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Increase of sample load without peak deterioration by careful selection of electrolyte in capillary zone electrophoresis

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Abstract

In this study it is demonstrated that much higher concentrations of bases dissolved in water can be injected in capillary zone electrophoresis without causing peak deterioration, e.g., peak splitting, if it is the co-ion that buffers instead of the counter-ion. Those findings can be utilised to control peak shapes and in this way an increase in the sample load and indirectly a decrease in the detection limits of impurities in the sample can be obtained. Good results were obtained with 4-aminobutyric and 6-aminocaproic acids as buffering co-ions. Another possibility evaluated successfully was that of using a dibasic acid, malic acid or succinic acid. With an electrolyte containing both succinic acid and 6-aminocaproic acid at pH 4.5, it was possible to load at least 10–20 times more of the test substances imidazole, creatinine or 2-aminopyrimidine dissolved in water than with an electrolyte at the same pH containing acetic acid and tris(hydroxymethyl)aminomethane.
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Keywords: Sensitivity; Peak shape; Background electrolyte composition; Imidazole; Creatinine; Aminopyrimidine

1. Introduction

Capillary zone electrophoresis at low pH is an electrophoretic method chosen for the separation of hydrophilic bases. A commonly used buffer solution is phosphate at pH 2.5 [1]. If the resolution is not sufficient with this background electrolyte (BGE), the addition of cyclodextrin, an ion-pair reagent, an organic solvent or changes in the ionic strength may solve the problem [2]. A very powerful selectivity agent in capillary electrophoresis is the pH [3–5], as small changes in the electrolyte pH close to the pK_a of an analyte influence the charge and therefore the electrophoretic mobility of the analyte. Besides a phosphate buffer, citrate [1] or acetate [1,4], for example, can be used. It is important for the electrolyte to have a good buffering capacity [6,5] to avoid unacceptable changes in pH during the runs [7,8]. The sample can be dissolved in water [4,7], which maximises resolution and peak efficiencies [9], or in dilute buffer [7,10]. As the diluent used for the sample can have a large influence, it is important that the same diluent is used for both the sample and the standard [7]. Low ionic strength combined with a pH difference between sample and BGE can give a good stacking effect [11]. To obtain stacking effects, the pH of the sample and BGE should be chosen so that the mobility in the sample plug is higher than in the BGE [8]. Transient isotachophoresis–capillary zone electrophoresis can give stacking both inten-

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tionally and unintentionally [12,13]. Friedberg et al. found that the pH, ionic strength and type of buffer in the sample had a large effect on acetonitrile-induced stacking [10]. Stacking in capillary zone electrophoresis is one way of decreasing detection limits and is dealt with in Refs. [11,14], for example.

Ermakov et al. [15] reported peak splitting of pyridine (pK_a about 5.2) when analysed with 20 m*M* acetic acid adjusted with NaOH to pH 5. After the peak representing the ionised form the baseline shifted upward due to the presence of the analyte and a second peak with the uncharged form migrated with the electroosmosis. A necessary condition for this effect to occur was reported to be the presence of a strong co-ion in the electrolyte and an over-loaded system [15]. Peak splitting was also observed by Monson et al. [16] for acetic, propionic and butyric acids under some conditions. It occurred for a high load when the pH in the sample was lower than that in the electrolyte containing 20 m*M* potassium hydrogenphthalate adjusted to pH 6.0 and with 0.5 m*M* cetyltrimethylammonium chloride added as flow modifier. The solution of the problem was reported to be sample size reduction and dilution of the sample with the background electrolyte [16].

For the determination of impurities (related substances) in new medicinal products and substances, it is necessary to load a large amount of sample to enable quantification down to impurity levels of 0.05% of the main component, which is a common requirement according to the ICH guidelines [17,18].

In this study the importance of the choice of the electrolyte buffer in relation to the amount of sample (bases dissolved in water) that can be loaded without peak deterioration was examined. It was demonstrated that if the co-ion was buffering at the pH of the electrolyte or if the counter-ion was dibasic and had suitable pK_a values, at least about 10–20 times more could be loaded, compared with an electrolyte containing only acetate as buffering constituent.

2. Experimental

2.1. Chemicals

Imidazole, tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (5 *M*, Titrisol), succinic acid and acetic acid (Normapur analytical-reagent grade) were supplied by Merck, creatinine, 2-aminopyrimidine, formic acid and 4-aminobutyric acid by Fluka, and 6-aminocaproic acid (6-ACA) by Roth. The solvent used was Milli-Q+ water (Millipore).

2.2. Equipment

An Agilent instrument, HP^{3D}CE, was used. The electrophoretic separations were performed at 25 °C in bare fused-silica capillaries from Supelco, 35 cm (effective length approx. 26.5 cm)×75 μm I.D. The analyses were run in constant voltage mode with normal polarity at 15 kV after an initial ramp time of 0.2 min. The injection was carried out by pressure, 32 mbar for 2 s, immediately followed by injection of electrolyte for 3 s at the same pressure. The detector was used with a bandwidth of 4 nm at different wavelengths.

The pH of the electrolytes was measured potentiometrically with a PHM210 equipped with a combined pH electrode pHC2401 from Radiometer (Copenhagen, Denmark). All electrolytes were filtered through a Millex HV 0.45 μm filter with a plastic syringe and the first portion was discarded.

