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Use of microdialysis for the on-line coupling of capillary isoelectric focusing with electrospray mass spectrometry

M.H. Lamoree, 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, Netherlands

Abstract

The coupling of capillary isoelectric focusing (IEF) with electrospray mass spectrometry (MS) has been described for the model proteins horse heart myoglobin, carbonic anhydrase I and β-lactoglobulin A. An experimental set-up incorporating an IEF capillary and a transfer capillary which are connected by a dialysis fiber, enabling the implementation of microdialysis (MD), has been developed. Low-molecular-mass ampholytes that create the pH gradient in the capillary can be dialysed out of the system, while the proteins remain in the capillary, so that subsequent electrospray MS detection can be performed. To facilitate capillary IEF–MD–MS, acetic acid that can function both as anolyte and catholyte has been used. © 1997 Elsevier Science B.V.

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1. Introduction

In recent years, isoelectric focusing (IEF) has, in analogy to electrophoresis, undergone a transformation from the slab gel to the capillary format [1]. As a result modern detection techniques such as electrospray mass spectrometry (MS) have become available for analyte detection. IEF is traditionally used for the characterization of proteins, which are separated in a pH gradient according to their isoelectric point. In order to obtain protein separation in capillary IEF, a commercially available mixture of ampholytes is used to create a pH gradient in the capillary.

Alongside the separation power of capillary IEF, the relatively large injection volumes which lie in the order of microliters are a major advantage. Thus, analogous to capillary isotachophoresis, analytes are separated and concentrated in one step.

In capillary IEF great effort has been directed to the characterization of hemoglobin and its variants [2–5]. Another aspect that has gained a lot of attention is the dynamic or static coating of capillaries in order to reduce adsorption of the proteins to the capillary wall, as well as the electroosmotic flow in the separation capillary [6,7]. The separated proteins were mostly detected by UV absorbance although recently MS detection has been described [8,9].

The use of a mass spectrometer as detector for capillary IEF raises some limitations. Just as with micellar electrokinetic chromatography (MEKC) and chiral capillary zone electrophoresis (CZE), where large amounts of involatile buffer additives are used, the presence of an excess of ampholytes in the capillary effluent leads to electrospray instability and salt deposition in the interface. Likewise, the in-

*Corresponding author.

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volatility of the acids and bases that are habitually used to generate the pH gradient creates problems when used in combination with MS detection. These two aspects have to be dealt with in order to obtain a capillary IEF–MS system that operates under long term stable conditions.

In this paper on-line capillary IEF–MS is presented for some model proteins. To this end, the use of a volatile acid and base and the incorporation of a microdialysis device in the experimental set-up have been investigated. By means of microdialysis [10] the low-molecular-mass ampholytes are dialysed out of the separation system so that they do not interfere in the electrospray process, while the high-molecular-mass proteins are transported to the MS system.

2. Experimental

2.1. Experimental set-up

The experimental set-up is schematically represented in Fig. 1. For hydrodynamic injection and power supply at the inlet of the IEF capillary a programmable injector for capillary electrophoresis (Prince Technologies, Emmen, Netherlands) was used. In all the experiments, 75 μm I.D.×190 μm O.D. fused-silica capillaries have been used that were coated with polyvinylalcohol [11] in order to reduce the electroosmotic flow and the adsorption of analyte proteins to the capillary wall. The capillary system consisted of two capillaries (730 and 400 mm, respectively) that were coupled by means of a 3 mm piece of dialysis tubing from an artificial kidney, which had an inner diameter of approximately 200 μm. The molecular mass cut-off of this material is 5000. On both sides of the dialysis tubing the fused-silica capillaries were inserted, and glued meticulously to the dialysis tubing without creating a dead volume on either side.

