Journal of Chromatography, 589 (1992) 321–327
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 736

Capillary isoelectric focusing with electroosmotic zone displacement and on-column multichannel detection

Wolfgang Thormann*, Jitka Caslavska, Sarah Molteni and Josef Chmelík☆

Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH-3010 Berne (Switzerland)

(First received July 4th, 1991; revised manuscript received September 12th, 1991)

ABSTRACT

Isoelectric focusing (IEF) of proteins in uncoated, open-tubular fused-silica capillaries of 75 μm I.D. with on-column multiwavelength detection is reported. Small amounts of hydroxypropylmethylcellulose added to the catholyte are shown to provide column conditioning which allows rapid and high-resolution IEF analysis of proteins to be performed in the presence of an electroosmotic flow along the separation axis. The latter process displaces the developing zone pattern towards and across the point of detection. On-column multichannel zone detection is shown to be an efficient method for the simultaneous monitoring of the eluting proteins and carrier ampholytes. The absorbance profiles monitored at one location towards the capillary end and the temporal behaviour of the current under constant voltage conditions are shown to provide information on the degree of focusing at the time of detection.

INTRODUCTION

Traditionally, isoelectric focusing (IEF) has been carried out in gels, requiring tedious, time-consuming preparation and protein staining procedures [1]. In the past few years, however, attention has been given to developing IEF into a more instrumental format. For example, free fluid focusing has been studied in capillaries of rectangular cross-sections [2–6], in glass tubular capillaries [7–9] and in PTFE capillaries [5,10,11]. These approaches operated with minimized electroosmosis in which stationary steady-state zone patterns were established. The zones were detected either by array detection [2,3,5,6] or by UV absorption measurements towards the column end, which required that the proteins were mobilized and swept past a stationary detector after focusing [7–11]. Although these developments did not initiate a widespread use of capillary IEF, they provided an insight into the principles and dynamics of IEF and complemented theoretical descriptions based on computer simulation [2,5,6,12].

Modern capillary-type electrophoretic analysers feature fused-silica capillaries of 25–100 μm I.D. which can exhibit strong electroosmotic flows. Proteins are vulnerable to interactions with the column walls. Not surprisingly, IEF experiments with proteins in untreated fused-silica capillaries cannot, in general, be reproduced, whereas much improved data are obtained in capillaries coated with linear polyacrylamide [13–15] or, alternatively, in PTFE capillaries with minimized electro-osmosis [10,11]. None of these approaches, however, is suitable for routine IEF analysis. The first lacks reproducibility because of the instability of the coatings and the second is too inefficient. Moreover, owing to the mobilization of the zone pattern after focusing, the two procedures are lengthy.

In a recently performed study it was discovered that small amounts of hydroxypropylmethylcellulose (HPMC) added to the buffer allowed high-resolution isotachophoretic analyses of proteins to be performed in untreated, open-tubular fused-silica capillaries, *i.e.* in the presence of an electroosmotic flow [16]. Consequently, the same approach was ap-

☆ Permanent address: Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, CS-611 42 Brno, Czechoslovakia.

0021-9673/92/$05.00

plied to IEF. Independently, Mazzeo and Krull [17,18] investigated a similar method with the addition of methylcellulose. This group used a configuration which required the addition of a strong base to the sample to detect basic proteins. In this paper a fully dynamic approach to capillary IEF in the presence of an electroosmotic flow along the separation axis is reported and capillary IEF analysis with on-column multiwavelength detection is discussed.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical reagent grade. Cytochrome *c* from horse heart [CYTC, molecular weight (MW) 12 384, p*I* 9.3], carbonic anhydrase from bovine erythrocytes (CA, MW 31 000, p*I* 6.18) and HPMC (7509) were from Sigma (St. Louis, MO, USA). Ferritin from horse spleen (FER, MW 450 000, p*I* 4.2–4.5) and equine myoglobin from skeletal muscle (MYO, MW 17 800, p*I* 6.8–7.0) were from Serva (Heidelberg, Germany). Ampholine (pH 3.5–10) was obtained from Pharmacia-LKB (Bromma, Sweden).

