ELSEVIER

Journal of Chromatography A, 874 (2000) 121–129

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Development and robustness testing of a nonaqueous capillary electrophoresis method for the analysis of nonsteroidal anti-inflammatory drugs

Samir Cherkaoui, Jean-Luc Veuthey*

Laboratory of Pharmaceutical Analytical Chemistry, Pavillion de Isotopes, University of Geneva, Bd d'Yvoy 20, 1211 Geneva 4, Switzerland

Received 20 September 1999; received in revised form 20 December 1999; accepted 29 December 1999

Abstract

Nine non steroidal anti-inflammatory drugs were simultaneously separated by nonaqueous capillary electrophoresis with a methanol–acetonitrile (40:60, v/v) mixture containing 20 m*M* ammonium acetate. The effect of solvent composition, electrolyte nature and concentration on the electrophoretic behavior of the selected drugs was systematically studied. Investigated electrolytes were ammonium, lithium and sodium acetate. Modification of the solvent and/or the electrolyte composition was found to alter the migration order of the pharmaceutical drugs. Finally, to assess method robustness, three sensitive electrophoretic parameters as well as their interactions were evaluated using a full factorial design at two levels.

Keywords: Nonaqueous capillary electrophoresis; Background electrolyte composition; Pharmaceutical analysis; Nonsteroidal anti-inflammatory drugs

1. Introduction

In capillary electrophoresis (CE), selectivity can easily be improved by an appropriate choice of aqueous buffer pH, by adding surfactants and/or ion-pair agents, and by complexation with different agents such as, neutral or charged cyclodextrins, proteins, borate or some metal ions. But, good selectivity can also be achieved with organic modifier [1]. Introducing organic solvents into the running buffer is a well documented and largely used strategy in CE, both in capillary zone electrophoresis (CZE) [2,3] and in micellar electrokinetic chromatography (MEKC) [4,5]. In fact, organic solvents are often employed to influence selectivity and resolution, increase solubility of hydrophobic compounds, change micelle properties, modulate the separation window and in some cases improve the enantiomeric resolution. However, contradictory results, concerning erratic migration times and electric breakdown, have been reported at high organic solvent level. Therefore, organic modifier percentage was often limited at 40% [6,7].

Recently, nonaqueous capillary electrophoresis (NACE) has become an active area of study. NACE was found to be a good alternative for the analysis of

*Corresponding author. Tel.: +41-22-7026-336; fax: +41-22-7815-193.

E-mail address: jean-luc.veuthey@pharm.unige.ch (J.-L. Veuthey)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00052-2

pharmaceutical drugs and their metabolites, which are difficult to separate in aqueous media. Indeed, compared to those of water, the different chemical and physical properties of organic solvents (viscosity, dielectric constant, polarity, auto-protolysis constant, electrical conductivity, etc.), have shown to offer several advantages in terms of selectivity, efficiency, rapidity, MS compatibility and analyte solubility and stability. NACE was successfully applied to the analysis of a large number of pharmaceuticals including, acidic and basic drugs, chiral compounds, peptides, ions and preservatives. These applications are summarized in recent reviews [8–13].

Nonsteroidal anti-inflammatory drugs (NSAIDs) play an important role in modern therapy. Therefore, their determination in body fluids is needed for pharmacokinetic and toxicological studies. CE has been found to be an interesting alternative to chromatographic techniques for this class of drugs. Separation and determination of a number of NSAIDs by CZE [14,15] and by MEKC [16,17] have been described. Since NSAIDs are lipophilic acid compounds, with similar charge-to-mass ratio, NACE has been found suitable for their analysis [18,19]. NACE was recently applied to the separation of some NSAIDs, in a reverse polarity mode and with a methanolic solution containing 50 m*M* ammonium acetate and 13.7 m*M* ammonia [20]. Analysis of ibuprofen and its major metabolites in urine [13], as well as enantiomeric separation of some profens by cationic cyclodextrins dissolved in formamide [21] have also been reported. However, to the best of our knowledge, no data including the method robustness study have been reported.

In this paper, a systematic investigation of the potential of nonaqueous CE is presented for the separation of nine NSAIDs. The effect of the nature and concentration of the background electrolyte as well as the composition of the organic solvent are described. Optimized separation parameters are determined and method robustness is examined by applying a full factorial design at two levels. Three sensitive electrophoretic parameters, namely methanol percentage, ammonium acetate concentration and temperature are evaluated for their influence on separation time, resolution and normalized peak area.

2. Experimental

2.1. Chemicals

Ibuprofen, baclofen, indoprofen, indomethacin, ketoprofen, suprofen, fenoprofen, mefenamic acid and diclofenac were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate, sodium acetate and lithium acetate were obtained from Fluka (Buchs, Switzerland). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were provided by Romil (Kölliken, Switzerland).

