ELSEVIER

Journal of Chromatography A, 879 (2000) 219–233

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

On-line isotachophoretic sample focusing for loadability enhancement in capillary electrochromatography–mass spectrometry

M. Mazereeuw, V. Spikmans, U.R. Tjaden*, J. van der Greef

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

Received 1 October 1999; received in revised form 21 February 2000; accepted 21 February 2000

Abstract

The use of isotachophoretic (ITP) sample focusing to improve the detection limits for the analysis of charged compounds in capillary electrochromatography (CEC) is described. A coupled-column set-up was used with a 220-μm inner diameter capillary, in which counterflow ITP focusing was performed, connected via a T-junction to a 75-μm inner diameter CEC capillary. As is illustrated, the use of ITP focusing resulted in a dramatic reduction of the sample concentration detection limits. To demonstrate the performance of the ITP–CEC combination, several cationic low-molecular mass compounds in a plasma and urine matrix are analysed using UV-absorbance and mass spectrometric detection. A linear calibration curve was constructed over three decades and detection limits in the low nmol/l range were found for academic samples, using UV-absorbance detection.

Keywords: Isotachophoresis; Electrochromatography; Sample handling; Salbutamol; Scopolamine; Fenoterol; Neostigmine; Crystal Violet

1. Introduction

In recent years, capillary electrochromatography (CEC) gained a large interest as an analytical separation technique, which for charged compounds is due to the combination of the electrophoretic migration principles and the liquid chromatographic interaction with a stationary phase [1–9]. Separation of uncharged compounds in CEC is solely based on differences in distribution ratios between the mobile and stationary phase, as in liquid chromatography (LC). So far, often uncharged compounds, like polycyclic aromatic hydrocarbons (PAHs), are used as model compounds in CEC separations to determine the column packing quality. For charged compounds, however, electrophoretic separation principles are superimposed on the LC separation mechanism. It is this combination of electrophoretic properties and the added selectivity offered by the stationary phase that makes CEC such an attractive alternative to both capillary zone electrophoresis (CZE) and LC.

A technique which is related to CEC is pseudo-electrochromatography (p-EC) [10–12]. A p-EC set-up is in principle a HPLC system. However, an electric field can be applied over the analytical column as an electrophoretic counterpart of the organic modifier in the background electrolyte, resulting in a dramatic improvement in the separation or peak efficiency.

*Corresponding author.

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00259-4

The hyphenation with mass spectrometry (MS) offers added selectivity and the option of structure elucidation during the detection. Initially, the interfacing technique towards the MS was fast atom bombardment (FAB) [10,13]. However, since electrospray ionisation (ESI) became an established interfacing technique for capillary electrophoretic (CE) techniques and MS, the majority of the CEC systems are interfaced in this manner [11,12,14–18].

In capillary liquid separation techniques absolute detection limits can be very impressive, due to the necessity of very small sample volumes. However, the sample concentration detection limit determines the applicability of a technique and forms a serious drawback of, e.g., CZE and CEC. For uncharged compounds head-column stacking can be used [18] when the sample is dissolved with less modifier compared to the background electrolyte. Also, several researchers have developed a cartridge-like solid-phase extraction column that is implemented in the CZE capillary and loaded with sample prior to CZE analysis [19–22]. Using such pre-column devices, a significant decrease in the detection limit up to three orders of magnitude could be achieved. For charged compounds, however, a concentrating step like isotachophoresis prior to the separation step can be applied. Isotachophoresis (ITP) is an electrophoretic separation technique, in which a sample is separated into an array of highly concentrated and distinct analyte zones between a leading and terminating electrolyte. Under steady-state conditions, in which the zones migrate with equal velocity and have distinct borders, the concentrations of the analyte zones (C_A) are directly related to the concentration of the leading buffer (C_L), as is given by a simplified Kohlrausch equation: $C_A = C_L K$ [23]. In general the proportionality factor (K) varies between 0.2 and 0.9, indicating the enormous concentrating power offered by ITP.

In the past decade, ITP is combined by several researchers, using a single- [24–27] and coupled-column [28–33] approach. In the single-column approach both the ITP focusing and CZE separations are accomplished in one single capillary. Using a coupled-column system, ITP and CZE are performed in different capillaries, allowing the handling of very large sample volumes without compromising the CZE separation and buffer changing in between the ITP and CZE step. Another interesting feature of ITP is the possibility of a sample clean-up during the ITP focusing. Compounds with a mobility within the mobilities of the leading and terminating ions (ITP window) will be focused. All other compounds are excluded from the focused zones and diluted [24]. By performing carefully a heart-cut in the ITP zone, on-line sample clean-up can be achieved, which is beneficial for the column lifetime when complex samples are analysed. So far, two previous papers have reported on the separation of compounds present in bio-fluids [34,35]. In both cases, sample clean-up prior to CEC was necessary to extend the lifetime of the CEC column.

In this paper, ITP is used as a sample focusing technique prior to a CEC separation in a coupled-column set-up with UV-absorbance and mass spectrometric detection. To demonstrate the feasibility of the ITP–CEC combination in the analysis of charged compounds, several cationic drugs are used in academic samples and bioanalysis.

2. Experimental

2.1. Chemicals

Ammonium acetate and crystal violet were obtained from Merck (Darmstadt, Germany) and methanol from Biosolve (Barneveld, The Netherlands). Fenoterol, neostigmine and scopolamine were purchased from Sigma (St. Louis, MO, USA), β-alanine from Aldrich (Steinheim, Germany) and salbutamol was kindly donated by TNO Institute (Zeist, The Netherlands). All used chemicals were of analytical grade. Aqueous solutions were prepared with Milli-Q system purified water (Millipore, Bedford, MA, USA).

The leading buffer consisted of 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25). The terminating electrolyte consisted of 20 mmol/l β-alanine-buffer, pH 5.0, in methanol–water (75:25). Before use, the solutions were degassed by sonification in an ultrasonic bath and filtered over a 0.2-μm pore filter (Gelman Nylon Acrodisk, Ann Arbor, MI, USA).

The sheath liquid was composed at a mixture of methanol–water (80:20) containing 1% acetic acid.

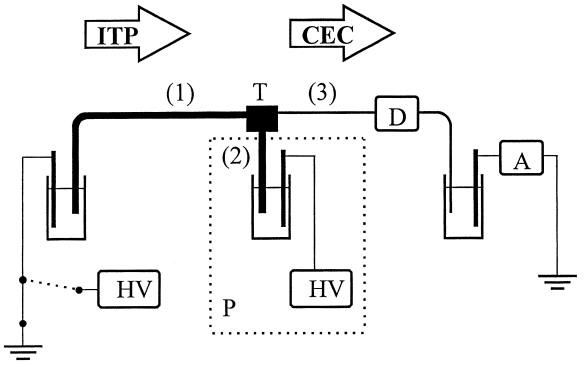

Fig. 1. Schematic representation of the ITP–CEC–UV set-up with a (P) programmable capillary injection system, (D) UV–Vis absorbance detector, (A) amperometer and (T) laboratory made polyethylene T-piece. Untreated fused-silica capillaries of 220 μm I.D. (1 and 2) and 75 μm (3) are used.

The stock solutions for the CZE and the CEC separations were prepared in 100% methanol and for ITP–CEC in terminating buffer at a concentration of 1 mmol/l. Sample solutions were further diluted down to nmol/l level.

2.2. Instrumentation

A schematic overview of the coupled-column ITP–CEC set-up is shown in Fig. 1. A programmable injection system for capillary electrophoresis (Prince, Prince Technologies, Emmen, The Netherlands) was used for hydrodynamic as well as electrokinetic injections and as a high voltage power supply for the ITP and the CEC separations.

