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Comparative studies on the analysis of glycosylation heterogeneity of sialic acid-containing glycoproteins using capillary electrophoresis

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Abstract

Comparative studies concerning glycoform analysis of sialoglycoproteins by capillary electrophoresis were performed using a few separation modes hitherto reported. Glycoprotein samples examined in the present study were successfully separated to their respective glycoforms using surface-modified capillaries commercially available for capillary gas chromatography in the running buffer near their isoelectric points. The analysis times were less than 50 min and reproducibilities in migration times were excellent (less than 2.0% RSD for both run-to-run and day-to-day analyses). We present a method for the glycoform analysis of α_1-acid glycoprotein in sera by simple pre-treatment as an application. The present technique will become one of the general methods for the evaluation of glycosylation heterogeneity of commercially available glycoprotein drugs.

Keywords: Glycoproteins; Proteins; Sialic acid; Erythropoietin; α_1-Acid glycoprotein

1. Introduction

Glycosylation of proteins is one of the most common and important post-translational modifications found in secretory proteins. In glycoproteins especially produced by biotechnology, glycosylation plays important roles for antigenicity [1], clearance [2] and biological activity [3]. Because the glycosylation is influenced by the type of cell line [4] and the culture conditions [5], structural analysis of glycans and control of glycosylation is essential for pharmaceutical reasons. Furthermore, carbohydrate chains of glycoproteins have distinct variations that are called heterogeneity. Two strategies have been developed for the analysis of such heterogeneity in carbohydrate chains. One is based on the separation of carbohydrate chains that were released from the protein core and labeled with chromogenic and fluorogenic reagents [6,7], the other is based on the

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direct separation of native glycoproteins [8], which is called glycoform analysis.

Capillary electrophoresis is one of the useful methods applicable for both strategies and a number of modes of separation have been developed for the direct resolution of glycoforms [8]. Complexation between borate ions and hydroxy groups of the carbohydrate chains was utilized to resolve glycoforms of intact ribonuclease B (bovine pancreas) [9]. The strategy of masking the silanol groups on the capillary inner-surface by amino compounds has also been adopted to separate glycoforms in bare fused-silica capillaries. Many studies have demonstrated the usefulness of 1,4-diaminobutane as an additive in the electrolyte solution. Landers et al. reported the remarkably enhanced resolution of ovalbumin glycoforms using 1,4-diaminobutane as an organic modifier [10]. The recombinant human erythropoietin (rhEPO) was resolved with high resolution into its glycoforms using a combination of 1,4-diaminobutane and a high concentration of urea. 1,4-Diaminobutane is used to diminish the interactions between the protein and the capillary wall and also to decrease electroosmotic flow. Urea is also added to inhibit protein aggregation [11]. Addition of zwitter ions was also proposed to suppress the undesirable interaction between the protein and capillary wall. Zwitter ions do not affect the conductivity of the electrolyte, hence high concentration of the zwitter ions inhibits non-specific adsorption of proteins. In addition, zwitter ions are considered to enhance the glycoform resolution due to the formation of ion pairs with glycoproteins. A zwitter ion, trimethylammonium propanesulfonate, was employed for the capillary electrophoresis of the monoclonal antibody in bulk form and in a therapeutic formulation [12], and also used to improve the resolution of ricin derived from *Ricinus communis* [13].

Isoelectric focusing on a slab gel has been widely used for glycoform analysis [14]. Its high-resolving power provides separation of complex mixtures and allows to estimate the isoelectric points (p*I*) of the constituents. However, the method using a slab gel is time-consuming and often lacks reproducibility of gels from batch to batch and staining–destaining procedures. Some of these limitations can be overcome when isoelectric focusing (IEF) is performed on a capillary format [15]. However, reproducible detection of the resolved glycoform seems to be an unsolved problem.

Chemically coated capillaries have been successfully employed for the glycoform separation of various glycoproteins since the early report by Hjerten in 1985 [16]. Recently, some papers reported glycoform separation using capillaries, of which inner-surfaces were covalently immobilized with the polymer layer by cross-linking. There is a wide choice of commercially available capillary gas chromatographic columns for such a purpose [17,18].

In the present study, we compare some hitherto reported methods and propose a general method for the evaluation of glycoprotein heterogeneity in chemically modified capillaries using the buffers near their isoelectric points. As an application, we developed a method for the glycoform analysis of α_1-acid glycoprotein in sera by a simple desalting procedure.

