Journal of Chromatography, 627 (1992) 263–271
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 592

Capillary isotachophoretic analyte focusing for capillary electrophoresis with mass spectrometric detection using electrospray ionization☆

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(Received July 15th, 1992)

ABSTRACT

An improvement in detectability in capillary electrophoresis–mass spectrometry (CE–MS) was realized using isotachophoretic analyte focusing. The practical approach is described for the on-line coupling of capillary isotachophoresis–capillary electrophoresis–mass spectrometry (ITP–CE–MS) using an electrospray interface. An equation was derived for the calculation of the splitting ratio of ITP zones into the CE system. The applicability of analyte focusing is demonstrated for the analysis of a mixture of anthracyclines. Initial experiments showed a 200-fold improvement in concentration detection limit for ITP–CE–MS compared with CE–MS.

INTRODUCTION

Capillary electrophoresis (CE) in combination with mass spectrometry (MS) can be a potentially powerful tool in the analysis of various compounds [1–9]. Especially in the area of protein and peptide analysis there is growing interest in a highly efficient separation technique such as CE in combination with the high selectivity of MS detection. CE–MS interfaces described in the literature are continuous-flow fast atom bombardment (CF-FAB), electrospray (ESP) and ionspray (ISP) types.

The application of CF-FAB has been realized by either a liquid-junction interface (LJI) or a coaxial approach for addition of the necessary make-up fluid for this type of ionization. In the coaxial approach [10] the separation capillary is inserted in the CF-FAB capillary and ends only a few millimetres before the tip of the CF-FAB probe. This minimizes the dead volume and facilitates highly efficient separations.

In the other approach of CE–CF-FAB-MS, an LJI for the electric decoupling and for addition of make-up fluid is used [2,11,12]. This type of interface utilizes a transport capillary between the CE capillary and the mass spectrometer. Transport of ions in this capillary is facilitated by means of a hydrodynamic flow generated by the vacuum in the ion source of the mass spectrometer. It is evident that this type of CE–MS coupling results in considerable more peak broadening than the coaxial approach.

On the other hand, the coaxial approach requires the use of 10 μm I.D. capillaries in order to prevent a hydrodynamic flow in the CE capillary because of the vacuum of the ion source at the CE capillary outlet. This means a considerable drawback with respect to the loadability and the corresponding sample concentration detection limits in CE–MS [9].

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☆ Presented at the *4th International Symposium on High Performance Capillary Electrophoresis, Amsterdam, February 1992*. The majority of the papers presented at this symposium were published in *J. Chromatogr.*, Vol. 608 (1992).

0021-9673/92/$05.00

Although the use of an LJI results in band broadening, the mass flow to the mass spectrometer will be superior for the LJI type of interfaces where 75 μm I.D. capillaries are used with a *ca.* 50-fold higher loadability than the 10 μm I.D. capillaries. Nevertheless, the loadability is limited to the nanolitre range, resulting in relatively high minimum detectable concentrations. An important improvement in detectability was realized by using a PATRIC (position- and time-resolved ion counting) array detector for CE–CF-FAB-MS [3].

The ESP interface for CE–MS described by Smith and co-workers [13,14] has several advantages over the CF-FAB interface. First, the CE capillary is inserted in the ion source and the migrating ions are electrosprayed from the capillary outlet directly into the mass spectrometer, implying that no additional peak broadening after the CE separation is introduced. Second, the use of a sheath flow with an approximately tenfold higher flow-rate allows the use of CE buffers with minor consideration of the ionization compatibility. In peptide analysis CE can be performed in buffers with high pH, while positive ions are detected in the mass spectrometer because of addition of acetic acid to the sheath flow. Third, the ion source is at atmospheric pressure, resulting in the absence of a hydrodynamic flow and 75 μm I.D. CE capillaries can be used. Finally, the ESP interface permits the analysis of high-molecular-mass compounds such as proteins. Because of multiple charges on the large molecules, the corresponding mass-to-charge ratios are within the detectable range of the mass spectrometer.

The coupling of CE–MS using electrospray with pneumatically assisted nebulization (ionspray) has been described utilizing either an LJI [15] or a coaxial approach [16]. In a recent investigation both approaches have been compared [17].