A Spellman CZE 1000 R (High Voltage Electronics, Plainview, USA) high voltage power supply was used for applying voltage over the ITP capillary (1) and the CEC capillary (3) during the transfer of the focused sample zone (see ITP–CEC procedure). Untreated fused-silica capillaries (SGE, Ringwood, Australia) of 220 μm inner diameter (I.D.) with a length of 42 cm (ITP capillary 1) and 27 cm (auxiliary capillary 2), respectively, were used. The packed column consisted of a 75-μm I.D. fused-silica capillary with a length of 37 cm (CEC capillary 3), packed with Nucleosil 100-5-C_{18} 5-μm particles (Macherey–Nagel, Düren, Germany) up to a length of 16 cm. The capillaries for ITP and CEC were connected by a transparent polyethylene T-piece with 300-μm channels [33].

On capillary detection was performed at a wavelength of 210 nm at 2 cm behind the outlet frit, using a Spectroflow 757 variable-wavelength UV detector (ABI Kratos, Ramsey, NJ, USA) which is equipped with a custom made detection cell. Registration was realised by a model BD 40 recorder (Kipp & Zonen, Delft, The Netherlands), and the current through the column was measured by an electronic galvanometer (John Fluke, Seattle, Washington).

Mass spectrometric analysis was performed on a Finnigan SSQ 710 single quadrupole instrument (Finnigan MAT, San Jose, CA, USA). The CEC column was positioned in the electrospray needle

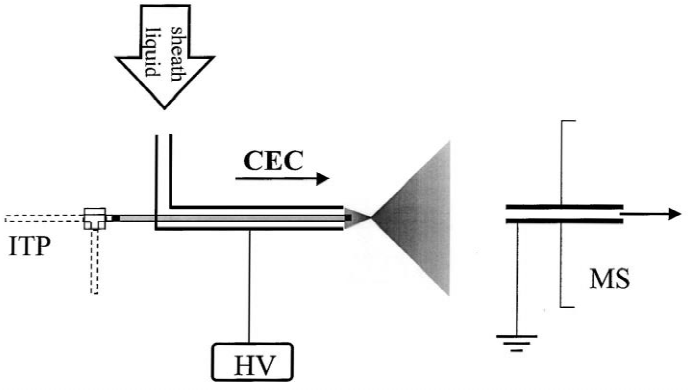

Fig. 2. Schematic representation of the entire ITP–CEC–MS set-up. The electrospray needle with the sheath flow contains the CEC column, which is directly connected with the electrospray. The spray is directed towards the inlet capillary of the interface on the SSQ 710 mass spectrometer (MS). HV is the electrospray power supply.

with the column outlet frit in the Taylor cone (Fig. 2), omitting additional band broadening due to transfer to the electrospray. The spraying voltage was approximately 3.5 kV. The sampling capillary (10 cm×0.5 mm) of the ESI interface was heated up to 200°C. A sheath flow-rate of 1 μl/min was applied by a syringe pump (model 2400, Harvard Apparatus, Edinbridge, UK).

2.3. ITP–CEC procedure

An overview of the four-step ITP–CEC procedure is shown in Fig. 3.

In the first step, the capillaries 1 and 2 are filled hydrodynamically and the CEC column (3) is rinsed electrokinetically with leading buffer. After levelling the vials, a 15–20-μl sample is introduced hydrodynamically (100 mbar for 25 s).

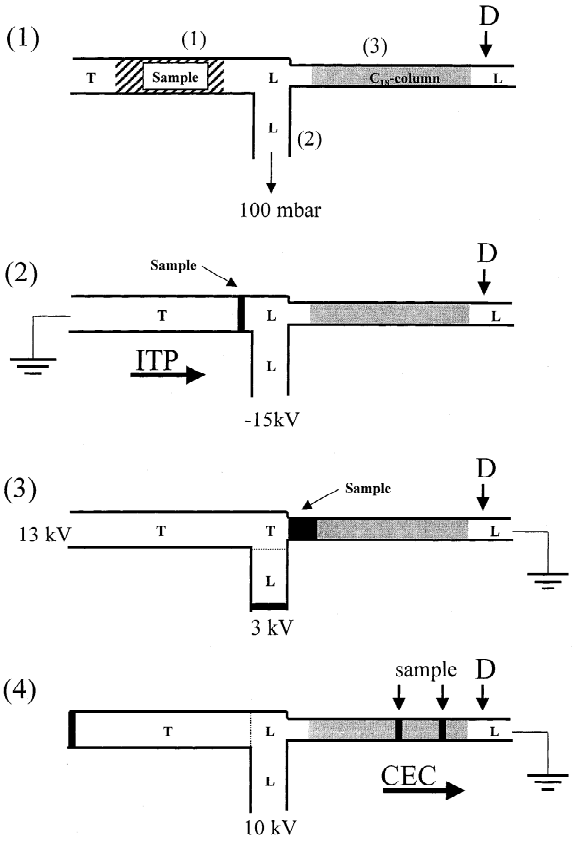

Fig. 3. Schematic representation of the ITP–CEC procedure. The sample loading, ITP focusing step, sample zone transfer and CEC separation are shown in step 1, 2, 3 and 4, respectively. The set-up contains a (D) UV–Vis absorbance or MS detection, (T) terminator buffer and (L) leading buffer. Untreated fused-silica capillaries of 220 μm I.D. (1 and 2) and 75 μm (3) are used.

In the second step, the sample vial is replaced by a vial with terminating buffer and the counterflow-ITP is started by applying 15 kV over the capillary, followed by a counterflow, which is induced by a pressure of 3 mbar. During the ITP focusing, the CEC capillary outlet is disconnected from the ground potential.

In the third step, the sample is transferred from the ITP capillary to the CEC column. When the focused sample zone reaches the T-piece, the voltage is switched off and capillary 2 is closed with a septum. A voltage of 13 kV is applied over capillary 1 and the CEC capillary. Simultaneously, 3 kV is applied at the inlet of capillary 2, to avoid an electrical current from the T-piece to the outlet of this capillary. After the transfer is completed, the capillaries 1 and 2 are flushed with leading buffer, to remove remaining compounds that can interfere during the CEC separation.

In the fourth step, the CEC separation is performed. When the focused sample zone is transferred into the CEC capillary, a voltage of 10 kV is applied over capillary 2 and the CEC capillary, while capillary 1 is blocked by a septum. Detection is performed at 2 cm behind the outlet frit with an UV absorbance detector at 210 nm. For the mass spectrometric detection, the electrospray needle is placed at a voltage of 3.6 kV and the selected ion monitoring mode was used to selectively detect the compounds. To maintain the field strength over the CEC column a voltage of 13.6 kV is applied during the CEC separation. As a sheath liquid 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25) is used.

2.4. CZE and CEC procedures

The CZE and CEC separations are performed with similar equipment as is used for the ITP–CEC separations. In CZE a 42-cm long fused-silica capillary of 75 μm I.D. (SGE) with a separation path length of 33 cm is used. The samples are electrokinetically introduced by applying 10 kV over the capillary for 10 s. The separation is performed with 20 kV over the capillary. The background electrolyte, in which the CZE separations are performed, is 20

mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25).

The CEC separations are performed with a 75-μm I.D. fused-silica capillary packed with Nucleosil 100-5-C_{18} 5-μm particles (Macherey–Nagel). The length of the packed bed is 17 cm. The samples are introduced by applying 10 kV over the capillary for 10 s. The separation is performed by applying 10 kV. The background electrolyte in which the CEC separations are performed is 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25). UV-absorbance detection is performed at 210 nm.

