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Separation of nonsteroidal anti-inflammatory drugs by capillary electrophoresis using nonaqueous electrolytes

The aim of the present work was to investigate the separation of nonsteroidal anti-inflammatory drugs (NSAIDs: niflumic acid, flufenamic acid, piroxicam, alclofenac, tiaprofenic acid, flurbiprofen, suprofen, ketoprofen, naproxen, indomethacin, carprofen, indoprofen, sulindac) in capillary electrophoresis (CE) using completely nonaqueous systems. The influence of different parameters such as nature and proportion of organic solvent (methanol, acetonitrile, 2-propanol), apparent pH (ranging from 7 to 9) and temperature (ranging from 25 to 40°C) on selectivity and migration times were studied systematically in an uncoated fused-silica capillary. A nonaqueous electrolyte made of 50 mM ammonium acetate – 13.75 mM ammonia in methanol proved to resolve 11 NSAIDs at 25°C and 13 NSAIDs at 36°C, both within 13 min and without a modifier besides the methanol itself. The same buffer containing 30% acetonitrile provides a satisfactory separation for 13 NSAIDs within 14 min at 25°C.

1 Introduction

Capillary electrophoresis (CE) has been used increasingly as separating analytical method over the last ten years. So far, the majority of CE separations have been realized in aqueous media. Organic solvents have been used extensively in micellar electrokinetic chromatography (MEKC) [1–4] and in capillary electrochromatography (CEC) [5, 6], most often to improve the separation of hydrophobic compounds. The use of nonaqueous electrolyte has gained attention in various publications because of the better solubility of hydrophobic analytes [7–23]. Already in 1984, a separation in an acetonitrile buffer containing hydrochloric acid and tetraethylammonium perchlorate was described by Walbroehl, for the analysis of quinoline derivatives [7]. Benson *et al.* used nonaqueous electrolytes containing ammonium acetate and acetic acid in methanol for the CE separation of hydrophobic metabolites of the antitumor drug pyrazoloacridine [8] and of the H_2 antagonist mifentidine [9, 10]. Tamoxifen and its metabolites have been separated using a mixture of methanol and acetonitrile (50:50) containing ammonium acetate [11]. Sahota and Khaledi [12] reported a separation of peptides in formamide.

Bjørnsdottir and Hansen have obtained important selectivity changes by using different organic solvents (formamide, *N*-methylformamide, *N,N*-dimethylformamide, dimethylsulfoxide, methanol, and acetonitrile) as separation media for cationic compounds, such as tricyclic antidepressives [13] and opium alkaloids [14], that would be difficult to resolve in aqueous buffers.

A tris(hydroxymethyl)aminomethane-acetate buffer in methanol has been described for the separation of aromatic and aliphatic acids [15] and *N*-methylformamide for the separation of carboxylic acids [16]. Two papers have reported the analysis of inorganic anions in nonaqueous systems, using dimethylformamide [17] and methanol [15] as organic solvents. The potential of nonaqueous electrolyte in CE for the separation of hydrophobic solutes has also been studied by Salimi-Moosavi and Cassidy [18] for a series of alkanesulfonates (C2–C16), alkylsulfates (C8–C18) and linear alkyl benzenesulfonates. Various acidic compounds (pharmaceuticals, including chiral separation of NSAIDs, dyes or surfactants) have been studied using running electrolyte containing different ratios of methanol and acetonitrile [20, 22].

Nonaqueous media extended the application range of CE to the analysis of compounds of poor solubility in water, and also improved the selectivity of the separation of compounds, which are characterized by similar electrophoretic mobilities in aqueous media. Organic solvents for separation media in CE are selected according to their dielectric constant, their viscosity, their UV-absorbance, and by their capability to increase hydrophobic interac-

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Abbreviation: NSAID, nonsteroidal anti-inflammatory drug

CE and CEC

tions between the solvent and the analytes, leading to significant changes of the selectivity of analytical separations. Solvents with high dielectric constants and low viscosity are the most suitable for the composition of buffer media [19].

Another interesting aspect of nonaqueous electrolytes is the complete evaporability of these separation media, which is highly suited to the coupling between CE and mass spectrometry [8–10]. CE with aqueous buffers containing additives (such as cyclodextrins, cellulose derivatives, surfactants) is not as convenient to mass spectrometric detection. Moreover, nonaqueous electrolytes can be prepared with volatile electrolytes, such as ammonium acetate, ammonia, or acetic acid. Applications of such media coupled to mass spectrometry were discussed by Tomlinson *et al.* [8–10].

The aim of the present work was to investigate the separation of nonsteroidal anti-inflammatory drugs (NSAIDs) in CE using methanol, acetonitrile, and a mixture of both as nonaqueous medium. NSAIDs are acidic compounds characterized by low solubility in water; some have similar charge densities, which makes their separation difficult in aqueous systems. The influence of different parameters such as apparent pH (*pH) and temperature of the buffer medium were also studied.

2 Materials and methods

2.1 Apparatus

All experiments were performed on a Spectraphoresis 1000 CE instrument (SpectraPhysics, San Jose, CA, USA) equipped with an automatic injector, an autosampler, a variable wavelength UV-visible absorbance detector (190–800 nm) and a temperature control system (15–60°C). An IBM PS/2 Model 90486 was used for instrument control and data handling. Electropherograms were printed on a Laserjet 4 printer (Hewlett-Packard, Avondale, PA, USA). A capillary cartridge was obtained from SpectraPhysics and fused-silica capillaries were provided by Polymicro Technologies (Phoenix, AZ, USA). The pH and apparent pH (noted *pH) of running buffers were measured by means of a model Delta 345 pH meter with a glass electrode from Mettler (Halstead, UK).

