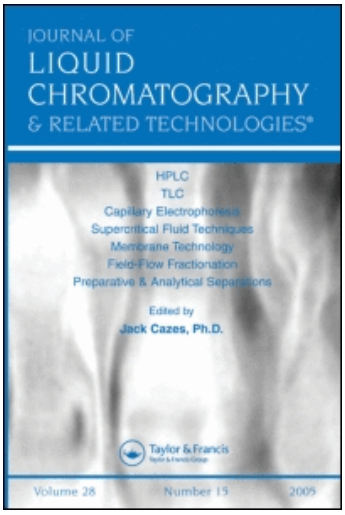

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Studies on System Peaks in Ion-Pair Adsorption Chromatography: I. Evaluation of Ion-Pair Adsorption Constants and Effects by the Injection of Finite Analyte Amounts

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STUDIES ON SYSTEM PEAKS IN ION-PAIR ADSORPTION CHROMATOGRAPHY. I. EVALUATION OF ION-PAIR ADSORPTION CONSTANTS AND EFFECTS BY THE INJECTION OF FINITE ANALYTE AMOUNTS

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ABSTRACT

Adsorption of a secondary amine, protriptyline, as phosphate ion-pair, to Nucleosil C_{18}, was studied. Two different techniques were compared; the traditional break-through and the system peak technique. Both techniques indicated that the adsorption of the ion-pair was best fitted to a 2-site adsorption model of the Langmuir type, indicating a heterogenous solid phase surface.

The reliability of the system peak technique was tested by comparing the retention volumes of the system peaks for different kind of equilibrium disturbances introduced into the system. When injecting finite amounts of analytes the system peak retention was affected by the analyte concentration, charge and hydrophobicity, keeping the eluent composition constant. The effects could qualitatively be explained in light of the developed model for the retention.

INTRODUCTION

In conventional chromatography used for analytical work, the theory for retention of analytes assumes that the retention time is independent of the analyte concentration. In ion-pair adsorption chromatography the retention can be expressed by assuming adsorption of the ion-pair according to the Langmuir adsorption isotherm (1,2). The theory outlined in these papers mainly treated the case for analytes in such low concentrations that the first approximately linear part of the adsorption isotherm was applicable. Also the theory dealing with the non-linear part was briefly outlined (2).

Early theoretical work on chromatography in general (3-6) included non-linear isotherms and interferences, which made the theory applicable both to high concentrations and multicomponent systems. This was a consequence of that liquid chromatography in those days was used mainly for preparative work and not analytical. In recent years there has been a renewed interest both in preparative chromatography, e.g. Horvàth et al. (7,8), and in multicomponent chromatography, e.g. Helfferich et al. (9,10). The theories have, however, treated general simple cases and have not been directly applicable to ion-pair chromatography. In this paper the theoretical part is limited to the treatment of the retention equations for ion-pair in one special retention model - a more thorough theoretical paper treating different retention models as well as response patterns will soon be published (11).

It is well known that the injection of samples in liquid chromatography may disturb the equilibria established by the mobile phase on the column and bring about the elution of composition disturbances. These phenomena can, however, only be observed if at least one of the mobile phase components is detectable. A second requirement for visualization is that the mobile phase component has a significant retention, otherwise it is obscured by general front disturbances. These so called system

peaks have constant retentions typical for the actual composition of the mobile phase, provided that the injected sample involves infinitesimal changes in the composition. As outlined by early theoreticians, DeVault (3) and Gluckauf (4) as well as by, more recently, Helfferich (9,10) and Kovats (12,13), the capacity ratios of system and analytical peaks are described by the partial derivative of the distribution isotherm of the corresponding component.

The aim of this paper was primarily to study the usefulness of system peaks for the estimation of retention and accompanying equilibrium constants with the established break-through technique as a reference method. The second goal was to exploit the reliability of the technique, which was performed by inducing system peaks in several principally different ways and then carefully register the chromatographic performance of the peaks.

A UV-absorbing tertiary amine, protriptyline, was a component of the mobile phase and served both as an ion pairing agent and as a probe. By use of a photo diode array detector operated with a spectral suppression technique, signals from both the probe and the analytes were obtained simultaneously. Substituted benzamides and organic sulphates and sulphonates were used as cat- and anionic analytes, respectively.

THEORY

In the present study the retention can be expressed by assuming that the solid phase is heterogenous, that is contains two kinds of adsorption sites (A_s and A_s^*) with different adsorption capacities (K_o and K_o^*) and affinities towards ion pairs (K_{QX} and K_{QX}^*, corresponding to the distribution constants) (2,14,15). The total amount of Q^+ adsorbed as ion-pair to the heterogenous surface may be expressed according to

$$C_{Q,s} = [QXA_s] + [QXA_s^*] = \frac{K_o K_{QX}[Q^+]_m[X^-]_m}{1 + K_{QX}[Q^+]_m[X^-]_m} + \frac{K_o^* K_{QX}^*[Q^+]_m[X^-]_m}{1 + K_{QX}^*[Q^+]_m[X^-]_m} \quad (1)$$

As mentioned in the introduction the retention of both a mobile phase component (k) and an analyte (j) is described by the partial derivatives, $\partial C_{k,s}/\partial C_{k,m}$ and $\partial C_{j,s}/\partial C_{j,m}$, respectively. However, the derivatives will give different solutions since the starting conditions deviate: $C_{j,m} = 0$, but $C_{k,m} \neq 0$ at the start of the elution.

The net retention volume ($V_{N,Q} = V_{R,Q} - V_m$) for the cation Q^+ pulse, which is a mobile phase component, can then be expressed by the partial derivative of eqn. 1 and by taking into account also the amount of solid phase in the column (W_s):

$$V_{N,Q} = W_s \frac{\partial C_{Q,s}}{\partial C_{Q,m}} = \frac{W_s K_o K_{QX}[X^-]_m}{(1 + K_{QX}[Q^+]_m[X^-]_m)^2} + \frac{W_s K^* K^*_{QX}[X^-]_m}{(1 + K^*_{QX}[Q^+]_m[X^-]_m)^2} \qquad (2)$$

However, with decreasing concentrations the denominator (N) can be successively simplified and will correspond to

1) $N_1 = 1 + 2K_{QX}[Q^+]_m[X^-]_m$; $N^*_1 = 1 + 2K^*_{QX}[Q^+]_m[X^-]_m$ (3)

when

$K^2_{QX}[Q^+]^2_m[X^-]^2_m < N_1$ (and corresponding term for the second site).

2) $N_2 = N^*_2 = 1$ (4)

when

$2K_{QX}[Q^+]_m[X^-]_m < 1$ (and corresponding term for the second site).

System peaks have been obtained in different ways; by injecting a higher or lower concentration of Q^+ and by injecting solutes of different characters, i.e. anionic and cationic. The system peaks will be positive or negative depending on the direction of the disturbance of the adsorption equilibria (16,17). The equations given above are, as pointed out before, only valid for the injection of amounts which causes infinitesimal changes in mobile phase components. In this study, however, in some experiments even finite concentration pulses have been introduced in different ways. For these cases no exact theory is yet available and the results have been interpreted only in a qualitative way from the above equations.

