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ANALYSIS OF GLYCOPROTEIN-DERIVED OLIGOSACCHARIDES BY HIGH-pH ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The technique of high-pH anion-exchange chromatography with pulsed amperometric detection has recently been shown to be a powerful method for resolving closely related oligosaccharides [M. R. Hardy and R. R. Townsend, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 3289–3293]. This report describes separations involving a total of nineteen different high-mannose, hybrid and complex-type oligosaccharides isolated after peptide:N-glycosidase F (PNGase F) or *endo*-β-N-acetylglucosaminidase H digestion of glycoproteins. Separations were carried out at a constant base concentration (0.1 *M* NaOH) using linear gradients from 0 to 0.2 *M* sodium acetate. The applicability of this chromatography for profiling the N-linked oligosaccharides of glycoproteins was demonstrated by generating "oligosaccharide maps" of PNGase F-liberated oligosaccharides from recombinant human tissue plasminogen activator, ribonuclease *b*, human transferrin, and bovine fetuin. Methods for recovering salt-free oligosaccharides after this chromatography were also investigated. On-line ion suppression with an anionic micromembrane suppressor cartridge was found to be capable of effective desalting up to a total sodium ion concentration of 0.15–0.2 *M* at a flow-rate of 1 ml/min. After high-pH anion-exchange chromatography with ion suppression, collected oligosaccharides were analyzed by fast-atom bombardment mass spectrometry after conversion to permethyl derivatives or after reductive amination with ρ-aminobenzoic acid ethyl ester.

INTRODUCTION

A significant recent development in carbohydrate analysis has been the application of high-pH anion-exchange chromatography (HPAEC) for monosaccharide analysis[1] and for the separation of oligosaccharides[2–4]. These separations take advantage of the fact that the hydroxyl groups of carbohydrates are weakly acidic[5] and, at pH values greater than 12, the resulting oxyanion derivatives can be separated by anion-exchange chromatography. When used in conjunction with pulsed amperometric detection[6] (PAD), HPAEC permits carbohydrate analysis to be carried out at the picomole level.

The use of HPAEC for separations of neutral oligosaccharides was described

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first by Hardy and Townsend[2], who reported on the separation of positional isomers of chemically synthesized, neutral oligosaccharides. They found that the separations were sensitive to molecular size, sugar composition and linkage of the monosaccharide units. In most cases, the elution order within a series of compounds was consistent either with the known hierarchy of acidities (hemiacetal > > 2-OH > > 6-OH > 3-OH > 4-OH) of the unsubstituted hydroxyl groups[5] or with the accessibility of the ionized groups to the functional groups of the anion exchange column. One limitation of that work was that many of the model oligosaccharides used were structurally related, but not identical, to typical N-linked oligosaccharides that would be obtained after digestion of a glycoprotein with peptide:N-glycosidase F (PNGase F) or *endo-β*-N-acetylglucosaminidase H (endo H). HPAEC has also been shown to be useful in the separation of sialylated and phosphorylated oligosaccharides[3] and in separating high-mannose and hybrid oligosaccharides[4].

In this paper we report on separations of a variety of N-linked oligosaccharides including high-mannose and hybrid oligosaccharides released by endo H, and di-, tri-, and tetra-antennary complex oligosaccharides released by PNGase F. We also describe methods for on-line desalting of oligosaccharides to permit subsequent identification by chemical or spectroscopic techniques.

EXPERIMENTAL

Materials

Recombinant human tissue plasminogen activator (rt-PA; Activase™) is the product of Genentech. Recombinant gp120 (rgp120) was produced by expression in Chinese hamster ovary (CHO) cells and immunoaffinity purified[7]. Ribonuclease *b*, human transferrin and bovine fetuin were purchased from Sigma. Endo H was obtained from Genzyme. PNGase F and α-fucosidase were purchased from Boehringer-Mannheim.

Model oligosaccharides

Compounds **1**–**4**, **15**, **18**, **19** and the sialylated counterparts of compounds **11**–**13** were isolated from CHO-expressed rt-PA. Their structures were confirmed by methylation analysis, fast-atom bombardment mass spectrometry (FAB-MS) and 500 MHz ^{1}H nuclear magnetic resonance (NMR) spectroscopy[8]. Compounds **5**–**7** were isolated from rgp120[9,10]. Compound **10** was purchased from Biocarb AB. Compound **17** was isolated from human transferrin. Compounds **8**, **9** and **11**–**13** were prepared by treatment of their sialylated counterparts with 0.02 *M* trifluoroacetic acid for 60 min at 80°C to remove sialic acid. Compounds **14** and **16** were prepared by treatment of compounds **13** and **15**, respectively, with α-fucosidase (40 mU) in 0.1 *M* sodium phosphate buffer (pH 4.5) at 37°C overnight.

