ELSEVIER

Journal of Chromatography A, 741 (1996) 139–145

JOURNAL OF CHROMATOGRAPHY A

Short communication

# Capillary electrophoresis of glycosylated proteins performed on a conventional capillary gas chromatographic column

R. Bonfichi[1]

*Lepetit Research Center, I-21040 Gerenzano (VA), Italy*

Received 12 January 1996; revised 29 February 1996; accepted 29 February 1996

**Abstract**

In this paper results are reported which document the suitability of a conventional poly(ethylene glycol) internally coated gas chromatographic capillary column for the capillary electrophoretic analysis of glycoproteins.

Although the observed migration times were not in the order of a few minutes and the efficiencies resulted were generally lower than 100 000 theoretical plates, the proposed method provides an easy tool for obtaining rapid and reproducible glycosylation profiles of various glycoproteins. The method applies very well even to complicated samples such as, for instance, heavily glycosylated/high-molecular-mass proteins. For the sake of simplicity, reference standard glycoproteins were used as samples.

*Keywords:* Capillary columns; Proteins; Glycoproteins

## 1. Introduction

Modern analytical chemistry pays ever-increasing attention to glycoproteins [1,2] because of their medical and biotechnological implications. The structural aspect which perhaps makes the analytical characterization of these complex biomolecules most difficult is their intrinsic "microheterogeneity". This heterogeneity, which is due to the presence of a series of different glycoforms, i.e., identical polypeptide chains with distinct glycan structures covalently attached [3,4], is of concern to protein manufacturers since it might affect protein biological activities and, therefore, their therapeutic use. Glycoprotein microheterogeneity has been the subject of numerous publications in the past [5–14] and, recently, carbohydrate analysis based on chromatographic and electrophoretic methods has been widely discussed in a thematic issue of *Journal of Chromatography A* edited by Professor Honda [15].

Protein separations by capillary electrophoresis (CE) are usually further hampered by adsorption onto the negatively charged fused-silica surface of the capillary. These problems can be partially overcome by using a high-ionic-strength buffer [16,17], a high-pH buffer [18], a low-pH buffer [19,20], buffer additives [21,22], dynamic coating agents [2] or internally coated capillaries [23,24]. Many coating types have been developed since the first proposed by Hjertén in 1985 [25] and they include, among others: polyvinylpyrrolidinone [19], maltose [26], pentafluoroaryl [27] and poly(ethylene glycol) (PEG) coatings [28,29]. The results for PEG coatings are

[1]Present address: SICOR S.p.A., Via Terrazzano 77, I-20017 Rho (Milan), Italy.


*PII* S0021-9673(96)00210-5

Table 1
Standard proteins considered in this study

| Protein | Origin | $M_r$ | pI | Carbohydrate content, g/100 g protein | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Neutral sugar | Hexose | Acetyl hexosamine | Sialic acid | Fucose | Total |
| Ribonuclease A (RNase A) | Bovine pancreas | ~13 500 | 9.7 | – | – | – | – | – | 0 |
| Ribonuclease B (RNase B) | Bovine pancreas | ~15 500 | 9.7 | ~6.3 | – | ~5.0 | – | – | ~11.3 |
| Ovalbumin | Hen egg | ~43 000 | 4.7 | ~2.0 | – | ~1.2 | 0 | – | ~3.2 |
| $\alpha_1$-Acid glycoprotein (AGP) | Human | ~41 000 | 2.7 | – | ~14.7 | ~13.9 | ~12.1 | ~0.7 | ~41.4 |

particularly attractive because, besides known advantages such as (i) hydrophilic surface structure, (ii) reduced electroosmotic flow and (iii) immobilization of the polymer layer by cross-linking, there is a wide choice of commercially available capillary gas chromatographic (GC) columns with immobilized PEG stationary phases.