2.3. Procedures

Capillaries (new and on exchange of the electrolyte) were rinsed with water for 2 min, 0.1 *M* sodium hydroxide for 5 min, water for 2 min and finally electrolyte for 7.5 min. Between runs rinsing with electrolyte for 2.5 min was performed. The capillaries were stored dry after rinsing with water for 5 min followed by air for 5 min.

3. Results and discussion

3.1. Imidazole and creatinine analysed with an electrolyte containing acetic acid adjusted with Tris to pH 4.5

A solution containing 20 m*M* acetic acid pH-adjusted to 4.48 with Tris was used as background electrolyte. Table 1 shows the pK_a values of all substances used in the study. Samples with imidazole (pK_a 6.99) dissolved in water and also in water and

Table 1
pK_a for substances used in this study

Substance	pK_a (approx. values) [21]
Acetic acid	4.756
4-Aminobutyric acid	4.03, 10.556
6-Aminocaproic acid (6-ACA)	4.37
2-Aminopyrimidine	3.45 (at 20 °C)
Creatinine	4.8, 9.2
Formic acid	3.75
Glutamic acid	2.13, 4.31, 9.67
Imidazole	6.99
Malic acid	3.40, 5.11
Succinic acid	4.21, 5.64
Tris	8.3

pH-adjusted to about 4.6 with hydrochloric acid, respectively, were analysed. When imidazole was dissolved in water, a poor peak shape was obtained (Fig. 1a), whereas when the substance was in its ionised form in the sample, the peak was a normal triangular one (Fig. 1b). Fig. 1c shows the electropherogram for a mixture of the imidazole solution analysed in Fig. 1b and creatinine (pK_a 4.8) dissolved in water. A split peak of creatinine was obtained (Fig. 1c). With this high concentration of creatinine in the mixture, the original baseline level was not reached after the first creatinine peak and a large amount of creatinine was found together with the electroosmotic flow peak. The spectra of the two peaks of creatinine representing the charged and uncharged molecules, respectively, are shown in Fig. 1d. Even if creatinine was added in a small amount to the pH-adjusted imidazole solution, the creatinine peak was broad and split (migration time about 1.5 min), but not the imidazole peak (Fig. 1e). This experiment shows that imidazole in its ionised form does not give any indication of a risk of overloading effects that will affect creatinine, for example, added in a small amount. Moreover, if the purity of a substance is analysed, although the peak performance of the main component is good, impurities at much lower concentrations could give split peaks due to unfavourable conditions for the impurities.

The phenomenon of an acceptable imidazole peak at the same time as the peak of creatinine is poor could be explained by the following simplified reasoning. When voltage is applied, the ionised form of creatinine and imidazole will leave the sample zone and Tris migrates into the zone. Tris has a much higher pK_a (8.3) than creatinine (4.8), which means that it behaves as a strong co-ion (non-buffering) in relation to creatinine. From the other side of the sample zone acetate migrates in and this ion is unable to donate any hydrogen ions. Hence, creatinine will not leave the initial sample zone properly. This situation is illustrated in Fig. 2a with $Tris^+$ as co-ion. However, imidazole has a higher pK_a (6.99) and Tris is not an equally strong co-ion in this case but a more buffering one, and so a proper peak can be obtained even at these high concentrations. Later on diffusion will take place [15]. Acetic acid diffuses into the sample zone, dissociates, hydrogen ions are formed and creatinine will become charged according to the equilibrium constant; naturally diffusion of creatinine into the BGE will also occur. Creatinine dissolved in water (no imidazole present) was also analysed at various concentrations. At the highest concentration (Fig. 3a), most creatinine migrates with the electroosmotic flow (EOF), whereas with decreasing concentration the peak shape gradually improves and migration time decreases (Fig. 3b–d). If creatinine was dissolved in water and was the only substance present in the sample, a proper peak was obtained with 2.3 m*M* (Fig. 3d). When imidazole was present in a large amount in the sample, the creatinine peak was split even if its concentration was as low as 0.9 m*M* (Fig. 1e).

3.2. Comparison of results for analysis of imidazole, creatinine and 2-aminopyrimidine dissolved in water with different electrolytes

In this section results from the analysis of imidazole, creatinine and 2-aminopyrimidine, respectively, dissolved in water are reported. According to the reasoning above, acetic acid pH-adjusted with 6-aminocaproic acid (pK_a 4.37) instead of Tris would be a better electrolyte for analysis of creatinine (pK_a 4.84). The ionised form of 6-aminocaproic acid migrates into the sample zone and, as creatinine has about the same pK_a, it will become ionised and a proper peak will result. Fig. 2b shows an example with an electrolyte containing 6-ACA pH-adjusted with hydrochloric acid. To obtain good peak shapes, it is here assumed to be necessary for some ions migrating into the sample zone to be able to disso-