Isotachophoresis (ITP) is a capillary separation technique which is capable of concentrating trace components and diluting of major constituents of the sample. The combination of these features makes ITP in principle an ideal technique for sample treatment. The use of ITP for sample pretreatment in CE has been described [18–21].

The degree of the concentration effect of ITP can be derived from the Kohlrausch equation:

$$C_A = C_L \cdot \frac{\mu_A}{\mu_A + \mu_R} \cdot \frac{\mu_L + \mu_R}{\mu_L} \quad (1)$$

where C_L is the molarity of the leading buffer, C_A the analyte concentration and μ the electrophoretic mobility (the subscript R refers to the counter ion). From this equation it can be seen that the final concentration of the analyte is proportional to the molarity of the leading buffer. This equation can be written as

$$C_A = C_L K \quad (1a)$$

where K is a proportionality factor. In the case of similar mobilities, K will have a value of about 1. Eqn. 1a clearly demonstrates the tremendous concentration potential of ITP. In this paper we describe the preliminary results of the on-line coupling of ITP–CE–MS with an ESP interface. For the coupling of ITP with CE we used the same set-up as described previously [18,19], where we demonstrated a decrease in the concentration detection limits by a factor of 100–1000. The CE–MS coupling was similar to that described by Smith *et al.* [13]. The potential of the ITP–CE–MS coupling is demonstrated with the analysis of a mixture of anthracyclines.

EXPERIMENTAL

Isotachophoresis

ITP was effected in a laboratory-made apparatus consisting of Plexiglas electrode vials and a PTFE capillary (150 × 0.32 mm I.D.). The terminating buffer consisted of 10 mmol/l histidine (HIS) (pH 7.2) in 60% (v/v) methanol (MeOH). Sodium phosphate (10 mmol/l, pH 7.2) in 60% MeOH was used as the leading buffer. Injection of 5 μl of analyte into the ITP system was done with a 100-μl injection syringe. No detector was used as the ITP zones of anthracyclines could be seen. For the determination of low concentrations one of the anthracyclines was used as a marker and added in a high concentration. The voltage was supplied by a Model RR100-1.5R power supply (Gamma High Voltage Research, Mt. Vernon, NY, USA) operating in the constant-current mode at 60 μA. For injection of ITP zones into the CE system by means of electrical splitting an additional power supply (Model RR40-1.5P; Gamma High Voltage Research) was used. The current was measured over a 350-Ω resistance placed in series with the capillary using a microamperometer (Model 134312; Goerz, Vienna, Austria).

Capillary electrophoresis

CE was carried out in a 700 × 0.075 mm I.D. (for CE–MS) or 700 × 0.040 mm I.D. (for CE–MS and ITP–CE–MS) fused-silica capillary (SGE, Ringwood, Australia) using an electrophoresis buffer composed of 10 mmol/l sodium acetate (pH 7.5)–60% MeOH (for CE–MS) or 10 mmol/l sodium phosphate (pH 7.2)–60% MeOH (for ITP–CE–MS).

The power supply was operated in the constant-voltage mode at 20 kV for CE. Samples were electrokinetically injected, applying 6 kV for 6 s.

On-capillary absorbance detection in CE took place at 350 mm from the anodic end, using a Spectra 100 UV–Vis absorbance detector (Spectra-Physics, Mt. View, CA, USA) at a wavelength of 210 nm.

Isotachophoresis–capillary electrophoresis

Coupling of ITP and CE was done as described previously [19] by inserting the CE capillary in the ITP capillary through a septum in the cathode compartment of the ITP system (Fig. 1). As the ITP zones approached the CE inlet, injection took place by means of electrical splitting proportional to the current distribution over the CE and ITP capillary. After injection the ITP capillary was carefully flushed with leading buffer and the CE run was started. The CE buffer was the same as the leading buffer.

