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Capillary electrochromatography and capillary electrochromatography–electrospray mass spectrometry for the separation of non-steroidal anti-inflammatory drugs

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Abstract

In this study capillary electrochromatography (CEC) was utilized for the separation of ten non-steroidal anti-inflammatory drugs (NSAIDs). Experiments were carried out in a commercially available CE instrument using a packed capillary with RP-18 silica particles where the stationary phase completely filled the capillary. The mobile phase consisted of a mixture of ammonium formate buffer pH 2.5 and acetonitrile. Selectivity and resolution were studied changing the pH and the concentration of the buffer, the acetonitrile content mobile phase and the capillary temperature. The optimum experimental conditions for CEC separation of the studied drug mixture were found using 50 m*M* ammonium formate pH 2.5–acetonitrile (40:60) at 25°C. The CEC capillary was coupled to an electrospray mass spectrometer for the characterization of the NSAIDs. A mobile phase composed by the same buffer but with a higher concentration of acetonitrile (90%) was used in order to speed up the separation of analytes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Electrochromatography; Background electrolyte composition; Non-steroidal anti-inflammatory drugs

1. Introduction

Capillary electrochromatography (CEC), firstly shown by Pretorius et al. in 1974 [1] is a new powerful electrophoretic technique useful for the separation of a wide number of compounds belonging to different classes such as explosives, herbicides, pharmaceuticals, etc. [2].

Analytes separation can be performed in fused-silica capillaries either packed with typical chromatographic material or wall modified [2–4].

In CEC the separation principles of both chromatography and capillary electrophoresis are combined achieving high efficiency and peak separations. Due to the presence of a wide number of free silanol groups on the stationary phase, a strong electroosmotic flow (EOF), depending on the experimental conditions used, is generated by applying a relatively high electric field. The EOF is the driving force responsible for the movement of both the mobile phase and the analytes along the capillary column. According to the chromatographic principle the analytes can selectively partition between the stationary and the mobile phase resulting in different migration times [2,5]. Compared to liquid chromatography (LC) where the mobile phase moves with a parabolic flow profile, in CEC the EOF has a flat flow profile responsible for the high efficiency that can be achieved with this analytical method.

In CEC separated zones are usually recorded by

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using on-column UV detection and recently the combination of mass spectrometry (MS) with CEC was also shown [6–9]. MS is a specific, sensitive and universal detection system able to provide important informations concerning the mass and the structure of the analyzed compounds. The combination of MS with CEC can allow the on-column analysis of separated zones avoiding very difficult recoveries after electrophoretic runs.

In this paper the capillary was packed with RP-18 silica gel stationary phase and ten non-steroidal anti-inflammatory drugs (NSAIDs) were analyzed in order to study the effect of several experimental parameters on resolution, efficiency and retention factor. The analytes used in this investigation belong to a class of drugs widely employed for the treatment of several inflammatory diseases. Due to their different chemical structures (see Fig. 1) they are an interesting group of compounds for studying and better understand the CEC separation mechanism.

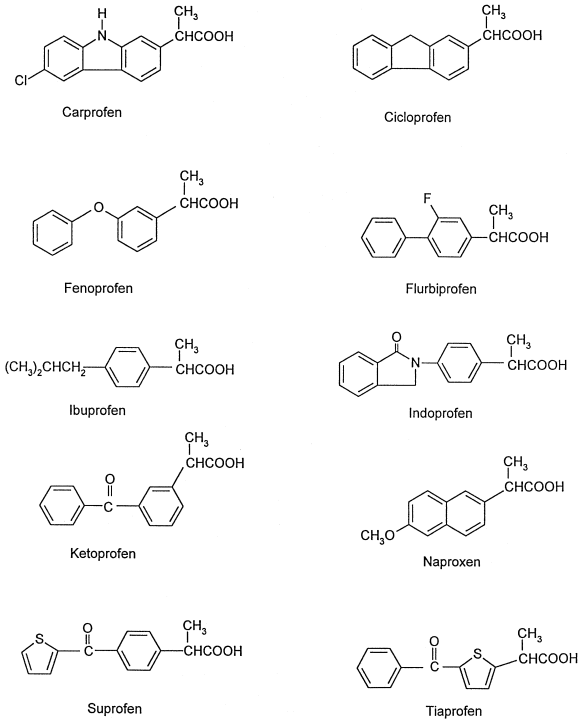

Fig. 1. Chemical structures of the studied non-steroidal anti-inflammatory drugs (NSAIDs).

