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Review

Analysis of glycoproteins, glycopeptides and glycoprotein-derived oligosaccharides by high-performance capillary electrophoresis

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Abstract

Recent developments in the analysis of glycoproteins by high-performance capillary electrophoresis are reviewed, with emphasis on their carbohydrate chains. Glycoforms of glycoproteins were directly separated from each other by careful optimization of the analytical conditions. Glycopeptides in tryptic digests were separated and the peptides carrying glycosylation sites were differentiated from others. Released oligosaccharide chains were separated from each other by direct or modified zone electrophoresis and directly detected by measuring the UV absorption at a low wavelength. Precolumn derivatization by various methods extended the utility of both the separation mode and detection technique. Dual mode analysis after derivatization permitted reliable identification and quantification without references.

Contents

1. Introduction

Advances in glycobiology and glycotechnology have made it possible to produce bioactive glycoproteins on an industrial scale [1,2], and various products have begun to be used for therapeutic purposes. Recombinant glycoproteins have also attracted the attention of glycobiologists, because they believe that there is a certain relationship between biological activity and carbohydrate structure [3]. In order to verify this relationship, it is necessary to examine the

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distribution of carbohydrate chains in the polypeptide core and to identify and quantify each carbohydrate chain. Various methods for these kinds of analyses have been reviewed by Dwek et al. [4].

High-performance capillary electrophoresis is a promising alternative tool for such analyses. However, it is fundamentally a method for ionic substances. Acidic carbohydrates that have charged residues such as carboxylate, sulfate and phosphate groups in their molecules allow direct separation by capillary electrophoresis. Details of the analysis of these groups of carbohydrates are reviewed in this issue by Linhardt and Pervin [5]. Their review also contains analysis of sialic acid-containing carbohydrates. Most classes of carbohydrates have no electric charge for direct separation; in addition, they have neither a chromophore nor a fluorophore for direct detection. Therefore, some devices are required to make capillary electrophoresis amenable to these classes of carbohydrate. Such devices are reviewed in this issue by Honda [6] and Paulus and Klockow [7].

In this review, we divide the discussion into three sections. The first section is focused on the analysis of glycoforms of glycoproteins. Mapping of tryptic digests and a discussion on microheterogeneity of carbohydrate chains are also included in this section. The second is the analysis of released oligosaccharides, either directly or after derivatization. Two-dimensional mapping is a special topic for the identification and quantification of released oligosaccharides, and hence is dealt in a separate section.

The papers for this review were collected through an on-line search of *Chemical Abstracts* using DIALOG and publications up to June 1994 were covered. Related papers were also collected from a PC-based commercial database, Reference Update (Research Information Systems). Readers can consult general reviews on capillary electrophoresis and micellar electrokinetic chromatography that have appeared in *Analytical Chemistry* [8,9]. Olechno and Ulfelder [10] wrote a chapter and Oefner and Chiesa [11] authored a review on carbohydrate analysis by capillary electrophoresis. General guides for glycobiology have also appeared in a recent volume of *Methods in Enzymology*. There are a few chapters on the electrophoresis of carbohydrate chains in glycoproteins and glycosaminoglycans [12]. Readers can also obtain practical protocols in glycobiology from a recent volume of *Methods in Molecular Biology* [13].

2. Carbohydrate-mediated separation of glycoproteins and glycopeptides

2.1. Analysis of glycoforms of glycoproteins

Glycoproteins contain amide bonds and sometimes aromatic amino acids in their molecules, hence they can be directly monitored by UV absorption measurements. Early work on analysis of glycoproteins was focused on separation of glycoforms of sialoglycoproteins. Wenisch et al. [14] compared capillary zone electrophoresis and analytical isoelectric focusing in immobilized pH-gradient gels with regard to speed, sensitivity, quantification, reproducibility and resolving power by using human monoclonal antibodies against the glycoprotein, gp-41 of HIV virus and human recombinant superoxide dismutase as model proteins. They stated that capillary zone electrophoresis is superior to isoelectric focusing in terms of analysis speed and quantification, but has a lower resolving power. Kilàr and Hjertén [15] first reported a fundamental study on the separation of transferrin glycoforms by capillary zone electrophoresis. They found that the separation occurred based on the difference in the number of the sialic acid residue(s). Wu et al. [16] reported a method using capillary zone electrophoresis to monitor charge heterogeneity in recombinant-DNA derived human growth hormone (rhGH), T4 receptor protein (rCD4) and tissue plasminogen activator (rtPA) by using a covalently bonded silica capillary at low pH. Tran et al. [17] also reported similar work on the study of carbohydrate-mediated microheterogeneity of recombinant human erythropoietin (rhEPO) by capillary zone electrophoresis. A systematic approach was presented to optimize the resolution by changing the pH, buffer type

and organic modifier. The best resolution was obtained by using a mixed buffer, 100 m*M* acetate–phosphate (pH 4.0). It was stated that a 10-h preincubation of the capillary tube with the running buffer was necessary to attain the best resolution. They ascribed the need for such a long preincubation to the slow equilibration rate between the capillary inner wall with the buffer. Grossman et al. [18] described an example of separation among ribonuclease A, B_1 and B_2, although identification of peaks was not complete.

Based on these earlier studies, recent work has achieved a much higher resolution by modifying the carrier electrolyte solution. Typical examples of glycoform separation are shown in the following examples.

Erythropoietin (estimated molecular mass ca. 34 000) is a glycoprotein hormone having both N- and O-glycosidically linked carbohydrate chains. Several companies have produced recombinant human erythropoietin from various cell lines, but the kinds and the amounts of carbohydrate chains vary among the products. Nevertheless, the presence of the carbohydrate chains is essential for in vivo biological activities. Watson and Yao [19] reported the separation of the glycoforms of a recombinant human erythropoietin, rhEPO. This variant, rhEPO, is a mixture of several glycoforms, each having three N-glycosylated sites and one O-glycosylated site. The number of the sialic acids at these sites varies among glycoforms. The resolution of these glycoforms was not optimum in 10 m*M* tricine–10 m*M* sodium chloride at pH 6.2, only a single fused peak being observed, as shown in Fig. 1A.