On the basis of these facts and the tasks of this laboratory being the consistency monitoring of glycoprotein production batches (i.e., the reproducibility of the glycoforms pattern from batch-to-batch) and the development of analytical methods which could be run anywhere without any particular material or expertise (such as that required for making

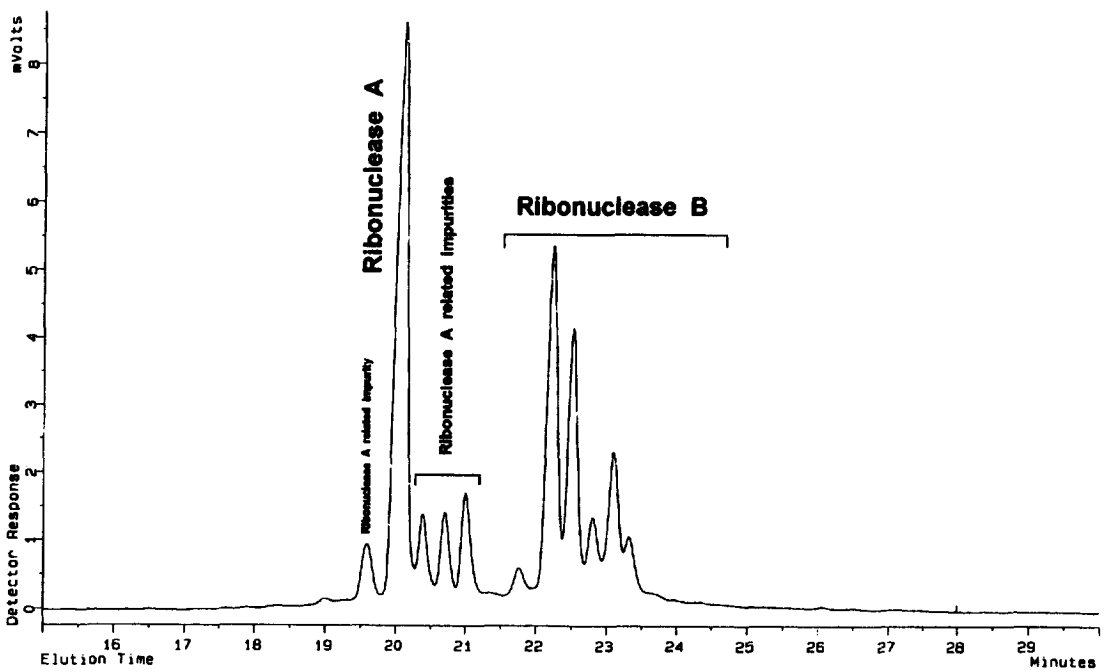


Fig. 1. Electropherogram of a mixture of ribonucleases A and B. Injection: electrokinetic, 15 kV, 0.2 min. BGE: 25 m*M* D,L-glutamic acid pH 3.3; 20 kV; ca. 1.3 μA; UV 210 nm; 25°C.