2.5. Pseudo-electrokinetic column packing

The set-up for the packing of the CEC columns is shown in Fig. 4. Fused-silica capillaries (SGE) of 75 μm I.D. are packed with Nucleosil 100-5-C_{18} 5 μm particles (Macherey–Nagel) for both CEC and ITP–CEC purposes, using a modified pseudo-electrokinetic (p-EK) packing procedure as is described before [36].

A temporary frit is made of sintered 8–10 μm bare silica particles (Merck, Darmstadt, Germany). Then, the capillary is placed in a slurry reservoir and flushed with 10 mmol/l trishydroxymethylaminomethane (Tris) buffer, pH 8.0, in methanol–water (90:10). A laboratory-made glass vial, to which a high voltage power supply is connected and that can be pressurised up to 5 bar, was used as the slurry reservoir. Using a stirring apparatus (Wilten, Breda, The Netherlands), a homogeneous liquid slurry is created. The capillary is pressure filled (2.5 bar) and electrokinetically packed with 30 kV. Following on, permanent frits are made by fusing the column material, while the old frits are removed. Then, the capillary is flushed with degassed water and filled with background electrolyte. A detection window is made by burning the polyimide coating from the capillary at a distance of 2 cm from the outlet frit.

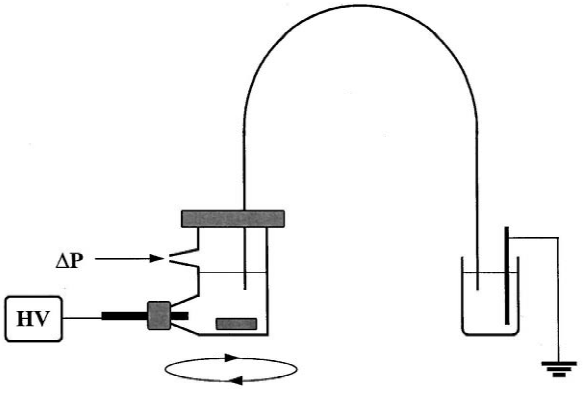

Fig. 4. Schematic representation of the packing set-up. A stirring apparatus was used to homogenise the slurry and a pressure of 2.5 bar (ΔP), simultaneously with a high voltage of 30 kV, was applied over a fused-silica capillary (75 μm I.D.) filled with background electrolyte.

2.6. Biosample preparation

The biosamples are prepared by spiking human plasma and urine with a mixture of neostigmine, salbutamol and fenoterol up to a concentration of 3.3, 3.3 and 1.6 μmol/l of the respective components. One millilitre of the spiked plasma sample is mixed with 0.3 ml concentrated perchloric acid and incubated for 30 min at 5°C. Following on, the sample is centrifuged for 5 min at 5000 *g*, to precipitate the proteins. Then, 500 μl of the supernatant is taken and brought to pH 11 with 2.3 mol/l NaOH.

For urine samples, 500 μl urine were brought to pH 11 using 2.3 mol/l NaOH.

Solid-phase extraction (SPE) of both sample types was performed on XAD-2 material (Serva, Heidelberg, Germany), which was preconditioned with 3 ml methanol containing 0.1% triethylamine, followed by rinsing with 3 ml water. The sample is slowly eluted through the SPE column, followed by a washing step with 3 ml of water. Then, the column is dried and elution of the sample components is achieved with 1 ml of methanol. The methanol is evaporated from the sample under vacuum and 30°C. The residue is dissolved in 0.5 ml of terminating buffer.

3. Results and discussion

3.1. Column packing

An important practical aspect in CEC is the production and stability of the packed column, which is strongly affected by the frits. During a CEC

separation, the frits may deteriorate due to a high electric current and consequently destroying the column.

During the packing of the capillary, a temporary frit was produced to support the silica-based C_{18}-material. The length of the frit strongly affects the packing time, since a long frit produces a high mechanical resistance and slows down the initial filling of the capillary. Also, a long frit produces a relative large electric resistance and, consequently, loss in electric field strength during the electrokinetic packing will occur [37]. On the contrary, a frit which is too short will be pushed out of the capillary when a high voltage or pressure is applied. For the described conditions an optimal frit length of 2–3 mm was found, which allowed relatively fast electrokinetic packing (15–20 min) of capillaries with a 20–30-cm packed bed.

The packing velocity is also influenced by the concentration of electrolyte in the packing buffer. When the concentration of the electrolyte is high, the generated EOF is reduced and the packing time will be extended. Therefore, a packing buffer of 10 mmol/l Tris buffer, pH 8.0, in methanol–water (90:10) is used. A lower concentration of the packing electrolyte did not increase the packing rate of the stationary phase.

3.2. CEC of charged compounds

During a CEC separation of charged compounds, electrophoretic and liquid chromatographic separation principles are combined and can result in a different separation pattern, when compared to a CZE separation (Fig. 5A). The most striking difference between the signals of a CZE and a CEC separation is the elution of crystal violet (peak not shown in Fig. 5B, see Fig. 6), which is a relatively hydrophobic compound (see Fig. 5C) and shows a strong affinity for the C_{18}-stationary phase. Also, the elution order of salbutamol and scopolamine changed due to the difference in interaction with the stationary phase. The double peaks produced by fenoterol are probably due to ageing of the sample. The obtained efficiencies in the CZE and the CEC separation were comparable and in the order of 140 000 plates/m.

3.3. ITP–CEC

To improve the loadability of electrophoretic systems, ITP has been a well studied and effective method [24–33]. Using ITP, the loadability of a CEC system for the analysis of ionic compounds can be enhanced considerably. A sample is focused between a leading and terminating buffer and up to the concentration which is similar to the leading buffer. In the steady-state situation the focused zones migrate with an equal velocity and have distinct borders, which will be maintained due to the ‘self correcting’ ability of the ITP zones. As a result, no analyte is lost, which is of particular use when the focused sample zone is transported through a channel system and exposed to band broadening influences [33].

To combine ITP with CEC and allowing the option of a sample clean-up, a coupled-column set-up as described by Kaniansky and co-workers [31,32] is used. ITP is performed in a wide bore (220 μm inner diameter) fused-silica capillary, which is connected to the analytical CEC column (75 μm inner diameter), via a T-piece [33]. Larger inner diameters can also be used [31,32], increasing the loadability to even higher limits while maintaining a high electric field strength during the ITP focusing. However, Joule heating effects and a hydrodynamic flow due to level differences during the ITP–CEC procedure will become more prominent.

In CEC, the sample is usually electrokinetically introduced which causes sample discrimination when ionic compounds are analysed. In contrast, by using a coupled-column system, the sample is hydrodynamically introduced in the ITP capillary, thus avoiding sample discrimination.

To reduce the ITP focusing time a high potential difference is applied in the range of 15–20 kV, limited by the CE apparatus or by the conductivity of the sample to avoid gas bubble formation. However, application of a high potential difference over the CEC column can result in column breakdown, which presumably originates from gas bubble formation due to a high electric current density in the column frits. Therefore, the CEC capillary is disconnected from ground potential during the ITP focusing.