2.2 Chemicals and reagents

Methanol of HPLC grade (maximum 0.02% of water) was obtained from Acros (Geel, Belgium). Ammonium acetate of analytical grade was purchased from Merck (Darmstadt, Germany), as were ammonia, acetic acid and ben-

zylic alcohol of analytical grade. Water was of Milli-Q quality (Millipore, Bedford, MA, USA). Piroxicam, indomethacine, niflumic acid, flufenamic acid, ketoprofen, suprofen, sulindac, carprofen, indoprofen, flurbiprofen and naproxen were obtained from Sigma Chemicals (St. Louis, MO, USA). Alclofenac was obtained from Continental Pharma (Brussels, Belgium) and tiaprofenic acid from Roussel (Brussels, Belgium). All drugs were used as received without further purification. The chemical structures and the pK_a values of these compounds are given in Fig. 1. All solutions were filtered using polytetrafluoroethylene (PTFE) membranes of 0.22 μm from Schleicher & Schuell (Dassel, Germany).

2.3 Electrophoretic technique

Electrophoretic separations were carried out with uncoated fused-silica capillaries having 50 μm internal diameter and 44 cm length (37 cm to the detector). Before each injection, the capillary was treated successively with alkaline solutions (1 M NaOH, 0.1 M NaOH), water and running buffer. At the beginning of each working day, the capillary was rinsed with running buffer for 10 min. Between each injection, the capillary was successively rinsed with water and methanol for 2 min (about four volumes of capillary), and with buffer for 3 min (about six volumes of capillary). The optimal separation buffer for NSAIDs consisted of 50 mM ammonium acetate – 13.75 mM ammonia in different organic solvents, preferably methanol. The applied voltage was –25 kV (detector at the anode end of the capillary) for methanolic buffers and 25 kV (detector at the cathodic end) for aqueous buffers. UV detection was performed at 280 nm. This wavelength was found to be a good compromise for the detection of the NSAIDs tested. Injections were made in hydrodynamic mode for a period of 2 s (corresponding to 5.3 nL). The capillary was thermostated at 25°C, unless otherwise stated. Test mixtures of NSAIDs were prepared in methanol, at a concentration of *ca.* 4×10^{-5} M (20 μg/mL) each. The migration order was determined by injection of individual solutions of each NSAID at the same concentration and by spectral comparison. The electroosmotic flow was measured with a neutral marker (0.01% solution of benzylic alcohol). The resolution (R_s) and plate number (N) were calculated according to the standard expressions based on the peak width at half height [24]. The asymmetry factor (A_s) was determined using the expression: $A_s = b/a$ where a is the distance between the perpendicular from the peak maximum to the leading edge of the peak at one-tenth of the peak height and b is the distance between the perpendicular from the peak maximum to the trailing edge of the peak at one-tenth of the peak height.

3 Results and discussion

3.1 Organic solvent

The influence of the nature and the concentration of organic solvents was studied first. As can be seen in Fig.
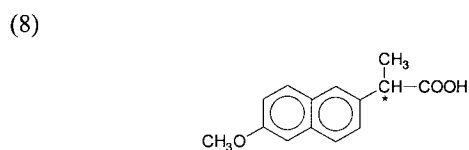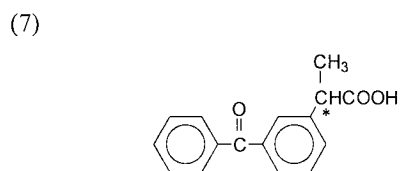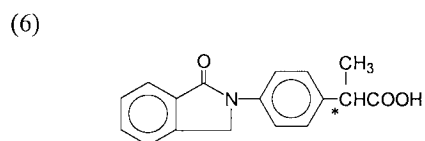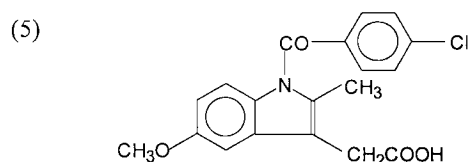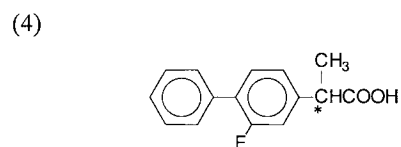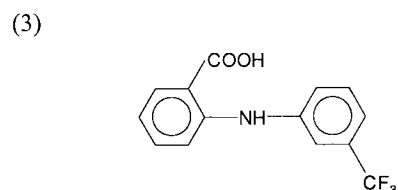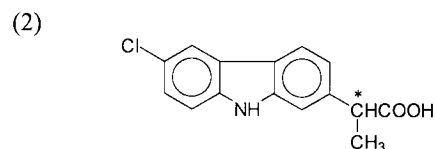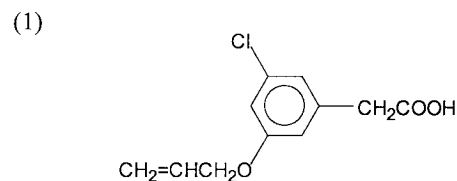
2, the addition of methanol, ethanol and 2-propanol to the running electrolyte significantly reduced the EOF. Acetonitrile does not have as strong an influence, probably due to its higher dielectric constant and the lower viscosity of the mixture. Methanol was also found to provide important
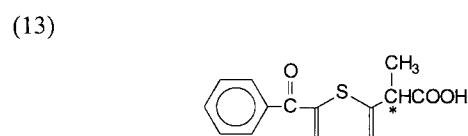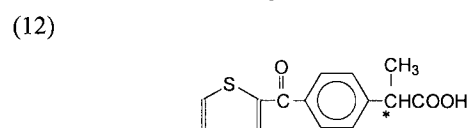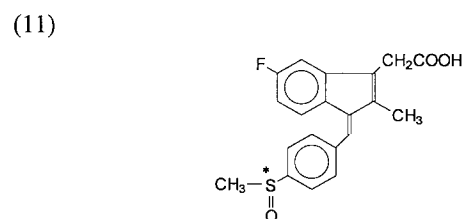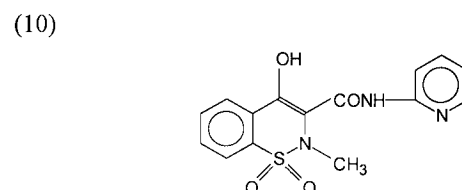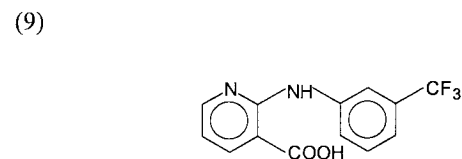