Watson and Yao [19] attempted to improve the resolution by the addition of various additives including water-soluble polymers (polyethylene glycol 200, 400 and 20 000), organic solvents (ethylene glycol, ethanol, acetonitrile, tetrahydrofuran) and organic cations (1,4-diaminobutane, morpholine, 1,5-diaminopentane, 1,7-diaminoheptane). The aim of the introduction of these additives was to decrease the electroosmotic flow (EOF). Balancing of the velocities of both EOF and electrophoretic migration enhanced the difference in charge-to-size ratio, resulting in a better resolution of glycoforms, as shown in Fig. 1B. They also found that a high concentration of urea (7 *M*) improved the resolution dramatically, as shown in Fig. 1C. The effect of urea was assumed to be the consequence of reduced adsorption of the solute on the capillary inner surface. On the other hand, digestion of rhEPO with neuraminidase caused a reduction in the number of peaks resolved. This means that the separation was mainly dependent on the number of sialic acid residues. They compared the separation profile obtained by capillary electrophoresis with that

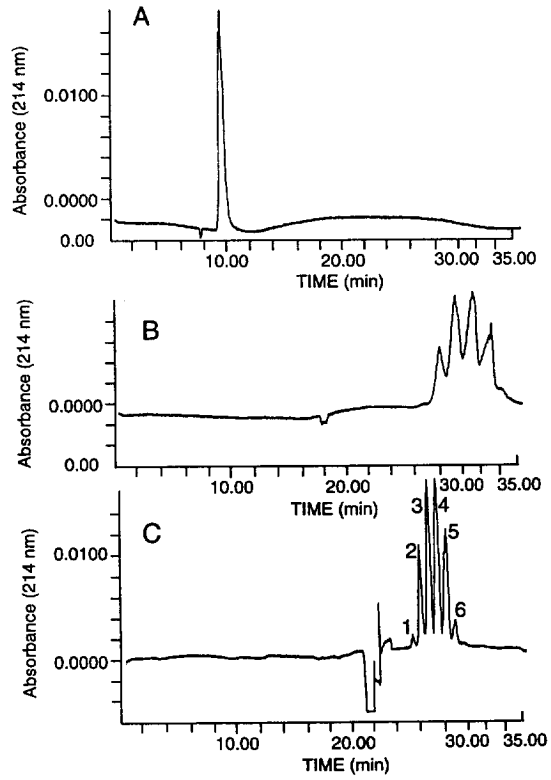

Fig. 1. Separation of the glycoforms of recombinant human erythropoietin by capillary electrophoresis. Sample concentration, 1 mg/ml. Capillary, uncoated fused silica (50 cm × 75 μm I.D.). Applied potential, 10 kV. Carrier solutions: (A) 10 m*M* tricine–10 m*M* NaCl (pH 6.2); (B) 10 m*M* tricine–10 m*M* NaCl–2.5 m*M* 1,4-diaminobutane (pH 6.2); (C) 10 m*M* tricine–10 m*M* NaCl–2.5 m*M* 1,4-diaminobutane–7 *M* urea (pH 6.2). The peak numbers designate glycoforms in the order of increasing number of sialic acid residues. From Ref. [19] (Copyright 1993, Academic Press).

obtained by the conventional technique of gel isoelectric focusing. Both separation methods showed almost the same electrophoretic profile and abundance of each glycoform.

Ribonuclease B (molecular mass 15 500) has a single N-glycosylation site at Asn-34, to which high-mannose type oligosaccharides having 5–9 mannose residues are attached. Rudd and coworkers [20,21] succeeded in separating its glycoforms, as shown in Fig. 2.

The buffer solution used for separation was 20 m*M* sodium phosphate (pH 7.2) containing sodium dodecyl sulfate (SDS) and sodium tetraborate at concentrations of 50 and 5 m*M*, respectively. They suggested that the separation was mainly based on the formation of complexes between the mannose residues and the borate ion. Although there are linkage isomers for high-mannose type oligosaccharides in ribonuclease B [22], no resolution occurred among the isomers. Suzuki et al. [23] reported that high-mannose type oligosaccharides have commonly three outermost mannose residues and complex with the borate ion to approximately the same magnitude. Consequently, their pyridylamino derivatives were poorly resolved in basic borate buffer. It seems that the separation of glycoforms of ribonuclease B is not only dependent on complex formation with the borate ion but also on another mechanism.

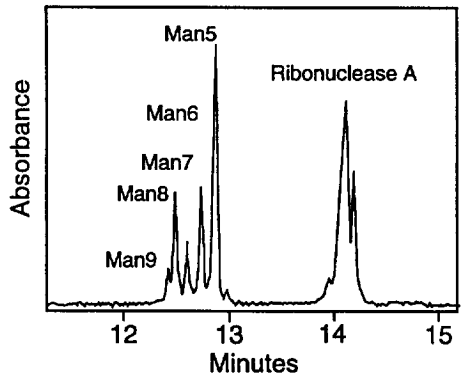

Fig. 2. Glycoform profiles of ribonuclease B. Capillary, uncoated fused silica (72 cm × 75 μm I.D.). Applied potential, 1 kV for 1 min, 20 kV for 19 min. Carrier, 20 m*M* sodium phosphate–50 m*M* SDS–5 m*M* sodium tetraborate (pH 7.2), 30°C. Detection, UV absorption at 200 nm. Commercially available ribonuclease B shows the presence of a non-glycosylated form of ribonuclease A. From Ref. [20] (Copyright 1992, Chapman and Hall).

Work on the separation of glycoforms of ovalbumin was reported by Landers et al. [24]. Ovalbumin (molecular mass ca. 43 000) from hen egg white contains a single glycosylation site at Asn-293, and its carbohydrate chain shows complex microheterogeneity [25,26]. Further, there are two potential phosphorylation sites in the ovalbumin molecule. By employing 100 m*M* borate buffer (pH 8.5) containing 1,4-diaminobutane (putrescine) at a concentration of 1 m*M*, the glycoforms of ovalbumin were well separated. Addition of putrescine caused a decrease in the velocity of electroosmotic flow, and made the separation of glycoforms prominent. Phosphorylation in the protein moiety did not effect resolution of ovalbumin glycoforms but caused retardation of the migration time.

