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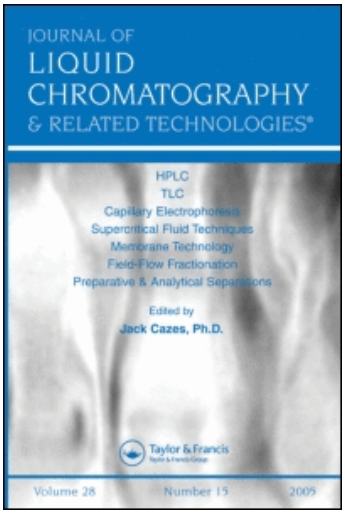

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Studies on System Peaks in Ion-Pair Adsorption Chromatography. II. Effects on Analyte Peak Compression and Deformation

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STUDIES ON SYSTEM PEAKS IN ION-PAIR ADSORPTION CHROMATOGRAPHY. II. EFFECTS ON ANALYTE PEAK COMPRESSION AND DEFORMATION

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ABSTRACT

Peak compression and deformation effects were studied in a reversed phase ion-pair adsorption chromatographic system, comprising silanized (C_{18}) silica as solid phase and an acidic mobile phase. An indirect detection technique was used, protriptyline (a secondary hydrophobic amine) being the probe, and substituted benzamides the analytes.

As the injection of large amounts of organic anions disturbed the column equilibria, system peaks were generated. The behavior was studied of analyte peaks co-eluting with the ion gradients in the negative probe (co-ion) peaks. Two principally different probe deficiency peaks were obtained: the negative system peak and the indirectly detected organic anion. Compression of a co-eluting analyte was obtained when low analyte concentrations eluted at certain positions in the negative probe peaks. Higher analyte concentrations or elution at other positions, produced deformation or

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splitting of the analyte peak. The variables studied were those governing the retention and shape of the analyte, organic anion and of the system peak.

INTRODUCTION

In gradient elution chromatography, the migrating velocity of the analytes in the column is increased, resulting in narrower analyte peaks. Thus the detection limit will be improved and the separation speed will increase, which is of particular value with regard to peaks eluting late in the isocratic system (1). The gradient can be introduced by changing the composition of the eluent, either as a continuous change or as a step change.

The shape of the eluted peaks also depends on the character of the injection solvent. Using solvents with weaker elution strength than the eluent can give rise to narrower analyte peaks, whereas stronger eluting injection solvents can give rise to broadening and even splitting of the analyte peaks (2-5). On the other hand, it has been observed that the use of an injection solvent with a retention similar to that of the analytes, resulted in more efficient analyte peaks (6).

In adsorption chromatography, gradient elution is usually performed by increasing the elution strength of the eluent. Thus in reversed phase adsorption liquid chromatography (LC), the concentration of the organic modifier is increased (1).

In reversed phase ion-pair adsorption chromatography narrow analyte peaks can be obtained by stepwise increase of the eluent concentration of the organic co-ion (an ion with the same charge as the analyte) (7-9). This was obtained by introducing a second eluent containing not only the counter ion, always present in ion-pair chromatography, but also the co-ion, resulting in a very steep gradient of the co-ion, a so called break-through front. A compression of the analyte peak was obtained when the analyte eluted in this front. This also strongly affected the analyte retention, resulting in great improvements in analysis time and

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detectability. However, before the next run, the column had to be re-equilibrated with the first eluent, which is time consuming and a drawback with the method.

The injection of a solvent deviating from the eluent, will disturb the equilibria in the column. This gives rise to migrating zones of mobile phase components, so called system zones. If one or more of the components can be detected, these zones will appear as positive or negative peaks (10-12). In the system zone there is a negative and positive gradient of the mobile phase component, which can be used to affect co-eluting analyte peaks. In ion-pair chromatography, system zones are easily created, by e.g., injecting a large amount of an organic anion simultaneously with a cationic analyte into a system, equilibrated with an organic co-ion present in the eluent. A very narrow analyte peak was observed when the analyte zone co-eluted with the negative system zone (i.e., a co-ion deficiency) (13).

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In reversed phase ion-pair LC, utilizing an organic ionic component in the mobile phase, both peak compression effects have been noted (7-9,13-15), as well as deformation or splitting effects (3,4,14,16-18). The peak compression is explained as a result of a co-ion competing effect (7-9,13,14) or as the effect of an ion-pairing agent (15). To explain peak splitting, have been proposed such events as interplay between two different chromatographic mechanisms (4,16-18) or between the positive and negative gradient in the system peak (14). Peak deformation or splitting is sometimes a problem in ion-pair chromatography, though in many papers this effect is merely mentioned as an incidental chromatographic artefact. Both when peak compression and peak splitting are obtained, retention of the analyte peak has been observed to change (4,7-9,13-18), as compared with the isocratic situation.

In this study are investigated the peak compression effect occurring when a cationic analyte co-eluted with a simultaneously injected organic anion or with a system peak of the probe (cationic). The aim was to ascertain how to obtain and to optimize this effect. The peak compression effect has been explained with reten-

tion models based on the ion-pair adsorption model (19-23). The deformation, splitting and retention effects were also studied.

MATERIALS AND METHODS

Apparatus, chromatographic technique and preparation of the eluent

see ref. 23 and 24.

Chemicals

Acetonitrile, Lichrosolv, and dichloromethane p.a. were obtained from Merck (Darmstadt, G.F.R.), and protriptyline (PT) from Merck Sharp and Dohme (Haarlem, Netherlands). The sodium salts of hexane- and octanesulfonic acid and pentyl- and octylsulfate were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). The sodium salts of hexyl- and nonylsulfate were obtained from Merck, and the sodium salts of heptane- and decanesulfonic acids from Fluka AG (Buchs, Switzerland). Phosphoric acid 99% and 98% crystalline p.a. quality, and Titrisol 1 M NaOH were obtained from Merck.

The substituted benzamides used as analytes throughout this work are denoted FLA combined with a number (cf. ref. 24). They were synthesized at the Department of CNS-medicinal chemistry, Astra Alab AB (Södertälje, Sweden), and kindly made available by L.B. Nilsson, Dept. of Bioanalytical Chemistry.

Detection technique

The technique has been described earlier (23,24). The benzamide signal was measured at a wavelength where only the benzamide had absorbance. At very large deviations in the PT signal the benzamide signal was disturbed, resulting in baseline inter-

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ference. However, the PT signal was measured at a wavelength where also the benzamide had absorbance. To compensate for the benzamide absorbance, another wavelength was used as reference, where the benzamide absorbance equaled that at the first wavelength, and where PT had no absorbance.

Determination of the retention volume

The retention volume, V_R, was determined at the peak maximum for positive peaks, and at the peak minimum for negative peaks.

To measure the asymmetry factor (asf), a perpendicular was dropped to the baseline from the vertex, formed by the two peak tangents. The back part of the peak baseline divided by the front part gives the asymmetry factor.

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Determination of the peak depth

Peak depth was measured as the distance between the baseline and the peak minimum, the value being given in milliabsorbance units (mAU).

Determination of the steepness

The tangent of the upslope or the downslope of a peak is a measure of slope steepness. The steepness is given as milliabsorbance units per milliliter (mAU/ml), see Fig. 1, and corresponds to a concentration change of the detectable compound in the peak.