Electrolyte systems and sample preparation

For all the experiments presented here 20 m*M* sodium hydroxide and 10 m*M* orthophosphoric acid were used as the catholyte and anolyte, respectively. In most instances 0.06–0.3% HPMC was added to the catholyte. The sample components were dissolved in 2.5 or 5% (w/v) Ampholine without the addition of HPMC. Protein concentrations of 0.06–1 mg/ml were used.

Instrumentation and running conditions

The laboratory-made instrument used in this work has been described previously [16]. It features a 75 μm I.D. fused-silica capillary of about 90 cm length (Polymicro Technologies, Phoenix, AZ, USA) with a fast-scanning multiwavelength detector (Model UVIS 206 PHD) and a capillary detector cell (No. 9550-0155) (Linear Instruments, Reno, NV, USA). The effective separation distance was about 70 cm. No cooling of the capillary was provided. Two 50-ml plastic bottles served as electrode vessels and a VacTorr 150 vacuum pump (CGA/Precision Scientific, Chicago, IL, USA) was used to rinse the capillary tube. Current was applied at constant voltage (20 kV) with a Model HCN 14-20000 power supply (FUG Elektronik, Rosenheim, Germany). Multiwavelength data were read, evaluated and stored using a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments) with windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Throughout this work the 206 detector was used in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals (26 wavelengths). New capillaries were first rinsed with 1 *M* sodium hydroxide solution (20 min) and then 0.1 *M* sodium hydroxide solution containing 0.3% HPMC (10 min). The latter solution was also used to condition the capillary at the beginning of a series of experiments (10-min wash). Before each run the capillary was cleaned with the catholyte for at least 10 min.

Sample application occurred manually via gravity through lifting the capillary end, dipped into the sample vial, some 34 or 65 cm for a specified time interval. With a 4-min injection at 34 cm height and a catholyte with 0.06% HPMC, an initial sample zone of about 5 cm length was obtained. The initial configuration of an experiment, a transient state and the detection phase are shown schematically in Fig. 1.

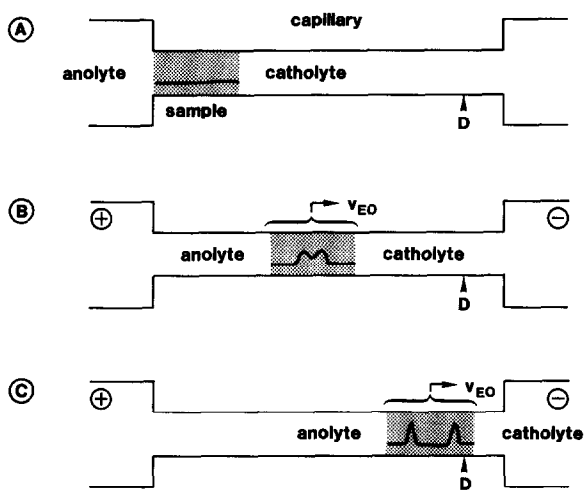

Fig. 1. Schematic representation of (A) the initial configuration, (B) a transient state and (C) detection for capillary IEF in the presence of electroosmosis. D represents the point of detection and v_{EO} the electroosmotic displacement. Carrier ampholytes are represented by the dotted area and the protein distribution by the solid line within that area.

RESULTS AND DISCUSSION

The experimental arrangement used in this work is shown schematically in Fig. 1. Experiments without the addition of HPMC to the catholyte were performed first. It was interesting to find that IEF experiments in the presence of electroosmosis and without proteins (*i.e.* with carrier ampholytes only) were reproducible. However, proteins added to the sample altered the system. Much longer elution times were observed and the experiments became irreproducible. This is similar to the situation reported in the isotachophoresis of proteins [16]. It is presumed that proteins are adsorbed onto the capillary walls, thereby forming a coating which changes the surface charge and thus the electroosmotic flow (reduction or even reversal of net sample flux). The additon of HPMC allowed the IEF analysis of proteins. Fig. 2 shows the focusing of CYTC and CA in 2.5% (w/v) Ampholine and with 0.06% HPMC in the catholyte. The concentrations of the two proteins in the sample were 0.16 and 0.27 mg/ml, respectively, and the initial sample zone length was about 5 cm. Fig. 2A shows the three-dimensional data obtained with on-column fast-scanning polychrome detection. The location of the two proteins is clearly visible as characteristic bands. Owing to the negative surface charge of untreated fused-silica, the entire pattern migrated towards the cathode. Basic proteins, such as CYTC, reach the detector before neutral and acidic proteins. The complex absorption pattern of the carrier ampholytes, seen particularly at low wavelengths, can also be seen (compare with Fig. 3A). The spectra in Fig. 2B represent time slices extracted from the eluting protein peaks. These normalized spectra were corrected for the background absorption of the carrier ampholytes, *i.e.* time slices taken adjacent to the protein zones were subtracted from those obtained within the protein peaks. Fig. 2C and D shows single-wavelength data for 200 and 280 nm, respectively. As a result of the strong absorption of the carrier ampholytes at 200 nm it is difficult to monitor the protein bands at this wavelength. With detection at 280 nm, however, specificity for proteins is obtained. Note that all the data presented in Fig. 2