Hirudin from leech, an anticoagulant glycoprotein, has a single O-glycosidic carbohydrate chain at Thr-45. The glycoforms of hirudin were distinguished from each other by using 105 m*M* borate buffer (pH 8.3) containing putrescine at a concentration of 0.2 m*M* [27].

2.2. Mapping and confirmation of glycopeptides

The separation of simple amino acids and peptides was one of the first examples to show the high capability of capillary zone electrophoresis, reported by Jorgenson and Lukacs [28]. Peptides and glycopeptides, having electric charges and chromophores, are appropriate analytes for capillary electrophoresis, although there are not many papers focusing on separations based on the difference in carbohydrate moieties in glycopeptides. Nashabeh and El Rassi [29] reported the capillary zone electrophoresis of α_1-acid glycoprotein fragments obtained by digestion with trypsin and N-glycanase. They first fractionated the tryptic peptides into several groups by high-performance affinity chromatography on a column of concanavalin A (Con A)-immobilized silica. Separation by capillary electrophoresis of the groups Con A-non-reactive, Con A-slightly reactive and Con A-strongly reactive by capillary electrophoresis was attempt-

ed using a capillary tube chemically modified with a hydrophilic coating. The oligosaccharide chains released from the glycopeptides with N-glycanase were also examined for separation after being labelled with 2-aminopyridine by reductive amination.

The separation of tryptic peptides derived from rhEPO was reported by Rush [30]. An example of a separation using 100 m*M* heptanesulfonic acid in 40 m*M* phosphate buffer (pH 2.5) as carrier is shown in Fig. 3. The tryptic map was divided into two sections of non-glycosylated and glycosylated peptides. Peaks due to non-glycosylated peptides were observed in the earlier region, then those of glycosylated peptides followed. The total analysis time required was about 80 min using a 75-cm capillary (effective length 50 cm). Rush [30] indicated that segregation into two groups in the electropherogram was based on the difference in formation of charged ion pairs between peptides and heptanesulfonic acid. Eighteen tryptic peptides within the first 30 min were successfully identified from 21 predicted peptides. Confirmation of each peak was performed by comparison of its elution time in reversed-phase high-performance liquid chromatography with that of the authentic specimen.

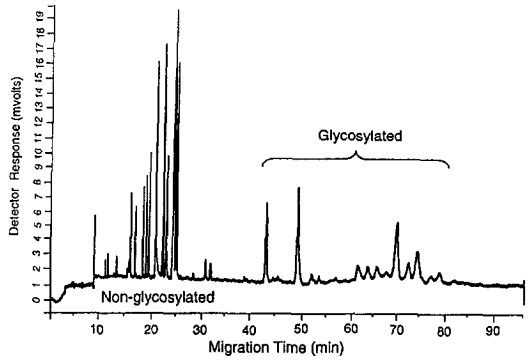

Fig. 3. HPCE profile of trypsin-digested recombinant human erythropoietin (3.75 mg in 10% glacial acetic acid, 1 ml). Capillary, uncoated fused silica [75 cm (effective length 50 cm) × 50 μm I.D.]. Applied potential, 16 kV. Carrier, 40 m*M* sodium phosphate buffer (pH 2.5) containing 100 m*M* heptanesulfonic acid, 30°C. Detection, UV absorption at 200 nm. The non-glycosylated peptide and the glycopeptide sections are as indicated. From ref. [30] (Copyright 1993, American Chemical Society).

The other three peptides were associated with three N-glycosylation sites (a peptide having Asn-24 and Asn-38 and a peptide having Asn-83) and an O-glycosylation site (Ser-126). These peptides were observed in the 40–70-min region and show considerable heterogeneity in the carbohydrate including region. If digestion of rhEPO with trypsin proceeded completely, the heterogeneity was obviously due to variation of the carbohydrate chains. By using neuraminidase or N-glycanase or a combination of both enzymes, they confirmed which were N- and/or O-glycosylated glycopeptides. They also tentatively identified the carbohydrate chain in each glycopeptide based on its relative peak abundance against the HPLC tryptic map. Comparison of the relative abundances of oligosaccharides derived from the glycopeptides with the elution profile in high-performance anion-exchange chromatography also served for the tentative identification of peaks.

If general conditions are established for the categorization of tryptic peptides from various glycoproteins into non-glycosylated and glycosylated peptides, this capillary zone electrophoretic method will be extremely useful for studies of microheterogeneity around the glycosylation sites of glycoprotein samples.

3. Analysis of carbohydrate chains released from the polypeptide core

3.1. Analysis with direct detection

Several methods have been established for the release of carbohydrate chains from the polypeptide core. For chemical and enzymatic release, see the reviews by Bruce [31] and, in this issue, O'Neill et al. [32]. The released oligosaccharides usually contain 2-acetamido-2-deoxyhexose residue(s) [33] and sialic acid residues [34], and hence can be detected by monitoring the low-wavelength absorption. In capillary electrophoresis, monitoring the UV absorption around 200 nm is possible with a commercially available apparatus. Detection at 185 nm was reported to give much higher sensitivities [35].

However, detection at such low wavelengths is not selective to oligosaccharides, and a careful clean-up procedure is necessary.

Separation with direct UV detection of the reducing oligosaccharides released from α_1-acid glycoprotein (human) by hydrazinolysis was reported by Hermentin et al. [36]. The buffer system was 80 m*M* ammonium sulfate–20 m*M* sodium phosphate–2 m*M* diaminobutane, adjusted to pH 7 with phosphoric acid. The migration profiles were similar to those observed for the separation by anion-exchange chromatography using Mono-Q (Pharmacia, Uppsala, Sweden) as the stationary phase. They also compared the migration profiles with the elution patterns of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD), although confirmation of the peaks was not performed. They reported that capillary electrophoresis–UV detection was almost 4000 times more sensitive than HPAEC–PAD in terms of injected amount. However, it should be noted that a much higher concentration of sample is required for analysis by capillary electrophoresis. In a subsequent paper, they built a database for N-linked glycans of sialylated complex type by using a spreadsheet program on a personal computer [37]. They also employed the database to confirm the structures of oligosaccharides released from recombinant human urinary erythropoietin, bovine serum fetuin and α_1-acid glycoprotein. They reported that the resolution of the peaks of α2,3- and α2,6-linked isomers of sialooligosaccharides was achieved by using the buffer system 80 m*M* ammonium sulfate–20 m*M* sodium phosphate–2.0 m*M* 1,5-diaminopentane, adjusted to pH 7.0 with 0.1 *M* sulfuric acid. The efficiency of the database was confirmed in a different laboratory and by a different analyst who used an HPCE system from a different manufacturer.