The ITP time is dependent on the volume and conductivity of the introduced sample, the applied

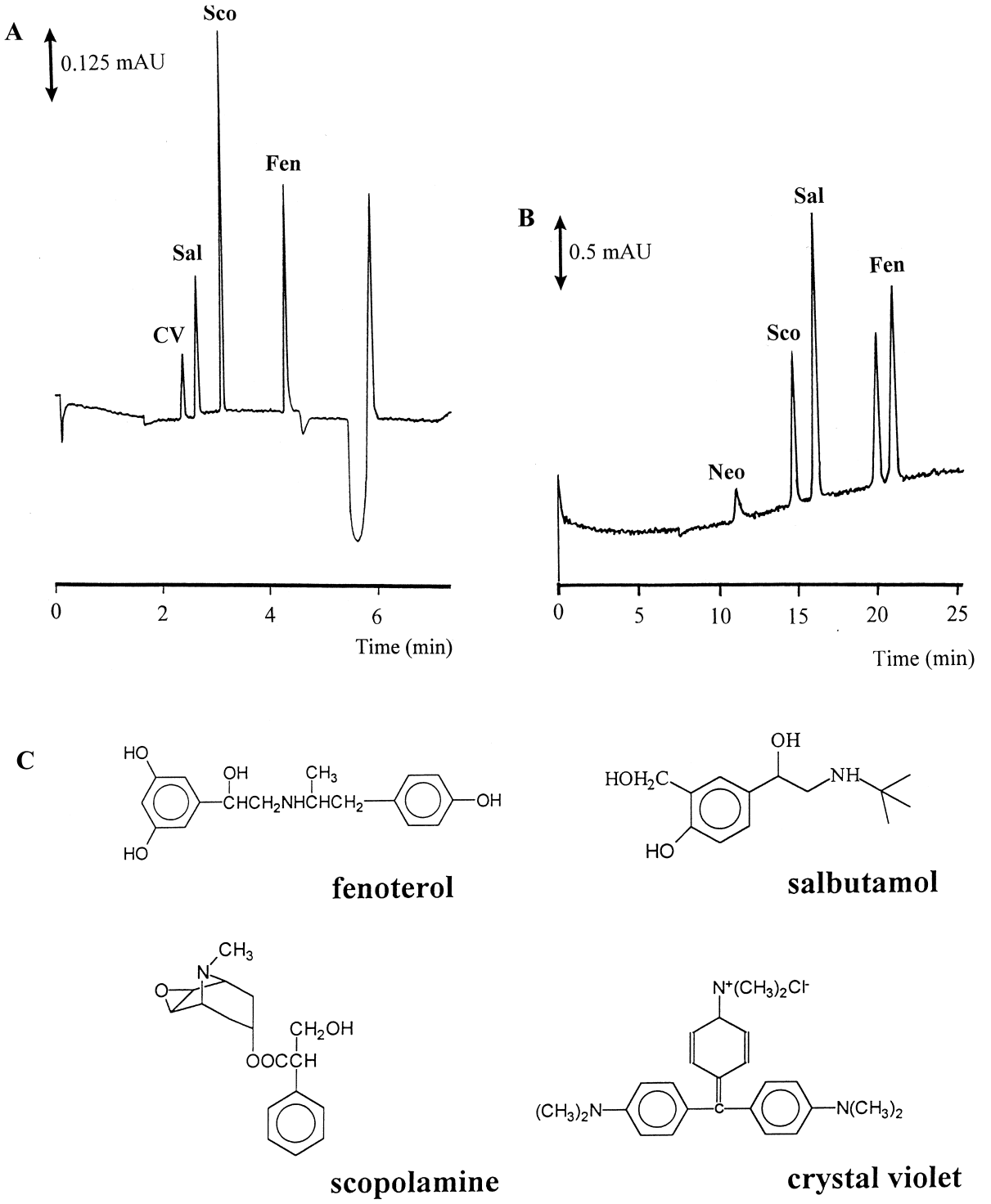

Fig. 5. (A) CZE electropherogram using 600 V/cm of 0.25 μmol/l crystal violet (CV), 0.83 μmol/l salbutamol (Sal), 13 μmol/l scopolamine (Sco) and 1.3 μmol/l fenoterol (Fen). (B) CEC electrochromatogram using 750 V/cm of 0.25 μmol/l neostigmine (Neo), 18 μmol/l scopolamine (Sco), 8.5 μmol/l salbutamol (Sal) and 5.0 μmol/l fenoterol (Fen). In both systems the samples were injected electrokinetically by applying 10 kV during 10 s, and the separation was performed in 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25).

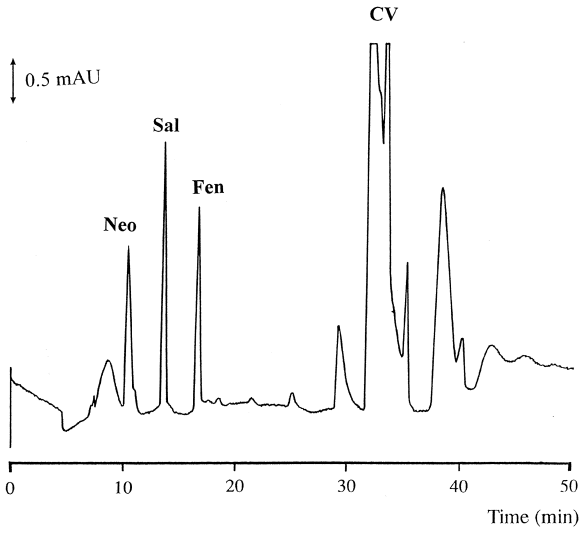

Fig. 6. ITP–CEC electrochromatogram of a mixture of 13 nmol/l neostigmine (Neo), 13 nmol/l salbutamol (Sal), 66 nmol/l fenoterol (Fen) and 5 μmol/l crystal violet (CV), dissolved in terminating buffer. The 15-μl sample was injected hydrodynamically and concentrated using the ITP buffers. The ITP step is performed during 13–14 min using 15 kV, with a counterflow of 3 mbar. The separation was performed in 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25), using a field strength of 500 V cm^{-1} (5.5 μA).

field strength, the leading buffer concentration, the mobility of the compounds and the migration path length. When a very large sample is introduced the focusing time of the sample often exceeds the migration time to the outlet of the ITP capillary. By applying a hydrodynamic counterflow the ITP focusing will continue while extending the migration towards the outlet of the ITP capillary. In general the ITP focusing step under counterflow conditions for academic samples of 15 μl is 13.5±2% min.

A critical parameter in the ITP–CEC procedure is the moment at which the focused sample zone reaches the outlet of the ITP capillary and the transfer can be started. Current monitoring, as developed by Reinhoud et al. [24,25], could not be used due to an unstable electric current with marginal differences when the focused zone reaches the T-piece.

The deviation in the ITP time is in principle small enough to accurately determine the moment of transfer between the ITP and CEC. However, variations in the size and the electrophoretic properties of the sample as well as the possibility of random experimental variations require a direct monitoring of the ITP process. Therefore, crystal violet, which is a dark blue cationic dye, was added to the sample to allow continuous visual monitoring of the focusing process. Also, an extra detector that is placed immediately in front of the T-piece can be used to