The coupled capillary construction was placed in a custom-made dialysis device (see Fig. 2) that was fabricated from two pieces of plexiglass that both had a slot drilled into it with a length of 30 mm, and a width of 1 mm. The two halves were fitted together with four screws, and the system was kept leak tight by means of two pieces of silicon rubber which were placed in between the two plexiglass parts. In both pieces of silicon rubber a space of the same dimensions as described above was cut away. The coupled capillaries were placed with the dialysis tubing in the center of the slot in the dialysis device. The volume

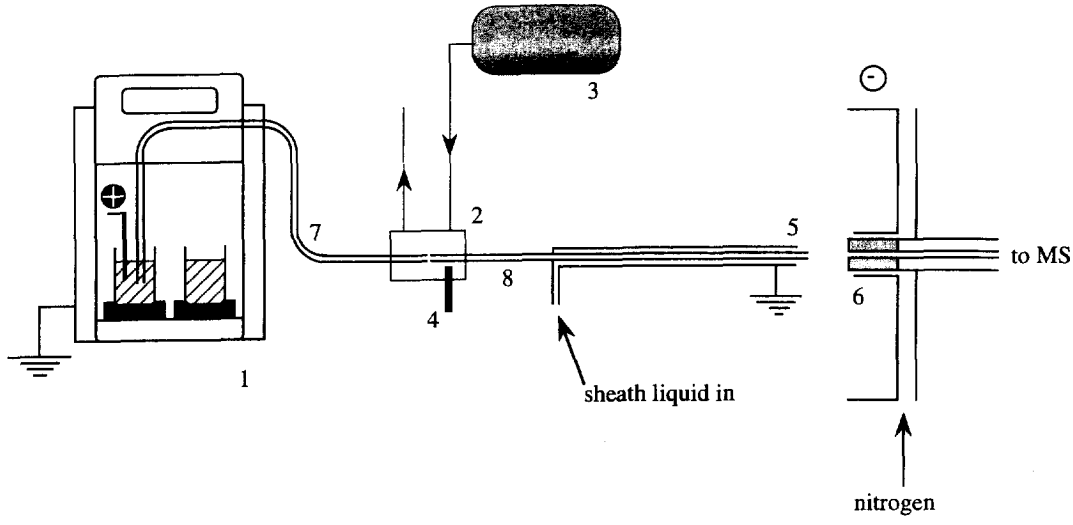

Fig. 1. Schematic representation of the experimental set-up: (1) programmable injector for CE with internal high voltage power supply; (2) custom-made plexiglass dialysis device (3) syringe pump for the delivery of dialysis liquid to the dialysis device; (4) electrode connection on the dialysis device;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