RESULTS AND DISCUSSION

The reversed phase ion-pair LC system used in this study, was equilibrated with the secondary amine protriptyline (PT), which is

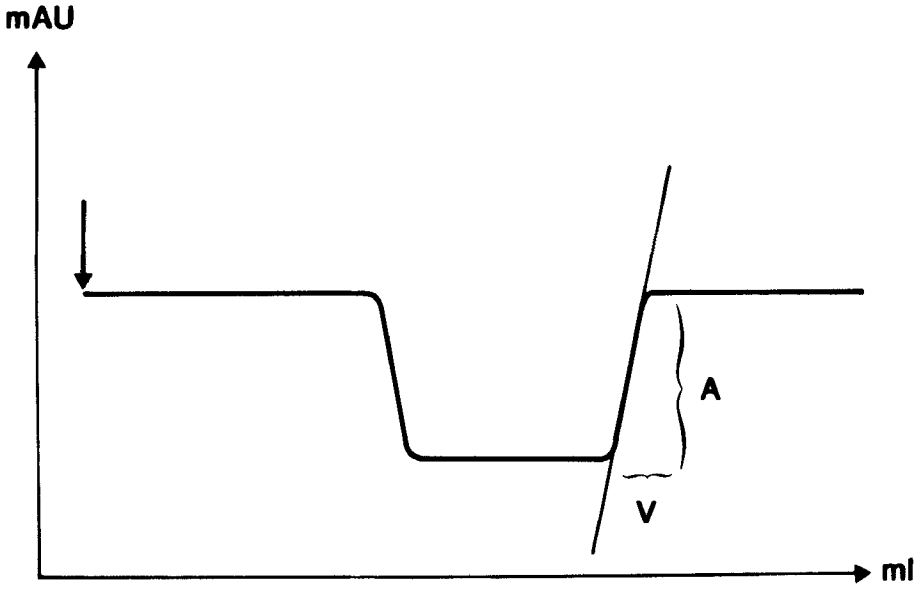

FIGURE 1

Principles for the measurement of steepness of gradient in a negative zone.
Steepness = A/v (mAU/ml)

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UV-absorbing and used as a so called probe. In a previous work the adsorption of PT to Nucleosil C_{18} was studied (24). A 2-site adsorption model of the Langmuir type was indicated, with the strong site covered to 5.5% and 37%, respectively, and the weak site covered to 0.4% and 4.2%, respectively, at the two PT concentrations mainly used in this work, 1.5×10^{-5} M and 1.5×10^{-4} M. The cationic analytes used for the compression studies were substituted benzamides (see ref. 24).

The retention models used are based on the ion-pair adsorption model (19-23). The adsorption of an ionic organic compound to the solid phase is accompanied by an ion of opposite charge. The

limited adsorption capacity of the solid phase gives rise to competition between the ions adsorbed (8,19-23). To simplify discussion, the surface is assumed to be homogenous.

The net retention volume of the cationic analyte, HA^+, with Q^+ as the organic cationic mobile phase component, may be described by (24):

$$V_{N,HA} = \frac{W_s K_o K_{HAX} [X^-]_m}{1 + K_{HAX}[HA^+]_m[X^-]_m + K_{QX}[Q^+]_m[X^-]_m} \qquad (1)$$

K_o, K_{HAX} and K_{QX} are constants describing the adsorption. The equation is only used for qualitative discussions, since it is quantitatively valid only at low concentrations of HA^+, when the term, $K_{HAX}[HA^+]_m[X^-]_m$, in the denominator is negligible. The retention of the cationic analyte HA^+, will increase with increasing concentration of the mobile phase anion, X^-, or decrease with increasing concentration of the mobile phase component, Q^+.

In reversed phase ion-pair chromatography, the injection of an organic ion will change the equilibria of the organic mobile phase components at the top of the column, here referred as the injection zone, resulting in migrating component zones. These zones will appear as positive or negative system peaks if at least one of the mobile phase components is detectable (10-12). The system peak retention is governed by an expression related to eqn. 1 (7,12,24).

In this study, the equilibria of the probe, protriptyline, were disturbed by injecting a large amount of an organic anion (sulfonate or sulfate). This gave rise to two peaks observed by the probe signal, a negative peak, followed later by a positive peak. When the anion had higher retention than the probe, the negative peak was the probe system peak. This situation is designated case 1 (Fig. 2). The non-detectable anion eluted together with a probe excess, and appeared as the positive probe peak (10-12). When the anion had lower retention than the probe, the

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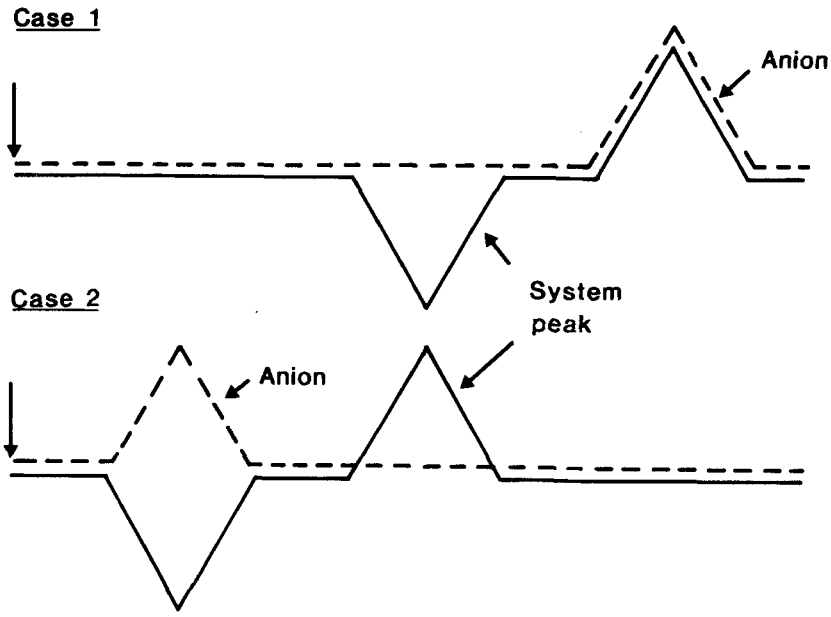

FIGURE 2

Principles for indirect UV-detection by a cationic probe after injecting a non-UV absorbing anion. Visible signal (———). The non-visible anion signal is indicated (-----).
Case 1. Anion retention is higher than probe retention.
Case 2. Anion retention is lower than probe retention.

anion was indirectly detected as a negative peak, this condition being designated case 2 (Fig. 2). The system peak then appeared as the positive probe peak (10-12).

Peak compression

The two kinds of negative probe-signal peaks, case 1 and case 2, were used for gradient purposes. The simultaneous injection of

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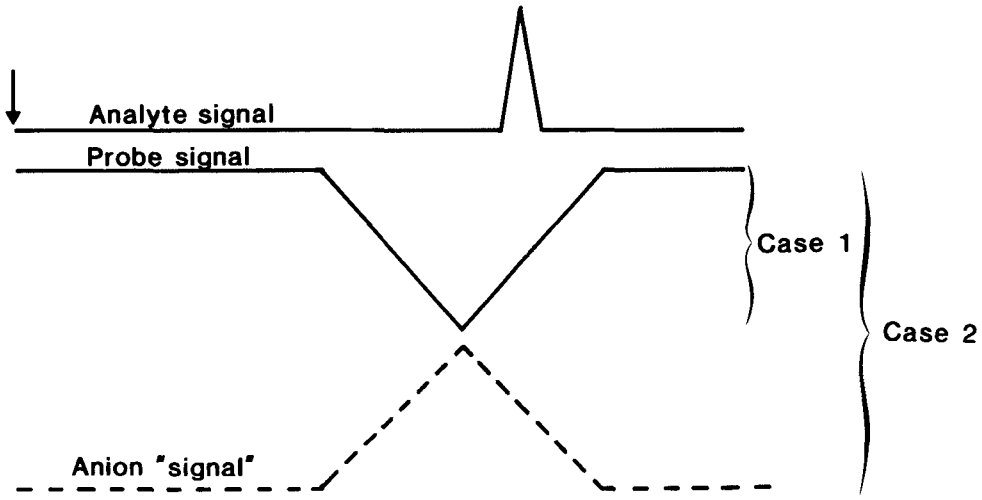

FIGURE 3

Schematic presentation of peak compression. The analyte peak co-elutes with the back part of the negative probe peak, case 1 or case 2 (cf. Fig. 2).

