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Application of Microdialysis for On-line Coupling of Capillary Isoelectric Focusing with Electrospray Mass Spectrometry on a Magnetic Sector Instrument

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The on-line coupling of capillary isoelectric focusing (CIEF) with electrospray mass spectrometry (MS) by means of microdialysis (MD) for application to three carbonic anhydrase isozymes is described. The dialysis device consisted of a donor and an acceptor compartment that were separated by a flat membrane, and served to clear the CIEF effluent that was directed to the mass spectrometer from ampholytes that were necessary for the establishment of a pH gradient in the IEF capillary. In addition to single-ion monitoring and full-scan experiments, a position- and time-resolved ion counting (PATRIC) detector was used in the static array mode. For CIEF/MD/MS of unknown proteins the minimum amount of analyte required for the analysis was estimated to be in the mid-femtomole range using full-scan MS data acquisition. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

In biochemical and clinical research, isoelectric focusing has proved to be a very valuable tool for protein characterization. Traditionally, the separation was carried out in a gel medium, but with the rapid development of capillary separation techniques isoelectric focusing was extended to the capillary format.[1] With commercially available mixtures of ampholytes a pH gradient is formed in the capillary in which the proteins to be analyzed are separated and focused according to their isoelectric point. The high resolving power of capillary isoelectric focusing (CIEF), as of any other capillary separation technique, in combination with the focusing characteristics analogous to isotachophoresis, has generated growing interest in this technique.[2–4]

An important aspect of the development of CIEF is the wide range of detection techniques that has become available. Whereas laborious staining procedures are inevitable for gel isoelectric focusing, on-line detection of the proteins focused in CIEF can take place in a very short time. UV absorbance detection is obviously the most widely used technique on a routine basis, but it suffers from a lack of sensitivity. Partly, this can be attributed to the presence of the pH gradient: the carrier ampholytes absorb strongly below 230 nm, whereas at higher wavelengths the transparency varies considerably, resulting in an unstable background signal.

The most desirable detection technique in combination with CIEF is probably mass spectrometry (MS) because of the additional information that can be obtained: together with the separation and the indication of the p*I*, the mass provides information about the nature of the proteins. Through the development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which are both very suitable for protein analysis, mass spectrometry has become accessible for the fields of biochemistry and biotechnology.[5,6] Off-line micropreparative CIEF with MALDI time-of-flight MS and on-line coupling of CIEF with electrospray MS detection have both been described for several proteins[7–11]

For on-line coupling, however, the nature of mass spectrometric detection places some limitations on the composition of the solution to be introduced into the mass spectrometer when electrospray stability and minimum interface contamination are required. High amounts of background electrolytes or involatile salts should preferably be avoided in order to ensure reliable operation of the mass spectrometer. In combination with ESI-MS of proteins and peptides, microdialysis (MD) has been used as a desalting technique prior to introduction of the sample into the mass spectrometer.[12] The use of microdialysis has also been described as a sample desalting technique for CIEF.[13]

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In this paper, on-line coupling of CIEF with MS is described using microdialysis as a tool to clear the CIEF effluent of the excess of ampholytes before introduction into the mass spectrometer, while maintaining the separation of the proteins. The dialysis device used in earlier work[14] for the coupling of CIEF with MS has been thoroughly revised and miniaturized. For MS detection a magnetic sector instrument with the option of position- and time-resolved ion counting (PATRIC) for static and scanning array detection was used.

As model proteins, some carbonic anhydrases (CAs) were used. The carbonic anhydrases are zinc-binding proteins[15] and represent a family of isozymes that are very widespread in plant and animal life.[16] In mammals, at least seven different isozyme forms designated CA-I to CA-VII have been described. The isozymes of CA are responsible for the reversible hydration–dehydration of carbon dioxide and water, with protons being transferred between the active site of the enzyme and a surrounding buffer. The proteins have a 10-stranded β-sheet that halves the molecule into a hydrophilic and a hydrophobic region. Because of their availability in sufficient quantities and at relatively low cost, these proteins are extremely suitable for on-line CIEF/MD/MS method development.