Figure 1. Chemical structures of NSAIDs and their pK_a values. (1) Alclofenac (pK_a 4.6); (2) Carprofen (pK_a 4.3); (3) Flufenamic acid (pK_a 3.9); (4) Flurbiprofen (pK_a 4.1); (5) Indomethacin (pK_a 4.5); (6) Indoprofen (pK_a 5.8); (7) Ketoprofen (pK_a 4.0); (8) Naproxen (pK_a 4.2); (9) Niflumic acid; (10) Piroxicam (pK_a 6.3); (11) Sulindac (pK_a 4.7); (12) Suprofen (pK_a 3.9); (13) Tiaprofenic acid (pK_a 3.0).
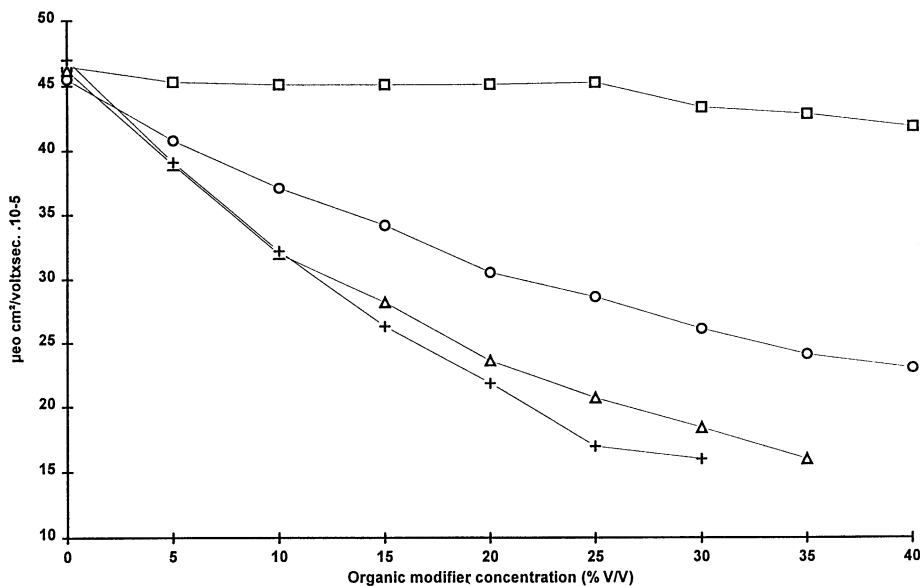
Figure 2. Influence of organic solvent concentration on electroosmotic mobility (μ_{eo}). Buffer: 75 mM glycine adjusted to pH 9.1 with triethanolamine containing an organic solvent in different proportions (0–40%). Voltage, 15 kV. Detection wavelength, 200 nm. Neutral marker, 0.01% solution of benzylic alcohol in water. Organic solvents: ($\square$) acetonitrile; ($\bigcirc$) methanol; ($\triangle$) ethanol; (+) 2-propanol.

Table 1. Electroosmotic mobilities and NSAID effective mobilities in aqueous and nonaqueous systems

Effective mobilities ($\mu_{ep} \times 10^{-5}$) cm^2/Vs			
Aqueous system (1)		Nonaqueous system (2)	
Alclofenac	−28.1	Niflumic acid	−26.0
Flufenamic acid	−27.0	Flufenamic acid	−24.2
Niflumic acid	−26.8	Piroxicam	−22.8
Tiaprofenic acid	−26.5	Alclofenac	−22.2
Flurbiprofen	−26.4	Tiaprofenic acid	−21.6
Naproxen	−26.4	Flurbiprofen	−21.3
Carprofen	−25.8	Ketoprofen	−20.7
Ketoprofen	−25.5	Naproxen	−20.1
Piroxicam	−24.0	Indomethacin	−19.6
Indomethacin	−22.1	Carprofen	−18.8
Sulindac	−21.3	Sulindac	−17.6
Electroosmotic mobilities ($\mu_{eo} \times 10^{-5}$) cm^2/Vs			
Aqueous system (1)		Nonaqueous system (2)	
μ_{eo}	63.0	μ_{eo}	7.5

