ELSEVIER

Journal of Chromatography A, 979 (2002) 11–25

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Sample matrix influence on the choice of background electrolyte for the analysis of bases with capillary zone electrophoresis

E.K. Margareta Andersson*, Ingela Hägglund

Product Analysis, Pharmaceutical and Analytical R&D, AstraZeneca, SE-151 85 Södertälje, Sweden

Abstract

Low levels of impurities need to be determined in drugs. Consequently, if UV detection is used, a large sample amount must be loaded and as narrow peaks as possible obtained. The sample matrix and the stability of the samples as well as the peak resolution should be considered when the electrolyte is chosen. In this study the influence of the sample matrix composition with varying background electrolytes on the peak appearance of model mixtures loaded in large amounts was investigated. A robust electrolyte for analysis of bases in a sample with varying pH was found to consist of a buffering co-ion and a buffering counter-ion (the pH was approximately 4.2 in the electrolyte). If a minor component has higher mobility than the macrocomponent and the co-ion, better peak shape can be obtained if, for instance, enough sodium chloride is added to the sample, i.e., sample self-stacking is exploited. The effect of addition of organic modifiers, isopropanol or acetonitrile, was examined and good linearity and precision have been shown for impurities in the concentration range tested, approximately 0.03 to 5 mol% of the main component, in model mixtures.

Keywords: Matrix effects; Background electrolyte composition; Peak shape; Basic drugs

1. Introduction

To be able to quantify low levels of impurities in a drug substance or formulation, down to 0.05% (w/w) or less according to the requirements set out in ICH guidelines [1,2], both the selectivity and the sensitivity must be good. In this study UV detection has been used and as the optical path length is much shorter than with LC, efforts must be made both to enable the loading of a large amount of the sample on the capillary and to have narrow peak widths for the impurities to obtain low detection limits [3].

We have focused on analysis of bases, an important group of drugs, by capillary zone electrophoresis after hydrodynamic injection of the sample. A commonly used background electrolyte (BGE) for the analysis of bases with capillary zone electrophoresis is phosphate buffer at pH about 2.5, which is recommended, among others, by Altria et al. [4]. If the selectivity is not satisfactory, the addition of an ion pair reagent, cyclodextrins or organic solvents might solve the problem [5,6]. Another possibility might be to change the ionic strength, which may be assumed to modify the selectivity if two unresolved components have different charge numbers [6,7]. The most powerful method is often to change the pH of the BGE [6,8]. A BGE at higher pH containing a weak anion as the buffering constituent can split a peak if the sample is a base dissolved in water and its concentration is high [9]. In such cases it has been

*Corresponding author. Fax: +46-8-553-29020.
E-mail address: margareta.andersson@astrazeneca.com (E.K.M. Andersson).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01252-9

reported that it is better to have a buffering co-ion in the BGE [10]. It is always important for the electrolyte to have a good buffering capacity to resist changes in pH caused by buffer depletion [11,12]. The magnitude and direction of the electroosmotic flow also influence the selectivity, as this is defined as the difference of the mobility of the two separands divided by the average mobility [7].

When electrolytes are used at low pH, there is a risk of system zones occurring (due to the hydrogen ion), which may interfere with the sample peaks and make quantitation impossible [13]. Also multivalent ions in the electrolyte, phosphate [14,15] or phthalate [15], histamine (a cation) [16], for example, can give system peaks under some conditions. The mobility of a system peak is determined by the composition of the BGE, although it is not easy to predict [17]. Gaš et al. developed a computational model which can be used for studying the features of different electrolytes and for optimization also at very high or low pH [14]. If a component in the electrolyte has UV absorption, it is possible to detect system zones by injecting distilled water [13].

At least during the latter part of the electrophoretic process band broadening of the zones will take place. Dispersive factors are described, for example, in Ref. [18]. The extent of peak broadening depends on, for instance, diffusion, although for triangular peaks the most important factor is the so-called electromigration dispersion caused by poor mobility matching. This dispersion is caused by a change in the migration velocity for the analyte with concentration [19] and depends on both conductivity and a pH effect and is proportional to the square root of the migration time [20]. The fronting and tailing characteristics of weak bases when the BGE contains a buffering co-ion, a buffering counter-ion, or both a buffering co-ion and a counter-ion are discussed in Ref. [21]. Peak shape diagram is explained in Ref. [19], where it is also stated that there is no simple relationship between effective or ionic mobility for a weak analyte and the co-ion that could be used to predict whether a peak is fronting or tailing. The effect of the choice of co-ion and counter-ion on the dispersion of analyzed anions and the concept of a modified peak shape diagram (MPSD) is discussed in Ref. [22].

Organic modifiers can be added to the electrolyte not only to obtain better resolution as mentioned above but also to increase the solubility of the substance [6] so that a higher concentration can be used in the sample. The solvents recommended when UV absorption is considered are lower alcohols and acetonitrile [23]. Organic modifiers can influence the mobility for different analytes to a varying extent [23]. An organic modifier can also affect the electroosmotic flow [24] as well as the pK_a, the pK_a values for acids being changed more than for bases [24].

To obtain narrow peaks, it is important for the starting zone of the analyte to be as narrow as possible when the migration in the BGE begins, i.e., when the analyte leaves the sample zone. A common recommendation is to dissolve the sample in water [11,25] or diluted BGE [8,11,26], which gives a higher field strength in the sample zone than in the electrolyte and therefore a stacking effect (provided that the conductivity of the sample is not too high), i.e., the conditions are chosen so that the mobility in the sample zone is higher than in the BGE [12]. Alternatively, the components (bases) could be uncharged in the applied sample zone but become charged when the co-ion of the electrolyte migrates into the zone and so pushes the analytes into narrow zones [10].

Actual sample solutions in the pharmaceutical industry are often pH-adjusted and could also contain sodium chloride (in samples from toxicological studies, for instance). The pH, buffer in the sample and the ionic strength have an effect on plate number, resolution and migration time [27]. A high ionic strength in the sample often gives poor peak shapes [11,28], and to avoid unnecessary peak broadening, the injected sample plug is kept as short as possible [11]. If the running conditions are appropriate, stacking can also be obtained in cases with high conductivity in the sample by sample self-stacking (transient isotachophoresis) [26,28–31]. This may also occur unintentionally if there is a high concentration of sodium ions present in the sample (analysis of bases). The transient isotachophoretic process occurs simultaneously with the electrophoretic one in this case [26]. The effects of the number, mobilities and concentrations of the macrocomponents in the sample on the stacking/destacking of other components are described in Refs. [26,31]. Schwer et al. described a two-buffer stacking system in which the capillary is first filled with a terminating electrolyte with a co-ion with a very low mobility

then a zone containing leading ion with high mobility and finally the sample are applied [32]. If stacking is performed with transient isotachophoresis caused by the separation medium isotachophoresis will precede the electrophoretic process [31]. A more efficient option is the use of column coupling [31].

The so-called acid stacking is described in Ref. [33,34]. Stacking when the sample contains both acetonitrile and a high salt concentration is described in Ref. [27]. Refs. [3,30,35,36] are review articles on stacking in CZE.