Capillary electrophoresis–mass spectrometry

A Finnigan (San Jose, CA, USA) MAT TSQ 70 triple-quadrupole mass spectrometer equipped with an ESP system (Finnigan MAT) was used in the positive-ion mode. A sheath flow-rate of 1 μl/min was applied by means of a syringe pump (Model 2400; Harvard Apparatus, Edinbridge, UK). For the on-line coupling of the CE and MS systems it is essential to make electrical contact between the ESP needle and the CE capillary outlet. The sheath liquid consisted of 1% glacial acetic acid in ethanol–water (3:2, v/v). The current in the CE capillary was monitored as described under the CE conditions. The counter electrode of the electrospray was set at −4 kV and the ESP needle was grounded. The distance from the outlet of the CE capillary to the counter electrode was *ca.* 20 mm. Height differences between electrode vials and electrospray were avoided to prevent any hydrodynamic flow. All experiments were performed in the multiple ion detection (MID) mode.

Chemicals

Doxorubicin (DOX), carminomicin (CAR) and epirubicin (EPI) were available as 1 mg/ml chlorohydrate solutions containing 5 mg/ml of lactose and were purchased from Carlo Erba (Nivelles, Belgium). Daunorubicin (DAU) was purchased from Rhone-Poulenc (Paris, France). Histidine, methanol, sodium hydrogenphosphate and sodium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade. Demineralized water was used in all experiments.

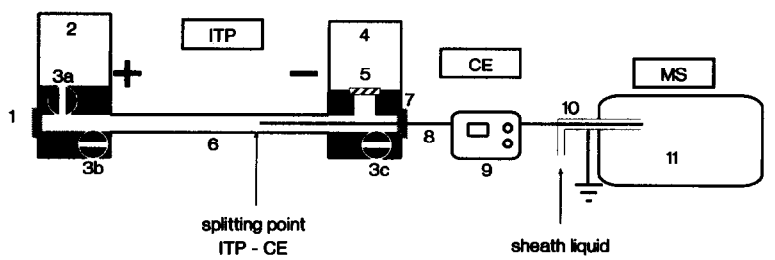

Fig. 1. Representation of on-line ITP–CE–MS coupling. Injection takes place through a silicone-rubber septum (1) by means of a syringe inserted up to valve 3b. The valves (3a–c) are used for flushing the ITP capillary with buffer. The terminating buffer vial (2) is connected to a positive voltage and the leading buffer (4) vial to earth during ITP and is raised to +2 kV at the time of injection. ITP takes place in a 150 × 0.320 mm I.D. capillary (6). The capillary is closed by a membrane (5) to prevent hydrodynamic flow. For the CE run the electrode in vial 2 is disconnected and the electrode in vial 4 is set at a positive voltage. CE takes place in either a 700 × 0.075 mm I.D. or 700 × 0.04 mm I.D. capillary (8) and was inserted into the ITP system through a septum (7). The UV absorbance detector (9) was placed 350 mm from the CE capillary end. The sheath needle (10) for electrospray MS (11) was always grounded.

RESULTS AND DISCUSSION

CE and ITP of anthracyclines

Several preliminary experiments were carried out for the optimization of the CE separation. Anthracyclines adsorb on the capillary wall, glassware and other laboratory materials, introducing a source of irreproducibility especially at low concentrations [22]. One way to overcome this problem is to use an organic modifier in the stock solutions and the electrophoresis buffers [23]. Therefore, either acetonitrile (ACN) or MeOH was added to the electrophoresis buffer.

Although the use of volatile buffers is often preferred in MS to prevent contamination of the ion source by salt formation, it was possible to work for several days with a 10 m*M* sodium phosphate or sodium acetate buffer before crystallization was notable and the ion source had to be cleaned. The low flow-rate in CE and the use of a volatile sheath liquid allowed the optimization of the CE buffer almost regardless of its MS compatibility.

The ITP separations of anthracyclines were performed in the cation mode because of their positive charge below pH 7.5. The ITP buffer system is a modification of the system described by Akedo and Shinkai [24], who used a leading buffer of sodium acetate at pH 6.0 and a terminating buffer of β-alanine in 60% MeOH for the determination of doxorubicin and doxorubicinol in plasma. Under these conditions, however, the mobility of some anthracyclines appeared to be higher than that of sodium. Therefore, the pH was increased to 7.2, thus lowering the mobility of the anthracyclines. Histidine was chosen as the terminating buffer because its p*I* of 7.5 is close to the working pH, resulting in the lowest mobility.