2. Experimental

2.1. Materials

Fetuin (fetal calf serum), transferrin (human serum) and α_1-acid glycoprotein (AGP) samples from human, bovine and sheep were obtained from Sigma (St. Louis, MO, USA). Two standard samples of rhEPO produced in a CHO cell line were provided by the European Pharmacopoeia as Biological Reference Product (BRP). Each sample vial contains about 32 500 units of EPO (about 250 μg). A pharmaceutical preparation of EPO was a gift from Ms. Nishiura of Kinki University, Department of Medicine (Osaka, Japan). Neuraminidase from *Arthrobacter ureafaciens* was a gift from Drs. Tsukada and Ohta of Marukin Shoyu (Uji, Kyoto, Japan). All aqueous solutions were prepared using water purified with a Milli-Q purification system (Millipore). A fused-silica capillary (50 μm I.D.) was purchased from Waters (Tokyo, Japan). Capillaries (50 or 100 μm I.D., film thickness 0.1 μm) coated with dimethyl polysiloxane or (50% phenyl)–methyl polysiloxane were obtained from GL Sciences (Tokyo, Japan) and are called DB-1 and DB-17 capillary, respectively. Preparations of hydroxypropylcellulose

(HPC) and hydroxypropylmethylcellulose (HPMC) having different viscosities were from Shin-etsu (Tokyo, Japan). Trimetylammonium propanesulfonate (Z1-Methyl) was purchased from Waters. Other reagents and solvents used were of the highest grade commercially available. The sialic acid contents in glycoprotein samples were assayed by HPLC according to the method reported by Hara et al. [19].

An AGP sample from rat sera was purified according to the procedures reported by Kishino et al. [20]. A 10-ml serum sample obtained from two rats was diluted to 100 ml with 0.01 *M* citrate–phosphate buffer (pH 4.0). The mixture was applied on a column of DEAE-Sephadex G-25 (Pharmacia, 10 ml of gel volume). The column was washed with the same buffer until the eluate showed a constant absorbance (below 0.05) at 280 nm. AGP was eluted with 0.1 *M* citrate–phosphate buffer (pH 4.0, 30 ml). The eluate was passed through a small column of SP-Sephadex C-25 (Pharmacia, 10 ml of gel volume), and dialyzed against distilled water for 48 h at 4°C, then lyophilized to afford AGP as an almost pure state.

2.2. Instruments

Capillary electrophoresis was performed with a P/ACE 5010 capillary electrophoresis system (Beckman, Fullerton, CA, USA). The detection window was made at 7 cm from the outlet of the capillary by carefully removing the polyimide coating with a razor under a microscope, and the transparent portion was fixed on the detector block. Detection was performed in the on-line mode while the UV absorption was monitored at 214 nm. The compositions of the running buffer are given below in detail. Injections were performed automatically in pressure mode for 2 s from the cathodic end, unless otherwise described. Data were collected and analyzed with standard System Gold software (Version 8.10) on an IBM personal computer.

2.3. Isoelectric focusing gel electrophoresis of rhEPO on a slab gel

The procedure for IEF of rhEPO on a slab gel was similar to that described in the previous paper with modification [21], and briefly as follows. The anodic electrolyte: glutamic acid (14.71 g) was dissolved in water (500 ml), and phosphoric acid (33 ml) was added to the mixture. The solution was diluted to 1000 ml. The cathodic electrolyte: β-alanine (8.9 g) was dissolved in water (300 ml) and the solution was diluted to 1000 ml. The slab gel: urea (9 g), a 30% aqueous solution (6.0 ml) of acrylamide–*N*,*N*′-methylenebis(acrylamide) (36.5:1, w/w), 1.05 ml of pH 3–5 ampholyte (Pharmacia), 0.45 ml of pH 3–10 ampholyte and water (15 ml) were mixed and the mixture was degassed under diminished pressure with an aspirator for a few minutes. A 15-μl portion of tetramethylenediamine and 0.3 ml of a freshly prepared solution of ammonium persulfate (100 g/l) were added. The solution was poured into a gel cassette with dimensions 15×15×0.05 cm and allowed to polymerize.

Pre-focusing took place for 1 h in horizontal mode at a constant power of 10 W with maximum voltage and current settings of 2000 V and 100 mA, respectively, using an Atto AE 3235 apparatus (Atto, Tokyo, Japan). A 15-μl portion of each EPO solution and PI marker (pH 3–5, Pharmacia) were applied. Focusing was carried out for 30 min at the same power setting. After focusing, the gel was incubated for 30 min in 200 ml of a solution containing 7 g of sulphosalicylic acid and 20 g of trichloroacetic acid. After carefully removing the fixing solution, the gel was incubated for 30 min in 200 ml of destaining solution (acetic acid–methanol–water, 1:5:5, v/v). The gel was then incubated in a solution of Coomassie Blue R (0.25 g, Sigma) in the destaining solution (200 ml). The gel was destained in the destaining solution until the bands were well visualized against a clear background with occasional changing of the destaining solution.

2.4. Digestion of fetuin with neuraminidase

A portion (100 μl) of an aqueous solution of fetuin (1 mg/ml) was diluted with 40 m*M* acetate buffer (pH 6.0, 100 μl). Into the solution, neuraminidase (0.5 mU, 5 μl) was added and the mixture incubated at 30°C. A portion (20 μl) was removed at specified intervals and analyzed by capillary electrophoresis.