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an analyte with a large amount of an organic anion, resulted in a compressed analyte peak when the analyte co-eluted with the back part of the negative probe signal peak.

In the back part of the negative probe signal peaks, the probe concentration increases continuously. When the cationic analyte elutes in this co-ion gradient, it is displaced by the probe, the displacement being larger in the back part of the analyte zone than in the front part (Fig. 3)(eqn. 1). The back part of the analyte zone thus migrates under conditions which increase the migration velocity, as compared with that of the front part, causing a narrower analyte peak to elute. The probe gradient in the negative probe signal peak in case 2, is complemented by a decreasing concentration of the anion (Fig. 3). This counter ion

gradient also causes a narrower analyte peak (eqn. 1). A basic requirement for obtaining the peak compression effect is thus that the analyte elutes in the so called upslope of the back part of the negative probe signal peaks, in case 1 or case 2. The greater the steepness of the gradient(s) in the upslope, the larger the peak compression effect is expected to be.

Retention regulation

To ascertain how to obtain the co-elution of the analyte with the back part of the negative probe peak, it was studied how the retention of these peaks might be regulated.

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Analyte retention

The retention regulation of the cationic analytes and the organic anions used in this system has been described elsewhere (24). It was shown that the retention of the cationic analyte decreased with increasing concentration both of the cationic probe (PT) and of the analyte itself (cf. ref. 20,21). It was also shown that the retention of the organic anion was dependent on the PT concentration in the eluent (cf. eqn. 1).

When the cationic analyte was injected simultaneously with a large amount of an organic anion, a counter ion, the situation is more complicated than when the organic anion is a regular eluent component. Since the analyte and the anion are injected simultaneously, they can affect each other both in the injection zone and during co-migration along the column, until the analyte and anion zones separate from each other. The system peak created by the anion injection, containing an excess or deficiency of the probe (PT), the co-ion, also affects analyte retention.

When the analyte FLA 870 was injected simultaneously with some organic anions of different hydrophobicities, octanesulfonate gave a higher analyte retention than the more hydrophobic decanesulfonate (Table 1). The reason is probably that the analyte and

TABLE 1

Retention of FLA 870 when simultaneously injected with different hydrophobic anions.
Inj. sample: 100 µl FLA 870, 5.0×10^{-4} M, and anion, 5.0×10^{-4} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.2×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

Anion	Anion ret./ml	Analyte ret./ml
hexanesulfonate	2.4	9.6
octanesulfonate	9.0	10.8
decanesulfonate	35.0	10.1

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octanesulfonate had similar retentions. The examples clearly indicate that not only the hydrophobicity of the anion is of importance for the effect on analyte retention, but also the retention difference between the analyte and the anion when eluted. The analyte and the organic anion affect each other already from the beginning of their migration along the column, resulting in ion-pair adsorption (24). The further they co-migrate, the greater the effect on analyte retention.

The probe system peak created by the injection of a large amount of a hydrophobic anion also strongly affects analyte retention. When 20 µl of a sample with a low concentration of the hydrophobic analyte, FLA 659 (i.e., a strongly retained analyte), was injected simultaneously with a high concentration of the anion decanesulfonate, the analyte co-eluted with the negative system peak. Analyte retention increased from 19.0 (isocratic) to 23.2 ml, and anion reten-

tion was 31.7 ml. Analyte retention was thus affected by the low co-ion concentration present in the negative system peak. The initial co-migration with the anion, the counter ion, also affected the retention.

The simultaneous injection of the cationic analyte and the organic anion not always results in retention changes when the anion concentration is changed. This is especially the case when injecting a rather hydrophilic analyte and anion. Table 2 shows the simultaneous injection of FLA 908 with the more retained anion octanesulfonate. Analyte retention remained unaffected even by a ten fold increase of the anion concentration.

On the other hand, anion retention can be affected by the analyte concentration. This is shown in Table 3 for a rather low concentration of octanesulfonate at increasing analyte concentrations.

The retention effects described indicate a complex retention behavior, when the equilibria in the system are disturbed by co-injecting the analyte with an organic anion.

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TABLE 2

Retention of FLA 908 when simultaneously injected with different concentrations of octanesulfonate (OS).
Inj. sample: 20 µl FLA 908, 5.0×10^{-4} M, and octanesulfonate in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

OS conc./M	OS ret./ml	FLA 908 ret./ml
5.0×10^{-5}	9.1	4.2
5.0×10^{-4}	8.5	4.2

TABLE 3

Retention of octanesulfonate (OS) when simultaneously injected with FLA 870 of different concentrations.
Inj. sample: 100 µl octanesulfonate, 5.1×10^{-5} M, and FLA 870 in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.2×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

FLA 870 conc./M	OS ret./ml
1.0×10^{-5}	9.0
1.0×10^{-4}	9.3
1.0×10^{-3}	10.0

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The system peak retention

The retention of large system peaks has not been studied in detail, and no retention equation has been developed. However, retention behavior can be predicted in a qualitative way from equations given earlier (24). In line with the Langmuir theory, large negative system peaks have a higher retention than positive ones (24), due to the different concentrations of the system peak component in the two types of peaks. Also the retention of the system peaks will be affected by the co-elution of organic anion or cations, but this has not yet been studied in detail.

The steepness of the probe upslopes

The steepness of the negative probe signal peak is an important variable for obtaining peak compressions. A large degree of

steepness of the upslope of the back part of the negative peak is essential. Therefore the factors governing the steepness of the negative probe peaks used for the gradient purposes in this system, case 1 and case 2, were carefully studied.

The steepness indicates the rate of change in probe concentration and was measured by dividing the absorbance by the volume occupied by the back part of the negative peak (Fig. 1). From this it follows that the steepness is dependent both on the depth, width and symmetry of the peak. Increased depth will favour a large steepness, while increased peak width and tailing will cause a lower steepness of the back part of the negative peak.

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Case 2

The shape of the indirectly detected organic anion peak, case 2, was studied (cf. Fig. 2), when changing the concentration or the hydrophobicity of the injected organic anion. Special attention was focused on studying the change in the steepness of the upslope of the negative peak.

The anion octanesulfonate was studied in the concentration range 1.0×10^{-6} M to 1.0×10^{-2} M (Table 4). Anion retention decreased, while the peak width and asymmetry factor increased with increasing octanesulfonate concentrations. This is a consequence of the limited adsorption capacity of the solid phase, and resulted in a sharp front and diffuseness at the back of the eluted peak. Despite increased peak width and tailing, the steepness of the peak upslope (the back) increased, due to the simultaneous increase in peak depth.

However, this increase in steepness was slight compared with the increase in steepness for the front part of the peak, the downslope (Table 4). Moreover, in line with the Langmuir equation, the retention of the peak front decreased much more than did that of the back part.

TABLE 4

Peak variables of octanesulfonate (OS) for increasing injection concentrations.
Inj. sample: 100 µl octanesulfonate in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.1×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

OS conc./M	Ret. /ml	Peak width/ml	asf	Depth /mAU	Steepness/mAU/ml Front	Back
1.0×10^{-6}	9.8	0.7	2.1	12	58	23
1.0×10^{-5}	9.6	0.7	2.1	15	105	33
1.0×10^{-4}	9.1	1.1	4.2	55	474	66
1.0×10^{-3}	8.0	2.2	7.0	139	1276	74
2.0×10^{-3}	6.8	2.4	9.3	189	1572	86
5.1×10^{-3}	5.8	3.2	12.8	269	1942	93
1.0×10^{-2}	4.9	4.1	20.6	361	3204	96

When organic anions with increasing hydrophobicity but lower retention than the probe were injected, the peak width and asymmetry factor increased (Table 5), as did the depth of the negative peak.

The increased hydrophobicity resulted in a closer elution between the anion and the system peak, affecting the apparent peak shape and peak depth by a phenomenon known as relative response (10-12).