CE–MS of anthracyclines

In the coupling of CE with MS, the electrical contact between the electrospray needle and the capillary outlet appeared to be very important. Disruption of this contact could lead to electroperforation of the capillary because of the grounded stainless-steel electrospray needle surrounding the CE capillary over a length of 15 cm. By addition of 1% of glacial acetic acid to the sheath liquid this problem could be overcome, possibly owing to improved wetting of the fused silica and the stainless-steel ESP needle and to the higher conductivity of the sheath flow solvent.

Tuning of the mass spectrometer was done using a continuous flow of leading buffer containing 184 μmol/l of DOX at a flow-rate of 10 nl/min using the syringe pump. During the tuning of the mass spectrometer it appeared that the signal was not only determined by the concentration but also by the hydrodynamic flow-rate.

Fig. 2 shows the UV signal and the mass electropherogram of a mixture of DAU, EPI and DOX at concentrations of 66, 83 and 83 μg/ml, respectively. The UV trace shows no resolution between the stereoisomers DOX and EPI. In the mass electropherogram a shoulder is formed on the peak at $m/z = 544$, indicating a small difference in electrophoretic mobility. Also, the resolution between the peak of DAU and the peak of DOX and EPI has improved with respect to the UV signal. The mass electropherogram was monitored at 35 cm from the UV detector, resulting in a larger difference in migration time. The efficiency calculated as the plate number from the UV absorbance and the mass spectrometric peak of DAU was in both instances *ca.* 120 000, indicating that the electrospray does not significantly contribute to peak broadening.

In Fig. 3 the separation of the stereoisomers EPI and DOX is more pronounced. By changing the pH of the electrophoresis buffer from 4.6 to 7.2, the pH is closer to the pK_a values of EPI and DOX which are 7.7 and 8.2, respectively. This permitted an almost baseline separation of both compounds.

ITP–CE—MS of anthracyclines

The diameter of the CE capillary in ITP–CE–MS experiments was smaller than that in CE–MS experiments. It was necessary to use a 40 μm I.D. CE capillary in the ITP–CE mode in order to reduce the hydrodynamic flow in the CE capillary when the ITP capillary was flushed with leading buffer. Flushing of the ITP capillary was done after injection of ITP zones into the CE capillary. A syringe filled with leading buffer was connected to valve 3c (Fig. 1), valves 3c and 3b were opened and valve 3a was closed. Pressure was applied to the syringe and the ITP tube was flushed to establish a continuous buffer system during the CE run. The pressure applied to the ITP system through valve 3c is distributed over the ITP capillary and the CE

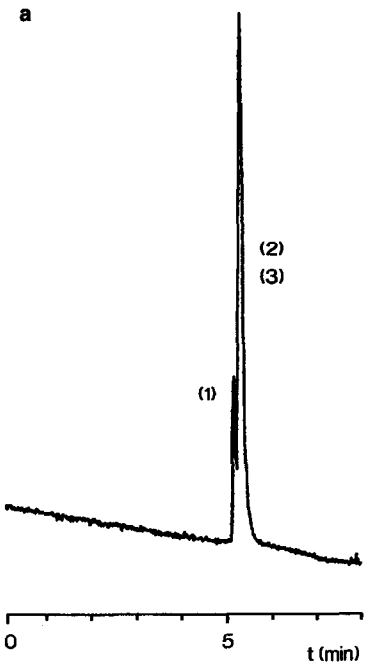

Fig. 2. Electropherograms of the UV absorbance (a) and mass spectrometric (b) detection of DAU (1), EPI (2) and DOX (3) (66, 83 and 83 μg/ml, respectively). CE took place in a 700 × 0.075 mm I.D. capillary using a 10 mmol/l sodium acetate buffer (pH 4.6) in 70% ACN, and a voltage of 20 kV.