2.5. Capillary zone electrophoresis of rhEPO and fetuin in a fused-silica capillary

The running buffer was prepared as follows. Urea (42.04 g), tricine (0.1792 g), and sodium chloride (0.0584 g) were dissolved in about 60 ml of water. Each 1-ml portion of an aqueous solution of 2.5 m*M* 1,4-diaminobutane and 2 *M* acetic acid was added to the mixture. The pH of the solution was adjusted to pH 5.5 at 30°C with an aqueous 2 *M* acetic acid solution. The solution was diluted to 100 ml with water using a volumetric flask, and a portion was used as the running buffer. The running buffer was not stable, hence it was prepared before analysis. A fused-silica capillary [107 cm (100 cm effective length)×50 μm I.D.] was previously washed with 0.1 *M* NaOH for 30 min and then with water for 30 min. Each analysis was performed after washing the capillary with water for 10 min and then rinsing with the running buffer for 10 min. Injections were conducted automatically in pressure mode at 3.45 kPa for 5 s. The separation was carried out at 30°C. The applied potential was 15 kV.

2.6. Capillary electrophoresis in a DB-1 capillary

A DB-1 capillary [27 cm (20 cm effective length)×100 μm I.D.] was previously washed with methanol for 10 min and then with water for 10 min. Each analysis was performed after washing the capillary with the running buffer for 10 min. Injections were performed in the same manner described above. The separation was carried out at 30°C. The applied potential was 15 kV. The values of electroosmotic flow were $2.38 \cdot 10^{-4}$, $2.61 \cdot 10^{-4}$, $3.00 \cdot 10^{-4}$, $4.68 \cdot 10^{-4}$ and $5.29 \cdot 10^{-4}$ $cm^2/V/s$ at pH 4.2, pH 5.0, pH 5.7, pH 7.0 and pH 8.5, respectively, using benzoin as the neutral marker.

2.7. Glycoform analysis of α_1-acid glycoprotein in rat sera

A serum sample was desalted by a modified microcentrifuge method [22]. A column packed with 1 ml of Sephadex G-25 (superfine grade) was washed with water (3 ml). The column was then washed with 1.0 ml of water containing 0.1% bovine serum albumin to prevent non-specific adsorption of AGP to the gel matrix. The column was centrifuged at 1000 rpm for 3 min. A serum sample (0.1 ml) was applied to the column, and the column was centrifuged at 1000 rpm for 3 min. A portion of the effluent was analyzed by capillary electrophoresis. The sample solution was injected by the electrokinetic method at 10 kV for 10 s. Other analytical conditions were the same as described above.

3. Results and discussion

3.1. Glycoform analysis by isoelectric focusing on a slab gel

The European department for the quality of medicine directed a collaborative study for the replacement of the IEF test by the CE test in the monograph on erythropoietin concentrated solution in early 1998. The procedures for IEF and CE (see below) were based on this protocol and were described in the Experimental section in detail. The authors also had examined IEF gel electrophoresis for the glycoform analysis of rhEPO [21]. An example of the IEF-gel electrophoresis patterns is shown in Fig. 1. Two standard samples of rhEPO derived from CHO cell lines were examined. One of the preparations (S-1) showed five distinct bands, and another sample (S-2) showed four bands. Although both samples had shown five bands during the staining procedure, the destaining procedure wiped out the band of the highest p*I* value in S-2 to obtain a clear background. It was technically difficult to reveal all the bands in good reproducibility.

3.2. Capillary zone electrophoresis using a fused-silica capillary in a buffer containing 1,4-diaminobutane as an organic modifier

Watson and Yao reported the glycoform separation of rhEPO using a fused-silica capillary in a buffer containing 1,4-diaminobutane at weakly acidic conditions [11]. Addition of 1,4-diaminobutane reduces the electroosmotic flow and results in enhancement of the resolution among glycoforms. We re-examined this separation mode to the analysis of rhEPO. A few examples after repetitive analyses of an rhEPO sample are shown in Fig. 2. The first analysis

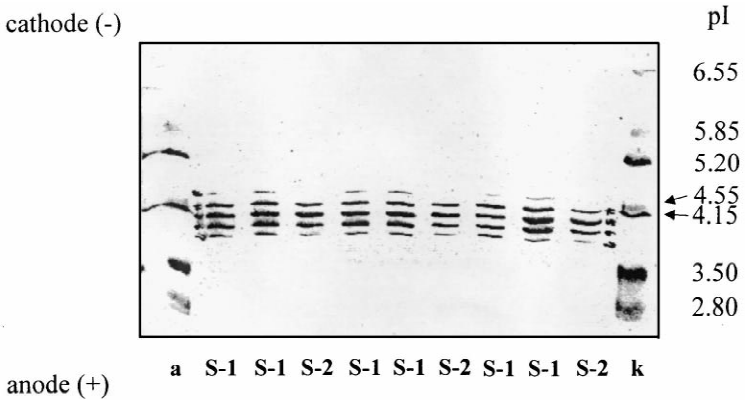

Fig. 1. Isoelectric focusing gel electrophoresis of rhEPO. (a and k), p*I* marker; S-1, sample 1; S-2, sample 2. Analytical conditions: see Experimental.

