ELSEVIER

Journal of Chromatography A, 737 (1996) 281–289

JOURNAL OF CHROMATOGRAPHY A

Non-native capillary isoelectric focusing for the analysis of the microheterogeneity of glycoproteins

Jörg Kubach, Rudolf Grimm*

Analytical Division Europe, Hewlett-Packard, Hewlett-Packard-Strasse 8, 76337 Waldbronn, Germany

Received 25 October 1995; revised 5 January 1996; accepted 5 January 1996

Abstract

A simple capillary isoelectric focusing method in bare fused-silica capillaries under denaturing conditions was developed for the separation of the glyco-isoforms of recombinant tissue plasminogen activator (rTPA) which have p*I* values ranging from pH 6 to 8. The glycoprotein was denatured in 8 *M* urea and separated in the presence of 0.75% carrier ampholytes, 0.4% hydroxypropylmethylcellulose and 4 *M* urea. Excellent separation of the glyco-isoforms of rTPA was achieved. Good reproducibilities for peak area and migration times were obtained using pressure mobilization of the focused protein zones. The method optimized for rTPA was also successfully applied to other glycoproteins (recombinant human erythropoetin and a monoclonal antibody) with different carbohydrate contents (3–55%).

Keywords: Protein heterogeneity; Capillary columns, coated; Glycoproteins; Urea; Erythropoetin; Tissue plasminogen activator

1. Introduction

Native capillary isoelectric focusing (cIEF) has been shown to be a very powerful method for the separation of many proteins [1–6]. The analysis time of an individual sample is much less in capillaries compared to IEF separations in gels. Analysis times of less than 4 min can be achieved [7]. Further advantages over IEF separations in gels include overall reduced running costs and direct UV detection of components. Using time-consuming staining and destaining procedures, sensitivity in cIEF is comparable with the sensitive silver staining procedures in gels. Using Servalyt carrier ampholytes, not only 280 nm, but also 200 nm can be used for more sensitive detection of peptides and proteins [7].

In addition to the analytical use of native cIEF, the micropreparative use of this technique has recently been demonstrated [8]. Proteins from a single run were collected automatically into vials containing carrier ampholytes and collected protein fractions were then successfully subjected to automated N-terminal protein sequence analysis without prior removing of the carrier ampholytes [8].

Successful matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of collected protein and peptide fractions from cIEF separations have also been shown recently [9,10].

Although native cIEF works well for a large variety of proteins, a common problem is that proteins tend to denature when they reach their p*I* value. Native cIEF also requires more or less salt-free protein and peptide samples. However, many

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00017-9

proteins need the presence of a certain amount of salt to maintain their solubility. These aspects often result in blockage of capillaries leading to high electric fields at a very small segment. Consequently, this causes high local Joule heating, which leads to breaking of the capillary.

To circumvent all solubility problems of proteins for their cIEF analysis we developed a simple standard cIEF method in bare fused-silica capillaries with various capillary lengths in the presence of urea. As a model protein the glycoprotein recombinant tissue plasminogen activator (rTPA) was used. cIEF separations of rTPA under denaturing conditions have been recently shown using coated capillaries and a complicated method [11]. Coated capillaries are generally expensive and have a limited lifetime. For our method, proteins were denatured in 8 *M* urea and analyzed with standard carrier ampholytes (Servalyt) containing 4 *M* urea. Various parameters, e.g. different concentrations of dynamic coating reagents like hydroxypropylmethylcellulose (HPMC) or poly(ethylene glycol) (PEG), and of carrier ampholytes were tested and optimized. Our method was then evaluated for its applicability to the separation of glyco-isoforms of other glycoproteins [recombinant human erythropoetin (rhEPO) and a monoclonal antibody] with different carbohydrate contents. Reproducibilities of peak area and retention times were determined for rTPA and rhEPO from cIEF separations performed in capillaries with an effective length of 56 cm (long method) and 25 cm (short method).

2. Materials and methods

2.1. Materials

Servalyt 3–10 and Servalyt 6–9 were purchased from Serva (Heidelberg, Germany). HPMC (2% solution gives 100 cps), PEG, neuraminidase and urea were from Sigma (St. Louis, MO, USA). Phosphoric acid and sodium hydroxide were from Merck (Darmstadt, Germany).