Among the experimental parameters that can influence the CEC separation, we selected the buffer type, concentration and pH, the concentration of the organic solvent present in the mobile phase and the capillary temperature. After selecting the optimum experimental conditions CEC was coupled with electrospray mass spectrometry (ESI-MS) in order to characterize the separated NSAIDs.

2. Experimental

2.1. Reagents and chemicals

All chemicals used in this study were of analytical grade and employed without further purification. Ammonia solution (30%) and formic acid (99%) were purchased from Carlo Erba (Milan, Italy). Acetonitrile (ACN) and methanol (MeOH) were from BDH (Poole, UK). Carprofen, cicloprofen and suprofen were kindly supplied by Dr. Cecilia Bartolucci (Istituto di Strutturistica Chimica, CNR, Montelibretti, Roma, Italy). Ibuprofen, indoprofen, fenoprofen, ketoprofen, naproxen were purchased from Sigma (St. Louis, MO, USA). Tiaprofen (tiaprofenic acid) was supplied by Roussel-Uclaf (Paris, France). Standard stock solutions (1 mg/ml) were prepared in methanol and diluted with water to the desired concentrations prior to injection (0.025–0.05 mg/ml).

2.2. Instrumentation

An HP^{3D} automatic electrophoresis apparatus (Hewlett-Packard, Waldbronne, Germany), equipped with an UV–visible diode array detector (operated at 205 nm) and an air thermostating system, was used for both CEC and CEC–MS experiments. The packed capillary was positioned into the appropriate cartridge after removing the polyimide layer (about 0.5 cm) for on-line UV detection. When CEC was coupled with MS the capillary, packed for all the length (53 cm), was directly inserted with the outlet frit into the mass spectrometer interface (the UV measurement was not done).

2.3. Electrochromatography

CEC packed columns were laboratory prepared. The fused-silica capillaries 100 μm I.D. (375 μm O.D.) were purchased from Composite Metal Services (Hallow, UK) and packed with LiChrospher 100 RP-18 (5 μm) (Merck, Darmstadt, Germany). The fused-silica capillary was connected with one end to an HPLC column frit (temporary frit) and with the opposite side to a peek HPLC pre-column, containing the slurry, connected to a LC 10 HPLC pump (Perkin-Elmer). The slurry was prepared by adding 30 mg of stationary phase to 1 ml of methanol. The pre-column and part of the capillary were dipped into an ultrasonic bath in order to keep in solution the particles of the stationary phase. Methanol was pumped at ~2000 p.s.i. (1 p.s.i.= 6894.76 Pa) until the capillary was packed (35 cm). Then, after removing the slurry reservoir, double distilled water was pumped (~3000 p.s.i.) into the capillary for about 1 h.

An heating coil was used for the preparation of both the inlet and the outlet frits by sintering the C_{18} particles at ~600°C for 60 s. Detection window was therefore made at 8.5 cm from the outlet frit by polyimide removal at ~300°C for 30 s. After removing the temporary frit, the column was cut close to the inlet and outlet frits. The total length (completely packed) used in this study was 32 cm while 23.5 cm was the effective length. For CEC–MS experiments the capillary was 53 cm long and packed for all the length.

The packed capillary was equilibrated with the aqueous-organic mobile phase for 1 h by using the HP^{3D} instrument, applying 12 bar pressure at the inlet end of the capillary and then both pressure (12 bar) and voltage (25 kV) until a stable current and baseline signal were monitored (about 15 min).

The mobile phase used for the experiments was 50 m*M* formic acid, titrated to pH 2.5 with ammonia solution, and different concentrations of acetonitrile as organic solvent. CEC experiments were carried out applying 25 kV and 5 bar pressure at both ends of the capillary. Injection was done at the anodic end of the capillary by high-pressure application (12 bar for 30 s) followed by a background electrolyte (BGE) plug (12 bar, 12 s). The capillary temperature was maintained at 25°C.

2.4. Coupling electrochromatography with electrospray mass spectrometry

The CEC apparatus was coupled with an LCQ ion trap mass spectrometer (Finnigan, San Josè, CA, USA) through an electrospray interface (Finnigan). A mixture of 1% ammonia in water–methanol (30:70, v/v) was used as sheath liquid delivered by a syringe pump at a flow-rate of 5 μl/min. The negative ion mode was used for analyte mass detection.

