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Peak distortion effects of suramin due to large system peaks in bioanalysis using ion-pair adsorption chromatography

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ABSTRACT

Sample injections in ion-pair adsorption chromatography lead to changes in the established equilibria of the eluent components at the top of the column, which in turn lead to migrating concentration changes in the column (system peaks). Large system peaks contain big concentration deviations of the mobile phase components compared with the bulk composition in the eluent. Analyte peaks are distorted upon combined elution with large system peaks. In bioanalysis, samples that deviate considerably from the eluent are often injected, resulting in large system peaks. An illustrative example of such analyte peak distortions in bioanalysis is given for the case of suramin, and guidelines are given for avoiding the effects.

INTRODUCTION

The injection of a sample into a chromatographic system may result in more peaks than there are components in the injected solution. When the sample is injected, the equilibria at the top of the column are disturbed, which in turn results in the development of zones corresponding to concentration changes of the eluent components (system zones) [1,2]. These excess or shortage zones migrate along the column and appear as positive or negative system peaks in the chromatogram, if at least one of the eluent components is detected [1,2].

Large system peaks contain big concentration deviations of the mobile phase components compared with the eluent composition. Analyte peaks will have distorted shapes and altered retention times following elution with such concentration gradients, compared with the situation when these gradients are not present during the chromatographic run (isocratic conditions) [3–6]. In most cases the distorted analyte peak will be deformed or split, but under certain conditions extremely narrow and well shaped peaks are obtained. Although a compressed peak may also be an undesirable phenomenon, it can be used to increase the sensitivity of the system, *i.e.* to detect lower analyte concentrations [6].

In reversed-phase ion-pair chromatography, the organic ionic component added to the eluent often has a retention volume similar to that of the analytes, so interactions between its system peak and the analytes are likely, In bioanalysis, one often injects samples that deviate considerably from the eluent, resulting in large system peaks and increasing the risk of distortions of analyte peaks. An illustrative example of peak deforma-

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tion and peak compression due to large system peaks is the bioanalysis of suramin (a polyvalent aromatic sulphonic acid).

Suramin has shown promising effects against different forms of cancer [7]. However, severe toxicity has frequently been encountered at serum suramin concentrations only marginally higher than those used for therapy. This narrow therapeutic window (*ca.* 200–300 mg/l), combined with an extremely high protein binding (>99.7%) and often low albumin levels in treated patients, makes suramin a prime candidate for therapeutic monitoring of free drug concentrations. For this purpose, a sensitive and reliable determination method in plasma water is essential.

The reported chromatographic methods are almost all based on reversed-phase ion-pair chromatography with octadecyl silica as stationary phase and an eluent containing tetrabutylammonium as counter-ion (0.2–10 m*M*) in a mixture of buffer and an organic modifier [8–15]. The plasma samples are worked-up by precipitating the plasma proteins with methanol and/or acetonitrile, often together with the addition of excess tetrabutylammonium [8,10,11,13–15] in order to increase the recovery. One of the methods (ultrafiltration) has also been used to determine the free drug fraction [14,15].

EXPERIMENTAL

Chemicals

Analytical-reagent grade chemicals were used unless indicated otherwise. Methanol (LiChrosolv), phosphoric acid (99% crystalline) and 1 *M* sodium hydroxide solution (Titrisol) were obtained from Merck (Darmstadt, Germany). Tetrabutylammonium bromide (TBABr) and sodium anthraquinone-2-sulphonate where obtained from Fluka (Buchs, Switzerland). Suramin (Germanine) was obtained from Bayer (Leverkusen, Germany).

Chromatographic system

A UV detector (Linear 206 PHD) and a refractive index (RI) detector (Beckman 156) were used in series; the UV detection wavelength was 313 nm. The volume between the UV detector and the RI detector was chromatographically determined as 0.07 ml. The pump was an LKB 2150 HPLC pump, and the sample injector was a Rheodyne 7125, with a 100-μl loop. A Kipp & Zonen dual-channel recorder (Model BD 112) was used.

