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Combined liquid–liquid electroextraction and isotachophoresis as a fast on-line focusing step in capillary electrophoresis

E. van der Vlis, M. Mazereeuw, U.R. Tjaden*, H. Irth, J. van der Greef

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, Netherlands

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Abstract

Combined liquid–liquid electroextraction and isotachophoresis as a fast on-line focusing step in capillary electrophoresis is described. Owing to the limited conductivity of the organic phase, high electric field strengths can be applied, resulting in high migration rates. Liquid–liquid electroextraction is used to focus the analyte ions from a large organic sample into a small volume in the terminating buffer zone, just above the liquid–liquid interface. Simultaneously, isotachophoresis is used to focus the analyte ions between the terminating buffer and the leading buffer. A steady state is reached within a few minutes, as isotachophoresis starts with a small sample volume. On-line capillary zone electrophoresis is used for the separation of the analytes. Propantheline, neostigmine, salbutamol and terbutaline were used as model compounds, and crystal violet was used to visualize the process for tuning the system. Concentration detection limits of pure solutions down to 10^{-9}–10^{-10} mol/l for the model compounds were obtained using simple UV absorbance detection.

1. Introduction

Capillary zone electrophoresis (CZE) is a strong separation technique owing to its high efficiency, short analysis times and ease of operation. However, injection volumes in the low nanolitre range combined with a short optical path length (10–100 μm) make CZE a challenging field to be explored. Different attempts have been made to enhance the sensitivity in CZE by improving detection techniques and employing sample pretreatment prior to the CZE separation [1].

A favourable technique for on-line sample enrichment is isotachophoresis (ITP) [2–10]. The focusing time in ITP depends mainly on the concentration of constituents and related the electric field strength, the mobility of the analyte and the migration path length. Focusing times of more than 1 h are reported for very large sample volumes [11,12]. In such cases, the sample throughput is determined by the time required for sample handling, rather than the CZE run time. Therefore, long focusing times for large sample volumes still limit the applicability of ITP in bioanalysis.

Since the beginning of this century it has been known that by applying an electric field over the two phases in a liquid–liquid extraction system, the extraction rate of ionic analytes is increased [13]. Primarily this principle was used as a tool in chemical engineering for the enhancement of

* Corresponding author.

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mass transfer through the liquid–liquid interface [14–19]. This so-called electroextraction (EE) permits the rapid extraction of charged compounds from large volumes of organic solvents (up to several millilitres) owing to the extremely high local electric field strength. Further, high recoveries are obtained when exhaustive EE is considered. As the whole procedure is performed in a single capillary, the method can easily be automated.

EE is a sample enrichment technique that permits the rapid extraction of analytes from a large volume of organic solvent into a small buffer volume, just above the interface. On-line ITP is used to focus the analyte ions between the terminating buffer and the leading buffer. As EE yields a very small buffer volume containing the analyte ions, only a few minutes of ITP are needed to reach a steady state. Finally, on-line CZE is used for the separation of the analytes. In this paper, qualitative and quantitative aspects of the EE–ITP–CZE method are presented and discussed.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical-reagent grade. All aqueous solutions were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, USA). Triethylamine, ethyl acetate (EtAc), potassium hydroxide and acetic acid were obtained from Merck (Darmstadt, Germany), methanol from Biosolve (Barneveld, Netherlands), β-alanine from Aldrich (Steinheim, Germany), crystal violet (CV) from Janssen Chimica (Beerse, Belgium), and terbutaline, neostigmine (N) and propantheline (P) from Sigma (St. Louis, MO, USA). Salbutamol was kindly donated by TNO Zeist (Netherlands).

The leading buffer consisted of 50 mmol/l triethylamine solution adjusted to pH 5 with acetic acid–methanol (20:80, v/v). The terminating buffer consisted of 10 mmol/l β-alanine solution adjusted to pH 5 with acetic acid–methanol (95:5, v/v). Standard solutions of the cationic species were made in so-called saturated EtAc. For this purpose, 10 mmol/l β-alanine solution, adjusted to pH 5 with acetic acid, was extracted with EtAc. Standard solutions of the model compounds at a concentration of 10^{-2} mol/l were prepared in methanol–water (50:50, v/v). Methanol was used for further dilution of the standard solutions to a concentration of 10^{-4} mol/l. Finally, saturated EtAc was used for further dilution of the methanol solutions obtained.