Taverna and co-workers [38,39] examined micellar electrokinetic chromatography for the separation of sialylated N-linked oligosaccharides derived from recombinant tissue plasminogen activator. They used 50 m*M* phosphate buffer (pH 7.0) containing sodium dodecyl sulfate (SDS) at a concentration of 100 m*M* as the carrier. Addition of divalent cations such as calcium or magnesium improved the resolution among peaks through an increase in the time window between a neutral marker and SDS micelles. More than 20 peaks were observed in 20 min under the optimum conditions, although confirmation of the peaks was not performed.

Kakehi et al. [40] reported an application of capillary electrophoresis to the analysis of O-glycosylated sialooligosaccharides in mucin-type glycoproteins. For quantitative release of O-glycosylated oligosaccharides, a standard method using alkali in the presence of sodium borohydride [41] was employed. In this method, the reducing termini of the released oligosaccharides were concurrently reduced to the hydroxyl group to give alditol derivatives. Therefore, the released oligosaccharides were detected directly by monitoring the absorption at 185 nm. Fig. 4 shows the separation of a mixture of some authentic monosialooligosaccharide alditols obtained from bovine submaxillary mucin and swallow nest materials. Table 1 lists the oligosaccharides examined, along with their electrophoretic mobilities calculated from the migration times in Fig. 4. Analysis was performed by using 200 m*M* borate buffer (pH 9.6) containing SDS

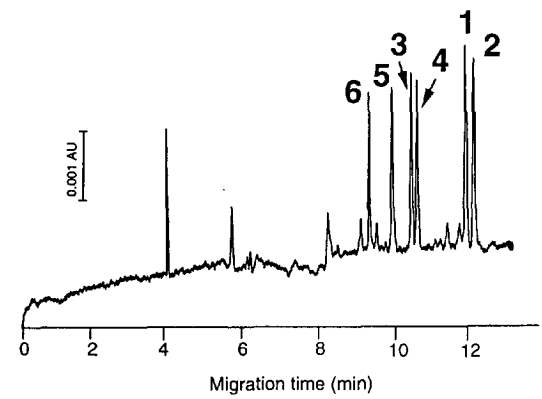

Fig. 4. Separation of an equimolar mixture of monosialooligosaccharide alditol standards. The peak numbers are identical with the oligosaccharide numbers in Table 1. Capillary, fused silica (50 cm × 50 μm I.D.). Applied potential, 17 kV. Detection, UV absorption at 185 nm. Carrier, 200 m*M* borate buffer (pH 9.6) containing SDS (0.1 *M*). From Ref. [40] (Copyright 1994, Elsevier Science Publishers).

Table 1
Electrophoretic mobilities of some sialooligosaccharides

Carbohydrate	μ_{ep} ($cm^2\ min^{-1}\ V^{-1} \times 10^3$)
NeuAc	14.44
NeuGc	14.66
NeuAcα(2→6)GalNAcOH (**1**)	13.04
NeuGcα(2→6)GalNAcOH (**2**)	13.26
GalNAcα(1→3)[NeuAcα(2→6)]GalNAcOH (**3**)	11.39
GalNAcα(1→3)[NeuGcα(2→6)]GalNAcOH (**4**)	11.57
Galβ(1→4)GlcNAcβ(1→6)GalNAcOH (**5**)	10.67
NeuAcα(2→3)Galβ(1→3)/[a]	
Galβ(1→4)GlcNAcβ(1→6)GalNAcα(1→3)GalNAcOH (**6**)	9.73
NeuAcα(2→3)Galβ(1→3)/[a]	

From Ref. [40] (Copyright 1994, Elsevier Science Publishers).
[a] / = Linkage between the Galβ(1→3) and GalNAcOH residues.

at a concentration of 100 m*M*. Larger sialooligosaccharides appeared earlier, and smaller ones followed. This means that the separation is mainly based on the charge of the carboxyl group in the sialyl residues relative to the molecular size. Resolution between N-acetyl- and N-glycolylneuraminic acids was improved by addition of SDS, presumably owing to a slight change in molecular size. It was also confirmed that the calibration graph for sialyllactose as a model oligosaccharide showed good linearity over the range 0.9–20 m*M*.

Fig. 5 shows an electropherogram of the product from bovine submaxillary mucin released with alkali–borohydride on an analytical scale. The procedure for analytical sample preparation was easy. After alkali–borohydride degradation, the reaction mixture was passed through a small column of cation-exchange resin and evaporated to dryness. The boric acid in the residue was removed as the volatile methyl ester. Two predominant peaks due to NeuAcα(2→6)-GalNAc-OH (peak 1) and GalNAcα(1→3)[NeuAcα(2→6)]GalNAc-OH (peak 3) were observed, accompanied by the peaks of the corresponding N-glycolyl analogues, NeuGcα-(2→6)GalNAc-OH (peak 2) and GalNAcα-(1→3)[NeuGcα(2→6)]GalNAc-OH (peak 4). The total peak areas of these major oligosaccharides were 91% of the overall peak areas. The small multiple peaks observed between 7 and 9 min were not identified, but assumed to be of larger sialooligosaccharides. The amount of the mucin sample subjected to alkali–borohydride degradation was 100 μg as glycoprotein. Assuming the sample volume introduced to be 4.0 nl (out of a 10-μl volume of the sample solution), the introduced sample amount was

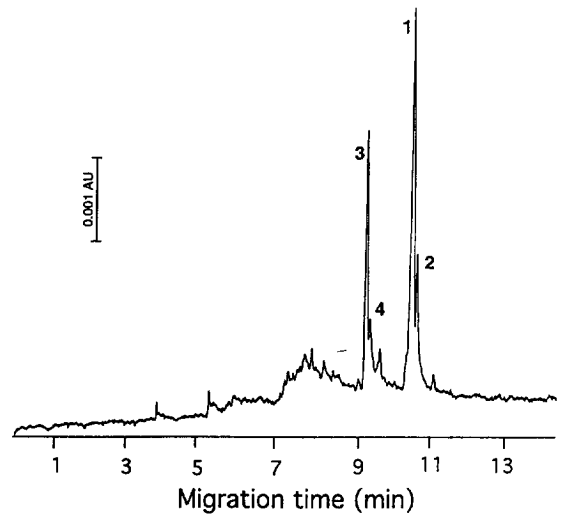

Fig. 5. Micro-scale analysis of O-glycosidically linked sialooligosaccharides in bovine submaxillary mucin. Analytical conditions as in Fig. 5. Peak numbers as in Table 1. From Ref. [40] (Copyright 1994, Elsevier Science Publishers).

calculated to be as small as 40 ng. Most of the samples could be recovered after analysis for further studies. The result obtained from swallow nest material, which contained a complex mixture of O-linked sialooligosaccharides, was also described [42]. More than 50 peaks were observed in the 20-min region, although only a few peaks could be identified.

