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Journal of Chromatography A, 929 (2001) 113–121

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Salt effects in capillary zone electrophoresis V. Adsorption and retention of electrically neutral analytes

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Received 16 November 2000; received in revised form 9 July 2001; accepted 17 July 2001

Abstract

Two electrically neutral analytes previously observed to be separated from the neutral marker in capillary zone electrophoresis (CZE) experiments [sulphanilamide (SAA) and sulphaguanidine (SGW)] have been examined to determine the basis for separation. The degree of separation increases markedly with buffer concentration and improves with increasing field strength. On the basis of the apparent electrophoretic mobilities in conventional CZE, migration times in a zero EOF environment were calculated for SAA, SGW and six other sulphonamides that were known to be ionized. These six markers were used to test the legitimacy of our predictions and to correct for small discrepancies between the predicted and observed migration times. It was concluded that SAA and SGW have negligible electrophoretic mobilities and that they are retained in the electrical double layer close to the capillary wall. A mechanism for adsorption is proposed and the generality of the phenomenon is discussed.

Keywords: Adsorption; Salt effects; Sulphanilamide; Sulphaguanidine

1. Introduction

Separations in capillary zone electrophoresis (CZE) are based upon finite differences in charge and size between analytes, and in the limit when there is no charge, there should be no separation. However, there are many mechanisms by which electrically neutral analytes may be separated. The majority of these depend upon the addition of modifiers to the background electrolyte (BGE). At one extreme the additive is a surfactant above its critical micelle concentration and we have MEKC [1,2]. Alternatively, if electrically neutral analytes have non-bonding electron pairs, they may chelate with multiply charged cations [3,4] added to the BGE and thus be separated on the basis of differences in average charge that arise from less than complete complexation. If all of the electrically neutral analytes are fully complexed, the resultant ions may be separated due to size differences between the complexes. In another variation, electrically neutral cyclodextrins have been analyzed by CZE by adding benzoate to form charged inclusion complexes [5]. Neutral analytes have also been found to bind to monomeric cationic surfactants to become separable in both non aqueous [6,7] and aqueous [6,8] mobile phases. But in the absence of these or other additives which may complex neutral

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)01135-9

analytes, there are very limited possible explanations for neutral analytes not moving at the speed of the EOF. Indeed, Boček et al. [9] have observed that: ''Generally, the weakest interaction agent is a buffer, . . . ''. One exception in a simple buffer arises due to borate complexation of diols and provides important applications to sugar and catechol analyses [10].

But when all forms of complexation can be discounted and neutral analytes emerge after the neutral marker (NM), there are few possible explanations and delay is probably due to interaction with the capillary wall. Adsorption is generally associated with large molecules and most frequently with proteins, although large electrically neutral molecules have been used to coat the capillary wall via adsorption [11] and small positively charged analytes might be expected to adsorb on the negatively charged silica surface. (For further information in the general area of adsorption, readers are referred to Refs. [12–15]). However, in simple phosphate buffers, the adsorption of small electrically neutral analytes might not be expected. Moreover, phosphate in particular is believed to interact strongly with the capillary wall, thus shielding the surface from protein adsorption [16] and presumably from some other potential adsorbants.

However, starting with our initial investigations of sulphonamide (SFA) separations [17], we found finite discrimination between the NM and the electrically neutral analytes. Subsequent investigations at higher salt concentrations (210 m*M* sodium phosphate buffer) revealed even greater separations [18]. Sulphanilamide (SAA, $pK_{a2}=10.4$) and sulphaguanidine (SGW, $pK_{a2}=11.3$) formed a band that was clearly centered at a time after the emergence of the NM. The effect was even more pronounced in 174 m*M* potassium phosphate buffer at the same pH (7.0) [19].

The aims of the current work were to investigate the extent and reproducibility of the observed effect and to demonstrate the presence or absence of electrophoretic mobility as a possible explanation.