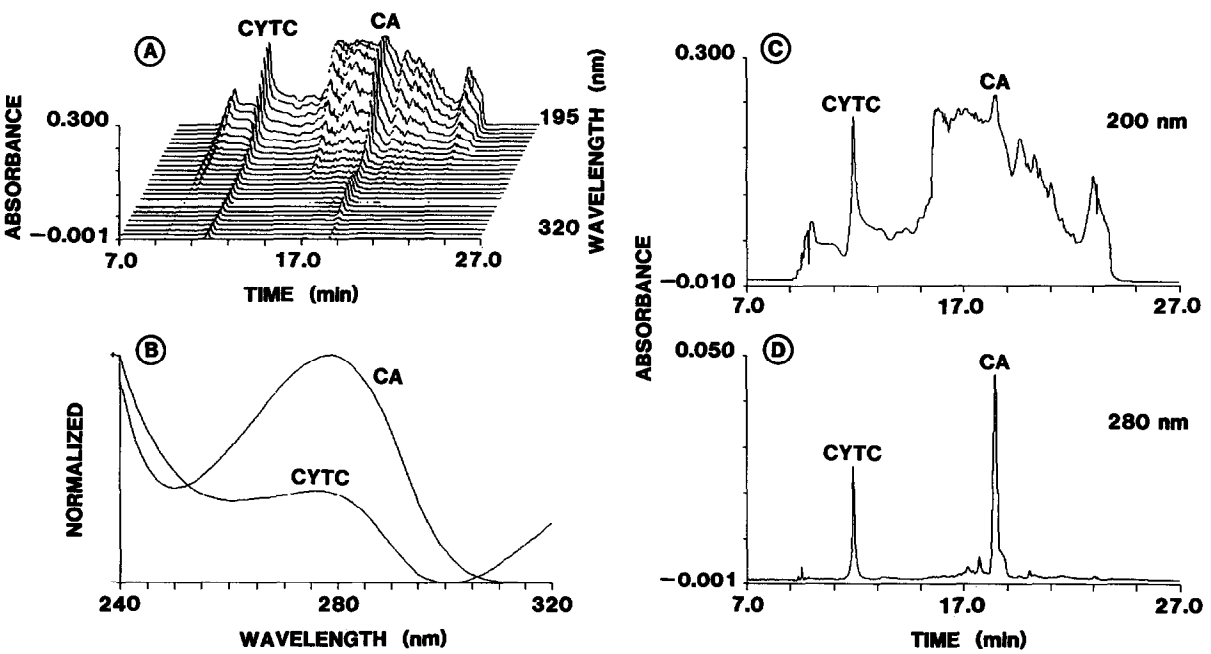

Fig. 2. Capillary IEF of CYTC (0.16 mg/ml) and CA (0.27 mg/ml) in 2.5% Ampholine. The HPMC concentration in the catholyte was 0.06%. Sampling occurred for 4 min at a height of 34 cm. The current values at the beginning, during protein detection and after 30 min of power application were about 25, 2 and 4 μA, respectively. (A) Three-dimensional data plot; (B) background corrected time slices (spectra) for the two proteins; (C) single-wavelength pherogram at 200 nm; (D) pherogram at 280 nm.