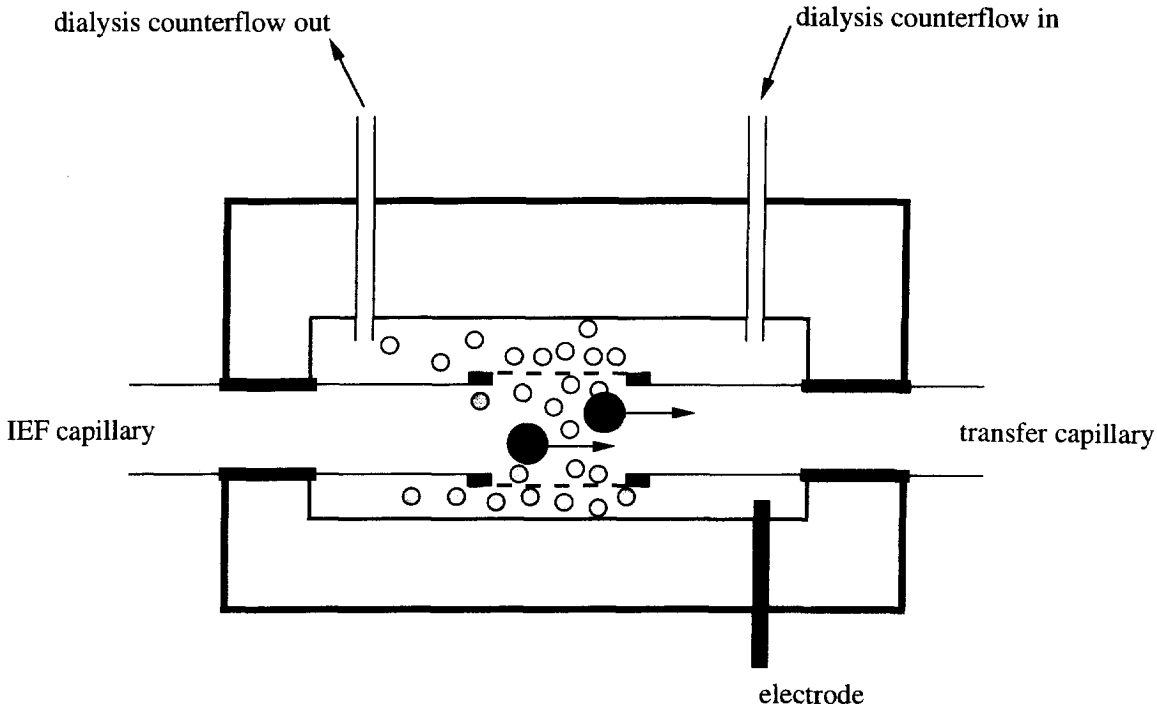

Fig. 2. Custom-made plexiglass dialysis device. See Section 2.1 for details.

of the dialysis chamber was approximately 25 μl. The lower part of the device had an electrode connection, while in the upper part two connections were made for a syringe pump (Model 2400, Harvard Apparatus, Edinbridge, UK) which was used to flush the dialysis device.

In the first capillary, before the dialysis tubing, IEF was carried out, while the second capillary functioned as a transfer capillary for transportation of the analytes towards the MS, after dialysis had taken place.

All MS experiments were performed on a Finnigan MAT SSQ 710 single-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray interface (Analytica, Branford, CT, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid, delivered at a flow-rate of 1 μl/min, which consisted of methanol–5% acetic acid (80:20, v/v). Nitrogen was used as a drying gas at a temperature of 50°C. The electrospray tip was held at ground potential, while the electrospray counterelectrode was set at −3.4 kV. Analyte detection was carried out in the multiple ion detection (MID) mode. Therefore, two m/z values from the mass envelope of each protein were incorporated in an MID procedure.

2.2. Capillary IEF

2% acetic acid was used both as (weak) acid and (weak) base to establish the pH gradient in the capillary. For capillary IEF either a 1 or a 2.5% Pharmalyte 5-8 solution was used. The sample consisted of a mixture of proteins that were dissolved in the same percentage of Pharmalyte 5-8 that was used for capillary IEF. Between runs, the system is flushed thoroughly with water and 2% acetic acid. After flushing with 2% acetic acid, the coupled capillary system is filled with Pharmalyte 5-8 solution. Subsequently, a known volume of sample in Pharmalyte solution was injected in such a way that there is still a zone containing 2% acetic acid present in the IEF capillary. Care was taken that the dialysis tubing and the transfer capillary were filled with 2%

acetic acid, so that transient capillary IEF could be carried out. The dialysis liquid that was pumped through the dialysis device by the syringe pump consisted of 2% acetic acid. To transport the focused protein zones in the capillary past the UV absorbance detector or towards the electrospray tip for MS detection a pressure of typically 25 mbar is applied.

To estimate the extent of zone broadening in the dialysis device, an experimental set-up was used that incorporated two UV absorbance detectors (Spectra-Physics, Mount View, CA, USA), one placed just in front and the other just after the dialysis device at a detection wavelength of 280 nm. The coupled capillary system was identical to the one described above. The outlet of the transfer capillary was placed in a vial containing 2% acetic acid, which was held at ground potential.

2.3. Chemicals

The mixture of ampholytes used to create a pH gradient in the separation capillary was Pharmalyte 5-8 (Pharmacia Biotech, Uppsala, Sweden). Acetic acid was purchased from Baker (Deventer, Netherlands) and methanol from Rathburn (Walkerburn, Scotland). As model proteins horse heart myoglobin [M_r 16 951, isoelectric points (pI values) 7.2 and 6.8], carbonic anhydrase I (M_r 28 783, pI 6.6) and β-lactoglobulin A (M_r 18 360, pI 5.3) were used, all purchased from Sigma (St. Louis, MO, USA). In all experiments, deionized water was used, obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

3. Results and discussion

As with combinations of virtually all separation methods, the coupling of capillary IEF to MS detection is governed by volatility requirements for the IEF medium. For real on-line measurements, the first problem already arises with the choice of catholyte, which is normally placed at the outlet of the separation capillary. When capillary IEF is directly coupled to electrospray MS detection, the outlet of the separation capillary is guided via the spray needle and sheath liquid in order to spray the capillary effluent towards the electrospray sampling capillary. The presence of sodium hydroxide, which is normally used as catholyte, in either the sheath liquid or the separation capillary is expected to seriously hamper the electrospray process by ion suppression and salt deposition in the interface.