Another benefit of sample stacking is the better precision for peak area because of a smaller integration error [37]. To enable correct quantitation, the sample diluents should be the same for sample and standard as they have a large effect on the results [11]. The transient isotachophoresis or isotachophoretic step will often affect the migration times of the components as the mobility of the zone during the isotachophoretic step can deviate substantially from the electrophoretic mobility [29,38]. When the corrected area is calculated, the peak area is divided by the migration time, but this does not reflect the real velocity with which the zone passes the detector in all cases [29], which may cause a quantitation error.

The aim of this study was to further investigate electrolytes that were found to be suitable for analysis of impurities in basic drug substances or formulations (where heavy loading is necessary) and to examine the influence of varying sample matrices on peak shapes. Often samples have to be pH-adjusted due to solubility or stability problems at high pH and it was found that the most robust electrolyte at lower pH contained both a buffering co-ion and a buffering counter-ion. The effect of the mobility of the macrocomponent on the performance of the other components in the sample has been investigated. Isopropanol and acetonitrile have been tested as organic modifiers in the electrolyte to increase the solubility of the drug, as this might sometimes be crucial.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Imidazole, hydrochloric acid (5 *M*, Titrisol), sodium chloride and potassium chloride (Suprapur), dipotassium phosphate trihydrate and succinic acid were supplied by Merck, acetic acid and phosphoric acid (Normapur) by Prolabo, 4-aminobutyric acid (4-ABA), 2-aminopyrimidine, benzimidazole, caproic acid, creatinine, formic acid, 2-morpholinoethanesulfonic acid (MES), propionic acid and isopropanol (IPA) for residue analysis by Fluka, potassium sulfate by Aldrich, potassium acetate (Analar grade) by BDH, 6-aminocaproic acid (6-ACA) by Roth and L-histidine by Sigma, β-alanine (β-Ala) by Serva, and acetonitrile (ACN), HPLC grade by Baker. 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, 75 μm I.D. or poly(vinyl alcohol) (PVA)-coated capillary, 75 μm I.D. from Agilent (G1600-67319), effective lengths approximately 26.5 cm and total length 35 cm. The analyses were run in constant voltage mode with normal polarity at 15 kV (20 kV with organic modifier) after an initial ramp time of 0.2 min. The injection was carried out by pressure, 32 mbar for 4 or 7 s unless otherwise stated, immediately followed by injection of electrolyte for 5 s at the same pressure. The detector was used at 207 nm with a bandwidth of 4 nm.

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

2.3. Procedures

A fused-silica capillary (new and on exchange of the electrolyte) was 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. The PVA-coated capillary was only rinsed with water and the electrolyte. In between the runs rinsing with electrolyte for 2.5 min was performed. The capillaries were

Table 1
pK_a of substances used in this study

Substance	pK_a (approx. values) [39]
Acetic acid	4.756
β-Alanine (β-Ala)	3.55
4-Aminobutyric acid (4-ABA)	4.03, 10.556
6-Aminocaproic acid (6-ACA)	4.37, 10.80
2-Aminopyrimidine	3.45
Benzimidazole	5.53
Caproic acid	4.85
Creatinine	4.8, 9.2
Formic acid	3.75
L-Histidine	1.80, 6.04, 9.33
Imidazole	6.99
2-Morpholinoethanesulfonic acid (MES)	Approx. 6.1 [40]
Propionic acid	4.87
Succinic acid	4.21, 5.64

stored dry after rinsing with water for 5 min followed by air for 5 min.

3. Results and discussion

3.1. Comparison of BGE with different buffering co-ions

In this study the concept bad peak shape is used for a peak that shows splitting or an appearance indicating two unresolved peaks. (A triangular peak is not considered abnormal.) Table 1 shows pK_a values for substances used in this study. An electrolyte containing 35 m*M* 4-ABA pH-adjusted with hydrochloric acid to 4.20 was compared to an electrolyte with 35 m*M* 6-ACA pH-adjusted in the same way to 4.20. The pK_a of 4-ABA is 4.03 and of 6-ACA 4.37, which means that the buffering capacity is approximately the same for both electrolytes, although the concentration of BH^+ is about 1.5 times higher in the 6-ACA electrolyte. The electrolytes were used for analysis of a model mixture containing imidazole, benzimidazole, and creatinine with varying sample matrices (Table 2 and 3). Imidazole was assumed to be the main component and the others to be impurities. The tables show that the presence of KCl also at a concentration that gave a higher conductivity in the sample than in the BGE did not disturb the analysis. On the contrary, the peak shape was better and the peak narrower, the more KCl was added up to at least 45 m*M* (results not shown) due to a stacking effect caused by the potassium ion and the weak co-ion migrating into the sample zone, pushing the analytes together into narrow zones [10].

Tables 2–4 also show that if the sample was pH-adjusted with acid (see examples with acetic acid in Tables 2 and 3 and propionic acid in Table 4), the amount added is important, i.e., the lower the pH, the more difficult it is to obtain normal peaks (benzimidazole, creatinine).

Table 3 shows that the creatinine peak is better if caproic acid is used instead of propionic acid in the sample, although the two acids have approximately the same pK_a and the pH of the samples were

Table 2
Influence of the sample concentration (KCl, acid, pH) on peak performance. Electrolyte: 35 m*M* 4-ABA pH-adjusted with hydrochloric acid to 4.20; silica capillary, injection time 7 s

Matrix	Sample pH	Sample conductivity/ BGE conductivity approx.	Peak appearance in mixtures with:		
			Imidazole (37 m*M*)	Benzimidazole (0.8 m*M*)	Creatinine (0.8 m*M*)
KCl (45 m*M*)	ca. 9.3	4.4	N	N	N
Propionic acid (60 m*M*)	5.06	2.1	N	Bad	Bad
Caproic acid (60 m*M*)	5.08	1.8	N	Bad	Bad
Acetic acid (30 m*M*)	6.51	2.1	N	N	N
Acetic acid (45 m*M*)	5.45	2.2	N	Bad	Bad
Acetic acid (60 m*M*)	4.95	2.3	N	Bad	Bad
Formic acid (30 m*M*)	6.50	2.0	N	N	N
MES (60 m*M*)	6.22	1.5	N	N	N

N, normal peak; Bad, abnormal peak.

Table 3
Influence of the sample composition (KCl, acid, pH) on peak performance. Electrolyte: 35 m*M* 6-ACA pH-adjusted with hydrochloric acid to 4.20; silica capillary, injection time 7 s

Matrix	Sample pH	Sample conductivity/ BGE conductivity approx.	Peak appearance in mixtures with:		
			Imidazole (37 m*M*)	Benzimidazole (0.8 m*M*)	Creatinine (0.8 m*M*)
KCl (45 m*M*)	ca. 9.3	3.0	N	N	N
Propionic acid (60 m*M*)	5.06	1.4	N	N	Bad
Caproic acid (60 m*M*)	5.08	1.2	N	N	N
Acetic acid (30 m*M*)	6.51	1.4	N	N	N
Acetic acid (45 m*M*)	5.45	1.5	N	N	Bad
Acetic acid (60 m*M*)	4.95	1.5	N	Bad	Bad
Formic acid (30 m*M*)	6.50	1.3	N	N	N
MES (60 m*M*)	6.22	1.0	N	N	N

N, normal peak; Bad, abnormal peak.

comparable. These results indicate that the mobility of the acid used for pH adjustment can be important and that better results could be obtained if the acid has lower mobility. Of the two electrolytes described in Tables 2 and 3, the electrolyte with 6-ACA is the better one. This might be due to the higher concentration of BH^+ as overloading effects as a rule are more easily obtained with lower concentrations in the electrolyte.