Fig. 3. CE–MS of the stereoisomers EPI (1) and DOX (2) (250 μg/ml each) in 10 mmol/l sodium acetate buffer (pH 7.5) and 60% methanol using a 700 × 0.040 mm I.D. capillary and a voltage of 20 kV at the anodic end.

capillary. This resulted in a hydrodynamic flow that is proportional to the capillary radius to the power 4 and to the reciprocal of the capillary length. This hydrodynamic flow was a major source of peak broadening of the CE zones after ITP focusing when 75 μm I.D. CE capillaries were used. By using 40 μm I.D. capillaries this peak broadening could be considerably reduced.

Injection of the ITP zones into the CE capillary was done by electrical splitting [25]. The CE inlet is positioned between the anode and cathode vial of the ITP, at *ca.* 3 cm from the cathode. The CE capillary outlet is always grounded by means of the ESP interface. This means that during the ITP run a potential drop exists over the CE capillary. By raising the cathode voltage of the ITP from 0 to +2 kV the splitting ratio was increased in favour of the CE.

The ITP–CE coupling can be represented as an electrical circuit. When the influence of the electro-

osmotic flow is negligible, the splitting ratio between the CE and the ITP capillary can be calculated by the current ratios I_2/I_3 (see Fig. 4) [25]. In order to manipulate the splitting ratio, it is convenient to know the parameters that are influencing I_2 and I_3. Therefore, an equation is derived giving the splitting ratio I_2/I_3 as a function of the dimensions of the capillaries using the resistance ratio R_3/R_2, and of the voltages applied at the time of injection. The currents I_2 and I_3 can be written as

$$I_2 = (V_1 - I_1R_1 - V_2)/R_2 \quad (2)$$

and

$$I_3 = (V_1 - I_1R_1)/R_3 \quad (3)$$

and the ratio as

$$\frac{I_2}{I_3} = \frac{R_3}{R_2} \cdot \frac{V_1 - I_1R_1 - V_2}{V_1 - I_1R_1} \quad (4)$$

where V_1 and V_2 are the potentials of the anode and cathode vial of the ITP, respectively, at the time of