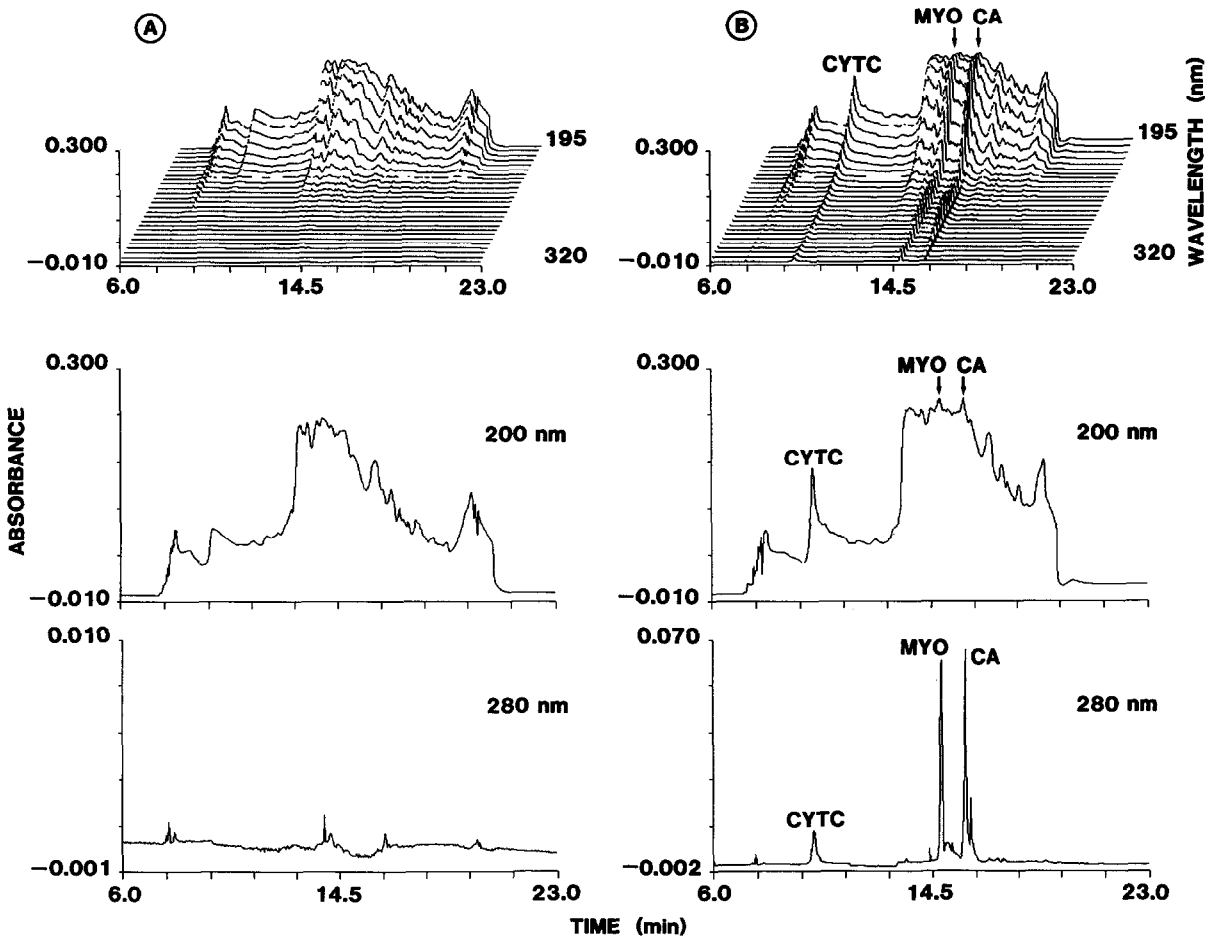

Fig. 3. Capillary IEF of (A) 2.5% Ampholine (blank) and (B) CYTC (0.13 mg/ml), MYO (0.20 mg/ml) and CA (0.22 mg/ml) in 2.5% carrier ampholytes. Other conditions as in Fig. 2.

were extracted from the multichannel data of one run. With multichannel detection the two protein zones and a characteristic IEF pattern of the carrier ampholytes are monitored simultaneously.