Table 4 shows that KCl is also tolerated in an electrolyte containing β-Ala pH-adjusted with hydrochloric acid, as was expected. The concentration of β-Ala^+ in this electrolyte is approximately the same as for 6-ACA^+ in the electrolyte described in Table 3. Nevertheless, bad peaks were obtained in more cases with the β-Ala than with the 6-ACA electrolyte, so it is reasonable to believe that the concentration of BH^+ is not the only important factor. If the pH of the sample is comparatively low, the analytes might begin to migrate out of the sample

Table 4
Influence of the sample composition (KCl, acid, pH) on peak performance. Electrolyte: 111 m*M* β-ALA pH-adjusted with hydrochloric acid to 4.22; silica capillary, injection time 7 s

	Matrix	Sample pH	Sample conductivity/ BGE conductivity approx.	Peak appearance in mixtures with:		
				Imidazole (37 m*M*)	Benzimidazole (0.8 m*M*)	Creatinine (0.8 m*M*)
a	KCl (45 m*M*)	ca. 9.3	3.0	N	N	N
b	Propionic acid (30 m*M*)	6.54	1.0	N	N	N
c	Propionic acid (60 m*M*)	5.06	1.4	N	Bad*	Bad
d	Caproic acid (60 m*M*)	5.08	1.2	N	N	N
e	Acetic acid (30 m*M*)	6.51	1.4	N	Bad	Bad?
	Acetic acid (45 m*M*)	5.45	1.5	N	Bad	Bad
	Acetic acid (60 m*M*)	4.95	1.5	N	Bad	Bad
	Formic acid (30 m*M*)	6.50	1.3	N	Bad	Bad
	MES (60 m*M*)	6.22	1.0	N	N	N
f	Phosphoric acid (30 m*M*)	6.03	1.2	N	Bad	Bad
	Sulfuric acid (15 m*M*)	6.61	1.4	N	Bad	Bad
	Sulfuric acid (22.5 m*M*)	2.78	2.1	Bad	Bad	Bad
	Potassium sulfate (31 m*M*)	–	3.4	N	N	N
	Potassium acetate (42 m*M*)	–	2.0	N	N	N
	Dipotassium phosphate (31 m*M*)	–	3.0	N	N	N

N, normal peak; Bad, abnormal peak. The electropherograms corresponding to a–f are shown in Fig. 1a–f.

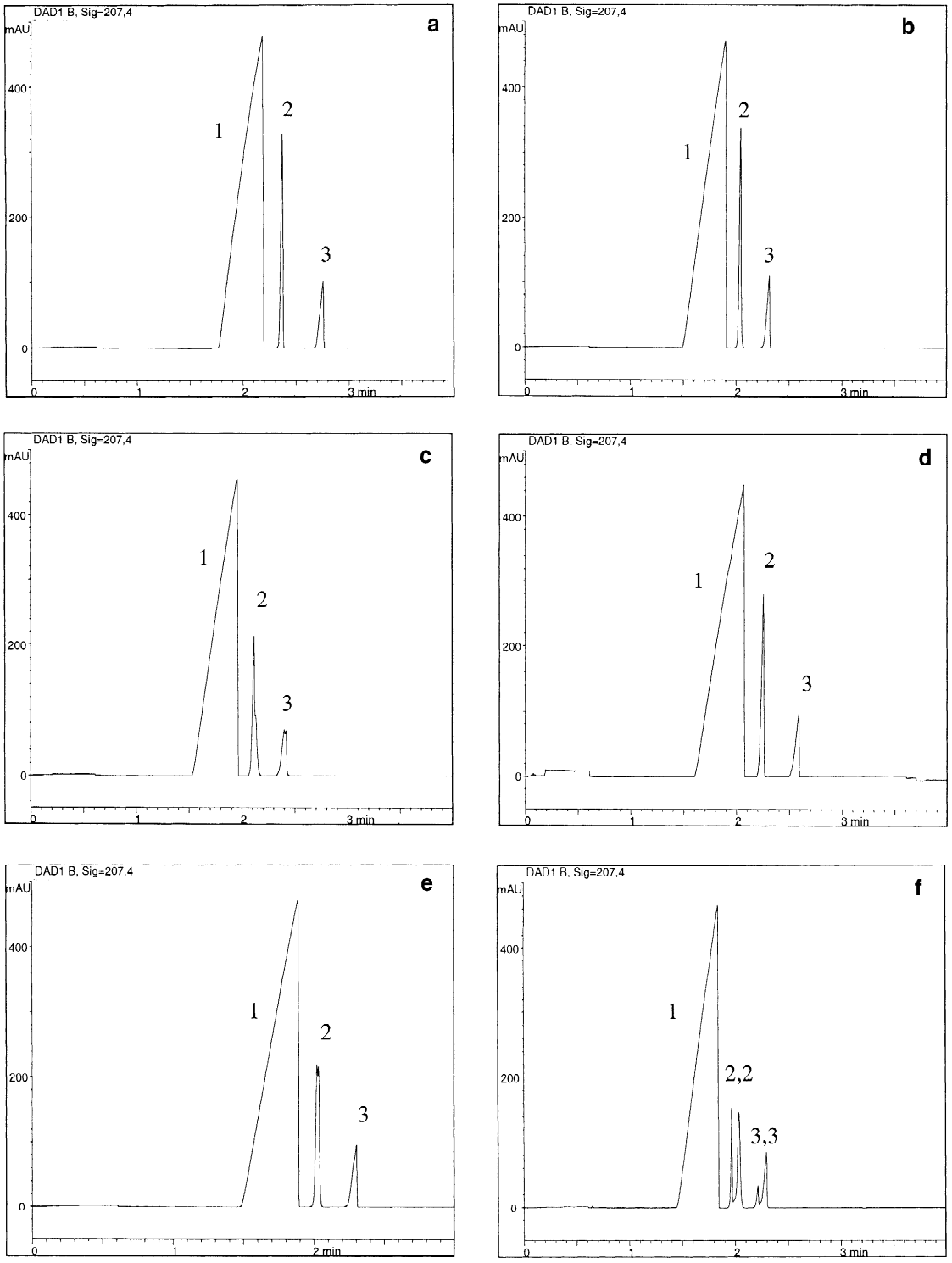

Fig. 1. Examples of normal and abnormal peaks obtained with varying sample matrices. The electrolyte and samples are described in Table 4. Migration order: imidazole (peak 1), benzimidazole (peak 2), creatinine (peak 3). Capillary: bare fused-silica.