Conventional-size columns (particle size 5 μm; 150 or 100 mm × 4.6 mm I.D.) were used. They were either obtained ready-packed from the supplier (Nucleosil 100-5 C_{18}, Spherisorb S5ODS-1 and Kromasil 100-5 C_{18}) or packed by a slurry technique (Nucleosil 100-5 C_{18}). The status of the packed columns was frequently tested with phenolic analytes and water–methanol as eluent, and the performance of the columns remained unchanged during this work. The system (column and eluent reservoir) was carefully thermostated in a water-bath (25.00 ± 0.01°C). When ultrafiltrate was injected a Nucleosil 100-5 C_{18} guard column (10 mm × 4.6 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) was installed in series with the analytical column.

The eluent was prepared by adding TBABr to methanol–phosphate buffer. The system peak of tetrabutylammonium was detected by RI detection. The flow-rate was 0.80 ml/min in all experiments. The eluent was not recirculated.

Sample pretreatment

The procedure of Tjaden *et al.* [14] was followed. Aliquots of 250-μl defrosted plasma samples were mixed in a polyethylene Eppendorf cup with 200 μl of 1 *M* TBABr for 30 s. Then 500 μl of methanol were added and the solution was mixed again for 30 s. After 30 min of incubation at 4°C, the tubes were centrifuged at 5000 *g* for 10 min; 25 μl of the supernatant were diluted with 1000 μl of water, and 100 μl of this solution were injected into the chromatographic system.

Ultrafiltration

Again, the procedure of Tjaden *et al.* [14] was followed. The incubated plasma samples were transferred to the sample reservoir of the micropartition ultrafiltration system (MPS-1) containing YMT membranes with cut-off values of

30 000 and 3000. Centrifugation was performed for 15 min at 37°C at 2000 *g* using a fixed-angle (28°) rotor, and 100 μl of the ultrafiltrate were injected into the chromatographic system.

RESULTS AND DISCUSSION

Distortions of analyte peaks due to large system peaks

In ion-pair adsorption chromatography, the front and rear of the large counter-ion or co-ion system peaks consist of concentration gradients giving distortion effects on co-eluting analyte peaks. In this work, effects on suramin by the system peak of tetrabutylammonium were studied and the latter component acted here as a counter-ion to suramin.

The effect on the analyte peak shape following combined elution with a counter-ion system peak depends on the position of the analyte in the system peak and on whether the system peak is positive or negative. The analyte peak is well shaped and narrower, compared with an isocratic run, at the rear of a positive counter-ion system peak (Fig. 1a, position II) or at the front of a negative one (Fig. 1b, position I) [4,5]. The analyte experiences a continuously weaker ion-pair effect in the decreasing counter-ion gradient, and its rear will therefore have a more accelerated velocity than its front.

Analyte elution at the front of the positive counter-ion system peak (Fig. 1a, position I) or at the rear of the negative one (Fig. 1b, position II) gives a broad and deformed analyte peak [4,5]. In the increasing counter-ion gradient, the analyte experiences a continuously stronger ion-pair adsorption effect and the front moves under conditions that speed it up more than the rear of the peak.

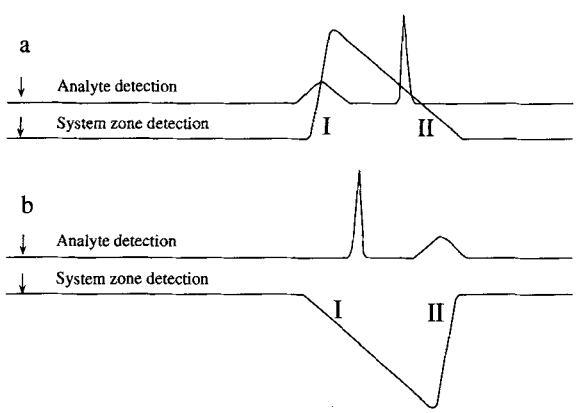

Fig. 1. Schematic representation of the effects on analyte peak co-elution with different parts of a large positive (a) or a large negative (b) counter-ion system peak.