3. Results and discussion

CEC is a separation technique particularly suitable for the analysis of neutral compounds, however it can provide efficient separation also for charged analytes including acidic ones [10–13]. For such compounds the selection of low buffer pH seems to be useful in order to obtain good separations by CEC. In fact in these experimental conditions the analyte dissociation is strongly reduced increasing the hydrophobicity of analytes and therefore maximizing the interaction with the stationary phase. Furthermore the self-mobility of the acidic compounds, opposite to the EOF at higher pH values, is not affecting the migration time. Thus based on our previous experience [12] we selected ammonium formate at pH 2.5 with the aim to suppress/minimize the dissociation of the studied NSAIDs and optimize a CEC method with a volatile buffer compatible for the coupling with the MS detector.

A 50 m*M* ammonium formate buffer at pH 2.5 containing acetonitrile as organic modifier was the BGE used for the preliminary CEC experiments. The capillary was pressurized at both inlet and outlet capillary ends at 5 bar and 25 kV was the applied voltage. Acetonitrile was selected as the organic modifier because in CEC it can generate a relatively high electroosmotic flow and possesses a low viscosity both useful to analyse the studied compounds in a relatively short time [14,15].

The effect of several physico-chemical parameters on the separation of ten NSAIDs (namely carprofen, cicloprofen, fenoprofen, ibuprofen, indoprofen, ketoprofen, naproxen, suprofen and tiaprofen) was studied and below discussed.

3.1. Effect of acetonitrile BGE concentration

In order to study the effect of organic modifier on the analytes separation by CEC different aliquots of acetonitrile, in the range 50–80% (v/v), were added to a 50 m*M* ammonium formate buffer at pH 2.5.

When the ACN buffer content increased a general decrease of the analytes separation was observed (Fig. 2). Particularly a loss of resolution was recognized for tiaprofen, ketoprofen and naproxen group of peaks (peaks 3, 4 and 5, respectively) and for fenoprofen, carprofen and flurbiprofen (peaks 6, 7 and 8, respectively). However at all the studied acetonitrile concentrations fenoprofen and carprofen comigrated. The increase of acetonitrile buffer content produced shorter analysis time according with both the increase of the electroosmotic flow mobility (from $7.52 \cdot 10^{-5}$ to $15.60 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ at 50 and 80% of acetonitrile, respectively) and the lower analyte retention on the stationary phase. In fact Fig. 3 reports the dependence of the logarithmic function of k' on acetonitrile concentration showing for all the compounds a decrease of the retention factor when the acetonitrile increased. The organic solvent content therefore influenced the analyte partitions between the stationary and the mobile phase.

It is noteworthy to remark that the interactions between the analytes and the stationary phase were not differently affected by the change of ACN concentration, in fact at all the studied organic solvent buffer content conditions the migration order of the compounds stayed the same.

As can be easily observed from the electrochromatograms depicted in Fig. 2 when the acetonitrile content increased sharper peaks were generally produced. This effect is important to obtain higher separation efficiency and higher method sensitivity for the increased analyte detectability. In fact the

