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Combined liquid–liquid electroextraction–isotachophoresis for loadability enhancement in capillary zone electrophoresis–mass spectrometry

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Abstract

Combined liquid–liquid electroextraction (EE) and isotachophoresis (ITP) as a fast on-line focusing step in capillary zone electrophoresis (CZE) prior to electrospray mass spectrometric (ESP-MS) detection is described. Very high electric field strengths can be applied owing to the low conductivity of the organic phase, which results in high migration rates. Liquid–liquid electroextraction enables the fast extraction of analyte ions into a small buffer volume, whereas ITP is used to focus the analytes away from the liquid–liquid interface. As a result, ITP starts with a small sample volume containing the extracted ions. After reaching the steady state within several minutes, CZE separation follows. Clenbuterol, salbutamol, terbutaline and fenoterol were used as model compounds. Concentration detection limits of pure solutions down to $2 \cdot 10^{-9}$ mol/l for clenbuterol, salbutamol and terbutaline, and $5 \cdot 10^{-9}$ mol/l for fenoterol have been achieved using on-line EE–ITP–CZE–ESP-MS.

1. Introduction

The coupling of capillary zone electrophoresis (CZE) to mass spectrometric detection (MS) is advantageous as it combines the high separation power of CZE and the high selectivity of MS detection. However, sample loadability and concentration sensitivity in CZE still limit its use as a separation technique prior to MS detection. Different attempts have been made to enhance sensitivity in CZE by employing sample concentrating techniques prior to the CZE separation, including chromatographic and isotachophoretic preconcentration [1].

Isotachophoresis (ITP) has proven to be a very useful technique for sample enrichment on-line with CZE [2–11]. As the focusing time in ITP is determined mainly by the concentration of constituents and related to this the electric field strength, the mobility of the analyte and the migration path length, the time needed for the concentration of very large sample volumes is generally longer than 1 h [12,13]. Such long focusing times are undesirable in routine analyses.

Another approach is the use of liquid–liquid extraction as an off-line sample-handling technique prior to CZE [14]. Low detection limits are obtained using chloroform for the extraction of doxorubicin from plasma. A back-extraction to 5 mmol/l phosphoric acid resulted in an extract of low ionic strength compared to the CZE buffer. As a result, analyte stacking during electrokinetic sample injection occurred.

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For several decades it has been known that by using an electric field in liquid–liquid extraction processes on an industrial scale the mass transfer in the extraction of ionic compounds is increased [15–21]. So far, the use of this so-called electroextraction has been limited to bulk processes only. Recently, this principle has been adapted and applied to a miniaturized scale in combination with CZE [22]. The on-line use of combined EE–ITP enabled the fast extraction of charged compounds from large volumes of organic solvents (up to several millilitres) into a 100-μm I.D. fused-silica capillary owing to the extremely high local field strength. Automation could easily be accomplished, as the whole procedure was performed in a single capillary.

This paper describes the use of on-line EE–ITP as a novel focusing technique for the improvement of sample concentration detection limits in CZE–MS. Some β-agonists have been used as test compounds. After EE into the terminating buffer of the ITP system only a few minutes are required to reach the steady state.

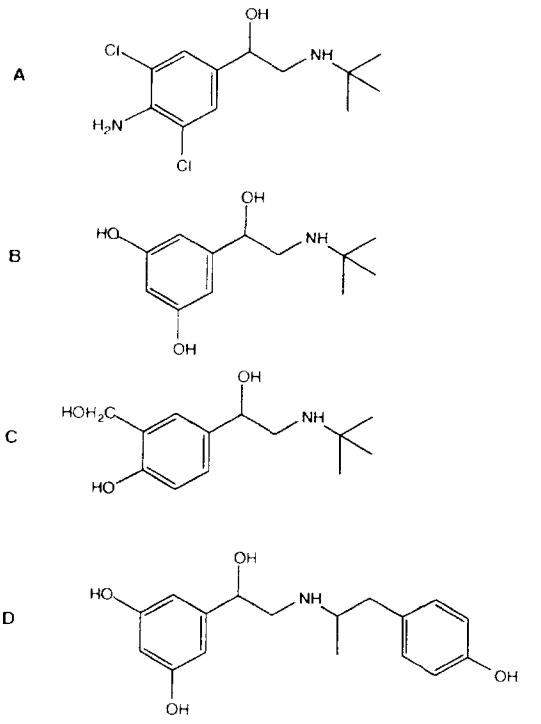

Fig. 1. Chemical structures of clenbuterol, M_r 276.1 (A); terbutaline, M_r 225.1 (B); salbutamol, M_r 239.2 (C); fenoterol, M_r 303.1 (D).