Table 5
Influence of type of buffering ion on peak performance of bases with varying composition in sample

	Buffering co-ion	Buffering counter-ion	Buffering co-ion and counter-ion
Sample pH high	OK	Not OK	OK
Sample pH high, considerable amount of KCl present	OK	Not OK	OK
Low pH	Not OK	OK	OK

OK, normal peak shape; Not OK, bad (abnormal) peak shape.

zone immediately the voltage is applied and not after being ''pushed'' from the rear side which is the case if the pH of the sample is high, i.e., if the analytes are uncharged initially [10]. The rear portion of the split peak obtained at low pH could be due to the weak co-ion migrating into the sample zone from the rear side giving a pushing effect on the analytes (for example creatinine) as the equilibrium towards BH^+ of the analyte is favoured. The mobility of β-Ala^+ in the BGE and at the pH of the sample is lower than that of 6-ACA^+ and a much larger portion of the analytes become charged when β-Ala migrates through the sample zone due to the lower pK_a for β-Ala compared to 6-ACA. These two factors will probably contribute to the splitting observed.

When the sample had been pH-adjusted with caproic acid (denoted d in Table 4), the peaks for benzimidazole and creatinine were better than if propionic acid (denoted c in Table 4) had been used. Propionic acid (denoted b in Table 4) at pH 6.54 was better than acetic acid at pH 6.51 (denoted e in Table 4). These results confirm those of Table 3 that it is better if the acid in the sample has a lower mobility. When MES was used for pH adjustment of the sample to 6.22, the results were better than if formic or acetic acid was used at pH 6.50, although the tendency is for the higher pH to be better when electrolytes with a buffering co-ion is used. The conclusion is, therefore, that not only the pH but also the choice of acid is important. A low mobility of the acid used for pH-adjustment could infer that the sample zone is better buffered as the acid does not migrate through the sample zone so rapidly, hence, giving a better result.

The conductivity in the sample zone is high compared with the BGE and therefore stacking due to high field strength does not prevail. The stacking that could occur is transient isotachophoresis and this process should be performed utilizing a buffering counter-ion. In the electrolytes just mentioned, however, no buffering counter-ion is present that can supersede the acid added to the sample when it

Table 6
Influence of the sample composition (KCl, acid, pH) on peak performance. Electrolyte: 60 m*M* succinic acid pH-adjusted with Tris to 4.23; silica capillary, injection time 7 s

Matrix	Sample pH	Sample conductivity/ BGE conductivity	Peak appearance in mixtures with:		
			Imidazole (37 m*M*)	Benzimidazole (0.8 m*M*)	Creatinine (0.8 m*M*)
KCl (45 m*M*)	ca. 9.3	2.6	N	Bad	Bad
Propionic acid (60 m*M*)	5.06	1.2	N	N	Bad
Caproic acid (60 m*M*)	5.08	1.1	N	N	N?
Acetic acid (30 m*M*)	6.51	1.3	N	Bad	Bad
Acetic acid (45 m*M*)	5.45	1.3	N	N	Bad
Acetic acid (60 m*M*)	4.95	1.3	N	N	N
Phosphoric acid (30 m*M*)	6.03	1.0	N	N	Bad
Phosphoric acid (45 m*M*)	2.94	1.4	N	N	N
Phosphoric acid (60 m*M*)	2.45	1.8	N	N	N
Sulfuric acid (15 m*M*)	6.61	1.2	N	Bad	Bad
Sulfuric acid (22.5 m*M*)	2.78	1.9	N	N	N

N, normal peak; Bad, abnormal peak.

migrates towards the rear end and therefore bad peak shapes were obtained. To choose an acid with low mobility was therefore the best choice in this case to obtain as good peak shape as possible.

If the pH in the sample is high, other salts than KCl can also be used, e.g., potassium sulfate, potassium acetate, or dipotassium hydrogenphosphate. Therefore, it would be possible to adjust the pH of a sample with low pH containing, for instance, acetic acid with potassium hydroxide before the analysis if the base has a solubility and stability that are high enough to get good peak shapes. Fig. 1 shows electropherograms for some of the mixtures mentioned above. Fig. 1c,e,f illustrate split peaks, while in Fig. 1f the split peaks are almost baseline-separated. Table 5 summarizes the result. It should be remembered that the sample zone-length is important as systems can always become overloaded and the purpose was to elucidate if some experimental conditions could tolerate a heavy loading better than others.

3.2. BGE with a buffering dibasic acid

Succinic acid has two pK_a values, 4.21 and 5.64, and can be used as a buffering counter-ion in an electrolyte pH-adjusted to 4.23 with Tris. Succinic acid was used in Ref. [10] for analysis of bases dissolved in water. Table 6 shows that such an electrolyte does not work if a high concentration of KCl is present in the sample, in contrast to the results with buffering co-ions presented in Tables 2–4. With succinic acid the results became better and better with decreasing pH–the very opposite of the results obtained with BGE containing a buffering co-ion. The ionized form of succinic acid penetrates into the sample zone from the detector side and its mobility in the sample zone will be low if the concentration of KCl is high (high conductivity in the sample plug). It will therefore take a long time before all the analyte molecules are ionized and can move out of the sample plug. The bad peaks obtained with this electrolyte had a steep front side followed by a typically rather flat part and, finally, a tailing end. If, however, the pH in the sample plug is low enough to start with, the process is facilitated. See also Table 5.

3.3. BGE in which both the co-ion and counter-ion are buffering

A BGE containing 140 m*M* β-Ala was pH-adjusted with acetic acid to 4.22. (This electrolyte has a conductivity comparable to the 35 m*M* 6-ACA electrolyte mentioned in Section 3.1.) All the samples specified in Table 6 were analyzed and gave peaks that were normal. So if samples with a lot of different matrices are to be analyzed, this type of electrolyte seems to be a good choice. A disadvantage, however, as Table 7 shows, was that this electrolyte gave a lower plate number than an electrolyte with the same concentration of β-Ala but

Table 7
Influence of concentration of KCl in sample on plate number for analytes with varying counter-ions in the electrolytes. Electrolyte: 140 m*M* β-ALA pH-adjusted with HCl and acetic acid, respectively, to 4.22; silica capillary, injection time 7 s

Electrolyte	Sample	Benzimidazole		Creatinine	
		Migration time (min)	Plate number	Migration time (min)	Plate number
β-ALA+HCl	In water	2.25	64700	2.53	32700
140 m*M*	11.3 m*M* KCl	2.37	66000	2.71	31500
	22.6 m*M* KCl	2.32	63000	2.66	29900
	22.5 m*M* HCl (pH 6.72)	2.28	61100	2.62	29700
β-ALA+HAc	In water	2.67	23600	2.96	19400
140 m*M*	11.3 m*M* KCl	2.51	26000	2.76	22100
	22.6 m*M* KCl	2.52	27300	2.76	22100
	22.5 m*M* HCl (pH 6.72)	2.48	24800	2.74	21000

The concentrations in the samples are 37 m*M* imidazole, 0.8 m*M* benzimidazole, and 0.8 m*M* creatinine.

pH-adjusted with HCl to the same pH. When β-Ala is pH-adjusted with HCl and the sample pH is high, the analytes will be pushed into narrow zones when they become charged [10]. The potassium ion will have a stacking effect on benzimidazole (the first eluted analyte), so benzimidazole will migrate in the stack for a relatively longer time than creatinine. If the electrolyte is pH-adjusted with acetic acid, acetate/acetic acid will move into the sample plug and facilitate the migration of analyte out of the sample plug before the zone sharpening from the rear side is completed, which causes lower plate numbers. See also Table 5.