Reduction and S-carboxymethylation of glycoproteins

Samples of rt-PA, rgp120, ribonuclease B, human transferrin and bovine fetuin were reduced and S-carboxymethylated prior to glycosidase digestion, as follows. Samples were dialyzed into 0.36 *M* Tris–HCl buffer (pH 8.6) containing 8 *M* urea and 3 m*M* EDTA. Dithiothreitol (DTT) was added to a final concentration of 10 m*M*. After 4 h at room temperature, iodoacetic acid was added to a final concentration of

25 m*M* and the samples were incubated at 25°C for 30 min in the dark. Samples were then quenched with excess DTT, dialyzed into 100 m*M* NH_4HCO_3 and lyophilized.

Digestion with PNGase F

Reduced and S-carboxymethylated glycoprotein samples to be digested (typically 0.5 mg) were reconstituted in 0.25 *M* sodium phosphate buffer (pH 8.6) containing 10 m*M* EDTA and 0.02% (w/v) sodium azide. PNGase F (3.2 units) was added, and the samples were incubated overnight at 37°C.

Digestion with endo H

Reduced and S-carboxymethylated glycoprotein samples (typically 0.5 mg) were reconstituted in 0.05 *M* sodium phosphate buffer (pH 6) containing 0.02% sodium azide. Endo H (0.05 units) was added and the samples were incubated overnight at 37°C.

Chromatography on Sephadex G-15

Glycosidase digestion mixtures were applied to a column (47 × 1 cm I.D.) of Sephadex G-15 equilibrated in 0.1 *M* NH_4HCO_3. The flow-rate was 0.6 ml/min. Carbohydrate-containing fractions were detected by the phenol–sulfuric acid assay[11].

C_{18} Sep-Pak

Glycosidase digestion mixtures were applied to C_{18} Sep-Pak cartridges that had been preconditioned with ethanol and acetonitrile[12] and then equilibrated in water. After sample application the cartridge was washed with 0.3 ml of water. Oligosaccharides were eluted with 0.5 ml of water followed by 1 ml of 40% (v/v) aqueous acetonitrile and then dried on a centrifugal evaporator.

Ethanol precipitation

At the termination of glycosidase digestion, samples were mixed with three volumes of ice-cold ethanol[13] and centrifuged at 11 600 *g* for 10 min at 4°C. Pellets were washed three times with ice-cold 75% ethanol. The combined supernatants were evaporated under a stream of nitrogen and then reconstituted in water for chromatography.

HPAEC conditions

HPAEC was carried out on a Dionex BioLC system (Dionex, Sunnyvale, CA, U.S.A.) equipped with a pellicular anion exchange column (AS6) and a pulsed amperometric detector (Dionex PAD-2). The detector potentials used were 0, +0.75 and −0.85 V with a duration of 180 ms at each potential. Detection was facilitated by the addition of 0.5 *M* NaOH (at a flow-rate of 0.75 ml/min) to the column effluent upstream of the detector cell. Semipreparative runs were made without any post-column addition of base, and the column effluent was passed through an anion micromembrane suppressor unit (Dionex AMMS) for on-line desalting. The regenerant solution for the AMMS unit was 0.03 *M* H_2SO_4 at a flow-rate of 6.5 ml/min.

The chromatograms shown here were generated using either of two gradient programs. For both gradient programs, solvent A was 0.1 *M* NaOH, solvent B was 0.1 *M* NaOH containing 0.5 *M* sodium acetate and the flow-rate was 1 ml/min.

Gradient program 1: the column was pre-equilibrated in 100% solvent A; after a 5-min hold, oligosaccharides were eluted with a linear gradient from 0 to 20% solvent B in 40 min. *Gradient program 2:* the column was pre-equilibrated in 5% solvent B; after a 2-min hold, oligosaccharides were eluted with a two-stage linear gradient from 5% to 15% solvent B in 5 min, and from 15% to 40% solvent B in 25 min.

Methylation analysis

Collected oligosaccharides were prereduced with sodium borohydride and permethylated using methyl iodide and methylsulfinyl-carbanion in methyl sulfoxide[12]. The permethylated oligosaccharides were recovered by chromatography on C_{18} Sep-Pak cartridges (Waters Assoc.).

Derivatization with p-aminobenzoic acid ethyl ester (ABEE)

Oligosaccharides collected after HPAEC with ion suppression were lyophilized and then reconstituted in 0.01 ml of water. To this was added 0.04 ml of a reagent consisting of 165 mg of ABEE, 35 mg of sodium cyanoborohydride, 0.041 ml of acetic acid and 0.35 ml of methanol[14,15]. Samples were incubated at 80°C for 30 min. After cooling, the samples were partitioned between water (0.3 ml) and chloroform (0.3 ml). The aqueous phase was subjected to reversed-phase high-performance liquid chromatography (HPLC) on a Novapak C_{18} column (15 cm × 4.6 mm I.D., Waters Assoc.) using an acetonitrile–water gradient system to recover the ABEE derivatives. The HPLC column effluent was monitored for absorbance at 254 nm.