2.2. Instrumentation and electrophoretic procedure

CE data were generated in a HP Hewlett-Packard CE system (Waldbronn, Germany) equipped with an on-column diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. The total capillary length (Composite Metal Services, Hallow, UK) was 48.5 cm, while the length to the detector was 40 cm, with a 50 μm internal diameter. An alignment interface, containing an optical slit matched to the internal diameter, was used and the detection wavelength was set at 200 nm with a bandwidth of 10 nm. A CE Chemstation (Hewlett-Packard) was chosen for instrument control, data acquisition and data handling.

All experiments were performed in cationic mode (anode at the inlet and cathode at the outlet). The capillary was thermostated at 20°C, unless otherwise stated. A constant voltage of 30 kV, with an initial ramping of 500 V s^{-1}, was applied during analysis. Sample injections (8-nl injection volume) were achieved using the pressure mode for 10 s at 25 mbar. The nonaqueous buffer was prepared by dissolving an appropriate amount of the electrolyte in a methanol–acetonitrile mixture. The apparent pH* was measured by a pH meter equipped with a combined glass–calomel electrode. The pH electrode was calibrated with standard aqueous buffer solutions.

The capillary was rinsed daily with 0.1 *M* sodium hydroxide, followed by water and acetonitrile for 5 min each. This flushing procedure is expected to remove any trace of water in the capillary. Between analyses, the capillary was flushed with the running

buffer for 3.5 min, and when not in use, it was washed with acetonitrile, water and then stored in air.

Since NSAIDs are acidic drugs (Fig. 1), in the normal mode, they migrate in the opposite direction to the electroosmotic flow (EOF) and appear in the electropherogram after the neutral peak.

2.3. Sample preparation

2.3.1. Standard solutions

Stock standard solutions of NSAIDs were prepared by dissolving each compound in methanol in order to give a concentration of 1 mg ml^{-1}. The studied mixture was prepared by dissolution of individual compounds in methanol to give a final concentration of 20 μg ml^{-1} each.

2.4. Software

A statistical graphic software package, Statgraphics (version 6) for windows (Manugistics, Rockville, USA) was used to generate and manipulate factorial design data.