2. Experimental

2.1. Instrumental

All separations were performed using a model 270A CZE System (Applied Biosystems, Forster City, CA, USA). Electropherograms were analysed and data integrated using a model 600 Data Analysis System (Applied Biosystems) interfaced to an Apple Macintosh computer. UV detection was carried out at 254 nm and the detector time constant was set to 0.3 s. Bio-Rad, (Hercules, CA, USA) donated the capillary coated with a hydrophilic poly-AAEE (polyacryloylaminoethoxyethanol) layer. To avoid disturbing the coating, it was used as supplied, viz. length: 108.2 cm; injection to detection length: 85.2 cm, I.D.: 50 μm and O.D.: 360 μm. An uncoated fused-silica capillary of the same I.D. and O.D. (SGE, Ringwood, Australia) was cut to length and a window burned to match the critical lengths of the coated capillary. The sample was introduced by means of vacuum injection in which a 1-s injection corresponded to a 4-nl injection volume (for dilute aqueous buffers). All measurements were taken at pH 7.0 and 30°C.

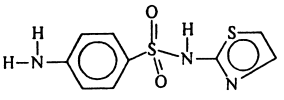
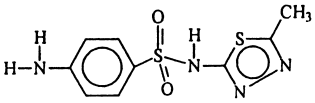
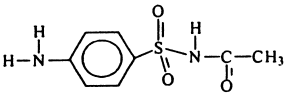
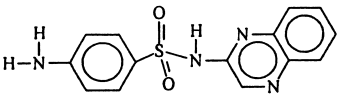
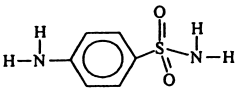
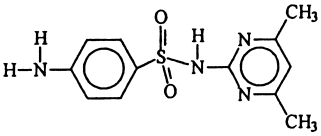
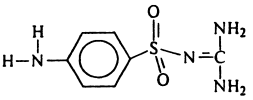
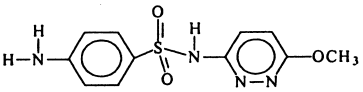
2.2. Chemicals and materials

The eight sulphonamides [sulphacetamide (SAC), sulphamethizole (SMI), sulphaquinoxaline (SQ), sulphamethoxypyridazine (SMOP), sulphathiazole (ST), sulphanilamide (SAA), sulphaguanidine (SGW) and sulphamethazine (SMZ)] were obtained from Sigma (St. Louis, MO, USA). Table 1 shows the structures and pK_a values. Cetyltrimethylammonium bromide (CTAB) was supplied by Aldrich (Milwaukee, WI, USA) and mesityl oxide by Hopkin and Williams (High Wycombe, UK).

Stock solutions of the sulphonamides were prepared by dissolving exactly 0.1 g of each in 80 cm^3 of methanol (BDH, 99.8%). The solutions were sonicated for up to 2 h to ensure complete dissolution and subsequently made up to 100 cm^3. Aliquots were then taken from each stock solution and diluted with Milli-Q water to give a 96:4 (H_2O–MeOH, v/v) mixture. The methanol causes a baseline disturbance and hence serves as a neutral marker. The final concentration of each SFA was 25 ng/μl. Sample solutions were filtered and degassed using a Millipore 0.22-micron filter prior to injection.

The potassium phosphate buffers (65, 101, 138, 174 and 250 m*M*) were prepared using KH_2PO_4 (99% Ajax Chemicals, Victoria, Australia) and $K_2HPO_4 \cdot 3H_2O$ (BDH). The mass of each salt re-

Table 1
Abbreviations for structures and pK_a data for the analytes

SFA	Structure	pK_{a1}	pK_{a2}	SFA	Structure	pK_{a1}	pK_{a2}
ST			7.2	SMI			5.4
SAC		1.8	5.4	SQ			5.5
SAA		2.4	10.4	SMZ		2.4	7.4
SGW			11.3	SMOP			6.7

quired was calculated using the Henderson–Hasselbach equation. Buffer pH was adjusted to exactly 7.0 using 20% KOH (Ajax Chemicals) or 20% H_3PO_4 (BDH) as required. Milli-Q water was used to make up the buffers and both solutes were of AR grade. The buffers were degassed by filtration through a Millipore 0.22-micron cellulose acetate filter.

A 3% mesityl oxide solution was prepared by dilution of 99% mesityl oxide with Milli-Q water. This served as a neutral marker for runs where dynamic coating was used. A 3% acetone solution was prepared for use as a neutral marker for runs performed on the coated capillary. Acetone was diluted and made up to volume with Milli-Q water.

CTAB stock solution (1 m*M*) was prepared by weighing out the appropriate mass of cetyltrimethylammonium bromide and making up to volume in 65 m*M* potassium phosphate buffer (prepared as indicated above). CTAB standards in the range 20–80 μ*M* were prepared by dilution of the stock solution. The pH of each standard was then adjusted to pH 7.0 using the method described above.