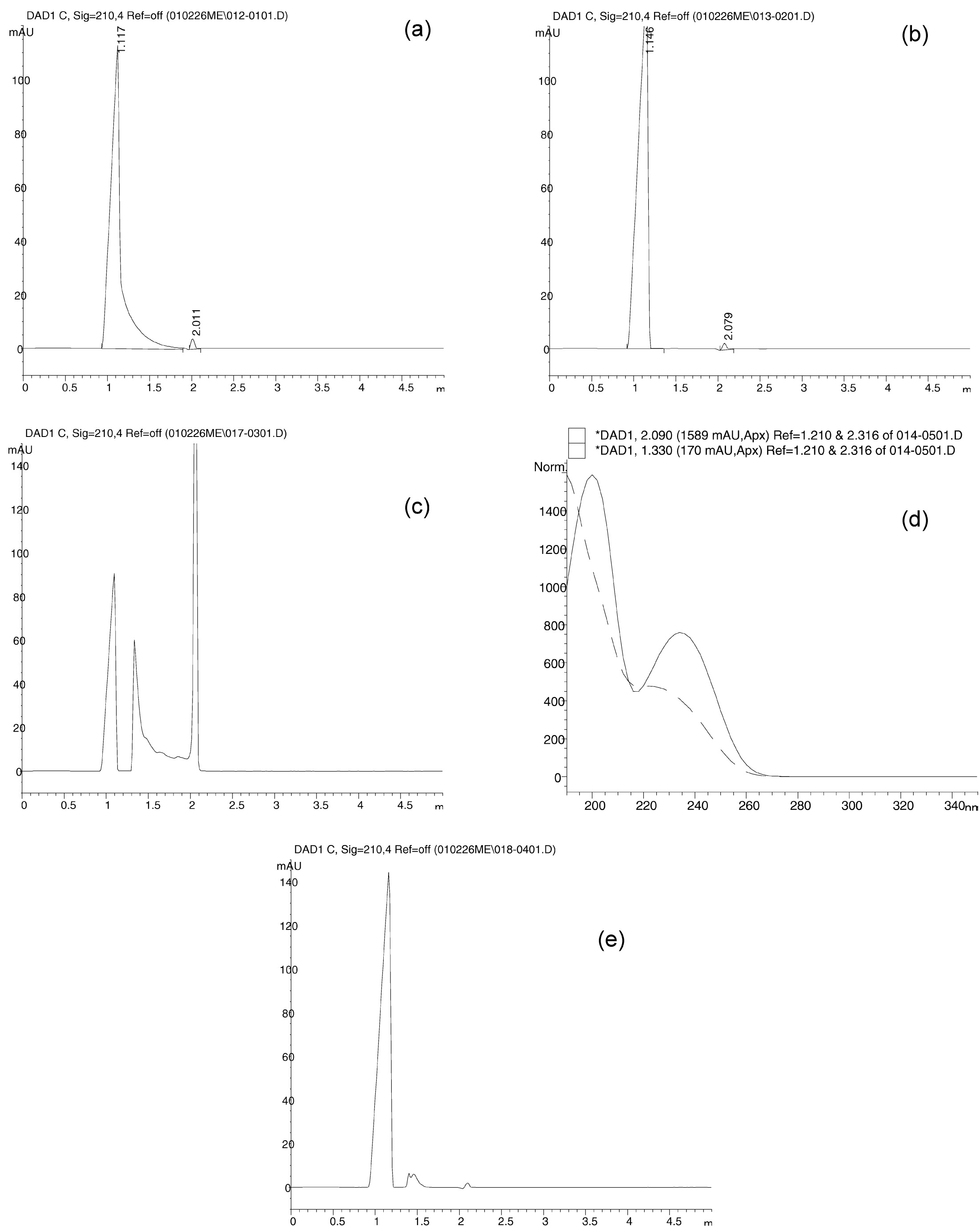

Fig. 1. Electropherograms illustrating the influence of the pH and other components in the sample on peak shape. Electrolyte: 20 m*M* acetic acid pH-adjusted with Tris to 4.48. Other conditions are given in Section 2.2. (a) Imidazole in water, concentration 38.9 m*M*. (b) Imidazole in water, pH-adjusted to 4.59 with hydrochloric acid, concentration 38.9 m*M*. (c) As (b) but with equal parts of imidazole solution and creatinine dissolved in water. Concentrations: imidazole 19.5 m*M* and creatinine 21.6 m*M*. (d) Spectra of the two peaks in (c). (e). Mixture of 98 parts of imidazole in water, pH-adjusted to 4.59 [analysed in (b)] and two parts of creatinine in water [analysed in (c)]. Concentrations: imidazole 38.1 m*M* and creatinine 0.9 m*M*.

