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Protocols in Biotechnology

Analysis of Carbohydrates in Glycoproteins by High-Performance Liquid Chromatography and High-Performance Capillary Electrophoresis

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ABSTRACT

We describe two methods for the analysis of oligosaccharide chains in glycoproteins by high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE). *O*- and *N*-glycosidically linked oligosaccharides released from glycoproteins can be identified as their borohydride-reduced forms by anion-exchange HPLC with pulsed amperometric detection. *N*-Glycosidically linked oligosaccharides can also be analyzed as 2-aminopyridine derivatives by HPCE in direct zone electrophoresis mode in an acidic phosphate buffer and zone electrophoresis mode as borate complexes in an alkaline buffer.

We also present a convenient procedure for the analysis of the constituent monosaccharides of these oligosaccharides chains by HPLC based on reversed-phase partition mode as 1-phenyl-3-methyl-5-pyrazolone derivatives.

Index Entries: Glycoproteins; oligosaccharide chains; high-performance anion exchange chromatography; pulsed amperometric detection; 2-aminopyridine; capillary zone electrophoresis; borate complexes; monosaccharides; reversed-phase high-performance liquid chromatography; 1-phenyl-3-methyl-5-pyrazolone.

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INTRODUCTION

Analysis of Oligosaccharide Chains in Glycoproteins

Analysis of the diverse species of carbohydrate chains released from glycoproteins inevitably requires high-resolution methods. In addition, because of the relative nonvolatility even after derivatization, high-performance liquid chromatography (HPLC), rather than gas chromatography, becomes the technique of choice. Various separation modes are employed for HPLC, such as normal phase partition (on amine-bonded silica) and ligand exchange (on sulfonated styrenedivinylbenzene copolymers). Reversed-phase partition (RP-HPLC) as reductively pyridyl aminated derivatives or condensation products with 1-phenyl-3-methyl-5-pyrazolone (*1*) is also useful.

Another convenient procedure has been developed for direct analysis of carbohydrate chains by anion-exchange mode of HPLC (AE-HPLC) with the pulsed amperometric detection. We present here some applications of HPLC based on this mode to a few *N*- and *O*-glycosidically bound carbohydrates, using thyroglobulin and bovine submaxillary mucin as glycoprotein models (*2*).

High-performance capillary electrophoresis (HPCE) is a merging method for separation, and commercial automated apparatus have just become available. This article also describes a series of HPCE procedures for analysis of oligosaccharide derivatives using ovalbumin as a model glycoprotein.

Analysis of Constituent Monosaccharides

Analysis of constituent monosaccharides in glycoproteins offers fundamental information on the type of carbohydrate chain and the number of chains per molecule. Prior to analysis of constituent monosaccharides, they must be released by acid hydrolysis or enzyme digestion. Excellent reviews have been recently published by two groups (*3,4*). Procedures for acid hydrolysis to release neutral monosaccharides and amino sugars described in this article are the standard procedures employed in our laboratory, and are similar to those described by Hardy (*3*).

Neutral monosaccharides, including galactose, mannose, and fucose, which are ubiquitous in glycoproteins, can be quantitatively released with 2*M* trifluoroacetic acid in nitrogen atmosphere at 100°C. On the other hand, amino sugars should be hydrolyzed in 4*M* hydrochloric acid in nitrogen atmosphere. The *N*-acetyl group is incidentally removed under these conditions; hence, re-*N*-acetylation is necessary prior to analysis. This can be easily accomplished by adding acetic anhydride in an aqueous sodium bicarbonate solution. Hydrolysis in 4*M* hydrochloric acid causes serious degradation of neutral monosaccharides. For these reasons, a sample has to be hydrolyzed under two different conditions mentioned

earlier, one for neutral sugars and the other for amino sugars. Sialic acids must be hydrolyzed under much milder conditions using dilute acetic or sulfuric acid at a lower temperature, since they are labile to acid. Determination of sialic acid content is usually performed separately, for instance, by colorimetry (*5*); direct HPLC analysis of neuraminic acids by using malononitrile as a postcolumn labeling reagent is an alternative method (*6*).

Various methods for analysis of released neutral monosaccharides and amino sugars have been reported by many groups. We describe herein an example based on RP-HPLC with UV detection after precolumn labeling with 1-phenyl-3-methyl-5-pyrazolone (PMP, ref. *1*) developed in our laboratory.