showed excellent resolution of seven glycoforms around 60 min as shown in Fig. 2a. However, repetitive analyses of the same sample deteriorated the resolution. At the fourth analysis, glycoform peaks appeared earlier around 45 min and some of them were fused (Fig. 2b). Finally, only a broad peak was observed around 45 min (Fig. 2c) at the twelfth analysis. Washing of the capillary with 1 *M* sodium hydroxide, 1 *M* hydrochloric acid or several organic solvents did not recover the resolution. This was probably due to non-specific adsorption of the protein to the surface of the silica capillary by electrostatic interaction. We examined the effect of 1,4-diaminobutane concentration on the improvement of

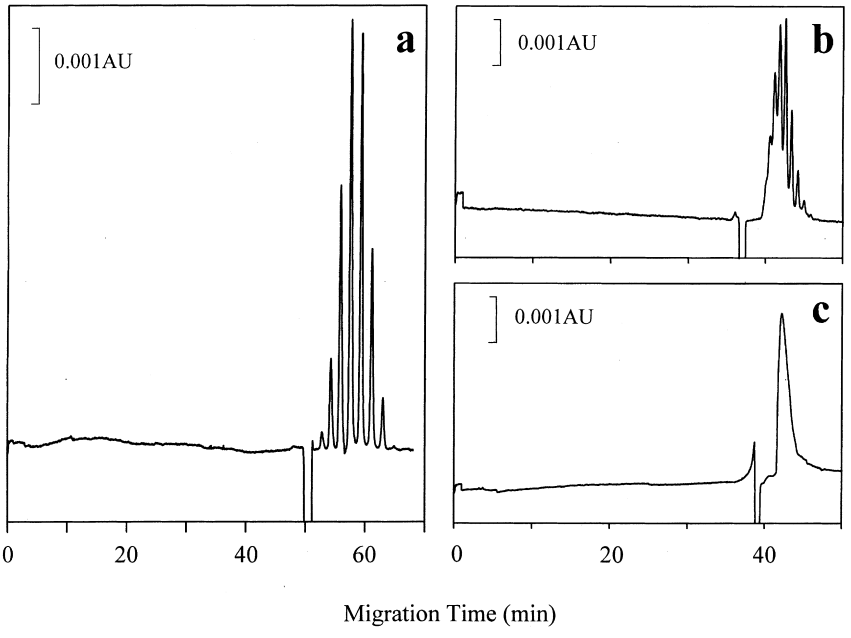

Fig. 2. Repetitive analysis of rhEPO using a fused-silica capillary in a buffer containing 1,4-diaminobutane and urea. Analytical conditions: capillary, fused-silica capillary [107 cm (effective length 100 cm)×50 μm I.D.]; running buffer, 0.01 *M* tricine–0.01 *M* NaCl–0.01 *M* sodium acetate–7 *M* urea–0.025 m*M* putrescine (pH 5.5); applied potential, 15.4 kV; detection, UV absorption at 214 nm. (a) First analysis, (b) fourth analysis, (c) twelfth analysis.

reproducibility. However, we found that the life of the capillary was limited at any concentration of 1,4-diaminobutane (data not shown). The separation occurs mainly based on their charge in both separation modes of IEF and CE and the migration order in IEF and CE is considered to be the same. Small peaks could be detectable in CE, but hardly detectable in IEF in clear background.

3.3. Capillary zone electrophoresis using a fused-silica capillary in a buffer containing a zwitter ionic substance

Non-specific adsorption of proteins to the silica surface has been a serious problem in CE. To circumvent the protein adsorption, use of an additive that acts as a dynamic coating of the capillary surface shields the silica wall from interacting with proteins. Zwitter ionic compounds such as trimethylammonium propanesulfonate (Z1-Methyl) have been used for such a purpose [23]. Z1-Methyl forms ion-pairs with protein glycoforms and may contribute to the enhancement in the resolution of their respective glycoform. Fig. 3 shows the results of the analyses of two rhEPO preparations using Z1-Methyl as an additive. The operation current was as low as 45 μA in the running buffer containing a high concentration (0.5 *M*) of Z1-Methyl, because Z1-Methyl is electrically neutral due to the mutual interaction between the quaternary ammonium ions and sulfonate ions. Although resolutions among glycoforms were not satisfactory, the analysis was completed within 15 min, and indicated that the peak profiles observed for each preparation showed characteristic patterns. Furthermore, this mode of separation will make it possible to determine the ingredients simultaneously as observed for the peaks at 10 and 17 min in Fig. 3a. The reproducibility in migration times was excellent (less than 0.5%; $n=5$).