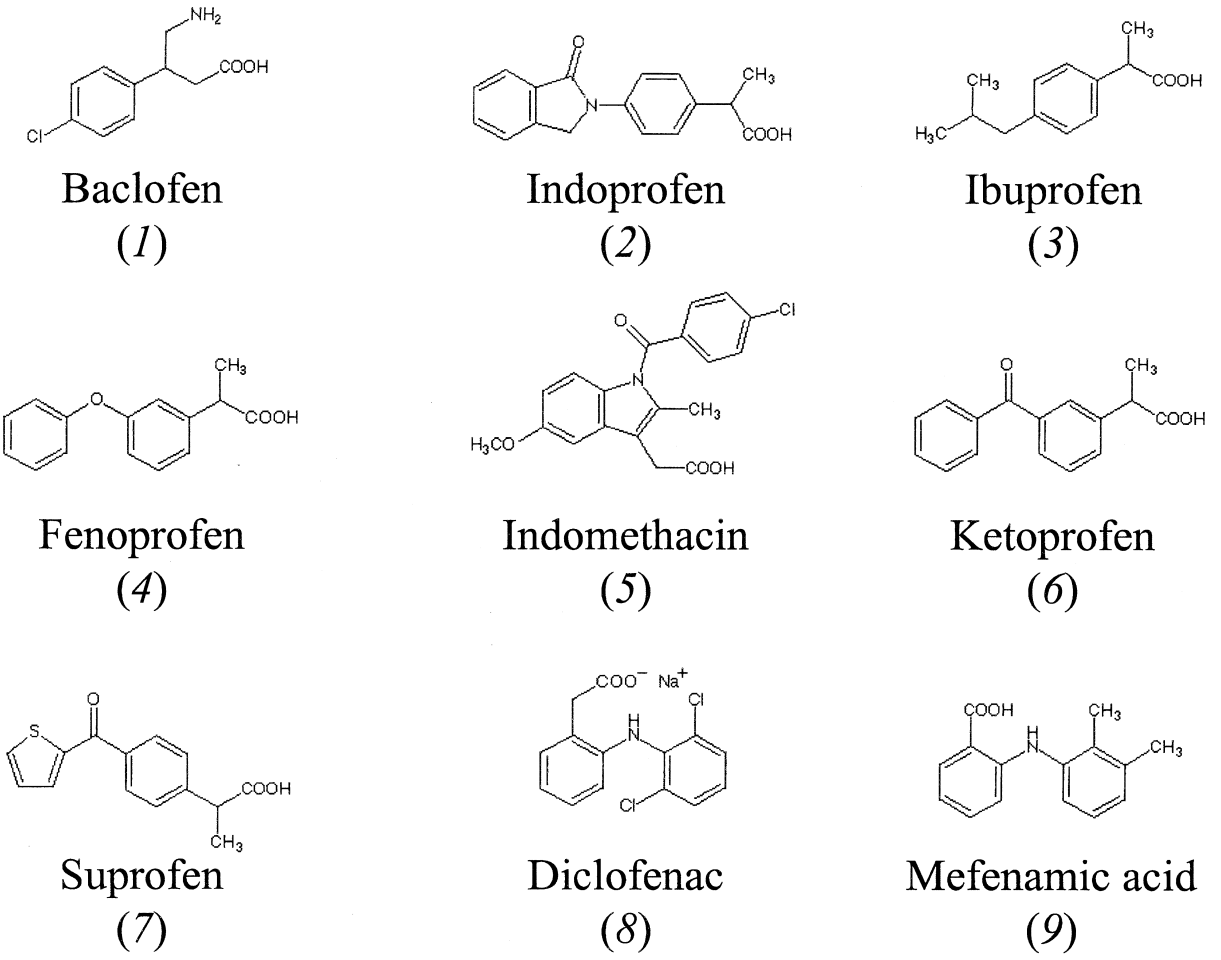

Fig. 1. Structures of investigated nonsteroidal anti-inflammatory drugs and their identification numbers.

3. Results and discussion

3.1. Method development

Among the different requirements for the successful use of NACE, the organic solvent should present a low UV absorbance at the wavelength of interest. Lower detection limits in CE are generally achieved at very low wavelength, which considerably limits the choice of the organic solvent to be used as electrophoretic medium. Publications report that methanol and acetonitrile are the best solvents when UV detection is selected [12,22,23]. Using neat methanol will result in longer migration times, since the dielectric constant to viscosity ratio is much lower in methanol (60.6) than in water (89.9) or acetonitrile (110.3). In addition, electrophoretic medium containing a mixture of solvents was found particularly advantageous to achieve high selectivity. By mixing methanol, with amphiprotic properties, and acetonitrile, generally classified among dipolar aprotic solvents, good separation performances can be expected Therefore, the first investigations were carried out using a mixture of methanol–acetonitrile (50:50, v/v).

3.1.1. Electrolyte composition

To evaluate the role of the electrolyte cation on the electrophoretic separation, different electrolytes, namely ammonium acetate, lithium and sodium acetate, were selected. As illustrated in Fig. 2, the same electrolyte concentration (10 m*M*), induced different migration behaviors for the investigated acidic drugs. Moreover, an increase in the size of the monovalent cation resulted in a decrease in electrophoretic mobility [24,25]. It is noteworthy that a different separation was observed when ammonium was used, instead of lithium or sodium. Indeed, mefenamic acid and diclofenac comigrate in the presence of ammonium while they are well resolved in presence of lithium or sodium. In addition, baclofen, which possesses both amine and carboxylic functions, migrates close to the EOF in ammonium solution while it is negatively charged in the other solutions. Such behavior can be mainly attributed to the different apparent pH* of the ammonium (8.14), sodium (9.21) and lithium (9.18) solutions. The strength of the ion interactions between electrolyte cations and NSAID molecules have also to be considered. Therefore, ammonium acetate was selected for further investigations due to these results and because this electrolyte is volatile and thus suitable for subsequent NACE coupling to mass spectrometry using an electrospray ionization interface. Work is in progress in our laboratory to highlight such successful coupling. In addition, electrophoretic medium containing ammonium acetate was found more stable than other tested systems.

3.1.2. Organic solvent composition

In previous studies concerning the application of NACE to the analysis of pharmaceutical drugs, it was demonstrated that the organic solvent composition has a critical effect on resolution, efficiency and migration time [22,23]. Thus, the acetonitrile percentage in methanol was varied between 25 and 75%. Neat acetonitrile was excluded because of the limited solubility of the electrolyte while neat methanol results in excessive migration times as mentioned above. As shown in Fig. 3, the electrophoretic mobilities of the investigated compounds were considerably decreased when the methanol percentage in acetonitrile was increased. This behavior is mainly due to the modification of dielectric constant to viscosity ratio. Indeed, the ϵ/η ratio was reported to be high at low MeOH concentration with a maximum value at 20% MeOH. Thus, since the electrophoretic mobility is directly proportional to the ϵ/η ratio, methanol–acetonitrile mixtures with lower ϵ/η values exhibit lower electrophoretic mobilities. Furthermore, it has to be noted that the migration order of indomethacin and fenoprofen above 50% methanol is inverted. Because electrophoretic mobility is mainly governed by the size and shape of negatively charged compounds, migration order inversion can be attributed to changes in the solvation degree. The diclofenac/mefenamic acid pair remains unresolved in the investigated methanol–acetonitrile mixtures. Fig. 3 shows that the methanol–acetonitrile mixture (40:60, v/v) is a good compromise for a rapid separation with a high resolution.