FAB-MS

FAB mass spectra were acquired on a JEOL HX110HF/HX110HF tandem mass spectrometer operated in a normal two-sector mode. All other conditions were as previously described[8].

RESULTS AND DISCUSSION

Separation of neutral oligosaccharides

HPAEC provides efficient separation of neutral oligosaccharides of the types found attached to asparagine residues of glycoproteins. Chromatograms obtained by HPAE analysis of high-mannose oligosaccharides are shown in Fig. 1. Fig. 1A shows the separation of a mixture of $Man_5GlcNAc$ (**1**), $Man_6GlcNAc$ (**2**) and $Man_7GlcNAc$ (**3** and **4**) structures released by endo H digestion of CHO-expressed rt-PA. These oligosaccharides have been characterized by other methods[8] and shown to have the structures indicated in Table I. In particular, ^1H NMR analysis indicated that the $Man_7GlcNAc$ structures consisted of a mixture of two isomers. These isomers are partially resolved by HPAEC (retention time of 32 min, Fig. 1A). Fig. 1B shows the high-mannose oligosaccharides released by endo H digestion of CHO-expressed rgp120. In this case, the size distribution of the high-mannose oligosaccharides is in good agreement with that determined by gel-permeation chromatography of the high-mannose oligosaccharides released by hydrazine treatment of rgp120[9,10]. The rgp120 sample contains the $Man_5GlcNAc$ (**1**), $Man_6GlcNAc$ (**2**) and both of the $Man_7GlcNAc$ (**3** and **4**) structures found in the rt-PA sample. In addition, the rgp120 sample contains another major $Man_7GlcNAc$ peak (**5**), at 31.5 min, which is well

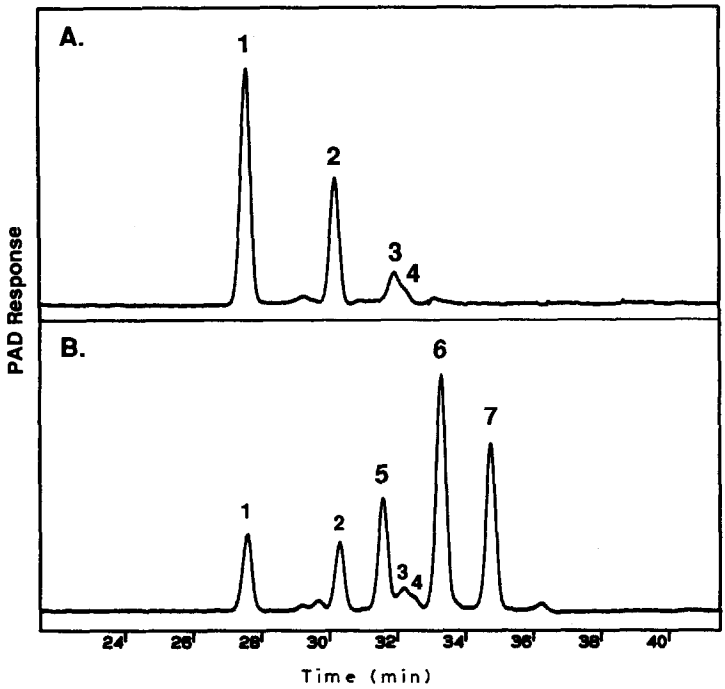


Fig. 1. Separation of high-mannose oligosaccharides. Oligosaccharides from (A) rt-PA and (B) rgp120 were liberated by treatment of the glycoproteins with endo H, recovered by ethanol precipitation, and analyzed by HPAEC using gradient program 1. Peak identifications are given in Table I.

resolved from the other two $Man_7GlcNAc$ isomers. Compounds **6** and **7** are $Man_8GlcNAc$ and $Man_9GlcNAc$, respectively.

The separation of three, closely related, asialo diantennary complex oligosaccharides is shown in Fig. 2. Compounds **8** and **9** differ in that the former structure has a fucose residue attached $\alpha(1\rightarrow6)$ to the reducing GlcNAc residue. The addition of fucose to an oligosaccharide has been shown with other model compounds[2] to cause a shift to earlier retention time in HPAEC. Compound **10**, which lacks the innermost GlcNAc residue of compound **9**, elutes latest of the three diantennary structures. The elution order of the three compounds in Fig. 2 is the opposite of that predicted for a purely size-based separation. Therefore, although the general trend in HPAEC is that oligosaccharides of a homologous series elute in order of increasing size, certain chain extensions can actually result in decreased retention presumably either by substitution of a particularly acidic position or by limiting accessibility of ionized groups to the functional groups of the column.