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade. Aqueous solutions were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, USA). Ethyl acetate (EtOAc), potassium hydroxide, ammonium acetate and acetic acid were obtained from Merck (Darmstadt, Germany), methanol from Biosolve (Barneveld, Netherlands), β-alanine from Aldrich (Steinheim, Germany), crystal violet from Janssen Chimica (Beerse, Belgium), clenbuterol hydrochloride, terbutaline sulphate and fenoterol hydrobromide (Fig. 1) from Sigma (St. Louis, MO, USA). Salbutamol sulphate (Fig. 1) was kindly donated by TNO Institute (Zeist, Netherlands).

The leading buffer and sheath liquid consisted of 50 mmol/l ammonium acetate solution adjusted to pH 5 with 3.3 mol/l acetic acid solution–methanol (20:80, v/v). The terminating buffer consisted of a 12 mmol/l β-alanine solution adjusted to pH 5 with 3.3 mol/l acetic acid solution–methanol (85:15, v/v). Standard solutions of the β-agonists were made in saturated EtOAc. For this purpose a 10 mmol/l β-alanine solution, adjusted to pH 5 with 3.3 mol/l acetic acid solution, was extracted with EtOAc. Stock solutions of the cationic species were made in water–methanol (50:50, v/v) to a concentration of 10^{-2} mol/l. Methanol was used for further dilution of the stock solutions to a concentration of 10^{-4} mol/l. Finally, saturated EtOAc was used for further dilution of the obtained methanol solutions.

2.2. Apparatus

Fig. 2 is a schematic representation of the experimental setup described hereafter. A programmable injection system (Prince, Lauerlabs, Emmen, Netherlands) with the possibility of regulating and fine-tuning both the pressure and

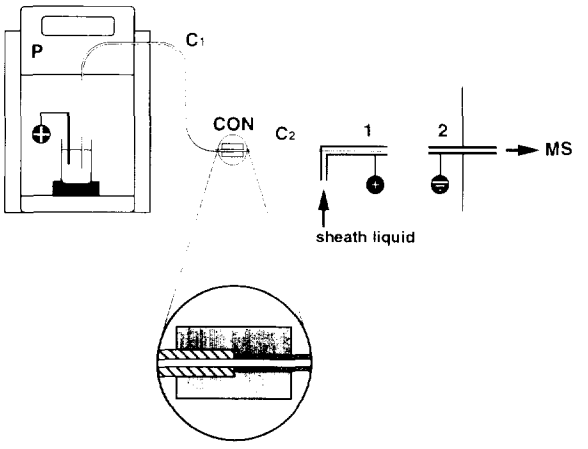

Fig. 2. Schematic representation of the EE–ITP–CZE–MS set-up consisting of a programmable injector (P), a 100-μm I.D., 375-μm O.D. untreated fused-silica capillary (C1) connected to a 100-μm I.D., 170-μm O.D. untreated fused-silica capillary (C2) by means of a laboratory-made transparent polyethylene connector (CON). Capillary C2 is inserted into the stainless-steel needle assembly of the electrospray needle (1) which is positioned opposite to the heated sampling capillary (2).

the voltage. The current was recorded in the low μA-range. EE–ITP–CZE took place in a 70 cm × 100 μm I.D. untreated fused-silica capillary (SGE, Ringwood, Vic., Australia) with an O.D. of 375 μm, which was coupled to a 20-cm length of untreated fused-silica capillary (BGB Analytik) with equal I.D., but with an O.D. of 170 μm. The coupling of the two capillaries was achieved using a laboratory-made connector of transparent polyethylene (Fig. 2). The 170-μm O.D. capillary was inserted into the stainless-steel needle assembly of the electrospray needle, after burning off several millimetres of the polyimide-coating of the end of the capillary (Fig. 3).