2.3. Capillary conditioning

Fused silica capillaries were conditioned with 1.0 *M* NaOH for 30 min to ensure a clean capillary surface and improved reproducibility. Between runs, a 3-min rinse with 0.1 *M* NaOH followed by Milli-Q water for 3 min then 10 min with the running buffer was found to provide good reproducibility.

For the coated capillary, conditioning was not performed. It is generally not considered necessary and may affect the integrity of the coating. A 5-min rinse with the running buffer between runs was found to give reasonably consistent results.

For the dynamic coating procedure, a detailed wash procedure was required for consistent results. Rinsing with 0.1 *M* HCl was reported to remove essentially all CTAB from silica surfaces [20]. Acid–base conditioning was performed between runs. This consisted of the following sequence: 2 min Milli-Q, 3 min 0.2 *M* HCl, 1 min Milli-Q, 3 min 1.0 *M* NaOH, 2 min Milli-Q. This wash was then followed by a 10-min rinse with the running buffer.

2.4. Calculations

Apparent mobilities (μ_{app}) were calculated from the apparent migration times (t_{app}):

$$\mu_{app} = \frac{L_D L}{V t_{app}} \quad (1)$$

Hence, for injection at the anode, the apparent electrophoretic mobilities of analytes, μ'_{ep}, were calculated according to:

$$\mu'_{ep} = \mu_{app} - \mu_{eo} \quad (2)$$

Thus, in a zero EOF environment, provided the experimental conditions are otherwise held constant, the electromigration times of the analytes in a reversed polarity field (t'_{ep}) would be:

$$t'_{ep} = \frac{L_D L}{V \mu_{ep}} \quad (3)$$

3. Results and discussion

3.1. Salt effects

Given the observed increases in migration times of the apparently electrically neutral SFAs (SAA, SGW) as the buffer concentration increased [18,19], we decided to systematically investigate this aspect first. Potassium phosphate was chosen as it yielded better separation from the NM than sodium phosphate buffers. Fig. 1 shows the data. It is immediately obvious that there is a strong salt effect and that, as with the obviously charged SFAs [18,19], resolution of MeOH, SAA and SGW improves with increasing buffer concentration. The resolution is considerably better at all concentrations than we have previously observed, but the major contribution to this was the increased capillary length utilized (85.2 cm compared to 49.0 cm used previously).

3.2. Effect of applied voltage

Fig. 2 shows the variation in resolution of MeOH (NM) and the first SFAs as a function of the applied voltage. Resolution clearly improves up to about +18 kV, but after that baseline noise becomes unacceptable.

3.3. CZE in the poly-AAEE coated capillary

From the means of repeated experiments in the uncoated capillary the apparent electrophoretic mobilities (μ'_{ep}) of the eight SFAs were calculated according to Eqs. (1) and (2) and are shown in Table 2. The migration times of the analytes in the reversed polarity field (injection at the cathode) and in the absence of EOF (t'_{ep}) were then calculated according to Eq. (3). For the analytes of particular interest (SGW and SAA), the t'_{ep} were excessive and could not be utilized. However, to test the EOF of the poly-AAEE coated column, the most mobile six SFAs were run under reversed polarity. The observed migration times were greatly in excess of the predicted t'_{ep} and non-zero EOF was apparent. To verify this, we reverted to injection at the anode on