Separation of a more complex mixture of desialylated di-, tri- and tetraantennary complex oligosaccharides is shown in Fig. 3. Once again, the effect of fucosylation of the reducing GlcNAc residue is a shift to earlier retention time, as is illustrated by the diantennary oligosaccharides plus/minus fucose (peaks **8** and **9**) and the tetraantennary oligosaccharides plus/minus fucose (peaks **13** and **14**). Two branching isomers of fucosylated triantennary oligosaccharides were also resolved by this technique: the fucosylated 2,4-branched triantennary structure (compound **11**) eluted at

TABLE I

STRUCTURES OF THE MODEL OLIGOSACCHARIDES USED

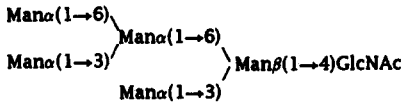
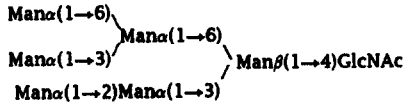
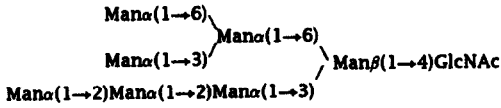
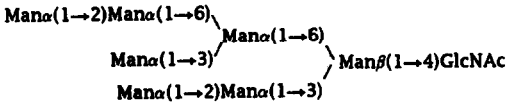
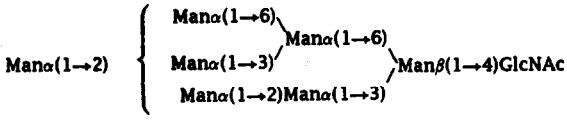
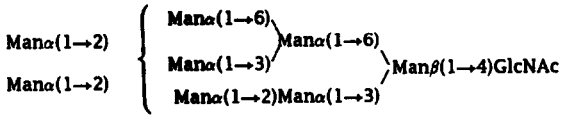
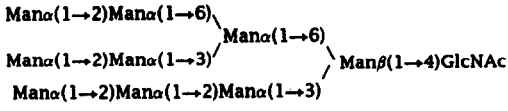
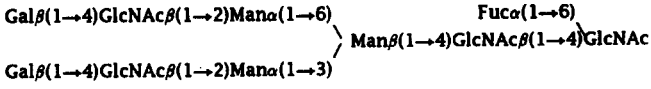
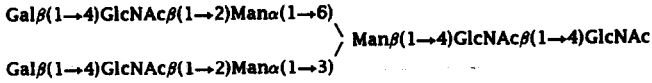
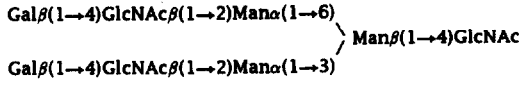
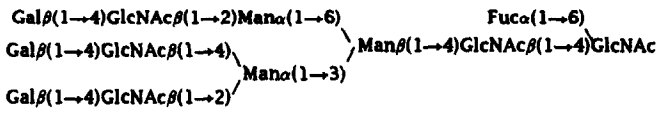
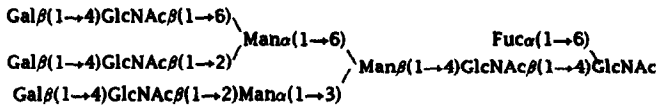
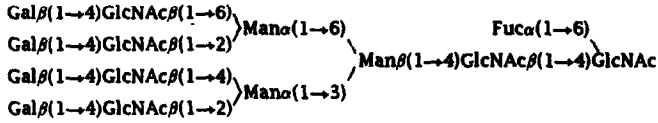
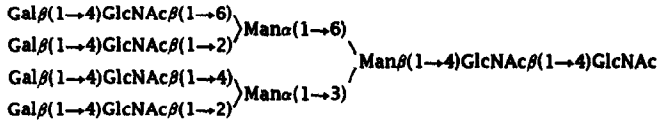
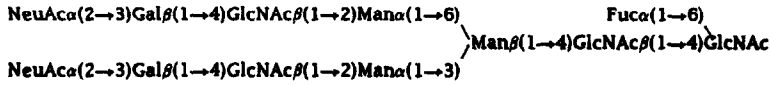
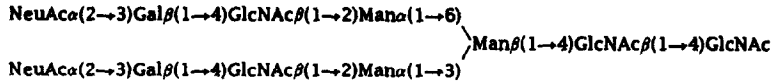
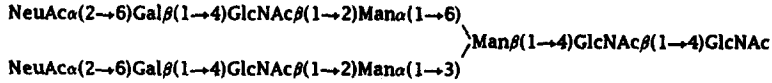
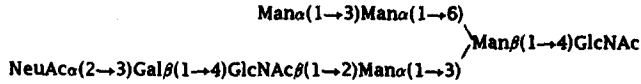
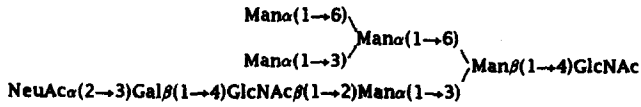
Compound	*Structure*
1	
2	
3	
4	
5[a]	
6[a]	
7	
8	
9	
10	

TABLE I *(continued)*

Compound	*Structure*
11	
12	
13	
14	
15	
16	
17	
18	
19	

[a] The attachment positions of the peripheral mannose residues have not been determined for these compounds.