3.1.3. Electrolyte concentration

In order to investigate the effect of the electrolyte ionic strength, the concentration of ammonium acetate was varied over the range 5–20 m*M*, while

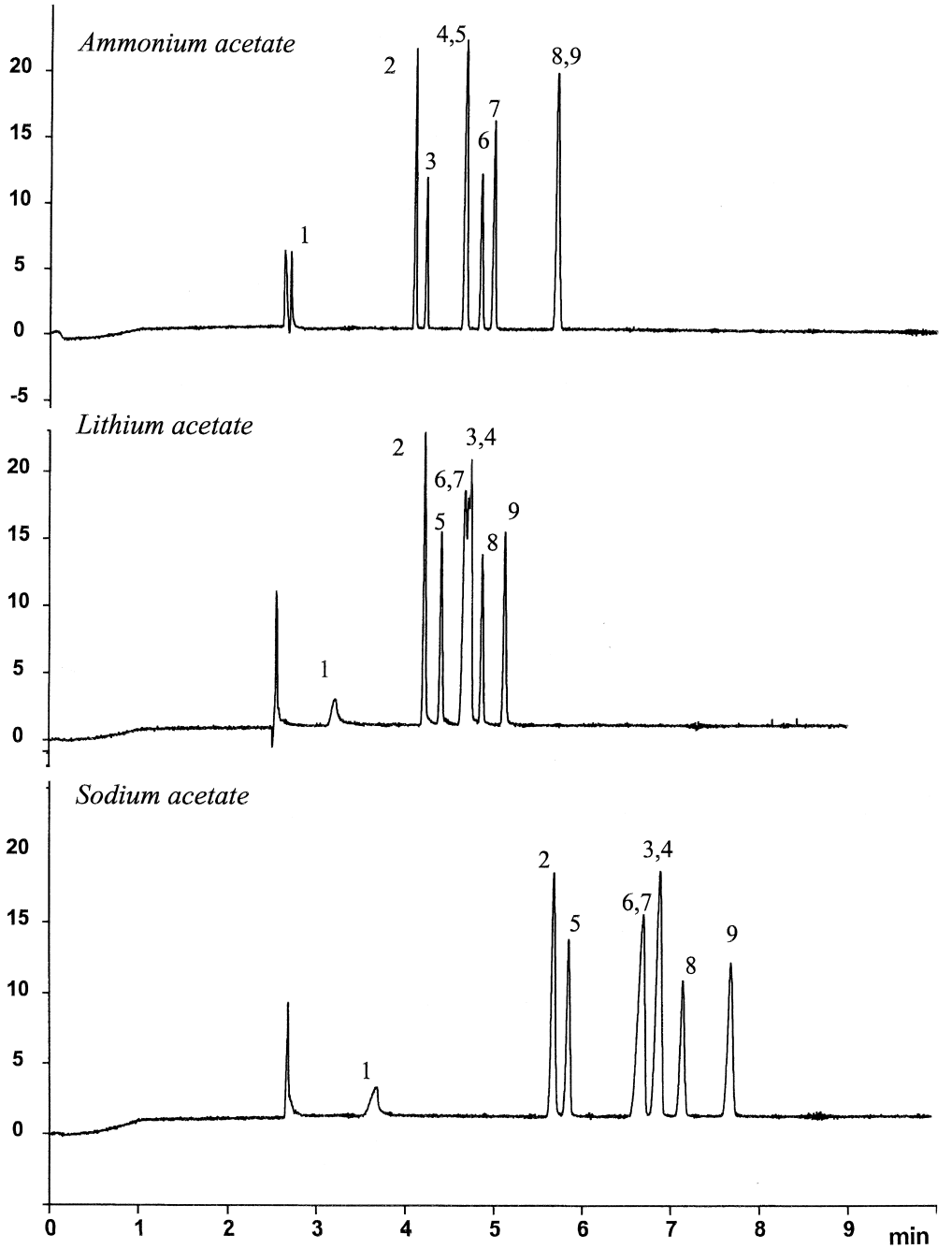

Fig. 2. Effect of electrolyte cation on separation of investigated NSAIDs. Electrophoretic medium; MeOH–MeCN (50:50, v/v) containing: (A) 10 m*M* ammonium acetate, (B) 10 m*M* lithium acetate, (C) 10 m*M* sodium acetate. Applied voltage; 30 kV. Uncoated fused-silica capillary: 48.5 cm (effective length 40 cm)×50 μm I.D. Sample injection; 25 mbar for 10 s. Temperature; 20°C. Detection at 200 nm. Peak assignment as in Fig. 1.