MATERIALS

Analysis of Oligosaccharide Chains in Glycoproteins

AE-HPLC

1. *N*-glycosidically bound oligosaccharides (*see* Note 1):
   a. Sample: borohydride-reduced oligosaccharide mixture from porcine thyroglobulin (5 mg): prepared from porcine thyroids by hydrozinolysis, re-*N*-acetylation and borohydride reduction according to the method of Ui and Tarutani (*7*).
   b. Sodium hydroxide (analytical grade) (*see* Note 2).
   c. Sodium acetate.
   d. Water: double-distilled and filtered through a membrane filter (0.2 μm).
   e. The HPLC apparatus with gradient elution: set up from a Dionex pump capable of programmable gradient elution, a Rheodyne 7125 sample injector equipped with a Tefzole seal with a 20-μL loop, a Dionex HPIC-AS-6 analytical column (4 mm id, 25 cm) with an HPIC-AS-6 guard column (4 mm id, 5 cm), and a Dionex triple-pulsed amperometric detector equipped with a gold electrode (PAD II).
2. *O*-glycosidically bound oligosaccharides:
   a. Bovine submaxillary mucin (1 mg): obtained from fresh bovine submaxillary glands by the method of Tettamanti and Pigman (*8*).
   b. Sodium hydroxide (0.05*M*) containing sodium borohydride to a concentration of 1*M*.
   c. Acetic acid.
   d. A column of Amberlite CG-120 (H^+ form, 10 mL).
   e. The HPLC system with isocratic elution: built up from a Hitachi 655 dual-plunger pump. Other equipment is the same as that used in HPLC with gradient elution described earlier.

Analysis by HPCE as Reductively Pyridylaminated Derivatives

1. Direct electrophoresis (CZE-HPCE) in an acidic carrier.
   a. The HPCE system: a Bio-Rad HPE 100 apparatus.
   b. Carrier: 100 m*M* phosphate buffer (pH 2.5) (*see* Note 3).
   c. A capillary tube: a cassette-mounting polyacrylamide-coated tube (25 μm id, 20 cm) designed for this apparatus; available from Bio-Rad (Hercules, CA).
2. Zone electrophoresis as borate complexes (ZEBC-HPCE).
   a. The HPCE system (*9*): a handmade apparatus constructed from a high-voltage power supply (Matsusada Precision Devices, Model HEL-30-13), a pair of PTFE electrode vessels (1-mL vol), a Hitachi 650-10 LC fluoromonitor, and an SIC Chromatocorder 12 (*see* Note 4).
   b. A fused silica capillary tube (Scientific Glass Engineering, Melbourne, Australia; 50 μm id, 95 cm) (*see* Note 5).
   c. Borate buffer as carrier: prepared by adding sodium hydroxide pellets to a solution (200 m*M*) of boric acid, pH being adjusted to 10.5.

Analysis of Constituent Monosaccharides

Hydrolysis

1. Trifluoroacetic acid.
2. Hydrochloric acid.
3. Amberlite CG-120 (H^+ form, 3 mL).
4. Acetic anhydride.
5. A centrifugal concentrator.
6. A block heater (70°C, 100°C).
7. A polypropylene tube with a screw cap (1.5 mL).
8. A glass tube (7 mm id, 12 cm).

Precolumn Conversion to PMP Derivatives

1. 1-Phenyl-3-methyl-5-pyrazolone: available from Kishida (Doshomachi, Osaka). The same reagent is also available from Aldrich (Milwaukee, WI) in the name of 3-methyl-1-phenyl-5-pyrazolone. (Note 6).
2. An aqueous sodium hydroxide solution: prepared by diluting standardized 1.0*N* sodium hydroxide to 0.3*M* concentration.
3. Diluted hydrochloric acid: prepared by dilution of standardized 1.0*N* hydrochloric acid to 0.3*M* concentration.
4. Chloroform.
5. A centrifugal concentrator.
6. A polypropylene tube with a screw cap (1.5 mL).

Analysis of PMP Derivatives in RP-HPLC

1. The HPLC apparatus: set up from a Hitachi 655A-12 dual-plunger pump, a Rheodyne 7125 injector with a 20-μL loop, a Shimadzu SPD-6A-UV detector, and a Hitachi D-2000 data processor. Volume of detector cell, 8 μL; wavelength for detection, 245 nm; column (4.6 mm id, 25 cm), Capcell Pak C-18 (Shiseido, Ginza, Chuo-ku, Tokyo); eluent, phosphate buffer (70 m*M*, pH 6.8) containing acetonitrile to a concentration of 18%; flow rate, 1.0 mL/min.
2. Acetonitrile (HPLC grade).
3. Phosphate buffer (70 m*M*, pH 6.8): Prepare the buffer by dissolution of KH_2PO_4 (18.15 g) and $Na_2HPO_4 \cdot 12H_2O$ (47.75 g) in 4.0 L of double-distilled water followed by filtration through a membrane filter (0.2 μm).