3.3.1. Electrolytes with a buffering co-ion and counter-ion used for analysis of samples with varying mobility of the macrocomponent

With a pH in the sample zone that is relatively low, the analytes are partly charged and can start migrating out of the sample plug as soon as the voltage is applied. If then only the co-ions of the electrolyte are buffering, they cannot migrate fast enough through the sample plug (if it is too long) to maintain charging of the analytes and therefore the peak might split (Tables 2–4). However, if the electrolyte contains acetate/acetic acid, the acid will migrate into the sample plug from the detector side and the analytes will become charged. The disadvantage of the fact that the process occurs at the same time at both boundaries of the sample plug is that the plate number will be lower and the plug must be shorter than if the pH of the sample is high and only the co-ion of the electrolyte is buffering. But, if there is a need to have a moderate pH of the sample (for stability or solubility reasons), it is desirable to have both co-ion and counter-ion buffering since risk of split peaks is then lower.

In the experiments below a smaller sample plug was injected than in the above-mentioned experiments. Two electrolytes with the same magnitude of BH^+ in the electrolyte were studied more thoroughly for analysis of samples containing phosphoric, acetic, formic, or hydrochloric acid. One of the electrolytes containing 140 m*M* β-Ala pH-adjusted with acetic acid to 4.23 was used for analysis of samples in Table 6. From the results it can be concluded that if the macrocomponent (imidazole) has the highest mobility of the components, all the peak shapes are normal. When creatinine (pK_a about 4.8) or histidine (pK_a about 6.0) was the macrocomponent, the peaks of components with higher mobilities (shorter migration time), i.e., imidazole and benzimidazole, gave bad peak shapes (the co-ion of the BGE has low mobility). The reason for this result may be that when imidazole is the component with the highest concentration, it causes transient isotachophoresis. If the macrocomponent has a sufficiently high concentration and migrates more slowly than some minor component, the latter will have bad peak shapes as there will then be no transient isotachophoresis and the conductivity in the sample plug will be high. The 2-aminopyrimidine peak with the longest migration time performed well in all cases. If the injected volume was lowered (from 4 to 1 s at 32 mbar), normal peak shapes were obtained under all conditions, although the aim of being able to load a substantial amount to obtain a low detection limit for impurities will then have been given up.

The other electrolyte containing 45 m*M* 6-ACA pH-adjusted to 4.24 with acetic acid confirmed the results in Table 8. This electrolyte was also used to determine the effect of varying the concentration of sodium chloride (a component often present in pharmaceutical preparations) and acid used for pH adjustment of the sample on the corrected area% (with correction for ramp time) obtained for benzimidazole and 2-aminopyrimidine (pK_a 5.53 and 3.45, respectively) when the macrocomponent in the sample was creatinine. Table 9 shows that the area% of 2-aminopyrimidine calculated on creatinine (the major component) is independent of the concentration of sodium chloride at the concentrations used, while benzimidazole (shortest migration time) gave about a 3% lower area% when the concentration of sodium chloride increased from 3.2 to 6.4 mg/ml. The choice or the amount of acid in the sample did not affect the result under the experimental conditions used. The benzimidazole peak was most narrow with the highest concentration of salt due to a longer transient isotachophoretic process.

The 2-aminopyrimidine peak became broader when sodium chloride was added, although the migration time only changed by approximately 1%. The plate number was about 1.5 times higher with the lower than with the higher concentration of sodium chloride. This is presumably due to lower mobility of 2-aminopyrimidine than of 6-ACA and is the reason why the 2-aminopyrimidine peak is

Table 8
Influence of the properties of the main component on peak performance. Electrolyte: 140 m*M* β-ALA pH-adjusted with acetic acid to 4.23; PVA capillary, injection time 4 s

Macro-component	Acid for pH adjustment	Acid total (m*M*)	pH	Comments
Imidazole[a]	Phosphoric	45	2.75	All peaks normal
	Acetic	90	4.41	
		150	4.12	
	Formic	60	3.75	
		90	3.42	
	Hydrochloric	36	2.93	
		60	ca. 1.8	
Creatinine[b]	Phosphoric	45	2.67	Creatinine and 2-aminopyrimidine:
	Acetic	90	4.20	normal peaks. Imidazole,
		150	4.01	benzimidazole: bad peaks
	Formic	60	3.66	
		90	3.38	
	Hydrochloric	36	2.80	
		60	1.85	
Histidine[c]	Phosphoric	45	2.99	Histidine and 2-aminopyrimidine:
	Acetic	90	4.46	normal peaks. Imidazole,
		150	4.15	benzimidazole: bad peaks
	Formic	60	3.78	
		90	3.45	
	Hydrochloric	36	3.38	
		60	2.07	

[a] Concentration in sample, components in migration order: 33 m*M* imidazole, 0.5 m*M* of each of benzimidazole, creatinine, and 2-aminopyrimidine.
[b] Concentration in sample, components in migration order: 0.6 m*M* imidazole, 0.5 m*M* benzimidazole, 33 m*M* creatinine, and 0.5 m*M* 2-aminopyrimidine.
[c] Concentration in sample, components in migration order: 0.6 m*M* imidazole, 0.5 m*M* benzimidazole, 33 m*M* histidine, and 0.5 m*M* 2-aminopyrimidine.

Table 9
Influence of the sample composition (acids, pH and NaCl) on obtained area%. Electrolyte: 45 m*M* 6-ACA pH-adjusted with acetic acid to 4.24; PVA capillary, injection time 4 s

Acid for pH adjustment	pH	NaCl (mg/ml)	Benzimidazole (area%)	2-Aminopyrimidine (area%)
Phosphoric	2.45	6.4	3.99	1.40
Acetic	4.09	6.4	3.95	1.38
	3.87	6.4	3.95	1.40
Formic	3.54	6.4	3.97	1.40
	3.13	6.4	3.96	1.41
Phosphoric	2.45	3.2	4.10	1.39
Acetic	4.23	3.2	4.09	1.39
	3.92	3.2	4.10	1.39
Formic	3.56	3.2	4.10	1.39
	3.18	3.2	4.10	1.40
Acetic	4.03	0	4.23	1.38

The samples contained 0.5 m*M* benzimidazole, 33 m*M* creatinine, and 0.6 m*M* 2-aminopyrimidine. Area% calculated as corrected area% on the creatinine peak (ramp time has been considered).

adversely affected by the high concentration of sodium ions in the sample [31]. Such an effect on the 2-aminopyrimidine peak was not observed with the electrolyte containing β-Ala mentioned in the previous paragraph (results not shown), and the β-Ala electrolyte was therefore chosen for further experiments. Furthermore, the peak of 2-aminopyrimidine was more symmetric (and narrower) with the β-Ala electrolyte.