2.2. Proteins

rTPA was kindly provided by Dr. Billy Wu (Genentech, San Francisco, CA, USA), rhEPO by Dr. Andre Wetter (Cilag, Schaffhausen, Switzerland) and a monoclonal antibody by Dr. Brian Thomas (Institute of Horticultural Research, Littlehampton, UK).

2.3. CE instrumentation

All cIEF separations were performed using the Hewlett-Packard G1600 HP^{3D}CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany). The system is equipped with a diode-array detector and controlled by a HP ChemStation. Separations were performed on 50 μm I.D. fused-silica capillaries (Polymicro Technology) with an effective length of either 56 or 25 cm.

2.4. cIEF method optimization for capillaries with an effective length of 56 cm (long method)

2.4.1. Dynamic coating: HPMC and PEG concentration

HPMC and PEG stock solutions were made up to 0.2, 0.4, 0.6 and 0.8% (w/v) in water and mixed 1:1 with 8 *M* urea. Further, 0.75% (v/v) Servalyt was prepared in each stock solution. A 2 mg/ml solution of rTPA in 8 *M* urea was diluted in all these solutions by a factor of 1:10. For the analysis the capillary was completely filled with sample diluted in HPMC containing 4 *M* urea or PEG containing 4 *M* urea. Focusing was performed with 10 m*M* phosphoric acid containing 4 *M* urea as anolyte and 20 m*M* sodium hydroxide containing 4 *M* urea as catholyte in the corresponding HPMC or PEG solutions at 30°C with 30 kV for 16 min. After focusing, protein bands were mobilized by applying a pressure of 50 mbar to the inlet vial whilst maintaining the voltage at 30 kV.

2.4.2. Carrier ampholyte concentration (pH 3–10)

The following series of carrier ampholyte concentrations was prepared in 0.4 *M* HPMC, 4 *M* urea: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 3.0 and 4.0% (v/v).

rTPA was diluted again 1:10 in the corresponding carrier ampholyte solutions and focused as in Section 2.4.1.

2.4.3. Mixtures of carrier ampholytes (pH 3–10+ 6–9)

Two different carrier ampholytes with the pH range 3–10 and 6–9 were mixed to give a final concentration of 0.75% (v/v) in 4 *M* urea, 0.4% HPMC and tested as follows: 100%, 75%, 50%, 25% and 0% pH 3–10. cIEF conditions were as in Section 2.4.1.

2.4.4. Concentration of rTPA

rTPA was diluted to final concentrations of 1.94, 1.55, 0.58, 0.39 and 0.2 mg/ml in 0.75% Servalyt (1:1 of pH 3–10 and 6–9), 0.4% HPMC and 4 *M* urea. Focusing was performed as described in Section 2.4.1.

2.5. Focusing of rhEPO and the monoclonal antibody in 56-cm capillaries

rhEPO and the antibody, denatured in 8 *M* urea, were diluted to give a final concentration of 0.2 mg/ml each in 0.75% Servalyt 3–10, 0.4% HPMC and 4 *M* urea and focused at 30°C with 30 kV for 10 min using 50 m*M* phosphoric acid containing 4 *M* urea as anolyte and 20 m*M* sodium hydroxide containing 4 *M* urea as catholyte. Protein bands were mobilized with pressure as described in Section 2.4.1. Between runs the capillary was flushed for 5 min with water and for 5 min with carrier ampholyte solution containing 0.4 *M* HPMC and 4 *M* urea.

2.6. Short cIEF method

rTPA and rhEPO (final concentration: 0.2 mg/ml each) were diluted into 0.75% carrier ampholyte 3–10, 0.4% HPMC and 4 *M* urea. Capillary conditioning between runs and focusing was essentially the same as described for the longer capillary except the focusing time was reduced to 4.5 min.

2.7. IEF or rhEPO in agarose gels

Gel IEF separations of rhEPO were kindly performed by Dr. Wetter (Cilag, Switzerland) according to the method of O'Farrell [12]

2.8. Neuraminidase treatment of rTPA

Sialic acid was cleaved from rTPA by incubation with 0.25 U/mg neuraminidase for 20 h at 37°C.