For the analyte HA^+, with Q^+ as the mobile phase component and assuming symmetrical peaks, the derivation will give

$$V_{N,HA} = \frac{W_s K_o K_{HAX} [X^-]_m}{1 + K_{QX}[Q^+]_m[X^-]_m} + \frac{W_s K_o^* K_{HAX}^* [X^-]_m}{1 + K_{QX}^*[Q^+]_m[X^-]_m} \qquad (5)$$

However, when the analyte concentrations are high and influence the retention and peak shape no exact theory is yet available. Simplified models relating the capacity ratios directly to distribution may be applicable (cf. 2), but then only for qualitative discussions about retentions.

Cationic analyte, HA^+

$$V_{N,HA} = \frac{W_s K_o K_{HAX} [X^-]_m}{1 + K_{QX}[Q^+]_m[X^-]_m + K_{HAX}[HA^+]_m[X^-]_m} + \frac{W_s K_o^* K_{HAX}^* [X^-]_m}{1 + K_{QX}^*[Q^+]_m[X^-]_m + K_{HAX}^*[HA^+]_m[X^-]_m} \qquad (6)$$

Anionic analyte, Z^-

$$V_{N,Z} = \frac{W_s K_o K_{QZ} [Q^+]_m}{1 + K_{QX}[Q^+]_m[X^-]_m + K_{QZ}[Q^+]_m[Z^-]_m} + \frac{W_s K_o^* K_{QZ}^* [Q^+]_m}{1 + K_{QX}^*[Q^+]_m[X^-]_m + K_{QZ}^*[Q^+]_m[Z^-]_m} \qquad (7)$$

An equation derived in an analogous way for the probe, Q^+, was used for the evaluation of break-through studies (14,18). It was assumed that the adsorption of buffer components to the solid phase was negligible, similar to the above derivations.

$$V_{N,Q} = \frac{W_s K_o K_{QX} [X^-]_m}{1 + K_{QX}[Q^+]_m[X^-]_m} + \frac{W_s K_o^* K_{QX}^* [X^-]_m}{1 + K_{QX}^*[Q^+]_m[X^-]_m} \qquad (8)$$

In this study the convention is taken that the retention is assumed to be on the linear part of the adsorption isotherm when the terms including the distribution constants in the denominator are < 0.1. This means that a maximum of 10% of the adsorption sites are covered with the ion of interest. Since the retention model is expressed by assuming the existence of two different sites it means that the linear parts of the two adsorption isotherms will have different limiting concentrations of the two ions participating in the equilibria.

MATERIALS AND METHODS

Apparatus

The pumps were an LDC 711-47 Milton-Roy Minipump and an Altex model 100. The detector was a HP 1040 A photo diode array UV-detector. The sample injector was a Rheodyne 7125, with a 20, 100 or 200 µl loop. A Valco CV-6-HPax injector was used when changing eluent from one pump to the other in the break-through experiments. The column was of stainless steel with a polished inner surface, equipped with Swagelok connectors and Altex 250-21 filters. The columns were 100 mm in length with 4.6 mm inner diameter and packed with Nucleosil C_{18}, 5 µm, Batch No. 4081 or 5012 (Machery-Nagel, Düren, FRG). A water bath, HETO type 02 PT 923 (Birkerød, Denmark), was used to thermostate the chromatograph. The pH was measured with a Beckman 71 pH meter equipped with a Beckman combined electrode type 39831, or with a Metrohm 632 pH meter equipped with a combined Metrohm glass electrode type AG 9100 Herisan. The spectrophotometric measurements were made with a Zeiss PM Q II Spektralphotometer.

Chemicals

Acetonitrile Lichrosolv and dichloromethane p.a. were from Merck (Darmstadt, G.F.R.). Protriptyline (PT) (Fig. 1) as chloride

salt, was from Merck Sharp and Dohme (Haarlem, Netherlands). The sodium salts of hexane-(HS), octane-(OS) sulfonic acid and pentyl-(PSA), octyl-(OSA) sulfate were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). The sodium salts of butane sulfonic acid (BS), hexyl-(HSA) and nonyl-(NSA) sulfate were obtained from Merck. The sodium salts of heptane-(HepS) and decane-(DS) sulfonic acid were obtained from Fluka AG (Buchs, Switzerland). Phosphoric acid 99% and 98% crystalline p.a. quality and Titrisol 1 M NaOH were from Merck.

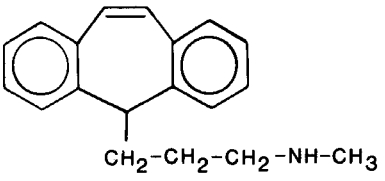
The substituted benzamides (Fig. 1) were synthetized at the Department of CNS-medicinal chemistry, Astra Alab AB (Södertälje, Sweden) and kindly supplied by L.B. Nilsson, Dept. of Bioanalytical Chemistry at this company.

Preparation of the eluent

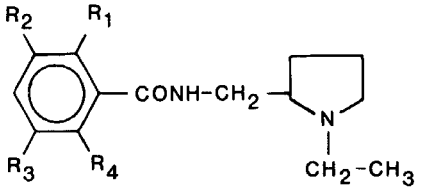
The eluent was prepared by mixing 3 parts of phosphate buffer solution pH 2.0 with one part of acetonitrile, (3+1), if not otherwise stated. The buffer solution was of ionic strength 0.05 and had a total phosphate concentration of 1.3×10^{-1} M. The phosphate buffer was prepared by mixing phosphoric acid 1 M and sodium hydroxide 1 M. The actual concentrations of ionic species in the eluents were corrected for the volume decrease of 1.9%, which occurs when acetonitrile is mixed with phosphate buffer. The total concentration in the eluents of phosphate then was 9.9×10^{-2} M and of sodium ion 3.4×10^{-2} M (without PT in the eluent). An equivalent amount of the NaOH was exchanged for protriptyline (PT) when preparing the phosphate buffers containing PT, in order to keep the ionic strength constant.

Protriptyline (PT) in the eluent was prepared by dissolving PT HCl 5.0×10^{-3} M in dichloromethane saturated with water. By shaking equal volumes of the dichloromethane solution and a NaOH solution pH 12.5 the PT was extracted as a base to the organic phase and Cl^- was distributed to the aqueous phase. PT was then reextracted to an aqueous phase by

Protriptyline



Substituted benzamides



	R_1	R_2	R_3	R_4
FLA 870	OH	Cl	Cl	OCH_3
FLA 965	OH	C_2H_5	H	OCH_3
FLA 659	OCH_3	Br	Br	OH
FLA 131	OCH_3	Cl	C_2H_5	OH
FLA 986	OH	C_3H_7	H	OCH_3
FLA 966	OCH_3	Br	C_2H_5	OH

FIGURE 1

Structures of protriptyline and the benzamides used in the study.

shaking equal volumes of the dichloromethane solution and phosphate buffer pH 2.0. The phosphate buffer solution was, after separation, bubbled with N_2, in order to remove remaining dichloromethane.

The concentration of protriptyline (PT) in the eluent was measured spectrophotometrically (λ = 291 nm, $\varepsilon = 1.34 \times 10^4$) using 1 or 5 cm cuvettes. The lowest PT concentrations were too low to measure accurately and were calculated.