The applicability of capillary IEF in the presence of an electroosmotic displacement along the separation axis is further shown by the data presented in Fig. 3. Multichannel and single-wavelength pherograms of a blank (A) and a protein sample (B) are shown. The experimental conditions were essentially the same as in Fig. 2, the exceptions being that MYO was added to the sample as a third protein and that the runs were performed on another day. The pherograms are essentially equal, the retention times, however, are shorter than the previous day. This change is attributed to a different electroosmotic pumping rate, which indicates a slightly different conditioning state of the fused-silica surface. Such a variability is not considered to be a problem as long as an internal standard is used and/or the eluting carrier ampholytes are also monitored. The monitored zones are characterized by comparing time slices, *e.g.* the spectra of the protein zones. Background-corrected, normalized protein time slices of Fig. 3 are shown in Fig. 4A and B. As is shown in Fig. 4C and D, the spectra are in good agreement with those of Fig. 2B.

These simple experiments show that CYTC, MYO and CA can easily be analysed in presence of 0.06% HPMC. Other proteins, including bovine se-

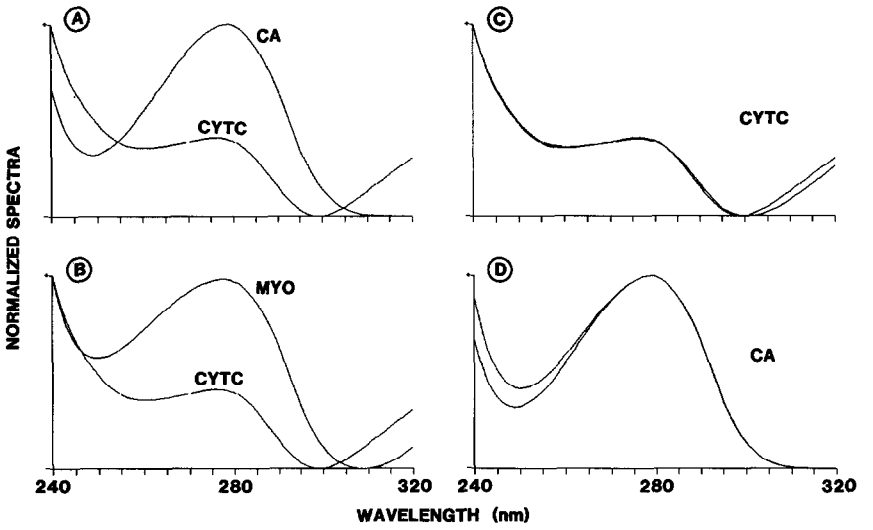

Fig. 4. Background-corrected time slices for (A) CA and CYTC and (B) MYO and CYTC extracted from the data presented in Fig. 3. Panels C and D show the comparison of background corrected normalized spectra of Figs. 2B and 4A for CYTC and CA, respectively.

rum albumin, FER and ribonuclease A, increased the retention times of proteins and carrier compounds, which is considered to be an indication of residual interaction between these proteins and the capillary walls. An increase of the HPMC concentration in the catholyte to 0.3% improved the behaviour of these proteins. With this simple sampling device, however, sample application became difficult. Experiments were therefore continued with 0.1% HPMC. The capillary IEF analysis of a sample containing CYTC, MYO, CA and FER in 2.5% Ampholine and with 0.1% HPMC in the catholyte is shown in Fig. 5A. Sampling occurred for 4 min at a height of 65 cm. All four proteins separated well, as is shown with the single-wavelength pherogram (centre panel) obtained at 280 nm. The trace denoted by S is believed to be a spike originating from particles (protein precipitates) which are transported through the detection cell. Similar spikes were observed in many other experiments.

The experiment was performed at a constant voltage of 20 kV and the current was monitored for the entire run. The temporal change of the current is shown in the bottom graph. It compares well with those published for free solution IEF systems with minimized electroosmosis [4,5]. The slight increase in current after about 15 min of power application was also observed in the experiments reported earlier (see, *e.g.*, the current values of the legend of Fig. 2) and is attributed to the gradual filling of the capillary with the anolyte after focusing has been attained. In all the experiments presented (Figs. 2, 3, 5A), it is assumed that full separation was attained prior to detection of the protein zones.