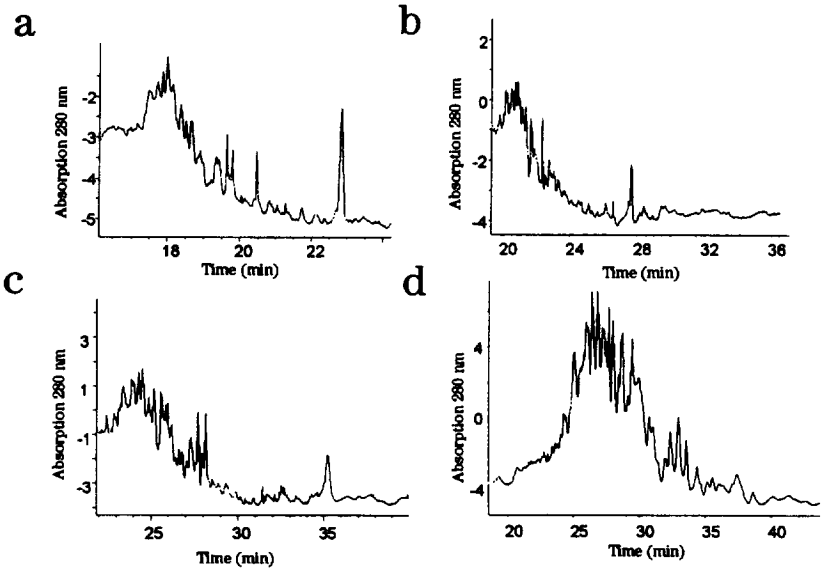

Fig. 1. Optimization of the HPMC concentration for dynamic coating in % (v/v): (a) 0.1, (b) 0.2, (c) 0.3 and (d) 0.4 as additive to all solutions.

2.9. MALDI-TOF-MS

Native rTPA and neuraminidase treated rTPA were analyzed by MALDI-TOF-MS using the HP G 2025 A MALDI-TOF-MS system. Sinapic acid containing diammoniumhydrogencitrate was used as matrix.

3. Results and discussion

For the optimization of a powerful non-native capillary isoelectric focusing method we used the glycoprotein rTPA as a model protein. The following parameters were tested: (i) concentration of dynamic coating reagents HPMC and PEG: 0.1, 0.2, 0.3 and 0.4% (w/v); (ii) concentration of carrier ampholytes p*I* 3–10: 0.25–4.0% (v/v); (iii) mixtures of carrier ampholytes p*I* 3–10 and p*I* 6–9 and (iv) protein concentration.

Using inexpensive bare fused-silica capillaries for protein separations with cIEF, potential protein–capillary wall interactions have to be suppressed. Further, the electroosmotic flow has to be significantly reduced. To solve these problems dynamic coating reagents were routinely used and must be added to all solutions. We tested various concentrations of HPMC, which has very similar coating behavior as methylcellulose, generally used in native