All experiments were performed on a TSQ-70 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) using a laboratory-made electrospray interface which was positioned in the thermospray ion source. Fig. 3 is a schematic representation of the interface. The electrospray needle was kept at +3 kV, whereas the heated sampling capillary was kept at ground potential. The temperatures of the sampling capillary and of the ion source were 175

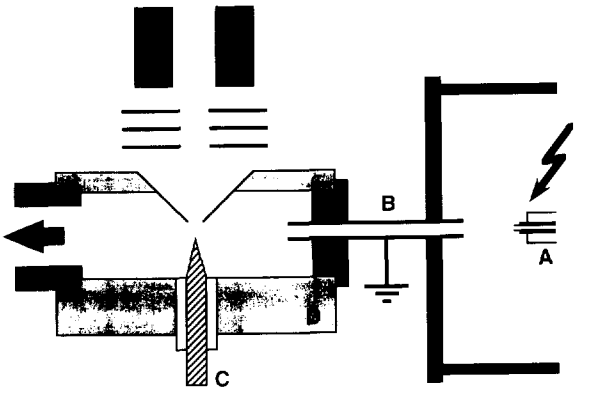

Fig. 3. Schematic representation of the laboratory-made electrospray interface showing the electrospray needle assembly (A), the heated sampling capillary (B), the conical-shaped repeller (C) and the thermospray ion source (D).

and 150°C, respectively. The repeller electrode was set at +30 V. A sheath flow of 1 μl/min, delivered by a Model 2400 syringe pump (Harvard Apparatus, Edinbridge, UK), was used for optimal electrospray conditions. Protonated analytes were monitored in selected-ion monitoring (SIM) mode, using 0.2 s per mass.

2.3. EE–ITP–CZE procedure

Fig. 4 shows the different stages of the procedure, as well as 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, respectively. Next, the capillary was filled with leading buffer. The 4-ml 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 at the anodic side of the capillary.

Step 2

The capillary inlet is placed in the sample vial containing the EtOAc sample solution (Fig. 4, A1). EE is started by applying a voltage of +10 kV during 1 min (Fig. 4, A2), followed by an additional 9 min at +10 kV in combination with

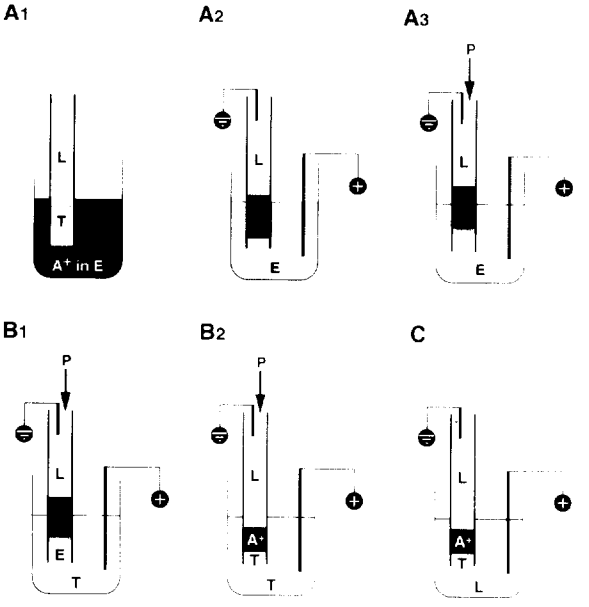

Fig. 4. Step-wise representation of the EE–ITP–CZE procedure. (A1) The capillary, filled with leading buffer (L) and terminating buffer (T), is placed in the ethyl acetate (E) sample solution containing the cationic analyte molecules (A^+). (A2) Electroextracton (EE) is performed by applying a voltage. (A3) During EE, using a pressure-induced counterflow (P) in the direction of the arrow, the sample solution is electroextracted. (B1) The analytes are focused by means of pressure-induced counterflow ITP, after the capillary is placed in the terminating buffer vial. (B2) During ITP the remaining EtOAc 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.

an 8-mbar pressure-induced counterflow to avoid entrance of EtOAc into the capillary (Fig. 4, 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 terminating buffer and leading buffer. Thus, ITP is performed simultaneously and in conjunction with EE.