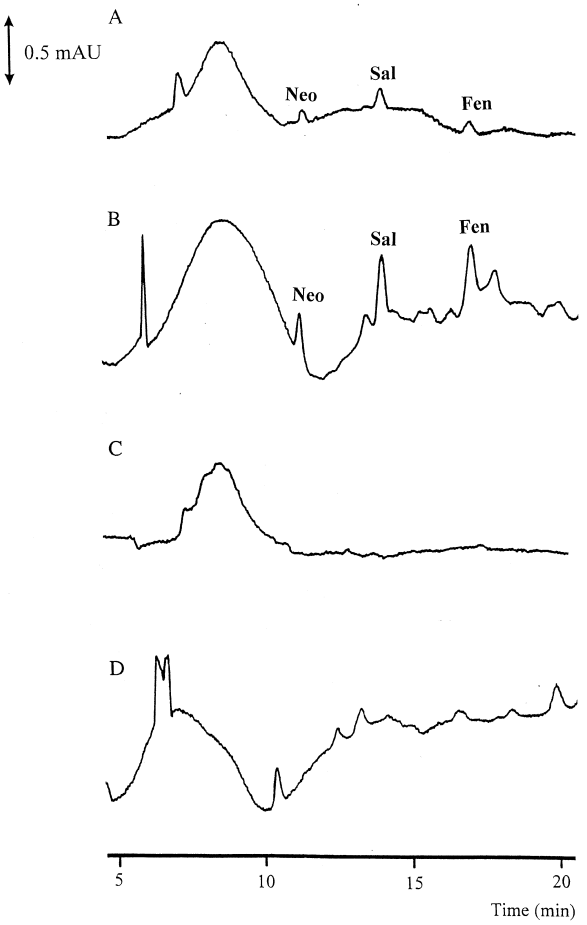

Fig. 7. Electrochromatograms of the detection limits of neostigmine (Neo), salbutamol (Sal) and fenoterol (Fen) with (A) hydrodynamic and (B) electrokinetic injection up to a volume of 15 μl and the accompanying blank signals (C,D). The concentration of the sample components in (A) are 5.0 nmol/l for neostigmine and salbutamol and 2.5 nmol/l for fenoterol. In (B) the concentrations are 3.0 nmol/l l for neostigmine and salbutamol and 1.5 nmol/l for fenoterol. The samples were dissolved in terminating buffer and the separation was performed in 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25); 0.1 mmol/l crystal violet was used as a visual marker of the ITP process. Experimental conditions as in Fig. 5.

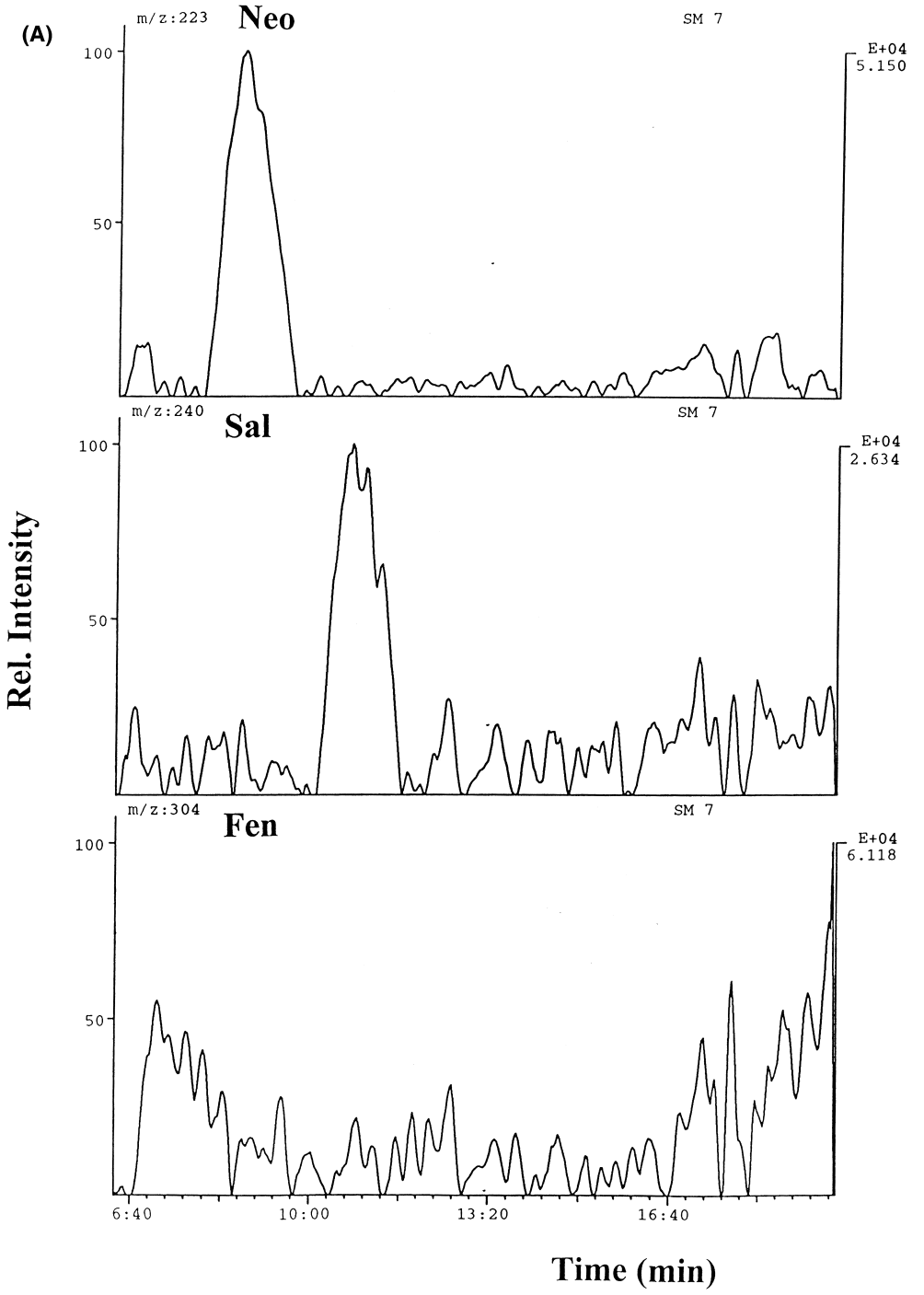

Fig. 8. Selected ion trace electrochromatograms of a (A) CEC–MS analysis of 3.3 μmol/l neostigmine (Neo), 3.3 μmol/l salbutamol (Sal) and 1.7 μmol/l fenoterol (Fen). The sample is dissolved in terminating buffer and electrokinetically introduced with 10 kV during 5 s. In (B) the electrochromatograms of an ITP–CEC–MS analysis of 11.0 nmol/l neostigmine, 11.0 nmol/l salbutamol and 5.5 nmol/l fenoterol are shown. The sample is dissolved in terminating buffer and 15 μl was hydrodynamically introduced. Crystal violet was used as a visual marker. The background electrolyte during the CEC separation was in all cases 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25).