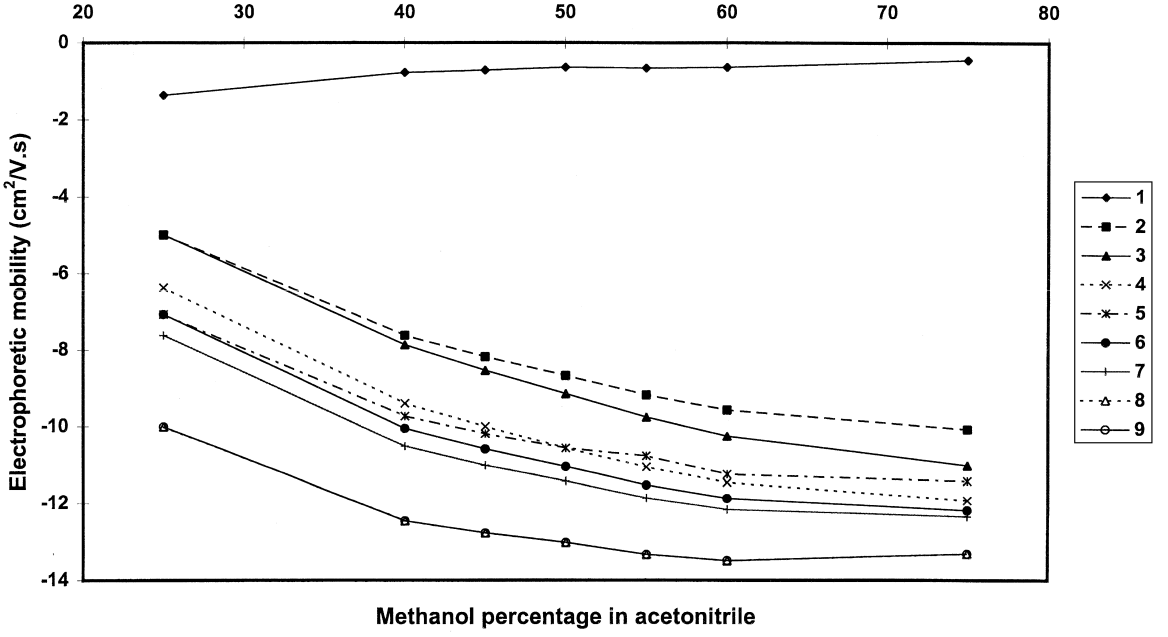

Fig. 3. Effect of methanol percentage on electrophoretic mobility of studied compounds. Electrophoretic medium: 10 m*M* ammonium acetate in different methanol–acetonitrile mixtures. Other conditions as in Fig. 2.