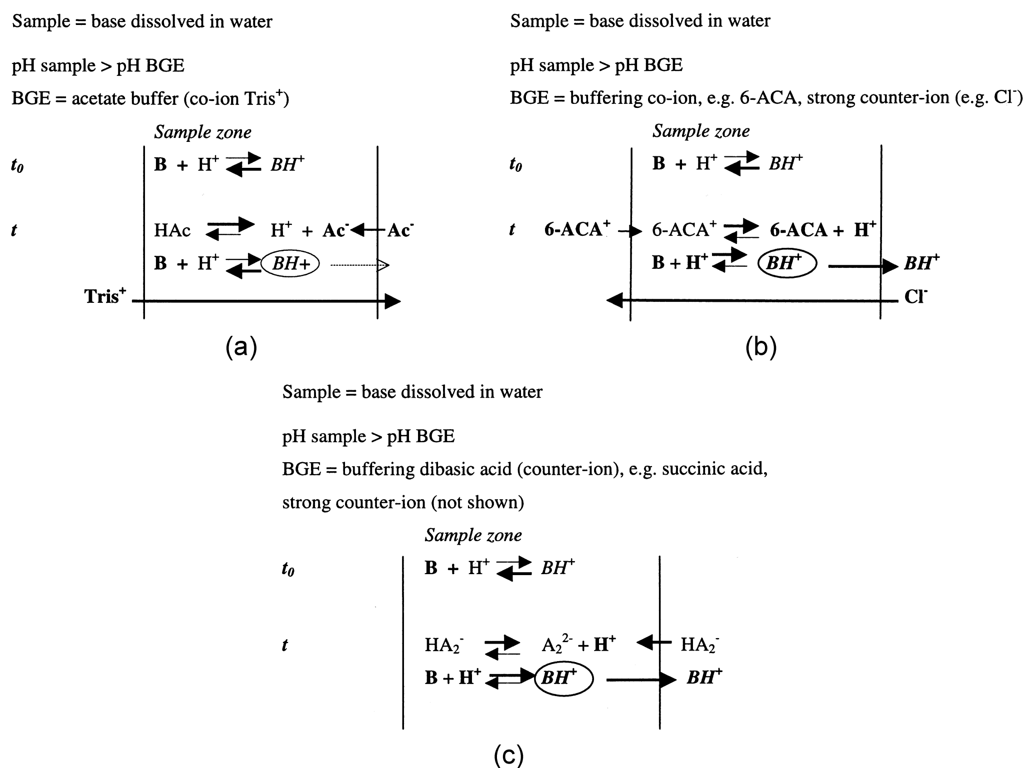

Fig. 2. (a) Scheme for electrolyte with buffering counter-ion. (b) Scheme for electrolyte with buffering co-ion. (c) Scheme for electrolyte with buffering dibasic counter-ion.

ciate to form hydrogen ions in order for the base in the sample to become positively charged. Fig. 4a–d show the results with an electrolyte containing 20 m*M* acetic acid pH-adjusted to 4.48 with 6-ACA. Compare Fig. 4a with Fig. 1a (imidazole dissolved in water). Imidazole, creatinine and also 2-aminopyrimidine gave normal peaks (Fig. 4a–d), although 2-aminopyrimidine has a pK_a about one unit lower than 6-ACA. An electrolyte containing 20 m*M* hydrochloric acid adjusted with 6-ACA to pH 4.48 was also tested and it was found that acetic acid is not necessary to obtain normal peaks. See Table 2, where summarised data are presented.

It seemed reasonable also to assume that an acid with two ionisation constants could be used as a buffering substance, provided that the difference between the constants is not too large and the electrolyte pH is suitable. Malic acid and succinic acid were tested for this purpose. When the ionised form of the acid (charge −1) migrates into the sample zone (with higher pH), the acidic ion will dissociate further and the conjugate acid of the sample base will be formed according to the equilibrium constant (see Fig. 2c). Table 3 shows the results of different electrolytes tested with imidazole, creatinine and 2-aminopyrimidine, respectively, all dissolved in water. The electrolytes represent three pH values: approx. 3.25, 4.0 and 4.5; the cations of the electrolytes were Tris, 4-aminobutyric acid or 6-aminocaproic acid; the acids used were formic, acetic, malic, succinic or glutamic acid. The following conclusions can be drawn from Table 3. Electrolyte 2 containing 4-aminobutyric acid was better than No. 1 containing Tris as more 2-aminopyrimidine could be loaded without adverse effects. Electrolyte 7 with 6-ACA and hydrochloric acid gave a better result than acetic acid with Tris (No. 6) for the test substances, although the buffering capacity is of the same magnitude for the two electrolytes. Electrolyte 5 shows the possibility of using a dibasic