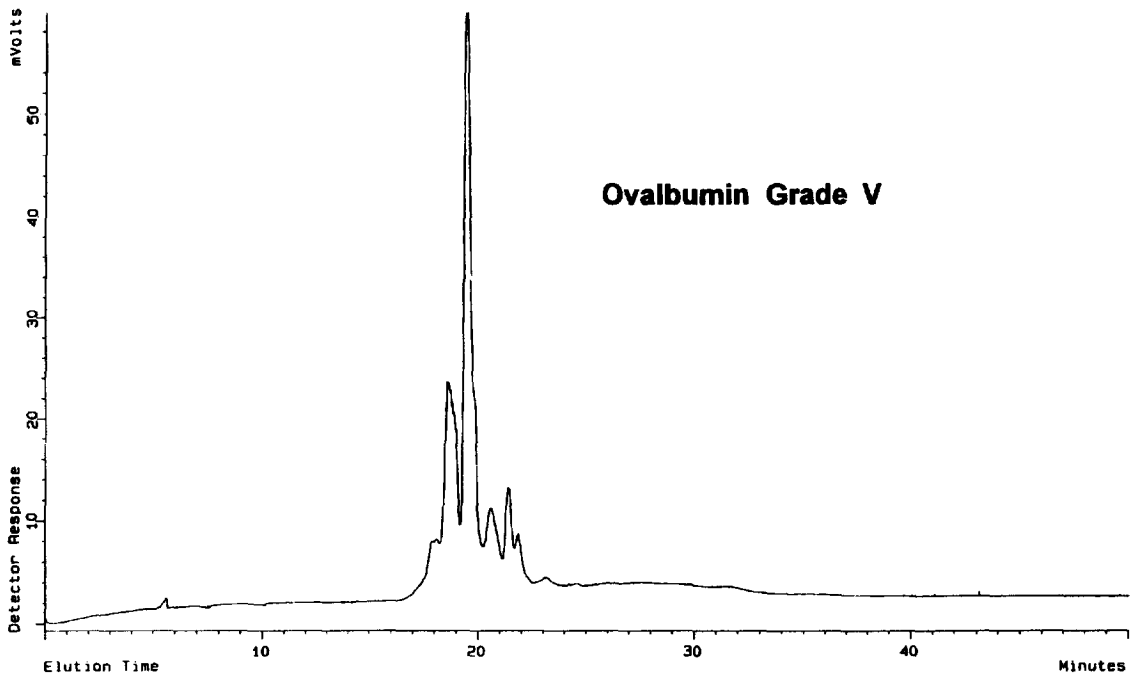


Fig. 2. Electropherogram of ovalbumin [albumin, chicken egg, Grade V: minimum 98% (agarose electrophoresis)]. Injection: electrokinetic, −15 kV, 0.1 min. BGE: 25 m*M* MOPS at pH 7 using Tris; −30 kV; ca. −3.6 μA; UV 210 nm; 25°C.

derivatized capillaries), I chose to investigate the use of a conventional PEG-coated GC capillary column for the CE separation of several glycoproteins. In particular, for this study, a Durawax fused-silica open tubular (FSOT) capillary GC column was used, internally coated with a 0.1 μm thick film of DB-WAX stationary phase. For the sake of simplicity, reference standard glycoproteins were used as samples.

## 2. Experimental

### 2.1. Apparatus

CE separations were carried out using a Crystal 310 (ATI-UNICAM, Cambridge, UK) instrument equipped with a variable-wavelength UV–Vis detector. For all separations a Durawax FSOT (J & W Scientific, Folsom, CA, USA) capillary GC column was used, with an effective length of 62 cm, total length 80 cm, and 50 μm I.D., internally coated with a 0.1 μm thick layer of DB-WAX stationary phase. UV detection was at 210 nm.

### 2.2. Reagents

All the proteins used during this study, i.e., ribonucleases A (type III-A: from bovine pancreas) and B (type III-B: from bovine pancreas), β-lactoglobulins A and B (from bovine milk), $\alpha_1$-acid glycoprotein (from bovine serum, 99%), $\alpha_1$-acid glycoprotein (human, purified from Cohn Factor VI, 99%), ovalbumin [albumin, chicken egg, Grade V: minimum 98% (agarose electrophoresis)], ovalbumin [albumin, chicken egg, Grade VI: approx. 99% (agarose electrophoresis)], were from Sigma (St. Louis, MO, USA). Biological buffers [i.e., Tris, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPS), etc.] were also from Sigma.