The concentration of $H_2PO_4^-$ in the eluent

The dielectric constant of acetonitrile is close to that of methanol (19). In a methanol-buffer mixture (1+1), there was no change in the dissociation degree of H_3PO_4 (14). In analogy it is assumed that the dissociation degree of H_3PO_4 is unchanged when mixing the phosphate buffer solution with acetonitrile (3+1). The $H_2PO_4^-$ concentration was thus calculated from the concentration in the phosphate buffer with respect only to the dilution with acetonitrile. The calculated concentration of $H_2PO_4^-$ in the eluent was 3.4×10^{-2} M.

Chromatographic technique

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

The flow rate of the mobile phase usually was 0.80 ml/min. No recirculation of the eluent was used.

Break-through technique and detection technique

The studies were performed according to Sokolowski (14). The corresponding absorbance spectra of protriptyline (PT) and a substituted benzamide can be seen in Fig. 2. The signal for this benzamide was registered at 343 nm, using 550 nm as reference. The PT signal was registered at 281 nm and to compensate for the benzamide absorbance, 355 nm was used as reference, since the absorbance of the benzamide at 355 nm equals the absorbance at 281 nm. The compensation was only made when a benzamide was present. In all other chromatographic runs the PT signal was registered at 291 nm.

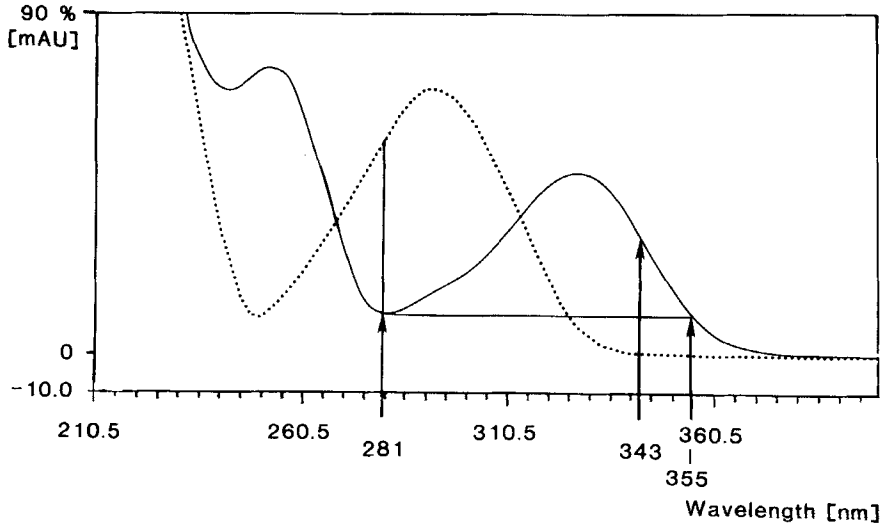


FIGURE 2

Absorbance spectra of protriptyline (·····) and the substituted benzamide FLA 950 (————).

Determination of the hold-up and retention volumes

The hold-up volume of the column, V_m, was determined from the peak obtained when sodium nitrate (0.5 g/l) was injected into the system when equilibrated with methanol-water (6+4). Equilibrating the system with phosphate buffer pH 2.0 and acetonitrile (3+1) containing protriptyline (PT) or injecting samples into the equilibrated system, gave rise to early eluting peaks. The first one of these peaks had a retention volume corresponding to sodium nitrate in the system mentioned above.

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

The asymmetry factor, asf, was measured by drawing a perpendicular from the vertex, formed by the two peak tangent

lines, to the baseline. The back part of the peak baseline was divided by the front part.

Determination of the peak height

The peak height was measured at the peak maximum or minimum, for positive and negative peaks, respectively. The peak height is given in absorbance units (A.U.) or in concentration units (M). A positive or a negative sign after the units above, indicate a positive or a negative peak, respectively.

Determination of the PT concentration change in the system peak

The PT-concentrations in the system peaks were measured from the chromatograms (see Fig. 3). In a positive system peak the PT-concentration is; $C_{sp} = C_b + \Delta C^+$, where C_b equals the concentration in the bulk and ΔC^+ represents the increase in concentration relative the bulk and in analogy for a negative system peak; $C_{sp} = C_b - \Delta C^-$.

A relative measure on the magnitude of the deviation in PT-concentration is obtained by taking the ratio C_{sp}/C_b. An infinitesimal change of the system peak concentration is assumed when $0.99 < C_{sp}/C_b < 1.01$.

Non-linear curve fitting.

The non-linear regression analysis was performed by use of the Minuit program, version D506 from CERN, which contains the minimizing procedures Simplex and Migrad.

The concentrations of the ionic species in the eluents were used as the independent variables. In the adsorption study, the amount of organic ions adsorbed to the solid phase, determined by

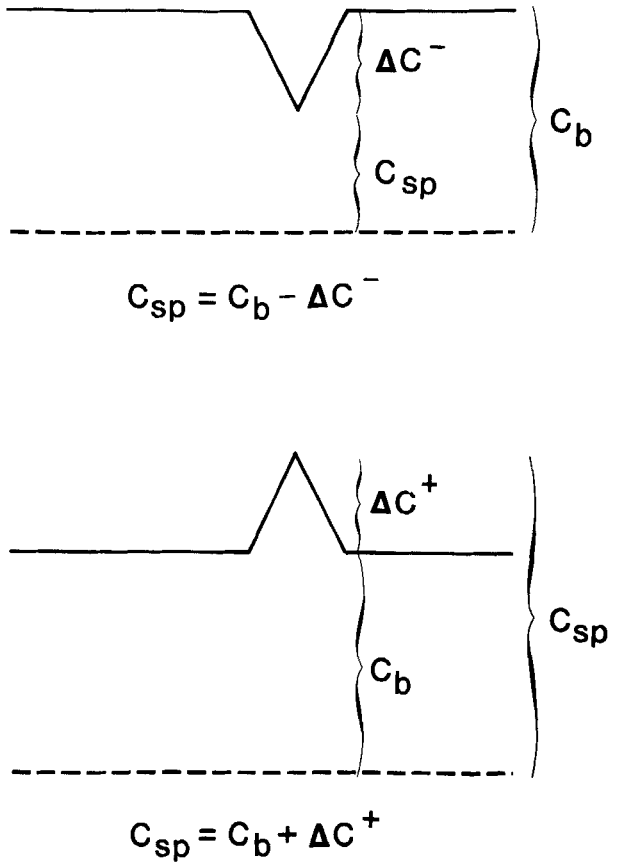


FIGURE 3

Determination of the PT-concentration in the system peak. ΔC^- is the PT-concentration corresponding to the maximum depth in the negative system peak and ΔC^+ the PT-concentration corresponding to the maximum height in the positive system peak. C_{sp} is the total PT-concentration in the system peak and is obtained either as $C_b - \Delta C^-$ or $C_b + \Delta C^+$, depending on if the system peak is negative or positive.

the break-through retention volumes, was used as the dependent variable. In the system peak method, the net retention volume of the system peak was used as the dependent variable. The weight factor was set to 1, assuming constant absolute error. The minimizing criterion was the sum of the squares of the deviation between the observed and calculated values of the dependent variable divided by the degrees of freedom, the residual mean square (s^2) (20).