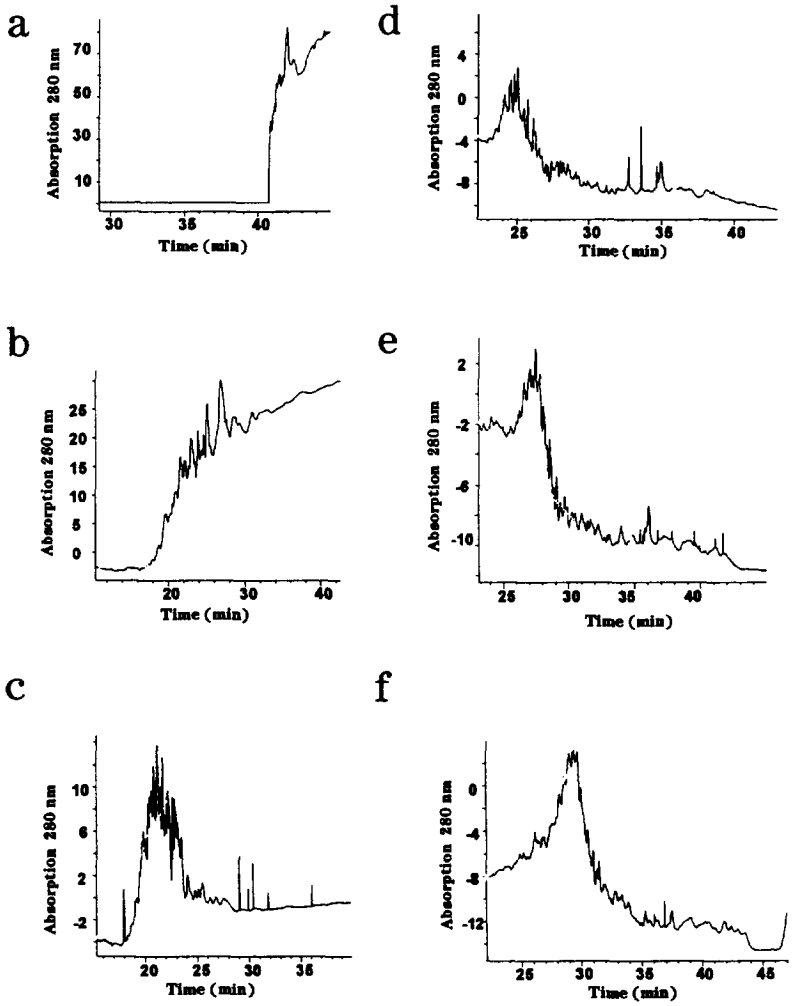

Fig. 2. Optimization of carrier ampholyte concentration: (a) 0.25%, (b) 0.5%, (c) 0.75%, (d) 2%, (e) 3% and (f) 4%.

cIEF, and PEG in the presence of 4 *M* urea for this purpose. As shown in Fig. 1, the addition of 0.4% (w/v) HPMC to all solutions resulted in the best cIEF separation of rTPA.

The use of PEG for dynamic coating of the capillaries was tested using the same concentrations as with HPMC; however, cIEF using PEG was not successful (data not shown). Hence, 0.4% HPMC in the presence of 4 *M* urea was used for dynamic coating in all further optimization steps.

The carrier ampholyte (pH range 3–10) concentration was then varied from 0.25 to 4% Servalyt whilst all other parameters were kept constant. From all concentrations tested the best separation was achieved using 0.75% (v/v), as shown in Fig. 2.

Since the method was optimized for the cIEF separation of the glycoprotein rTPA and the glycoisoforms range in their p*I* value between pH 6 and 8, we also tested various mixtures of commercially available carrier ampholyte pH ranges. pH 3–10 was mixed with pH 6–9 and tested for optimal separation of the TPA glyco-isoforms. The best combination was found to be a 1:1 mixture of both pH ranges (Fig. 3).

A further parameter which was considered as important for the method optimization was the concentration of rTPA. Glycoproteins very often split into several components depending on the heterogeneity of the carbohydrate moiety and their content of negatively charged sugar molecules. The ideal final concentration of rTPA in the carrier ampholyte–HPMC–urea mixture was found to be in the range 300–600 $\mu g/\mu l$ (see Fig. 4).

Finally, we combined all tested parameters, which suggested the following optimized conditions: Dilution of denatured rTPA to a final concentration of 300–600 $\mu g/\mu l$ in 0.75% carrier ampholytes (1:1 mixture of Servalyt 3–10 with Servalyt 6–9) containing 0.4% HPMC and 4 *M* urea. Focusing is performed with 20 m*M* sodium hydroxide containing 0.4% HPMC and 4 *M* urea at the cathodic side and 50 m*M* phosphoric acid containing 0.4% HPMC and 4 *M* urea at the anodic side. Focused protein bands of rTPA glyco-isoforms are mobilized by concomitant application of 50 mbar pressure to the inlet vial. Between runs, capillaries are flushed for 5 min with water and for 5 min with 0.75% (v/v) Servalyt solution in 0.4% HPMC and 4 *M* urea. A typical current profile is shown in Fig. 5a. Up to 25 glycoisoforms of rTPA varying in their amount of sialic acid molecules with p*I* values ranging from pH 6 to 8 could be separated (Fig. 5b). Using the short method (capillaries with an effective length of 25 cm) analysis time was decreased from 45 min to less