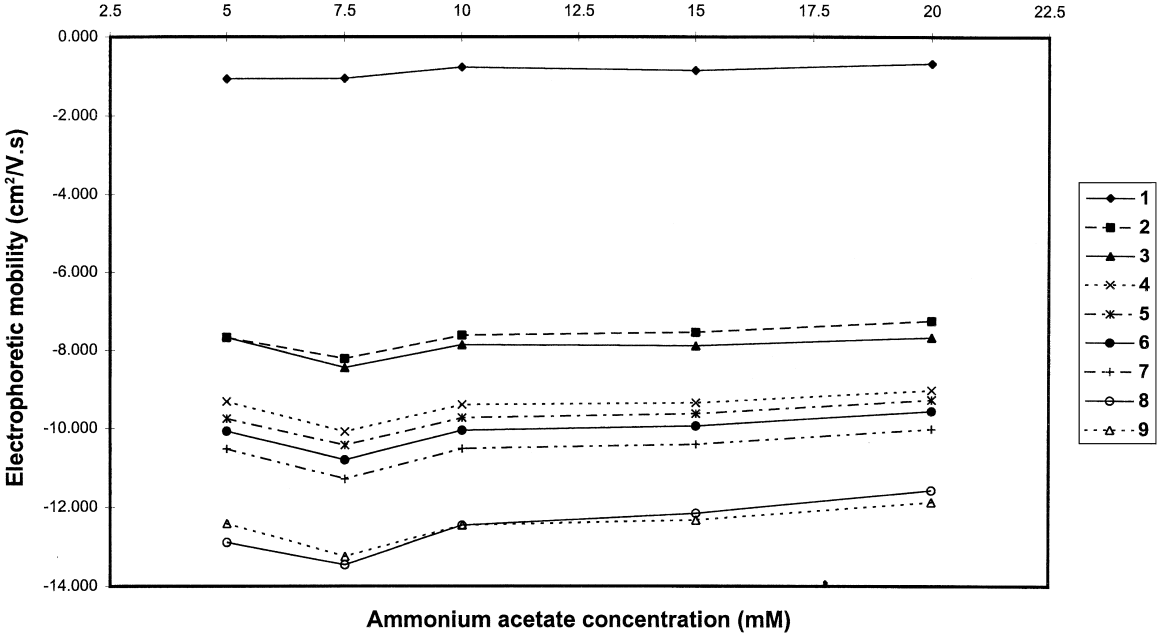

Fig. 4. Effect of ammonium acetate concentration on electrophoretic mobility of investigated drugs. Electrophoretic medium; methanol–acetonitrile (40:60, v/v) containing different amounts of ammonium acetate. Other conditions as in Fig. 2.

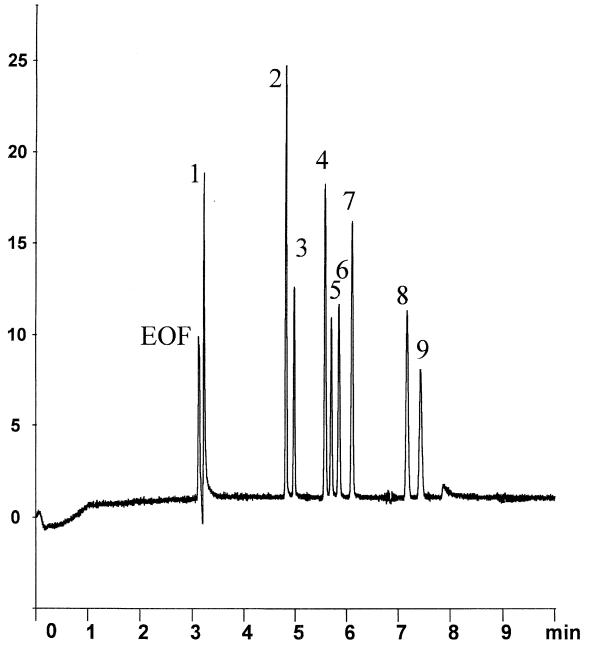

Fig. 5. Typical electropherogram of NSAIDs. Electrophoretic medium; methanol–acetonitrile (40:60, v/v) containing 20 m*M* ammonium acetate. Other conditions as in Fig. 2.

keeping the acetonitrile percentage at 60%. EOF generally decreases when the electrolyte ionic strength increases, which results in improving the resolution. As shown in Fig. 4, the separation of acidic compounds is less affected by ammonium acetate in the studied concentration range, except diclofenac and mefenamic acid. Indeed, an inversion of the migration order of these latter compounds was observed from 10 m*M* and a further increase of the ammonium acetate concentration improves the resolution. This behavior may be explained by the complex formation between the electrolyte cation (NH_4^+) and diclofenac [26,27]. Therefore, as shown in the electropherogram (Fig. 5), a methanol–acetonitrile mixture (40:60, v/v) containing 20 m*M* ammonium acetate yielded the best compromise in terms of analysis time, selectivity and separation efficiency.

3.2. Method robustness

Robustness is an important aspect of method validation. It is defined as the capability of an analytical procedure to remain unaffected by small but deliberate variations in the method parameters. Therefore, due to the risk of volatile solvent evaporation and its effect on method reproducibility [28], the robustness of a nonaqueous system has to be assessed. The most sensitive electrophoretic parameters that could affect separation performances were examined: (1) methanol percentage, (2) ammonium acetate concentration, and (3) temperature. The selected experimental factors and their level ranges are summarized in Table 1.

Given the number of investigated electrophoretic parameters, a 2^3 full factorial design was applied. Five points at the optimized conditions (central values) were included in the design and 13 measurements were thus randomly performed. The measured responses were separation time (last migrating compound, i.e., mefenamic acid), resolution between indoprofen and ibuprofen and indoprofen normalized peak area (peak area divided by the migration time). Responses from the design were statistically analyzed using Statgraphics and the effects were plotted as standardized Pareto charts, which are graphical representations of the size of experimental parameters as well as their second order interactions estimated effects. A parameter is considered to be statistically significant at a 5% level if its standardized effect is greater than the critical *t*-value. As shown in Fig. 6a, separation time is mainly influenced by the ammonium acetate concentration,