The risk of protein adsorption onto the capillary wall is not the only fundamental aspect of this IEF technique and experimental configuration used in this work. With a stationary, on-column detector placed towards the capillary end and electroosmotic zone displacement across its location, no complete confirmation of separation can be obtained in a single experiment. This is illustrated with the data shown in Fig. 5B and C. The sample load for the run shown in Fig. 5B was twice that of Fig. 5A; the sample zone lengths at the beginning of the two experiments, however, were equal. Comparison of the monitored absorbance profiles shows that the separation stages at the time of detection were different. Furthermore, the detection time intervals were shorter and the electric current higher with an increased sample concentration, the latter being caused by a smaller resistance across the developing gradient. The higher current produces a larger electroosmotic pump rate such that the characteristic ampholyte pattern could not be completely established prior to detection. The transient IEF data

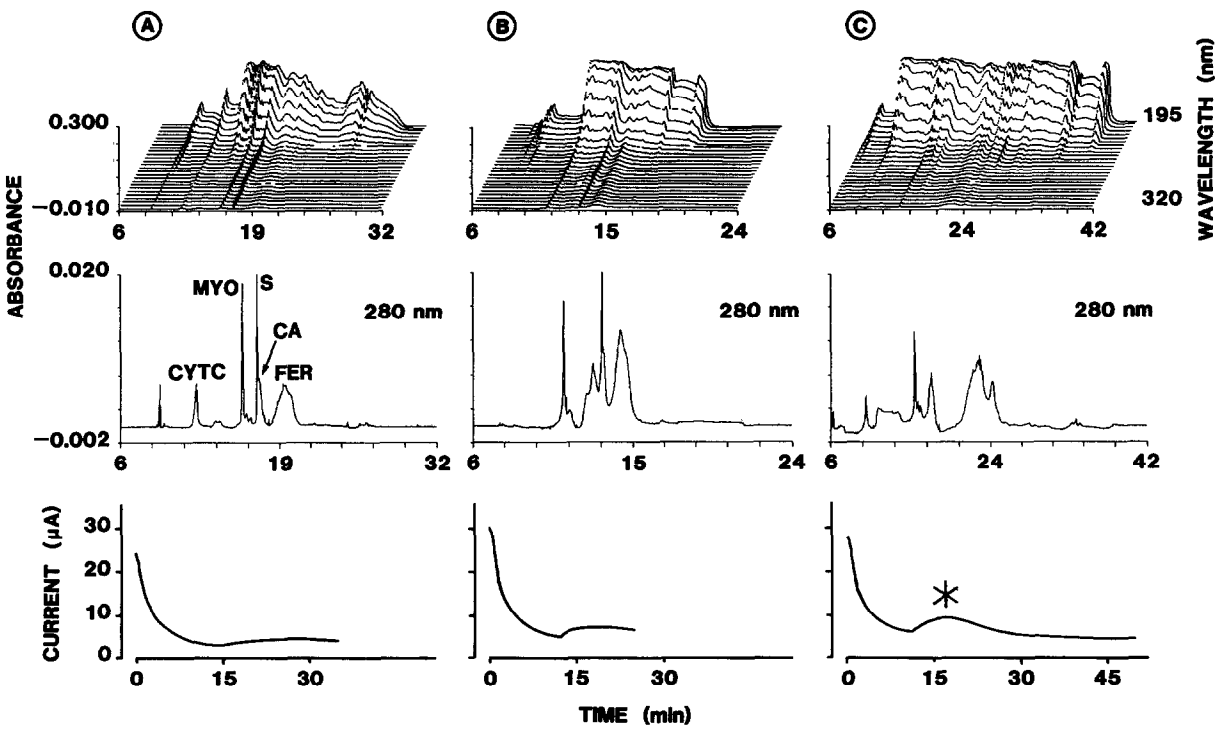

Fig. 5. Focusing of CYTC, MYO, CA and FER with 0.1% HPMC in the catholyte and sampling at a height of 65 cm. S represents a spike from a particle. (A) 4-min sampling of a 2.5% Ampholine solution containing 0.06 mg/ml of each protein. (B) 4-min sampling of a 5% Ampholine solution with 0.12 mg/ml of each protein. (C) 6-min sampling of a 5% Ampholine solution with 0.06 mg/ml of each protein. Three-dimensional pherograms (top panels), single-wavelength pherograms at 280 nm (centre graphs) and temporal behaviour of the current (bottom panels) are shown. Note that the absorbance data for the first 6 min are not depicted, whereas the complete current *versus* time graphs are presented. For other explanations, refer to text.