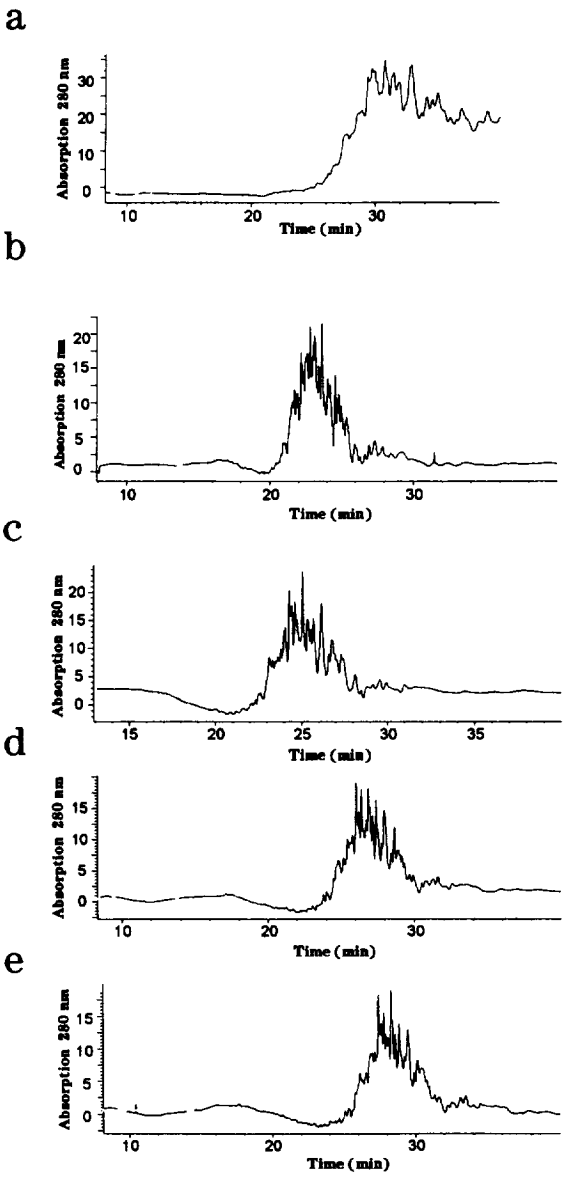

Fig. 3. Optimization of mixtures of carrier ampholytes pH 3–10 and pH 6–9: (a) 100% pH 3–10, (b) 75% pH 3–10, 25% pH 6–9, (c) 50% of each pH range, (d) 25% pH 3–10, 75% pH 6–9 and (e) 100% pH 6–9.