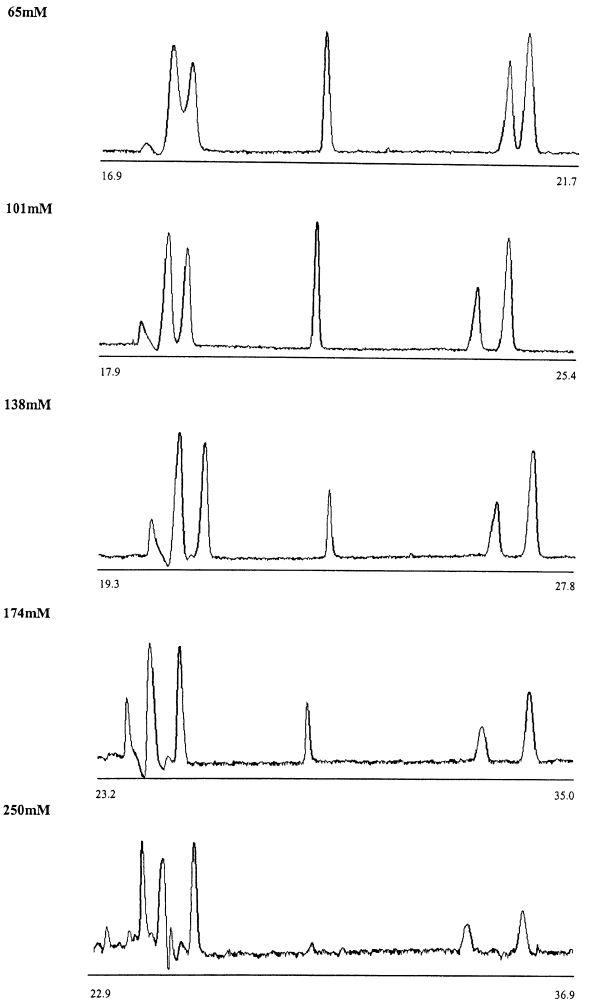


Fig. 1. The effect of potassium phosphate buffer concentration upon separation in untreated fused-silica at +18 kV. The first three peaks are the neutral marker (MeOH), SAA and SGW. The other peaks shown are SMZ, SMOP and ST, respectively.