All the other chemicals used during this study

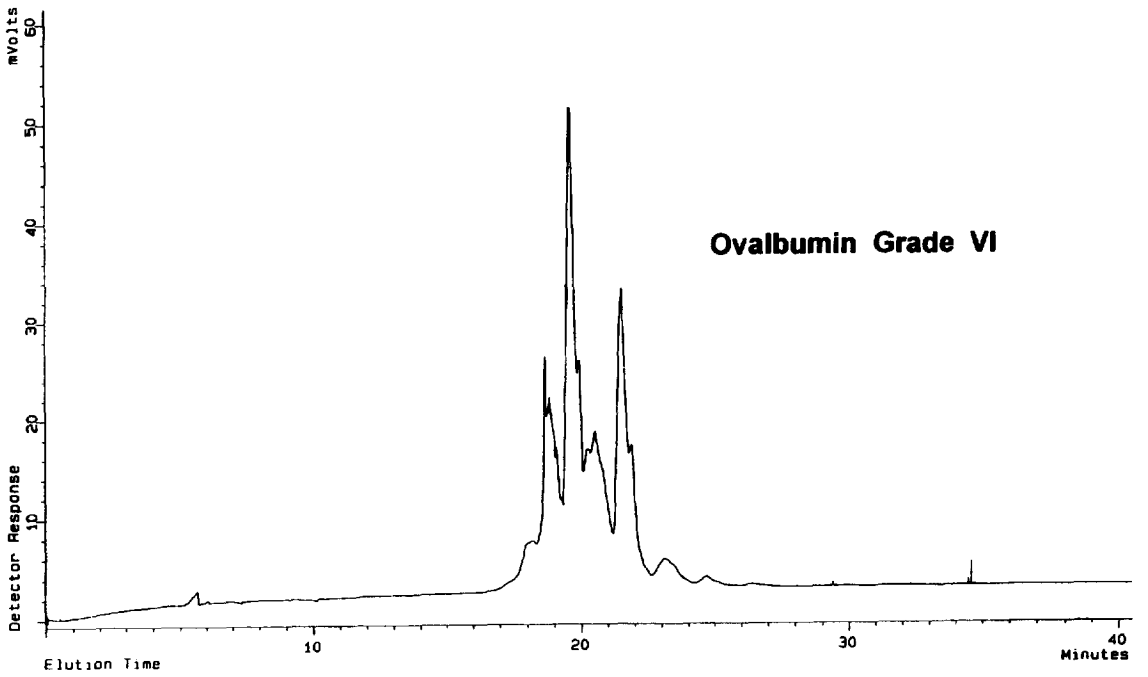


Fig. 3. Electropherogram of ovalbumin [albumin, chicken egg, Grade VI: approx. 99% (agarose electrophoresis)]. Conditions as in Fig. 2.