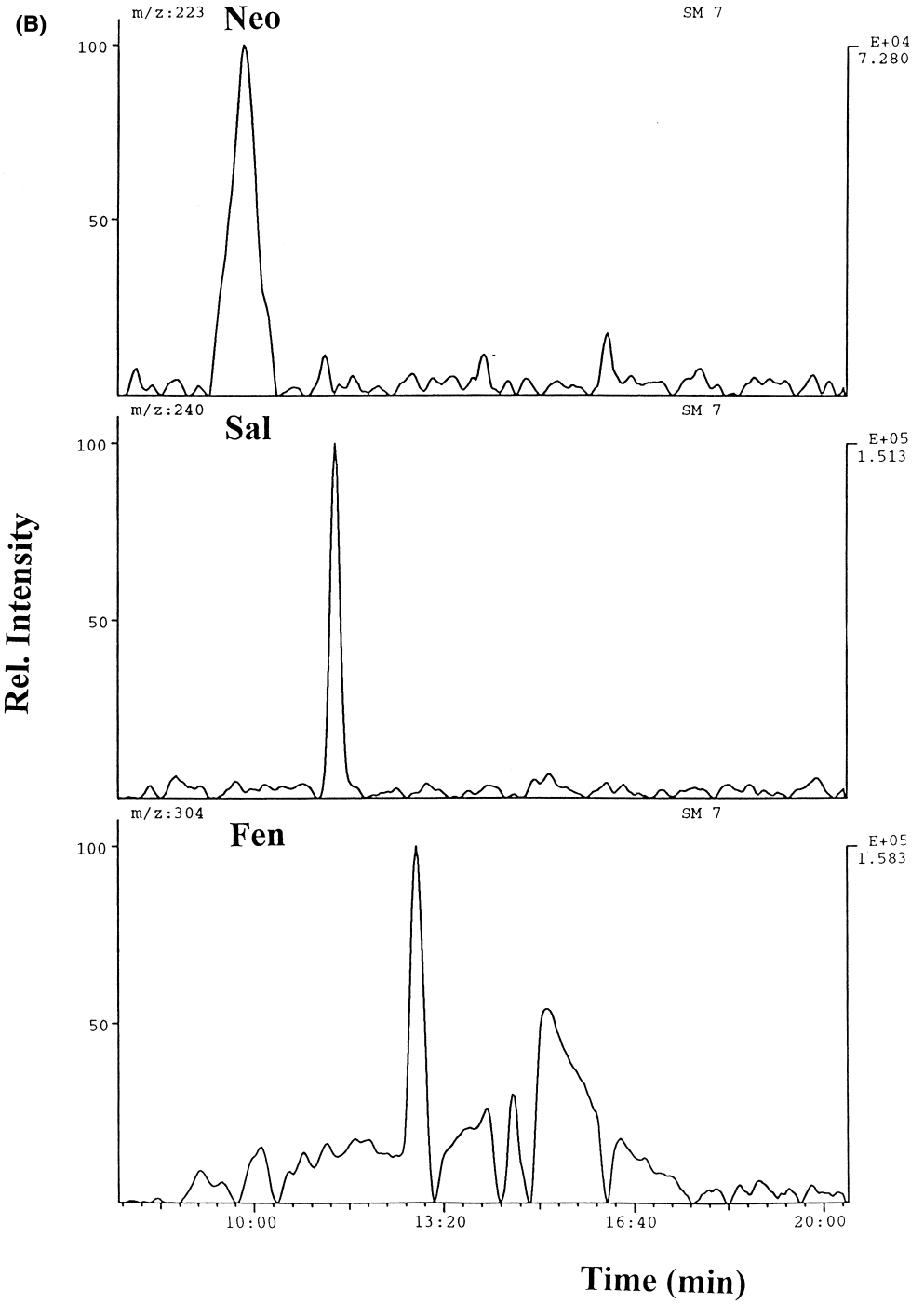

Fig. 8. (*continued*).

determine the passage of the focused zone [28] and avoid the use of crystal violet.

The transfer of the sample zone from the ITP capillary into the CEC capillary is solely electrophoretic by applying a potential difference over the respective capillaries. The auxiliary capillary 2 is blocked with a septum to avoid a hydrodynamic flow through the ITP capillary. Also a small potential difference of 3.0 kV was applied at the auxiliary capillary to avoid electrophoretic migration of the sample into the auxiliary capillary. During the transfer through the T-piece, the sample zone maintains focus due to the self-correcting properties of the ITP steady-state situation and no sample is lost.

Immediately after a complete transfer of the sample zone into the CEC capillary, the ITP and auxiliary capillary are flushed with fresh leading buffer and the CEC separation is started. The electric current during the CEC separation was approximately 5.5 μA (10 kV) and is limited by the packed bed and frits of the column. High electric current densities result in gas bubble formation and column breakdown.

In Fig. 6 an ITP–CEC separation of a 15-μl sample containing three small drugs and crystal violet is shown with a sample concentration in the range of 13–66 nmol/l. Crystal violet, however, was added to the sample at a concentration of 5 μmol/l to visualise the entire ITP and transfer procedure. The signal is thus a result of overloading and extended the total CEC separation time dramatically.

3.4. Quantitative aspects

The reproducibility of six ITP–CEC separations, of 15 μl hydrodynamic injections of 110 nmol/l salbutamol and scopolamine and 55 nmol/l fenoterol, was measured over a time span of 3 days. Using crystal violet as a visual marker, relative standard deviations in the electro-elution time of the separations were in the range of 1.2–1.4%. The surface areas, however, vary up to 13.1%, which is mainly induced by the manual adjustment of the inlet vial and blocking the capillary with a septum.

Calibration plots were constructed for salbutamol and fenoterol in the concentration range 1–500 nmol/l and resulted in the respective equations $y=0.099x-0.839$ ($r^2=0.970$) and $y=0.048x-0.291$ ($r^2=0.996$) in which y is the peak area and x is the injected sample concentration in nmol/l.

ITP–CEC detection limits were determined for neostigmine, salbutamol and fenoterol and shown in Fig. 7, for a hydrodynamic (A) as well as electrokinetic injection (B) into capillary 1. The detection limits are in general 1–5 nmol/l, which is a factor 100–1000 lower compared to ordinary CEC. Electrokinetic injection was performed by inserting the inlet of the ITP capillary in the sample vial and applying 15 kV, until the sample zone reached a similar volume as in hydrodynamic injections (visual check

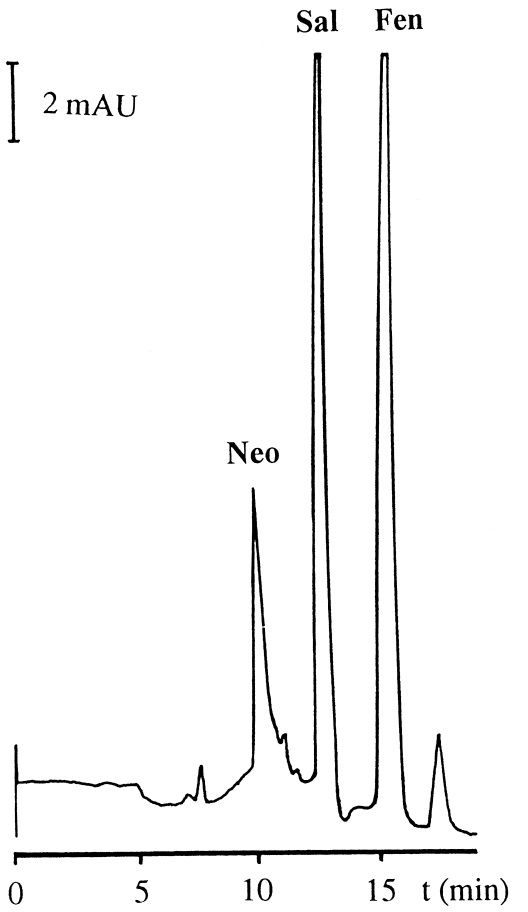

Fig. 9. ITP–CEC electrochromatogram of urine spiked with 3.3 μmol/l neostigmine (Neo) and salbutamol (Sal) and 1.6 μmol/l fenoterol (Fen). A sample volume of 15 μl is hydrodynamically introduced and the CEC separation was performed in 20 mmol ammonium acetate buffer, pH 5.0, in methanol–water (75:25). Crystal violet was used as a visual marker. Sample pretreatment as is described in Section 2 was used.