2.2. Apparatus

Fig. 1 is a schematic representation of the experimental set-up described hereafter. A programmable injection system for CE (PRINCE; Lauerlabs, Emmen, Netherlands) with the possibility of pressurized and/or electrokinetic injection was used for injection and control of the procedure. The current was registered in the low-μA range. EE–ITP–CZE took place in an untreated 100-μm fused-silica capillary (SGE, Ringwood, Victoria, Australia) with a total length of 90 cm. At 70 cm from the capillary inlet a detection window was obtained by burning off the polyimide coating. On-capillary UV absorbance detection was performed at 200 nm, using a Spectra 100 UV–Vis absorbance detector (Spectra-Physics, Mount View, CA, USA). The signal was digitized using a laboratory-made 12-bit A/D converter operating at a sampling frequency of 20 Hz. The A/D converter was connected to a computer (Mega ST4; Atari,

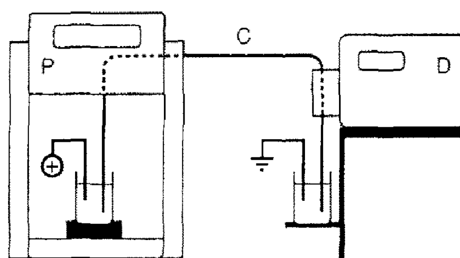

Fig. 1. Schematic representation of the EE–ITP–CZE set-up, consisting of a programmable injector (P), a 100-μm I.D. untreated fused-silica capillary (C) and a UV–Vis absorbance detector (D).

Sunnyvale, CA, USA) controlling the sampling frequency, collecting the data and handling the data using laboratory-written software.

2.3. EE–ITP–CZE procedure

Fig. 2 shows the different stages of the procedure and the positioning of the capillary and the electrode. The capillary is conditioned for 10 min daily with water, aqueous potassium hydroxide (0.25 mol/l), water and leading buffer. The 300-μl sample vials were made of glass. Platinum rod electrodes were used for both the anode and the cathode.

Step 1

A terminating buffer zone of approximately 15 mm is introduced hydrodynamically at 30 mbar for 18 s.

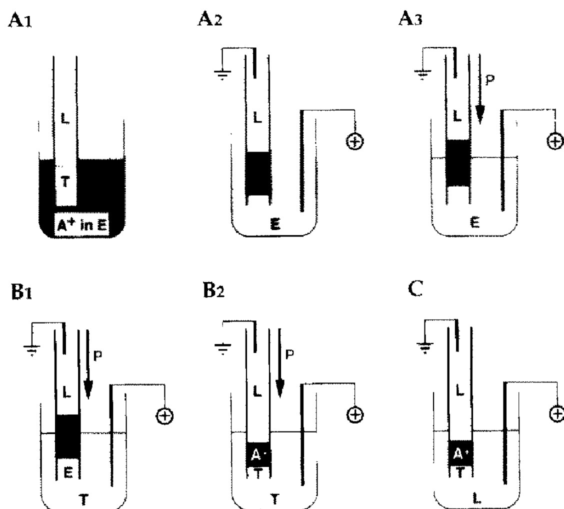

Fig. 2. Stepwise representation of the EE–ITP–CZE procedure. (A_1) The capillary, filled with leading buffer (L) and terminating buffer (T), is placed in the ethyl acetate (E) sample solution containing the cationic analyte molecules (A^+); (A_2) electroextraction (EE) is performed by applying a voltage; (A_3) during EE, using a pressure-induced counterflow (P) in the direction of the arrow, the sample solution is exhausted; (B_1) the analytes are focused by means of pressure-induced counterflow ITP, after the capillary is placed in the terminating buffer vial; (B_2) during ITP the remaining EtAc plug is pushed out of the capillary, while reaching the steady-state; (C) the capillary is placed in the leading buffer vial and CZE starts.

Step 2

The capillary inlet is placed in the sample vial containing the EtAc sample solution (A1). EE is started by applying a voltage of +10 kV for 1 min (A2), followed by an additional 9 min at +10 kV in combination with a 30-mbar pressure-induced counterflow to prevent more EtAc from entering the capillary (A3). The analyte molecules start to migrate through the interface between the organic phase and the buffer system and continue to migrate through the terminating buffer zone. Simultaneously, the analytes are focused between the terminating buffer and leading buffer.