Step 3

Next, the capillary inlet is placed into the terminating buffer vial. By applying a voltage of + 15 kV during 1 min using an 8-mbar pressure-induced 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 EtOAc and the buffer system.

Step 4

ITP continues at + 9 kV during 6 s. A 50-mbar pressure-induced counterflow is used to push the EtOAc plug still present in the capillary towards the capillary outlet (Fig. 4, B1).

Step 5

Until the current reaches 4.0 μA, ITP proceeds at + 9 kV combined with a 23-mbar pressure-induced counterflow to push out the greater part of the terminating buffer while maintaining a steady state. This step usually takes less than 1 min (Fig. 4, B2).

Step 6

CZE is started and performed in leading buffer [23] by applying a voltage of + 21 kV (Fig. 4C).

3. Results and discussion

3.1. Theoretical aspects of electroextraction–isotachophoresis

The power of EE–ITP is a result of the high local electric field strength in EtOAc. Pure EtOAc hardly conducts electric current. By saturating EtOAc with an aqueous electrolyte solution, the conductivity of the organic phase is slightly increased while the solubility of ionic species improves considerably. This results in a somewhat higher conductivity of the organic phase. Still, the conductivity is about 1000 times lower than that of the terminating buffer of the ITP system [22]. 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. As the conductivity of EtOAc compared to that of the buffer system is low, the greater part of the applied potential difference exists over the EtOAc zone. This results in a high local electric field strength which is the driving force of migration of ions (Eq. 1),

$$v = \mu \cdot E \quad (1)$$

in which v is the ion migration rate, μ the electrophoretic mobility and E the electric field strength. As can be calculated [22], the electric field strength in the EtOAc sample can reach 45 kV/cm when applying a voltage of + 10 kV over a 90-cm length fused-silica capillary with an I.D. of 100 μm. As the electric field strength is the driving force behind ion migration, the consequence of mass transfer in general is an electric current, usually referred to as driving current. All ionic compounds in an electric field contribute to the total current. Although the driving current during EE–ITP is generally lower than 0.8 μA using a voltage of + 10 kV, the extraction rate of the analyte ions from the organic solvent is high owing to the high electric field strength. Even ITP is already in an advanced stage after 10 min of EE with a driving current below 0.8 μA, owing to the short migration path length of 15 mm. As described earlier [22,24], the number of ions $[N(t)]$ of a single species i extracted from the organic phase during time t considering a zero electroosmotic flow and a constant electric field strength is

$$N(t) = S \cdot E_{\mathrm{EtOAc}} \mu_i c_i t \quad (2)$$

where c_i is the total concentration of ionic species i, E_{EtOAc} is the constant electric field strength in the EtOAc zone and S is the area of the liquid–liquid interface, which equals the cross section of the capillary. During EE, the electroosmotic flow is counterbalanced by a pressure-induced counterflow and can thus be neglected. As reported previously [22], in extreme situations the electric field strength in the EtOAc zone at the end of exhaustive EE will be 6% higher than the initial value. This justifies the assumption that the electric field strength is independent of time and can be considered constant.

During EE EtOAc enters the capillary as a result of the induced electroosmotic flow in the capillary. As EtOAc enters the capillary the electric current, and consequently the mass transfer, declines. Thus, a maximum mass transfer is obtained at a maximum driving current. Hence, a pressure-induced counterflow is applied to counterbalance the electroosmotic flow, hereby preventing the entrance of EtOAc into the capillary. In order to obtain reproducible EE performance, a stable liquid–liquid interface is required [22]. Therefore, EtOAc is allowed to enter the capillary during the first minute of EE. Then, a pressure-induced counterflow is applied to push the liquid–liquid interface back to several millimetres above the tip of the capillary during the remaining 9 min of EE. Initial optimization was carried out using the cationic dye crystal violet, allowing visual monitoring of the whole procedure.