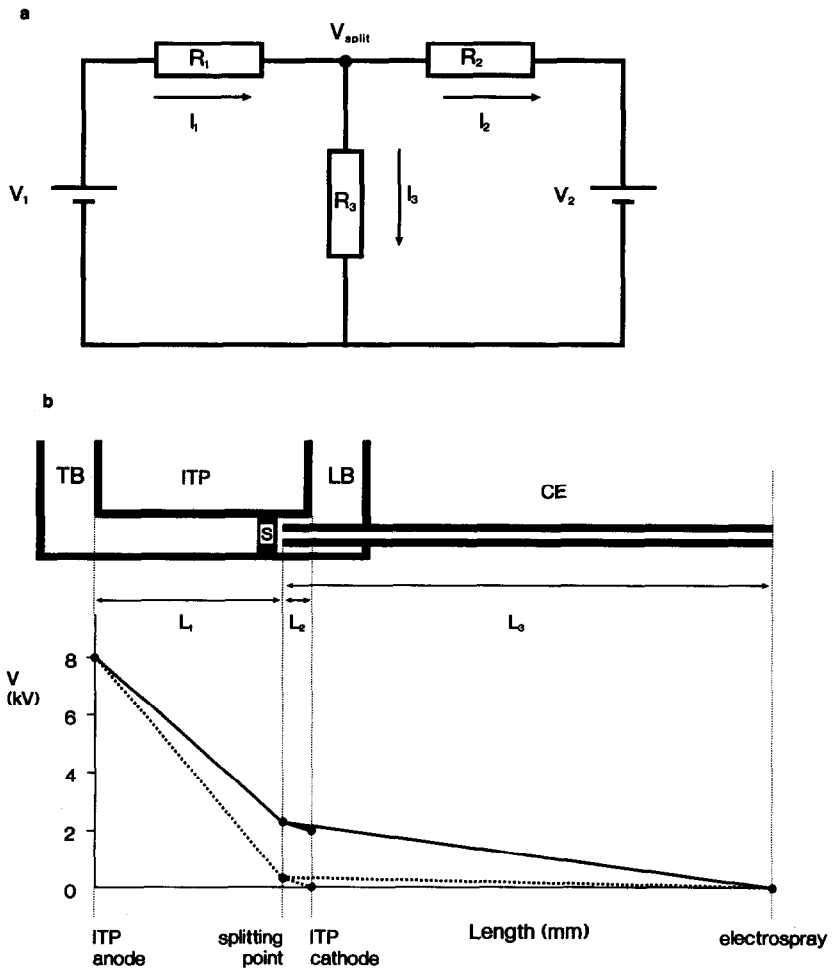

Fig. 4. (a) Schematic representation of the electrical circuit in ITP–CE–MS at the time of injection. R_1, R_2 and R_3 are the electrical resistances of the ITP capillary up to the point of injection (V_{split}), the ITP capillary from V_{split} to the cathode vial with correction for the inserted CE capillary outer diameter and the CE capillary, respectively. I_1, I_2 and I_3 are the corresponding currents through the capillaries. V_1 is the ITP anode voltage and V_2 is the ITP cathode voltage at the time of injection. (b) The voltage distribution over the capillaries at the time of injection is given for V_2 = 0 kV (dashed line) and for V_2 = 2 kV (solid line) at a constant voltage V_1 of 8 kV. L_1, L_2 and L_3 represents the lengths of the corresponding capillaries as used in eqns. 8, 12 and 13. S is the sample zone and TB and LB are the terminating and leading buffer, respectively.

injection and R_1, R_2 and R_3 are the electrical resistance of the ITP capillary from the anode to the splitting point and of the ITP capillary from the splitting point to the cathode and of the CE capillary, respectively.

As $V_1 - I_1R_1$ represents the voltage at the splitting point at the time of injection (V_{split}), eqn. 4 becomes

$$\frac{I_2}{I_3} = \frac{R_3}{R_2}\left(1 - \frac{V_2}{V_{split}}\right) \quad (5)$$

When the cathode of the ITP is grounded at the time of injection ($V_2 = 0$), the equation is simplified and the splitting ratio is

$$I_2/I_3 = R_3/R_2 \quad (6)$$

In this instance the splitting ratio is independent of V_{split}, which means that the filling of the ITP capillary with terminating buffer does not influence the splitting ratio. It should be noted that the current I_2 (Fig. 4a) can become negative. When V_2 approaches V_1 and $V_2 > V_{split}$ then the direction of the current is reversed, the ions will migrate in the opposite direction and the ITP conditions no longer exist. Just before that, when $V_2 = V_{split}$, the current ratio $I_2/I_3 = 0$, which means that theoretically 100% of the analyte will migrate into the CE system. However, in this instance small changes in the voltage will cause large differences in the splitting ratio. In our experiments the splitting ratio was considerably lower in order to prevent overloading of the CE system and to have a reproducible splitting ratio. The capillary resistance R can be calculated from

$$R = L/\pi r^2 K \quad (7)$$

where L is the capillary length, r is the capillary radius and K is the conductivity of the buffer. As both the CE capillary and the ITP capillary from the splitting point to the cathode are filled with the same leading buffer, the resistance ratio R_3/R_2 can be written as

$$\frac{R_3}{R_2} = \frac{L_3}{L_2} \cdot \frac{r_{ITP}^2 - r_{CE,o}^2}{r_{CE,i}^2} \quad (8)$$

where L_3/L_2 is the ratio of the total CE capillary length to the ITP capillary length from splitting point to the cathode. The ITP capillary radius r_{ITP} is corrected for the inserted CE capillary outer radius $r_{CE,o}$; $r_{CE,i}$ is the inner radius of the CE capillary. The voltage at the splitting point at the time of injection is calculated from

$$I_3 = I_1 - I_2 \quad (9)$$

$$\frac{V_{split}}{R_3} = \frac{V_1 - V_{split}}{R_1} - \frac{V_{split} - V_2}{R_2} \quad (10)$$

$$V_{split} = \frac{V_1 + \frac{R_1}{R_2} \cdot V_2}{1 + \frac{R_1}{R_3} + \frac{R_1}{R_2}} \quad (11)$$

The resistance ratios are

$$\frac{R_1}{R_2} = \frac{L_1}{L_2} \cdot \frac{K_L}{K_T} \cdot \frac{r_{ITP}^2 - r_{CE,o}^2}{r_{ITP}^2} \quad (12)$$

and

$$\frac{R_1}{R_3} = \frac{L_1}{L_3} \cdot \frac{K_L}{K_T} \cdot \frac{r_{CE,i}^2}{r_{ITP}^2} \quad (13)$$