METHODS

Analysis of Oligosaccharide Chains in Glycoproteins

AE-HPLC

1. *N*-glycosidically bound oligosaccharides:
   a. Dissolve a sample of borohydride-reduced oligosaccharide mixture from thryoglobulin (5 mg) in distilled water (1 mL).
   b. Inject an aliquot (20 μL) to the HPLC column.
   c. Elute the column with eluent prepared by continuously adding 0.1*M* sodium hydroxide containing sodium acetate (0.5*M*) into a solution of 0.1*M* sodium hydroxide in linear gradient mode for 200 min at a flow rate of 1.0 mL/min. Set the applied potentials and pulse durations of the detector as follows: $E_1 = +0.045$ V (0.6 s), $E_2 = +0.60$ V (0.12 s), $E_3 = -0.80$ V (0.42 s). The elution profile of oligosaccharide mixtures from porcine thyroglobulin is shown in Fig. 1. Each peak is assigned to the structure given in Scheme 1 by comparing the elution volume to that of the authentic specimen. It is indicated that high mannose-type oligosaccharides are eluted with low alkali concentrations. Increase of the mannose residue results in more retardation. Sialooligosaccharides have longer retention times, and the retardation becomes larger as the number of sialic acid residues increased (*see* Note 7).

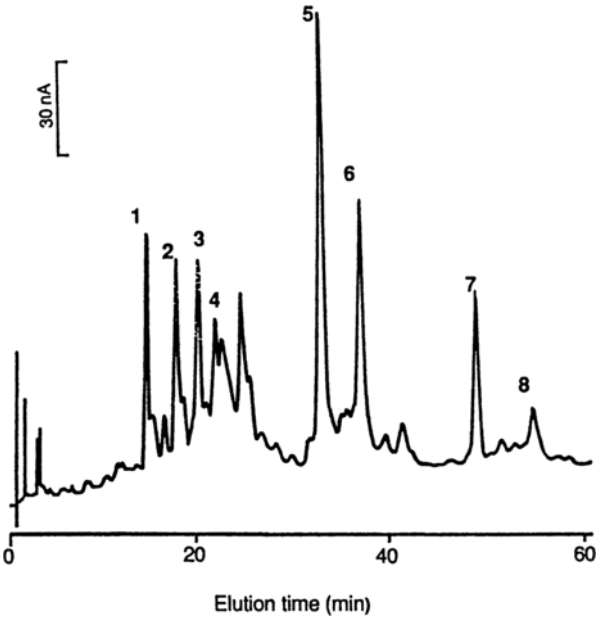
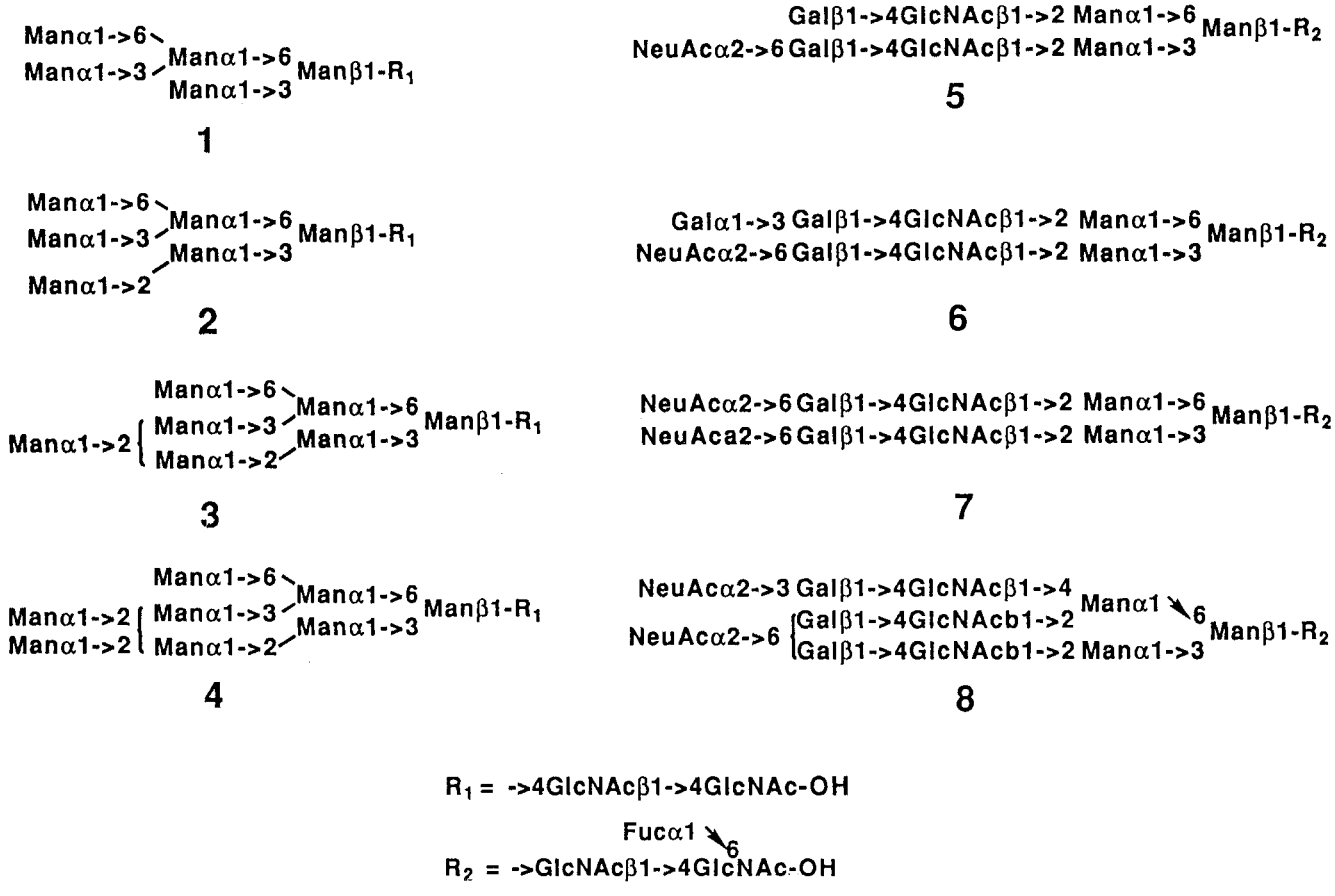