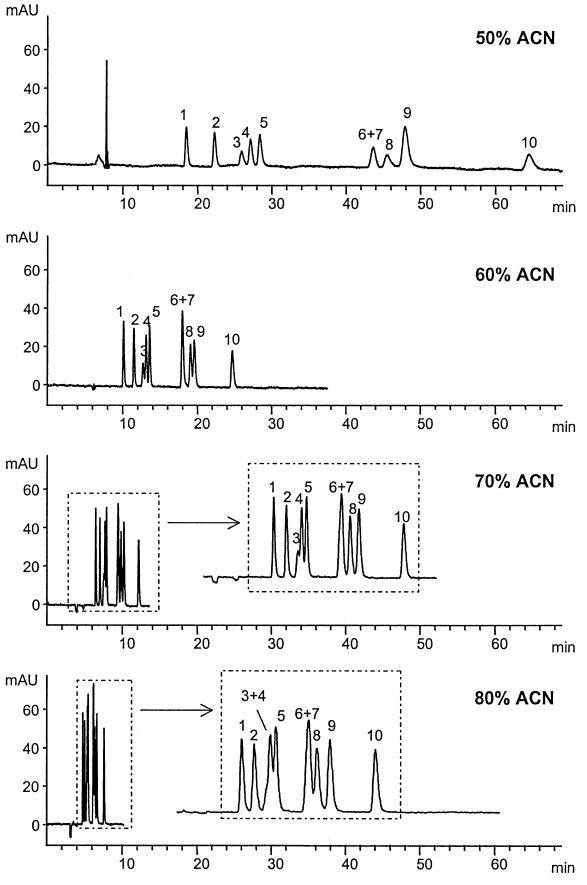

Fig. 2. Separation of ten NSAIDs by CEC using different concentrations of acetonitrile (ACN). Capillary 32 cm (effective length 23.5 cm)×100 μm I.D. packed with LiChrospher 100 RP-18 (5 μm); mobile phase 50 m*M* ammonium formate pH 2.5 and different concentrations of ACN; applied voltage, 25 kV, applied pressure (both sides) 5 bar; injection, 12 bar for 30 s of 0.025–0.05 mg/ml of each NSAIDs. For other experimental conditions see text. (1) Indoprofen, (2) suprofen, (3) tiaprofen, (4) ketoprofen, (5) naproxen, (6) fenoprofen, (7) carprofen, (8) flurbiprofen, (9) cicloprofen, (10) ibuprofen.

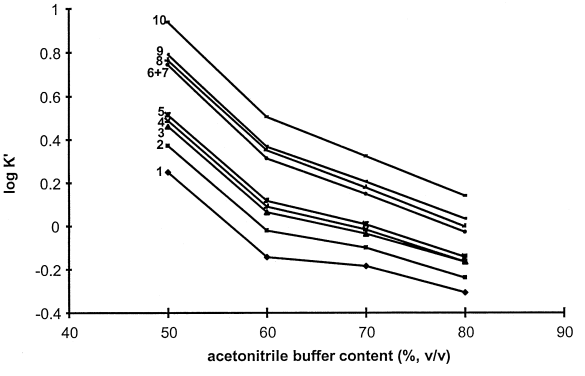

Fig. 3. Effect of acetonitrile concentration added to the buffer (50 m*M* ammonium formate pH 2.5) on logarithmic function of retention factor (log k').

raise of ACN concentration caused a general increase of efficiency (data not shown) recording, for most of the studied compounds, the highest value of number of theoretical plates per meter (in the range 70 115–100 259) at 70% of ACN.

From the reported results seems that 60% of acetonitrile provided the best compromise in terms of analyte separation, peak shape, method sensitivity and analysis time.

3.2. Effect of buffer pH

In order to study the effect of buffer pH on NSAIDs separation the analyte mixture was run in the pH range of 2.5–4.5 with 50 m*M* ammonium formate buffer–acetonitrile. The increase of pH from 2.5 to 4.5 produced a noticeable increase of the analysis time, which can be explained according to the analytes charge (negative) responsible for a self-mobility of studied compounds opposite to the electroosmotic flow. pH values higher than 4.5 were not investigated due to the too long analysis time already recognized at pH 4.5 (40 min of total analysis time). At pH 3.5 the migration order of studied NSAIDs was the same than that observed at pH 2.5 with the exception of tiaprofen (peak 3)