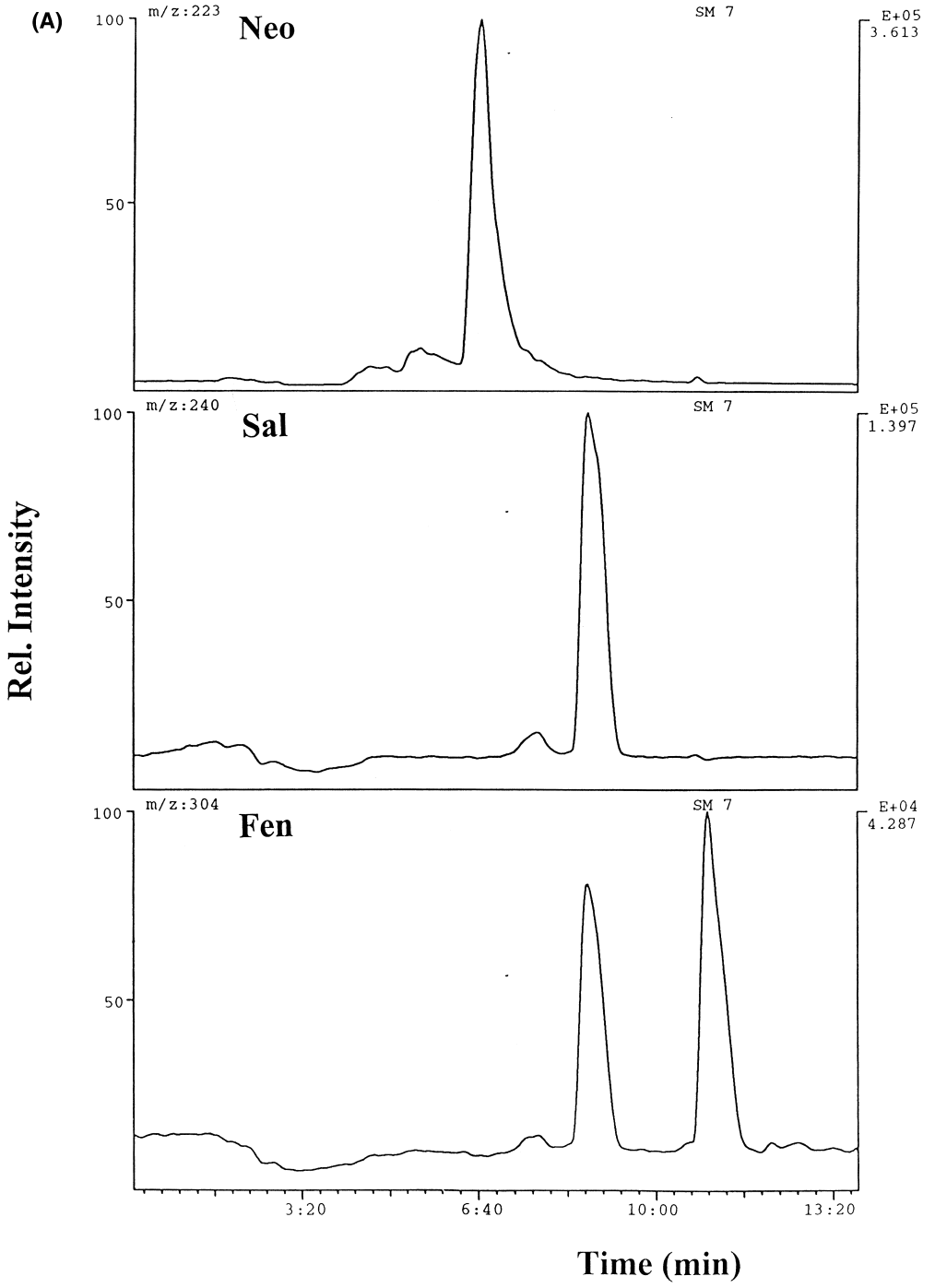

Fig. 10. ITP–CEC–MS electrochromatograms of 15 μl human (A) urine and (B) plasma sample, spiked with 3.3 μmol/l neostigmine (Neo), 3.3 μmol/l salbutamol (Sal) and 1.7 μmol/l fenoterol (Fen). The separation buffer was 20 mmol/l ammonium acetate buffer, pH 5.0, in methanol–water (75:25).

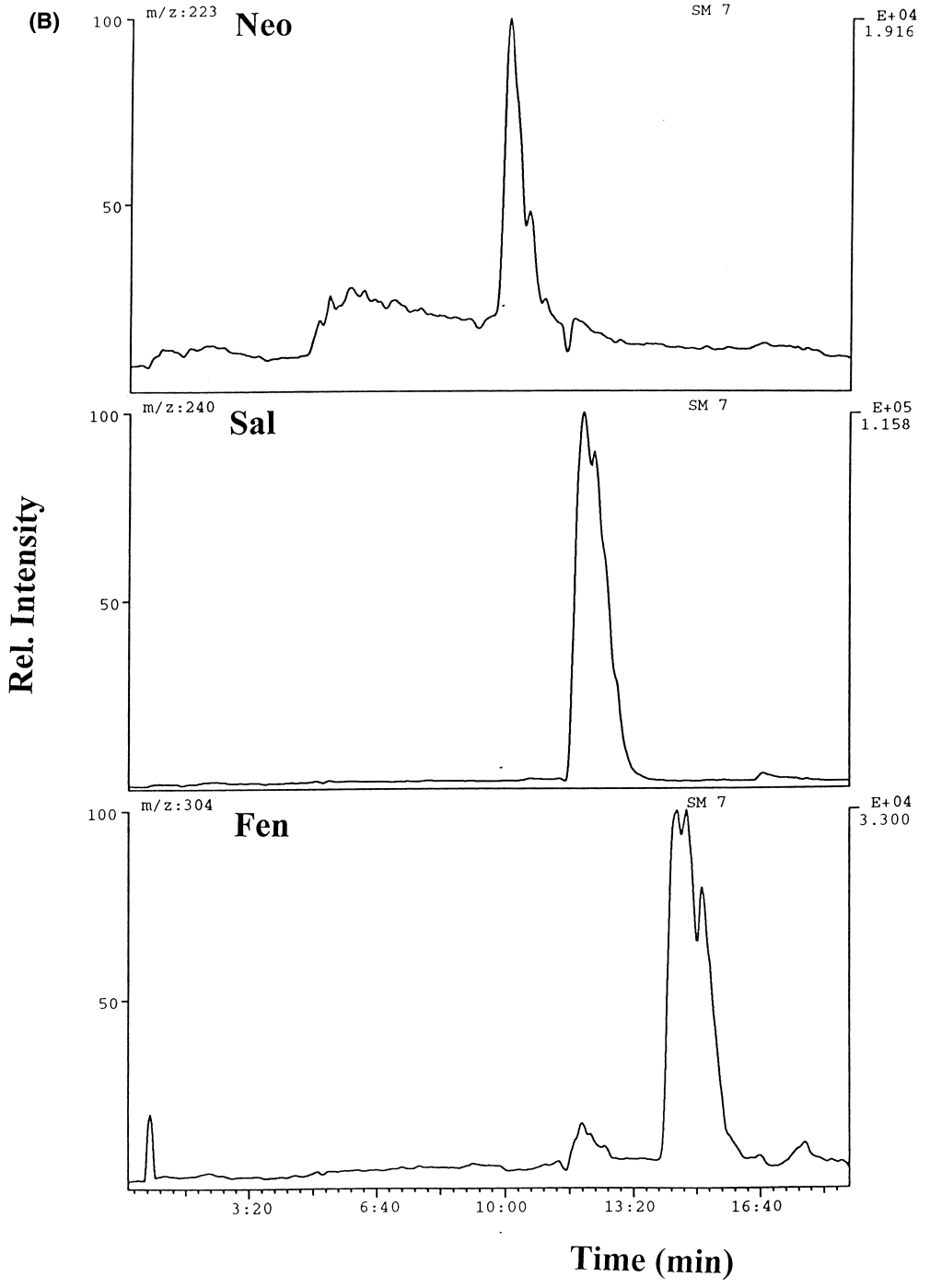

Fig. 10. (*continued*).

with crystal violet). The sample ions migrate, based on their mobility and the electroosmotic flow (EOF), into the ITP capillary as large overlapping zones. After the injection is completed, the inlet of the ITP capillary is placed in a terminating buffer, the sample zone is focused and the ITP–CEC procedure is continued as described. Under the given conditions, the detection limit that is obtained with electrokinetic injection is approximately a factor 2 lower compared to the hydrodynamic approach, which is due to more efficient sample ion introduction. However, matrix compounds are also apparent in higher concentrations as can be observed in Fig. 7B,D from the baseline.