Although the analyte peak may be separated from the large system peak at the column outlet, the analyte zone has passed through the large system zone at an earlier stage of its migration along the column. The distortions therefore also occur (peak broadening and compression), albeit with less pronounced effects, if the analyte is eluted before or after the system peak concentration gradient at the column outlet [5].

Large negative system peaks will have larger retention volumes than large positive ones (*cf.* Fig. 1a and b) and these effects can be explained via the Langmuir adsorption isotherm [4]. This is the reason why large negative system peaks show fronting, which means that the rear is steeper than the front (*cf.* Fig. 1b), the opposite shape to that obtained for large positive system peaks, which show tailing (the front is steeper than the rear) (*cf.* Fig. 1a) [4].

The analyte peaks that are affected by the positive or negative counter-ion system peak will show an increase or decrease in retention volume, respectively, compared with the corresponding isocratic situations. The compression and broadening effects on co-eluting analyte peaks of organic co-ion system peaks, are the reverse of Fig. 1a and b [4,5].

Distortions in the bioanalysis of suramin

The eluent used contained *ca.* 4 m*M* TBABr in phosphate buffer (pH 7.5, $I = 0.02$)–methanol (42.5:57.5). The main difference from the system recommended by Tjaden *et al.* [14] was that 4.0 m*M* TBABr was used instead of 5.0 m*M*. The suramin peak and the counter-ion (tetrabutylammonium) system peak eluted close to each other. Injection of the worked-up plasma (total concentration) gave a large positive system peak of the counter-ion (Fig. 2). The suramin peak was eluted together with the rear of the counter-ion sys-

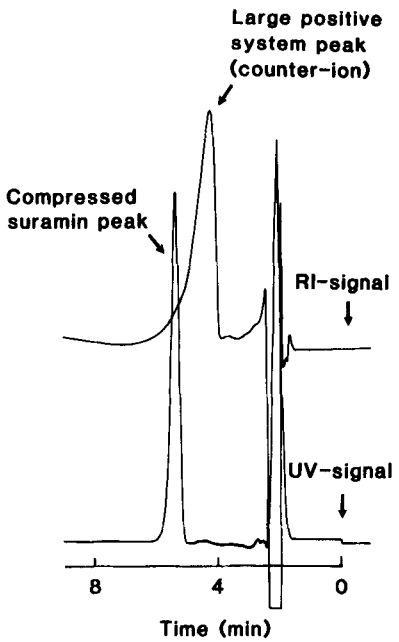

Fig. 2. Peak compression of suramin. Sample, 100 μl of worked-up plasma spiked with suramin (1.0 μg/ml); eluent, 3.9 m*M* TBABr in phosphate buffer (pH 7.5, I = 0.02)–methanol (42.5:57.5); column, Nucleosil C_{18} (batch 1) (150 mm × 4.6 mm I.D.).

tem peak as a slightly compressed peak (*cf.* Fig. 1a, position II) compared with the isocratic run (the injection of suramin dissolved in eluent). The width of the suramin peak was reduced by 18% compared with the corresponding isocratic run, and its retention volume was increased from 3.89 ml (isocratic) to 4.17 ml. The peak compression effect may be a problem if its existence is unknown. The worked-up standard samples induced larger system peaks than the simple aqueous standard samples and therefore created a steeper counter-ion gradient, which resulted in a greater peak-width reduction for suramin. This will result in incorrect absolute recovery determinations if peak-height measurements are used.

Although the peak compression effect in this case was rather weak, the effect was an advantage in quantitative assays. The calibration graphs based on peak-height measurements for the worked-up standard samples were linear over the range studied, *i.e.* between 5.024 and 502.4 μg/ml of plasma. The correlation coefficients (r) were better than 0.999 and the equation of the line was $y = 0.11 + 0.148x$. The intra-assay precision was comparable with isocratic conditions, and the coefficient of variation (C.V.) was 1–2% (n = 10), except for the lowest concentration, when it was 5% (n = 10).