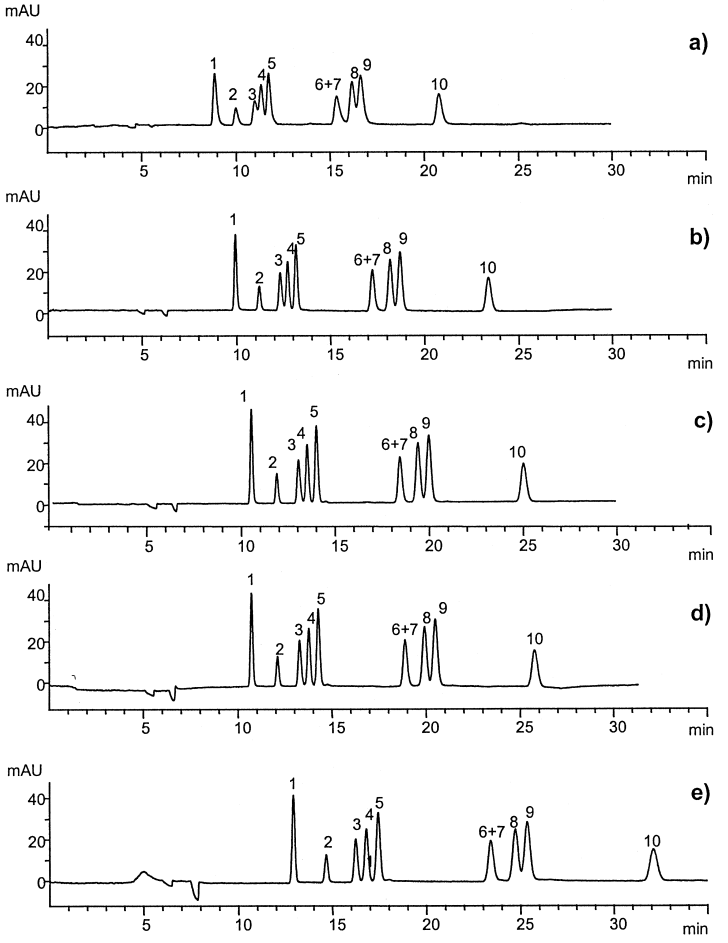

Fig. 4. Effect of buffer concentration on CEC separation of studied compounds. Mobile phase ammonium formate pH 2.5–ACN (40:60). Ammonium formate concentration (m*M*): (a) 25, (b) 50, (c) 75, (d) 100, (e) 150.

which migrated behind ketoprofen and naproxen compounds at pH 3.5. Furthermore the increase of pH also produced a loss of resolution for ketoprofen–naproxen and for flurbiprofen–cicloprofen (results not shown).

Therefore the lower was the pH the higher was the analytes separation and the lower the analysis time: at acidic pH the very reduced analytes ionization maximized the interaction of the analytes with the stationary phase producing the highest separation and hydrophobic discrimination.

3.3. Effect of buffer concentration

The influence of the buffer concentration on anti-inflammatory drugs separation was studied using a BGE composed by 60% of acetonitrile with different ammonium formate buffer concentrations at pH 2.5, in the range 25–150 m*M*.

As can be observed in Fig. 4 higher buffer concentration produced a general increase of the analytes migration times. A decrease of both resolution and efficiency (data not shown) were obtained at both the highest and the lowest buffer concentration investigated (25 and 150 m*M*, respectively). No noticeable differences were instead recognized at 50, 75 and 100 m*M* all providing good analytes separation in reasonable analysis time. This effect can be explained considering that the change of ionic strength or buffer concentration is modifying the double layer on the silica surface changing the EOF of the CEC system [16].

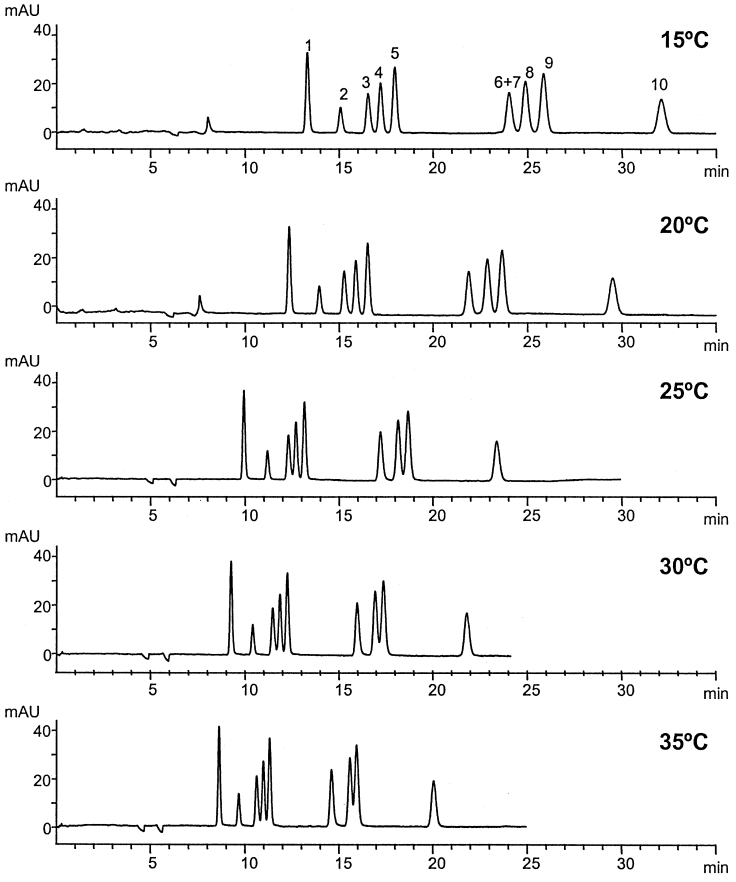

Fig. 5. Influence of capillary temperature on the CEC separation of the studied NSAIDs.