3.2. Analysis after derivatization

Several methods for precapillary derivatization are available for the analysis of oligosaccharides. All the derivatives in these methods have both ionic and chromophoric and/or fluorophoric tags. Derivatization of carbohydrates is described in detail in Ref. 6.

Precolumn derivatization using 2-aminopyridine (PA) was originally developed for the derivatization of oligosaccharides for HPLC. The ionic character of the imino group in PA derivatives allows their separation by capillary zone electrophoresis. Honda and co-workers reported the usefulness of the PA derivatives for the separation of monosaccharides [43] and oligosaccharides obtained from ovalbumin [44]. Since one imino group is introduced to each reducing sugar, each derivative has the same positive charge in an acidic medium, whereas the molecular size varies with the degree of polymerization (d.p.). Thus, ovalbumin-derived PA-oligosaccharides were separated into five major peaks in 100 m*M* phosphate buffer (pH 2.5) in a linear polyacrylamide-coated capillary tube. Peaks were assigned to hepta- to undecasaccharides in order of increasing d.p. Each of the octa-, nona- and decasaccharide peaks contained a multiple number of oligosaccharides having the same d.p. but differing in monosaccharide composition and sequence. Much better separation of ovalbumin-derived PA-oligosaccharides was achieved by using an alkaline borate buffer as carrier. In this system the PA-oligosaccharides were separated as borate complexes and oligosaccharide species more easily complexed with the borate ion migrated more slowly. Almost all major oligosaccharides were separated by this mode. The PA derivatives can be detected by measurement of the UV absorption at 240 nm. Fluorescence detection at 395 nm with irradiation at 316 nm gave a much higher sensitivity. Nashabeh and El Rassi [29] reported similar work on the capillary electrophoresis of sialooligosaccharides derived from α_1-acid glycoprotein as their PA derivatives, although they did not identify the peaks.

Liu et al. [45] reported the analysis of fetuin-derived oligosaccharides after derivatization by the CBQCA method. After releasing the carbohydrate chains by hydrazinolysis, the mixture of carbohydrate chains was reductively aminated with ammonium sulfate or ammonium chloride in the presence of sodium cyanoborohydride. The mixture was then reacted with 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) in alkali containing potassium cyanide. Fluorescent isoindole derivatives could be separated in alkaline phosphate–borate buffer and detected at the attomole level using argon-ion laser-induced fluorescence. They could not identify the peaks and did not mention whether the sialyl residues were removed or not during derivatization. The derivatization requires two-step reactions, i.e., reductive amination and labelling with CBQCA, but they omitted purification after reductive amination of carbohydrates. Some problems with quantification seem to remain.

Derivatization with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) is a variation of reductive amination. This reagent was developed for precolumn labelling of oligosaccharides for the slab gel electrophoresis of complex oligosaccharides [46–48], and applied to capillary electrophoresis of glycoprotein-derived oligosaccharides by Oefner and Chiesa [11]. After releasing high-mannose type oligosaccharides from bovine pancreatic ribonuclease B by enzymatic digestion with endoglycosidase H, the released oligosaccharides were derivatized with ANTS. The ANTS derivatives of high-mannose type oligosaccharides were successfully separated within 16 min. Although details of the experimental conditions were not described, some positional isomers were reported to be partially resolved.

4. Two-dimensional mapping of carbohydrate chains

There are numerous species of oligosaccharides in glycoproteins, hence it is impractical to analyse all species by a single separation mode. Using high-performance liquid chromatography, Tomiya et al. [26] reported a two-dimensional technique to cover approximately 200 species of oligosaccharides. A similar approach was reported by Suzuki et al. [23] using high-performance capillary electrophoresis. In this case the reproducibility of mapping was higher, provided that relative mobility was used as a migration index. Parallel analysis of reference compounds is unnecessary. In addition, dual-mode separation was easily achieved by simply changing the buffer. A buffer change is much easier if an automated apparatus is used. The same capillary can be used for several months or more without a decrease in efficiency.

The dual separation mode used by Suzuki et al. involved zone electrophoresis in an acidic phosphate buffer (100 m*M*, pH 2.5, containing 0.1% hydroxypropylcellulose) and zone electrophoresis as borate complexes in an alkaline borate buffer (200 m*M*, pH 10.5). N-Glycosidically linked oligosaccharides were released from glycoproteins by hydrazinolysis and derivatized with PA. The sialic acid residue(s) were removed by digestion of the PA derivatives with neuraminidase for simplicity of data interpretation. The first mode allowed the separation of PA-oligosaccharides on the basis of molecular size, because each derivative has one imino group, i.e., the same electric charge. The second mode converted PA-oligosaccharides in situ into ionic borate complexes, which moved to the cathode by a combination of electroosmotic flow and electrophoretic migration. The separation is based on configurational differences in oligosaccharides. They investigated the migration profiles of fifteen complex-type, eleven high-mannose-type and six hybrid-type oligosaccharides in both separation modes. The relative mobilities of each PA-oligosaccharide with respect to PA-glucose were plotted on both axes of the two-dimensional map, as shown in Fig. 6. Table 2 lists the structures of the oligosaccharides examined.