EXPERIMENTAL

Experimental set-up

The experimental set-up is shown schematically in Fig. 1. A programmable injector for capillary electrophoresis (Prince Technologies, Emmen, The Netherlands) was used for hydrodynamic injection and power supply at the inlet of the IEF capillary. To reduce the electroosmotic flow and the adsorption of the proteins on the capillary wall, fused-silica capillaries (100 μm i.d., 245 μm o.d.; BGB Analytik Zürich, Switzerland) were coated with poly(vinyl alcohol).[17] The separation system consisted of two capillaries with lengths of 750 and 350 mm, respectively, that were connected by means of a custom-made dialysis device. In Fig. 2(a) a side-view of the dialysis device is presented. It is constructed from Plexiglas and consisted of two pieces that fit perfectly together. In both pieces, a slot has been drawn with a width of 140 μm and a length of 15 mm. The slot in the donor compartment had a depth of 100 μm, resulting in an approximate volume of 0.15 μl, while that in the acceptor compartment was 300 μm deep. In the acceptor compartment a platinum electrode was incorporated. A cross-section of the dialysis device is shown in Fig. 2(b). The first capillary, in which IEF was carried out, comes into the donor compartment via a piece of PTFE tubing and a finger-tight connector, and at the end of the slot the capillary for transfer of the analytes to the mass spectrometer is connected. The acceptor compartment is flushed by means of a syringe pump (Model 2400, Harvard Apparatus, Edinbridge, UK). Between the two parts of the dialysis device a flat synthetic cellulose ester dialysis membrane with a thickness of about 30 μm and a molecular mass cut-off of 5 kDa was placed (Spectrum, Houston, TX, USA). The two pieces were screwed tightly together while ensuring the right position of the donor and acceptor compartments.

The MS experiments were performed on a Finnigan MAT 900 magnetic sector instrument that was equipped with an API ion source operated in the electrospray mode (Finnigan MAT, San Jose, CA, USA) and a PATRIC detector. The mass spectrometer was optimized with respect to the temperature of the sampling capillary, which appeared to be 200 °C. Electrical contact at the electrospray needle tip was established via a sheath liquid that was delivered at a flow rate of 5 μl min^{-1}. With the composition of the sheath liquid of methanol–5% acetic acid (80:20, v/v) the formation of ionic boundaries and distortion of the electrophoretic zones in the transfer capillary were avoided.[18] The electrospray tip was kept 3 kV above the accelerating voltage of the mass spectrometer, which was 5 kV. All spectra were obtained in the positive ion mode, using either single-ion monitoring (SIM), full-scan or PATRIC in the static array mode. In this mode the

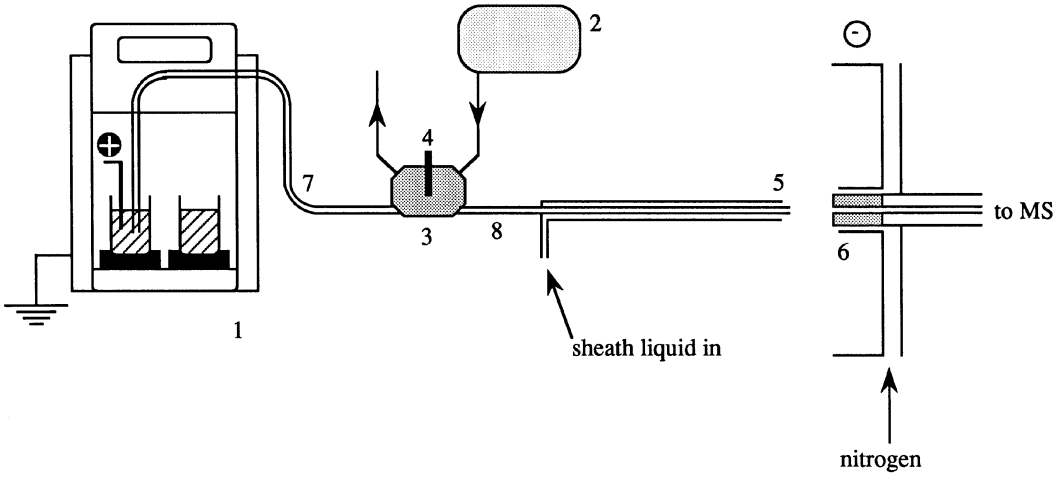

Figure 1. Schematic diagram of the experimental set-up: (1) programmable injector for CE with internal high-voltage power supply; (2) syringe pump for the delivery of dialysis liquid to the dialysis device; (3) custom-made Plexiglas dialysis device; (4) electrode connection on the dialysis device; (5) electrospray needle at 8 kV; (6) electrospray sampling capillary at 5 kV; (7) IEF capillary; (8) transfer capillary.