Although the force of habit often leads to the choice of sodium hydroxide as the catholyte and phosphoric acid as the anolyte for capillary IEF, these chemicals can be replaced by other acids and bases that are more compatible with MS detection. Acetic acid was found to be a very attractive alternative because, in addition to its volatility, it can function as both anolyte and catholyte.

Another feature of capillary IEF is the use of relatively high amounts of Pharmalyte which forms the pH gradient. Apart from volatility considerations, constant introduction of such a load of buffer additives is extremely unattractive due to its influence on the sensitivity on one hand, and to source fouling and consequently long term stable operation on the other. The difference in molecular size of the Pharmalyte constituents [12] and the protein analytes opens up possibilities for the incorporation of microdialysis (MD) in the experimental set-up. By introduction of a MD step after capillary IEF of the proteins has taken place, the low-molecular-mass (~650) Pharmalyte constituents can be dialysed out of the separation system. The much larger analyte proteins are not able to pass through the dialysis membrane and are subsequently transported via the transfer capillary to the electrospray tip for introduction into the MS system.

In order to achieve this, a capillary IEF–MS system that incorporates a MD step has been developed, using a two capillary system that is connected by means of a dialysis fiber from an artificial kidney. In capillary IEF–MD–MS, three stages can be discerned. At first, IEF of the analyte proteins is carried out in the IEF capillary. In the second step the focused zones are mobilized hydrodynamically. By passing through the short piece of dialysis fiber in the dialysis device, the Pharmalyte constituents are dialysed out of the separation system, and the analyte proteins enter the transfer capillary. In the third step, the analyte proteins are transferred to the electrospray tip for introduction into the MS.

In Fig. 3 the procedure is represented schematically. Throughout the whole analysis, 2% acetic acid is