Injection of the ultrafiltrate (free fraction) led to extreme deformation of the suramin peak (Fig. 3). A large negative system peak appeared, and the suramin peak was eluted at the rear of the negative counter-ion system peak and was therefore deformed (*cf.* Fig. 1b, position II). It should be mentioned that deformations also can develop when drugs with high protein binding are chromatographed after direct injection of plasma samples [16,17]. However, the recommended cut-off value of the ultrafiltrate membrane was 30 000 [14], and the use of membranes with values of 3000 gave the same result. This indicates that protein binding is not the cause of the deformation of suramin in this case.

The separation factor (α) concerning the suramin peak and the system peak was 1.3 in this

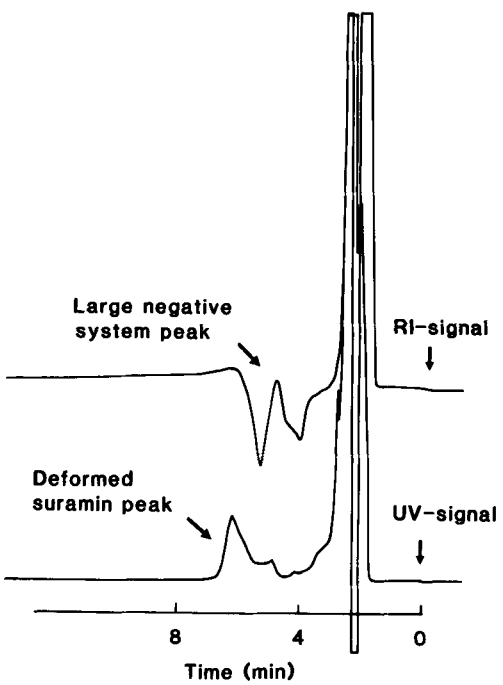

Fig. 3. Peak deformation of suramin. Sample, 100 μl of ultrafiltrate (cut-off 3000) spiked with suramin (1.0 μg/ml); eluent and column as in Fig. 2, but the eluent contained 4.1 m*M* TBABr.

case. The effects may be eliminated by using a smaller injection volume and/or dissolving the analyte in eluent. However, this approach is often not possible in bioanalysis, where small amounts of analytes are analysed, and certainly not for the free fraction of the highly protein-bound drug suramin. One must therefore increase the separation between the analyte peak and the system peak. The most important parameters determining the selectivity between the suramin peak and the system peak in this chromatographic system are the counter-ion concentration, the methanol content, the ionic strength, the pH and the column temperature. Finally, the character of the solid phase, including batch variations, is essential.

Counter-ion concentration and methanol content

In ion-pair adsorption chromatography the retention of the analyte can be regulated by changing the nature and/or the concentration of the counter-ion [4,5]. The capacity factor of suramin increased strongly with increasing concentration of the counter-ion [14] (Fig. 4), whereas the capacity factor of the system peak decreased [4]. At a counter-ion concentration of *ca.* 3–4 m*M*, the capacity factors of the suramin peak and the system peak are close, and distortions of the suramin peak occurred when the different types of worked-up plasma samples were injected (see above). With increasing counter-ion concentration the separation between the suramin peak and the counter-ion system peak continuously improved, and this approach was used to eliminate the distortions of suramin in this case. At a counter-ion concentration of 5 m*M* the suramin peak was sufficiently separated from the system peak and no distortions occurred when the different types of suramin samples were injected.

The effect of small changes of the methanol content (55–60%) was investigated (Fig. 5). The capacity factors of both the suramin peak and the system peak decreased with increasing methanol content in the eluent. The dependence of the suramin peak was much stronger, however, giving improved separation from the system peak at decreasing methanol content.

Ionic strength and pH

The capacity factor of the suramin peak decreased rapidly and that of the system peak increased when the ionic strength was varied from 0.01 to 0.2 (Fig. 6). The same tendency among anionic analytes and the system peak of the organic counter-ion in the eluent has been observed earlier [18]. The capacity factor of the analyte peak changes rapidly as the ionic strength is changed from low to high values. However, when the ionic strength reaches 0.1 the changes seem to level off (*cf.* Fig. 6) [18].