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TABLE 5

Peak variables for anions of different hydrophobicities.
Inj. sample: 100 µl anion in phosphate buffer, pH 2.0
Eluent: Protriptyline in phosphate buffer, pH 2.0, + acetonitrile (3+1)

Anion concentration: 1.0×10^{-3} M.
Protriptyline concentration: 1.2×10^{-4} M.

Anion	Ret./ml	Peak width/ml	asf	Depth /mAU	Steepness Back /mAU/ml
hexanesulfonate	2.3	0.4	4.0	105	390
octanesulfonate	7.5	2.0	6.8	185	104
Anion concentration: 1.2×10^{-3} M. Protriptyline concentration: 1.1×10^{-4} M.					
pentylsulfate	2.6	0.2	2.8	26	209
hexylsulfate	4.5	0.9	5.5	122	172

This relative response consists of an increase in response in indirect detection as analyte retention approaches the system peak. However, despite the increased peak depth, the increased peak width and tailing resulted in a decreased steepness of the upslope, the back, of the negative probe peak (Table 5).

The greatest steepness of the probe gradient at the rear slope of the negative probe peak, case 2, was obtained when injecting a large amount of a low-retained anion at the actual PT concentration.

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The positive system peak was observed to follow the same tendencies as the injected organic anion, when the peak was made larger. Thus peak width and tailing increased (i.e., a sharp front and a diffuse rear slope of the eluted peak), with increasing concentration of the organic anion injected.

Case 1

When anion retention was higher than probe retention, the system peak was negative, case 1. Injecting 1.0×10^{-4} M to 1.0×10^{-2} M of the organic anion nonylsulfate into the system (Table 6) showed that the depth of the negative peak to increase markedly until the nonylsulfate concentration was 1.0×10^{-3} M. At higher concentrations, the depth was almost constant, though the peak increased in width and was transformed into a zone, which means that the minimum probe concentration appeared as a plateau instead of as a peak (cf. Fig. 1 and ref. 23). The depth of the zone was of the same magnitude as the absorbance given by the actual PT concentration in the eluent, indicating that the nonylsulfate concentration required for a zone to appear instead of a peak is strongly dependent on the PT concentration. When the nonylsulfate concentration was increased at the low concentrations still giving rise to a peak, both retention and peak width increased, while the asymmetry factor decreased (Table 6). The increased leading gave a diffuse front and a sharp back of the larger negative system peak. Together with the increased depth, this resulted in a markedly increased steepness of the upslope (i.e., the back slope), of the negative system peak (Table 6). This is opposite to the case with indirect detection of the anion, case 2 (cf. Table 4). With a further increases in anion concentration, the increase in depth and steepness for the negative system zone was reduced.

The increase in width of the system peak, with increasing concentration of injected nonylsulfate, is shown in Fig. 4. The retention volumes for half the downslope of the front part and

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half the upslope of the back part of the negative system peak or zone, were plotted versus the nonylsulfate concentrations. Initially, the retention of the back part increased markedly while the retention of the front part remained fairly constant. This is in contrast to the indirectly detected anion (case 2) or the positive system peak, where the retention of the back remained fairly constant, while the retention of the front decreases largely. However, at the anion concentration, 3.4×10^{-3} M, retention both of the back and front parts of the system zone began to decrease (Fig. 4). This is probably due to disturbance from the anion that eluted closely following the system zone. At this anion concentration, the retention of the back part of the negative system zone reaches the retention for the front part of the indirectly detected positive anion peak.

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Peak compression in case 1

The peak compression effect obtained for the analyte when eluted in the upslope of the negative system peak (case 1) was studied.

The hydrophobic analyte, FLA 659, was injected together with the hydrophobic anion decanesulfonate (Fig. 5). The analyte concentration was 1.0×10^{-5} M, and the anion concentration 5.0×10^{-3} M. The high anion concentration gave rise to a large negative system zone in which the analyte peak eluted. However, only the back part of the analyte peak eluted in the upslope of the back part of the zone. As compared with the isocratic situation, the efficiency increased from N = 4100 to "N" = 22500.
The symbol "N" is used to indicate the non-isocratic situation (cf. ref. 23). The analyte retention volume increased from 19.2 to 24.9 ml (cf. analyte retention above), and the peak height from 2.0 to 3.5 mAU. Peak width decreased from 1.2 to 0.7 ml, and the asymmetry factor from 2.0 to 1.3.

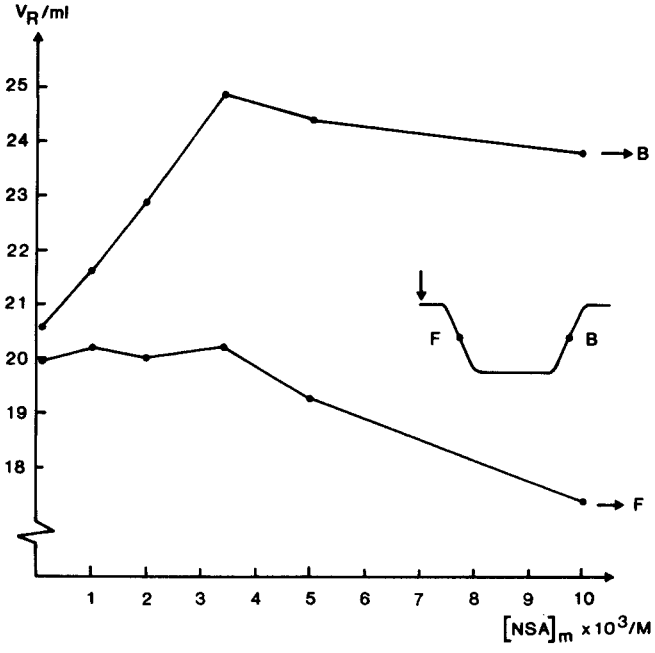

FIGURE 4

Shape of the negative system peak or zone (case 1) at increasing nonylsulfate concentrations.
Injected sample and eluent, see Table 6.

The indirectly detected positive anion peak eluted close after the negative system zone. At lower analyte concentrations, analyte retention increased further, and eluted closer to the anion. At analyte concentrations of 5.0×10^{-6} M and below, the analyte peak was deformed and showed extreme tailing (Fig. 6), indicating that the analyte and anion partly co-eluted. Thus the tailing was a result of the retaining effect of the anion zone. Disturbances of the analyte signal is due to incomplete compensation for the probe absorbance (cf. detection technique).

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TABLE 6

System peak (negative) variables for increasing injection concentrations of nonylsulfate (NSA).
Inj. sample: 200 µl nonylsulfate in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-5} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

NSA conc./M	Ret./ml	Peak width/ml	asf	Depth/mAU	Steepness Back/mAU/ml
	System peak				
1.0×10^{-4}	20.3	1.0	0.9	7.7	20
1.0×10^{-3}	21.3	2.4	0.3	79,7	203
	System zone				
2.0×10^{-3}				82.7	220
3.4×10^{-3}				84.6	228
5.0×10^{-3}				85.1	271
1.0×10^{-2}				87.3	247

The possibility was examined of adjusting the analyte peak within the upslope of the negative system peak by increasing the anion concentration. The analyte, FLA 659, was injected together with three different nonylsulfate concentrations: 1.0, 2.0 and 3.3 $\times 10^{-3}$ M. The analyte eluted in the negative system peak created by the anion. Analyte peak width was plotted together with the system zone width versus the nonylsulfate concentration (Fig. 7), giving the position of the analyte peak in the system zone. Both