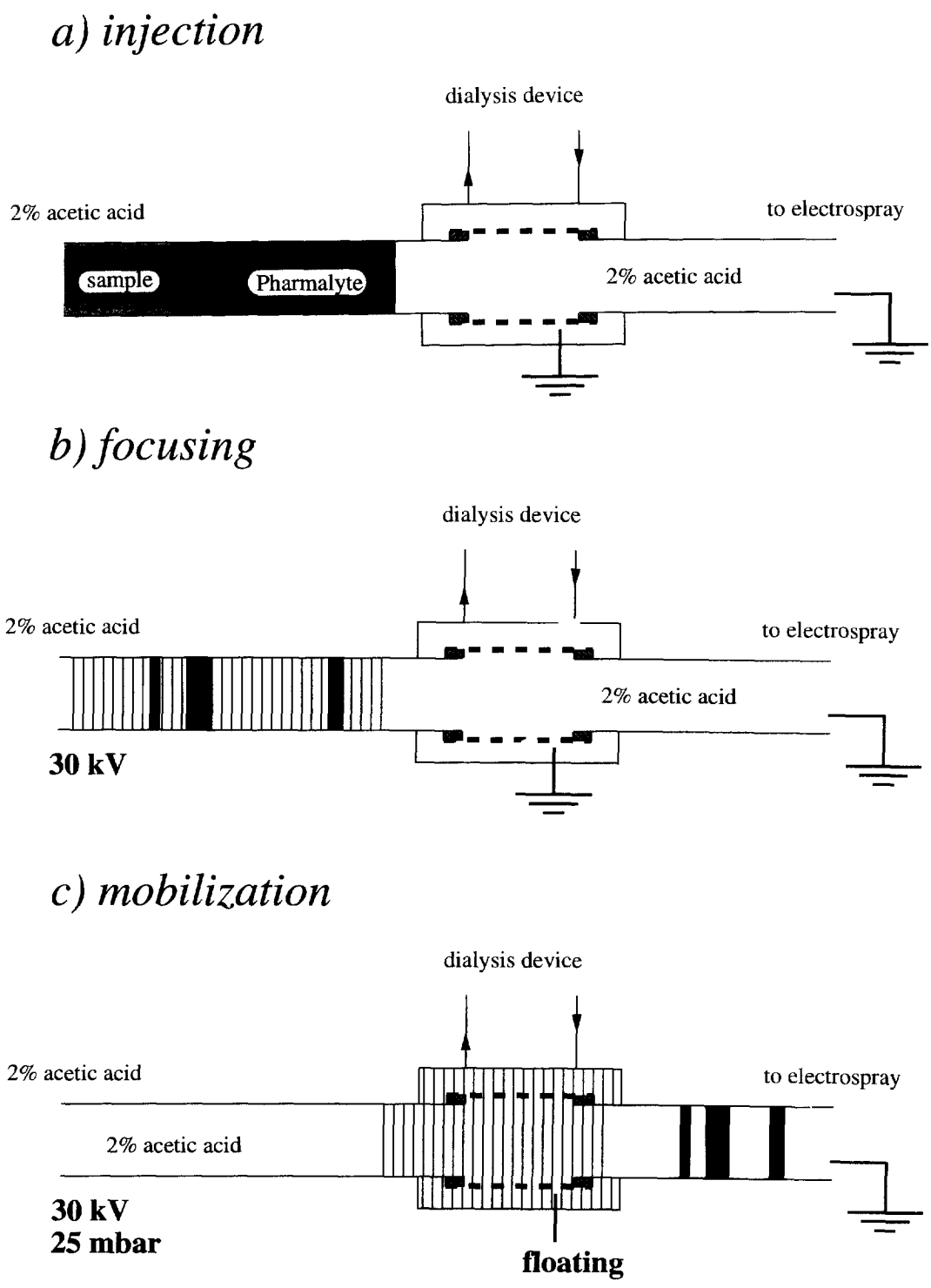

Fig. 3. Schematic representation of the principle of capillary IEF–MD–MS: (a) injection; (b) focusing; (c) mobilization.

pumped through the dialysis device, and the electrospray tip is held at ground potential. For injection, the capillary system is rinsed with 2% acetic acid. Subsequently, the IEF capillary is filled with Pharmalyte and sample, in such a way that there is still a zone containing acetic acid present in the IEF capillary (Fig. 3a). IEF is started by applying 30 kV to the inlet of the capillary system, while keeping the dialysis device at ground (Fig. 3b). After 6 min of focusing, the ground connection on the dialysis device is removed and the focused zones are mobilized by application of a hydrodynamic pressure of 25 mbar at the capillary inlet, while maintaining a voltage of 30 kV on the inlet of the capillary system (Fig. 3c). When the Pharmalyte constituents and the focused proteins reach the dialysis fiber, the low-molecular-mass species pass through the pores of the fiber. The large analyte proteins, that cannot be dialysed out of the system, are transported via the transfer capillary to the electrospray tip, so that MS detection can be performed.

In order to assess the extent of band broadening that is introduced by the dialysis step, UV experiments have been performed with the coupled capillary system. Two UV absorbance detectors were used, one of which was placed just in front and the other just after the dialysis device. The outlet of the transfer capillary was placed in a vial containing 2% acetic acid, which was held at ground potential. Capillary IEF was carried out with 1% Pharmalyte, according to the procedure described above. In Fig. 4 the two electropherograms are shown. Elec-