3.4. Effect of capillary temperature and column-to-column repeatability

Based on the results above reported the optimum BGE composition was 50 m*M* ammonium formate buffer at pH 2.5 containing 60% of acetonitrile. Under these operating conditions the effect of capillary temperature on the separation of NSAIDs was therefore investigated thermostating the capillary at 15, 20, 25, 30 and 35°C.

The electrochromatograms, reported in Fig. 5, show that the temperature influenced both the analysis time and the analyte separation. The highest separation was obtained at 15°C but in a relatively long analysis time. The increase of the temperature shortened the analysis time but a loss of resolution was observed for flurbiprofen and cicloprofen (peaks 8 and 9). According to the obtained data 25°C was selected as capillary temperature of the optimized method.

In order to test the column to column repeatability two capillaries were packed with the same silica material (effective and total lengths were the same) and used for CEC experiments for the separation of the selected NSAIDs. The analysis repeatability obtained is documented by the two electrochromatograms compared in Fig. 6.

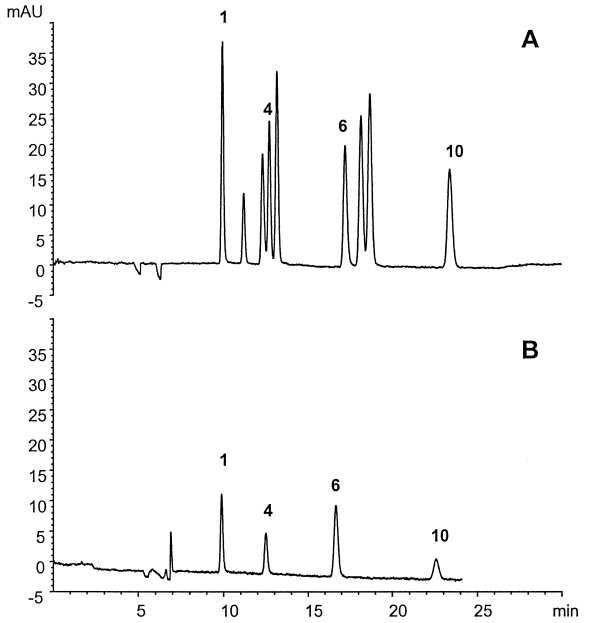

Fig. 6. Column-to-column packing repeatability. Analysis of a selected mixture of NSAIDs performed in columns A and B identically prepared in March and July 1999, respectively. (1) Indoprofen, (4) ketoprofen, (6) fenoprofen, (10) ibuprofen.

3.5. CEC–MS coupling

In order to couple the CEC technique with the mass spectrometer a longer capillary (53 cm) was packed for all the length, using the same packing procedure as previously described. The capillary was therefore inserted in the cartridge of the capillary electrophoresis apparatus and the outlet end was directly introduced into the mass spectrometer electrospray interface. Due to the longer length of the packed capillary stronger analytes retention in the stationary phase was expected under the same experimental condition. In fact using the optimized experimental conditions the analytes were strongly retained in the stationary phase and no peaks appeared after 50 min of analysis. For this reason the BGE composition was opportunely modified by adding a higher content of organic solvent (90% of acetonitrile). Fig. 7 shows the relative electrochromatogram and the mass spectra of the characterised peaks. Although a loss of resolution was observed for all the compounds in mixture the use of mass spectrometer in ion track mode allowed to identify each studied analyte. The mass spectrometer is therefore very helpful in characterising and identifying compounds in complex mixture providing a high degree of specificity.