3.4. Selection of chemically-modified capillaries

A number of reports concerning glycoform analysis have been reported using chemically modified capillaries for CE and capillary columns for capillary gas chromatography. These capillaries show little non-specific adsorption of protein to the capillary

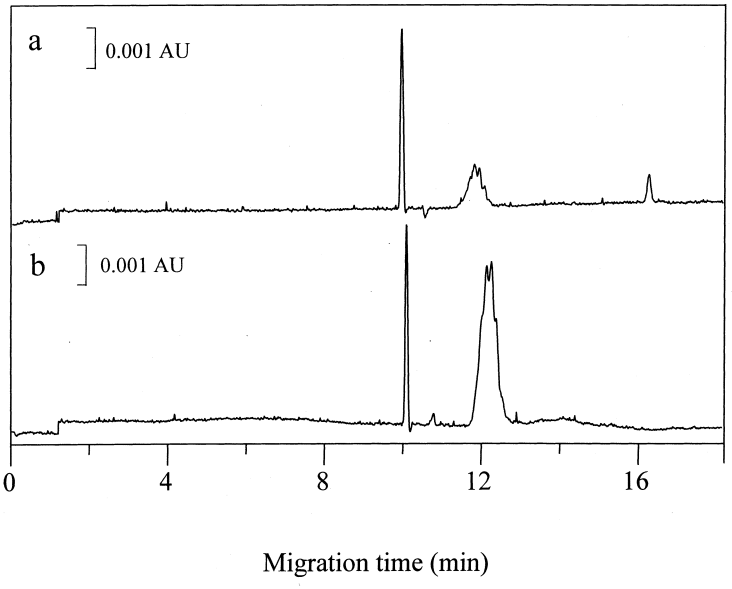

Fig. 3. Analysis of two different rhEPO preparations in a running buffer containing Z1-Methyl. Analytical conditions; capillary, fused-silica capillary [97 cm (effective length 90 cm)×75 μm I.D.]; running buffer, 25 m*M* borate buffer (pH 8.4) containing 500 m*M* Z1-Methyl; applied potential, 25 kV; detection, UV absorption at 200 nm. (a) S-1 and (b) S-2 as in Fig. 1. A peak observed at 10 min for both electropherograms is due to chloride ions.

inner surface. In the present study, we examined DB-1, DB-17 and DB-WAX capillaries, of which the inner-surfaces are modified with dimethylpolysiloxane, 50% phenyl–50% methyl polysiloxane and polyethyleneglycol, respectively. A DB-WAX capillary is not stable under neutral or slightly alkaline conditions, and not appropriate for repetitive analysis of glycoproteins. The analysis of human α_1-acid glycoprotein using a DB-1 capillary showed the better baseline than that using a DB-17 capillary (data not shown). This is probably because of the background noise in detection due to phenyl groups of a coating material of the DB-17 capillary. Therefore, we employed a DB-1 capillary column (100 μm I.D.) throughout the work.

3.5. Optimization of analytical conditions using a DB-1 capillary

Resolutions among fetuin glycoforms were greatly dependent on the pHs of the running buffer as shown in Fig. 4. A group of slightly resolved peaks was observed around 13 min at pH 9.0 (Fig. 4a). At the lower pH values, migration times became larger and resolution of glycoforms more obvious. Finally, more than ten distinguishable peaks were observed in 20 m*M* acetate buffer at pH 5.0 using a DB-1 capillary of 50 cm effective length (Fig. 4d). However, no peaks were observed at pH values lower than five.

Although the resolution of glycoforms was not sufficient in the absence of HPMC (data not shown), addition of a neutral polymer enhanced the resolution of fetuin glycoforms. A small electroosmotic flow of $2.61 \cdot 10^{-4}$ $cm^2/V/s$ was observed at this pH in the DB-1 capillary, but the flow became negligible when a small amount of a neutral polymer was added in the running buffer. By adding a neutral polymer in the running buffer, the polymer covers the inner-surface of the capillary, and shields the surface from the undesirable interaction between the protein and capillary inner-surface. However, higher concentrations than 1.6% of HPMC as a polymer did not improve the resolution but fused the resolved peaks (data not shown). Increase in viscosity of the buffer probably disturbed the migration of each glycoform, and hampered their separation.

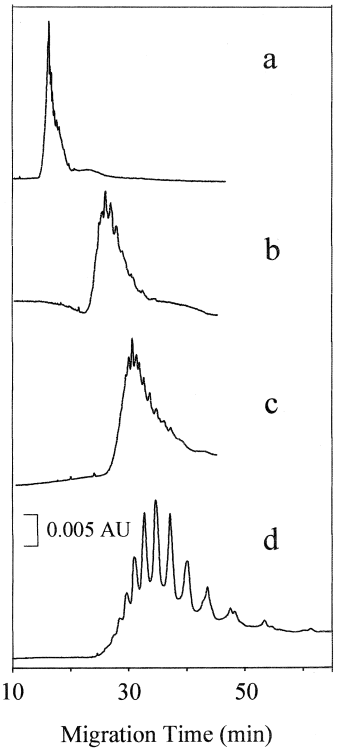

Fig. 4. Effect of pH on the resolution of fetuin glycoforms using a DB-1 capillary. Analytical conditions: capillary, DB-1 capillary [57 cm (effective length 50 cm)×100 μm I.D.]; running buffer, (a) 25 m*M* Tris–borate (pH 9.0), (b) 25 m*M* Tris–HCl (pH 7.4), (c) 20 m*M* acetate buffer (pH 6.0), (d) 20 m*M* acetate buffer (pH 5.0); applied potential, 15 kV; detection, UV absorption at 200 nm. All buffers contain HPMC at 0.4% w/v concentration.