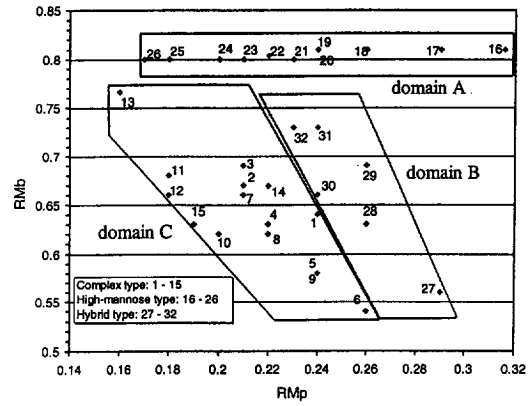

Fig. 6. Two-dimensional map of PA-oligosaccharides derived from various glycoproteins. The plot numbers are identical with those in Table 2. The relative electrophoretic mobility in the phosphate buffer, $(RM)_p$, was calculated as $(t_{PA\text{-}Glc})/t$, where t and $t_{PA\text{-}Glc}$ are the migration times of the PA-oligosaccharide in question and PA-glucose, respectively. The relative mobility in the borate buffer, $(RM)_b$, was calculated as $[(t_{PA\text{-}Glc})/t][(t - t_0)/(t_{PA\text{-}Glc} - t_0)]$, where t_0 is the migration time of the neutral marker. From Ref. [23] (Copyright 1992, Academic Press).

It is notable that the oligosaccharides examined were positioned into three domains. High-mannose-type oligosaccharides were found in domain A, and their extension along the abscissa is mainly dependent on their molecular size. In alkaline borate buffer, complexation with the borate ion occurred but to the same extent for all structures, because these oligosaccharides have the same number (three) of peripheral mannose residues, which are the most reactive to the borate ion. PA-oligosaccharides of hybrid type are found in the area ranging from the lower right corner to the upper middle (domain B). Those of complex type are located in domain C on the left of domain B. These general tendencies were the same as those described in a previous paper [44]. The time required for this dual-mode analysis was less than 2 h, including buffer change.

Table 2
PA-oligosaccharides used for two-dimensional mapping

No.	Structure	$(RM)_b$	$(RM)_p$
Complex type			
1	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.64	0.24
2	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\searrow4\quad\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow4N\beta1\nearrow2M\alpha1$	0.67	0.21
3	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\searrow4\quad\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow3N\beta1\nearrow2M\alpha1$	0.69	0.21
4	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6\qquad F\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.63	0.22
5	$G\beta1\rightarrow4\begin{cases}N\beta1\rightarrow2M\alpha1\searrow6\qquad F\alpha1\searrow6\\ N\beta1\rightarrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N\end{cases}$	0.58	0.24
6	$N\beta1\rightarrow2M\alpha1\searrow6\qquad F\alpha1\searrow6$ $N\beta1\rightarrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.54	0.26
7	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6\qquad F\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\nearrow3$	0.66	0.21
8	$G\beta1\rightarrow4\begin{cases}N\beta1\rightarrow2M\alpha1\searrow6\qquad F\alpha1\searrow6\\ \qquad N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N\\ N\beta1\rightarrow2M\alpha1\nearrow\end{cases}$	0.62	0.22
9	$N\beta1\rightarrow2M\alpha1\searrow6\qquad F\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $N\beta1\rightarrow2M\alpha1\nearrow$	0.58	0.24
10	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\searrow4\quad M\beta1\rightarrow4N\beta1\rightarrow4N$ $F\alpha1\nearrow3\quad M\alpha1\nearrow3$ $G\beta1\rightarrow3N\beta1\nearrow2$	0.62	0.20
11	$G\beta1\rightarrow4N\beta1\searrow4$ $G\beta1\rightarrow3N\beta1\nearrow2M\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\searrow4\quad M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow3N\beta1\nearrow2M\alpha1\nearrow3$	0.68	0.18
12	$G\beta1\rightarrow4N\beta1\searrow4$ $G\beta1\rightarrow3N\beta1\nearrow2M\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\searrow4\quad M\beta1\rightarrow4N\beta1\rightarrow4N$ $F\alpha1\nearrow3\quad M\alpha1\nearrow3$ $G\beta1\rightarrow3N\beta1\nearrow2$	0.66	0.18

Table 2 *(continued)*

No.	Structure	$(RM)_b$	$(RM)_p$
13	$G\beta1\rightarrow4N\beta1\rightarrow3\begin{cases}G\beta1\rightarrow4N\beta1\searrow4\\ G\beta1\rightarrow3N\beta1\nearrow2\end{cases}{}^{M\alpha1\searrow6}_{M\alpha1\nearrow3}M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow4N\beta1\searrow4$ $G\beta_1\rightarrow3N\beta1\nearrow2$	0.77	0.16
14	$G\alpha1\rightarrow3G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6$ $F\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\nearrow3$ $M\beta1\rightarrow4N\beta1\rightarrow4N$	0.67	0.22
15	$G\beta1\rightarrow4N\beta1\rightarrow2M\alpha1\searrow6$ $F\alpha1\searrow6$ $G\beta1\rightarrow4N\beta1\searrow4$ $G\beta1\rightarrow4N\beta1\nearrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.63	0.19
High-mannose type			
16	$M\alpha1\searrow6$ $M\alpha1\nearrow3M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\beta1\rightarrow4N\beta1\rightarrow4N$	0.81	0.32
17	$M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $M\alpha1\rightarrow2$ $M\alpha2\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.81	0.29
18	$M\alpha1\rightarrow2\begin{cases}M\alpha1\searrow6 & M\alpha1\searrow6\\ M\alpha1\nearrow3 & M\alpha1\nearrow3\\ M\alpha1\rightarrow2\end{cases}M\beta1\rightarrow4N\beta1\rightarrow4N$	0.81	0.26
19	$(M\alpha1\rightarrow2)_2\begin{cases}M\alpha1\searrow6 & M\alpha1\searrow6\\ M\alpha1\nearrow3 & M\alpha1\nearrow3\\ M\alpha1\rightarrow2\end{cases}M\beta1\rightarrow4N\beta1\rightarrow4N$ (including 20)	0.81	0.24
20	$M\alpha1\rightarrow2M\alpha1\searrow6$ $M\alpha1\nearrow3M\alpha1\searrow6$ $M\alpha1\rightarrow2M\alpha1\rightarrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.81	0.24
21	$M\alpha1\rightarrow2M\alpha1\searrow6$ $M\alpha1\rightarrow2M\alpha1\nearrow3M\alpha1\searrow6$ $M\alpha1\rightarrow2M\alpha1\rightarrow2M\alpha1\nearrow3M\beta1\rightarrow4N\beta1\rightarrow4N$	0.81	0.22
22	$M\alpha1\searrow6$ $M\alpha1\nearrow2M\alpha1\searrow6M\alpha1\searrow6$ $M\alpha1\nearrow3\quad{}^{3}M\beta1\rightarrow4N\beta1\rightarrow4N$ $M\alpha1\rightarrow2M\alpha1\rightarrow2M\alpha1\nearrow3$	0.80	0.23
23	$M\alpha1\rightarrow2M\alpha1\searrow6$ $M\alpha1\nearrow2\ M\alpha1\searrow6\ M\alpha1\searrow6$ $M\alpha1\nearrow3\ M\beta1\rightarrow4N\beta1\rightarrow4N$ $M\alpha1\rightarrow2\ M\alpha1\rightarrow2\ M\alpha1\nearrow3$	0.80	0.21