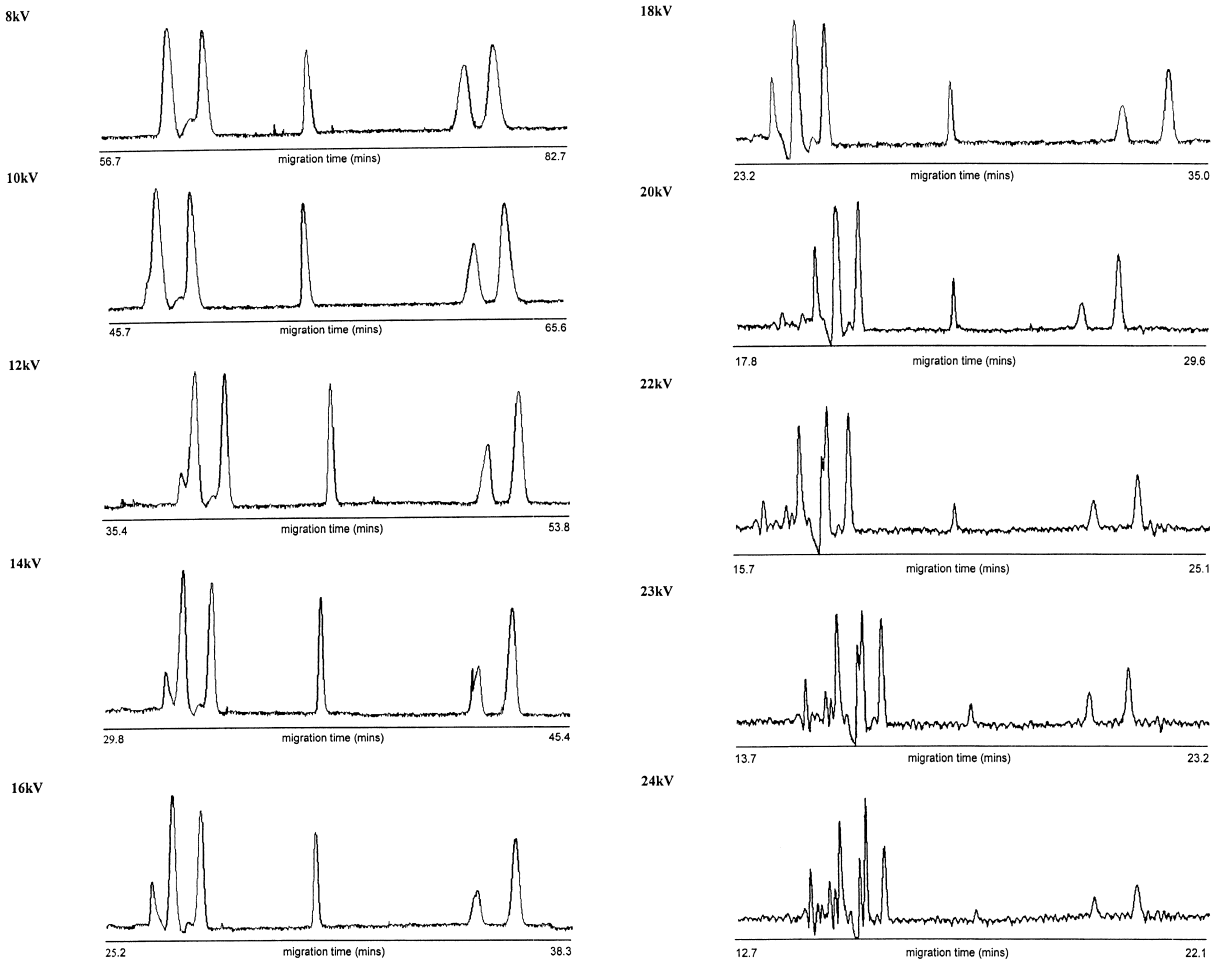


Fig. 2. Electropherograms in untreated fused-silica with 174 m*M* potassium phosphate buffer showing the effect of applied voltage upon the separation of MeOH, SAA, SGW, SMZ, SMOP and ST, respectively.

Table 2
Average apparent electrophoretic mobilities ($\mu'_{ep}/10^{-6}$cm^2/s/V) and standard deviations calculated from 12 measurements

Analyte	174 m*M*		65 m*M*	
	Average mobility	SD	Average mobility	SD
SAA	7.7	0.4	7.4	0.5
SGW	17.6	0.5	12.6	0.4
SMZ	51.5	2.4	45.8	1.2
SMOP	89.5	3.2	84.5	1.7
ST	97.7	3.6	88.1	1.8
SQ	186.2	3.8	180.0	1.1
SMI	215.0	4.1	208.8	1.0
SAC	231.5	3.2	226.5	1.1

the poly-AAEE-coated column. At the extended run times, it was not possible to unequivocally locate the baseline disturbance due to the MeOH in the sample solvent. Hence acetone was added as an approximate EOF marker and Fig. 3a shows that it emerged at 95 min. Hence the poly-AAEE coating does not fully cover the surface silanols and does not give rise to a zero EOF. Fig. 3b shows SAA and SGW emerging at 186 and 220 min, respectively, and the discrimination mechanism between SAA and SGW (and the NM) are strongly reinforced. Fig. 3b also shows that peaks may be readily detected up to about 300 min.

3.4. EOF suppression by dynamic coating

Given the excessive run times calculated in the absence of EOF, experimental conditions were modified to allow the observation of the migration of SGW and SAA, in the event that their separation from the neutral marker was due to electrophoresis. The uncoated capillary was cut down to 42.0 cm, the minimum length possible to connect the buffer reservoirs. The detection window was then located at 21.9 cm and the lowest ionic strength phosphate buffer (65 m*M*) was used. Whilst the 65 m*M* buffer provided the poorest resolution available (see Fig. 1), SAA and SGW are clearly resolved from each other and the NM at long run times (Fig. 3b). Injection times were reduced to 5.0 s. Due to the reduced capillary length, the applied voltage was also reduced. It was found that a maximum of +10 kV (238.1 V/cm) could be applied without anomalous effects such as temperature and current fluctuations. This field strength was then used for all subsequent runs. The t'_{ep} were calculated as described previously and are shown in column 2 of Table 3. As the longest t'_{ep} is 208 min (for SAA), the chosen experimental conditions allowed us to comfortably examine electropherograms up to t'_{ep} for SAA+50%.

In the face of variation in CTAB concentrations reported to cause zero EOF, we chose the value from the line of best fit drawn by Altria and Simpson [21] to their plot of μ_{eo} vs. ln[CTAB]. This yielded a value of about 37 μ*M*. Hence, with other reported values as high as 150 μ*M* (pH 7.2, 10 m*M* phosphate with the ionic strength adjusted to 50 m*M* with KCl [20]), we decided to examine the range from 20 μ*M* CTAB, upwards. To track the EOF variation as a function of [CTAB], mesityl oxide was chosen [20,22]. Up to 50 μ*M* in CTAB, the EOF could be seen, but at 60 μ*M* with respect to CTAB we were unable to detect any EOF (Fig. 4).