Step 3

Next, the capillary inlet is placed in the terminating buffer vial. By applying a voltage of +15 kV for 1 min without applying a counterflow, the analytes that are still present in the terminating buffer zone are accelerated in the direction of the leading buffer, away from the interface between EtAc and the buffer system.

Step 4

ITP continues at +10 kV for 6 s. A 60-mbar pressure-induced counterflow is used to push the EtAc plug still present in the capillary towards the capillary outlet (B1).

Step 5

Until the current reaches 1.1 μA, ITP proceeds at +4 kV combined with a 40-mbar pressure-induced counterflow. This step usually takes less than 1 min (B2).

Step 6

CZE is started and performed in leading buffer [20] by applying a voltage of +30 kV (C).

3. Results and discussion

The anodic end of the separation capillary filled with leading buffer and a small plug of terminating buffer is placed in the organic sample solution (Fig. 2). By applying an electric field over the organic solvent and the buffer system a

mass transfer is induced. Analyte molecules of the same charge sign migrate from the organic solvent into the terminating buffer, in the direction of the oppositely charged electrode. On arrival in the terminating buffer zone, the analyte molecules continue to migrate towards the leading buffer. Thus, ITP is performed simultaneously and in conjunction with EE. Finally, the analyte molecules are focused between the terminating buffer and the leading buffer. While maintaining the steady state, the organic solvent that entered the capillary during EE is removed by using a pressure-induced counterflow. CZE is performed subsequently.

3.1. Theoretical aspects and optimization of electroextraction

Pure EtAc cannot conduct electric current and can therefore be regarded as an insulator. The conductivity of pure EtAc can be increased by saturation with an aqueous carrier electrolyte solution. As a consequence of the application of an electric field, transport of charge via the migration of ions originating from the aqueous phase takes place. The relationship between mass transfer and the electric current for an ionic species i is given by [21]

$$J_i = \frac{I_i}{Fz_i} = E\mu_i c_i S \qquad (1)$$

where J_i is the ion flux across a capillary with cross section S, F the Faraday constant, z_i the ion charge, I_i the contribution of each type of ionic compound to the total current, E the electric field strength, μ_i the ion mobility and c_i the ion concentration; the subscript i refers to any ion in solution. The total current (I_{tot}) is the sum of the contributions of all ionic compounds:

$$I_{\mathrm{tot}} = \sum_i I_i \qquad (2)$$

The relationship between the current and the transfer of ions through an interface with cross-section S can be described as [21]

$$I = SFE \sum_i z_i \mu_i c_i \qquad (3)$$

At low analyte concentrations, e.g., 10^{-8}–10^{-9} mol/l, no current was measured with the available equipment. Therefore, EtAc was saturated with a 10 mmol/l solution of β-alanine (pH 5), which acts as carrier electrolyte in order to conduct the current. This resulted in a significant increase in the amount of analyte extracted and an improvement in the overall reproducibility. Experiments in which β-alanine was replaced with crystal violet showed similar results with respect to analyte extraction recovery and reproducibility.

Compared with the buffer system, EtAc itself hardly induces a ζ potential and related to this hardly any electroosmotic flow (EOF). As a result of the EOF induced by the buffer system, EtAc is dragged into the capillary during EE. A counterflow is used during EE (step 3) to counterbalance the EOF in order to prevent further entry of EtAc into the capillary. To maintain a stable interface during the EE, a short EtAc zone should be present in the capillary inlet. However, when the EOF predominates over the counterflow, EtAc continues to enter the capillary. As the applied voltage (V) is constant, the electric current decreases gradually according to Ohm's law. The resistance of each zone is given by

$$R_{\mathrm{zone}} = \frac{L_{\mathrm{zone}}}{\pi r^2 \kappa_{\mathrm{zone}}} \qquad (4)$$

where L_{zone} is the zone length, r is the radius of the capillary and κ_{zone} is the specific conductivity in the zone. The total resistance over the three zones in the capillary with a constant radius is given by

$$R_{\mathrm{tot}} = \left(\frac{L_{\mathrm{E}}}{\kappa_{\mathrm{E}}} + \frac{L_{\mathrm{TB}}}{\kappa_{\mathrm{TB}}} + \frac{L_{\mathrm{LB}}}{\kappa_{\mathrm{LB}}}\right)\frac{1}{\pi r^2} \qquad (5)$$