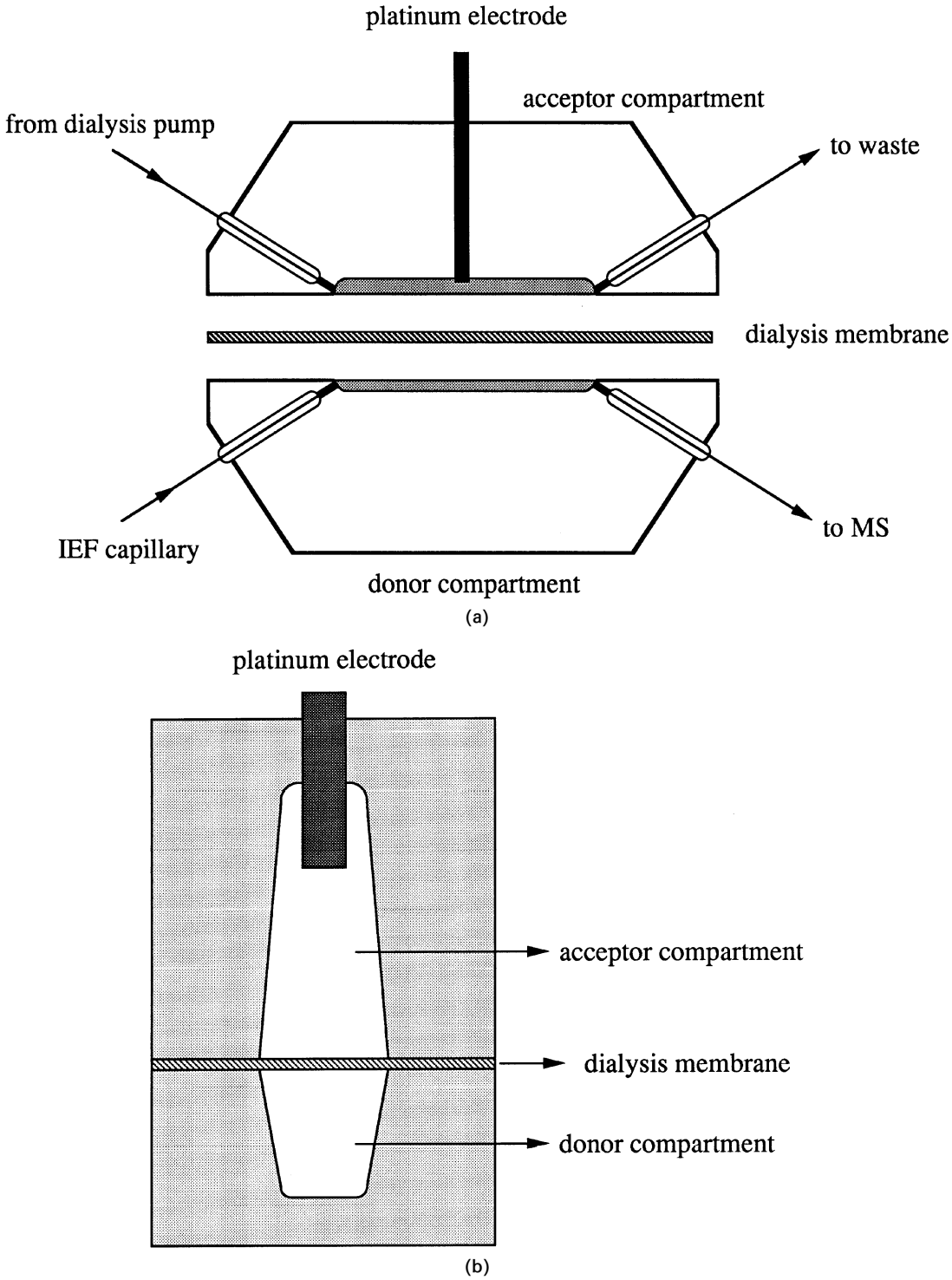

Figure 2. (a) Side-view of the custom-made Plexiglas dialysis device. (b) Cross-section of the dialysis device. See text for details.

PATRIC detector was set at a certain predefined mass with a mass window of 4%. With the deconvolution program BIOMASS (Finnigan MAT), experimental masses of the proteins were calculated from the full-scan mass spectra.

Capillary IEF

As in previous work,[14] 2% acetic acid was used as both anolyte and catholyte, because of its compatibility with MS detection. Under the influence of an electric field, 1% Pharmalyte 5–8 solution served to generate the pH gradient in the separation capillary. For sample preparation, a mixture of three proteins was dissolved in the 1% Pharmalyte solution used for IEF. To avoid any memory effect, the whole capillary system with the dialysis device was flushed thoroughly with water and 2% acetic acid between runs. After the flushing procedure, a plug of 1% Pharmalyte solution was hydrodynamically injected into the IEF capillary. Subsequently, the protein sample was injected so that there was still a

plug consisting of 2% acetic acid, serving as the catholyte, present in the IEF capillary. The dialysis device and the second capillary for transfer of the proteins to the MS are still filled with 2% acetic acid, so that transient capillary IEF between the separation capillary inlet and the dialysis device could be carried out. For this purpose, a voltage of 30 kV was applied at the inlet of the separation capillary, while the dialysis device was held at ground potential via the electrode in the acceptor compartment. The acceptor phase consisting of 2% acetic acid was pumped through the acceptor compartment at a flow rate of 5 $\mu l\ min^{-1}$. For mobilization of the focused protein zones past the detector (UV absorbance; Spectra-Physics, Mount View, CA, USA), through the dialysis device and towards the electrospray tip, a pressure of typically 5 mbar was applied. Throughout the mobilization step, an electric field was maintained in the capillary system. The voltage on the electrospray tip was only applied after CIEF was finished to avoid any flow from the electrospray tip towards the dialysis device during CIEF. The UV absorbance detector, operating at a wavelength of 280 nm, was located at a distance of 630 mm from the IEF capillary inlet, just in front of the dialysis device.