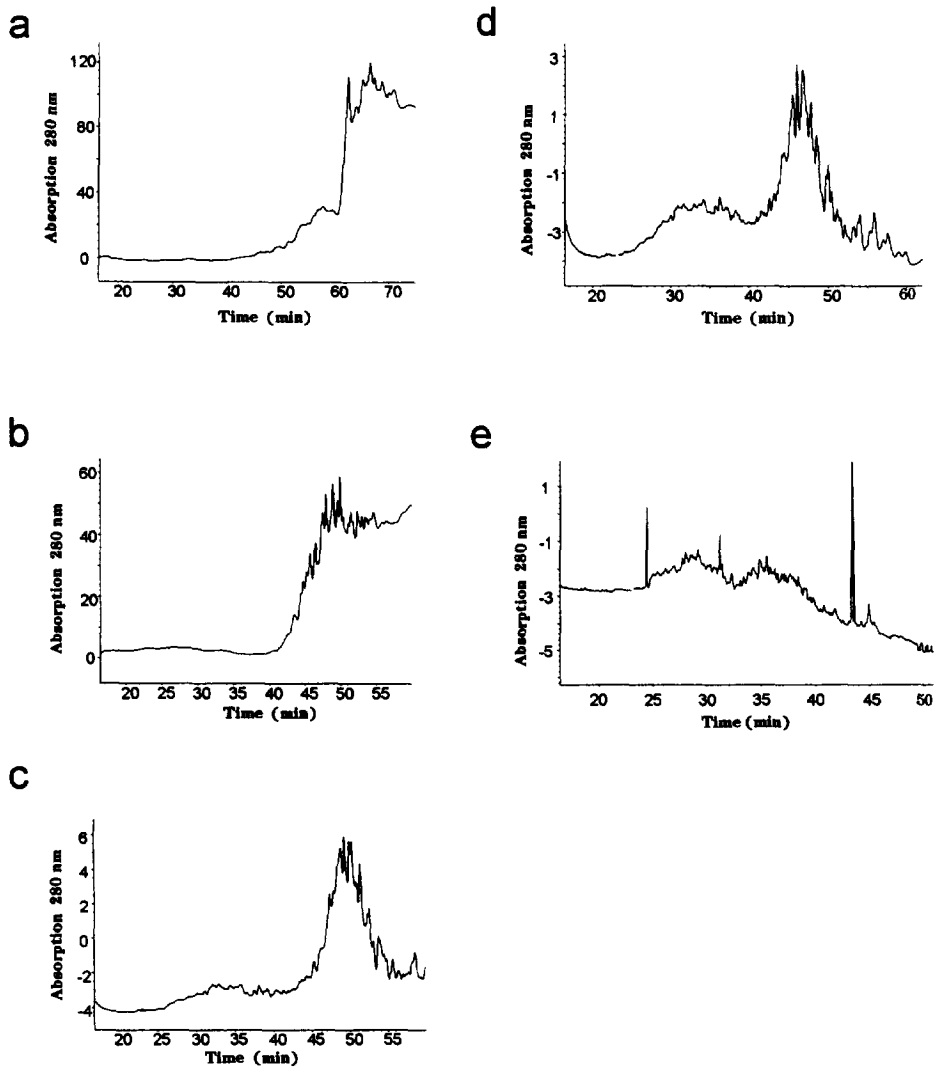

Fig. 4. Optimization of rTPA concentration: (a) 1940 μg/μl, (b) 1550 μg/μl, (c) 580 μg/μl, (d) 390 μg/μl and (e) 200 μg/μl.

than 9 min (Fig. 6), however, resolution of the rTPA glyco-isoforms decreased significantly (Fig. 6).

Using the optimized method, peak area and retention time precision were determined for rTPA in capillaries with an effective length of 56 and 25 cm. Using the standard method in a 56-cm capillary the R.S.D. (relative standard deviation) of the total peak area calculated from five subsequent cIEF separations was less than 7.4%. The R.S.D. of the retention time was less than 3.8%. In 25-cm capillaries the R.S.D. for peak areas was less than 2.0% and for retention times less than 1.7%.

Applying neuraminidase treatment to rTPA the negatively charged sialic acid molecules were removed from the carbohydrate moiety. Hence the overall net charge of the protein has to become more positive, resulting in a p*I* value higher than pH 8.0. Also the microheterogeneity caused by the negatively charged sugar molecules should disappear. Fig. 7 clearly shows both effects. Only one protein peak of desialyted rTPA is observed with a retention time of 18 min instead of 25–35 min determined for the various glyco-isoforms.

To test the more general applicability of the

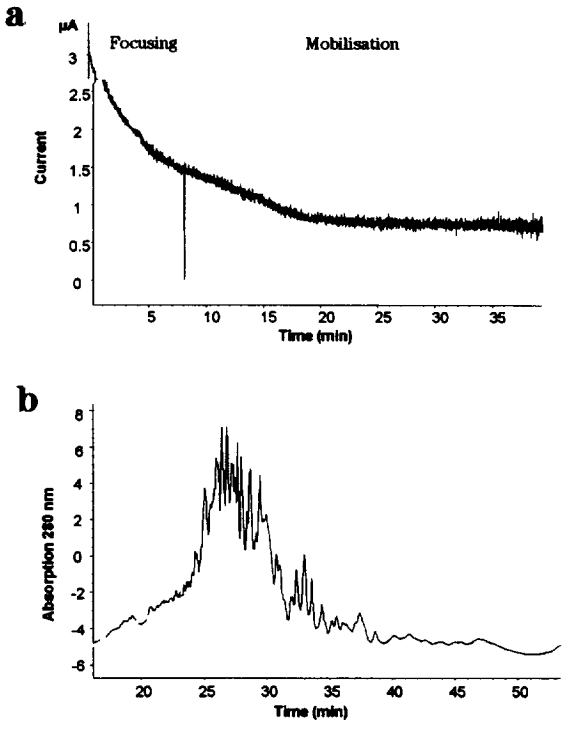

Fig. 5. Non-native cIEF separation of rTPA under optimized conditions: (a) current profile during cIEF analysis, (b) rTPA separation.