3.6. Application to glycoform separation of some glycoproteins

Based on the optimization studies using chemically modified capillaries as described above, we applied the present technique to glycoform analysis of some glycoproteins.

Fetuin showed interesting changes in glycoform during digestion with neuraminidase as shown in Fig. 5, where acetate buffer (pH 5.0) was used as the running buffer. Glycoform peaks disappeared gradually, and finally there were no peaks after 12 h (Fig. 5f). A small peak observed at 15 min (indicated by the arrow) was due to *N*-acetylneuraminic acid (NeuAc) released from fetuin. Release of NeuAc residues affords asialo-fetuin. Asialo-fetuin still has

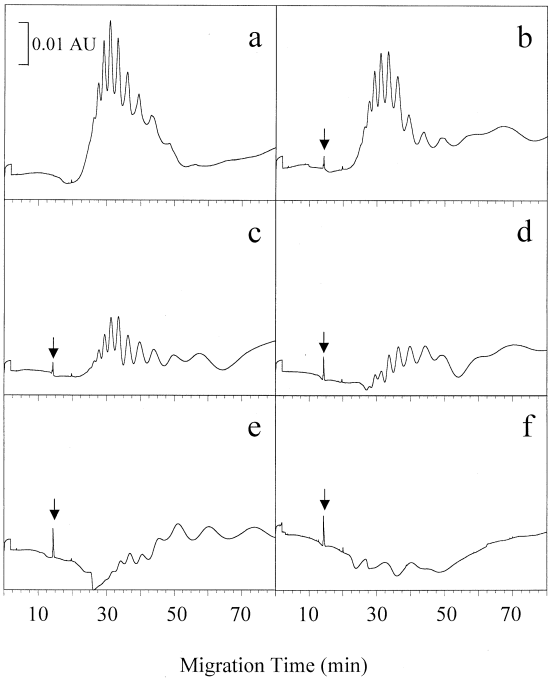

Fig. 5. Course of neuraminidase digestion of fetuin examined in the acetate buffer (pH 5.0). Analytical conditions are the same as in Fig. 5b. Digestion time is (a) 0 h, (b) 0.5 h, (c) 1 h, (d) 2 h, (e) 3 h and (f) 12 h, respectively. The arrows indicate the peak of NeuAc.

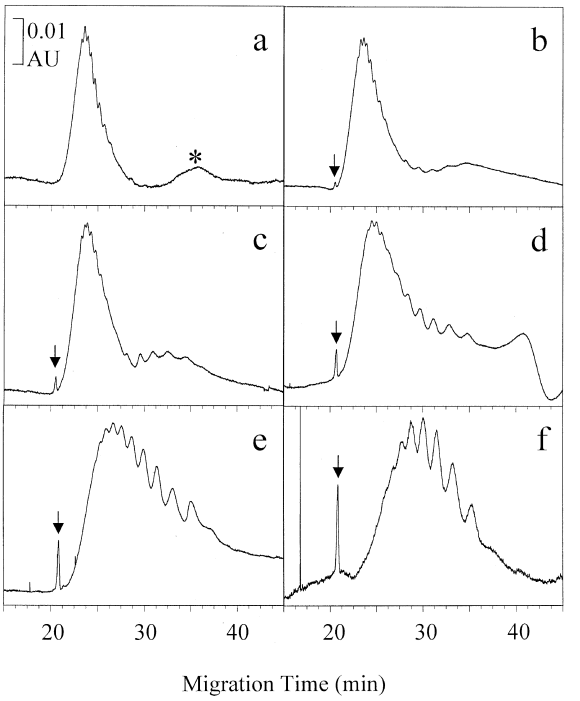

Fig. 6. Course of neuraminidase digestion of fetuin examined in Tris–borate buffer (pH 8.5). Analytical conditions: Capillary, DB-1 capillary [57 cm (effective length 50 cm)×100 μm I.D.]; running buffer, 25 m*M* Tris–borate (pH 8.5) containing 1.0% (w/v) of HPMC; applied potential, 12 kV; detection, UV absorption at 200 nm. Digestion times were the same as in Fig. 6. The arrows indicate the peak of NeuAc. The asterisk indicates a contaminating protein in the fetuin sample.

glycosylation heterogeneity based on neutral carbohydrate chains, but remaining carbohydrate chains do not have charges nor show any effect on resolution of glycosylation heterogeneity at this pH. Furthermore, asialo-fetuin has higher p*I* values due to a lack of acidic NeuAc residues, and migration velocities of asialo-fetuin become smaller and finally no peaks are observed. The course of digestion with neuraminidase was also monitored using 50 m*M* Tris–borate buffer (pH 8.5) containing 1% HPMC as the running buffer (Fig. 6). The fetuin peaks at 0-time digestion showed an incompletely resolved broad peak around 23 min. After prolonged digestion, the peak was gradually observed later and finally around 30 min as partially resolved peaks. Retardation of migration times was due to a decrease in negative charge, because NeuAc residues were released. This was also certified by the increase of the NeuAc peak at 21 min (indicated by the arrow). In borate buffer, hydroxy groups of carbohydrate chains form complexes with borate ions. Splitting of the fetuin peak observed after neuraminidase digestion is due to such complex formation between asialo-carbohydrate chains in fetuin molecules and borate ions. Hence, a combination of acetate buffer and Tris–borate buffer is a useful strategy for the analysis of sialic acid-containing glycoproteins.