Combining Eq. 5 with Ohm's law results in

$$I = \frac{\pi r^2 V \kappa_{\mathrm{E}}\kappa_{\mathrm{TB}}\kappa_{\mathrm{LB}}}{\kappa_{\mathrm{TB}}\kappa_{\mathrm{LB}}L_{\mathrm{E}} + \kappa_{\mathrm{E}}\kappa_{\mathrm{LB}}a + \kappa_{\mathrm{E}}\kappa_{\mathrm{TB}}[(1-a) - L_{\mathrm{E}}]} \qquad (6)$$

where L_{E}, L_{TB} and L_{LB} are the lengths of the EtAc zone, the terminating buffer zone and the leading buffer zone, respectively, and κ_{E}, κ_{TB}

and κ_{LB} are the specific conductivities of the EtAc zone, the terminating buffer zone and the leading buffer zone, respectively. In Eq. 6, L_{TB} is replaced by a, which equals 0.02 as the terminating buffer zone length is approximately 2% of the total capillary length (Section 2.3, step 1). The current, i.e., mass transfer, and the length of the organic solvent zone are related according to Eq. 6. Fig. 3 is a graphical representation of Eq. 6, each theoretical curve (A–E) representing a different specific conductivity of the organic solvent in relation to the ITP buffer system. An increase in the organic solvent zone length results in a decrease in the current. Fig. 3E is based on measured relative conductivity data. The relative conductivities for saturated EtAc, terminating buffer and leading buffer are 1, 1040 and 8640, respectively. A large difference in specific conductivity between the organic solvent and the terminating buffer strongly decreases the slope of the curve at short organic solvent zone lengths. Therefore, mass transfer is closely determined by the applied pressure-induced counterflow. As small differences in the organic solvent zone length affect the current dramatically, the mass transfer and the related reproducibility are influenced accordingly.

Fig. 4 shows the relationship between the pressure-induced counterflow and the peak area

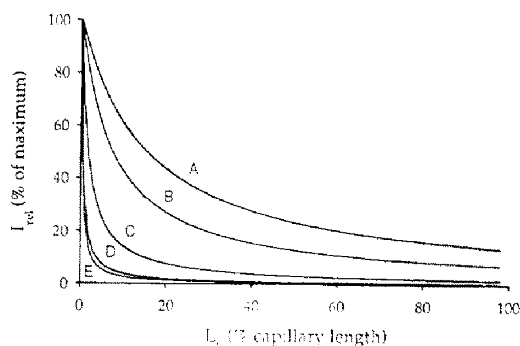

Fig. 3. Relationship between the current (I_{rel}) and the organic solvent zone length (L_E) during EE. The relative specific conductivities for the organic solvent zone, the terminating buffer zone and the leading buffer zone are as follows (κ_E, κ_{TB}, κ_{LB}), respectively: (A) 1000, 1040, 8640; (B) 500, 1040, 8640; (C) 100, 1040, 8640; (D) 10, 1040, 8640; (E) 1, 1040, 8640.

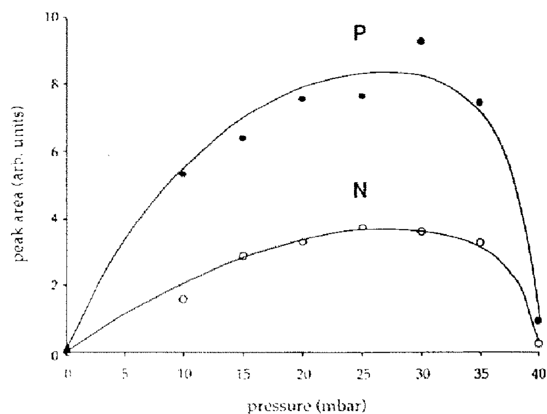

Fig. 4. Peak area vs. counterflow pressure using 300-μl samples of a 10^{-8} mol/l neostigmine (N) and propantheline (P) solution in saturated EtAc.