method for the analysis of the microheterogeneity of glycoproteins, we analyzed rhEPO, which has about 55% carbohydrate moiety of the total molecular mass, compared to 5–18% for rTPA. Using the standard method in a 56-cm capillary all six isoforms with p*I* values ranging from pH 4.6 to 5.1 could be baseline separated (Fig. 8a). The resolution of the rhEPO isoforms in cIEF is very similar to that

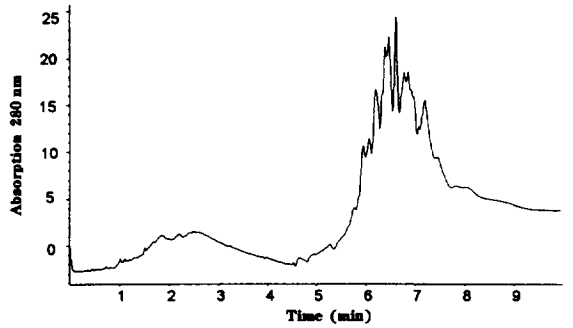

Fig. 6. Non-native cIEF separation of rTPA in a 25-cm capillary.

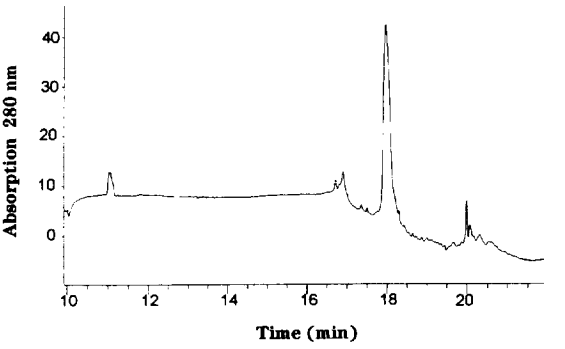

Fig. 7. cIEF separation of rTPA after neuraminidase treatment.

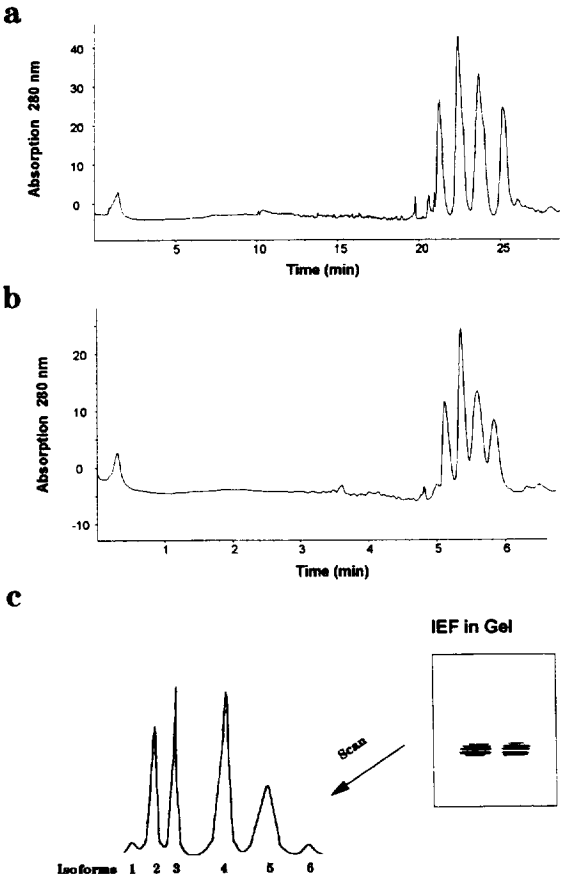

Fig. 8. (a) cIEF separation of rhEPO using optimized conditions in a 56-cm capillary; (b) cIEF separation of rhEPO in a 25-cm capillary; (c) IEF separation of rhEPO in an agarose gel with densitometric scan.