shown in Fig. 5C were obtained with an even higher sample load than that of Fig. 5B and with a longer initial sample zone. This example clearly illustrates that incomplete focusing is not only seen by the monitored absorbance profiles but also by the temporal behaviour of the current (bottom graph of Fig. 5C). The current–time relationship observed in Fig. 5C, particularly the characteristic current bump marked by the asterisk, was found to be typical for transient states which were not close to complete separation. With the increased sample loads of Figs. 5B and 5C, longer capillaries or capillaries with a reduced surface charge would have to be used to reach complete separation before detection.

CONCLUSIONS

The data presented in this paper prove the feasibility and show the complexity of performing protein IEF in untreated fused-silica capillaries with an electroosmotic flow along the separation axis. HPMC added to the catholyte, with which the capillary is filled prior to sample and power application, is believed to form a dynamic coating and to reduce the interaction between the proteins and the walls. It provides a simple conditioning effect which allows the IEF analysis of many proteins (at the nanogram level) in plain, open-tubular fused-silica capillaries of small I.D. in presence of an electroosmotic flow. The electroosmotic flow is responsible for the transport of the developing zone pattern through the capillary and across the point of detection. Fast-scanning polychrome detection is shown to be an interesting way of characterizing the protein zones and the carrier ampholytes. The absorbance profiles of the carrier ampholytes monitored at one location toward the capillary end and the temporal behaviour of the current provide useful

information on the degree of focusing at the time of detection. However, the confirmation of complete separation in one run can only be obtained with multiple detectors placed along the capillary. Further work has to be performed before this capillary IEF method can be adopted for routine analyses.

ACKNOWLEDGEMENTS

The generous loan of the UVIS 206 detector by its manufacturer, Linear Instruments (Reno, NV, USA), and of the HCN power supply by Mettler-Toledo (Greifensee, Switzerland) is gratefully acknowledged. This work was supported by the Swiss National Science Foundation.

REFERENCES

1 P. G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983.
2 W. Thormann, R. A. Mosher and M. Bier, *J. Chromatogr.*, 351 (1986) 17.
3 W. Thormann, N. B. Egen, R. A. Mosher and M. Bier, *J. Biochem. Biophys. Methods*, 11 (1985) 287.
4 W. Thormann, A. Tsai, J. P. Michaud, R. A. Mosher and M. Bier, *J. Chromatogr.*, 389 (1987) 75.
5 R. A. Mosher, W. Thormann and M. Bier, *J. Chromatogr.*, 436 (1988) 191.
6 R. A. Mosher, W. Thormann, R. Kuhn and H. Wagner, *J. Chromatogr.*, 478 (1989) 39.
7 S. Hjertén and M. Zhu, *J. Chromatogr.*, 346 (1985) 265.
8 S. Hjertén, J. L. Liao and K. Yao, *J. Chromatogr.*, 387 (1987) 127.
9 S. Hjertén, K. Elenbring, F. Kilàr, J. Liao, J. C. Chen, C. J. Siebert and M. Zhu, *J. Chromatogr.*, 403 (1987) 47.
10 M. A. Firestone and W. Thormann, *J. Chromatogr.*, 436 (1988) 309.
11 W. Thormann and M. A. Firestone, in J. C. Janson and L. Ridén (Editors), *Protein Purification*, VCH, Weinheim, 1989, pp. 469.
12 W. Thormann and R. A. Mosher, *Adv. Electrophoresis*, 2 (1988) 45.
13 F. Kilàr and S. Hjertén, *Electrophoresis*, 10 (1989) 23.
14 F. Kilàr and S. Hjertén, *J. Chromatogr.*, 480 (1989) 351.
15 M. Zhu, D. L. Hansen, S. Burd and F. Gannon, *J. Chromatogr.*, 480 (1989) 311.
16 P. Gebauer and W. Thormann, *J. Chromatogr.*, 558 (1991) 423.
17 J. R. Mazzeo and I. S. Krull, *BioTechniques*, 10 (1990) 638.
18 J. R. Mazzeo and I. S. Krull, presented at the *15th International Symposium on Column Liquid Chromatography, June 3–7, 1991, Basel*, Abstract P226/2.