of neostigmine and propantheline obtained after EE–ITP–CZE of a 300-μl sample of a 10^{-8} mol/l mixture in saturated EtAc. The pressure used to induce a counterflow during EE at +10 kV (Section 2.3, step 3) is varied from 0 to 40 mbar. Three stages can be distinguished on each of the curves. In the first stage (0–5 mbar), the pressure-induced counterflow does not fully counterbalance the EOF. Hence EtAc is dragged into the capillary, resulting in a diminished mass transfer as explained above. In the second stage of the curve (15–35 mbar), a maximum is reached in which maximum mass transfer is obtained. Finally, above 35 mbar, the amount extracted is decreased dramatically as the EtAc zone together with the analyte molecules are pushed out of the capillary. At any given voltage during EE the pressure-induced counterflow should be chosen to compensate the EOF.

Based on the conductivity data mentioned above, the relationship of the electric field strengths in the three different zones (Fig. 5) can be calculated. The resistances of the three zones are related according to Eq. 4. Considering a constant current through the capillary applying a voltage of +10 kV over a 90-cm capillary, the electric field strengths in the EtAc zone, the terminating buffer zone and the leading buffer zone are 47250, 47 and 5 V/cm, respectively.

The number of ions [$N(t)$] of a single species i

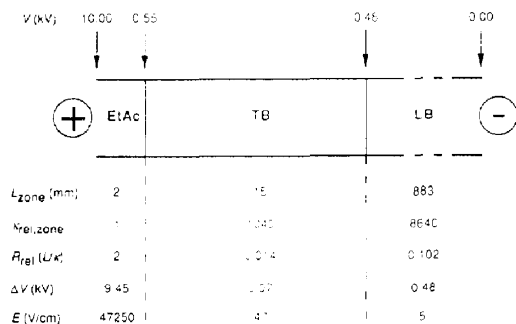

Fig. 5. Schematic representation of the ethyl acetate sample zone (EtAc), terminating buffer zone (TB) and leading buffer zone (LB) in a capillary, in which L_{zone} represents the zone length, $\kappa_{rel,zone}$ the relative conductivity of the zone, R_{rel} the relative resistance, ΔV the voltage drop over the zone and E the electric field strength in the zone.

extracted from the EtAc sample during time t is described by

$$N(t) = SE_{EtAc}\mu_i C_i t \tag{7}$$

This equation is similar to the relationship described by Chien and Burgi [22], considering a zero EOF, a constant EtAc zone length (2 mm) and a constant electric field strength over the EtAc zone, E_{EtAc}.

During the EE, the ion concentration in the EtAc zone will decrease owing to mass transport, which consequently enhances the E_{EtAc}. However, the electric field strength in the EtAc zone cannot exceed 50 kV/cm, which is an increase of approximately 6%. This extreme situation will only occur when the EtAc sample is completely exhausted. This justifies the assumption that the electric field strength in the EtAc zone is independent of time and can be considered constant.

Fig. 6 shows the relationship between the peak area and EE time, obtained after EE of a 300-μl sample of a 10^{-8} mol/l crystal violet solution in saturated EtAc for 0, 1, 3, 6, 12 and 20 min. In the first part of the curve the relationship between EE time and amount extracted is linear, which is in agreement with Eq. 7. During EE the analyte concentration and also the concentration of background electrolyte decrease gradually. A plateau is reached when the total ion concentration in the organic phase approaches zero.

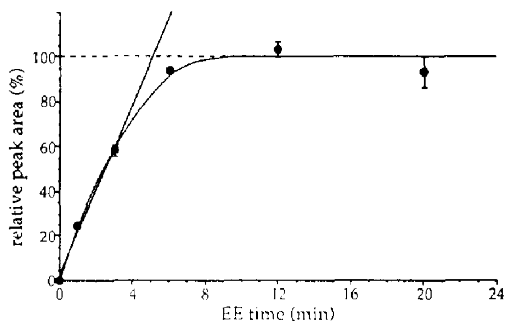

Fig. 6. Relative peak area vs. EE time using 300-μl samples of a 10^{-8} mol/l crystal violet solution in saturated EtAc ($n = 3$).

This was observed after 10 min of EE, applying a voltage of +15 kV combined with a 45-mbar pressure-induced counterflow.