The resistance ratios are corrected for differences in conductivities using the conductivity ratio K_L/K_T because at the time of injection the ITP capillary is filled to the splitting point with terminating buffer. The conductivity ratio can easily be measured by the ratio of the voltage drop over the ITP capillary before the ITP run when the capillary is filled with leading buffer and at the end of the ITP run when the capillary is filled with terminating buffer at a constant current. The measured difference in voltage drop is caused by a proportional difference in conductivity of the leading buffer with respect to the terminating buffer.

When in the described system the ITP cathode is grounded ($V_2 = 0$), the current ratio can be calculated from eqns. 6 and 8, using the capillary lengths L_3, L_2 and radius r_{ITP}, $r_{CE,o}$ and $r_{CE,i}$ which are 70 cm, 3 cm, 160 μm, 100 μm and 20 μm, respectively (Fig. 4b). This results in a current ratio of 910, which means that 0.1% of the total current is split to the CE.

Raising the voltage in the ITP cathode compartment appeared to be a convenient way to manipulate the splitting ratio. At a cathode voltage, V_2, of 2 kV, the splitting ratio was calculated to be 128, implying that 0.8% was split into the CE system (L_1 = 12 cm,

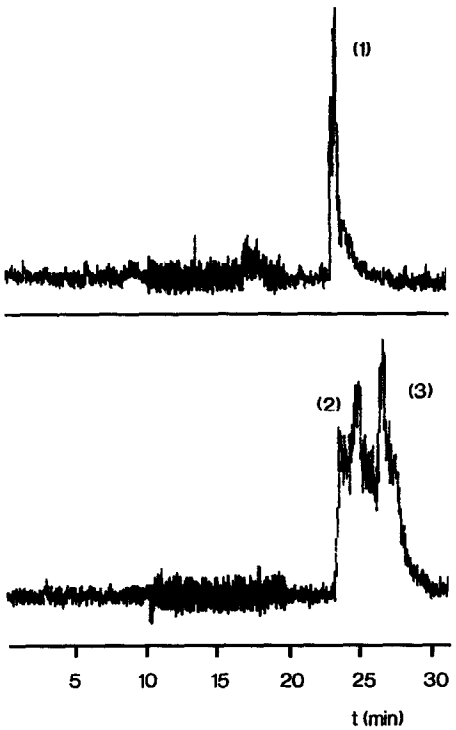

Fig. 5. Mass spectrometric electropherogram obtained in ITP–CE–MS of DAU (1), EPI (2) and DOX (3) (3, 8 and 8 μg/ml, respectively).

$K_L/K_T = 7$, $V_1 = 8$ kV). This splitting ratio was used in further experiments. Although this splitting ratio is low, V_2 was not increased further because this would lead to overloading and unwanted effects caused by the electroosmotic flow in the CE capillary. An increase in V_2 with a constant V_1 reduces the total current in the ITP system and slows the ITP process at the time of injection. At the same time, however, the voltage drop over the CE capillary will cause an increased migration rate in the direction of the CE cathode and will result in peak broadening during injection. This phenomenon and the effect of electrical splitting using a constant current in ITP are still under investigation.

To study the potential of isotachophoretic analyte focusing in CE–MS, several ITP–CE–MS experiments were performed. The standard mixture of anthracyclines was diluted to 5, 20, 50 and 200 times lower concentrations than in the CE–MS experiments. A 5-μl volume of a mixture of DAU, EPI and DOX at concentrations of 3, 8 and 8 μg/ml, respectively, was injected into the ITP system. CAR was added as a marker at a concentration of 10 μg/ml. The mass electropherogram after ITP–CE separation (Fig. 5) shows considerable peak broadening due to overload of the CE capillary after the ITP step.

Lowering the concentration by a factor of 10 improved the peak shape considerably (Fig. 6). In this instance the concentration of the marker CAR was 30 μg/ml.