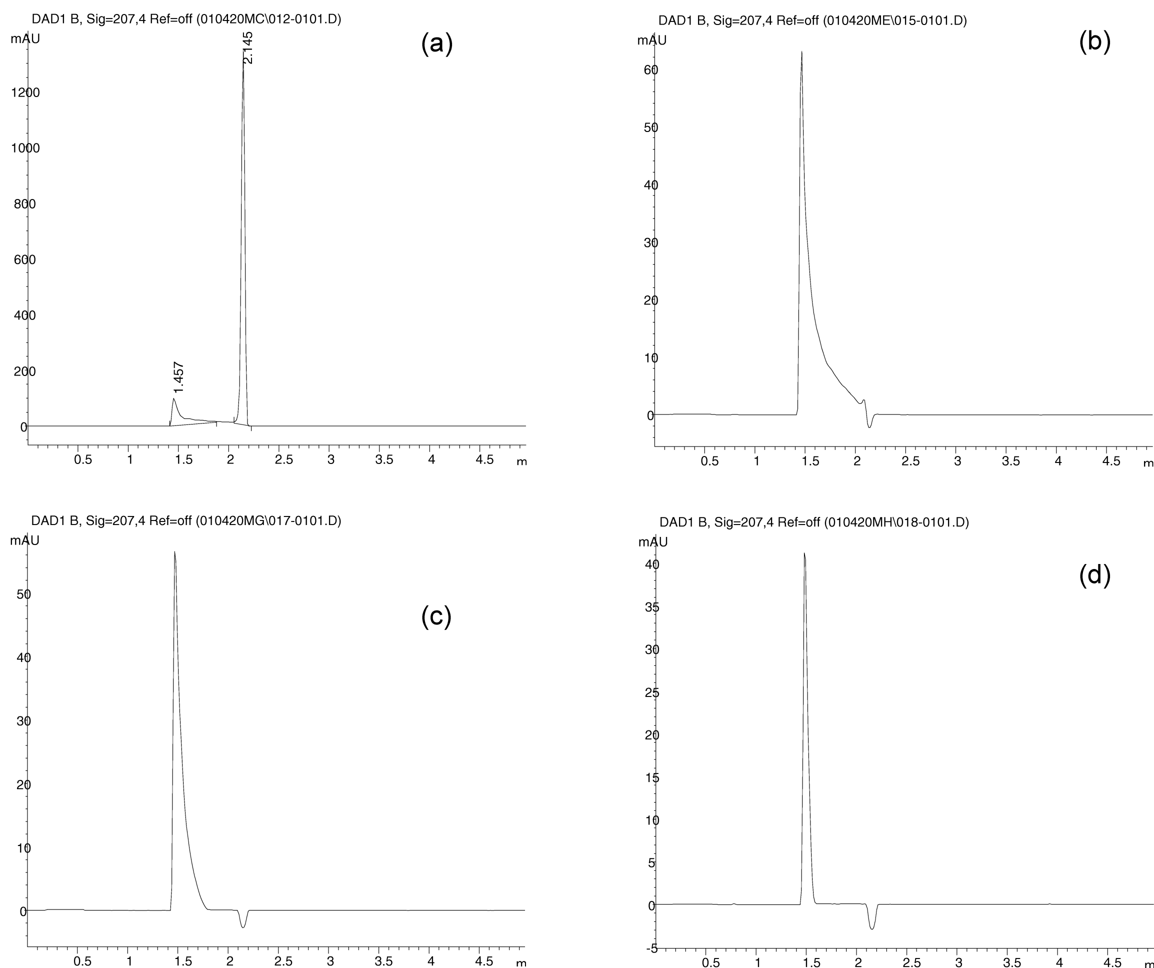

Fig. 3. Electropherograms illustrating an example of overloading effects on peak shape. Electrolyte as in Fig. 1. Other conditions are given in Section 2.2. (a) Creatinine in water, concentration 46 m*M*. (b) Creatinine in water, concentration 9 m*M*. (c) Creatinine in water, concentration 5.5 m*M*. (d) Creatinine in water, concentration 2.3 m*M*.

acid (malic acid) with good results compared with electrolytes 3 and 4 containing acetic/formic acid and Tris. Another dibasic acid, succinic acid, was used in Electrolyte 8 with a good result compared with No. 6. Electrolyte 9 with both a buffering counter-ion, a dibasic acid, and a buffering co-ion, 6-ACA, was tested with very high loads, 100 m*M* of imidazole and creatinine, respectively, both dissolved in water. Despite these high concentrations (five times higher than the electrolyte), expected triangular peaks were obtained but no other peak irregularities. A solution with 16.6 m*M* 2-aminopyrimidine (a higher concentration was not tested) also gave a normal peak shape. Compare this with the results obtained for electrolyte 6. Electrolyte 10 containing glutamic acid and Tris gave the same results as No. 6 containing acetic acid and Tris, due to the high pK_{a2} (9.67) of glutamic acid. All the results are as expected considering the simple model described above.

3.3. Experiments under real life conditions

Experiments were performed with mixtures because the sample components can influence each other, as was shown in Fig. 1e. This is also what