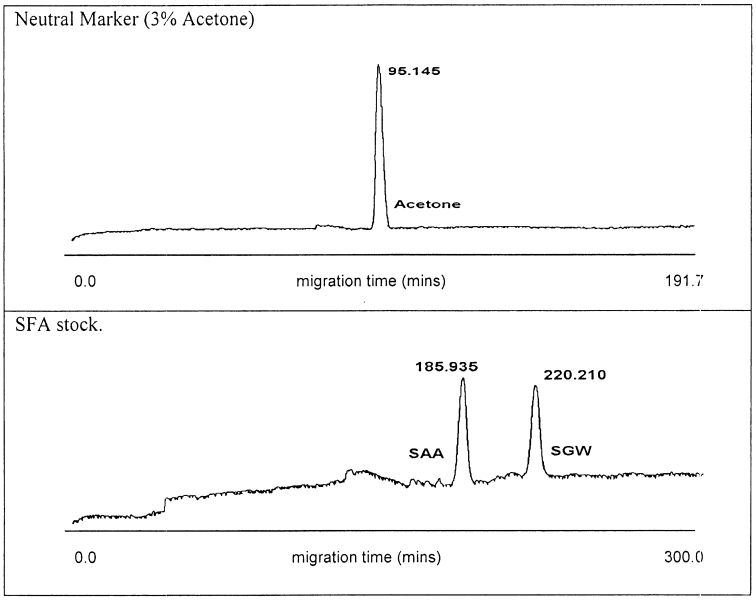


Fig. 3. Confirmation of residual EOF in the poly-AAEE coated capillary at +18 kV in 65 m*M* potassium phosphate buffer.

Table 3
Calculated migration times for the sulphonamides in a zero-EOF environment

Sulphonamide	Calculated migration times (min)		
	t'_{ep}	Projected	
		Solid line	Dashed line
SAC	6.77		
SMI	7.34		
SQ	8.52		
ST	17.39		
SMOP	18.14		
SMZ	33.44		
SGW	121.61	116.2	121.4
SAA	208.29	197.5	207.6

Conditions: 65 m*M* potassium phosphate buffer 60 μ*M* with respect to CTAB, injector-to-detector length 21.9 cm, applied voltage −10 kV over 42.0 cm.

At 60 μ*M* CTAB (65 m*M* phosphate buffer, pH7 and −10 kV), the six obviously ionized SFAs were detected and their observed migration times are plotted versus the expected migration times in Fig. 5. The solid line of best fit is drawn to the points for the six SFAs, has a gradient significantly different to 1.0 and the observed and expected times are not well correlated. When the outlier in the plot (♦, SQ) is removed, the resultant dashed line of best fit is close to ideal, with the gradient close to 1.0 (0.994), the intercept small and the correlation close to 1.0 ($R^2 = 0.997$). Hence we believe that our use of the previously calculated electrophoretic mobilities were a legitimate predictor of migration times under the prevailing experimental conditions.

We have projected both of these lines of best fit forward to the calculated migration times (t'_{ep}) for SGW and SAA (Table 3). For SAA, with t'_{ep} expected around 200 min, no peak was observed for about an additional 50% of that time, whereas the variation in estimates of expected times of emergence was only around 5% (last line, Table 3). In the case of SGW, there was a less than 5% variation between the highest and lowest estimates of t'_{ep} and the runs were followed for another 150% of that time interval (120 min). To apply a more demanding test, the outlier in Fig. 5 (SQ) could be taken as an extreme indicator of the likely times of emergence of SGW and SAA. With SQ emerging at 13.23 min, compared to 8.52 min being the time predicted on the basis of the measured mobilities, there was a 55% delay experienced for the emergence of this analyte. For SAA we have not detected any peaks for almost this additional percentage of the expected time and for SGW we have failed to observe any peaks for three times this percentage delay.

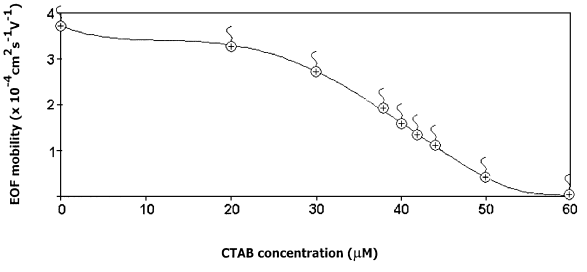


Fig. 4. Electroosmotic flow as a function of CTAB concentration in 65 m*M* potassium phosphate buffer at +10 kV in the untreated capillary.