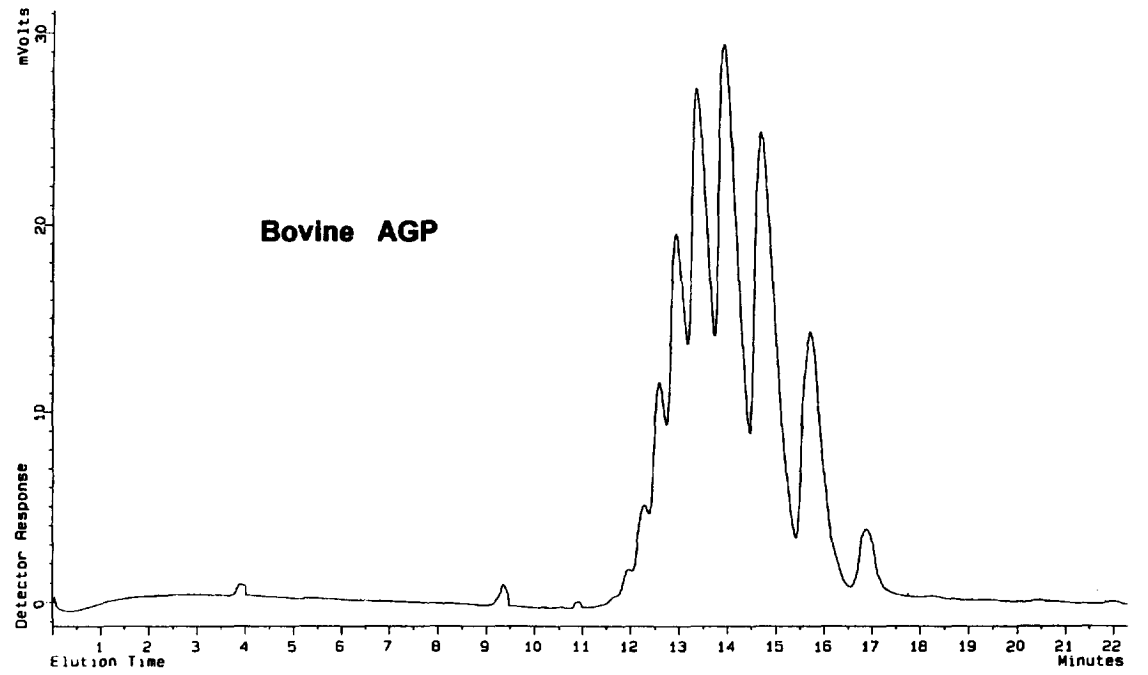


Fig. 4. Electropherogram of $\alpha_1$-acid glycoprotein (bovine). Injection: electrokinetic, −15 kV, 0.5 min. BGE: 25 m*M* MES at pH 5.6 using Tris; −30 kV; ca. −2.5 $\mu$A; UV 210 nm; 25°C.

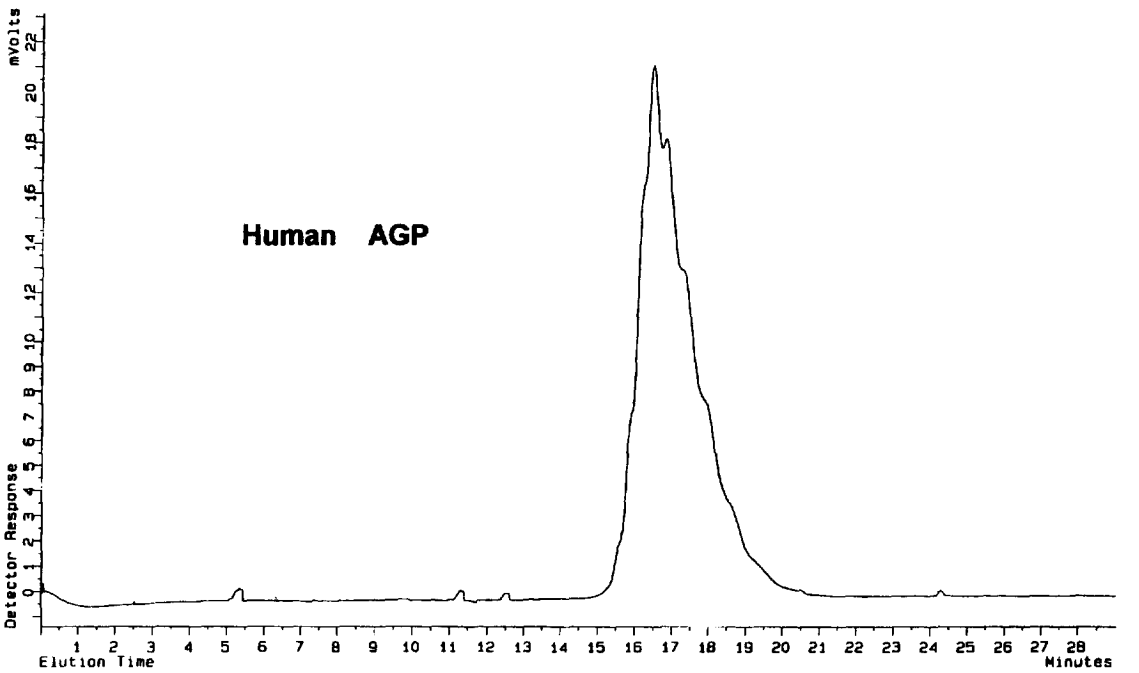


Fig. 5. Electropherogram of $\alpha_1$-acid glycoprotein (human). Conditions as in Fig. 4.

were of analytical or HPLC grade (Carlo Erba, Milan, Italy). Water was from a Milli-Q Plus purification system (Millipore, Bedford, MA, USA).