Further lowering of the detection limit is feasible by introducing more sample in the system along with increasing the volume of the ITP capillary or electrokinetic sampling time, possibly extended by using a hydrodynamic counterflow.

In Fig. 8 the $[M+H]^+$ selected ion currents of neostigmine, salbutamol and fenoterol in CEC–MS (Fig. 8A) at μmol/l level and ITP–CEC–MS (Fig. 8B) in the nmol/l range are compared. As is demonstrated a signal enhancement in the range of a factor 1000 can be achieved, while the ITP–CEC separation efficiencies are between 120 000 and 140 000 plates/m. Fenoterol was not visible in the CEC–MS analysis.

3.5. Bioanalysis

An ITP–CEC analysis of a 15-μl urine sample spiked with 3.3 μmol/l neostigmine and salbutamol and 1.6 μmol/l fenoterol is shown in Fig. 9. A SPE sample clean-up procedure as described in Section 2 was used to desalt the sample. However, owing to the high amount of matrix ions, the ITP focusing time was extended significantly and could take up to 40 min. An important aspect in the handling of complex samples in ITP–CEC is the length of the sample zone. Due to the large amount of compounds, the total length of the sample zone can increase dramatically and can result in sample loss during the transfer step, as was observed for fenoterol, when the transfer time was shortened.

In Fig. 10 the ITP–CEC–MS signals of the urine and a plasma sample are shown. Due to the relatively high analyte concentration, some overloading of the CEC column occurred, yet the separation was maintained. The signal at $m/z=304$ (Fig. 10A) contains two peaks. The latter peak is, based on the migration time, identified as the fenoterol peak. The signal at 8:40 could not be recognised.

Remarkable differences in the neostigmine trace between the plasma and urine signals are present. Analyte loss of the neostigmine due to an incomplete transfer can be excluded since the transfer step is tuned on the visual marker, crystal violet, which migrates in front of neostigmine and was quantitatively transferred. Possibly, the signal variations originate from analyte loss during the SPE sample pretreatment or other factors such as ionisation suppression in the electrospray due to co-eluting compounds.

4. Conclusions

The isotachophoretic focusing and CEC separation of charged compounds is demonstrated in a coupled-column set-up with mass spectrometric detection. A 15-μl sample of small cationic drugs was introduced, focused and separated within approximately 30 min. Lowering of the detection limit of a factor 100–1000 was achieved, while an average separation efficiency up to 140 000 plates/m was maintained. Using UV-absorbance or MS detection the obtained detection limits were in the range of 1–5 nmol/l for neostigmine, salbutamol and fenoterol. The ITP–CEC analysis of the small drugs in bio-fluids was successfully demonstrated for plasma and urine.

References

[1] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[2] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[3] M.M. Dittman, G.P. Rozing, in: J.P. Landers (Ed.), Handbook of Capillary Electrophoresis, CRC Press, New York, 1996, pp. 139–153.
[4] J.H. Knox, I.H. Grant, Chromatographia 32 (1991) 317.
[5] C. Yan, R. Dadoo, H. Zhao, R.N. Zare, Anal. Chem. 67 (1995) 2026.
[6] N.W. Smith, M.B. Evans, Chromatographia 41 (1995) 197.
[7] H. Rebscher, U. Pyell, Chromatographia 42 (1996) 171.

[8] R.M. Seifar, W.Th. Kok, J.C. Kraak, H. Poppe, Chromatographia 46 (1997) 131.
[9] C.G. Huber, G. Choudhary, C. Horváth, Anal. Chem. 69 (1997) 4429.
[10] E.R. Verheij, U.R. Tjaden, W.M.A. Niessen, J. van der Greef, J. Chromatogr. 554 (1991) 339.
[11] M. Hugener, A.P. Tinke, W.M.A. Niessen, U.R. Tjaden, J. van der Greef, J. Chromatogr. 647 (1991) 375.
[12] S.E.G. Dekkers, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 712 (1995) 201.
[13] D.B. Gordon, G.A. Lord, Rapid Commun. Mass Spectrom. 8 (1994) 544.
[14] K. Schmeer, B. Behnke, E. Bayer, Anal. Chem. 67 (1995) 3656.
[15] G.A. Lord, D.B. Gordon, L.W. Tetler, C.M. Carr, J. Chromatogr. A 700 (1995) 27.
[16] S.J. Lane, R. Boughtflower, C. Paterson, T. Underwood, Rapid Commun. Mass Spectrom. 9 (1995) 1283.
[17] S.J. Lane, R. Boughtflower, C. Paterson, M. Morris, Rapid Commun. Mass Spectrom. 10 (1996) 733.
[18] J. Ding, P. Vouros, Anal. Chem. 69 (1997) 379.
[19] N.A. Guzman, M.A. Trebilcock, J.P. Advis, J. Liq. Chromatogr. 14 (1991) 997.
[20] A.J.J. Debets, M. Mazereeuw, W.H. Voogt, D.J. van Iperen, H. Lingeman, K.-P. Hupe, U.A.Th. Brinkman, J. Chromatogr. 608 (1992) 151.
[21] A.J. Tomlinson, L.A. Benson, W.D. Braddock, R.P. Oda, S. Naylor, J. High Resolut. Chromatogr. 18 (1995) 381.
[22] A.J. Tomlinson, L.A. Benson, S. Jameson, R.P. Oda, S. Naylor, Electrophoresis 17 (1996) 1801.
[23] F.M. Everaerts, J.L. Beckers, T.P.E.M. Verheggen, in: Isotachophoresis: Theory, Instrumentation and Practice, Elsevier, Amsterdam, 1976.
[24] N.J. Reinhoud, U.R. Tjaden, J. van der Greef, J. Chromatogr. 641 (1993) 155.
[25] N.J. Reinhoud, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 653 (1993) 303.
[26] F. Foret, E. Szoko, B.L. Karger, Electrophoresis 14 (1993) 417.
[27] T.J. Thompson, F. Foret, P. Vouros, B.L. Karger, 65 (1993) 900.
[28] D.S. Stegehuis, U.R. Tjaden, J. van der Greef, J. Chromatogr. 591 (1992) 341.
[29] F. Foret, E. Szoko, B.L. Karger, J. Chromatogr. 608 (1992) 3.
[30] N.J. Reinhoud, A.P. Tinke, U.R. Tjaden, W.M.A. Niessen, J. Van der Greef, J. Chromatogr. 627 (1992) 263.
[31] D. Kaniansky, J. Marák, J. Chromatogr. 498 (1990) 191.
[32] D. Kaniansky, I. Zelensky, A. Hybenova, F.I. Onuska, Anal. Chem. 66 (1994) 4258.
[33] M. Mazereeuw, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 677 (1994) 151.
[34] C.J. Paterson, R.J. Boughtflower, D. Higton, E. Palmer, Chromatographia 46 (1997) 599.
[35] M.R. Taylor, P. Teale, S.A. Westwood, Anal. Chem. 69 (1997) 2554.
[36] R. Stol, M. Mazereeuw, U.R. Tjaden and J. van der Greef, J. Chromatogr. A 873 (2000) 293.
[37] M.T. Dulay, C. Yan, D.J. Rakestraw, R.N. Zare, J. Chromatogr. A 725 (1996) 361.