3.5. Interpretation

Under the conditions of experimentation, in zero EOF — or close to zero EOF environment — SAA and SGW do not have measurable mobilities. It is therefore concluded that — commensurate with their published pK_{a2} values — SAA and SGW have negligible electrophoretic mobilities at pH 7 and that any delay in emergence of these analytes after the NM and any separation from each other must be due to a retention mechanism. As retention can only take place within the shear plane in the vicinity of the capillary walls, the process may be referred to as adsorption. The particular mechanism of adsorption seems most likely to involve hydrogen bonding, with the ionized surface silanoate groups providing the lone pairs of electrons for interaction with the unionized protons to which the pK_{a2} values refer for SAA and SGW. This suggests a generalized mechanism for adsorption and retention of electrically neutral analytes. All analytes containing acidic protons become potential candidates. However, we are unaware of other examples of this phenomenon. This may well be because high ionic strength (or some alternative driving force) is required to provide a suitable environment to facilitate the process and most CZE analyses are done in buffers much less concentrated those that we have used.

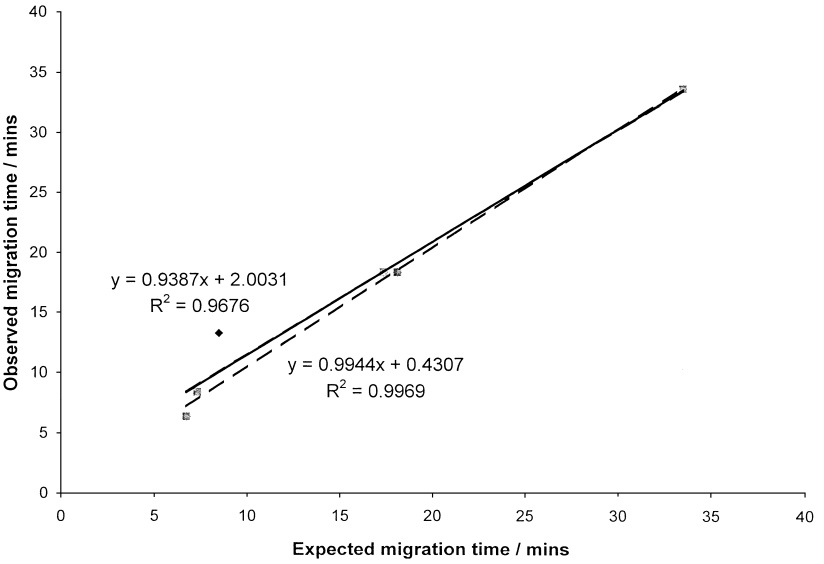


Fig. 5. Plots of the observed migration times of the ionized SFAs versus those predicted on the basis of the mobilities calculated in conventional CZE (Table 2). Conditions of analysis were in 65 m*M* potassium phosphate buffer 60 μ*M* with respect to CTAB concentration at −10 kV in the untreated capillary cut to 21.9 cm from injector to detector. The solid regression line is drawn to the all data points (equation and R^2 given above the solid line) and the dashed regression line excludes the outlier SQ: ♦ (equation and R^2 given below the dashed line).

What has been clear from our previous observations and has been strongly supported in this work is that increasing electrolyte concentrations cause increasing adsorption. That is, a salt effect is operating. As the buffer concentration increases, the chemical potentials and the activity coefficients of SAA and SGW increase in the bulk solution and the distribution of these analytes is pushed towards the alternative phase; the interphase between the bulk solution and the solid capillary wall. And if hydrogen bonding at the surface is the mechanism of adsorption and retention, the distribution of the analytes is specifically between the solution and the interface.

As we have discussed before [23], the activity coefficients of electrically neutral analytes have a complex dependence upon their own molecular structure, the nature of the ions in the salt solution and the concentration of that electrolyte. One of the consequences of this is that salting out (or the absence of it) depends upon the total analyte molecule and not just one small part. Thus some acidic analytes will be salted out and will display similar behaviour to that of SAA and SGW, whilst others will not. And in the presence of different buffer ions, the observed effects will change for some analytes but not for others.

Another possible consequence of our findings concerns the migration of the other partially ionized SFAs and indeed the migration of all acidic analytes that are not fully deprotonated. These electrically neutral conjugates of ionically migrating species will also be susceptible to the same effects as those experienced by SAA and SGW. Thus a normal component of the apparent migration times of some analytes may be due to adsorption and retention. However, at the lower buffer concentrations generally used, this component of the analysis time may often be negligible.

Acknowledgements

We would like to thank Dr Tim Wehr and Bio-Rad for the kind donation of the coated capillaries.

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