RESULT AND DISCUSSION

Analyte retention

In ion-pair chromatography, the analyte is retained as ion-pair with an ion of the opposite charge, the counter ion, due to adsorption of the ion-pair onto the alkylbonded silica of the solid stationary phase. The retention of the analyte can be regulated by changing the nature and/or the concentration of the counter ion (eqn. 5) (1). Because of the limited adsorption capacity of the solid phase, an organic ion of the same charge as the analyte, a co-ion, will compete with the analyte for the adsorption sites when the two ions have affinity for the same sites. Thus the retention of the analyte also can be regulated by the nature and/or concentration of the co-ion (eqn. 5) (2,14,21).

Organic anion

The cationic mobile phase component protriptyline (PT) will act as a counter ion for anionic analytes. An increasing counter ion concentration will enhance the retention of an anionic analyte (Table 1). However, at very high concentration and equilibrium constant of the counter ion, the analyte retention will decrease because of the limited adsorption capacity of the solid phase. The retention of the analyte will also change with its own

TABLE 1

Retention of octylsulfate.
Analyte: 20 µl eluent containing octylsulfate (OS)
Counter ion: protriptyline (PT).

[OS]/M	Counter ion conc./M	Analyte ret./ml
2.7×10^{-5}	4.9×10^{-6}	16.4
2.5×10^{-5}	1.7×10^{-5}	17.0
- " -	1.4×10^{-4}	22.7

concentration (2), provided the nonlinear part of the adsorption isotherm is applicable (see Fig. 4) (cf. eqn. 7). The analyte peak asymmetry increased with increasing analyte and counter ion concentration. Peak asymmetry may indicate overloading of the adsorption sites of lowest capacity, which makes the retention equation unvalid. The addition of a co-ion, which competes with the analyte for the sites, will decrease the peak asymmetry described above (2).

Organic cation

For cationic analytes, the protriptyline (PT) in the mobile phase, will act as a co-ion, and an increasing co-ion concentration will decrease the analyte retention (Table 2) (cf. eqns. 5 and 6). The cationic analyte retention decreased with increased analyte concentration in analogy with the anion (Fig. 5). The analyte peak asymmetry increased with increasing analyte concentration.

Adsorption of protriptyline-phosphate

Break-through technique

The amount of protriptyline (PT) adsorbed as phosphate ion-pair, was determined by using the break-through technique, as

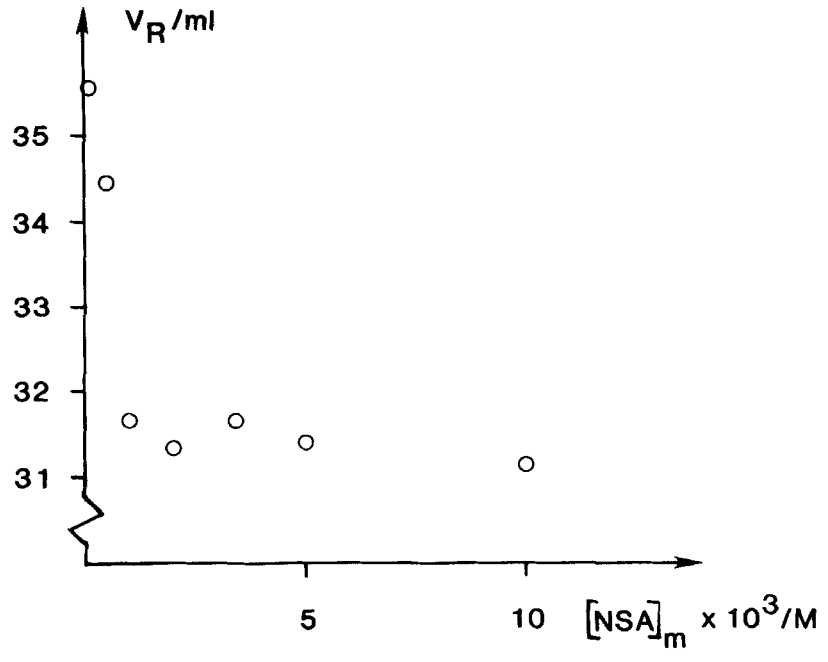


FIGURE 4

Retention volumes of nonylsulphate (NSA); dependence on injected concentration. Injected sample: 200 µl eluent containing NSA. Eluent: protriptyline 1.5 x 10^{-5} M.

TABLE 2

Retention of a substituted benzamide
Analyte: 20 µl eluent containing FLA 870 5.0 x 10^{-5} M
Co-ion: Protriptyline (PT)

[PT]/M	Analyte retention/ml
1.6 x 10^{-5}	12.0
8.8 x 10^{-5}	10.4

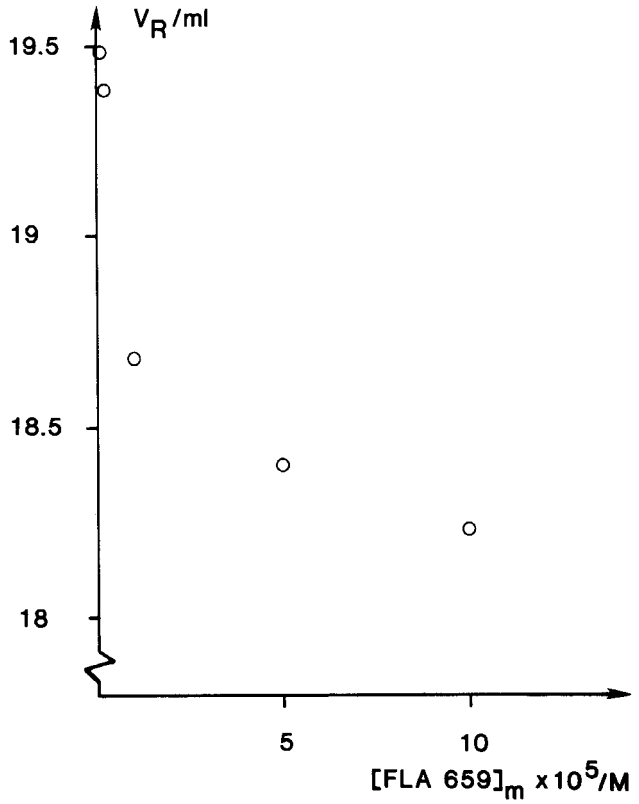


FIGURE 5

Retention volumes of a substituted benzamide, FLA 659; dependence of injected concentration. Injected sample: 200 µl eluent containing FLA 659. Eluent: protriptyline 1.5 x 10^{-5} M.

described elsewhere (14,22). Initially the column was equilibrated with an eluent lacking the PT, followed by a second eluent containing PT, in this study 17 different PT concentrations in the range 4.7 x 10^{-7} - 2.0 x 10^{-4} M were used. The amounts adsorbed were fitted to different adsorption models and the best fitting was obtained for the 2-site model (eqn. 1). The residual mean square, S^2, the distribution constant and the monolayer capacity, K_o, are shown in Table 3. The equilibrium constant for the strong adsorption site was 13 times stronger

TABLE 3

Determined constants for protriptyline adsorbed as phosphate ion-pair. Langmuir 2-site adsorption model (eqn. 1).
W_s = 0.9186 g ; n = 17

			90% confidence limit
S^2		7.4×10^{-10}	
K_o	(mol/g)	4.0×10^{-6}	$\pm 7.0 \times 10^{-9}$
K_{PTP}	(L^2/M^2)	1.1×10^{5}	$\pm 1.0 \times 10^{3}$
K^*_o	(mol/g)	3.0×10^{-5}	$\pm 1.3 \times 10^{-7}$
K^*_{PTP}	(L^2/M^2)	8.7×10^{3}	● 32

Concentration range of protriptyline: $4.7_2 \times 10^{-7}$ - 2.0×10^{-4} M
Concentration of phosphate ion: 3.4×10^{-2} M

than for the weak site, while its capacity is about 7 times lower which is in agreement with other observations (14,15). The values of the amount adsorbed for each site were computed (eqn. 1), and given in Figure 6, together with the isotherms for the total calculated and determined amount of PT-phosphate adsorbed.