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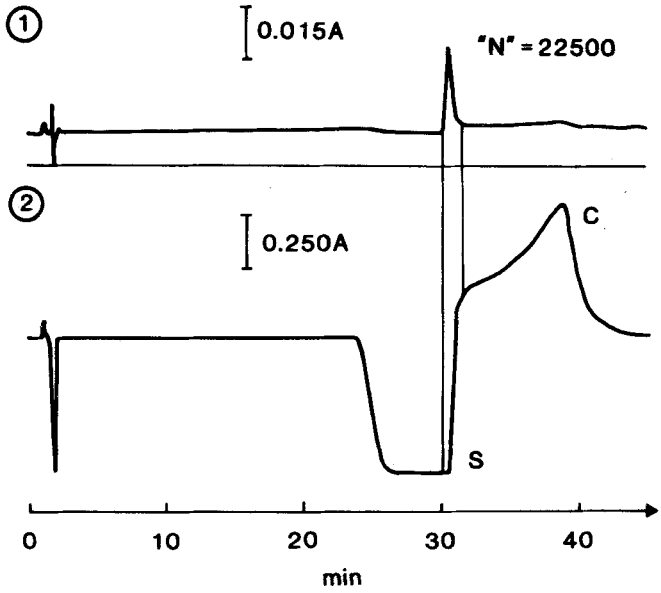

FIGURE 5

Peak compression of FLA 659 when injected with decanesulfonate, case 1.
1. The analyte signal
2. The probe (PT) signal, S = system peak, C = organic anion
Inj. sample: 200 µl FLA 659, 1.0×10^{-5} M, and decanesulfonate, 5.0×10^{-3} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.4×10^{-5} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

the retention of the back part of the negative system zone and analyte retention increased substantially with increasing anion concentrations. An important feature is that the increase in analyte retention was greater, resulting in displacement of the analyte peak from the plateau of the system zone to the upslope. As a consequence, the efficiency increased from "N" = 4300 to "N" = 20600. The steepness of the upslopes was similar (cf. Table 6). At the highest anion concentration, the analyte eluted very close

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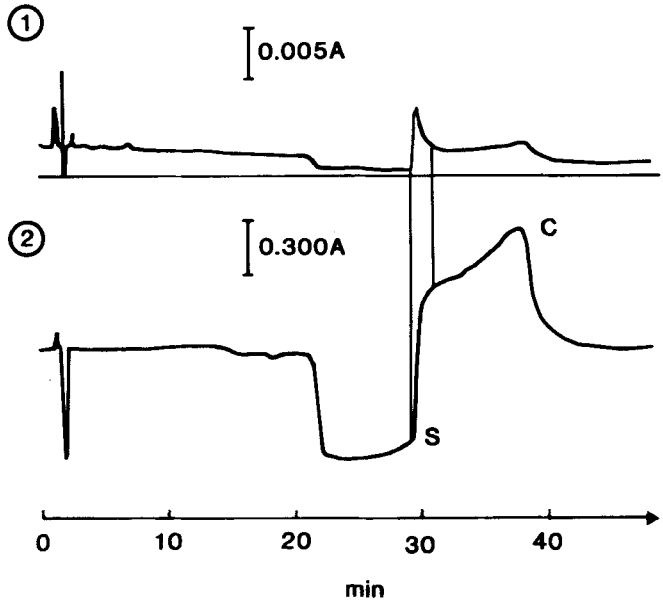

FIGURE 6

Peak tailing of analyte when its back co-elutes with the anion. Injection sample and eluent, see Fig. 5. The FLA 659 concentration was 5.0×10^{-6} M.

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to the anion, affecting the analyte peak shape negatively by inducing tailing (cf. Fig. 6).

Peak compression in case 2

The peak compression effect for the analyte when eluted in the upslope of the negative probe peak (case 2) was studied. The upslope of the back of this peak consists both of an increasing probe (co-ion) concentration and a decreasing anion (counter ion) concentration, either (or both) of which contribute to peak comp-

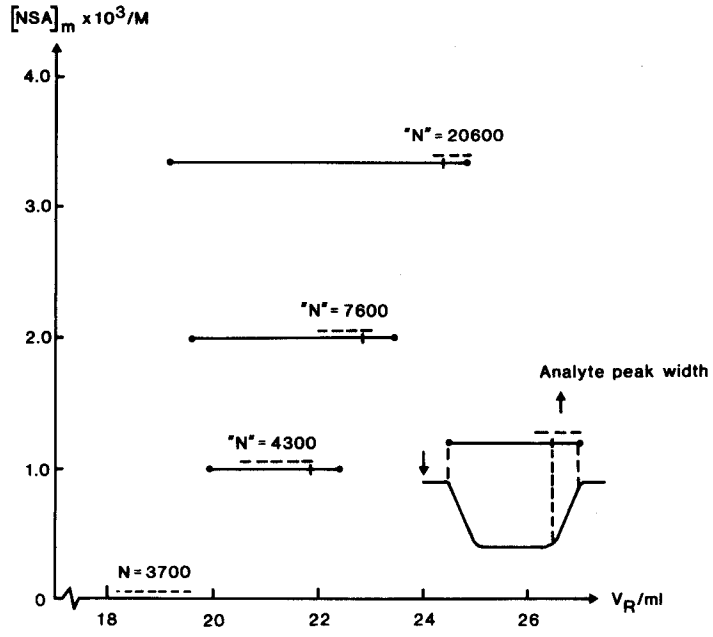

FIGURE 7

Regulation of the FLA 659 position in the negative system zone (case 1) by variation of the injected nonylsulfate concentration.
Inj. sample: 200 µl FLA 659, 1.0 x 10^{-5} M, and nonylsulfate in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5 x 10^{-5} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

ression. Different combinations of analytes and organic anions with retentions lower than that of the probe, were injected simultaneously, in order to obtain a co-elution of the analyte with the back of such a case 2 peak.

The analyte, FLA 908, was simultaneously injected with the anion octanesulfonate (Fig. 8). The analyte concentration was 1.0 x 10^{-5} M, and the anion concentration 5.0 x 10^{-3} M. The analyte peak eluted in the upslope of the negative probe peak

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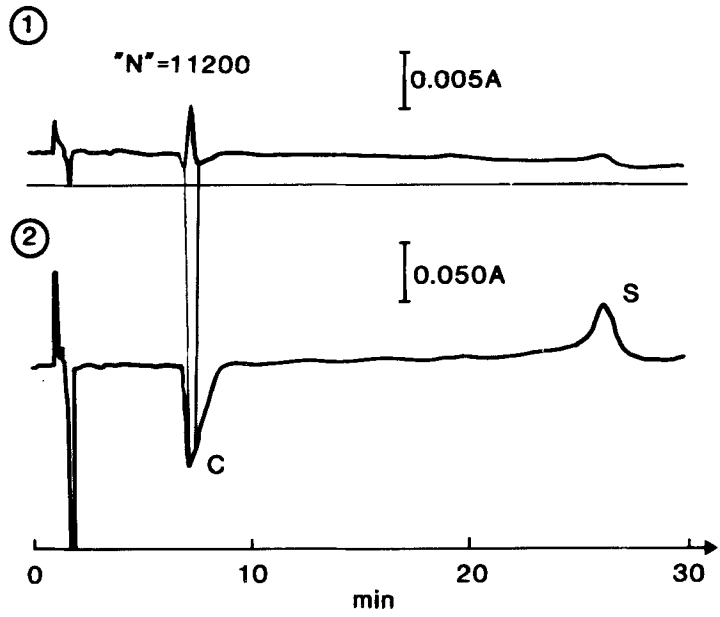

FIGURE 8

Peak compression of FLA 908 at co-injection with octanesulfonate (case 2).
Inj. sample: 20 µl FLA 908, 1.0×10^{-5} M, and octanesulfonate, 5.0×10^{-3} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-5} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

resulting in an efficiency of "N" = 11200, as compared with the isocratic situation where N = 5000. The retention volume of the analyte peak increased from 5.2 to 6.0 ml, and the peak width decreased from 0.29 to 0.23 ml, whereas the asymmetry factor was similar to that of the isocratic run.

The peak compression effect was studied when the injection volume and the hydrophobicity of the analyte and anion combination were changed (Table 7), using the same concentrations as above. The analyte, FLA 908, was injected simultaneously with the anion

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TABLE 7

Analyte efficiency at different injection volumes and hydrophobicity of analyte and anion.
Inj. sample: Analyte, 1.0×10^{-5} M, and anion, 5.0×10^{-3} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-5} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)
FLA 870 and octylsulfate is the more hydrophobic combination.