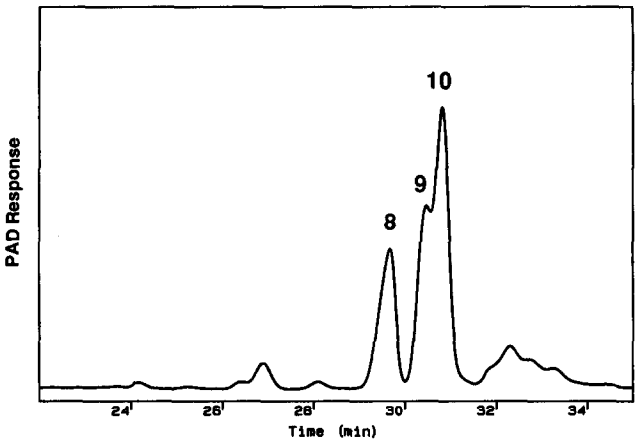


Fig. 2. Separation of three asialo-diantennary complex oligosaccharides. Chromatography was carried out using gradient program 1. Peak identifications are given in Table I.

29.5 min, and the fucosylated 2,6-branched triantennary oligosaccharide (compound **12**) eluted at 31 min. On the basis of the pK_a values of the unsubstituted hydroxyl groups of these two compounds, one would predict that the 2,4-branched oligosaccharide should elute after the 2,6-branched oligosaccharide (the 2,4-branched structure has an unsubstituted 6-OH, which is more acidic than the unsubstituted 4-OH of the 2,6-branched structure). The observed elution order is the opposite of that predicted solely on the basis of pK_a values, suggesting that the elution of these two compounds is dominated by the accessiblity of the ionized groups to the column matrix. These results are similar to those reported by Hardy and Townsend[2] for a pair of related compounds. Fig. 3 shows only the fucosylated forms of the two trian-

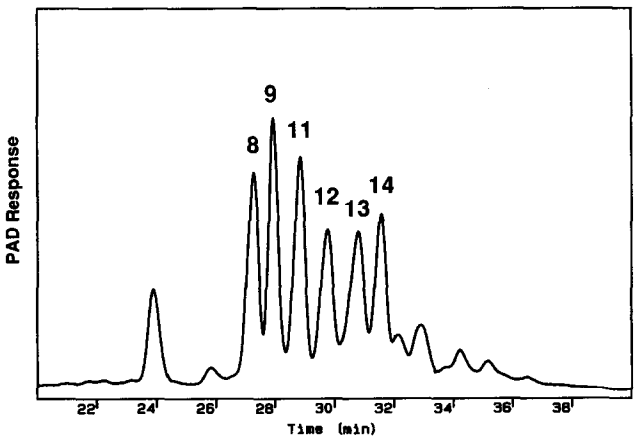


Fig. 3. Separation of asialo-complex oligosaccharides. Chromatography was carried out using gradient program 1. Numbered peaks are identified in Table I. Peaks not numbered are oligosaccharides present as contaminants in the model compounds.

tennary structures. Defucosylation of the triantennary structures caused the expected shift to later retention times. In the elution program used to generate Fig. 3, the defucosylated 2,4-branched triantennary oligosaccharide coelutes with the fucosylated 2,6-branched structure, and the defucosylated 2,6-branched oligosaccharide coelutes with the fucosylated tetraantennary structure (not shown).

Separation of sialylated oligosaccharides

Several experiments were performed to examine the ability of HPAEC to resolve structurally related sialylated oligosaccharides. As would be expected, the separation is dominated by the number of residues of sialic acid attached to an oligosaccharide. However, significant resolution was still attainable among related structures with the same formal charge. Fig. 4 shows that a fucosylated di-α(2→3)-sialylated diantennary oligosaccharide (compound **15**) is well resolved from the equivalent, non-fucosylated, di-α(2→3)-sialyl-diantennary oligosaccharide (compound **16**). The separation is also affected by the attachment position of sialic acid to galactose, as shown in Fig. 5. Both of the compounds in Fig. 5 are non-fucosylated diantennary complex oligosaccharides; they differ only in that compound **17** has sialic acid attached to the 6 position of galactose whereas the sialic acid in compound **16** is attached to the 3 position of galactose.

The separation of two (monosialyl) hybrid oligosaccharides is shown in Fig. 6. Compound **19** contains one more mannose residue than compound **18**, which causes a shift to later retention time. The elution characteristics of these hybrid oligosaccharides are similar to those reported previously for oligosaccharides isolated from ovalbumin[4].