Table 1
Comparison of the relative quantities of the glyco-isoforms of rhEPO determined by gel IEF–densitometry and by cIEF

Isoforms	Intensities measured with densitometry (%)	Intensities measured with 280-nm UV signal (%)
Isoform 1	less than 5	5
Isoform 2	5–30	20
Isoform 3	15–40	28
Isoform 4	25–40	25
Isoform 5	10–35	20
Isoform 6	less than 10	2

obtained with IEF separations in gels (Fig. 8c). Using the short cIEF method the resolution of the isoforms is somewhat reduced, but analysis time is also reduced to less than 7 min (Fig. 8b). A comparison of the quantitation of each isoform was performed by scanning the stained protein bands from the gel and comparing this to integration of the UV peaks obtained in cIEF generated with the ChemStation. Quantitative results were in very good agreement, as shown in Table 1. Reproducibility of the peak areas and retention times of the isoforms were also determined for separations in 56- and 25-cm capillaries. The relative standard deviation of peak areas was less than 5.5% and of retention times less than 7% in a 56-cm capillary. In 25-cm capillaries the relative standard deviations were less than 5% for peak areas and less than 6% for retention times.

Finally, the cIEF method was applied to another glycoprotein, a monoclonal antibody. As shown in Fig. 9, several isoforms of the antibody could be separated.

The non-native cIEF method which was originally developed to separate as many isoforms of rTPA as possible is also applicable to other glycoproteins to demonstrate their glyco-microheterogeneity (as shown for rhEPO and a monoclonal antibody). The method is simple and works properly and reproducibly in bare fused-silica capillaries. All potential solubility problems of proteins in cIEF separations are obviated by the presence of 4 *M* urea in all buffers. UV detection allows faster and easier quantitation of detected components compared to densitometric quantitation of stained protein bands in gels.

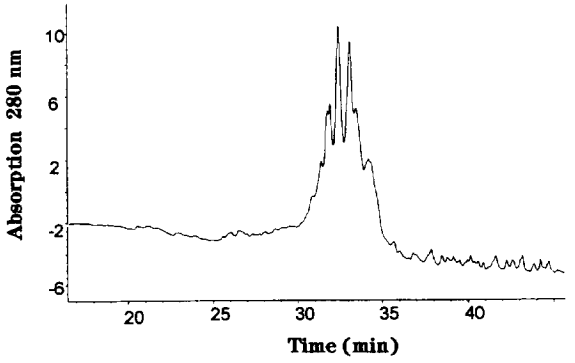

Fig. 9. cIEF separation of a monoclonal antibody in a 56-cm capillary.

Acknowledgments

We are very grateful to Dr. Billy Wu (Genentech, San Francisco, CA, USA) for the generous gift of rTPA, to Dr. Andre Wetter (Cilag, Schaffhausen, Switzerland) for providing rhEPO and for carrying out the IEF separation of rhEPO and also to Dr. Brian Thomas (Institute of Horticultural Research, Littlehampton, UK) for the monoclonal antibody. We also thank Gordon Ross (Hewlett-Packard, Waldbronn, Germany) for critical reading of the manuscript.

References

[1] J.F. Mazzeo and I.S. Krull, Anal. Chem., 63 (1991) 2852–2857 .
[2] S.-M. Chen and J.E. Wiktorowicz, Anal. Biochem., 206 (1992) 84–90.
[3] J.R. Mazzeo and I.S. Krull, J. Chromatogr., 606 (1992) 291–296
[4] M. Zhu, R. Rodriguez, T. Wehr and C. Siebert, J. Chromatogr., 608 (1992) 225–237.
[5] S. Molteni, H. Frischknecht and W. Thormann, Electrophoresis, 15 (1994) 22–30.

[5] T.-L. Huang, P.C.H. Shieh and N. Cooke, Chromatographia, 39 (1994) 543–548.
[7] R. Grimm, Application Note 12-5963-6809E, Hewlett-Packard, Waldbronn, Germany, 1995.
[8] R. Grimm, J. Cap. Electrophoresis, 2 (1995) 111–115.
[9] R. Grimm, G. Ross, M. Herold and F. Bek, Presented at the 7th International Symposium on High Performance Capillary Electrophoresis, Würzburg, 29 January–2 February 1995, Poster P-633.
[10] O. Müller, F. Foret and B.L. Karger, Presented at the 7th International Symposium on High Performance Capillary Electrophoresis, Würzburg, 29 January–2 February 1995, Poster P-634.
[11] K.G. Moorhouse, C.A. Eusebio, G. Hunt and A.B. Chen, J. Chromatogr. A, 717 (1995) 61–69.
[12] P.H. O'Farrell, J. Biol. Chem., 250 (1975) 4007–4021.