3.2. EE–ITP–CZE–MS

Measurement of the protonated β-agonists was performed using a SIM method [23]. Fig. 5 shows mass electropherograms obtained after CZE–MS analysis of a hydrodynamically injected volume of 50 nl of a 10^{-5} mol/l solution of clenbuterol, salbutamol, terbutaline and fenoterol in water. When a voltage of + 26 kV was applied at the anodic electrode, an increase in the voltage of the electrospray needle tip from + 2.6 kV to + 5.1 kV was observed. This phenomenon was reported earlier [25]. The peak height of fenoterol, as can be seen from Fig. 5, is 2–3 times lower than the peak heights of the other β-agonists. This can be explained by the lower proton affinity and related to this a lower ionization efficiency of fenoterol. Coupling of the two fused-silica capillaries by means of a laboratory-made transparent polyethylene connector (Fig. 2) did not influence the separation efficiency significantly. Plate numbers up to 100 000 could be achieved, which is comparable to the separation efficiency reported in a similar setup using a single 100-μm I.D. capillary in combination with the same leading buffer and sheath liquid [23]. This enables the use of capillaries with a thick silica wall at the injector side of the CE system. When applying EE, the use of fused-silica capillaries with thick capillary walls is recommended as electric field strengths of up to 50 kV/cm can be obtained, which may easily lead