Buffer: 50 mM ammonium acetate – 13.75 mM ammonia (1) in water or (2) in methanol
Other conditions as described in Section 2.3

changes in selectivity, compared to other solvents tested. In a completely methanolic system, the cathodic electroosmotic flow was strongly decreased in comparison to the analogous aqueous system (*cf.* Table 1), because of changes in the dielectric properties and the viscosity of the system. The electroosmotic mobility of methanolic medium was lower than the effective mobilities of NSAIDs, so that these anionic compounds could not reach the detector when the latter was located at the cathodic side of the capillary. Effective mobilities of most NSAIDs in nonaqueous systems were slightly reduced

compared to those obtained in aqueous systems (Table 1), whereas electroosmotic mobility was strongly decreased ($\mu_{eo} = 63 \times 10^{-5}$ cm^2/Vs in aqueous systems and $\mu_{eo} = 7.5 \times 10^{-5}$ cm^2/Vs in methanolic systems).

Consequently, the electroosmotic flow is too low to transport anionic NSAIDs towards the cathode located at the detector side, as is the case in aqueous systems [25]. Therefore, in methanolic systems the polarity of the electrical field had to be reversed and the NSAIDs were detected at the anodic end of the capillary. The electroosmotic flow then moves in the opposite direction.

If the reduction of the electroosmotic flow was the only effect when methanol was used as separation medium, a reverse migration order of NSAIDs should be observed, compared to that in aqueous systems. However, Table 1 shows several significant differences in the migration order of NSAIDs between the two systems. For example, sulindac exhibits the lowest effective mobility in both systems, while alclofenac, which should be the first migrating peak in methanolic systems (because of its higher effective mobility), appears as the fourth peak in methanolic electrolyte. Piroxicam appears among the analytes with a lower effective mobility in the aqueous system, while it has one of the highest effective mobilities in the nonaqueous system. In the aqueous buffer, a series of compounds (flufenamic acid, niflumic acid, tiaprofenic acid, flurbiprofen, naproxen, carprofen, and ketoprofen) exhibit similar electrophoretic mobilities and are difficult to resolve under such conditions. As shown in Table 1, the range of effective mobilities of NSAIDs is larger in the methanol buffer than in the aqueous buffer, so that greater differences in the mobilities of NSAIDs are achieved, particularly for the

group of compounds that are not resolved in the aqueous buffer. Methanol decreases the effective mobilities of NSAIDs but also changes the selectivity, depending on the chemical structure of the analyte. Different solvation properties of the analytes in this organic solvent can be made responsible for these changes in selectivity: the NSAIDs solvated by methanol may have greater differences in their molecular geometry.

Figures 3 and 4 show the separation of NSAIDs in water and methanol, respectively; both media have the same electrolyte composition. Due to the higher electroosmotic flow in the aqueous system, analysis times are considerably shorter than in methanol. As shown in Fig. 4, a methanol solution of ammonium acetate and ammonia gives rise to much better selectivity compared to that obtained in aqueous buffer, so that a baseline separation of eleven
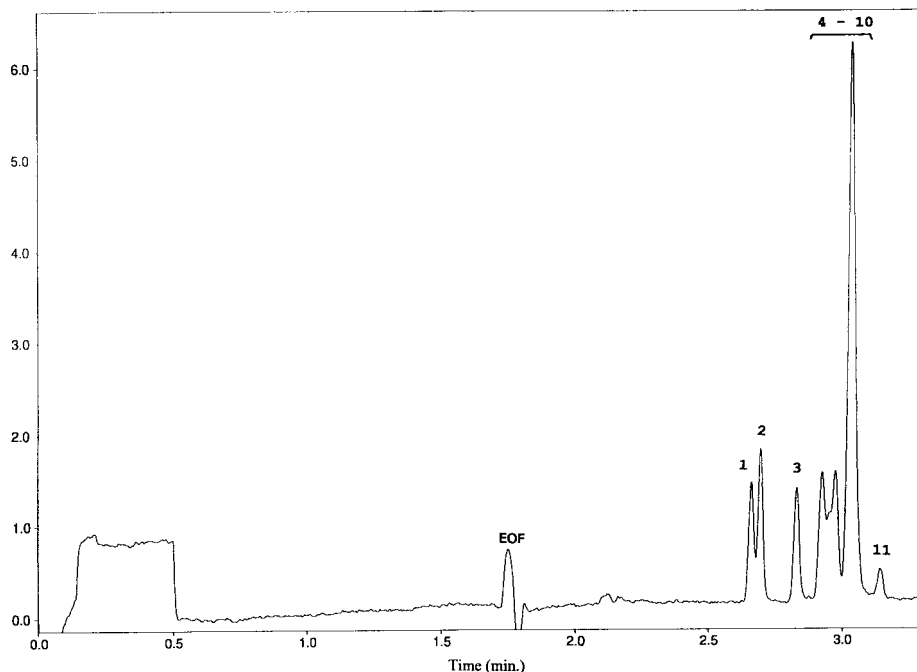