Chemicals

The ampholyte mixture used for the generation of the pH gradient in the IEF capillary was Pharmalyte 5–8 (Pharmacia Biotech, Uppsala, Sweden). Methanol was purchased from Rathburn (Walkerburn, UK) and acetic acid was obtained from Baker (Deventer, the Netherlands). Carbonic anhydrases I and II, both from human erythrocytes, were obtained from Sigma (St Louis, MO, USA). Apart from the original CA-I with a p*I* of 6.6, another chemically treated form of CA-I was used, which has a p*I* of 6.0. The isoelectric point of CA-II is 7.4. The accurate mass (in the region of 28 kDa) of these proteins was established during the MS experiments. In all experiments deionized water was used, generated with a Milli-Q system (Millipore, Bedford, MA, USA).

RESULTS AND DISCUSSION

The development of a technique such as electrospray MS is of great importance for the fields of biotechnology and protein analysis. Determination of the molecular mass, disulfide bonds and protein folding are only a few of the possibilities that mass spectrometric analysis of protein samples can offer. For the separation of mixtures of proteins IEF has a long tradition, and the transformation of this technique into capillary format facilitates the implementation of on-line MS detection. However, the coupling of such a separation technique is not completely straightforward because of the medium in which the separation is carried out. In CIEF, the proteins are separated and focused in a pH gradient according to their isoelectric point. For the generation of the pH gradient in the capillary, commercially available mixtures of ampholytes are used that have a relatively low molecular mass (<1000 Da) and limited volatility. Although the flow rate after mobilization in CIEF is low, the introduction of a constant flow of ampholytes into the mass spectrometer is not desirable because of electrospray instability, source contamination and a negative influence on the sensitivity. To achieve a decrease in ampholyte content in the CIEF effluent so that less ampholyte is directed to the electrospray, the difference in size of the ampholytes and the proteins offers the possibility of dialysis. By incorporating a dialysis step between CIEF and MS detection, the low molecular mass (~680 Da) Pharmalyte constituents can be dialyzed out of the separation system. The much larger analyte proteins are not able to pass through the dialysis membrane and are transported via a transfer capillary to the electrospray tip for introduction into the mass spectrometer.

As in earlier work,[14] for the incorporation of microdialysis into CIEF/MS two capillaries were coupled by means of a dialysis device. The dialysis device used here consisted of a donor and an acceptor compartment that are separated from each other by a flat membrane. In CIEF/MD/MS there are three stages to be distinguished (see Table 1). In the first step, after completion of the injection procedure in which the capillary system is successively filled with 2% acetic acid, 1% Pharmalyte 5–8 solution and the protein sample, IEF is carried out in the separation capillary. Care is taken that there is still a zone of 2% acetic acid present in the IEF capillary in front of the dialysis device in order to carry out transient CIEF. The voltage is applied between the IEF capillary inlet and the electrode in the acceptor compartment of the dialysis device, which is flushed with 2% acetic acid serving as the catholyte. The presence of the membrane in the dialysis device does not affect the electrical circuit, because it is completely permeable to small ions. In the second step, the focused zones are mobilized by application of a hydrodynamic pressure of 5 mbar at the inlet of the IEF capillary, while the voltage is applied between the IEF capillary inlet and the electrospray needle tip. During this stage, the electrode on the dialysis device is physically disconnected because otherwise it will serve as ground. By passing the total content of the IEF capillary through the donor compartment of the dialysis device, the low molecular mass ampholytes can be dialyzed out of the separation system into the acceptor phase. The large proteins that cannot penetrate the dialysis membrane are transported to the mass spectrometer via the transfer capillary in the third step.