Table 1
Values of experimental factors

Coded values:	Low value (−1)	Central value (0)	High value (+1)
Methanol percentage (%)	39	40	41
Ammonium acetate concentration (m*M*)	19	20	21
Temperature (°C)	19	20	21

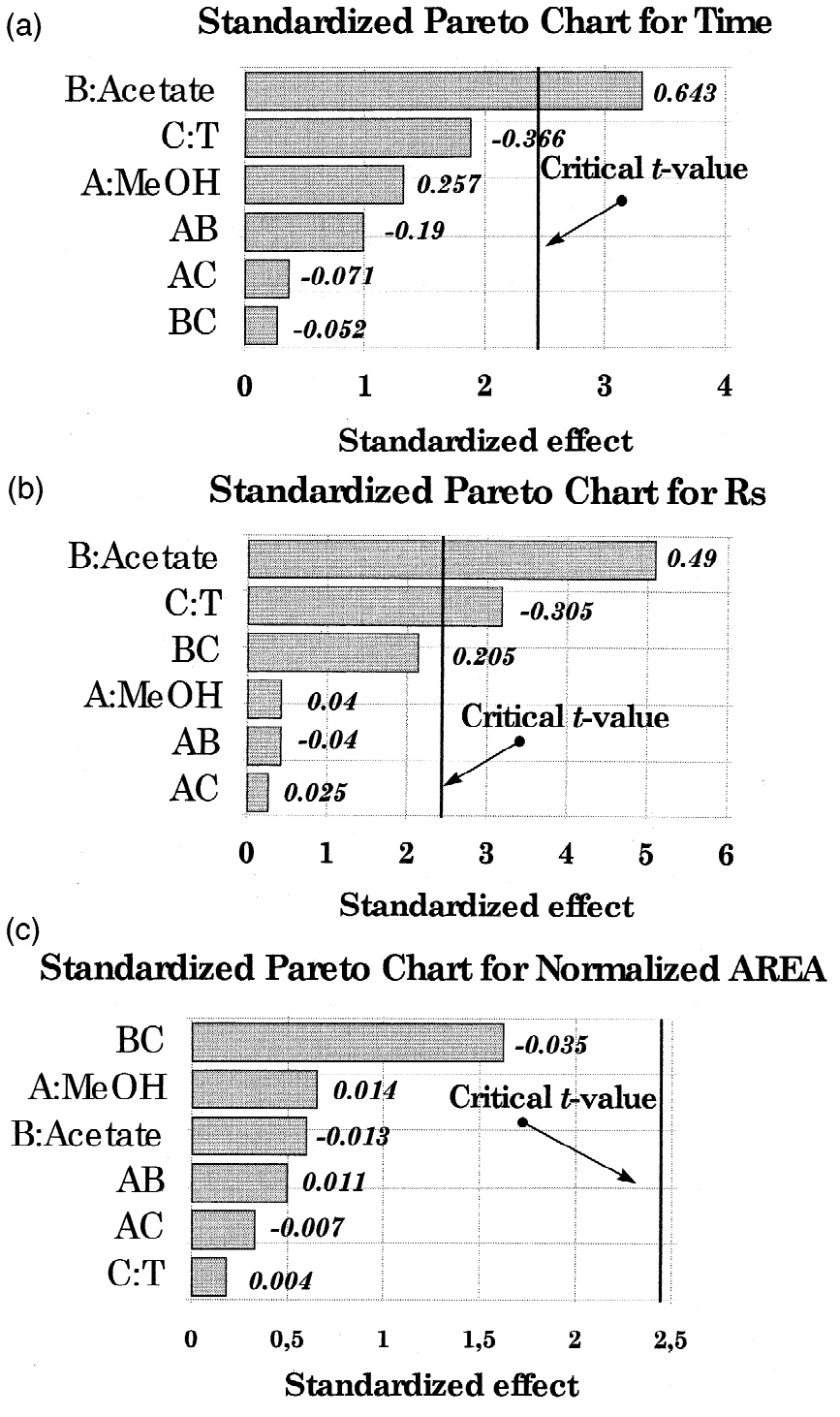

Fig. 6. Standardized Pareto charts, representing the estimated effects of parameters (*A*, *B*, *C*) and parameter interactions (*AB*, *AC*, *BC*) on (a) separation time, (b) resolution between indoprofen and ibuprofen and (c) indoprofen normalized peak area.

which has a positive effect on the response. This means that the separation time will increase with an increasing amount of ammonium acetate. Fig. 6b shows that both ammonium acetate concentration and temperature have a significant effect on the resolution between indoprofen and ibuprofen.