The improvement in detectability is clearly illustrated in Fig. 7, which shows the CE–MS results for 66 μg/ml DAU (a) in comparison with ITP–CE–MS results for 0.3 μg/ml DAU (b). The improvement in detectability is at least a factor of 200.

CONCLUSIONS

This work has demonstrated the possibility of analyte focusing using on-line ITP–CE–MS. An improvement in detectability of at least a factor 200 has been demonstrated for the analysis of DAU. Although in the ITP–CE–MS mode the concentra-

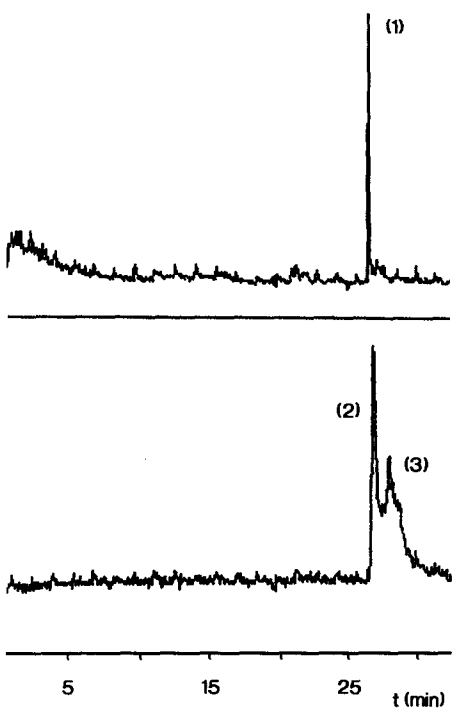

Fig. 6. Mass spectrometric electropherogram obtained in ITP–CE–MS of DAU (1), EPI (2) and DOX (3) (0.3, 0.8 and 0.8 μg/ml, respectively).

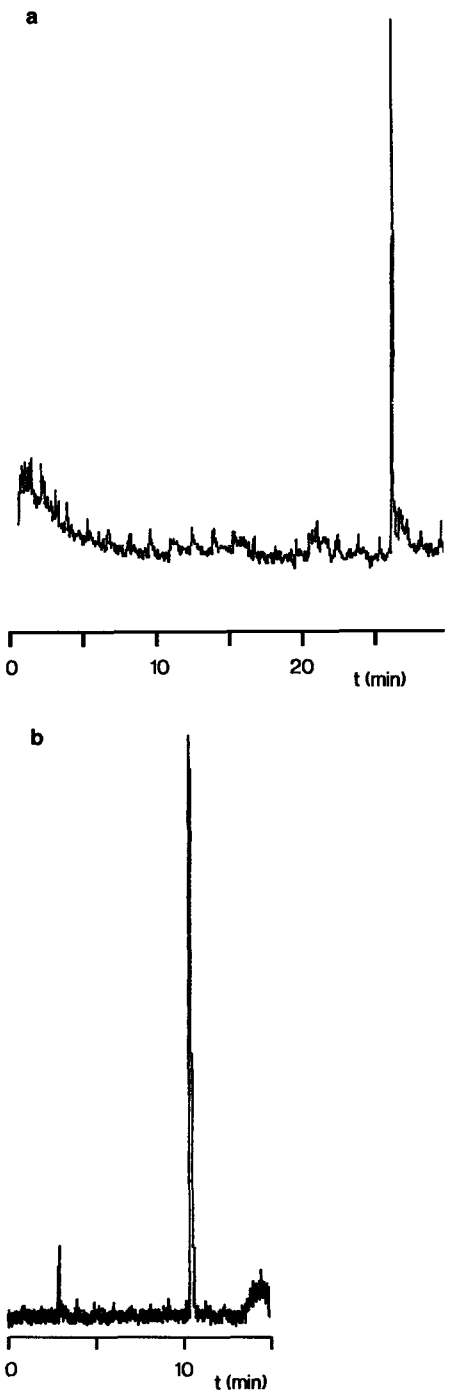

Fig. 7. Mass spectrometric detection of (a) 0.3 μg/ml of DAU by ITP–CE–MS and (b) 66 μg/ml of DAU by CE–MS.

tion of the anthracyclines was lowered by a factor of 5–200, some overloading of the CE system could still be seen.

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