Figure 3. Separation of NSAIDs with aqueous buffer. Buffer: 50 mM ammonium acetate –13.75 mM ammonia (pH 9) in water. Voltage, 25 kV. Other conditions as described in Section 2.3. Peaks: 1, sulindac; 2, indomethacin; 3, piroxicam; 4–10, unresolved peaks (ketoprofen, carprofen, naproxen, flurbiprofen, tiaprofenic acid, niflumic acid; flufenamic acid); 11, alclofenac.
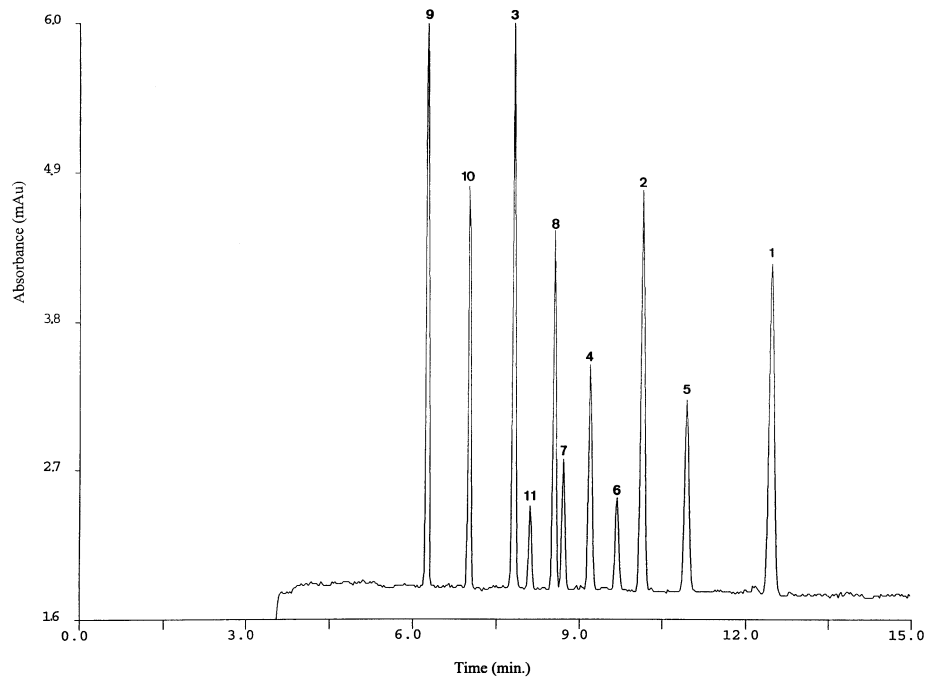

Figure 4. Separation of NSAIDs with methanolic buffer. Buffer: 50 mM ammonium acetate –13.75 mM ammonia (*pH 8.5) in methanol. Voltage: –25 kV. Other conditions as described in Section 2.3. Peaks: 1, niflumic acid; 2, flufenamic acid; 3, piroxicam; 4, alclofenac; 5, tiaprofenic acid; 6, flurbiprofen; 7, ketoprofen; 8, naproxen; 9, indomethacin; 10, carprofen; 11, sulindac.

NSAIDs could be achieved without addition of any other component to the running buffer.

As shown in Table 2, high efficiency and excellent peak symmetry were found for all NSAIDs tested in the nonaqueous methanol system, in spite of a relatively wide range of migration times. The peaks seem to be less sensitive to deformation due to electromigration dispersion in such nonaqueous system than with aqueous or mixed aqueous-organic electrolytes. For the next experiments, three analytes (indoprofen, suprofen, and fenoprofen) were added to the previous mixture to compose a more complex test sample. The influence of gradual replacement of methanol by acetonitrile on the separation of 13 NSAIDs is shown in Fig. 5. A slight increase in migration

Table 2. Efficiency and peak symmetry of NSAIDs in nonaqueous buffer

Analyte	Efficiency (N)	Asymmetry factor (A_s)
Niflumic acid	158 800	1.0
Flufenamic acid	131 200	1.0
Piroxicam	134 500	1.0
Alclofenac	122 300	1.1
Tiaprofenic acid	132 100	1.1
Flurbiprofen	119 600	1.1
Ketoprofen	111 900	1.1
Naproxen	96 200	1.2
Indomethacin	114 000	1.1
Carprofen	94 500	1.0
Sulindac	116 400	1.0

Buffer: 50 mM ammonium acetate – 13.75 mM ammonia in methanol (*pH 8.5).
Other conditions as described in Section 2.3

times was observed when the concentration of acetonitrile was increased. This tendency is certainly related to the concomitant increase of the electroosmotic flow (μ_{eo} = 7.5×10^{-5} cm^2/Vs without acetonitrile and μ_{eo} = 46×10^{-5} cm^2/Vs with 90% acetonitrile). This increase in electroosmotic mobility with acetonitrile concentration can be partly explained by changes of both viscosity and dielectric constant of the separation buffer.

Some inversions of the migration order occurred when the concentration of acetonitrile was varied, probably by changes of solvation of the analytes. The separation of the critical pair sulindac-indoprofen is improved by this inversion; it is completely resolved at concentrations greater than 25% of acetonitrile in the buffer. However, for several pairs, such as naproxen-indomethacin, alclofenac-tiaprofenic acid and piroxicam-flufenamic acid, which were baseline-resolved in methanol, the resolution decreased in buffers with high concentrations of acetonitrile. Figure 6 shows a separation of 13 NSAIDs obtained with a 50 mM ammonium acetate – 13.75 mM ammonia buffer prepared in 70% methanol and 30% acetonitrile. Under these conditions, which seem to be the best compromise for the model mixture applied, satisfactory separation was achieved for most compounds (all compounds were baseline-resolved except the pair flufenamic acid-piroxicam).