### 2.3. Protein samples preparation

In all cases a ca. 1.2–1.5 mg/ml water solution of each protein was used.

## 3. Results and discussion

The reference standard proteins considered during this study are shown in Table 1 [30]. In particular, two commercially available analytical grades of ovalbumin (i.e., Grades V and VI) have been taken into account and, besides human $\alpha_1$-acid glycoprotein (AGP), bovine AGP has been also investigated. Table 1 lacks data concerning bovine AGP because of their unavailability at the time of this study.

To minimize typical undesired analyte–wall interactions which occur when proteins are analyzed by CE, the electrophoretic separations have been performed on a commercially available PEG-internally coated capillary GC column. In order to achieve high efficiencies, all separations have been carried out using low-ionic-strength background electrolytes (BGEs) [31]. All proteins have been separated at pH values far enough from their isoelectric points (p*I* values). In particular, basic proteins (i.e., RNases A and B) have been analyzed at a pH value of ca. 3.3, which is definitely below their p*I* values. On the contrary, all other glycoproteins (i.e., ovalbumin and AGP) have been separated at pH values above their p*I* values and, therefore, these separations were carried out under reversed polarity conditions.

In Fig. 1, the CE–UV profile of a mixture of ribonuclease A (RNase A) and ribonuclease B (RNase B) is shown, obtained by using D,L-glutamic acid (pH ca. 3.3) as BGE. This mixture has been prepared ad hoc to show the capability of the proposed approach to distinguish between two proteins (RNase A, RNase B) characterized by similar molecular masses (ca. 13 550 vs. ca. 15 500) but different glycosylation percentages (0 vs. ca. 11.3%). On the most intense peak of the ribonuclease B cluster shown in Fig. 1, ca. 100 000 theoretical plates can be measured. This result can be accepted if it is