(Continued on p. 388)

Table 2 *(continued)*

No.	Structure	$(RM)_b$	$(RM)_p$
24	$M\alpha1\rightarrow2M\alpha1\searrow6$ $M\alpha1\nearrow2$ $M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $M\beta1\rightarrow4N\beta1\rightarrow4N$ $M\alpha1\rightarrow3M\alpha1\rightarrow2M\alpha1\rightarrow2M\alpha1\nearrow3$	0.80	0.20
25	$M\alpha1\rightarrow3M\alpha1\rightarrow2M\alpha1\searrow6$ $M\alpha1\rightarrow3M\alpha1\nearrow2$ $M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $M\beta1\rightarrow4N\beta1\rightarrow4N$ $M\alpha1\rightarrow3M\alpha1\rightarrow2M\alpha1\rightarrow2M\alpha1\nearrow3$	0.80	0.18
26	$M\alpha1\rightarrow3M\alpha1\rightarrow2M\alpha1\searrow6$ $M\alpha1\rightarrow3M\alpha1\nearrow2$ $M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $M\beta1\rightarrow4N\beta1\rightarrow4N$ $M\alpha1\rightarrow3M\alpha1\rightarrow2M\alpha1\rightarrow2M\alpha1\nearrow3$	0.80	0.17
Hybrid type			
27	$M\alpha1\rightarrow3M\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $N\beta1\rightarrow2M\alpha1\nearrow3$	0.56	0.29
28	$M\alpha1\rightarrow3M\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $N\beta1\searrow4$ $N\beta1\nearrow2$ $M\alpha1\nearrow3$	0.63	0.26
29	$M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $N\beta1\rightarrow2M\alpha1\nearrow3$	0.69	0.26
30	$M\alpha1\rightarrow3M\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow4N\beta1\searrow4$ $M\alpha1\nearrow3$ $N\beta1\nearrow2$	0.66	0.24
31	$M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $N\beta1\searrow4$ $N\beta1\nearrow2$ $M\alpha1\nearrow3$	0.73	0.24
32	$M\alpha1\searrow6$ $M\alpha1\nearrow3$ $M\alpha1\searrow6$ $N\beta1\rightarrow4M\beta1\rightarrow4N\beta1\rightarrow4N$ $G\beta1\rightarrow4N\beta1\searrow4$ $N\beta1\nearrow2$ $M\alpha1\nearrow3$	0.73	0.23

G = Galactose; F = fucose; M = mannose; N = N-acetylglucosamine. $(RM)_b$ and $(RM)_p$ are relative electrophoretic mobilities in borate and phosphate buffer, respectively. From Ref. [23] (Copyright 1992, Academic Press).

Table 3
List of analytical conditions for separation of carbohydrate chains mentioned in this review

Glycoprotein	Capillary[a]	Electrolyte	Applied potential (kV)	Detection	Analysis time (min)	Notes	Ref.
2.1. Separation of glycoforms of glycoproteins							
Transferrin (human)	Glass (20 cm × 0.1 mm I.D.) pretreated with polyacrylamide	18 m*M* Tris–18 m*M* boric acid–0.3 m*M* EDTA (pH 8.4)	8	UV at 280 nm	6		[15]
Transferrin (human)	Glass (185 mm × 0.1 mm I.D.) pretreated with polyacrylamide	Ampholyte (2% Bio-lyte 5/7)	5 or 6	UV at 280 nm	20		[15]
Recombinant soluble T4 receptor protein (rCD4)	Fused silica coated with a covalently bonded polymer (20 cm × 25 μm I.D.)	Phosphate buffer (pH 4.5 or 5.5)	12	UV at 200 nm	12		[16]
Recombinant tissue plasminoge activator (rtPA)	Fused silica coated with a covalently bonded polymer (20 cm × 25 μm I.D.)	Phosphate buffer (pH 4.5 or 5.5)	12	UV at 200 nm	10		[17]
Recombinant human erythropoietin (rhEPO)	Fused silica (20 cm × 75 μm I.D.)	100 m*M* acetate–phosphate buffer (pH 4.0)	10 (120 μA)	UV at 214 nm	30	The capillary was filled with the buffer and equilibrated	[17]
Recombinant human erythropoietin (rhEPO)	Fused silica (50 cm × 75 μm I.D.)	10 m*M* tricine –10 m*M* NaCl–2.5 m*M* 1,4-diaminobutane–7 *M* urea	10	UV at 214 nm	35		[19]
Ribonuclease A and B	Fused silica (120 cm × 50 μm I.D.)	20 m*M* CAPS (pH 11.00)	30	UV at 200 nm	10		[18]
Ribonuclease A and B	Fused silica (72 cm × 75 μm I.D.)	20 m*M* sodium phosphate–50 m*M* SDS–5 m*M* sodium tetraborate (pH 7.2)	1 for 1 min then 20	UV at 200 nm	15		[20,21]
Ovalbumin	Fused silica (87 cm × 50 μm I.D.)	100 m*M* borate buffer–1 m*M* 1,4-diaminobutane (pH 8.5)	25	UV at 200 nm	50		[24]

(Continued on p. 390)