Fig. 1. Analysis of borohydride-reduced oligosaccharides obtained from porcine thyroglobulin. Column, Dionex HPIC As-6 (4 mm id, 25 cm); flow rate, 1.0 mL/min; detection, pulse amperometry on a gold electrode ($E_1 = +0.045$ V, 0.6 s; $E_2 = +0.60$ V, 0.12 s; $E_3 = -0.80$ V, 0.42 s); elution, linear gradient (0.1*M* sodium hydroxide–0.1*M* sodium hydroxide containing sodium acetate, 0.5*M*, in 200 min); sample scale, 50 μg as glycoprotein. Peak numbers are corresponding to the compound numbers in Scheme 1. (Reproduced from ref. 2 with permission.)

2. *O*-glycosidically bound oligosaccharides:
   a. Release the *O*-glycosidically linked oligosaccharides by incubation of bovine submaxillary mucin (1 mg) in 0.05*M* sodium hydroxide (1 mL) containing sodium borohydride to a concentration of 1*M*, for 24 h at 45°C (*see* Note 8).
   b. Add acetic acid into the mixture carefully to decompose excess borohydride.
   c. Pass the reaction mixture through a column of Amberlite CG-120 (H^+ form, 10 mL), and wash the column with water (50 mL). Evaporate the combined eluate and washing fluids. Dissolve the residue in a small volume of methanol, and evaporate the solution. Repeat the procedure several times to remove boric acid as the volatile borate ester.
   d. Dissolve the final residue in water (1 mL), and inject an aliquot (20 μL) into the HPLC column.
   e. Elute the column with 0.3*M* sodium hydroxide using a Hitachi 655 dual-plunger pump for isocratic elution under similar conditions to those described in analysis of *N*-glycosidically bound oligosaccharides (*see* Note 9).



Scheme 1. Borohydride-reduced oligosaccharides from porcine thryoglobulin.

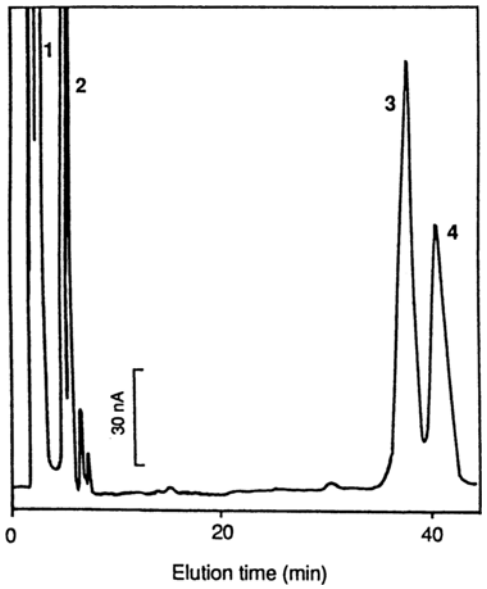
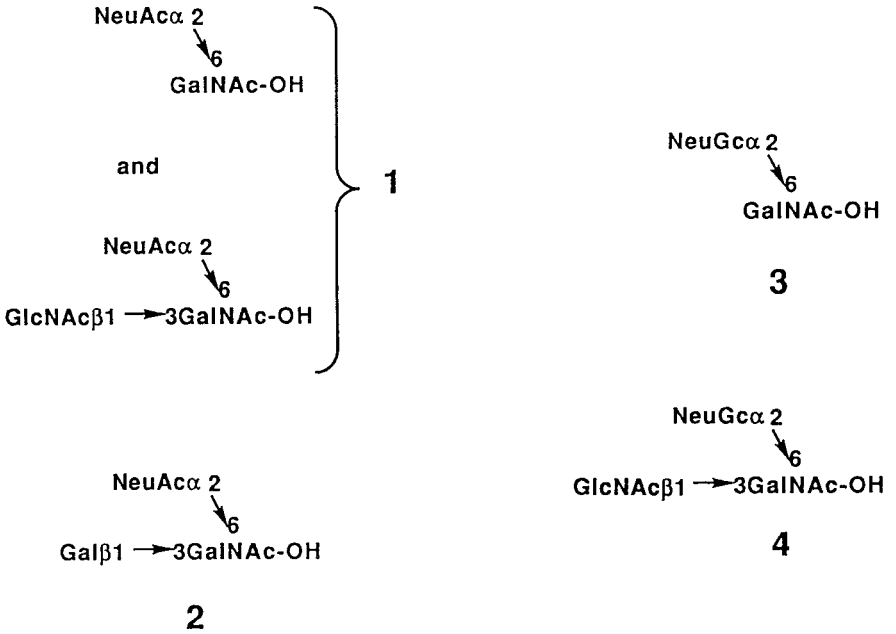