"Mapping" of oligosaccharides released from glycoproteins

The speed, sensitivity and selectivity of HPAEC make it a promising technique for profiling the oligosaccharides of glycoproteins. This is illustrated in Fig. 7, which

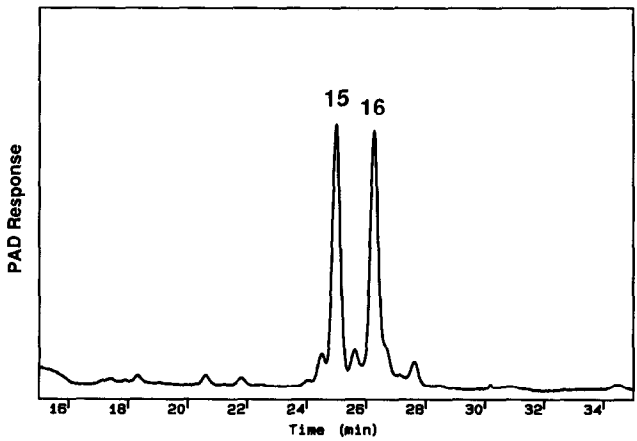


Fig. 4. Influence of fucosylation on the elution of sialylated diantennary oligosaccharides. Both compounds are di-α(2→3)-sialyl diantennary oligosaccharides. They differ by the presence (**15**) or absence (**16**) of fucose attached to the reducing GlcNAc residue. Elution was with gradient program 2.

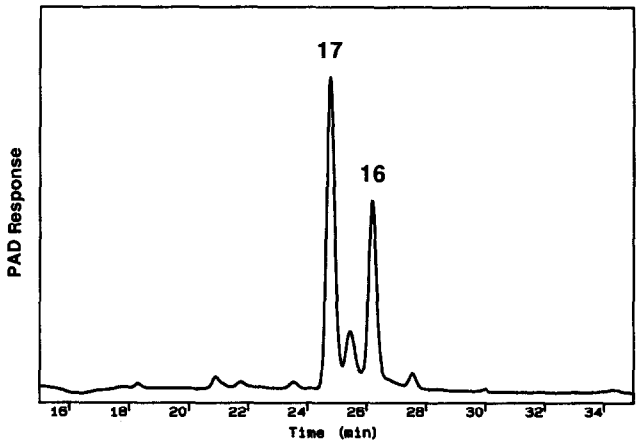


Fig. 5. Influence of sialylation position on the elution of diantennary oligosaccharides. Both compounds are disialyl diantennary oligosaccharides. Sialic acid is linked α(2→3) to galactose in compound **16** and α(2→6) to galactose in compound **17**. Elution was with gradient program 2.

shows the HPAEC profiles obtained after PNGase F treatment of four glycoproteins to release their N-linked oligosaccharides. As described above, the separations are dominated by the number of sialic acid residues on a particular oligosaccharide; as a result, the chromatograms contain regions where neutral, mono-, di-, tri- and tetrasialyl oligosaccharides elute (designated N, 1, 2, 3 and 4, respectively, in Fig. 7). For each glycoprotein, the HPAEC profile agrees well with what has been published previously on the N-linked oligosaccharides of the glycoprotein. rt-PA (Fig. 7A) is known to contain attached high-mannose, hybrid, and mono-, di-, tri- and tetrasialyl complex oligosaccharides[9]. Ribonuclease *b* (Fig. 7B) contains exclusively neutral (high-mannose) oligosaccharides[16]. The predominant oligosaccharide structure of

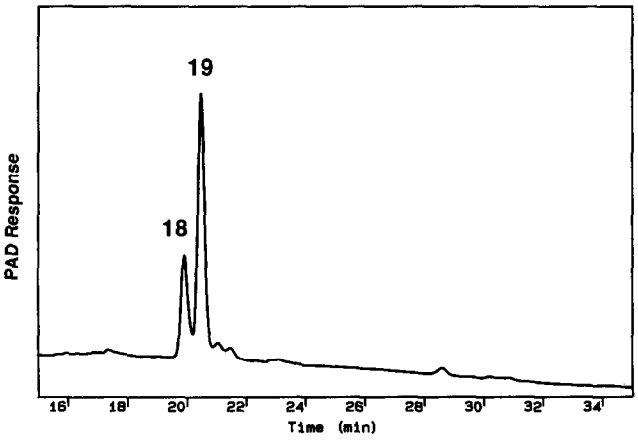


Fig. 6. Separation of hybrid oligosaccharides. Compounds **18** and **19** were released by endo H digestion of rt-PA. Elution was with gradient program 2.