3.3.2. Electrolyte with buffering co-ion and counter-ion used for analysis of samples containing a macrocomponent with lower mobility than one of the minor components: linearity, precision, and detection limit

As the most difficult case was found to be when a minor component has higher mobility than the macrocomponent (and the co-ion), this case has been further studied. The concentration of sodium chloride (sodium ion used as a stacker) was shown to be important, although the choice of acid was not found to be crucial (see above), hence acetic acid was chosen in the experiments below.

The electrolyte contained 142 m*M* β-Ala pH-adjusted with acetic acid to 4.25. Linearity was shown with samples containing a constant amount of creatinine (33 m*M*) and varying amounts of benzimidazole (eluted before creatinine) and 2-aminopyrimidine (eluted after creatinine). The total concentration of acetic acid was approximately 0.14 *M* and that of sodium chloride 77 m*M*, with pH 3.9 in all samples. The calibration line for benzimidazole gave $r^2=0.9996$, $n=7$ in the range 0.11–5.4 mol% (calculated on creatinine). The corresponding figure for 2-aminopyrimidine was 1.0000 with $n=7$ in the same range. Precisions on injection of a solution containing 0.11 mol% of each of benzimidazole and 2-aminopyrimidine were 1.1 and 3.0%, respectively, $n=5$. The detection limit was about 0.01 area% estimated from an impurity peak that had a signal-to-noise (peak-to-peak) ratio of 3 (corresponding to a concentration of approximately 3 μ*M*). The last mentioned electrolyte was also used to study the effect on quantitation of varying concentrations of acetic acid and sodium chloride in the samples. Five samples containing 33 m*M* creatinine, 0.3 m*M* benzimidazole, 1.5 m*M* 2-aminopyrimidine, and acetic acid diluted between 120 and 1170 times and two concentrations of sodium chloride, 3.5 or 4.4 mg/ml, were analyzed in duplicate. The area% values for benzimidazole and 2-aminopyrimidine were determined as area% of creatinine and the RSDs were 0.5 and 0.4%, respectively, for all samples pooled together ($n=10$). These results show that at least a variation of 20% in the concentration of sodium chloride was acceptable in this case.

3.4. Organic modifier added to an electrolyte with buffering co-ion and counter-ion used for analysis of samples containing a macrocomponent with lower mobility than one of the minor components

Table 10 shows results with an electrolyte containing β-Ala pH-adjusted with acetic acid to 4.25 and 9% acetonitrile when the sample matrix was varied: the acetonitrile concentration was between 0 and 19% and the sodium chloride concentration was 2.8 or 4.7 mg/ml. The samples also contained acetic acid diluted between122 and 1176 times. The RSDs for benzimidazole and 2-aminopyrimidine were 1.2 and 2.0%, respectively, for all results pooled together.

The precision was also determined by injecting two solutions containing 33 m*M* creatinine, 0.3 m*M* benzimidazole, and 1.5 m*M* 2-aminopyrimidine, of which one with 4.4 mg/ml of sodium chloride and acetic acid diluted 122 times was injected four times and the other with 3.5 mg/ml of sodium chloride and acetic acid diluted 184 times was injected 10 times (i.e., less variation in the matrix). The RSDs were 0.6 and 0.5%, respectively, for benzimidazole and 2-aminopyrimidine ($n=14$, no acetonitrile in the samples). These results show that even rather large variations in the sample matrices had no adverse effect on the results in this case. The effect of addition of sodium chloride on the benzimidazole peak when creatinine is the main component (transient isotachophoresis) can be seen if Fig. 2a and b are compared. No adverse effect on the 2-aminopyrimidine peak with migration time 11 min is observed.

3.4.1. Linearity for minor components with higher and lower mobility, respectively, than the macrocomponent analyzed with electrolytes containing various concentrations of organic modifiers

Table 11 shows the results with electrolytes

Table 10
Influence of varying concentration of ACN, NaCl and acetic acid in sample composition on obtained area%. Electrolyte: 129 m*M* β-ALA pH-adjusted with acetic acid to 4.25, 9% acetonitrile. PVA capillary, injection time 4 s

ACN in sample (%)	NaCl (mg/ml)	Acetic acid diluted (×)	pH	Benzimidazole (area%)	2-Aminopyrimidine (area%)
0	4.7	122	3.95	2.27	2.21
	2.8	184	4.19	2.34	2.20
	2.8	368	4.49	2.33	2.19
	4.7	588	4.78	2.27	2.23
	4.7	1176	5.13	2.26	2.20
9.6	4.7	122	NM	2.27	2.14
	2.8	184	NM	2.30	2.11
	2.8	368	NM	2.30	2.11
	4.7	588	NM	2.26	2.12
	4.7	1176	NM	2.28	2.15
19.2	4.7	122	NM	2.25	2.13
	2.8	184	NM	2.29	2.11
	2.8	368	NM	2.31	2.11
	4.7	588	NM	2.26	2.13
	4.7	1176	NM	2.26	2.13
			RSD (%)	1.2	2.0

The samples contained 0.3 m*M* benzimidazole, 33 m*M* creatinine, and 1.0 m*M* 2-aminopyrimidine, other constituents given above. Area% calculated as corrected area% on the creatinine peak area. NM, not measured.

containing β-Ala pH-adjusted with acetic acid to 4.25 containing organic modifiers. The linearity for benzimidazole is good in the concentration range 0.03 mol% (corresponds to 0.08 area%) to 4.1 mol% with acetonitrile in the electrolyte (main component creatinine, sodium chloride present in the sample). The combination of isopropanol in the electrolyte and a PVA-coated capillary seemed to be less good. In that case 2-aminopyrimidine also gave a significant intercept in repeated experiments, although no