rhEPO derived from BHK cell lines is a sialoglycoprotein having a molecular mass of 35 000 with a p*I* of 4.2. Glycoforms of this glycoprotein are well resolved in 20 m*M* acetate buffer (pH 5.7) with a DB-1 capillary as shown in Fig. 7a. At least, nine peaks were observed between 30 and 60 min. Although the resolutions among glycoforms were not as good as those observed in Fig. 2, a distinguishable number of peaks increased and reproducibilities were excellent as shown in Table 1. Fig. 7b shows an

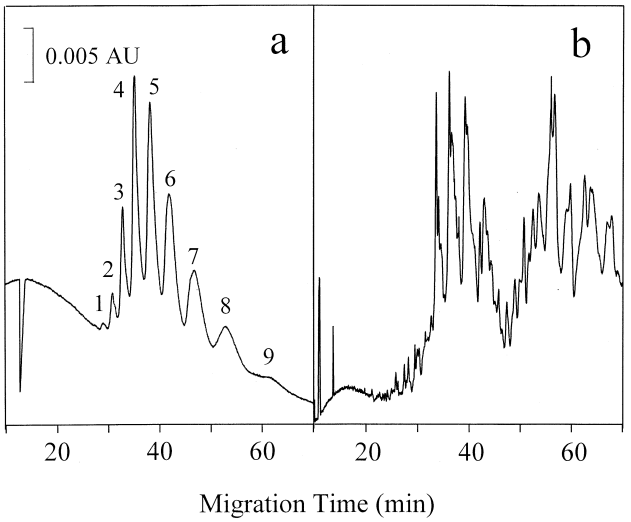

Fig. 7. Analysis of rhEPO glycoforms. Analytical conditions: Capillary, DB-1 capillary [57 cm (effective length 50 cm)×100 μm I.D.]; running buffer, 10 m*M* acetate buffer (pH 5.7) containing 0.5% (w/v) of HPMC; applied potential, 12.5 kV; detection, UV absorption at 200 nm. (a) Standard sample (S-1) and (b) pharmaceutical preparation.

example for the analysis of a pharmaceutical preparation, which contains hydrolysates of gelatin as the ingredient. Although a number of accompanying peaks were observed in the electropherogram, peaks observed from 35 to 45 min are due to those of EPO glycoforms.

Fig. 8 shows examples for the analysis of α_1-acid glycoprotein (AGP) derived from human, bovine, rat and sheep. In these analyses, a DB-1 capillary of 20 cm with inner diameter of 50 μm was employed. The time required for the analysis became rapid (within 30 min) and the resolutions among glycoforms were similarly excellent as observed in the analysis using a long capillary (50 cm). Nine well-resolved peaks were observed around 10 min for human AGP (Fig. 8a). More than ten peaks were observed for bovine AGP around 10 min (Fig. 8b) and for rat AGP around 8 min (Fig. 8c), respectively. However, AGP derived from sheep gave broad coalescent peaks around 11 min. The sialic acid contents of these AGP preparations showed similar values (about 10%, w/v) by the HPLC method [19]. However, glycoform analysis showed distinct variations among sources. This suggests that a variation in carbohydrate chains (not sialic acid content) of each AGP resulted in a difference in the glycoform separation, although precise studies should be necessary on the analysis of carbohydrate chains. AGP is an acute-phase glycoprotein with five *N*-linked glycans, exhibiting substantial heterogeneity in their structure [24]. During acute inflammation, a cytokine-induced transient decrease in the degree of branching of AGP occurs [25]. The degree of branching of AGP-glycan in chronic inflammation has been correlated with the duration and severity of

Table 1
Reproducibilities of migration times in glycoform analysis of r-HuEPO

Peak number	Migration time (min)[a]	Run to run[b] RSD (%)	Day to day[b] RSD (%)
1	28.1	1.05	1.01
2	29.9	1.19	1.12
3	31.8	1.04	1.23
4	34.0	1.21	1.29
5	36.7	1.29	1.34
6	40.0	1.67	1.64
7	44.4	1.54	1.99
8	49.8	1.57	1.95

[a] Analytical conditions: capillary, DB-1 (50 cm×100 μm I.D.); buffer, 10 m*M* acetate buffer (pH 5.7) containing 0.5% HPMC50; applied potential, 12.5 kV; injection, pressure method, 5 s; detection, UV absorption at 200 nm.

[b] Number of determinations was five. Peak number corresponds to those in Fig. 8a. Peak 9 was not determined because the integrator did not detect the peak in every analysis.