In Fig. 3, the mass electropherogram that is obtained by CIEF/MD/MS in the full-scan mode after injection of a mixture of the three CAs is represented. The injected amount was 6 pmol for each protein and the

Table 1. Different stages of CIEF/MD/MS

Stage	Inlet vial	Dialysis device	Electrospray tip	Time interval
CIEF	30 kV	Ground	Ground	10 min
Dialysis	30 kV	Floating	8 kV	>10 min
Transfer	30 kV	Floating	8 kV	>10 min

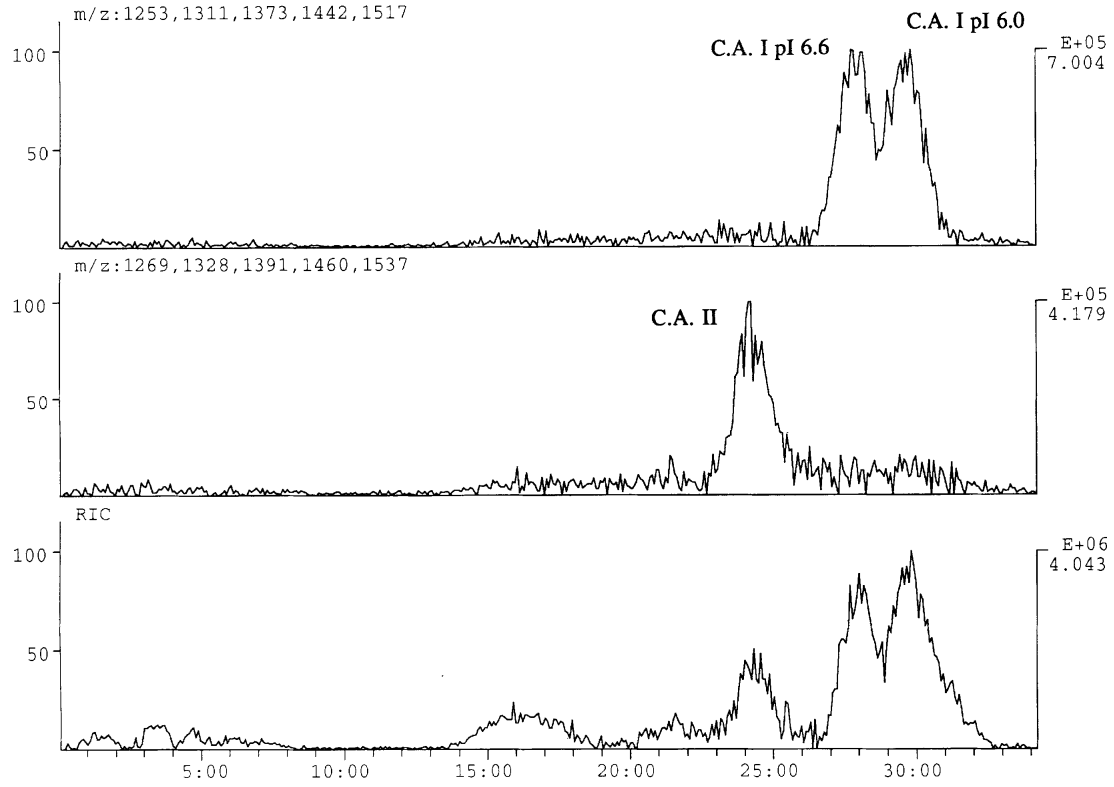

Figure 3. Mass electropherogram obtained by CIEF/MD/MS in full-scan mode, scanning from *m/z* 1000 to 2000 in 4.5 s. MS data acquisition was started simultaneously with the mobilization of the focused protein zones. The mobilization pressure at the IEF capillary inlet was 5 mbar, while a voltage of 22 kV was applied over the whole coupled capillary system. The amount injected was 6 pmol for each protein, with an injection volume of 2 μl. The uppermost trace was obtained by accumulation of the signals of the multiply charged ions with *m/z* 1253, 1311, 1373, 1442 and 1517 from the mass envelopes of CA-I (p*I* 6.0) and CA-I (p*I* 6.6). The middle trace was obtained after accumulation of the signals of the ions with *m/z* 1269, 1328, 1391, 1460 and 1537 from the mass envelope of CA-II. These ions were also used for data acquisition in the MID mode.

volume that was injected into the IEF capillary was 2 μl. After deconvolution of the spectra the masses of the proteins could be determined. For both p*I* forms (6.0 and 6.6) of CA-I the mass was established as 28 798 Da, and the mass for the whole protein of CA-II was 29 169 Da, both with a relative standard deviation of 0.02%. An interesting aspect of the mass spectrum of CA-II was the appearance of two other mass envelopes suggesting the formation of two fragments with masses of 7080 and 7638 Da. These ions were probably formed by nozzle/skimmer-induced fragmentation. CA-II consists of 259 amino acids, and the 7080 Da fragment corresponds to amino acids 199 (proline) to 259 (lysine, the *C*-terminus). The sequence of amino acids 193 (also proline) to 259 accounts for the fragment with a mass of 7638 Da.