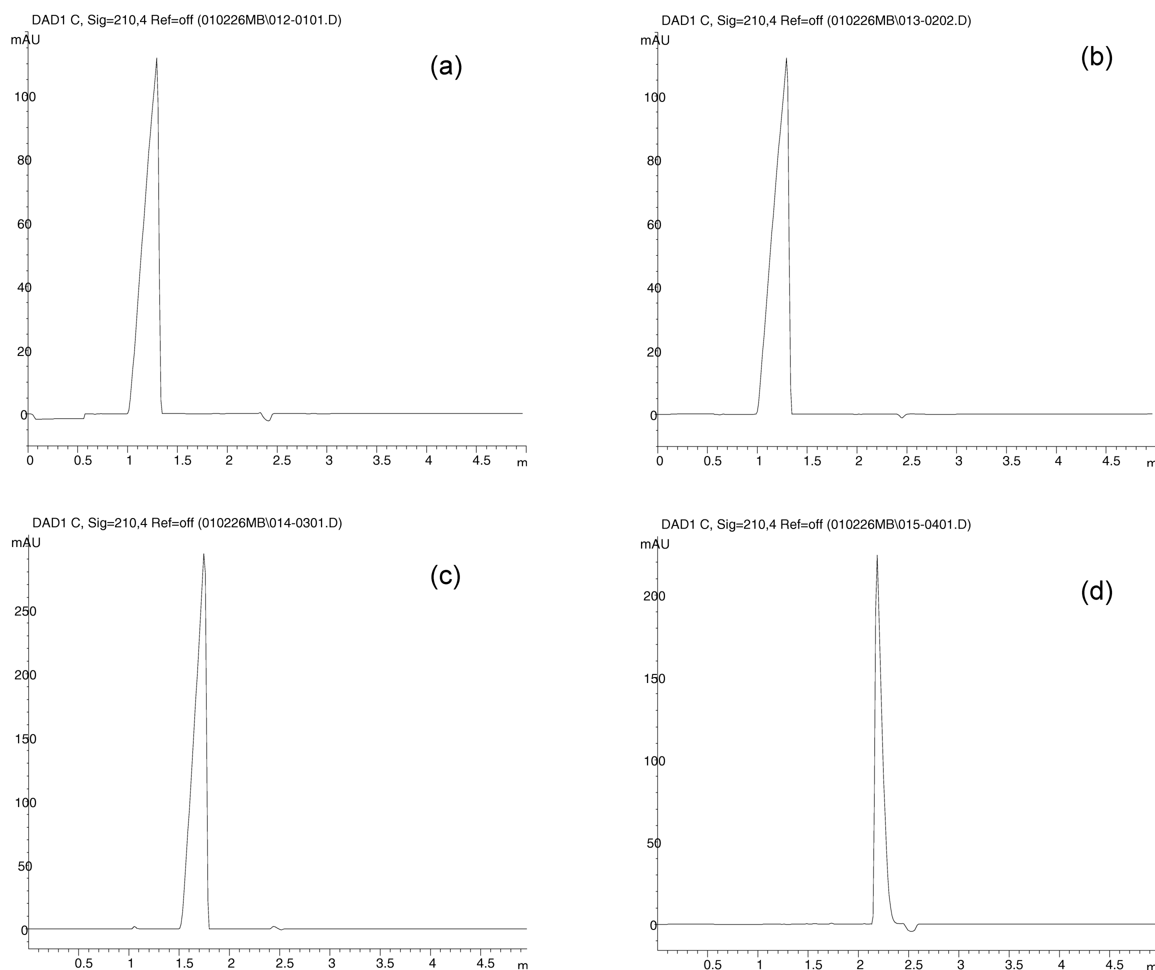

Fig. 4. Electropherograms showing normal peak shapes at high sample concentrations analysed with electrolyte containing both buffering co-ion and counter-ion. Electrolyte: 20 m*M* acetic acid adjusted with 6-ACA to pH 4.48. Other conditions are given in Section 2.2. (a) Imidazole in water, concentration 38.9 m*M*. (b) Imidazole in water, pH-adjusted to 4.59 with hydrochloric acid, concentration 38.9 m*M*. (c) Creatinine in water, concentration 43.2 m*M*. (d) 2-Aminopyrimidine in water, concentration 15.4 m*M*.

Table 2
Concentration of base dissolved in water analysed individually without peak deformation

	Electrolyte	Imidazole (m*M*)	Creatinine (m*M*)	2-Aminopyrimidine (m*M*)
(1)	20 m*M* HOAc+Tris, pH 4.48	<38.9	<43.2	<15.4
(2)	20 m*M* HOAc+6-ACA, pH 4.48	>38.9	>43.2	>15.4
(3)	20 m*M* HCl+6-ACA, pH 4.48	>38.9	>43.2	>15.4

>*a*, Concentration *a* gives a proper peak, higher concentration not tested.
<*a*, Concentration *a* gives an abnormal peak, lower concentration not tested.

Table 3
Concentrations of base dissolved in water analysed individually without peak deformation

	Electrolyte	Imidazole (m*M*)	Creatinine (m*M*)	2-Aminopyrimidine (m*M*)
(1)	20 m*M* HCOOH+Tris, pH 3.24	>17.4	>16.4, <21.9	<8.5
(2)	20 m*M* HCOOH+4-aminobutyric acid, pH 3.27	>17.4	Not analysed	>8.5
(3)	20 m*M* HOAc+Tris, pH 4.04	>9.7	<4.8	<4.8
(4)	20 m*M* HCOOH+Tris, pH 4.01	≈9.7	>1.2, <2.4	<1.0
(5)	20 m*M* malic acid+Tris, pH 4.04	Not analysed	>4.8	>4.8
(6)	20 m*M* HOAc+Tris, pH 4.52	≈9.7	>2.0, <4.9	<1.0
(7)	20 m*M* 6-ACA+HCl, pH 4.52	>19.3	>19.5	>16.6
(8)	20 m*M* succinic acid+Tris, pH 4.53	>48.3	>19.5	>16.6
(9)	20 m*M* succinic acid+6-ACA, pH 4.50	>100	>100	>16.6
(10)	20 m*M* glutamic acid+Tris, pH 4.53	≈9.7	>2.0, <4.9	≈1.0

>*a*, Concentration *a* gives a proper peak, higher concentration not tested.
<*a*, Concentration *a* gives an abnormal peak, lower concentration not tested.
≈*a*, Concentration *a* gives a peak that is only influenced to some extent.

could be expected considering the equilibrium constants, whereby the ionisation of a stronger base is favoured compared with a weaker one in a solution which is not acidic enough to ionise both. A mixture of 34 m*M* imidazole and 1 m*M* each of creatinine and 2-aminopyrimidine dissolved in water was analysed with an electrolyte containing 20 m*M* succinic acid adjusted with 6-ACA to pH 4.50 (composition as electrolyte 9, Table 3), the resulting electropherogram being shown in Fig. 5. The elution order is imidazole, creatinine, 2-aminopyrimidine (the EOF visible as a negative dip). As a comparison, an electrolyte containing 20 m*M* acetic acid pH-adjusted with Tris to 4.49 was used for analysis of the same sample (see Fig. 6a, which shows an extremely poor electropherogram). Fig. 6b shows the sample analysed after dilution and it should be noted that creatinine gives two peaks, at about 1.6 and 1.9 min, the identity being confirmed with spectra; 2-aminopyrimidine also gives two peaks, at 2.2 and 2.4 min, the last one at or very near the EOF. Imidazole, however, gives a normal triangular peak. The reason for this result is that imidazole is the strongest base and will therefore leave the starting zone more readily than the other two. This example illustrates that one component can be detected as two peaks under certain conditions and that the second one does not necessarily need to be eluted with the EOF. At still lower concentrations (see Fig. 6c) creatinine is detected as a double peak. Finally, Fig. 6d shows an electropherogram with normal peak shapes, although now the peaks have become quite small, the concentration in Fig. 6d being about 50 times lower than in Fig. 5. When the purity of a substance is to be determined, it is necessary to load a substantial amount to be able to quantify low amounts of impurities. If the pH of the sample is adjusted with hydrochloric acid or another suitable acid to a pH which is so acidic that the bases are charged, much better results are obtained with the acetic acid–Tris