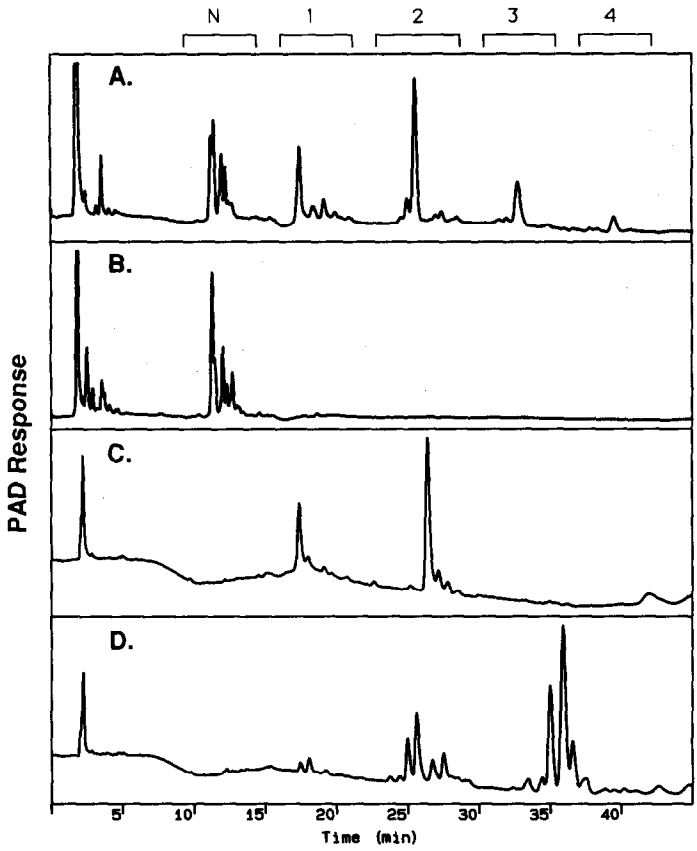


Fig. 7. Oligosaccharide profiles of four glycoproteins. The glycoproteins were reduced and S-carboxymethylated and then treated with PNGAse F. The oligosaccharides released were recovered by ethanol fractionation and analyzed by HPAEC using gradient program 2. (A) rt-PA; (B) ribonuclease *b*; (C) human transferrin; (D) bovine fetuin. The labels at the top indicate the elution positions of neutral (N), monosialyl (1), disialyl (2), trisialyl (3) and tetrasialyl (4) oligosaccharides.

human transferrin (Fig. 7C) is a non-fucosylated disialyl diantennary oligosaccharide in which sialic acid is attached to the 6-position of galactose[17]. The carbohydrate structures of bovine fetuin (Fig. 7D) are quite heterogeneous, differing in the extent of sialylation, the number of peripheral branches (*i.e.* diantennary *vs.* triantennary), and the linkage (β1,4 *vs.* β1,3) of galactose residues[18]. Hardy and Townsend[2] have demonstrated with fetuin glycopeptides that HPAEC is capable of resolving structures that contain β1,3-linked galactose from those with β1,4-linked galactose[2].

It should be noted that HPAEC is not able to resolve closely related acidic molecules as efficiently as it resolves their desialylated counterparts. For example, rt-PA contains nearly equimolar amounts of (trisialyl) fucosylated 2,4-branched and 2,6-branched triantennary oligosaccharides. These oligosaccharides coelute in Fig. 7A (retention time of 30.5 min), whereas their desialylated equivalents are well resolved by HPAEC (see Fig. 3).

Methods of preparing oligosaccharides for HPAEC

HPAEC separations depend upon ionizing oligosaccharides at high pH to promote their interaction with the anion exchange resin. As a consequence, the separations are easily perturbed by the presence of buffer salts in the sample matrix. We have investigated several techniques to recover oligosaccharides released by glycosidase digestion, with the goal of conveniently and reproducibly obtaining suitably salt- and protein-free samples for HPAEC analysis. Fig. 8 compares the HPAEC profiles obtained when oligosaccharides were recovered by gel-permeation chromatography (Fig. 8A), by chromatography on a C_{18} Sep-Pak cartridge (Fig. 8B) or by ethanol precipitation (Fig. 8C). The chromatograms obtained after ethanol precipitation were superimposable with those obtained after gel permeation. The C_{18} Sep-Pak cartridges did not prove to be efficient for this application, since desalting was incomplete (not shown) and varying recoveries were observed for different oligosaccharides (compare traces A and B in Fig. 8). As a result, we routinely use ethanol precipitation for sample preparation because it is simple, lends itself to the preparation of multiple samples in parallel, and gives results comparable to those obtained after gel-permeation chromatography.