Table 3 (*continued*)

Glycoprotein	Capillary[a]	Electrolyte	Applied potential (kV)	Detection	Analysis time (min)	Notes	Ref.
Hirudin	Fused silica (80 cm × 75 μm I.D.)	90 m*M* boric acid–15 m*M* sodium tetraborate–0.2 m*M* 1,4-diaminobutane (pH 8.3)	28	UV at 200 nm	50		[27]
2.2. Mapping of peptides and glycopeptides							
α_1-acid glycoprotein (human)	Fused silica (45 cm × 50 μm I.D.) modified with a hydrophilic coating	0.1 *M* phosphate solution (pH 5.0)	22.5	UV at 200 nm	60		[29]
Recombinant human erythropoietin (rhEPO)	Fused silica (50 cm × 50 μm I.D.)	40 m*M* sodium phosphate buffer (pH 2.5) containing 100 m*M* heptane sulfonic acid	16	UV at 200 nm	90		[30]
3.1. Direct analysis of released carbohydrate chains							
N-Linked sialooligosaccharides derived from α_1-acid glycoprotein (human)	Fused silica (100 cm × 50 μm I.D.)	80 m*M* ammonium sulfate–20 m*M* sodium phosphate–2 m*M* diaminobutane (pH 7.0)	20	UV at 190 nm	60	Released by hydrazinolysis	[36]
N-Linked sialooligosaccharides from rhEPO and α_1-acid glycoprotein	Fused silica (100 cm × 50 μm I.D.)	80 m*M* ammonium sulfate–20 m*M* sodium phosphate–2 m*M* diaminopentane (pH 7.0)		UV at 190 nm	60	Released by hydrazinolysis Database by Lotus 1-2-3 (about 80 carbohydrate chains)	[37]
N-Linked oligosaccharides derived from tPA	Fused silica (90 cm × 75 μm I.D.)	50 m*M* phosphate buffer (pH 7.0) containing SDS (100 m*M*) with/without $CaCl_2$ or $MgCl_2$	22	UV at 200 nm	30		[38,39]
O-Linked sialooligosaccharides as alditols (bovine submaxillary mucin)	Fused silica (50 cm × 50 μm I.D.)	200 m*M* borate buffer (pH 9.6–100 m*M* SDS	17	UV at 185 nm	12	Released by $NaOH–NaBH_4$	[40]

Table 3 (*continued*)

Glycoprotein	Capillary[a]	Electrolyte	Applied potential (kV)	Detection	Analysis time (min)	Notes	Ref.
O-Linked sialooligosaccharides as alditols (swallow nest material)	Fused silica (100 cm × 50 μm I.D.)	200 m*M* borate buffer (pH 9.6)–100 m*M* SDS	20	UV at 185 nm	40	Released by $NaOH–NaBH_4$	[40]
3.2. Analysis of released carbohydrate chains after derivatization							
N-Linked oligosaccharides (fetuin) as their CBQCA derivatives	Fused silica (60 cm × 50 μm I.D.)	20 m*M* Na_2HPO_4–20 m*M* $Na_2B_4O_7$ (pH 9.50)	20	Laser FL at 552 nm (excited at 457 nm)	25	Released by hydrazinolysis	[45]
High-mannose-type oligosaccharides (bovine pancreatic ribonuclease B) as their ANTS derivatives	Fused silica (72 cm × 50 μm I.D.)	50 m*M* triethylammonium phosphate buffer (pH 2.57)	20	UV at 235 nm	15	Released by endoglycosidase H	[11]
N-Linked asialooligosaccharides (transferrin, fetuin, immunoglobulin G, α_1-acid glycoprotein) as PA derivatives	Fused silica (30 cm × 50 μm I.D.)	100 m*M* phosphate buffer (pH 2.5)–0.1% (w/w) HPC	20	FL at 380 nm (excited at 320 nm)	30	Oligosaccharides from ovalbumin, ribonuclease B, invertase (yeast) throglobulin (porcine) also mentioned	[23]
N-Linked asialooligosaccharides (transferrin, fetuin, immunoglobulin G, α_1-acid glyco protein) as PA derivatives	Fused silica (30 cm × 50 μm I.D.)	200 m*M* borate buffer (pH 10.5)	12	FL at 390 nm (excited at 320 nm)	15	Oligosaccharides from ovalbumin, ribonuclease B, invertase (yeast), *throglobulin* (porcine) also mentioned	[23]

CAPS = (cyclohexylamino)propanesulfonic acid; SDS = sodium dodecyl sulfate; CBQCA = 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; FL = fluorescence; HPC = hydroxypropylcellulose; PA = pyridylamino; rhEPO = recombinant human urinary erythropoietin; tPA = tissue plasminogen activator.

[a] Capillary dimensions are expressed as effective length (length between the inlet and the detector window).

5. Conclusion

High-performance capillary electrophoresis of glycoproteins has been reviewed, focusing on the carbohydrate moiety. The analytical conditions mentioned in the present review are summarized in Table 3 for convenience.

In the earlier work on glycoproteins, the separation of glycoforms of glycoproteins employing their intrinsic charges was of most interest. Capillary electrophoresis solved this problem well, permitting the peak resolution of glycoforms from each other. Capillary electrophoresis was also a valuable tool for the confirmation of microheterogeneity of the glycopeptide structure around glycosylation sites by analysing tryptic digests of glycoproteins. The mass sensitivity in the direct analysis of glycoprotein-derived oligosaccharides seems better than that in HPLC, because the amount introduced is much smaller. However, much higher concentrations of sample solutions are required.

Precapillary derivatization of released oligosaccharides allowed much more sensitive detection, although the suitable separation mode varied depending on the ionic properties of the derivatives. Various methods for derivatization, including the PA, ANTS and CBQCA methods, have been briefly outlined. The CBQCA method is sensitive when the separated derivatives are detected by laser-induced fluorescence. Each method has its own advantages, but also drawbacks.

The manner of expressing migration data is important. The relative mobility with respect to an appropriate standard is highly reproducible and independent of the surroundings, including variation of electroosmotic flow. The assembly of such data on various carbohydrate chains in a two-dimensional fashion will make it possible to identify and quantify carbohydrate chains with much greater reliability than by high-performance liquid chromatography with gradient elution.

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