At high acetonitrile concentrations (above 50%), electroosmotic mobility becomes higher than the effective mobilities of NSAIDs, so that the analytes can not reach the detector at the anodic side. This nonaqueous medium is less favorable for high resolution than the completely
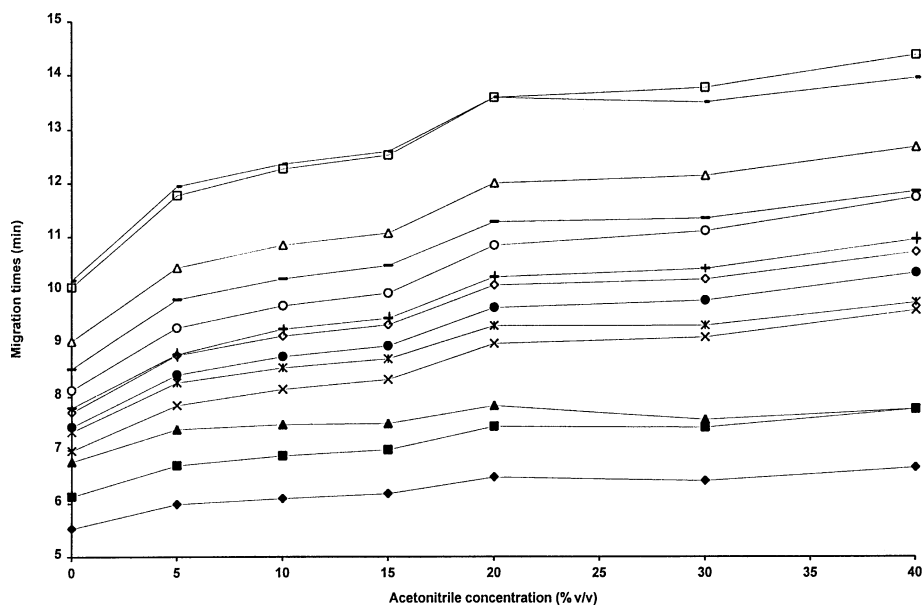

Figure 5. Influence of percentage of acetonitrile in methanol on migration times. Buffer: 50 mM ammonium acetate –13.75 mM ammonia in methanol-acetonitrile in varying proportions (0–40%). Other conditions as described in Section 2.3. Analytes: (♦) niflumic acid; (■) flufenamic acid; (▲) piroxicam; (×) alclofenac; (★) tiaprofenic acid; (●) flurbiprofen; (◇) suprofen; (+) ketoprofen; (○) naproxen; (–) indomethacin; (△) carprofen; (-)indoprofen; (□) sulindac.
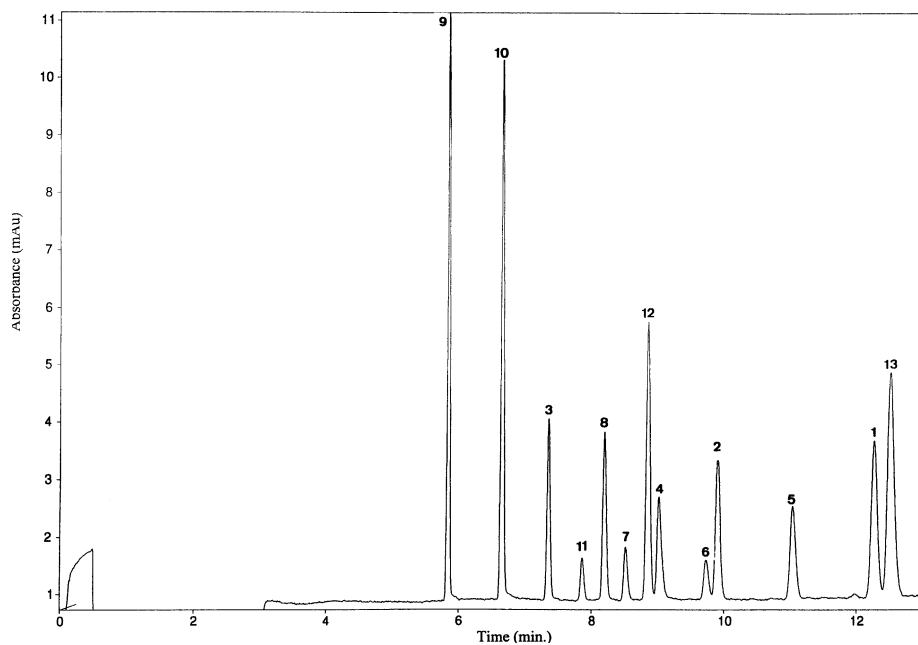
Figure 6. Separation of NSAIDs in a methanolic buffer containing 30% acetonitrile. Buffer: 50 mM ammonium acetate −13.75 mM ammonia in methanol/acetonitrile (70/30 v/v). Other conditions as described in Section 2.3. Peaks: 1, niflumic acid; 2, flufenamic acid; 3, piroxicam; 4, alclofenac; 5, tiaprofenic acid; 6, flurbiprofen; 7, suprofen; 8, ketoprofen; 9, naproxen; 10, indomethacin; 11, carprofen; 12, indoprofen; 13, sulindac.
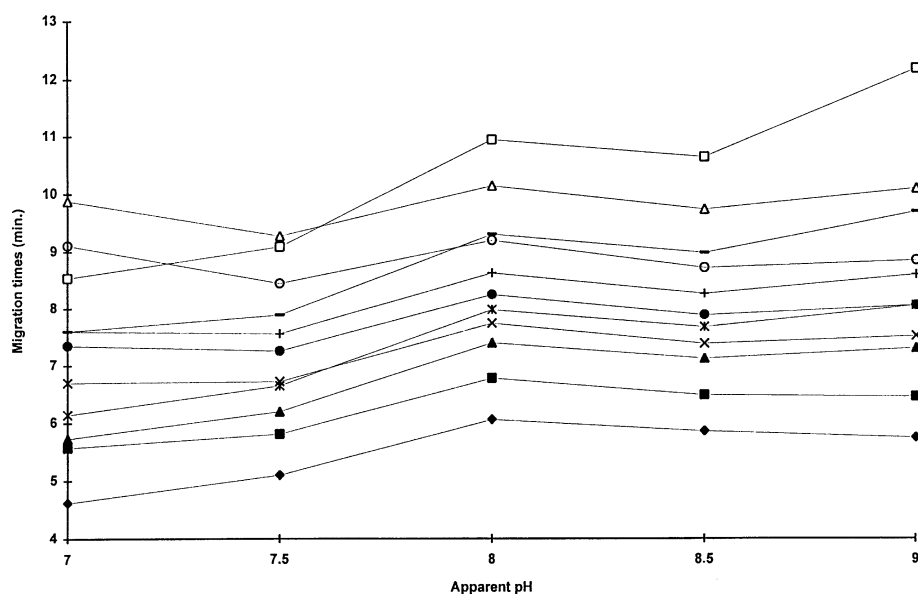