The same CIEF/MD/MS experiment was carried out in the SIM mode (Fig. 4), where for each protein five ions were selected from the mass envelopes obtained in the previous experiment. The added ion traces are represented as the reconstructed ion current. Both mass electropherograms show good resolution of CA-II (p*I* 7.4) and CA-I (p*I* 6.6), but the resolution between the two CA-I forms with p*I* 6.6 and 6.0 is limited. Initial experiments using a simplified system incorporating a second UV absorbance detector instead of the mass spectrometer, with the outlet of the second capillary placed in a vial containing 2% acetic acid at ground potential, indicated that in the dialysis device significant band broadening occurs. Still, the signals belonging to CA-I (p*I* 6.6) and CA-I (p*I* 6.0) could be distinguished as separate peaks. There are numerous factors that can contribute to the total band broadening caused by the dialysis device. Of course, the connections of the IEF and the transfer capillary are of major importance. Both capillaries approach the donor slot at a certain angle, necessitating careful polishing of the capillary tips so that they fit perfectly into the slot in order to avoid large dead volumes. Also, the shape of the donor slot has to be taken into account. The perfect circle of the IEF capillary cross-section goes over into a much more angular shape with one flat side [see Fig. 2(b)], and transforms back again into the circular cross-section of the transfer capillary. In addition, polishing of the Plexiglas surface inside the slot was impossible, leading to distortion of the flow profile caused by the rough surface. Furthermore, the Plexiglas material has different electroosmotic flow characteristics to the coated capillaries, which gives rise to band broadening, and adsorption of the analytes may also occur.

The exertion of a higher pressure on the dialysis membrane from within the acceptor compartment at higher dialysis flow rates did not lead to deformation of the membrane or distortion of the shape of the donor slot. As a result, the dialysis flow rate in the acceptor compartment did not affect the separation or the total analysis time. The dialysis flow rate was set at 5 μl min^{-1}, so that with a volume of ~0.5 μl the acceptor compartment was flushed 10 times per minute.

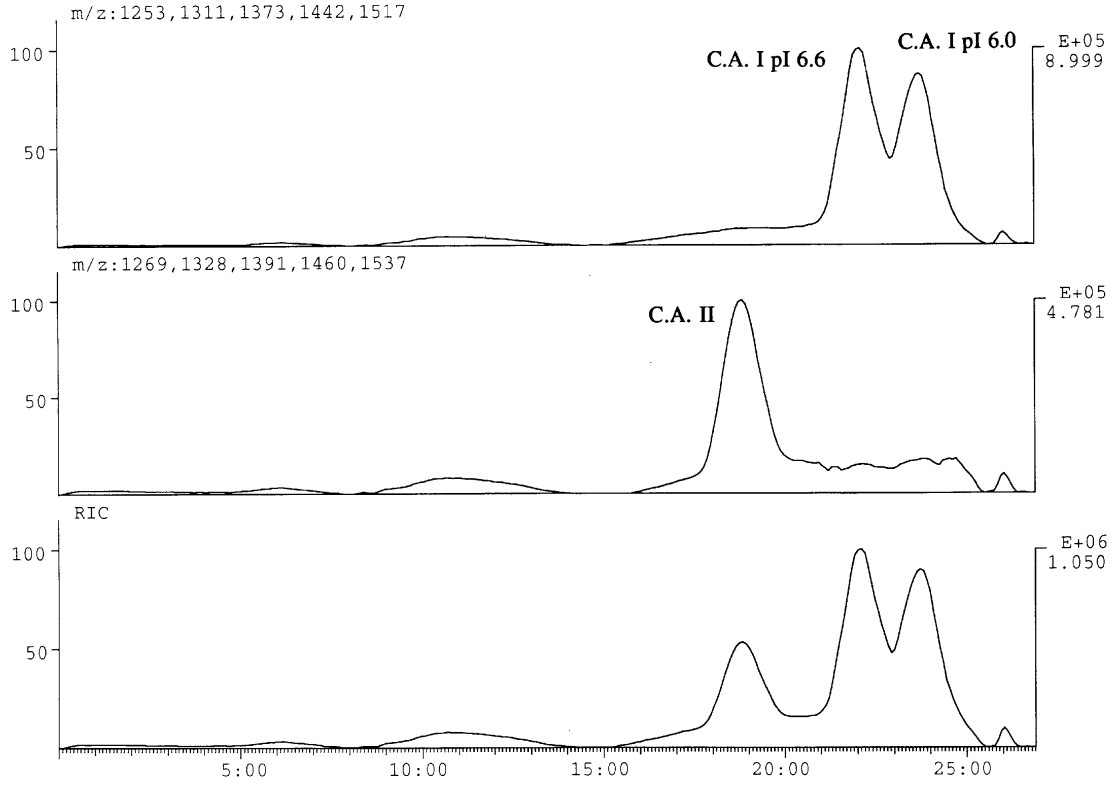

Figure 4. Mass electropherogram obtained by CIEF/MD/MS using SIM. For each protein five ions were selected from the mass envelopes obtained with full-scan data acquisition. The amount injected was 6 pmol for each protein, with an injection volume of 2 μl.