Fig. 2. Analysis of borohydride-reduced oligosaccharides obtained from bovine submaxillary mucin. Eluent, 0.3*M* sodium hydroxide. Other analytical conditions are the same as those described in Fig. 1. Peak numbers are corresponding to the compound numbers in Scheme 2. (Reproduced from ref. 2 with permission.)

Figure 2 shows the profile of isocratic elution of the mixture of *O*-glycosidically bound oligosaccharides obtained from bovine submaxillary mucin. The peaks are assigned to the structures in Scheme 2 by isolation of the compounds giving individual peaks, followed by examination of the isolated compounds by proton NMR spectroscopy at 500 MHz and fast atom bombardment mass spectrometry. Peaks are well separated from each other, and oligosaccharides containing *N*-acetylneuraminic acid (peaks 1 and 2) give much shorter elution times than those containing *N*-glycolylneuraminic acid (peaks 3 and 4).

HPCE (see Note 10)

1. CZE-HPCE in an acidic carrier:
   a. Introduce a sample solution (10 μL) to the inlet cavity with a microsyringe (*see* Note 11).
   b. Transfer pyridylaminated derivaties of carbohydrates in the cavity to the capillary tube by the electromigration method with application of a potential of 8 kV for 30 s.



Scheme 2. Borohydride-reduced oligosaccharides from bovine submaxillary mucin.

c. Perform HPCE at the potential of 15 kV (*see* Note 12). Figure 3 shows separation of *N*-glycosidically bound oligosaccharides based on their molecular sizes. Five major peaks are assignable to hepta-, octa-, nona-, deca-, and undecasaccharide derivatives, respectively, by comparison with isomaltooligosaccharide derivatives. Oligosaccharides having the identical degree of polymerizations (d.p.s) cannot be separated from each other by this mode.

2. ZEBC-HPCE:
   a. Introduce a sample solution to a capillary tube by the hydrodynamic method under conditions of a 10-cm rise for 10 s.
   b. The electrophoretogram is recorded at 395 nm (emission) with irradiation at 316 nm (excitation). Figure 4 shows an electrophoretogram of oligosaccharide derivatives. The first (11.7 min) and the last (24.3 min) peaks are owing to the remaining reagent (AP) and reductively pyridylaminated glucose (G-AP), respectively, added as an internal reference. The nine peaks 1–9 in the range of 14–17 min are assigned to the oligosaccharide derivatives. Scheme 3 gives proposed assignment based on comparison of their relative mobilities to the G-AP with those of authentic specimens.

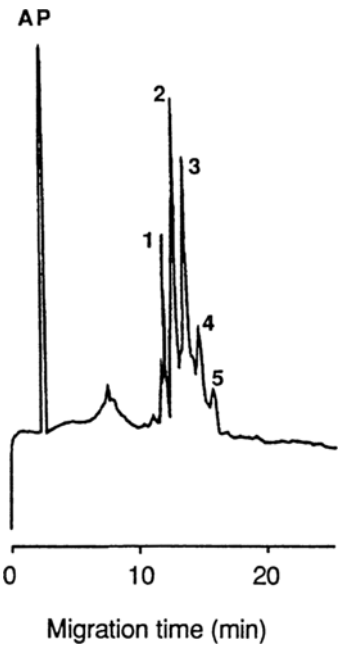


Fig. 3. Analysis of reductively pyridylaminated oligosaccharides derived from ovalbumin by ZE-HPCE. Capillary, fused silica coated with polyacrylamide (Bio-Rad, 25 μm id, 20 cm); carrier, 100 m*M* phosphate buffer (pH 2.5); applied voltage, 8 kV; detection, UV absorption at 240 nm. AP, 2-aminopyridine (excess reagent). Peaks 1, 2, 3, 4, and 5 are assignable to the derivatives of hepta-, octa-, nona-, deca-, and undecasaccharides, respectively, in Scheme 3. (Reproduced from ref. *9* with permission.)