The pH value of the buffer was varied between 6.1 and 7.9 (Fig. 7), and anthraquinone-2-sul-

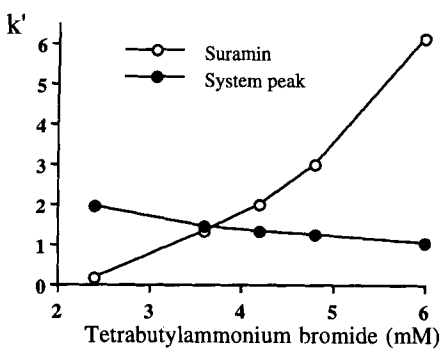

Fig. 4. Capacity factors of suramin and the system peak *versus* the tetrabutylammonium ion concentration in the eluent. Sample, 100 μl of suramin (1.0 μg/ml) in eluent; eluent, tetrabutylammonium in phosphate buffer (pH 7.5, I = 0.02)–methanol (42.5:57.5); column, Nucleosil C_{18} (batch 1) (150 mm × 4.6 mm I.D.).

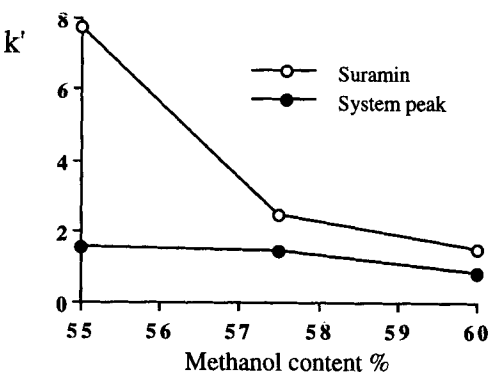

Fig. 5. Capacity factors of suramin and the system peak *versus* the methanol content in the eluent. Sample, eluent and column as in Fig. 4, but the eluent contained 5.0 m*M* TBABr and the methanol content was varied.

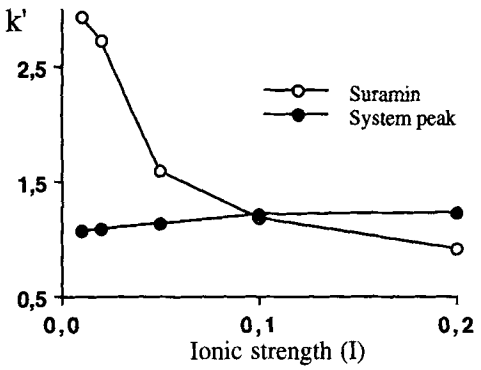

Fig. 6. Capacity factors of suramin and the system peak *versus* the ionic strength of the buffer. Sample, eluent and column as in Fig. 4, but the eluent contained 5.0 m*M* TBABr and the ionic strength of the buffer was varied.

phonate was included as an example of a univalent analyte. The capacity factors of both the anthraquinone-2-sulphonate peak and the suramin peak decreased almost linearly with increasing pH, but that of suramin decreased more rapidly. The capacity factor of the system peak increased with increasing pH. The effects cannot be explained as changes in the degree of ionization of those components, since the sulphonates are strong acids ($pK_a < 2$) and the system peak component is a quaternary ammonium component.

The pK_a for silanized silica has often been reported to be *ca.* 7 [19], which means that the number of dissociated silanol groups will be higher at pH 8 than at pH 6. A possible explanation for the result could therefore be that tetrabutylammonium ion binds to the dissociated silanol groups, and that the injected anions will be repelled from the surface at the higher pH values. It should also be mentioned that the concentration of the divalent buffer anions increases in relation to the univalent as the pH is shifted from 6 to 8. The pH and ionic strength values given are those of the buffer solution used for preparation of the eluent. However, even if the thermodynamically valid pH [20] and pK_a [21] values in the mixed media were used, the same discussion as above could be applied because the differences are rather small.