Apart from adsorption in the dialysis device, the adsorption of proteins on capillary walls is a well known problem with capillary electrophoretic separation techniques. In order to obtain good peak shapes after protein separation, the use of coated capillaries is essential. Various capillary coating procedures have been developed over the years, and in this work all capillaries were coated with poly(vinyl alcohol)[17] in order to minimize the adsorption of proteins. For the analysis of unknown proteins, often present in minute amounts, the MS data should be acquired in the full-scan mode because of a lack of any specific mass information beforehand.

Considering the mass electropherogram acquired in the full-scan mode (Fig. 3), where 6 pmol of each protein were injected, an estimated minimum amount of 500 fmol of protein is required in order to be able to assign the charge states for the mass envelopes and consequently to extract information concerning the protein mass.

The use of SIM or full-scan as the mode for MS detection has a significant influence on the UV signal in this experimental set-up. When using SIM as the scan mode for MS detection, the magnetic field of the magnetic sector instrument is kept constant while the electric field is varied. The UV absorbance detector is greatly influenced by the variable electric field, resulting in electronic noise in the UV signal. As expected this was not observed in the full-scan mode, where the electric field is kept constant and the magnetic field is variable.

In preliminary constant infusion experiments of the two forms of CA-I, a mass of 28 798 was found, with a standard deviation of 4 mass units (0.02%). The difference in p*I* between the two forms of CA-I raises the question of the nature of this difference. Surely the breakage of one or two disulfide bonds, with a change in mass of 2 or 4 units, might explain the p*I* shift. The breakage of disulfide bonds could result in a different folding of the protein with the possibility of a significant change in p*I* because of the higher accessibility of acidic residues that constitute ~12% of the protein. However, CA-I contains only one cysteine residue, thereby precluding this explanation. Another source of different folding and thus difference in p*I* might be found in the modification of some amino acid residues in the protein, leading to a minor difference in mass that is within the standard deviation.

To investigate this possibility, PATRIC detection in the static array mode was used. In the static detection mode all ions are detected simultaneously within a mass range of 4% around a central mass, with sensitivities comparable to SIM, but more mass spectrometric information is provided. In Fig. 5, the spectrum is shown that is obtained with static array detection with *m/z* 1824 as the center mass after CIEF/MD/MS in analogy with the experiments with full-scan and SIM detection. This spectrum is taken from the signal belonging to CA-I (p*I* 6.6) and corresponds to the 16^+ charge state of the protein. Comparing it with the spectrum taken from the signal belonging to CA-I (p*I* 6.0) (spectrum not shown), no significant mass difference between the two CAs is observed given the standard deviation of the high-resolution measurement. This leads to the conclusion that the p*I* difference is not caused by any amino acid modification that results in different protein