At the PT-concentration 2.0×10^{-4} M the strong site was covered to ~44% and the weak to ~6%. The non-linearity of the adsorption isotherm increases at higher concentrations of PT. By our convention the non-linear part of the adsorption isotherm is clearly observed when the concentration in the mobile phase gives a 10% coverage of the sites (2,14,23). At this concentration there will be a 10% decrease of the value of k', compared to the value of k' at infinitely low concentration, accompanied by a noticable peak asymmetry.

The net retention volumes of PT for each site and the total net retention volumes were computed (eqn. 8). These were plotted together with the determined values versus the PT-concentration in the mobile phase (Fig. 7). A steep increase in the net retention

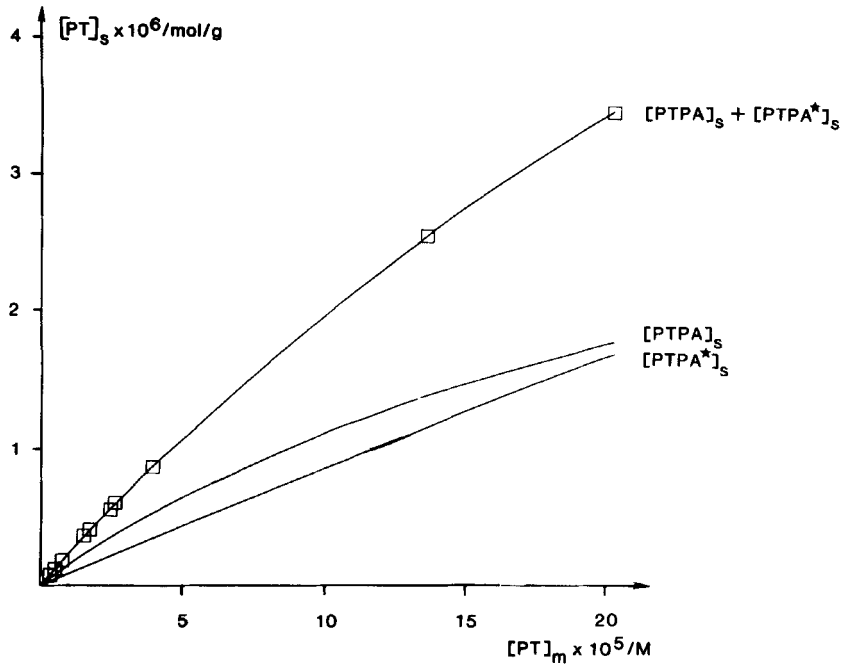


FIGURE 6

Adsorption of protriptyline as ion-pair with phosphate. Data fitted to the 2-site model (eqn. 1).
□ = total experimentally determined amount adsorbed.
——— = calculated amount adsorbed, $[PTPA^*]_s$ = amount adsorbed to the weak site, $[PTPA]_s$ = amount adsorbed to the strong site.

volume, originating from the strong site, was obtained when the PT concentration was decreased. The net retention volume originating from the weak site was nearly constant at the PT concentrations used. This indicates that the retention volume changes of the break-through curve, when decreasing the PT concentration, mainly was due to the overloading of the strong site.

The system peak method

The retention volume of the system peaks could also be used for evaluating the adsorption capacity and equilibrium constants

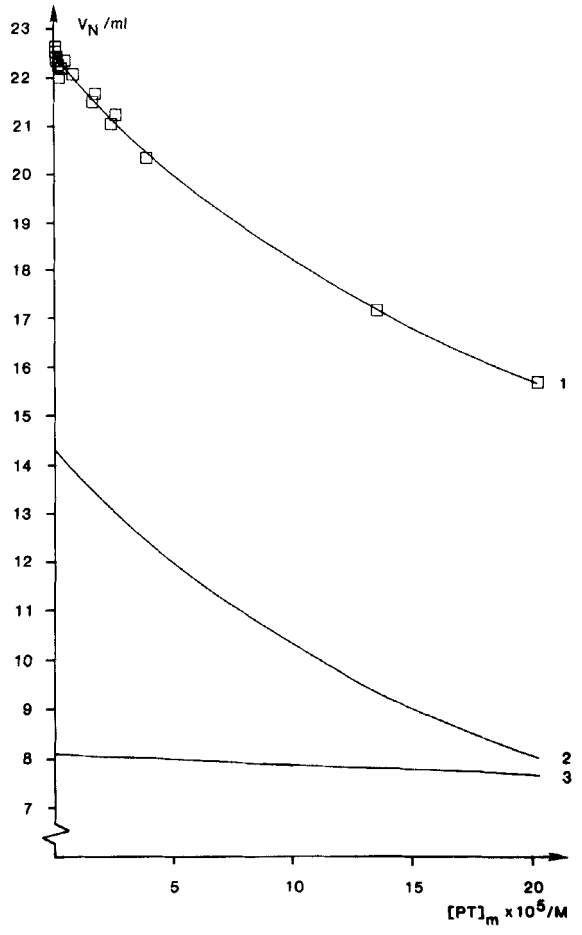


FIGURE 7

Break-through net retention volumes of protriptyline as phosphate ion-pair. Data fitted to the 2-site model.
□ = determined values, ——— = calculated values
1. Total calculated values, 2. Values originating from the strong site, 3. Values originating from the weak site

in the adsorption model, by using eqn. 2. This may have the advantage that a lot of experimental data easily can be obtained since every injection will give rise to such peaks.

The system peaks were created by disturbing the equilibrated PT-system (cf. refs. 16,17,24) in different ways. For each PT-concentration used in the adsorption studies, injection of different solutions, deviating in the eluent composition or containing anionic or cationic analytes were made. The equilibrium disturbance, creating the excess or deficiency of PT in the system peak, must be negligible for the equations to be valid and the intention was to make as small disturbances as possible. However, at low PT concentrations larger disturbances were necessary to introduce, in order to obtain detectable peaks. Despite the larger disturbances introduced, the PT concentration corresponding to the maximum or minimum peak height, (ΔC, see Fig. 3), decreased at low PT concentrations in the bulk eluent (cf. Table 4). With decreasing bulk PT-concentrations the C_{sp}/C_b-ratio increased for positive and decreased for negative system peaks, indicating an increasing deviation from the infinitesimal changes, which is a prerequisite for the theory. However, only peak asymmetries in the range 0.65 - 1.50 were accepted.