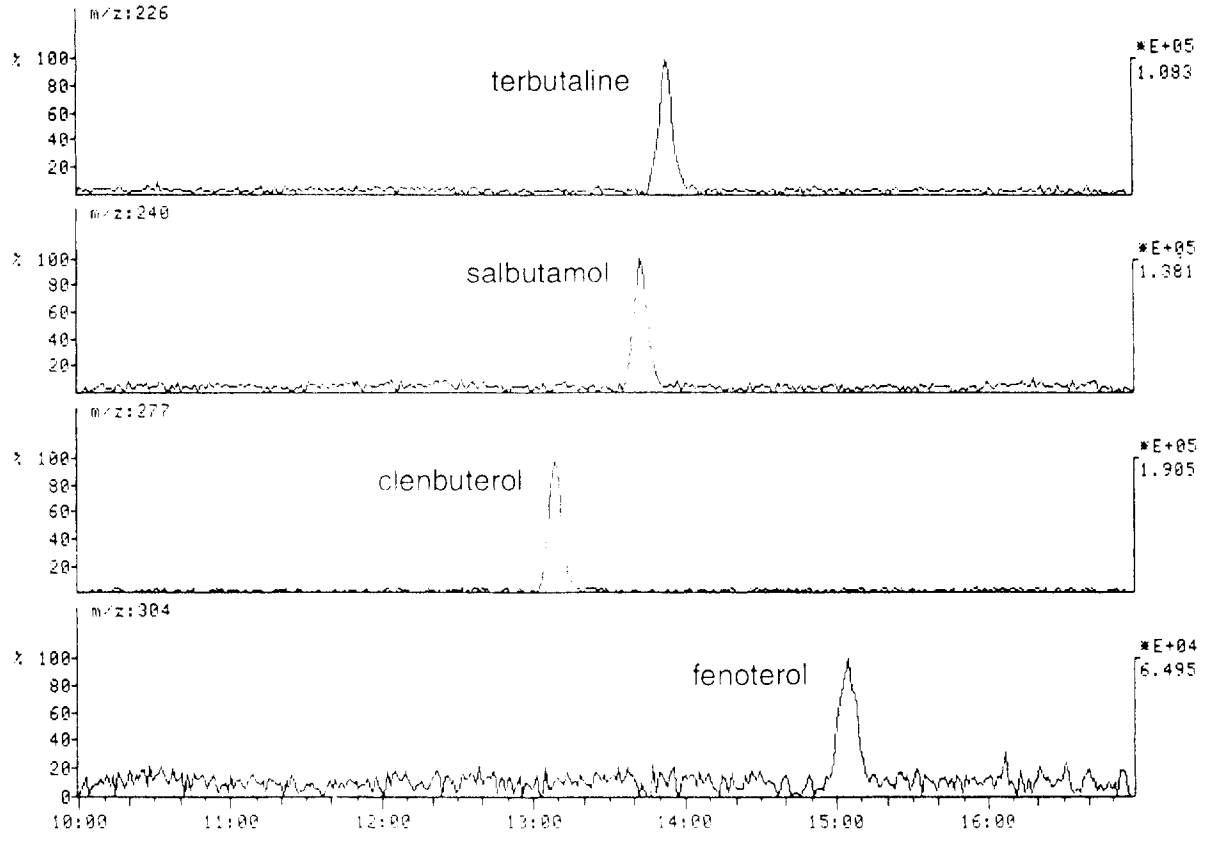

Fig. 5. Mass electropherograms of terbutaline (m/z 226), salbutamol (m/z 240), clenbuterol (m/z 277) and fenoterol (m/z 304) obtained after CZE–MS analysis of 50 nl of a 10^{-5} mol/l β-agonist mixture in water which was injected hydrodynamically.

to electric discharges through the capillary wall and subsequent damage of the capillary.

Fig. 6 shows mass electropherograms obtained after EE–ITP–CZE–MS of 4 ml of a $5 \cdot 10^{-9}$ mol/l β-agonists mixture in saturated EtOAc sample. Fenoterol has a migration time of 15 min 14 s. The peak at 13 min 11 s at m/z 304 is related to an impurity [23]. This was verified by EE–ITP–CZE–MS–MS of a 4-ml sample of a 10^{-7} mol/l agonist mixture in saturated EtOAc. Clenbuterol, salbutamol and terbutaline are characterized by water loss and subsequent loss of butene, whereas fenoterol is characterized by a fragment at m/z 135. This specific fragment from fenoterol is also obtained after performing collision-induced dissociation in a single quadrupole MS [26,27]. The peak at m/z 304 at 13 min 11 s did not show the specific m/z 135 fragment after collision. MS–MS fragmentation information could still be obtained after EE–ITP–CZE of a 4-ml sample at a concentration of 10^{-8} mol/l of the β-agonists in saturated EtOAc. Concentration detection limits for clenbuterol, salbutamol and terbutaline were $2 \cdot 10^{-9}$ mol/l, whereas $5 \cdot 10^{-9}$ mol/l could be obtained for fenoterol. In spite of the low recoveries (4–6%) the use of EE–ITP–CZE–MS (Fig. 6) clearly shows a gain in concentration sensitivity of about 2000 compared to CZE–MS (Fig. 5). From previous reports [22,23] it is demonstrated that the time needed (10 min) for complete EE and subsequent ITP-focusing of a 300-μl volume of a saturated EtOAc standard solution equals the time needed to focus an 870-nl aqueous standard solution using ITP–CZE. This clearly demonstrates the high concentrating power of EE.