In Fig. 7 the crystal violet recovery obtained after each of six repetitive extractions from an aliquot of 300 μl of 10^{-8} mol/l crystal violet solution in saturated EtAc at +15 kV and 45 mbar (Section 2.3, step 3) is presented. It shows that in the first EE cycle approximately 90% of the amount of crystal violet initially present in the sample is extracted from the organic phase. After five EE cycles no signal could be measured.

The amount of analyte extracted depends on several parameters, which simultaneously influence the electric current. These include local

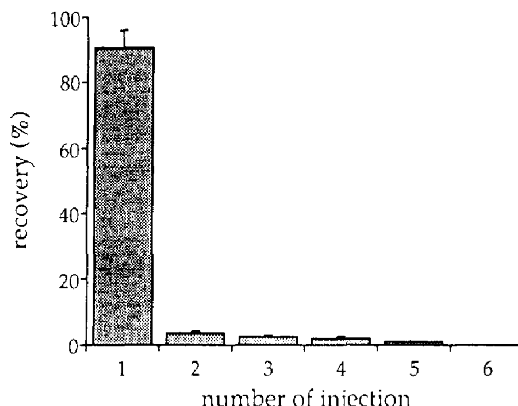

Fig. 7. Diagram of the recovery obtained after each of six repetitive extractions from a single 300-μl sample of a 10^{-8} mol/l crystal violet solution in saturated EtAc ($n = 3$).

differences in temperature, length of the EtAc plug in the capillary and variations in the EOF. These variables are difficult to control. During EE an alternating forward and backward movement of the interface was observed with 10^{-7} mol/l crystal violet in saturated EtAc. When the EtAc plug moves into the capillary, the electric field strength over the buffer zone diminishes and the EOF will be decreased accordingly. A steady state will be reached when the EOF is fully compensated by the pressure-induced counterflow. Fluctuations in the interface position in the capillary could be a consequence of an inhomogeneous distribution of anions and cations across the phase boundary [17]. Owing to electrophoresis, local accumulation of ions with the same charge occurs. The electric field over the capillary may induce an electrophoretic movement of the interface to the oppositely charged electrode. When the local accumulation of ions is neutralized, owing to repulsion and electromigration, the pressure-induced counterflow will predominate over the EOF. The capillary contents will be forced in the opposite direction hydrodynamically, until ions of the same charge sign accumulate again. Then the cycle starts all over.

The applied electric field strength has a significant influence on both the amount extracted and reproducibility. High electric field strengths will increase the amount of analyte extracted, but also disturb the interface and consequently affect the EE negatively. With 10^{-7} mol/l crystal violet in saturated EtAc a visible distortion and eventually segmentation of the interface zone in the capillary were observed using a voltage above +15 kV. In addition, a poorly conducting EtAc sample showed an identical distortion. This is in accordance with Thornton's theory, which suggests that droplet formation is enhanced in a system consisting of two immiscible phases that differ widely in conductivity [19].

In exhaustive EE the position and the geometry of the electrode with respect to the capillary inlet influence the EE recovery. Local exhaustion of the solution results in a dramatic decrease in conductivity and consequently a decrease in the ion transport in this section. The driving force behind analyte transport towards the exhausted section in the organic sample solution is determined by the concentration difference between the exhausted and the surrounding section, according to Fick's law. Important parameters are the viscosity of the organic solvent, the diffusion coefficient and diffusion path length, the vial geometry and the sample volume. The effects of these parameters are more pronounced when using large sample volumes for EE. EE of 3 pmol of crystal violet from a 300-μl saturated EtAc sample results in a three times larger peak area compared with the EE of 3 pmol of crystal violet from a 4-ml saturated EtAc sample with an equal EE time.

3.2. Quantitative aspects

Quantitative aspects of EE–ITP–CZE were investigated by generating calibration plots of crystal violet in the concentration range 10^{-10}–10^{-8} mol/l and of propantheline, neostigmine, salbutamol and terbutaline in the concentration range of 10^{-9}–10^{-7} mol/l. A typical calibration plot is characterized by $y = 1.5 \cdot 10^{12}x + 99$ ($r^2 = 0.999$), where y represents the peak area (arbitrary units) and x the analyte concentration (mol/l). The relative standard deviation (R.S.D.) of six extractions of a single batch of 10^{-8} mol/l crystal violet solution, without capillary conditioning between the runs, is 11.0%. Conditioning of the capillary between subsequent analyses shows no significant difference in reproducibility; the R.S.D. is 10.5% ($n = 6$).