Figure 7. Influence of apparent pH on migration times. Buffer: 50 mM ammonium acetate in methanol adjusted to *pH 7 with acetic acid and to *pH 8–9 with ammonia. Other conditions as described in Section 2.3. Analytes: (◆) niflumic acid; (■) flufenamic acid; (▲) piroxicam; (×) alclofenac; (★) tiaprofenic acid; (●) flurbiprofen; (+) ketoprofen; (○) naproxen; (−) indomethacin; (△) carprofen; (□) sulindac.

methanolic system, probably due to the high electroosmotic flow caused by acetonitrile as buffer component. The corresponding electroosmotic mobility (about 46 × 10⁻⁵ cm²/Vs) is close to that arising in the aqueous system (*cf.* Table 1). Moreover, even if the range of NSAID migration times is narrower than in methanol, the zones seem to be more sensitive to deformation due to electromigrative dispersion, as can be recognized from the asymmetry of the peaks. A separating medium with 100% acetonitrile could not be applied because the solubility of ammonium acetate is too low.

3.2 Apparent pH (*pH)

As can be seen from Fig. 7, changes in selectivity and in the migration order of NSAIDs were obtained by changing the apparent pH of the methanolic electrolyte, while migration times were not much affected. These changes could sometimes result in resolution improvement. The apparent pH was varied by an increasing ammonia concentration from 4.5 mM (*pH 8) to 35 mM (*pH 9). *pH 7 and *pH 7.5 were obtained by the addition of 35 and 15 mM of acetic acid, respectively. However, resolution

modifications observed with the apparent pH variation do not seem to be the only consequence of this increase in ionic strength, the observed effect probably depending on the nature of the analyte, as can be concluded from inversions of migration orders. Compared to the aqueous medium, NSAIDs could undergo a shift in their pK_a values in the methanolic buffer; this has been described previously for some acidic compounds [16]. A buffer with an apparent pH of 8.5, which corresponds to a concentration of 13.75 mM ammonia, allows for baseline resolution of the mixture of the eleven NSAIDs contained in the test mixture (see Fig. 4).

3.3 Temperature

Under the conditions described above, *i.e.*, 50 mM ammonium acetate and 13.75 mM ammonia in methanol (*pH 8.5) at 25°C, some of the 13 NSAIDs coelute, such as the pairs suprofen-ketoprofen and indoprofen-sulindac. As can be seen in Fig. 8, an increase in temperature leads to a slight decrease in migration times for all NSAIDs, due to a decrease in the viscosity of the separation medium. Some changes of the migration order occur as the temperature increases, resulting in improvement in resolution. Indoprofen migrates faster than sulindac at 25°C, but
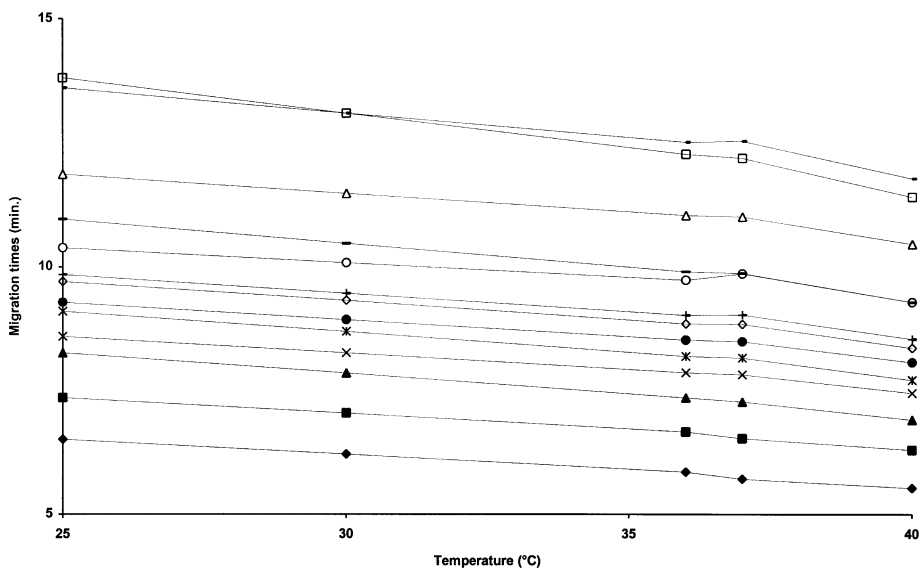