Analyte	Anion	Inj. vol./µl	"N"/N	Steepness Back/mAU/ml
FLA 908	octanesulfonate	20	2.2	12.8
		200	1.7	13.6
FLA 870	octylsulfate	20	1.6	11.7
		200	1.5	10.6

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octanesulfonate, and the analyte, FLA 870, was injected with the octylsulfate. Both FLA 908 and octanesulfonate are less hydrophobic (being less retained) than FLA 870 and octylsulfate. The less hydrophobic pair and the lower injection volume produced a higher peak compression effect.

With the analyte, FLA 870, at 1.0×10^{-5} M, and the anion octanesulfonate, at 5.0×10^{-5} M, the analyte eluted with a higher retention than the anion peak, and they were well separated (Fig. 9a). This gave a small peak compression effect "N" = 4800 and a small retention increase. In the corresponding isocratic run, N = 4000. The effect probably resulted from an initial analyte migration in the upslope of the negative probe zone, during a part

of the column elution. When the analyte concentration was increased 10 times, its retention decreased and it co-eluted with the end of the upslope of the negative probe peak (Fig. 9b). This gave an increased peak compression effect, "N" = 5000, as compared to the isocratic situation, where N = 1500 (this is another less efficient column, compared to that used in Fig. 9a).
Injecting an even higher concentration of the analyte and a higher concentration of the octanesulfonate also resulted in co-elution of the analyte with the end of the upslope (Fig. 9c), but in this case the efficiency was increased to "N" = 11400. The smaller injection volume, 20 µl, might also affect the peak compression positively as indicated above (cf. Table 7).

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Comparison between peak compression in case 1 and in case 2

The injection of large amounts of hydrophilic anions gave rise to the negative probe peak, case 2, whereas anions that were more hydrophobic produced the negative system peak, case 1. The upslopes of the two different types of negative probe peaks were both used for the compression of a co-eluting analyte peak. The case 2 peak, indicating the injected organic anion, showed tailing, and the large case 1 system peak leading. These effects were due to the limited adsorption capacity and resulted in much higher steepness for the case 1 upslope than for the case 2 upslope. The steepest probe gradient in case 2 was obtained by injecting high concentrations of low-retained anions into a system with a high PT concentration in the eluent. However, steepness increased only slightly with increased anion concentration. The steepest probe gradient in the case 1 situation was obtained when the system peak had the shape of a "zone" (see e.g., Fig. 1).

In the case 1 example shown in Fig. 5 the steepness of the upslope was about 300 mAU/ml. The injection of the low-retained analyte and anion combination (Fig. 8), resulted in a case 2 situation with a much lower steepness, 13 mAU/ml. The peak com-

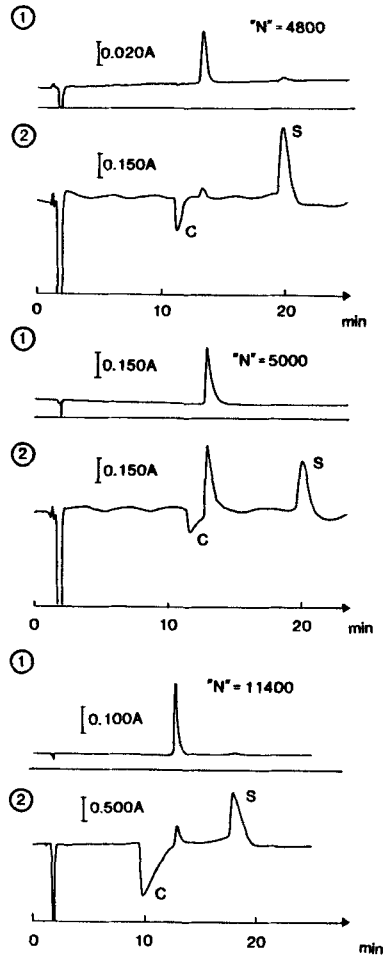

FIGURE 9 a,b and c

Peak compressions obtained by co-injecting FLA 870 and octanesulfonate. The analyte and the probe peak (case 2) eluted closely (a), or co-eluted (b and c).

a. Inj. sample: 100 µl FLA 870, 1.0×10^{-5} M, and octanesulfonate, 5.0×10^{-5} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.2×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

b. Inj. sample and eluent, see a. The conc. of FLA 870 is increased 10 times.

c. Inj. sample: 20 µl FLA 870, 4.8×10^{-4} M, and octanesulfonate, 5.1×10^{-3} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

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ression effect in Fig. 5 was negatively affected by the closely eluting anion (cf. peak compression in case 1, above). Despite this the efficiency increase was larger than in the case 2 example, probably due to the greater steepness of the probe upslope. The steepness of the case 2 upslope was much greater with a ten times higher PT concentration in the eluent (cf. Tables 4 and 5).

Peak deformation in probe upslopes

The compression effects described above were obtained for relatively low analyte concentrations. When the analyte concentration was increased, the compression effect was often transformed to a deformation effect, which was obtained when the analyte still eluted in the upslope of the negative probe peak.

In case 1 above (Fig. 5), a large peak compression effect was obtained. When the analyte concentration was increased five times a slight deformation occurred at the rear slope of the analyte peak (Fig. 10a). Simultaneously a deformation of the probe signal arose at the same retention value as did the deformation in the analyte peak. When the analyte concentration was increased another two times, the deformation in the analyte peak and the probe signal was much more pronounced (Fig. 10b).

The deformation effect was also observed for the case 2 situation. The peak compression effect seen in Fig. 8 transformed to peak deformation, when the analyte concentration was increased ten times and the anion concentration two times (Fig. 11a). However, in this case the deformation arose at the front side of the analyte peak. A similar deformation was also observed for the lower anion concentration. Also in these cases there was a corresponding deformation of the probe signal.

The deformation was also found to be dependent on the hydrophobicity and the injection volume. When the injection volume was increased, from 20 µl (Fig. 11a) to 200 µl (Fig. 11b), using the same analyte and anion concentrations (i.e., as in Fig. 11a), this

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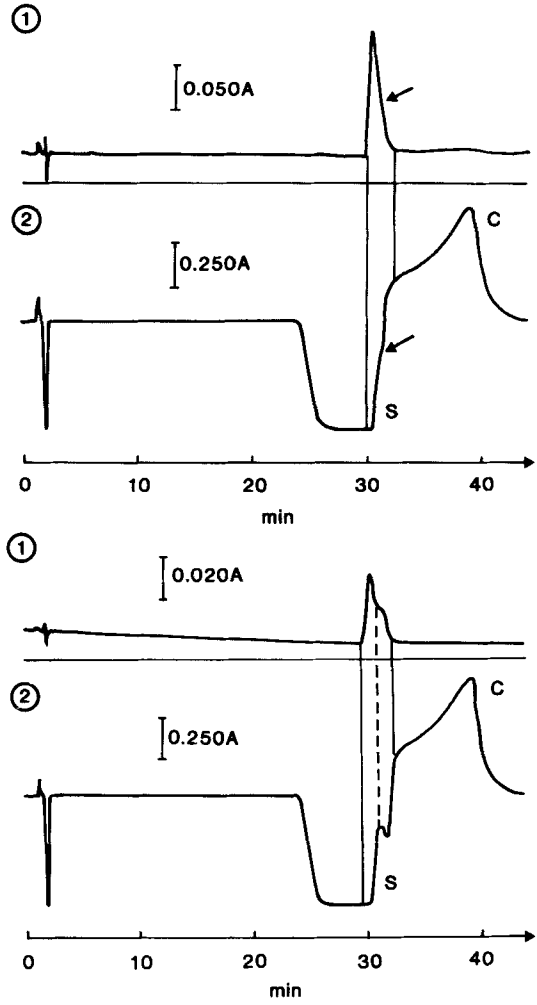