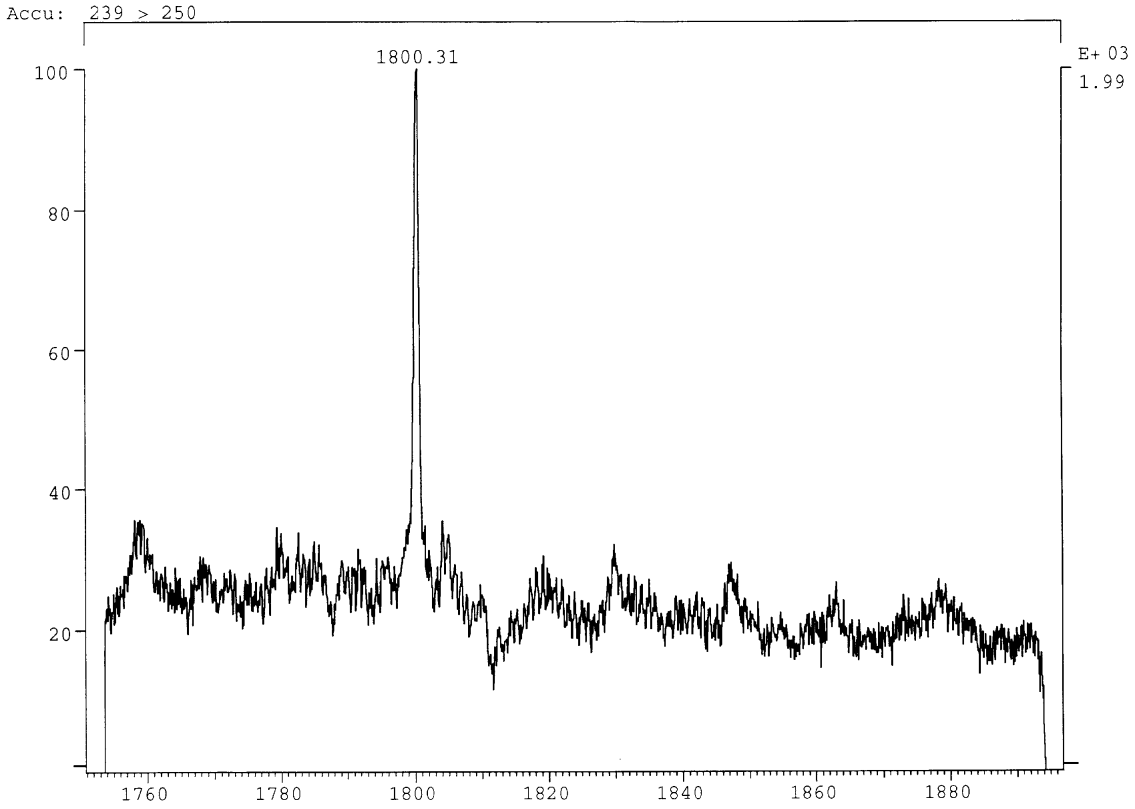

Figure 5. Mass spectrum of CA-I (p*I* 6.6) obtained with static array detection with *m/z* 1284 as the center mass after CIEF/MD/MS according to the procedure used for full-scan and SIM experiments. The signal belonging to *m/z* 1800.31 represents the 16^+ charge state of CA-I (p*I* 6.6). The mass spectrum for CA-I (p*I* 6.0) is identical.

folding, and that probably the observed mass difference in the two full-scan spectra can be attributed to the standard deviation in these measurements.

With the coupled capillary system incorporating the custom-made dialysis device, on-line CIEF/MD/MS could be carried out satisfactorily, but there are still some points to consider for optimization of the system. An aspect of CIEF/MD/MS that deserves attention is miniaturization of certain hardware parts of the analytical system. With regard to the high cost of interesting proteins such as the interleukins, it is advisable to use an injection device designed to handle sample volumes of only ~ 5 µl. Furthermore, the dimensions of the dialysis device have to be decreased for better adjustment of the separation and transfer capillaries. This necessitates the exploration of other techniques for the construction of such devices, such as silica etching. Equally important is the construction of an integrated set-up for the coupling of the dialysis device via the transfer capillary to the electrospray tip. The transfer capillary only serves as a connection between the dialysis device and the electrospray tip and is not required to contribute to the separation. In the ideal situation it should therefore be chosen as short as possible in order to minimize the total analysis time and zone broadening. In the set-up used throughout the experiments described here the transfer capillary had a length of 350 mm. Taking the length of the electrospray needle (210 mm) into account, this was the minimum workable length with which the electrospray interface could still be opened without limitations. This is desirable for checking the condition of the electrospray tip and for flushing purposes between days. Unfortunately, with most commercially available electrospray interfaces the length of the electrospray needle together with the size of the spray chamber will dictate the length of the transfer capillary. Another problem related to the use of a long transfer capillary arises when a protein with a low p*I* that is focused at the acidic side of the pH gradient has a high electrophoretic mobility and overtakes other proteins with lower electrophoretic mobility that have entered the transfer capillary first. This implies that the separation that is achieved with CIEF in the first capillary may be significantly reduced in the transfer capillary. With a long transfer capillary the consequences of this phenomenon should be addressed. All these considerations are important when a reliable and rugged CIEF/MD/MS method has to be developed for protein analysis on a routine basis.

CONCLUSIONS

The on-line coupling of CIEF with ESI-MS by means of a microdialysis device was demonstrated for three carbonic anhydrases. The implementation of the custom-made dialysis device incorporating a flat membrane separating the donor and acceptor compartment resulted in a stable electrospray and good mass spectral information from the mass electropherograms, owing to removal of part of the Pharmalyte. For future investigations, however, it remains necessary to further the design improve, especially with respect to the material

and the dimensions of the device, so that electrophoretic peak integrity can be maintained to a greater extent.

The spectral information obtained with the use of the PATRIC detector in the static array mode indicates that the large p*I* difference of the two forms of CA-I cannot be attributed to a modification in the amino acid sequence of the protein.

For the CIEF/MD/MS analysis of unknown proteins, MS data should preferably be acquired in the full-scan mode because of a lack of mass information beforehand. The minimum amount of analyte for full-scan MS detection was estimated to be of the order of 500 fmol.

With proteins being a very interesting group of cell constituents, the CIEF/MD/MS approach described here can lead to the successful combination of single cell analysis with MS detection. Apart from the separating power of CIEF, the considerable concentration of the sample during the analysis is an interesting aspect of this type of analysis.

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