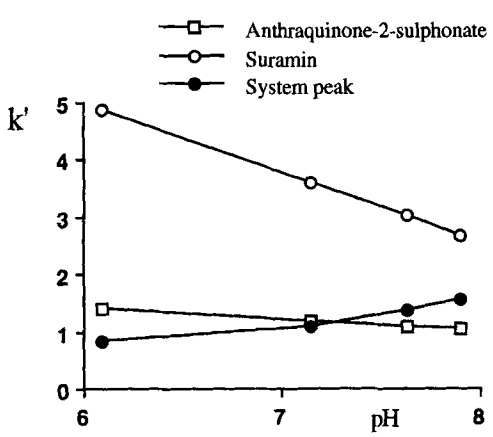

Fig. 7. Capacity factors of suramin and the system peak *versus* the pH of the buffer. Sample, eluent and column as in Fig. 4, but the eluent contained 5.0 m*M* TBABr and the pH of the buffer was varied.

Temperature

A change in column temperature affects the equilibria between the mobile phase and the solid phase (*cf.* Table I). The temperature was varied between 20 and 30°C, and this gave decreased capacity factors for both the suramin peak and the system peak. However, the capacity factor of the suramin peak decreased more, giving a decreased separation factor between the suramin peak and the system peak as the temperature was increased.

Character of the solid phase

Among four different solid-phase (Nucleosil C_{18}) batches tested, the separation factor between the suramin peak and the system peak varied between 1.3 and 7.5 (Table II). The selectivity differences between the suramin peak and the

TABLE I

VARIATIONS IN THE SELECTIVITY BETWEEN THE SURAMIN AND SYSTEM PEAKS DUE TO DIFFERENCES IN COLUMN TEMPERATURE

Sample, eluent and column were as in Fig. 4, but the eluent contained 5.0 m*M* TBABr

Temperature (°C)	Separation factor, α
20	2.9
25	2.2
30	1.6

TABLE II

VARIATIONS IN THE SELECTIVITY BETWEEN THE SURAMIN AND SYSTEM PEAKS DEPENDING ON THE SOLID-PHASE BATCH USED

Sample and eluent were as in Fig. 4, but the eluent contained 4.0 m*M* TBABr.

Batch	k' (suramin)	k' (system peak)	Separation factor, α
Nucleosil			
Batch 1	1.72	1.30	1.33
Batch 2	2.30	0.83	2.77
Batch 3	3.20	0.74	4.32
Batch 4	5.02	0.69	7.46
Kromasil	14.82	0.65	22.94
Spherisorb	0.98	1.84	0.53

system peak of the counter-ion were very large when octadecyl-bonded phases from different manufacturers were compared, *i.e.* Nucleosil C_{18}, Kromasil C_{18} and Spherisorb ODS-1 (*cf.* Table II). The selectivity variations between the univalent naphthalene-2-sulphonic acid and the counter-ion system peak were smaller than between the suramin peak and the system peak; the separation factor had values between 2.3 and 4.0 for the four different Nucleosil C_{18} batches.

CONCLUSION

It is of great importance to be aware of the risk of chromatographic distortions due to system peaks. This is especially important in the development of bioanalytical and related methods, where samples that deviate considerably from the eluent are injected, resulting in large system peaks. Knowledge and control of the parameters that determine the retention of the system peaks relative to the analyte peaks are therefore of paramount importance for fast and successful design of such methods.

The dependence of the selectivity between the suramin peak and the system peak on the batch variation of the solid phase is not possible to predict. All the solid-phase batches intended for use in the chromatographic system have therefore to be tested regarding the separation from the system peak. If the suramin peak elutes close to the system peak, it is easiest to change the counter-ion concentration in order to improve the separation distance between them.

When an adequate separation between the analyte peak and the system peak has been achieved, the selectivity between the analyte peak and the system peak should remain unchanged. It is therefore important to control all the parameters that affect the selectivity between the two peaks *i.e.* the counter-ion concentration in the eluent, the pH and the ionic strength of the eluent, and the column temperature.

A change in ionic strength has bigger effects on the suramin peak retention at low values of the ionic strength than at high values. A low ionic strength in the eluent is therefore preferable if the ionic strenght is to be used to improve the separation from the system peak. However, if adequate separation is achieved by other means, a high ionic strength will give a more robust chromatographic system against slight changes in ionic strength when new eluents are prepared.

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