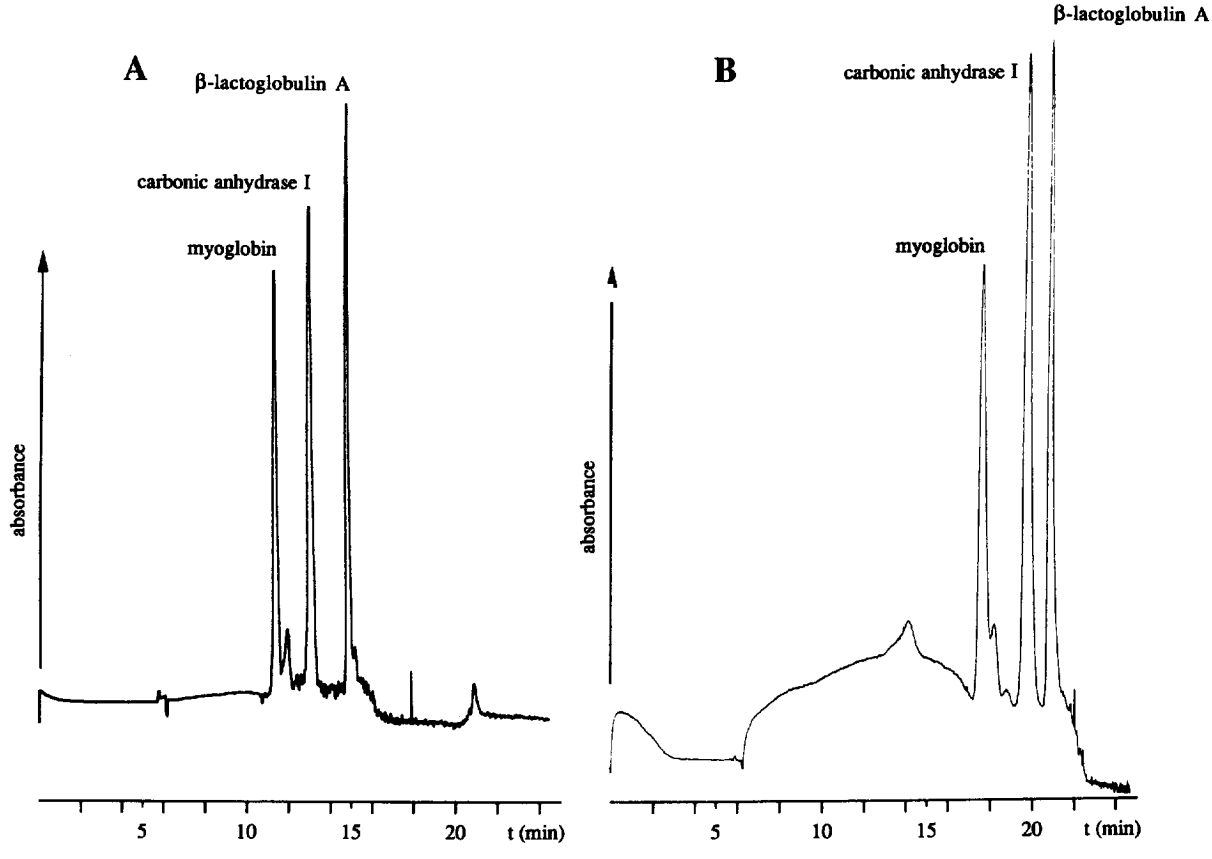

Fig. 4. To assess the performance of the dialysis device with regard to zone broadening, two UV absorbance electropherograms (λ=280 nm) were recorded. For electropherogram A, which was obtained just in front of the dialysis device, the sensitivity was set at 0.02, while for electropherogram B, obtained just after the dialysis device, the sensitivity was set at 0.005. The small peak migrating just behind the large myoglobin peak is the small fraction of myoglobin with p*I* 6.8.

tropherogram A was obtained just before the dialysis device, while in electropherogram B the zones are detected after passing the dialysis device. The injected amounts for myoglobin, carbonic anhydrase I and β-lactoglobulin A measured 8, 6 and 12 pmol, while the injected volume was 0.85 μl. Although band broadening occurs the system performs satisfactorily, because peak integrity is maintained. It can be seen from the increase in background signal in electropherogram A that some Pharmalyte passes the dialysis fiber and enters the second capillary, but this does not disturb UV absorbance detection. For MS detection however, the influence of such a small amount of Pharmalyte constituents has to be investigated.

It should be noted that after capillary IEF the Pharmalyte constituents and the proteins do not carry a net charge. This is inherent to the nature of IEF and forms the basis for separation of the different analyte proteins. However, in the dialysis fiber and in the transfer capillary some mixing with the acidic environment will occur, so that gradually the analyte proteins will become positively charged again. This can be seen when the two UV absorbance electropherograms are compared. In electropherogram A, the relative distance between the peaks corresponding to carbonic anhydrase I and β-lactoglobulin A becomes much smaller than the relative distance between the peaks corresponding to myoglobin and carbonic anhydrase I. An explanation for this phenomenon is given by the migration order of these three proteins in CZE when using a 2% acetic acid running buffer. Under these circumstances, which are more or less identical to those in the transfer capillary, the electrophoretic mobility of β-lactoglobulin A is higher than that of the other two proteins. Practically, this means that β-lactoglobulin A catches up with the carbonic anhydrase I and myoglobin, and that the resolution between β-lactoglobulin and the other two proteins decreases.