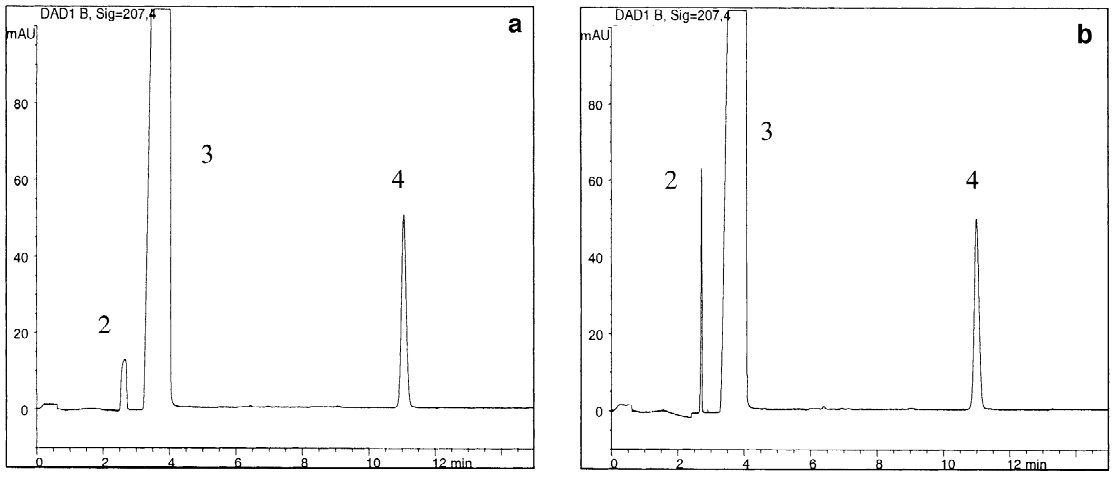

Fig. 2. The figure illustrates the effect of sodium chloride in the sample on the peak shape of an early-eluted minor component. The electrolyte was a mixture of a buffer containing 142 m*M* β-ala pH-adjusted with acetic acid to 4.25 and acetonitrile in the proportions 50:5 (v:v). Capillary: PVA-coated. (a) The sample contained 0.3 m*M* benzimidazole (peak 2), 33 m*M* creatinine (peak 3), 1.0 m*M* 2-aminopyrimidine (peak 4), acetic acid diluted 122 times, and 16% acetonitrile. (b) The sample as in Fig. 2a but with 4.7 mg/ml of NaCl added.

Table 11
Linearity for benzimidazole and 2-aminopyrimidine with varying composition of sample analyzed with PVA and silica capillaries. Electrolytes containing β-ALA pH-adjusted with acetic acid to 4.25 and organic modifier. Injection time 4 s

Capillary	Final conc. β-ALA (m*M*)	Organic modifier	Benzimidazole[a] r^2	2-Aminopyrimidine[a] r^2	*n*
PVA	129	ACN, 9%	1.0000[b]	0.9998[b]	18
PVA	122	ACN, 20%	0.9999[c]	0.9999[c]	11
PVA	131	IPA, 14%	0.9934[d]	0.9866[d]	9
Silica	131	IPA, 14%	1.0000[d]	0.9999[d]	6
Silica	122	ACN, 20%	0.9999[c]	1.0000[c]	5

[a] The samples contained 0.03–4 mol% benzimidazole, 33 m*M* creatinine, and 0.04–6 mol% 2-aminopyrimidine calculated on the creatinine concentration.
[b] 10% ACN, 4.7 mg/ml of sodium chloride and acetic acid diluted 122–735 times in the samples.
[c] 20 or 30% ACN, 4.7 mg/ml of sodium chloride and acetic acid diluted 122–735 times in the samples.
[d] 15% IPA, 4.7 mg/ml of sodium chloride and acetic acid diluted 184 times in the samples.

peak could be detected when 2-aminopyrimidine was not present in the sample. The same solutions and electrolyte were then tested on a fused-silica capillary on the same instrument on the same day. The results were good and the 95% confidence interval included the origin. The baseline was also better with the silica capillary than with the PVA-coated capillary.

Figs. 3 and 4 show that the resolution between the 2-aminopyrimidine peak and the disturbance due to the electroosmotic flow is lower with ACN than with IPA. When 40% acetonitrile was used, 2-aminopyrimidine could not be analyzed as it migrated too close to the electroosmotic flow (results not shown). The pH was measured in the aqueous phases throughout the study and the organic modifier has an effect not only on the pH value of the electrolyte but also on the pK_a of 2-aminopyrimidine, for instance. As the pK_a of β-Ala at 3.55 is the equilibrium constant for carboxylic acid–carboxylate and the

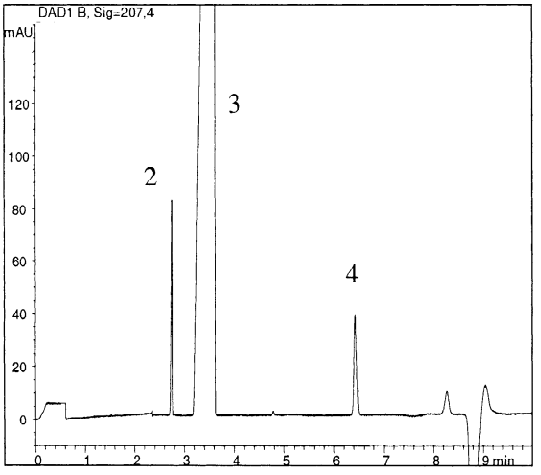

Fig. 3. The electrolyte was a mixture of a buffer containing 153 m*M* β-ALA pH-adjusted with acetic acid to 4.25 and isopropanol in the proportions 30:5 (v/v). The sample contained 0.3 m*M* benzimidazole (peak 2), 33 m*M* creatinine (peak 3), 1.4 m*M* 2-aminopyrimidine (peak 4), 4.7 mg/ml of NaCl, acetic acid diluted 184 times, and 15% isopropanol. Capillary: bare fused-silica.

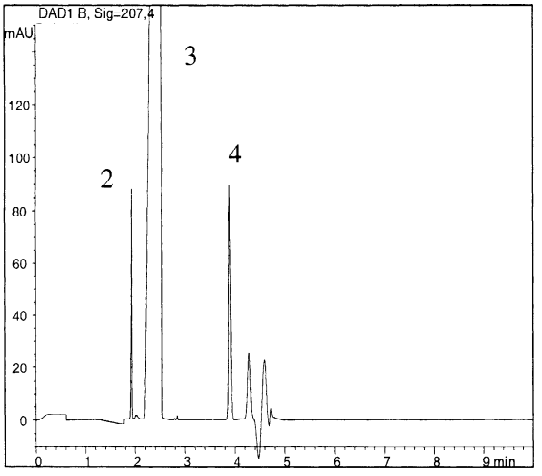

Fig. 4. The electrolyte was a mixture of a buffer containing 153 m*M* β-Ala pH-adjusted with acetic acid to 4.25 and acetonitrile in the proportions 20:5 (v/v). The sample contained 0.3 m*M* benzimidazole (peak 2), 33 m*M* creatinine (peak 3), 1.4 m*M* 2-aminopyrimidine (peak 4), 4.7 mg/ml of NaCl, acetic acid diluted 122 times, and 20% acetonitrile. Capillary: bare fused-silica.

other component in the electrolyte is acetic acid, the pH of the electrolyte will presumably be increased comparatively more than the pK_a value for 2-aminopyrimidine (a base) [23], which would give a lower mobility for 2-aminopyrimidine in the presence of the organic modifier.

3.5. Organic modifier added to an electrolyte with buffering co-ion

A solution consisting of 112 m*M* β-Ala pH-adjusted with hydrochloric acid to 4.25 was prepared. An electrolyte containing 30 ml of this solution and 5 ml of IPA was used for analysis of a sample containing 32 m*M* creatinine, 0.4 m*M* benzimidazole, and 1.4 m*M* 2-aminopyrimidine dissolved in water and 15% of IPA. All components gave normal peak shapes. Benzimidazole showed a good peak shape in this case although it migrates in front of the macrocomponent, although it has a higher pK_a than the macrocomponent and is therefore more easily positively charged and there will also be a stacking effect when β-Ala moves into the sample zone [10]. Addition of potassium chloride to the sample, 1.3 mg/ml, gave somewhat more narrow peaks, as expected.