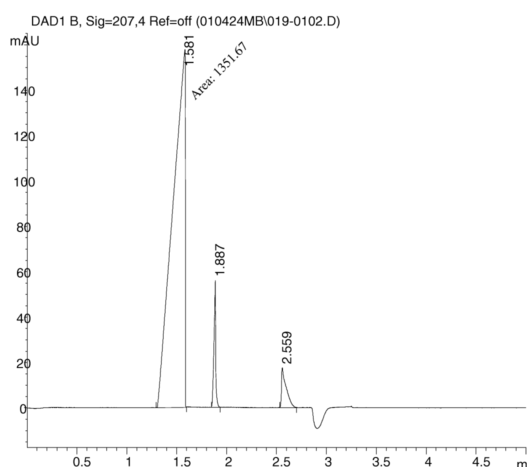

Fig. 5. Electrolyte: 20 m*M* succinic acid adjusted with 6-ACA to pH 4.50. Other conditions are given in Section 2.2. Sample: a mixture of 34 m*M* imidazole, 1 m*M* each of creatinine and 2-aminopyrimidine dissolved in water.

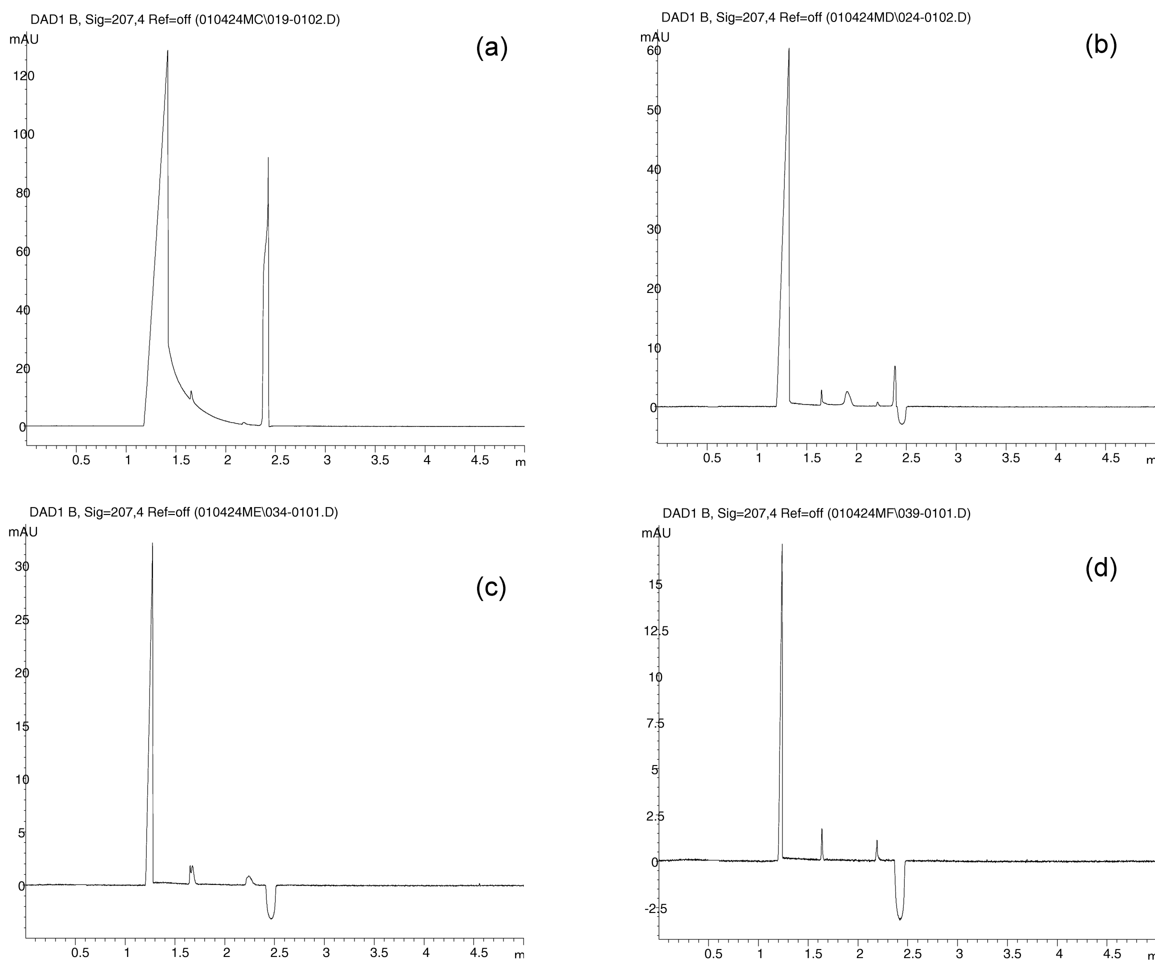

Fig. 6. Electropherograms obtained from analysis of a mixture of three bases dissolved in water at varying concentrations. Electrolyte: 20 m*M* acetic acid adjusted with tris to pH 4.49. Conditions as in Fig. 5. (a) Sample as in Fig. 5. (b) Sample containing 6.6, 0.2, 0.2 m*M* of imidazole, creatinine and 2-aminopyrimidine, respectively, in water. (c) Sample containing 2.0, 0.06, 0.05 m*M* of imidazole, creatinine and 2-aminopyrimidine, respectively, in water. (d) Sample containing 0.6, 0.02, 0.02 m*M* of imidazole, creatinine and 2-aminopyrimidine, respectively, in water.