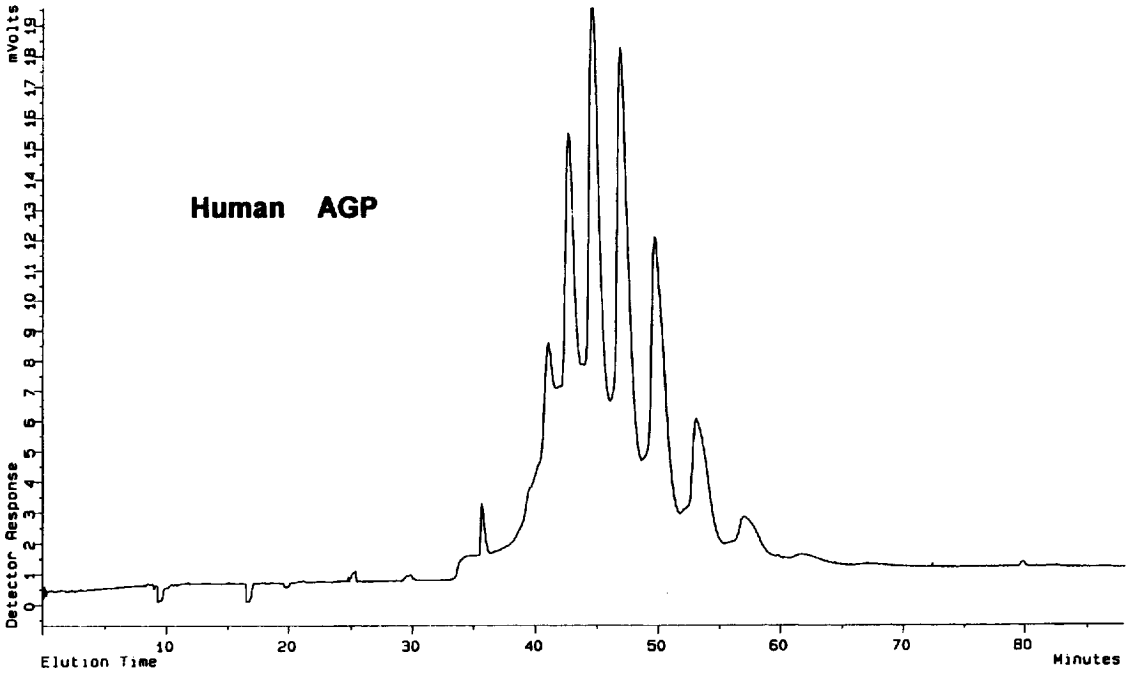


Fig. 6. Electropherogram of $\alpha_1$-acid glycoprotein (human). Injection: electrokinetic, −30 kV, 0.5 min. BGE: 50 m*M* MES at pH 5.6 using Tris, 8 *M* urea; −30 kV; ca. −1.1/−1.6 μA; UV 210 nm; 25°C.

taken into account that these theoretical plates were generated by a GC capillary column and not by an especially developed CE capillary.

The electrophoretic profile observed for ribonuclease B in Fig. 1, corresponds to that already described by Bonfichi et al. [2] and by Kelly et al. [32] obtained under completely different electrophoretic conditions.

Fig. 2 and Fig. 3 depict the CE–UV profiles corresponding to two different analytical grades of ovalbumin, a high-molecular-mass glycoprotein with a low glycosylation percentage (see Table 1). From the comparison of the two figures it emerges that the proposed method allows to clearly distinguish between the glycosylation patterns of the two glycoprotein samples.

In order to investigate glycoproteins characterized by a combination of high molecular mass and a high percentage of glycosylation, AGP has been considered as a model protein.

In Fig. 4 the CE–UV profile of bovine AGP is shown, obtained by using 25 m*M* MES–Tris pH 5.6 as BGE. When analyzed under the same electrophoretic conditions, human AGP displays the unresolved glycosilation pattern reported in Fig. 5. By doubling the MES concentration (i.e., using 50 m*M* instead of 25 m*M*) and adding urea to the BGE to a final concentration of 8 *M*, a well resolved glycoform profile can be obtained for human AGP (Fig. 6). This last separation represents a highly valuable result because of its complexity. In fact, according to my knowledge, no equivalent separation to that reported in Fig. 6 has ever been obtained in uncoated capillaries.

## 4. Conclusions

The CE method proposed in this paper, which combines the use of a conventional PEG-capillary GC column with low ionic strength buffers, has been demonstrated to provide highly valuable glycosylation profiles of various glycoproteins.

In particular, it has been shown that this procedure allows one to obtain distinctive and reproducible CE–UV patterns for both lightly (e.g., ribonuclease

B, ovalbumin) and heavily glycosylated (e.g., bovine and human AGP) high-molecular-mass proteins.

The method also applies to non-glycosylated proteins (e.g., ribonuclease A) and peptides.

Further experimental work, still in progress in this laboratory, has shown that the CE method described here also works very well for many other glycoproteins (of recombinant origin or isolated from natural sources) which will be reported elsewhere.

## Acknowledgments

The author gratefully acknowledges Dr. J.E. Coutant and Dr. L.F. Zerilli for reviewing the manuscript and for helpful comments.