FIGURE 10 a and b

Peak deformations obtained at co-elution of high concentrations of FLA 659 and the back part of the negative system zone, case 1 (cf. Fig. 5).
Inj. sample and eluent, see Fig. 5.
a. The FLA 659 concentration is 5.0×10^{-5} M
b. The FLA 659 concentration is 1.0×10^{-4} M

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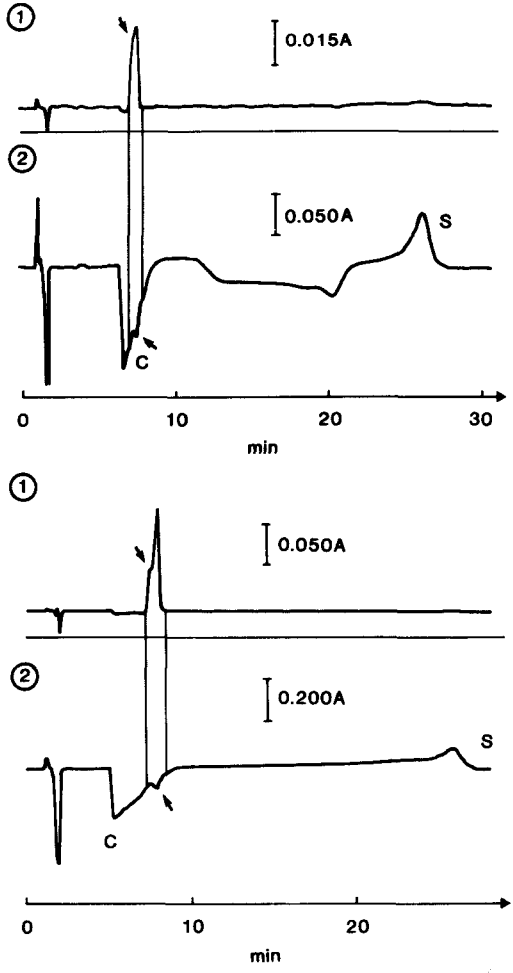

FIGURE 11 a and b

Peak deformation obtained at co-elution of a high concentration of FLA 908 with the back part of the negative probe peak, case 2 (cf. Fig. 8).
Inj. sample: FLA 908, 1.0×10^{-4} M, and octanesulfonate, 1.0×10^{-2} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-5} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)
a. Injected volume: 20 µl
b. Injected volume: 200 µl

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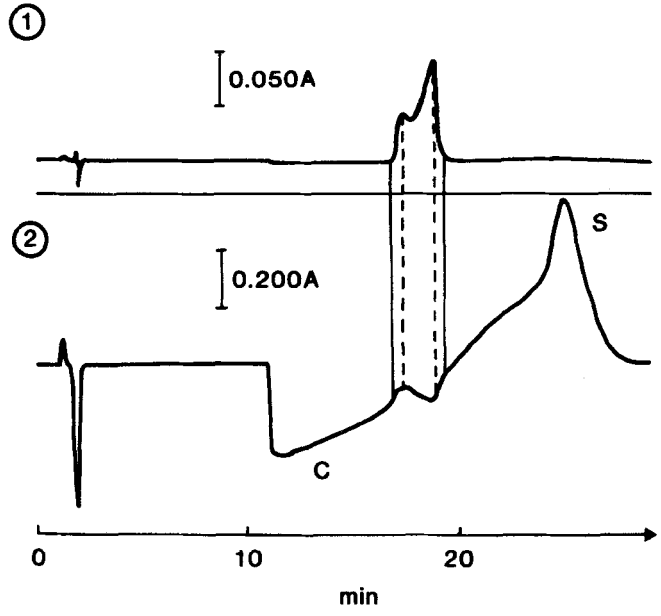

FIGURE 12

Peak deformation obtained at co-elution of FLA 870 and the probe peak, case 2.
Eluent: see Fig. 11b
Inj. sample: 200 µl FLA 870, 1.0×10^{-4} M, and octylsulfate, 1.0×10^{-2} M, in phosphate buffer, pH 2.0

resulted in an increased deformation of the analyte peak (Fig. 11b). When the hydrophobicity of the analyte and anion combination was also increased, the deformation increased even more, resulting in a split analyte peak (Fig. 12). The increased injection volume and hydrophobicity also resulted in a more deformed probe signal. The differences in steepness between these examples were small, and therefore assumed to be negligible.

The peak compression effects predominated when analyte concentrations and hydrophobicity were low and the injection volumes

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small, whereas peak deformation effects were predominant when the reverse was true. The increase in deformation of the analyte peak and probe signal with increasing analyte concentration and hydrophobicity is probably a reflection of competition between the analyte and the probe. The deformation effect might be due to the greater ability of the analyte to displace the probe in the probe gradient.

Other peak deformations

Any other position of the analyte peak in the negative probe signal peak resulted in deformation, even at low analyte concentrations. Deformations were observed especially for co-elution with the downslope (the front) of the negative probe peak, but also for the co-elution with the minimum probe concentration in the center of the probe peak. When high analyte concentrations were injected, extreme analyte peak deformations could be observed when the analyte co-eluted with the system peak created by the analyte itself.

In the downslope of the front part of the negative probe peaks, there is a continuously decreasing concentration of the probe. When the analyte elutes in this co-ion gradient, the probe displaces the front part of the analyte zone more than the back part. Thus, the front part of the analyte zone migrates under conditions which increase the elution speed as compared with the back part, causing a broader and more deformed analyte peak when eluted. This is the reverse of the effect in the upslope (cf. Fig. 3) (eqn. 1).

When a low concentration of the analyte, FLA 870, was injected simultaneously with a large amount of decanesulfonate, the analyte peak co-eluted with the downslope of the large negative system zone, case 1 (Fig. 13a). In line with the above discussion, the analyte peak was then very broad and markedly deformed. When the same concentration of a slightly more retained analyte, FLA 965, was injected with decanesulfonate, the analyte peak eluted in

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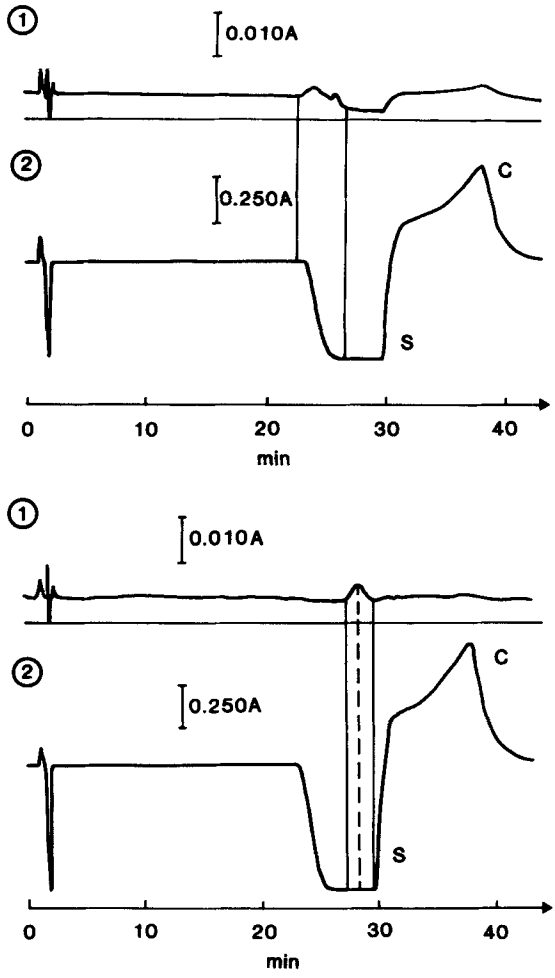

FIGURE 13 a and b

Effects on analyte peak shape when FLA 870 co-eluted with the front, the downslope, of the negative system zone, case 1
Effects on peak shape when the more retained FLA 965 (cf. a) co-eluted with the plateau of the negative system zone, case 1
. sample and eluent: see Fig. 5 (though FLA 659 is exchanged).

the plateau of the low probe concentration in the negative system zone (Fig. 13b). This also resulted in a broad analyte peak, though it was less deformed. It is noteworthy that in the two examples shown, the analyte concentration was the same as that which produced peak compression in the upslope of the negative system peak (cf. Fig. 5).