Analysis of Constituent Monosaccharides

Hydrolysis of Glycoprotein Samples

1. Neutral monosaccharides:
   a. Add 2*M* trifluoroacetic acid (200 μL) to a glycoprotein sample (100–500 μg) in a glass tube (7 mm id, 120 mm).
   b. Flush the solution with nitrogen, and seal the tube.
   c. Keep the tube in the block heater at 100°C for 4 h.
   d. Cool the tube, and open it.
   e. Transfer the solution and the washing fluid (300 μL) to a polypropylene tube.
   f. Evaporate the solution to dryness (*see* Note 13).
2. Amino sugars:
   a. Add 4*M* hydrochloric acid (200 μL) to a glycoprotein sample (100–500 μg) in a glass tube (7 mm id, 120 cm).
   b. Flush the solution with nitrogen, and seal the tube.

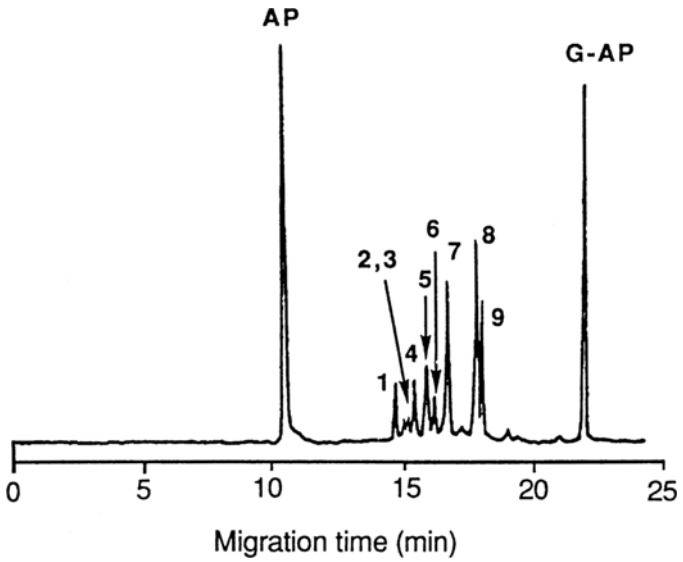


Fig. 4. Analysis of reductively pyridlyaminated oligosaccharides derived from ovalbumin by ZEBC-HPCE. Capillary, fused silica (Scientific Glass Engineering, 50 μm id, 95 cm); carrier, 200 m*M* borate buffer (pH 10.5); applied voltage, 20 kV; detection, fluorescence at 395 nm (irradiated at 316 nm). AP, 2-aminopyridine; G-AP, reductively pyridylaminated glucose (internal standard). Peak numbers are corresponding to the compound numbers in Scheme 3, though the assignment is tentative. Peaks 2 and 3 could not be assigned. (Reproduced from ref. *9* with permission).

c. Keep the tube in the block heater at 100°C for 4 h.
d. Cool the tube, and open it.
e. Transfer the solution and the washing fluid (300 μL) to a polypropylene tube.
f. Evaporate the solution to dryness.
g. Add an aqueous saturated solution of sodium bicarbonate (500 μL) and acetic anhydride (20 μL).
h. Keep the solution overnight in the refrigerator.
i. Pass the solution through a column of Amberlite CG-120 (H^+ form, 3 mL), and wash the column with water (20 mL).
j. Evaporate the combined eluate and the washing fluid to dryness.
k. Add methanol (2 mL) to the residue, and evaporate the mixture to dryness. Repeat the procedure several times.
l. Transfer the residue to a polypropylene tube with a small volume of water.
m. Evaporate the solution to dryness.

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                    Manα1↘
          Manα1→3↗       6
               GlcNAcß1→4Manß1→4GlcNAcß1→4GlcNAc-R
1                         3
                    Manα1↗
        GlcNAcß1→2


                    Manα1↘
            Manα1→3       6
               GlcNAcß1→4Manß1→4GlcNAcß1→4GlcNAc-R
4       GlcNAcß1→4        3
                    Manα1↗
        GlcNAcß1→2


                         Manα1↘
                Manα1→3       6
5                  GlcNAcß1→4Manß1→4GlcNAcß1→4GlcNAc-R
    Galß1→4GlcNAcß1→4         3
                         Manα1↗
           GlcNAcß1→2

           Manα1→6
                    Manα1↘
           Manα1→3       6
6               GlcNAcß1→4Manß1→4GlcNAcß1→4GlcNAc-R
        GlcNAcß1→4        3
                    Manα1↗
        GlcNAcß1→2
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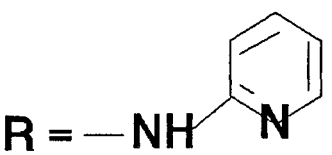


Scheme 3. Oligosaccharide derivatives from ovalbumin.