## References

[1] R.P. Oda, B.J. Madden and J.P. Landers, Adv. Chromatogr., 36 (1996) 163.
[2] R. Bonfichi, C. Sottani, L. Colombo, J.E. Coutant, E. Riva and D. Zanette, J. Mass Spectr, in press; and references cited therein.
[3] R.B. Parekh, A.G.D. Tse, R.A. Dwek, A.F. Williams and T.W. Rademacher, EMBO J., 6 (1987) 1233.
[4] T.W. Rademacher, R.B. Parekh and R.A. Dwek, Annu. Rev. Biochem., 57 (1988) 785.
[5] J.P. Landers, R.P. Oda, B.J. Madden and T.C. Spelsberg, Anal. Biochem., 205 (1992) 115.
[6] M. Taverna, A. Baillet, D. Biou, M. Schlüter, R. Werner and D. Ferrier, Electrophoresis, 13 (1992) 359.
[7] K.W. Yin, J. Chromatogr., 559 (1991) 401.
[8] W. Nashabeh and Z. El Rassi, J. Chromatogr., 536 (1991) 31.
[9] P. Hermentin, R. Witzel, R. Doenges, R. Bauer, H. Haupt, T. Patel, R.B. Parekh and D. Barzel, Anal. Biochem., 206 (1992) 419.
[10] F. Kilar and S. Hjertén, J. Chromatogr., 480 (1989) 351.
[11] E. Watson and F. Yao, Anal. Biochem., 210 (1993) 389.
[12] H.B. Hines and E.E. Brueggemann, J. Chromatogr. A, 670 (1994) 199.
[13] P.M. Rudd, I.G. Scragg, E. Coghill and R.A. Dwek, Glycocon. J., 9 (1992) 86.
[14] D.C. James, R.B. Freedman, M. Hoare and N. Jenkins, Anal. Biochem., 222 (1994) 315.
[15] S. Honda (Guest Editor), Chromatographic and Electrophoretic Analysis of Carbohydrates, J. Chromatogr. A, 720 (1996).
[16] F.A. Chen, L. Kelly, R. Palmieri, R. Biehler and H. Schwartz, J. Liq. Chromatogr., 15 (1992) 1143.
[17] F.A. Chen and J. Zang, J. Assoc. Offic. Anal. Chem., 75 (1992) 905.
[18] H.H. Lauer and D. McManigill, Anal. Chem., 58 (1986) 166.
[19] R.M. McCormick, Anal. Chem., 60 (1988) 2322.
[20] A. Vinther, S.E. Bjørn, H.H. Sørensen and H. Søeberg, J. Chromatogr., 516 (1990) 175.
[21] A. Emmer, M. Jansson and J. Roeraade, J. Chromatogr., 547 (1991) 544.
[22] W.G.H.M. Muijselaar, C.H.M.M. de Bruin and F.M. Everaerts, J. Chromatogr., 605 (1992) 115.
[23] K.A. Cobb, V. Dolnik and M. Novotny, Anal. Chem., 62 (1990) 2478.
[24] J.K. Towns and F.E. Regnier, Anal. Chem., 63 (1991) 1126.
[25] S. Hjertén, J. Chromatogr., 347 (1985) 191.
[26] G.J.M. Bruin, R. Huisden, J.C. Kraak and H. Poppe, J. Chromatogr., 480 (1989) 339.
[27] S.A. Swedberg, Anal. Biochem., 185 (1990) 51.
[28] G.J.M. Bruin, J.P. Chang, R.H. Kuhlman, K. Zegers, J.C. Kraak and H. Poppe, J. Chromatogr., 471 (1989) 429.
[29] J.A. Lux, H. Yin and G. Schomburg, J. High Resolut. Chromatogr., 13 (1990) 145.
[30] H.A. Sober (Editor), Handbook of Biochemistry, Chemical Rubber Co., Cleveland, OH, 2nd ed., 1970.
[31] D. Schmalzing, C.A. Piggee, F. Foret, E. Carrilho and B.L. Karger, J. Chromatogr. A, 652 (1993) 149.
[32] J.F. Kelly, S.J. Locke, L. Ramaley and P. Thibault, J. Chromatogr. A, 720 (1996) 409.