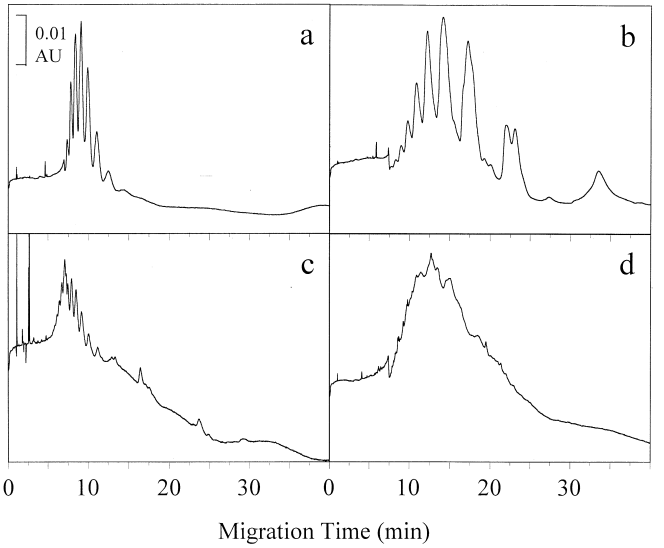

Fig. 8. Analysis of AGP glycoforms from different animal species. Analytical conditions: Capillary, DB-1 capillary [27 cm (effective length 20 cm)×50 μm I.D.]; running buffer, 20 m*M* acetate buffer (pH 4.1) containing 0.5% (w/v) of HPMC; applied potential, 15 kV; detection, UV absorption at 200 nm. AGP preparations: (a) human, (b) bovine, (c) rat and (d) sheep.

disease [26]. Fig. 9 shows an example of AGP from a rat serum sample at inflammation state induced by injection of carageenan.

The peaks observed for normal AGP (Fig. 9a) show a distinct difference from those observed for that in the inflammation state (Fig. 9b). The slow moving peaks observed in Fig. 9b apparently increased. This is probably due to a decrease of NeuAc residues or an increase of higher branched carbohydrate chains, although further studies should be necessary. These data were easily obtained by a simple desalting procedure as described in the Experimental section and did not need other purification procedures. However, the sample solution still contains various substances. In acetate buffer (pH 5.0), only AGP, which has a negative charge at this pH

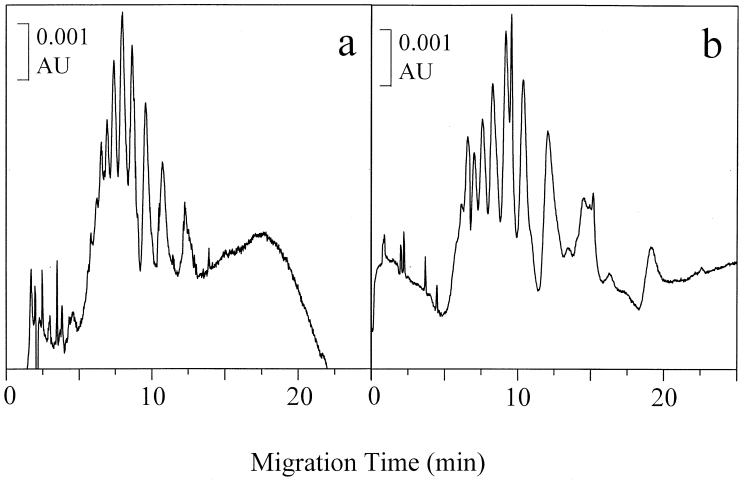

Fig. 9. Analysis of AGP samples obtained from rat sera in normal (a) and inflammation (b) state. Analytical conditions are the same as in Fig. 8, but the sample injection was performed by electrokinetic method, at 10 kV for 10 s.

Table 2
List of glycoproteins examined in the present study

Glycoprotein	Source	Molecular mass	p*I*	Recommended pH of the running buffer
Erythropoietin	BHK cell	35 000	4.2–4.3	5.7
Fetuin	Bovine serum	50 000	3.2–3.8	5.0
α_1-acid glycoprotein	Human serum	32 000	2.8	4.2
α_1-acid glycoprotein	Bovine serum	40 000	1.8–2.7	4.2

(see Table 2), was injected into the capillary by electrokinetic injection and other proteins which have higher isoelectric points, were not moved into the capillary at this pH. The difference in appearance of electropherograms between Fig. 9a and Fig. 8c was probably due to the injection method and sample purity. The method for the analysis of AGP in sera described in this paper will be useful for routine analysis of clinical purpose.

4. Conclusion

A comparative study concerning glycoform analysis of sialoglycoproteins was performed using a few separation modes hitherto reported. The use of a chemically modified capillary showed good reproducibility in repetitive analysis. The best separation was achieved in the buffer at about 1.5 higher pHs than their isoelectric points with a DB-1 capillary as shown in Table 2. In a buffer solution having a pH around their isoelectric points, each glycoform was successfully distinguished. We applied the present method to the analysis of α_1-acid glycoprotein in serum samples, and found that the method was suitable for routine glycoform analysis.

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