Precolumn Conversion to PMP Derivatives and Analysis of the Derivatives

1. Into a hydrolyzate obtained by one of the procedures described in Hydrolysis of Glycoprotein Samples or a mixture of standard aldoses (*see* Note 14), add a 0.3*M* aqueous solution of sodium hydroxide (50 μL) and a 0.5*M* methanolic solution (50 μL) of PMP.
2. Stand the mixture for 30 min at 70°C (*see* Note 15).
3. Cool the mixture to room temperature, and add an equivalent volume of 0.3*M* hydrochloric acid (50 μL) for neutralization.

Scheme 3. *continued*.

4. Evaporate the solution to dryness. Add water (200 μL) and chloroform (200 μL) to the residue, and shake the mixture vigorously.
5. Discard the chloroform layer, and repeat extraction with chloroform to remove the excess reagent from the aqueous layer (*see* Note 16).
6. Evaporate the aqueous layer to dryness, dissolve the residue in a small vol (200 μL) of eluent for HPLC, and inject an aliquot (20 μL) onto the HPLC column.

Elution profiles obtained from the hydrolysates of some glycoprotein samples are shown in Fig. 5, and the monosaccharide contents are summarized in Table 1. The accuracy and precision of this method are sufficiently high when rhamnose is used as the internal standard. For example, the calibration

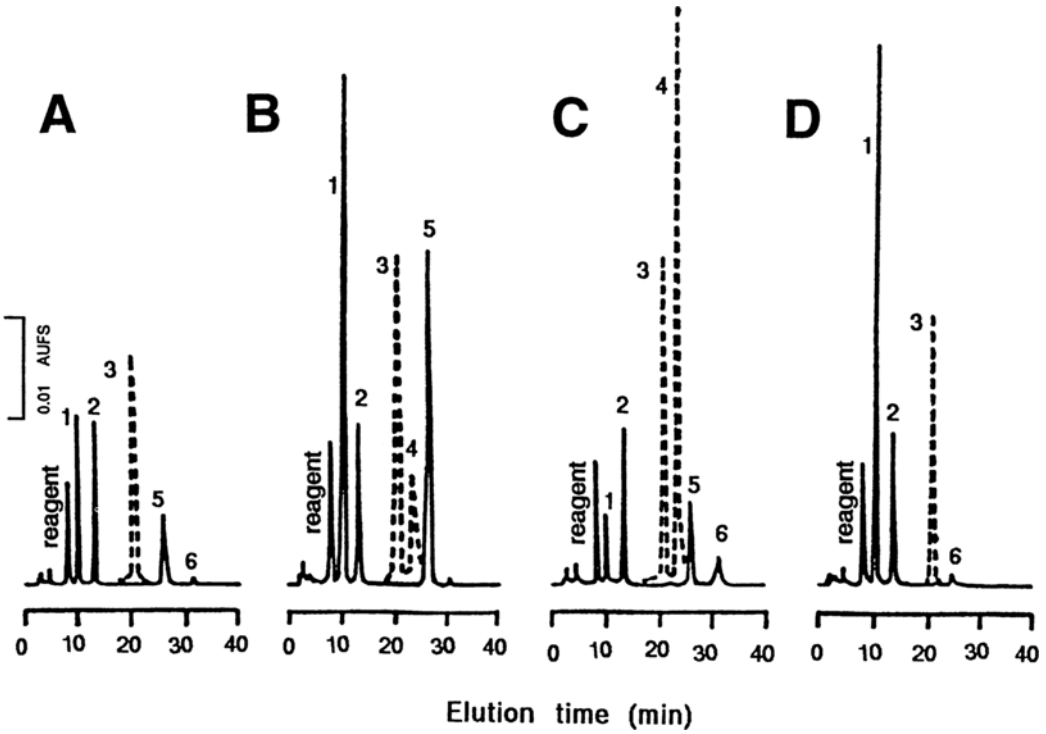


Fig. 5. Analysis of constituent monosaccharides of human serum transferrin (a), calf serum fetuin (b), bovine submaxillary mucin (c), and hen egg ovalbumin (d). Sample amount injected, 20 μg each as protein. Solid and dotted lines represent the results obtained with the trifluoroacetic acid and hydrochloric acid hydrolysates respectively. Column, Capcell Pak C-18 (4.6 mm, id, 25 cm); eluent, 0.1*M* phosphate buffer (pH 7.0) containing acetonitrile (18%); flow rate, 1.0 mL/min; sample amount, 20 μg each as protein. Peak assigment: 1, mannose; 2, rhamnose (internal standard); 3, *N*-acetylglucosamine; 4, *N*-acetylgalactosamine; 5, galactose; 6, fucose. (Reproduced from ref. *1* with permission.)

curve of glucose shows excellent linearity in the range of 5–1000 pmol. The lower limit of detection at the signal-to-noise ratio of 5 was ca. 1 pmol. Repeated determination ($n=7$) of glucose-PMP gave SD of 1.9, 1.1, and 2.3% at the 10-, 200-, and 1000-pmol levels, respectively.