4. Conclusion

Ten non-steroidal antiinflammatory drugs were successfully analyzed using capillary electrochromatography with a C_{18} reversed stationary phase. Almost all the compounds were baseline separated in mixture using a mobile phase composed of ammonium formate buffer at pH 2.5 containing 60% of acetonitrile. The influence of several physico-chemical parameters on the separation of the studied analytes was studied in order to understand the CEC separation mechanism. According to the partition chromatographic principle of reverse stationary

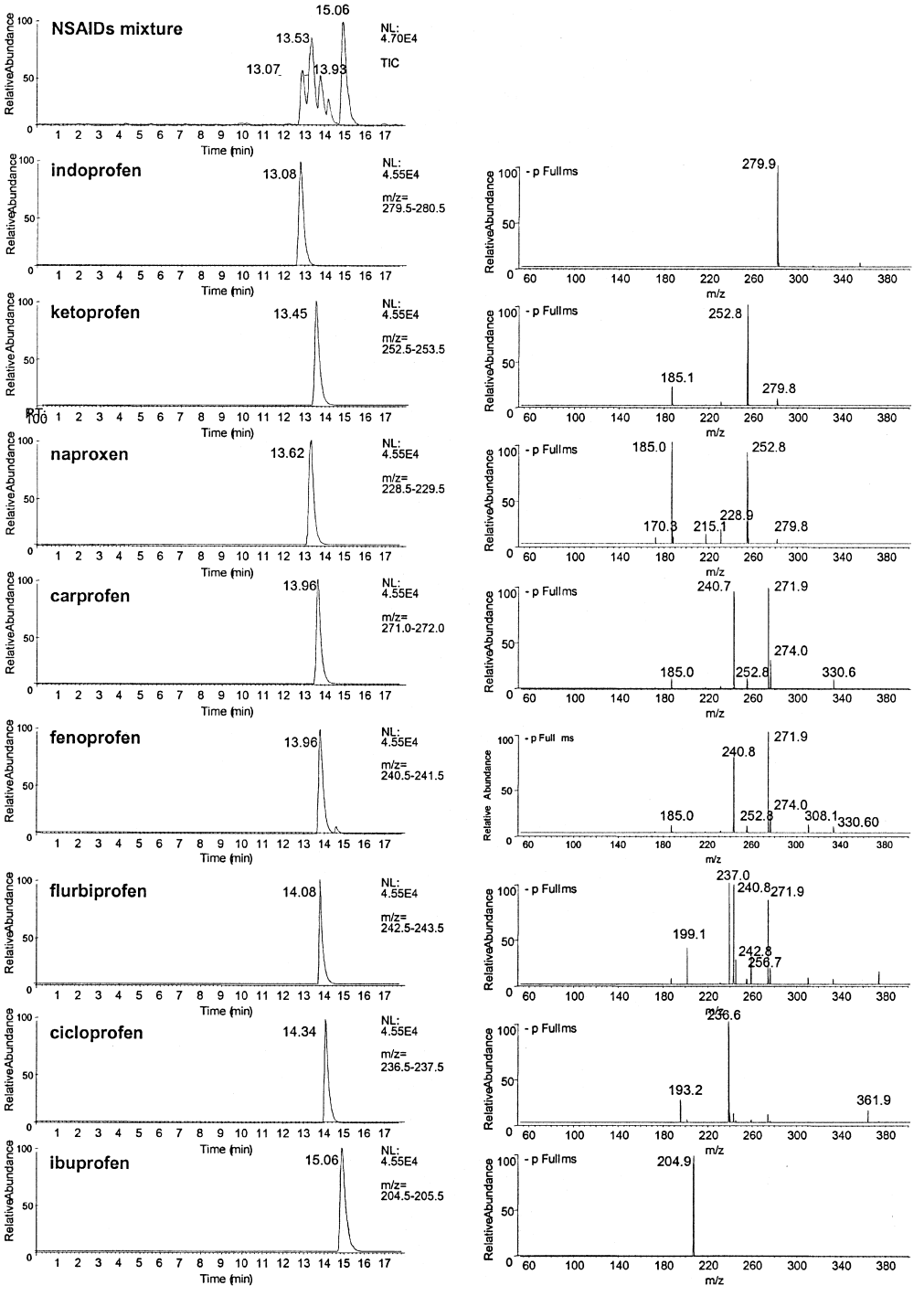

Fig. 7. CEC total ion mass track and single ion monitoring of a mixture of NSAIDs with the relative full scan mass spectrum. Capillary packed with C_{18}, total length, 53 cm; mobile phase 50 m*M* ammonium formate pH 2.5–ACN (10:90); applied voltage 30 kV. Detection, ESI-MS; polarity, negative; source voltage, 4.03 kV; sheath gas flow, 19.33 arbitrary units; sheath liquid, 1% ammonia–methanol (30:70), 5 μl/min, 12 bar inlet side pressure.

phase and to the analyte charge strong effects on the separation were found varying the acetonitrile content of the BGE and the buffer pH.