Based on a signal-to-noise ratio of 3, the limit of detection (LOD) for EE–ITP–CZE of a 300-μl sample of crystal violet was 10^{-10} mol/l. The LOD for both neostigmine and propantheline was $5 \cdot 10^{-10}$ mol/l and for salbutamol and terbutaline 10^{-9} mol/l. Noise was measured peak-to-peak.

3.3. Application of EE–ITP–CZE

In Fig. 8, a comparison is made between (A) EE–ITP–CZE and electrokinetic injection CZE electropherograms from (B) water and (C) terminating buffer. Aliquots of 300 μl of a 10^{-8}

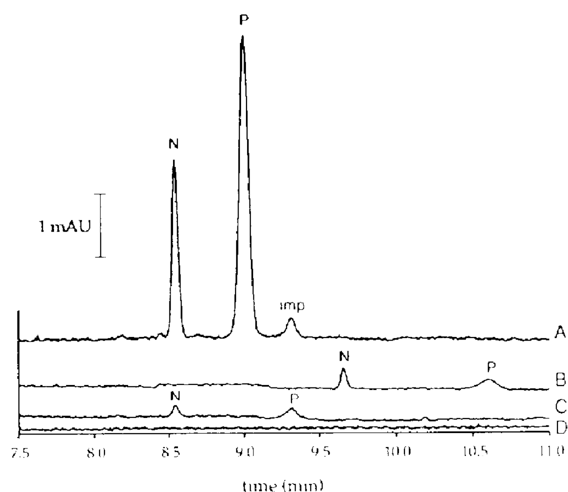

Fig. 8. Electropherograms obtained after (A) EE–ITP–CZE of a saturated EtAc solution, (B) ITP–CZE of a terminating buffer solution, (C) stacking of an aqueous solution and (D) electrokinetic injection for 20 s at +10 kV of 10^{-8} mol/l neostigmine (N) and propantheline (P) from a 300-μl sample. Electrokinetic sample injection, as used to obtain electropherograms A–C, was performed at +10 kV using an 8-mbar pressure-induced counterflow for 10 min. The peak marked "imp" is related to a chemical impurity. More experimental details are given in the text.

mol/l mixture of neostigmine and propantheline were used. Counterflow ITP [9,23] for 10 min at +10 kV combined with an 8-mbar pressure-induced counterflow was used to inject the analytes from a terminating buffer and water solution. Further, Fig. 8D shows an electropherogram obtained after an electrokinetic injection at +10 kV for 20 s from a 300-μl sample of a 10^{-8} mol/l mixture of propantheline and neostigmine in water. Owing to the different conditions in the three stacking procedures (Fig. 8A–C), different migration times of neostigmine and propantheline occur. A critical part of the EE procedure with respect to efficiency and resolution is the ITP step. A short ITP step results in poor efficiency and resolution, owing to the relatively long analyte zone prior to CZE. An optimum situation is obtained by increasing the duration of the ITP step until the steady-state situation is reached. Analyte recoveries in the counterflow ITP–CZE analyses from terminating buffer and water were significantly lower (approximately 10–30 times) than in the EE–ITP–CZE analyses from saturated EtAc, which can be explained by the difference in the conductivities and related large differences in the electric field strength.

Application of EE–ITP–CZE to the analysis of real samples containing high concentrations of matrix ions requires an additional sample clean-up step. However, this applies with any electrokinetic sample introduction technique, as discrimination between matrix ions and analyte ions during sample introduction occurs. Future research will be devoted to this subject.

4. Conclusions

It has been demonstrated that electroextraction in combination with isotachophoresis is a fast and effective technique for the on-line focusing of very large samples in capillary electrophoresis. Owing to the high concentrating power of electroextraction, the analytes are extracted in a small sample volume, just above the liquid–liquid interface, prior to ITP. As a result, ITP takes only a few minutes. Application of EE–ITP–CZE yields 10–30-fold higher sensitivities for the model compounds during equal time periods from equal sample volumes compared with sample stacking using a hydrodynamic counterflow. However, a requirement for the use of EE is that the analytes of interest should be dissolvable in the organic solvent used. In addition, a background electrolyte has to be added in order to obtain electric current, i.e., mass transfer.

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