Figure 8. Influence of temperature on migration times. Buffer: 50 mM ammonium acetate – 13.75 mM ammonia (*pH 8.5) in methanol. Other conditions as described in Section 2.3. Analytes: (◆) niflumic acid; (■) flufenamic acid; (▲) piroxicam; (×) alclofenac; () tiaprofenic acid; (●) flurbiprofen; (◇) suprofen; (+) ketoprofen; (○) naproxen; (−) indomethacin; (△) carprofen; (-) indoprofen; (□) sulindac.
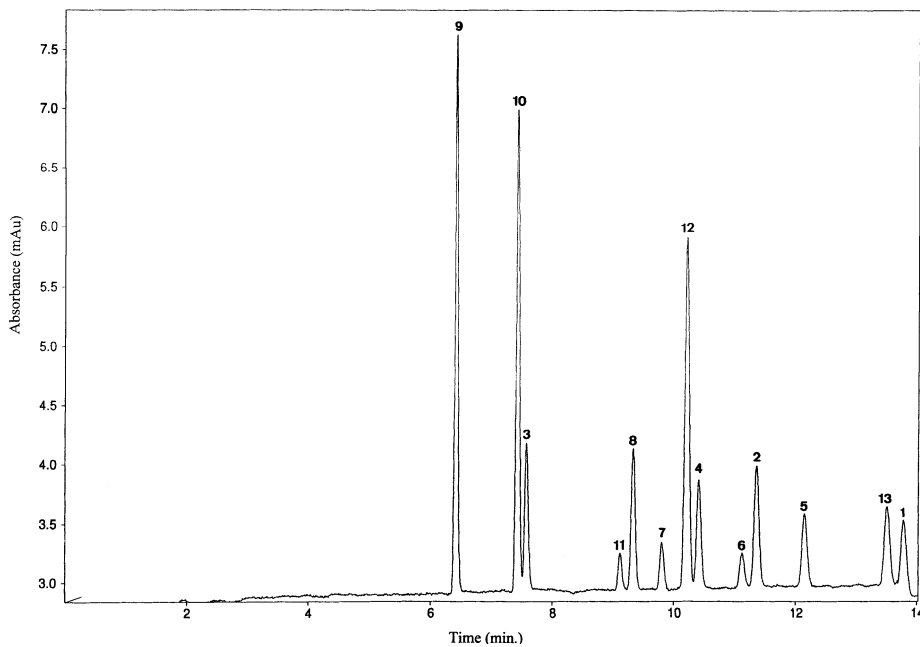

Figure 9. Separation of NSAIDs in nonaqueous buffer at 36°C. Buffer: 50 mM ammonium acetate – 13.75 mM ammonia (*pH 8.5) in methanol. Temperature, 36°C. Other conditions as described in Section 2.3. Peaks: 1, niflumic acid; 2, flufenamic acid; 3, piroxicam; 4, alclofenac; 5, tiaprofenic acid; 6, flurbiprofen; 7, suprofen; 8, ketoprofen; 9, naproxen; 10, indomethacin; 11, carprofen; 12, sulindac; 13, indoprofen.

these compounds coelute at 30°C, whereas sulindac becomes more mobile than indoprofen above 30°C. This inversion of migration order results in a resolution improvement from 1.2 at 25°C to 2.4 at 40°C. For the pair suprofen-ketoprofen, the resolution increases from 1.2 at 25°C to 1.6 at 40°C. In contrast, for the pair naproxen – indomethacin, which can be resolved with an R_s value of 4.1 at 25°C, the resolution decreases as the temperature increases. Naproxen moves increasingly closer to indomethacin, and the two analytes coelute at 37°C. Figure 9 represents the separation of 13 NSAIDs at 36°C. This temperature was found to be the best compromise for the model mixture of NSAIDs, and allows a good separation of 13 analytes in about 13 min. The effect of temperature on the selectivity in this nonaqueous system provides an additional parameter that may improve the separation of analytes with similar charge densities which are difficult to resolve even in nonaqueous media. However, it appears from this study that the electrophoretic behavior of some analytes is sensitive to very slight changes of temperature: an increase in temperature of only 1°C may have a strong influence on resolution, as for example for the pair naproxen-indomethacin. It appears from Fig. 9 that peak shape and peak efficiency remain good at higher temperatures (average N: 113 000 at 25°C and 102 000 at 36°C). This observation indicates that Joule heating produced in nonaqueous systems is lower than with aqueous buffers, making it possible to use higher temperatures in nonaqueous systems without a significant loss of efficiency, compared to aqueous buffers.

4 Concluding remarks

A nonaqueous electrolyte made of 50 mM ammonium acetate and 13.75 mM ammonia in methanol was suited to resolve eleven NSAIDs at 25°C and 13 NSAIDs at 36°C, without a modifier besides the methanol itself. The same buffer containing 30% acetonitrile provides a satisfactory separation for 13 NSAIDs at 35°C. The apparent pH, the addition of acetonitrile, and temperature are parameters which can be optimized for improvements of selectivity in nonaqueous systems. Not all effects of changes in absolute and relative migration times (selectivity) observed in the reported experiments can be explained. The use of nonaqueous electrolytes seems to be an effective means to change and increase separation selectivity in CE, especially for more hydrophobic analytes with similar charge densities, which are often difficult to resolve in aqueous media.

5 References

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