The net retention volumes of the system peaks at each PT-concentration were fitted to different retention models and also in this study the best fitting was obtained for the 2-site model (eqn. 2)(Fig. 8). In the figure, calculated values of the system peak based on the constants from the break-through adsorption studies, are compared with the obtained ones. At low PT-concentrations there was a large scatter in the experimentally determined values, but the mean values come close to the calculated ones. The fitting was not as good as for the break- through runs.

The deviations between the retention volumes from the break-through runs and system peaks might be due to uncertain retention volumes of the system peaks. At low PT-concentration, where baseline noise is noticable, it might be difficult to

TABLE 4

Injection of different amounts of water added to the eluent. The system is equilibrated with different protriptyline concentrations. The system peaks are positive.
Injection volume: 20 µl. Cuvette length: 0.6 cm.

H_2O added (%)	$\Delta C^+/M$	C_{sp}	C_{sp}/C_b
3.0	1.7×10^{-6}	2.1×10^{-4}	1.008
2.5	8.2×10^{-7}	1.4×10^{-4}	1.006
2.5	5.2×10^{-7}	8.8×10^{-5}	1.006
3.2	3.8×10^{-7}	6.0×10^{-5}	1.006
5.0	2.8×10^{-7}	3.9×10^{-5}	1.007
5.0	2.9×10^{-7}	3.8×10^{-5}	1.008
5.0	1.9×10^{-7}	2.5×10^{-5}	1.008
5.0	1.9×10^{-7}	2.6×10^{-5}	1.008
10.0	2.2×10^{-7}	1.6×10^{-5}	1.014
10.0	2.3×10^{-7}	1.7×10^{-5}	1.013
10.0	1.3×10^{-7}	1.1×10^{-5}	1.012
30.0	1.9×10^{-7}	8.0×10^{-6}	1.025
40.0	1.4×10^{-7}	5.1×10^{-6}	1.029
50.0	9.6×10^{-8}	3.1×10^{-6}	1.032
100.0	5.5×10^{-8}	2.4×10^{-6}	1.023
100.0	3.5×10^{-8}	2.0×10^{-6}	1.018
100.0	2.1×10^{-8}	2.1×10^{-6}	1.020
50.0	1.9×10^{-8}	7.2×10^{-7}	1.027
25.0	9.6×10^{-9}	4.8×10^{-7}	1.021

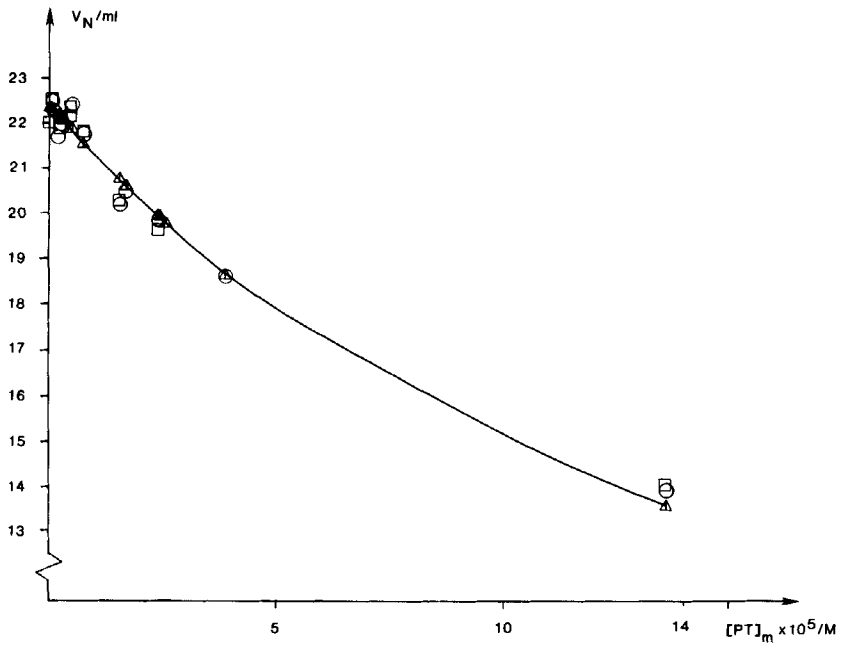


FIGURE 8

Net retention volumes of system peaks for different concentrations of protriptyline in the eluent.
△-△-△ = calculated values of the retention volumes, based on the constants from the break-through adsorption study (2-site model). Determined values when injecting different solutions deviating from the eluent. ○ = water, □ = FLA 870

measure the correct peak maximum/minimum. Further the system peaks are less distinct at low PT-concentration, which contributes to the low precision. A higher detector sensitivity might decrease such problems.

Changes of system peak retention

When the disturbance of the PT equilibrated system, made to induce the system peak, is not infinitesimal, the retention of the

system peak may change, provided that the probe concentration is so high that a non-linear adsorption isotherm is applicable. The change in the system peak retention will depend on the magnitude of the disturbance. Therefore the retentions of the system peaks induced, when injecting organic analytes with different concentrations and hydrophobicities of organic analytes, were studied.

The retention volume of the system peak, in a given chromatographic system, is determined by the PT-concentration in the system peak, C_{sp}, which is decisive for the position on the adsorption isotherm. The PT-concentration in the system peak, C_{sp}, is the PT-concentration in the bulk eluent, C_b, plus or minus the PT-concentration corresponding to the maximum or minimum peak height, ΔC, depending on a positive or a negative system peak as described earlier (see Methods and Fig. 3). When the disturbance induced on the equilibrated PT system is infinitesimal, the system peak will be very small and the PT-concentration in the system peak, C_{sp}, will not deviate significantly from the PT-concentration in the bulk eluent. Then eqn. 2 is valid for determining the retention volume of the system peak, at different PT-concentrations in the bulk eluent. In this case the slope of the tangential point on the adsorption isotherm, corresponding to the PT-concentration in the bulk eluent, will determine the retention of the system peak (Fig. 9).

If the system peak is induced by a large disturbance, the PT concentration in the system peak, C_{sp}, will deviate from the PT-concentration in the bulk eluent. For a positive system peak it is the sum of the PT-concentration in the bulk eluent, C_b, and the PT-concentration corresponding to the maximum peak height, ΔC^+ (see Fig. 3). This gives a position on the adsorption isotherm above the one for the PT-concentration in the bulk eluent (Fig. 9). The slope of the tangential line drawn from this position is lower and now determines the retention volume of the system peak. Of importance is that these measurements give the concentrations in the system peaks when eluted from the column.