Another aspect of the use of a dialysis device is the influence of the dialysis pump flow-rate. The influence of a low and a high dialysis pump flow-rate, 6 and 60 μl/min, respectively, was investigated for 1% and 2.5% Pharmalyte as the IEF medium. The ratio of the analyte signal to the background signal in MS detection was taken as a measure to represent the influence of the dialysis pump flow-rate on the dialysis (see Table 1). For these experiments the capillary IEF–MD–MS set-up was used. Increasing the dialysis pump flow-rate has two effects: the background decreases while the signal intensity increases. This phenomenon may be attributed to a decrease of analyte suppression when more Pharmalyte is dialysed out of the separation system at higher dialysis pump flow-rates. Consequently, the optimal condition for IEF–MD–MS is a high dialysis pump flow-rate, i.e., 60 μl/min. From the increasing electronic noise observed when 2.5% instead of 1% of Pharmalyte is used as the IEF medium it can be concluded once more that dialysis is not complete. Therefore, to limit contamination of the interface, Pharmalyte concentrations as low as possible should be used.

The mass electropherogram obtained by capillary IEF–MD–MS is shown in Fig. 5. For each protein, the traces of the two corresponding ions that were selected for the MID procedure are added, and MS data acquisition was started simultaneously with IEF. The injected amount of myoglobin was 0.78 pmol, for carbonic anhydrase I 2.3 pmol and for β-lactoglobulin A 14 pmol, with an injection volume of 0.66 μl. As with the capillary IEF–UV experiments, the resolution between carbonic anhydrase I and β-lactoglobulin A is reduced, due to the high electrophoretic mobility of the latter in the transfer capillary. Actually, with the use of acetic acid as the catholyte and as sheath flow constituent, it is possible to do on-line capillary IEF– MS with the same set-up as conventional CZE–MS without removing

Table 1
Influence of the dialysis pump flow-rate on the ratio of analyte signal intensity to background for 1 and 2.5% Pharmalyte as the IEF medium

Dialysis pump flow-rate (μl/min)	1% Pharmalyte Analyte signal intensity/background	2.5% Pharmalyte Analyte signal intensity/background
6	6.8	6.2
60	12.7	14.0