4. Conclusions

To be able to detect a low amount of impurities in a sample, in this case a base, a large amount of sample is injected which gives a potential risk of overloading the system. Under some conditions a component can then give a split peak, sometimes even baseline separated. To verify if a split peak (or two peaks) consists of more than one component it is useful to inject a shorter sample zone and to control if the chromatogram is as expected. If this is not the case some type of overloading effect is probably the reason. It should also be noted that the peak performance of injection of one component could not be used to judge whether the system is overloaded with regard to other components. In order to obtain good peak shapes, the sample matrix should be considered when the electrolyte is chosen. If the stability of the sample (bases) makes this feasible, a buffering co-ion can be used provided that the pH of the sample solutions is high enough. If the solubility of the base is not high enough, an organic modifier can be added to the electrolyte. Such experimental conditions allow the largest loaded sample plug with a high concentration without an adverse effect on the analyte peak and the narrowest zone as the analyte is pushed into a narrow zone from the rear side of the sample plug by the buffering co-ion.

The electrolyte most robust for variation of the sample matrix, pH and salts when a large amount of sample was loaded contained both a buffering co-ion and a buffering counter-ion (pH approx. 4.2 in the electrolytes used in the experiments). This makes it possible to keep the sample preparation simple and to choose a pH in the sample that gives the highest stability and/or solubility for the components. If a minor component has mobility higher than the macrocomponent and the co-ion, a better peak shape can be obtained if enough of sodium chloride is added in the sample, using the so-called sample self-stacking effect. It should be noted that as far as quantitation is concerned, a high concentration of a macrocomponent might influence the migration times as the migration velocity during the isotachophoretic and zone electrophoretic steps differ. If the solubility is crucial, it may be increased by adding an organic modifier, isopropanol or acetonitrile, both of which were used in this study. Isopropanol gave a lower electroosmotic flow in a fused-silica capillary than acetonitrile and can therefore be a useful tool to increase the resolution for the sample components. Good linearity and precision was shown for impurities in model mixtures in the concentration range 0.03 to approximately 5 mol% calculated on the main component when the sample self-stacking effect was utilized.

Acknowledgements

We are most grateful to Joanna Oreskär for valuable discussions during the work and regarding the manuscript. This project was made possible thanks to the support and resource allocation kindly offered by Astrid Arbin and Anna-Maria Tivert at Analytical Development R&D, Södertälje.

References

[1] ICH (International Conference of Harmonisation of Technical Requirement for Registration of Pharmaceuticals for Human Use) Guideline: Impurities in New Drug Substances, Step 5, 1995.
[2] ICH (International Conference of Harmonisation of Technical Requirement for Registration of Pharmaceuticals for Human Use) Guideline: Impurities in New Medicinal Products, Step 4, Nov 1996.
[3] M.C. Breadmore, P.R. Haddad, Electrophoresis 22 (2001) 2464.
[4] K.D. Altria, M.A. Kelly, B.J. Clark, Trends Anal. Chem. 17 (1998) 204.
[5] K.D. Altria, M.A. Kelly, B.J. Clark, Trends Anal. Chem. 17 (1998) 214.
[6] P. Boček, R. Vespalec, R.W. Giese, Anal. Chem. 72 (2000) 587A.
[7] E. Kenndler, in: M.G. Khaledi (Ed.), High Performance Capillary Electrophoresis, Theory, Techniques, and Applications, Wiley, New York, 1998, Chapter 2.
[8] H. Wätzig, M. Degenhardt, A. Kunkel, Electrophoresis 19 (1998) 2695.
[9] S.V. Ermakov, M.Y. Zhukov, L. Capelli, P.G. Righetti, Anal. Chem. 66 (1994) 4034.
[10] E.K.M. Andersson, I. Hägglund, J. Chromatogr. A 953 (2002) 227.
[11] K.D. Altria, F. Campi, LC·GC Eur. 13 (2000) 16.
[12] W.T. Kok, Chromatographia (Suppl.) 51 (2000) S16–23.
[13] J.L. Beckers, P. Gebauer, P. Boček, Electrophoresis 22 (2001) 3648.
[14] B. Gaš, P. Coufal, M. Jaroš, J. Muzikář, I. Jelínek, J. Chromatogr. A 905 (2001) 269.
[15] J.L. Beckers, P. Gebauer, P. Boček, J. Chromatogr. A 916 (2001) 41.
[16] J.L. Beckers, Electrophoresis 22 (2001) 2684.
[17] J. Beckers, P. Boček, Electrophoresis 20 (1999) 518.
[18] F. Foret, L. Křivánková, P. Boček, in: B.J. Radola (Ed.), Capillary Zone Electrophoresis, VCH, Weinheim, 1993.
[19] P. Gebauer, P. Boček, Anal. Chem. 69 (1997) 1557.
[20] X. Xu, W.T. Kok, H. Poppe, J. Chromatogr. A 742 (1996) 211.
[21] J.L. Beckers, J. Chromatogr. A 741 (1996) 265.
[22] M. Horká, K. Šlais, Electrophoresis 21 (2000) 2814.
[23] K. Sarmini, E. Kenndler, J. Chromatogr. A 806 (1998) 325.
[24] K. Sarmini, E. Kenndler, J. Chromatogr. A 792 (1997) 3.
[25] K. Altria, J. Chromatogr. A 735 (1996) 43.
[26] P. Gebauer, L. Křivánková, P. Pantůčková, P. Boček, W. Thormann, Electrophoresis 21 (2000) 2797.
[27] M.A. Friedberg, M. Hinsdale, Z.K. Shihabi, J. Chromatogr. A 781 (1997) 35.
[28] J. Boden, K. Bächmann, J. Chromatogr. A 734 (1996) 319.
[29] P. Gebauer, W. Thormann, P. Boček, J. Chromatogr. 608 (1992) 47.
[30] J.L. Beckers, P. Boček, Electrophoresis 21 (2000) 2747.
[31] L. Křivánková, P. Pantůčková, P. Boček, J. Chromatogr. A 838 (1999) 55.
[32] C. Schwer, B. Gaš, F. Lottspeich, E. Kenndler, Anal. Chem. 65 (1993) 2108.
[33] S. Park, C.E. Lunte, J. Microcol. Sep. 10 (6) (1998) 511.
[34] D.J. Weiss, K. Saunders, C.E. Lunte, Electrophoresis 22 (2001) 59.
[35] Z.K. Shihabi, J. Chromatogr. A 902 (2000) 107.
[36] J.P. Quirino, S. Terabe, J. Chromatogr. A 902 (2000) 119.
[37] B.X. Mayer, J. Chromatogr. A 907 (2001) 21.
[38] M. Mazereeuw, U.R. Tjaden, N.J. Reinhoud, J. Chromatogr. Sci. 33 (1995) 686.
[39] D.R. Lide (Ed.), CRC Handbook of Chemistry and Physics, 81st edition, CRC Press, Boca Raton, FL, 2000/2001.
[40] D.D. Perrin, B. Dempsey, Buffers for pH and Metal Ion Control, Science Paperbacks, Chapman and Hall, London, 1979.