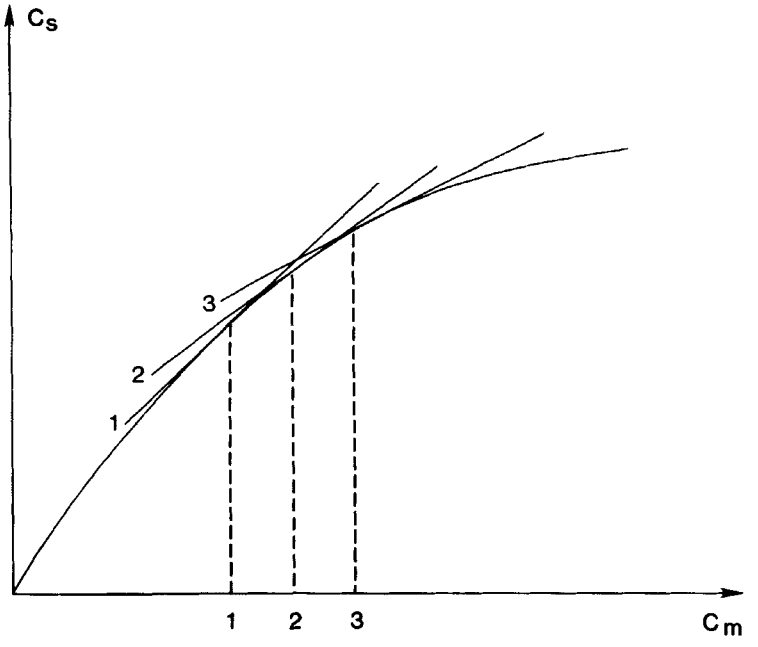


FIGURE 9

A schematic Langmuir adsorption isotherm, as a background to the interpretation of the system peak retention.
1. The PT-concentration in the finite negative system peak and the corresponding tangent., 2. The PT-concentration in the bulk eluent and the corresponding tangent for the infinitesimal system peak., 3. The PT-concentration in the finite positive system peak and the corresponding tangent.
The slope of the tangent determines the retention.

When created, the concentration differences between the system peak and the bulk eluent are larger, but due to the band broadening in the column the differences are decreasing, the extent depending on the column efficiency. For the negative system peak, induced by a large disturbance, the value of the PT-concentration in the system peak, C_{sp}, is determined by the PT-concentration in the bulk eluent, C_b, minus the PT-concentration corresponding to the depth of the system peak, ΔC^-

(Fig. 3). In this case the retention volume for the system peak will be higher compared to the system peak for an infinitesimal disturbance (cf. Fig. 9). Consequently for a given PT-concentration in the eluent, positive system peaks will have smaller retention volumes compared to the negative ones, provided that the induced difference in probe concentration is finite. However, when the linear part of the isotherm is applicable, the difference is negligible.

The peaks observed when injecting organic analytes into the equilibrated PT-system is a result of a competing processes due to binding and displacement of the UV-absorbing ion, PT. Depending on the charge and hydrofobicity of the injected analyte, the system peak will be positive or negative (17). Injection of a cationic analyte with analyte retention volume smaller than the system peak, gave rise to a negative system peak. When the analyte retention volume was higher, the system peak was positive. For the anionic analytes the reversed was obtained.

For all studies but the study of changing the injection volume, 100 µl was injected. The PT concentration used was 1.2×10^{-4} M; relating to the adsorption isotherm of PT as phosphate ion-pair, the strong adsorption site is thus covered to 28% and the weak to 3% (cf. Fig. 6). This corresponds to a point on the non-linear part of the adsorption isotherm of the strong site.

Different analyte concentration

When there is a change in the finite amount of the solute injected, according to the discussion above, this will result in a change of the PT-concentration in the system peak, C_{sp}, which governs the retention of the peak. The effects of a variation in the injected amounts of analyte thus was studied.

Decreasing the concentration of a cationic analyte, eluting before the system peak, resulted in decreasing negative system peak absorbance, corresponding for an increase of C_{sp}. If this PT-concentration is on the non-linear part of the adsorption

TABLE 5

Effects of varying cationic analyte concentrations on the system peak retention. The analyte elutes before the system peak. Injected sample: 100 µl FLA 870 in phosphate buffer pH 2.0. Eluent: Protriptyline (PT) 1.2×10^{-4}M

[FLA 870]/M	System peak absorbance/A.U.	System peak pos./neg.	System peak retention/ml
1.0×10^{-2}	0.315	negative	17.35
5.2×10^{-3}	0.264		16.61
1.0×10^{-3}	0.139		16.45
1.0×10^{-4}	0.020	positive	15.71
1.0×10^{-5}	0.051		15.29
1.0×10^{-6}	0.054		15.34

isotherm, the system peak retention volume will also decrease, as demonstrated in Table 5 for the three highest concentrations. The change in direction of the system peak, for the lower concentrations, is interpreted as to be effects of the phosphate buffer as discussed later.

The retention volumes of the positive system peaks, obtained when injecting different concentrations of anionic analytes, were also studied (Table 6). Decreasing the anionic analyte concentration gave decreased system peak absorbance. If the PT concentration in the system peak, C_{sp}, is on the non-linear part of the adsorption isotherm, a lower anionic analyte concentration gives a higher system peak retention. Two other anions, butanesulfonate and heptanesulfonate showed the same tendency.

As demonstrated in Table 6, the injection of only phosphate buffer gave a positive system peak. An explanation to this may be the following. When the phosphate buffer is introduced into the

TABLE 6

Effects of varying anionic analyte concentrations on the system peak retention The analyte elutes before the system peak. The system peak is positive.
Injected sample: 100 µl octanesulphonate (OS) in phosphate buffer pH 2.0.
Eluent: protriptyline (PT) 1.2×10^{-4}M

[OS]/M	System peak absorbance/A.U.	System peak retention/ml
1.0×10^{-2}	0.364	13.90
5.0×10^{-3}	0.267	14.24
2.0×10^{-3}	0.197	14.47
1.0×10^{-3}	0.160	15.73
1.0×10^{-4}	0.086	16.38
1.0×10^{-5}	0.056	16.50
1.0×10^{-6}	0.056	16.68
phosph.buff.	0.054	15.78

system, the PT will diffuse from the bulk mobile phase into the phosphate buffer zone. Because of the high difference in polarity between the phosphate buffer and the eluent, there will be an increased adsorption of PT in the phosphate buffer zone. The increased adsorption of PT will result in a positive system peak. The phosphate buffer zone has a negligible retention in this system and will appear as a negative zone in the front.

The retention volumes of the cation, FLA 870, and the anion, OS, are similar for about equal analyte concentrations. Therefore a rough comparison of the system peak retention volumes in the two series was made. At high analyte concentrations the negative system peak obtained by injecting the cation, had clearly higher retention volume than the positive system peak obtained by

injecting the anion (cf. Table 5 and 6). This is due to the differences in the position on the non-linear part of the adsorption isotherm, and is an evidence for the correctness of the interpretation presented above.

The analytes were dissolved in pure phosphate buffer. At a certain low concentration of the analyte the increased adsorption of the PT, caused by the phosphate buffer, will dominate in comparison to the binding or displacing effect obtained by the analyte. When injecting the cation (Table 5), the system peaks were negative only for the three highest analyte concentrations injected. With a further decrease in the concentrations they became increasingly positive. Comparing the system peaks induced by injecting only phosphate buffer (Table 6), with the system peaks induced by injecting low cation concentration, both the absorbance and the retention volume of the positive system peaks were similar to each other. This indicates that, when injecting a low cationic analyte concentration the system peak obtained is mainly induced by the phosphate buffer. However, when injecting a low concentration of the anion, the positive system peak obtained had a distinctly higher retention, despite of similar absorbance (Table 6). This indicates that the anion has some additional effect in retarding the system peak. This might be due to an initial attraction giving an increased ion-pair adsorption between the PT and the anion zone, before they separate from each other.