Fig. 6c shows that none of the investigated electrophoretic parameters have a significant effect on the normalized peak area. It is noteworthy that for the three responses, the methanol percentage is insignificant and that no important interaction between parameters is observed. It can be concluded that appropriate control of the separation temperature and use of air-tight vials are efficient in avoiding solvent evaporation during the electrophoresis process.

4. Conclusion

A simple, efficient and selective NACE method is reported for the analysis of several NSAIDs. Organic solvent composition as well as electrolyte nature and concentration have a significant effect on the electrophoretic mobility of investigated compounds. Separation behavior was considerably affected by the nature of the electrolyte cation. Changes in migration orders also occurred, which can be mainly explained by differences in solvation and apparent pH* values, as well as possible complex formation between electrolyte cations and the anti-inflammatory drugs. The method robustness was statistically examined by means of a full factorial design. In this context, the influence of relevant electrophoretic parameters on qualitative as well as quantitative responses was studied. The method was not robust with respect to ammonium acetate concentration as well as separation temperature and thus a careful attention has to be paid during the method application. Finally, the main advantage of this method resides in its MS compatibility which is due to the absence of non-volatile additives, generally used to enhance the selectivity of closely related compounds in aqueous media.

References

[1] P.D. Grossman, J. Colburn (Eds.), Capillary Electrophoresis – Theory and Practice, Academic Press, San Diego, CA, 1992.
[2] G.H. Janini, Chromatographia 35 (1993) 497–502.
[3] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801–1807.
[4] A.T. Balchunas, M.J. Sepaniak, Anal. Chem. 60 (1988) 617–621.
[5] S. Cherkaoui, L. Mateus, P. Christen, J.-L. Veuthey, Chromatographia 46 (1997) 351–357.
[6] S. Fujiwara, S. Honda, Anal. Chem. 59 (1987) 487–490.
[7] Y. Walbroehl, J.W. Jorgenson, Anal. Chem. 58 (1986) 479–481.
[8] I.E. Valko, H. Siren, M.L. Riekkola, LC·GC Int. 3 (1997) 190–196.
[9] S.H. Hansen, J. Tjornelund, I. Bjornsdottir, Trends Anal. Chem. 15 (1996) 175–180.
[10] K. Sarmini, E. Kenndler, J. Chromatogr. A 792 (1997) 3–11.
[11] M.L. Riekkola, S.K. Wiedmer, I.E. Valko, H. Siren, J. Chromatogr. A 792 (1997) 13–35.
[12] I. Bjornsdottir, J. Tjornelund, S.H. Hansen, Electrophoresis 19 (1998) 2179–2186.
[13] J. Tjornelund, S.H. Hansen, J. Biochem. Biophys. Methods 38 (1999) 139–153.
[14] I. Bechet, M. Fillet, Ph. Hubert, J. Crommen, J. Pharm. Biomed. Anal. 13 (1995) 497–503.
[15] M.G. Donato, E. Van Den Eeckhout, W. Van Den Bossche, P. Sandra, J. Pharm. Biomed. Anal. 11 (1993) 197–201.
[16] C.W. Maboundou, G. Paintaud, M. Berard, P.R. Bechtel, J. Chromatogr. B 657 (1994) 173–183.
[17] R. Weinberger, M. Albin, J. Liq. Chromatogr. 14 (1991) 953–972.
[18] S.H. Hansen, M.E. Jensen, I. Bjornsdottir, J. Pharm. Biomed. Anal. 17 (1998) 1155–1160.
[19] K. Altria, S.M. Bryant, Chromatographia 46 (1997) 122–130.
[20] M. Fillet, I. Bechet, V. Piette, J. Crommen, Electrophoresis 20 (1999) 1907–1915.
[21] F. Wang, M.G. Khaledi, J. Chromatogr. A 817 (1998) 121–128.
[22] S. Cherkaoui, E. Varesio, P. Christen, J.-L. Veuthey, Electrophoresis 19 (1998) 2900–2906.
[23] S. Cherkaoui, J.-L. Veuthey, Analusis 127 (1999) 765–771.
[24] M. Chiari, M. Nesi, P.G. Righetti, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996.
[25] H.J. Issaq, I.Z. Atamna, G.M. Muschik, G.M. Janini, Chromatographia 32 (1991) 155–161.
[26] S.P. Porras, I.E. Valko, P. Jyske, M.L. Riekkola, J. Biochem. Biophys. Methods 38 (1999) 89–102.
[27] J. Tjornelund, M.E. Jensen, S.H. Hansen, presented at the 13th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Granada, poster PA7.
[28] S. Cherkaoui, L. Mateus, P. Christen, J.-L. Veuthey, J. Pharm. Biomed. Anal. 21 (1999) 165–174.