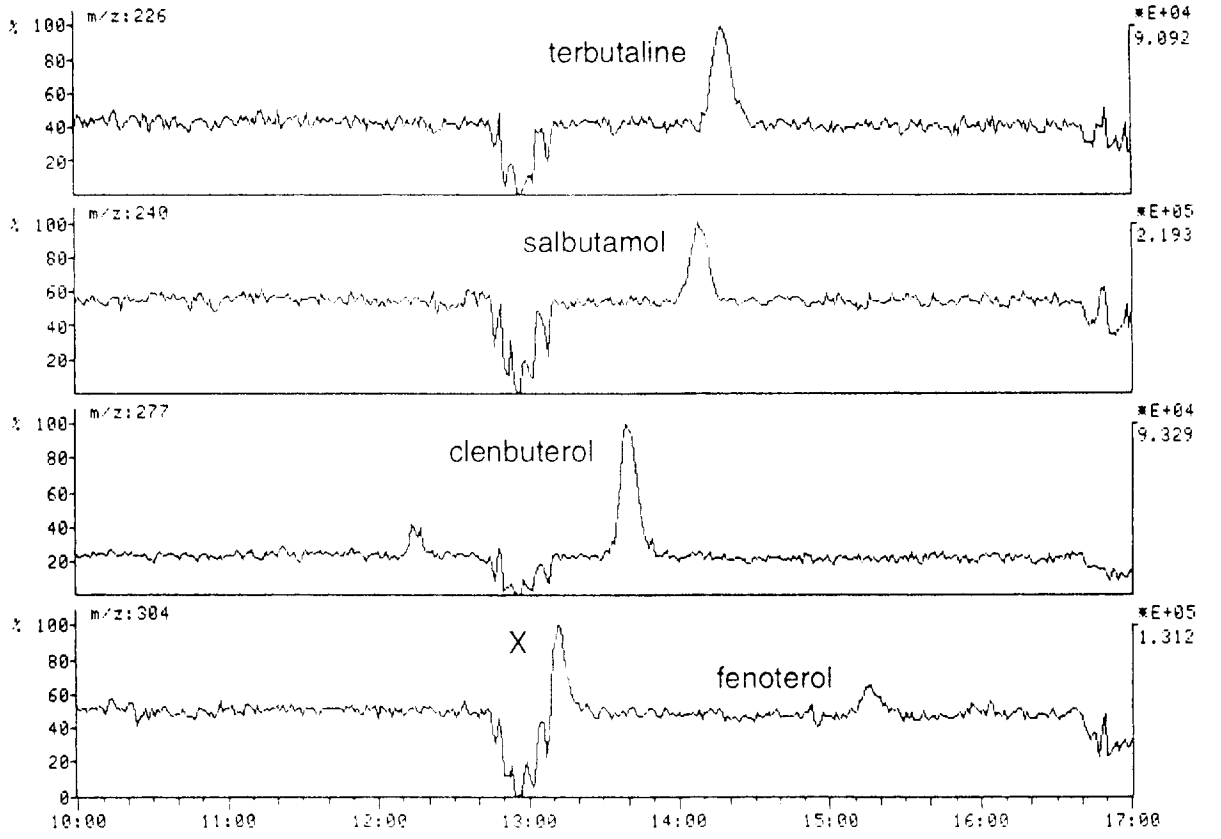

Fig. 6. Mass electropherograms of terbutaline (m/z 226), salbutamol (m/z 240), clenbuterol (m/z 277) and fenoterol (m/z 304) obtained after EE–ITP–CZE–MS analysis of 4 ml of a $5 \cdot 10^{-9}$ mol/l β-agonist mixture in saturated EtOAc. The peak indicated as "X" is related to an impurity.

Relative standard deviations (R.S.D.s) for EE of a 4-ml sample of a single batch of a $5 \cdot 10^{-9}$ mol/l mixture of the four β-agonists in saturated EtOAc are less than 12% ($n = 3$). When using sample volumes of more than 1 ml, the diffusion rate of the analyte ions in the EtOAc sample will be the limiting factor for the EE recovery. As EE of ions from the EtOAc sample occurs along the electric field lines, only the sample section between the tip of the capillary and the electrode is extracted. As a result, local exhaustion of only a small part of the total sample volume occurs. Diffusion of ions from the surrounding section into the exhausted section will eventually increase the EE recovery. However, diffusion is a rather slow process. Mixing of a sample of several millilitres during EE might be an option to increase the EE recovery within an equal period of time.

4. Conclusions

It has been demonstrated that electroextraction in combination with isotachophoresis is a fast technique for sample concentration in capillary electrophoresis–mass spectrometry, with great potential. The high concentrating power of electroextraction enables the extraction of ions into a small volume of terminating buffer prior to ITP. Therefore, only several minutes of ITP is needed to focus the extracted ions between terminating buffer and leading buffer. Application of EE–ITP–CZE prior to MS detection

results in a high concentration sensitivity, as it enables the use of very large sample volumes. Although only standard solutions were used, the results obtained clearly illustrate the concentrating power of EE–ITP. The application of EE–ITP–CZE–MS to the analysis of biological samples is under investigation.

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