is gratefully acknowledged.

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