When using a PT concentration 10 times lower in the mobile phase no retention changes could be noticed for the system peaks, when changing the analyte concentration. At the lower PT concentration the strong site is covered to only 3.5% and thus the system peak concentrations are on the linear part of the adsorption isotherm.

Different analyte hydrofobicities

A second way to change the magnitude of the disturbance, is to change the hydrophobicity of the injected analyte. The chosen analytes have different abilities to interact with PT by displacement or adsorption.

Among cations with less retention than the negative system peak, the one with the largest retention gives a negative system peak with higher absorbance and higher retention volume (Table 7). A prerequisite is, as pointed out earlier, that C_{sp} is on the non-linear part of the adsorption isotherm.

Anionic analytes of different hydrophobicities with less retention than the system peak were also injected. In four series the hydrophobicity was increased, resulting in increased absorbance of the positive system peak obtained. The PT-concentration in the positive system peak, C_{sp}, is on the non-linear part of the adsorption isotherm and it is expected that the retention volume of the positive system peak would decrease when the hydrophobicity of the anion increased. However, only in one series the retention of the system peak decreased (Table 8), which might indicate, as mentioned above, that the anionic analyte has some additional effect on the retention of the system peak. Possibly it is ion-pair adsorption in the injection zone, during the co-elution of the probe and anion zones along the column.

Hydrophobic cations with retention volumes higher than for the system peak, gave positive system peaks (Table 9). A straight forward interpretation of the theory would predict that the higher

TABLE 7

Effects of changing the hydrophobicity of cations on the system peak retention. The analyte elutes before the system peak. The system peak is negative.
Injected sample: 100 µl benzamide 1.0×10^{-3} M in phosphate buffer pH 2.0. Eluent: protriptyline (PT) 1.2×10^{-4} M.

Analyte	Analyte ret./ml	Syst.peak ret./ml	Syst.peak absorb./A.U.
FLA 870	8.65	16.45	0.139
FLA 965	11.85	17.71	0.203

TABLE 8

Effects of anion hydrophobicities on the system peak retention. The analyte elutes before the system peak. The system peak is positive. Injected sample: 100 µl organic anions in phosphate buffer pH 2.0. Eluent: Protriptyline (PT) 1.2×10^{-4} M (3+1).

Serie	Analyte	conc./M	Analyte ret./ml	Syst.peak ret./ml	Syst.peak absorb./A.U.
1	PSA	1.1×10^{-3}	2.77	15.32	0.069
	HSA	1.2×10^{-3}	4.44	16.37	0.103
2	HS	1.0×10^{-3}	2.24	15.39	0.082
	OS	1.0×10^{-3}	7.96	15.73	0.160
3	BS	5.0×10^{-3}	-	16.23	0.077
	HepS	5.0×10^{-3}	3.19	14.67	0.151
	OS	5.0×10^{-3}	5.85	14.24	0.267
4*	BS	1.0×10^{-3}	-	32.51	0.029
	HS	1.0×10^{-3}	4.47	32.87	0.078
	OS	1.0×10^{-3}	23.92	35.74	0.295

*Eluent: phosphate buffer + acetonitrile (4+1) containing protriptyline (PT) 1.3×10^{-4} M.

TABLE 9

Effects of cation hydrophobicities on the system peak retention. The analyte elutes after the system peak. The system peak is positive. Injected sample: 100 µl benzamide in phosphate buffer pH 2.0. Eluent: protriptyline (PT) 1.2×10^{-4} M

Analyte	conc./M	Analyte ret./ml	Syst.peak ret./ml	Syst.peak absorb./A.U.
FLA 131	1.0×10^{-3}	25.24	14.43	0.168
FLA 986	1.2×10^{-3}	27.00	15.17	0.227
FLA 966	1.0×10^{-3}	30.25	14.76	0.123

absorbance in the system peak, the lower should the retention volume be. The results deviates from this assumption indicating that other effects, like selective displacement at one of the two sites, as well may be of importance.

When the anionic analyte retention volume is larger than for the system peak, the latter is negative and has a significantly higher retention volume than the positive one, obtained by injecting an anionic analyte with a smaller retention volume than for the system peak (Table 10) (cf. Table 8 series 1,2). The extra effect on the retention of the system peak caused by the injected anion, will increase with the hydrofobicity of the anion (cf. Table 8), and add to the effects obtained from the difference in PT-concentration between the positive and negative system peaks.

The effects observed when injecting analytes of different hydrofobicity were not observed using 10 times lower PT-concentration since then the linear part of the adsorption isotherm is applicable.

Different injection volumes

In another series of experiments the amount of the analyte injected was varied by changing the injection volume, maintaining the same analyte concentration. This will also result in a change of the magnitude of the disturbance. The analyte injected was

TABLE 10

Effects of anion hydrophobicities on the system peak retention. The analyte elutes after the system peak. The system peak is negative. Injected sample: 100 µl sulfonate/sulfate in phosphate buffer pH 2.0. Eluent: protriptyline (PT) 1.2×10^{-4} M.

Analyte	conc./M	Analyte ret./ml	Syst.peak ret./ml	Syst.peak absorb./A.U.
DS	1.0×10^{-3}	>45	17.81	0.368
NSA	1.0×10^{-3}	45.57	17.23	0.323

TABLE 11

Effect of injection volume on the system peak retention. The analyte elutes before the system peak. The system peak is negative. Injected sample: 20 or 200 µl FLA 870 4.8×10^{-4} M in phosphate buffer pH 2.0. Eluent: protriptyline (PT) 1.5×10^{-4} M.

Injection volume/ l	System peak retention/ml	System peak absorbance/A.U.
20	14.58	0.029
200	15.06	0.044

cationic and eluted before the negative system peak and the injection volumes used were 20 and 200 µl, respectively. The system peak obtained when the injection volume was 200 µl had, as expected, higher absorbance and retention volume (Table 11).

Thus, changing the injection volume of the injected cation gave an effect, similar to that obtained when changing the cation hydrofobicity or concentration, resulting in a change of the position on the adsorption isotherm. However, there are some complications. The phosphate buffer zone in the front is much larger with the high injection volume. Even if the diffusion of PT from the bulk mobile phase to the phosphate buffer zone does not increase, this will have consequenses on the appearance of the system peak.

CONCLUSIONS

The adsorption of protriptyline as phosphate ion-pair, measured with the break-through technique, follows the Langmuir adsorption isotherm. The solid phase (Nucleosil C_{18}) behaved as having a heterogenous surface consisting of two sites, in accordance with earlier studies.

A comparison of the system peak technique with the break-through, indicated that equal results, regarding equilibrium constants, were obtained. To obtain reliable results with the system peak technique, the disturbance creating the system peak must be infinitesimal, when being on the non-linear part of the adsorption isotherm.

When creating system peaks by injecting finite amounts of an analyte, the system peak retention varied with the analyte concentration, charge and hydrophobicity. A careful experimental design must therefore be maintained when using the system peak technique to evaluate ion-pair adsorption constants.

Being on the so called linear part of the isotherm no changes of the system peak retentions were obtained.

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