If only the analyte was injected, large amounts of the cationic analyte itself also gave rise to a large probe system peak, in the same way as did large amounts of the injected anion, though now the direction of the system peak was reversed (10-12). When the analyte eluted close to and behind this system peak, the analyte peak shape was deformed. Injecting the analyte, FLA 659, at 5.3×10^{-4} M, using a high probe concentration, 1.5×10^{-4} M (Fig. 14a), the front part of the analyte peak was extremely deformed, due to the close elution with the system peak. Analyte retention was slightly higher than probe retention, resulting in the positive system peak. The back part of the system peak contained a gradient of decreasing probe concentrations in which the front part of the analyte peak eluted. The front part of the analyte peak was thus extremely deformed due to the larger displacement effect by the probe (cf. Fig. 13a).

At slightly less retention of the analyte than of the system peak (e.g., in the case of FLA 965), the result was a negative system peak (Fig. 14b). In this situation the front part of the analyte peak was not deformed but had the same shape as the probe. However, the analyte peak was highly tailing, owing to the high analyte concentration (24).

The examples described above all concern analyte co-elution with the system peak. However, the probe peak corresponding to an injected compound, may also be responsible for analyte peak deformation when co-eluted with the analyte. When the analyte, FLA 870, was injected simultaneously with octanesulfonate, this resulted in a split analyte peak (Fig. 15). The analyte concentration was ten times higher than that of the anion. The system peak obtained was negative, owing to the low analyte retention. The anion retention,

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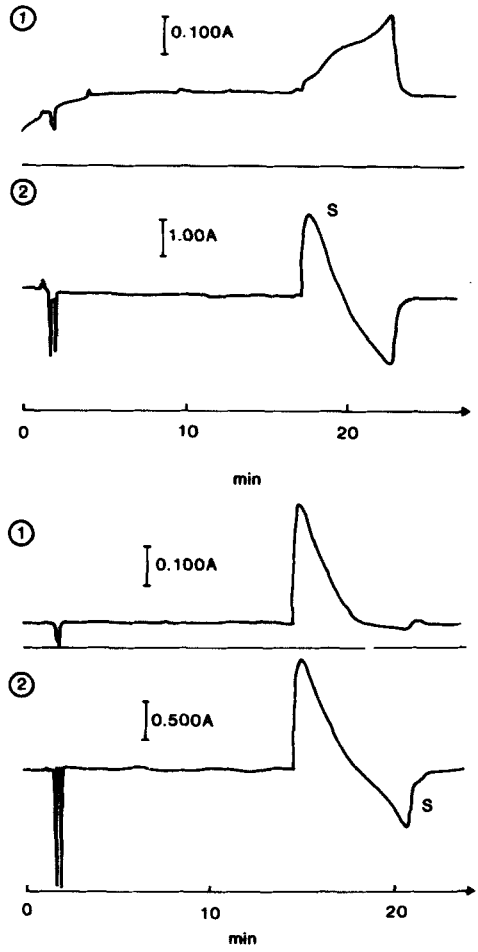

FIGURE 14 a and b

a. Effects on peak shape when FLA 659 eluted with a slightly higher retention than the positive system peak
b. Effects on peak shape when FLA 965 eluted with a slightly lower retention than the negative system peak

Inj. sample: 100 µl analyte, 5.3×10^{-4} M, in phosphate buffer pH 2.0

Eluent: Protriptyline in phosphate buffer, pH 2.0, + acetonitrile (3+1)

a. Protriptyline concentration 1.5×10^{-4} M
b. Protriptyline concentration 1.1×10^{-4} M

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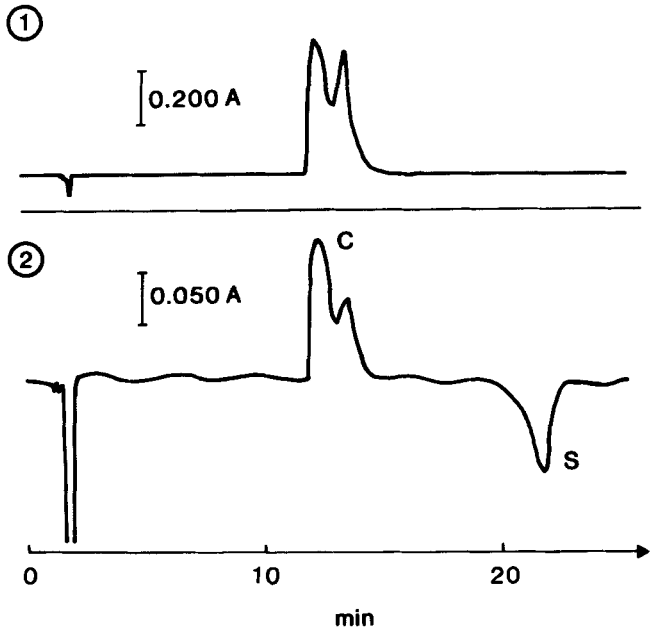

FIGURE 15

Splitting of FLA 870 peak when injected simultaneously with a low octanesulfonate concentration.
Inj. sample: 100 µl FLA 870, 4.8×10^{-4} M, and octanesulfonate, 5.1×10^{-5} M, in phosphate buffer, pH 2.0
Eluent: Protriptyline, 1.5×10^{-4} M, in phosphate buffer, pH 2.0 + acetonitrile (3+1)

however, was similar to that of the analyte, and appeared as a smaller negative probe peak in the middle of the larger positive peak for the analyte, when following the probe signal. The splitting of the analyte peak was probably an effect of the deformed positive probe signal peak, giving probe gradients with opposite directions (cf. ref. 14).

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CONCLUSIONS

Optimal conditions for peak compression

When low analyte concentrations eluted in the back part of the negative system peak, a larger compression effect was obtained than the corresponding elution with the (indirectly detected) anion peak, owing to the much stronger gradient effect in the back part of the system peak. Maximal steepness of the system peak upslope was obtained when the depth of the system peak approached levels corresponding to the actual probe concentration in the eluent. High analyte concentrations displaced the probe resulting in analyte peak deformations (see below), indicating that a strong displacement effect of the probe is essential for optimal peak compression. It is also important to avoid close elution of the analyte peak with the simultaneously injected compound used to induce the system peak, otherwise peak deformation occurs.

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Conditions for peak deformation and splitting

The compression effect was transformed to a deformation effect at high analyte concentrations. Deformation increased with the hydrophobicity and the injection volume, probably due to the displacement of the probe. This phenomenon can be avoided by the injection of low analyte amounts.

Elution of the analyte at other positions of the negative system peak, or the negative probe peak for the anion, resulted in peak deformation or splitting even for low amounts of analyte. This was due to a gradient of a decreasing, or constantly low co-ion concentration.

This kind of analyte peak deformation, due to migrating system peaks, is sometimes a problem in similar ion-pair systems. In bioanalytical work, the risk of deformation increases when

using systems with complex samples and rather hydrophobic organic ions in the eluent. This is especially true of coupled column systems using different eluents (25).

Regulation of retention, and the achievement of suitable effect

The deformation is often easily eliminated, by the injection of a cleaner sample and/or a lower amount of the sample, dissolved in the eluent, or by changing the concentration of an eluent component. The compression effect requires a more careful adjustment of the eluent components, utilizing the difference in retention changes for the analyte and the system peak. If the peaks already elute close to each other, the adjustment can often be made simply by changing the concentration of the simultaneously injected component used to induce the system peak.

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