NOTES

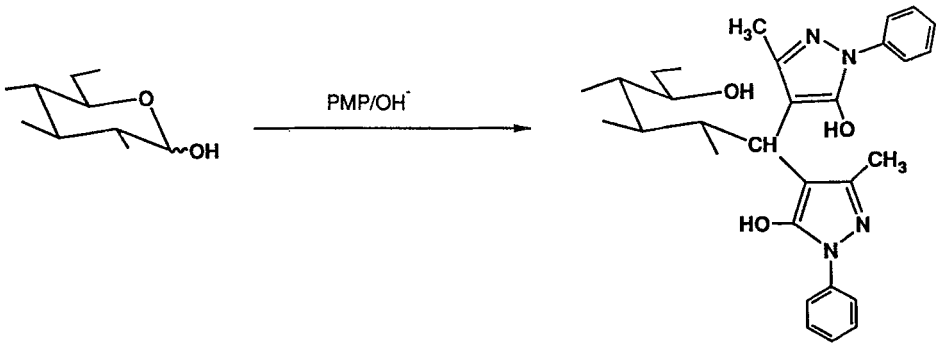
1. Readers should refer to ref. (*12*) for release of *N*-glycosidically bound oligosaccharides by a combination of hydrazinolysis, re-*N*-acetylation, and borohydride reduction.

Table 1
Determination of Component Monosaccharides in Glycoproteins[a]
(Reproduced from ref. *1* with permission.)

Glycoprotein	Content, w/w%				
	Fucose	Mannose	Galactose	Glucosamine	Galactosamine
Transferrin[b] (human serum)	0.040 (0.031)	1.08 (1.11)	1.00 (0.86)	2.52 (1.79)	0.053 (–)
Fetuin[b] (calf)	0.033 (0.027)	2.45 (2.73)	3.49 (4.59)	2.62 (5.30)	– (0.674)
Thyroglobulin[c] (bovine thyroid)	0.38 (0.37)	2.26 (2.09)	1.49 (1.20)	5.55 (2.95)	1.38 (1.38)
Albumin[b] (hen egg)	– (–)	2.80 (2.40)	0.12 (0.15)	2.73 (1.28)	– (–)
Mucin[b] (bovine-submaxillary)	0.53 (0.95)	0.16 (0.21)	1.24 (1.52)	6.22 (6.92)	14.68 (16.80)

[a]The numbers in parentheses are reported values.
[b]Ref. *10*.
[c]Ref. *11*.

2. Make aqueous 50% sodium hydroxide as the stock solution. The eluent for AE-HPLC is prepared by appropriate dilution of the stock solution. Surface and bottom layers of the stock solution should not be used to avoid contamination with sodium carbonate.
3. An aqueous solution of sodium hydroxide (100 m*M*) was added to 100 m*M* phosphoric acid, and pH was adjusted to 2.5.
4. The fluoromonitor is slightly modified. A quartz convex lens is placed between the light source and the cell holder so as to focus the irradiation light on a capillary tube.
5. A 5-mm portion of the polyimide coating is removed by burining at a distance of 30 cm from the outlet of the tube, where the excitation light irradiates.
6. The reagent is crystallized from hot methanol before use. A 0.5*M* solution is prepared by dissolution of the reagent (79 mg) in methanol (1.0 mL). The solution stored in a refrigerator for several months can be used.
7. This system employing a pulsed amperometric detector on a gold electrode is sensitive to not only carbohydrates, but also amino acids. It is weakly positive to proteins, such as albumin.



Scheme 4. Conversion of monosaccharides to PMP derivatives.

The problem of interference by these substances can be simply solved by clean up of the sample solutions on a small column of Sephadex G-25 column (1.0 cm id, 30 cm) with water as eluent.

8. Under these conditions, removal of the *N*-acyl groups on the sialic acid and hexosamine residues is negligible, whereas *O*-acyl groups are completely removed.
9. A programmable gradient pump may also be used for separation of *O*-glycosidically linked oligosaccharides.
10. Precolumn labeling of reducing oligosaccharides with 2-aminopyridine by reductive amination should be referred to Chapter 6 in ref. (*12*).
11. Oligosaccharide mixtures are prepared from ovalbumin (10 mg). Hydrazinolysis, re-*N*-acetylation, and pyridylamination (*see* Chapters 5 and 6 in ref. [*12*]) affords a pyridylaminated-oligosaccharide mixture. The final residue is dissolved in water (200 μL).
12. This mode allows separation based on molecular size, because all oligosaccharide derivatives have commonly one amino group and, accordingly, the same electric charge. The smaller species migrate faster, giving earlier peaks.
13. The dry-up procedure following hydrolysis seems to be important for reproducible determination and should be completed in a short period by using an efficient evaporator, such as a centrifugal concentrator.
14. Each monosaccharide in a range of 0.2 nmol–1 μmol can be determined.
15. Under these conditions, each monosaccharide molecule is condensed with two molecules of PMP, as indicated in Scheme 4.
16. Further repetition of extraction causes loss of 6-deoxyhexose derivatives.

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