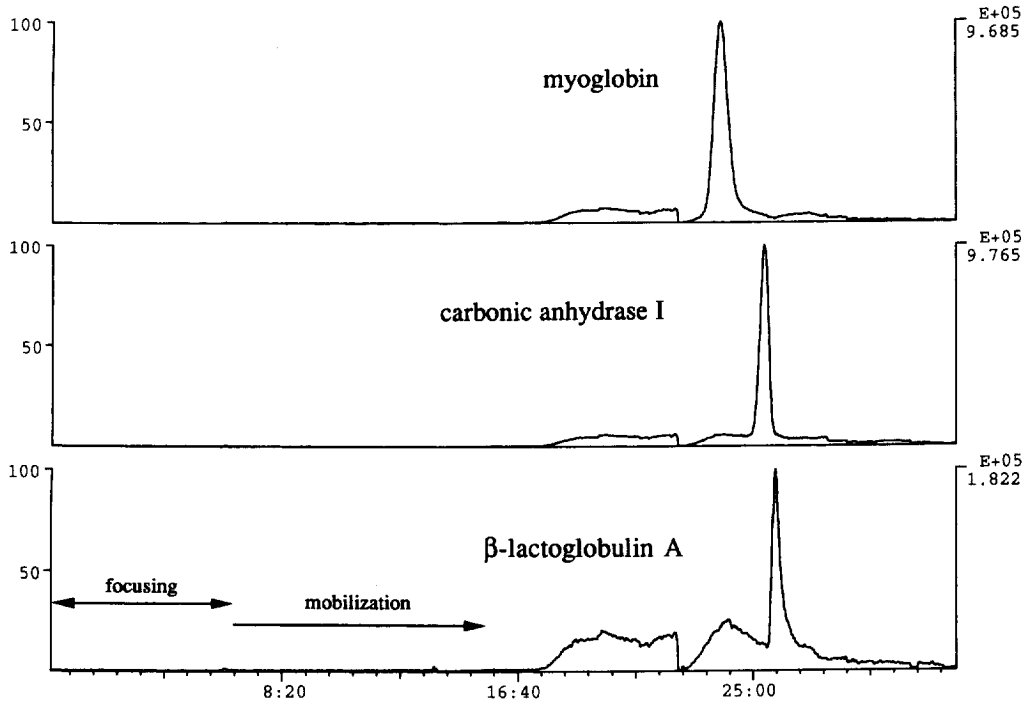

Fig. 5. Mass electropherogram obtained by capillary IEF–MD–MS. MS data acquisition was started simultaneously with capillary IEF, which was carried out with 1% Pharmalyte and a focusing time of 6 min at 30 kV over the IEF capillary. The mobilization pressure at the inlet was 25 mbar, while 30 kV was applied over the whole coupled capillary system. The dialysis pump flow-rate was 60 μl/min. The injection volume was 0.66 μl, with injected amounts of 0.78, 2.3 and 14 pmol for myoglobin, carbonic anhydrase I and β-lactoglobulin A, respectively.

the ampholytes (data not shown). Analyte resolution that was obtained on the basis of the differences in p*I* was maintained completely. However, such a system was found to be extremely unreliable, primarily because of the deposition of ampholytes as a white powder on the electrospray tip and the sampling capillary and an unstable electrospray. Thorough cleaning of the electrospray needle and the sampling capillary between runs was inevitable to perform two consecutive capillary IEF–MS runs. The capillary IEF–MD–MS system was much more reliable with regard to long term stable operation. Although dialysis with the custom-made dialysis device was not complete, it proved to be a very promising tool for on-line removal of undesirable buffer constituents. Nevertheless, optimization of the design remains essential for incorporation of microdialysis in capillary separation techniques.

4. Conclusions

The on-line coupling of capillary IEF with electrospray MS via microdialysis for three model proteins is achieved. The use of acetic acid, which functions both as anolyte and catholyte and meets the requirements of volatility necessary for reliable MS operation, facilitates the capillary IEF–MD–MS procedure. The custom-made dialysis device that was used to dispose of the large amount of Pharmalyte constituents worked satisfactorily. Improvement of the design remains necessary with regard to complete removal of the undesirable buffer constituents and with regard to minimization of the zone broadening.

Apart from the separating power, the large injection volumes for capillary IEF are an interesting feature. The injection volumes lie in the microliter range, which is about one order of magnitude higher

than for CZE. Therefore, capillary IEF is a very attractive analytical tool, because of the combination of concentration and separation of especially proteins in a single run.

References

[1] S. Hjertén, M. Zhu, J. Chromatogr. 346 (1985) 265.
[2] J.M. Hempe, R.D. Craver, Clin. Chem. 40 (1994) 2288.
[3] S.B. Harper, W.J. Hurst, C.M. Lang, J. Chromatogr. B 657 (1994) 339.
[4] S. Molteni, F.H. Frischknecht, W. Thormann, Electrophoresis 15 (1994) 22.
[5] J. Wu, J. Pawliszyn, Electrophoresis 16 (1995) 670.
[6] F. Kilár, CRC Handbook of Capillary Electrophoresis: A Practical Approach, CRC Press, Boca Raton, FL, 1994, Ch. 4, p. 95.
[7] J.R. Mazzeo and I.S. Krull, CRC Handbook of Capillary Electrophoresis: A Practical Approach, CRC Press, Boca Raton, FL, 1994, Ch. 18, p 495.
[8] Q. Tang, A.K. Harrata, C.S. Lee, Anal. Chem. 67 (1995) 3515.
[9] Q. Tang, A.K. Harrata, C.S. Lee, Anal. Chem. 68 (1996) 2482.
[10] Q. Wu, C. Liu, R.D. Smith, Rapid Commun. Mass Spectrom. 10 (1996) 835.
[11] M. Gilges, M.H. Kleemiss, G. Schomburg, Anal. Chem. 66 (1994) 2038.
[12] Isoelectric Focusing—Principles and Methods, Pharmacia Fine Chemicals, Uppsala.