electrolyte just mentioned (experiments were performed with imidazole and creatinine, results not shown). With the electrolyte succinic acid–6-ACA, pH adjustment of the sample is not necessary, whereas if some hydrochloric acid is added to the sample, more could be loaded. (If the conductivity of the sample is too high compared with the electrolyte, poor peak shapes can be obtained.) With an electrolyte containing 20 m*M* succinic acid and adjusted with Tris to pH 4.50, less can be loaded than with an electrolyte containing 20 m*M* 6-ACA adjusted with hydrochloric acid to the same pH. However, an electrolyte containing 20 m*M* succinic acid and 6-ACA at pH 4.50 was even better. It is an advantage that it is not necessary to acidify the sample so that all bases are charged, as the stability and pK_a of all components in a real sample are not known. A risk that should always be considered is that of a system peak if an electrolyte with low pH is used. In such a case there are (at least) two co-ions, the added one and also the hydrogen ion, and the system peak might influence the sample peak, see Refs. [19,20]. If

a buffering co-ion is used, it also follows from the reasoning above that this co-ion should be able to buffer the base with the lowest pK_a.

3.4. Experiments with tetrapentylammonium bromide and creatinine

An electrolyte with 20 m*M* acetic acid adjusted with Tris to pH 4.59 was used for analysis of creatinine and tetrapentylammonium bromide. The injection time for the sample was in this case 3 s, immediately followed by a second injection of tetrapentylammonium bromide and a third injection of electrolyte, both for 3 s (32 mbar). The sample contained 43.9 m*M* creatinine in water. The concentration of the tetrapentylammonium bromide solution was 16.9 m*M* in water. The electropherogram in Fig. 7a shows a small though normal peak which is eluted after 1.2 min, with a spectrum typical of the ionised form of creatinine and a much larger peak typical of the unionised form eluted after 1.9 min. A relatively small baseline disturbance can be seen between the two peaks. The experiment was repeated with an electrolyte containing 20 m*M* 6-ACA (buffering co-ion) adjusted with hydrochloric acid to pH 4.52. The electropherogram is shown in Fig. 7b, where all the creatinine is detected in its ionised form (shown by the spectrum). In real samples the tetrapentylammonium ion could be an unknown component in the sample with properties unfavourable for the separation, i.e., inducing peak splitting, and this example shows what might happen under certain conditions. An electrolyte with a buffering co-ion seems to give a more reliable separation system also in cases where a substance with the same effect as tetrapentylammonium is present.

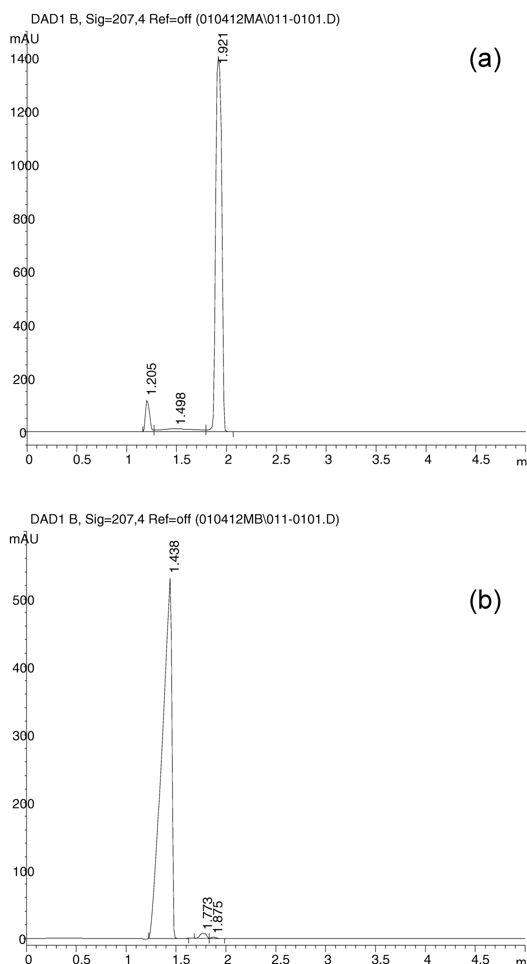

Fig. 7. Electropherograms showing different results with electrolyte containing buffering counter-ion and co-ion, respectively. (a) Electrolyte: 20 m*M* acetic acid adjusted with Tris to pH 4.59. Consecutive injections of sample (creatinine, 43.9 m*M* in water), tetrapentylammonium bromide solution (16.9 m*M* in water), electrolyte; all injections for 3 s with 32 mbar. (b) Electrolyte: 20 m*M* 6-ACA adjusted with hydrochloric acid to pH 4.52. Injections were made as in (a).

4. Conclusion

This study shows that how the electrolyte is buffered is very important to avoid peak deterioration and peak splitting that could easily be misinterpreted as two or more components, e.g., in a purity determination of a drug. An electrolyte containing a buffering co-ion or a suitable dibasic counter-ion made it possible to load at least 10–20 times more of a base dissolved in water compared with a buffering electrolyte containing a strong co-ion. Even more could be loaded if a combination of buffering co-ion and suitable dibasic buffering counter-ion was used. If an electrolyte with a strong co-ion is chosen, the pH of the sample becomes extremely important, and for bases a low pH in the sample is to be preferred so that the bases in the sample are charged. The importance of using a buffering co-ion in the electrolyte for obtaining good

peak shapes increases as the amount of unionised analytes increases and/or as sample concentration increases.

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