When the optimum conditions are found a strategy to shorten the analysis time is the use of pressure assisted CEC by applying the external pressure only at the inlet side of the capillary. As can be observed in Fig. 8 when pressure is applied only to the inlet side the analytes migrated faster (Fig. 8b) than with the pressure applied to both sides (Fig. 8a) without affecting their separation. Using the described set up (with the stationary phase present in the whole capillary) another strategy can be used in order to shorten the analysis time: the CEC run can be carried out reversing the polarity and using the shortest effective length. This is shown in Fig. 9a and b where a mixture of selected NSAIDs are separated in the shortest and longest effective length of the capillary, respectively. Here the analysis time is 3 times reduced and good resolution is observed.

If a non-complete separation of the analyte is obtained it was demonstrated that the coupling of CEC technique with the mass spectrometer detector could allow the characterization and detection of all the compounds in mixture by the ion track detection mode.

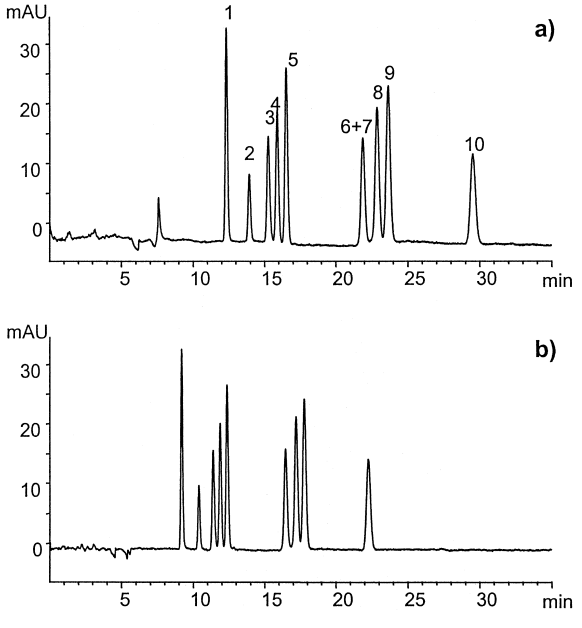

Fig. 8. Electrochromatogram of the NSAIDs separation with external pressure applied to (a) both sides (5 bar), (b) inlet side only (12 bar). Experimental conditions, 25 kV, 20°C. For other experimental conditions, see Fig. 2.

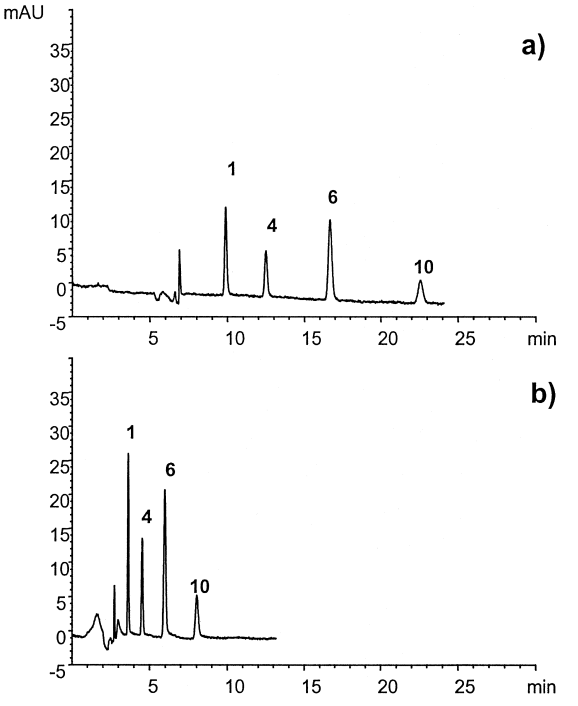

Fig. 9. Separation of selected NSAIDs by CEC by using two different effective lengths of the same capillary: (a) 23.5 cm, (b) 8.5 cm. Conditions 50 m*M* ammonium formate pH 2.5–60% ACN; total length of the capillary, 32 cm; applied voltage 25 kV. (1) Indoprofen, (4) ketoprofen, (6) fenoprofen, (10) ibuprofen.

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