On-line desalting of oligosaccharides

The high-resolution oligosaccharide separations attainable by HPAEC make

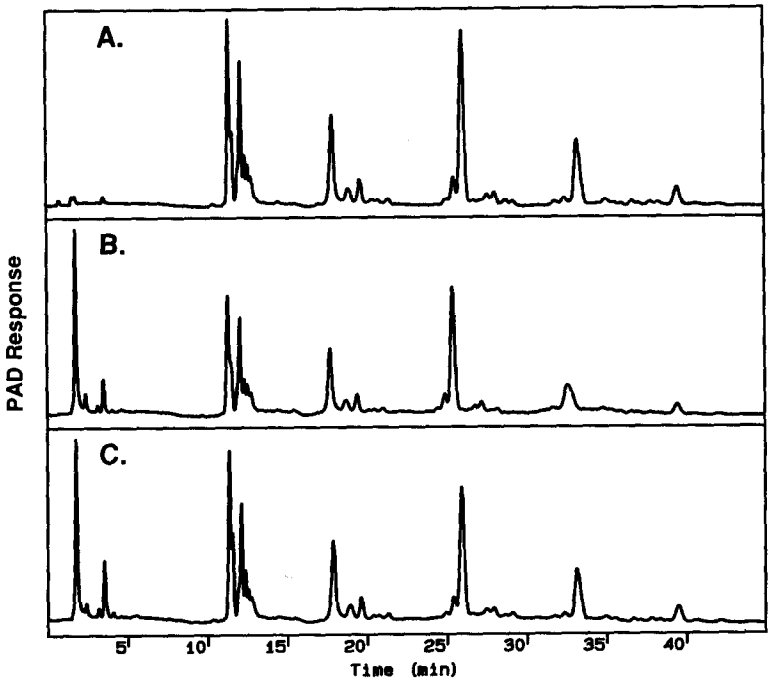


Fig. 8. Comparison between three methods of recovering oligosaccharides after PNGase F digestion. Identical aliquots of reduced and S-carboxymethylated rt-PA were treated with PNGase F, and the released oligosaccharides were recovered by (A) gel permeation chromatography, (B) reversed-phase chromatography on C_{18} Sep-Pak, and (C) ethanol precipitation. Elution was with gradient program 2.

the technique useful for general carbohydrate characterization. However, in the absence of supporting analytical results (*e.g.* mass spectrometry and/or NMR), chromatographic retention time alone is not sufficient to identify an oligosaccharide. One difficulty with HPAEC is that the eluents contain high concentrations of non-volatile salts (typically sodium acetate and sodium hydroxide); therefore, an unidentified oligosaccharide must be desalted prior to carrying out conventional methods of structure elucidation.

We have evaluated the use of an anionic micromembrane suppressor (AMMS; Dionex) downstream of the detector to achieve on-line desalting. Within the AMMS cartridge, the column effluent passes along one face of a polyanionic membrane, which is permeable to cations but impermeable to anions. Sodium ions in the effluent stream are exchanged for protons from an aqueous sulfuric acid regenerant solution on the other face of the membrane, thus converting the sodium hydroxide and sodium acetate to water and acetic acid, respectively. After "ion suppression", salt-free oligosaccharides can be recovered by lyophilization of column fractions. The main limitation of this approach is the exchange capacity of the AMMS cartridge: the cartridge is able to exchange *ca.* 0.15–0.2 *M* sodium ions at a flow-rate of 1 ml/min. Thus, although the capacity of the cartridge is generally satisfactory for desalting neutral oligosaccharides, it may be overwhelmed by the sodium acetate concentrations necessary to elute multiply charged oligosaccharides.

We have used this approach succesfully to desalt high-mannose and hybrid oligosaccharides, separated by HPAEC, and mono- di-, tri- and tetrasialyl complex oligosaccharides, separated by neutral-pH anion-exchange chromatography on a Mono Q column. The lyophilized oligosaccharides collected in this manner were then analyzed by ^{1}H NMR at 500 MHz, which yielded good spectra that contained only minimal acetate signals (not shown; see ref. 8).

Although oligosaccharides can be prepared succesfully for NMR analysis after HPAEC, the inherent insensitivity of NMR and the low capacity of the HPAEC columns make it desirable to be able to prepare samples for more sensitive analytical methods, most notably mass spectrometry. Preparation of samples for FAB-MS analysis requires thorough desalting, since even small amounts of contaminating salt adversely affect ionization and result in reduced sensitivity[19]. We have examined two methods for derivatizing oligosaccharides after HPAEC with ion suppression. The goal with both techniques was to make a derivative that could be purified by reversed-phase HPLC and would be suitable for FAB-MS analysis.

The first approach was to reduce and permethylate oligosaccharides that had been separated by HPAEC and desalted by on-line ion suppression (Fig. 9). Permethylated oligosaccharide alditols are suitably non-polar for reversed-phase chromatography and have been shown to exhibit good ionization and useful fragmentation in FAB-MS[19]. Fig. 9A shows the HPAEC separation of a mixture of high-mannose oligosaccharides released by endo H treatment of rt-PA. The peak indicated with the arrow was collected, recovered by lyophilization, reduced and permethylated. The resulting permethylated oligosaccharide alditol was recovered by reversed-phase chromatography on a C_{18} Sep-Pak cartridge[12] and analyzed by FAB-MS (Fig. 9B). The mass spectrum of this derivative contained abundant $[M+H]^+$ (*m/z* 1328.4) and $[M+Na]^+$ (*m/z* 1350.4) ions.

We were able to use this technique successfully to obtain mass spectra of a

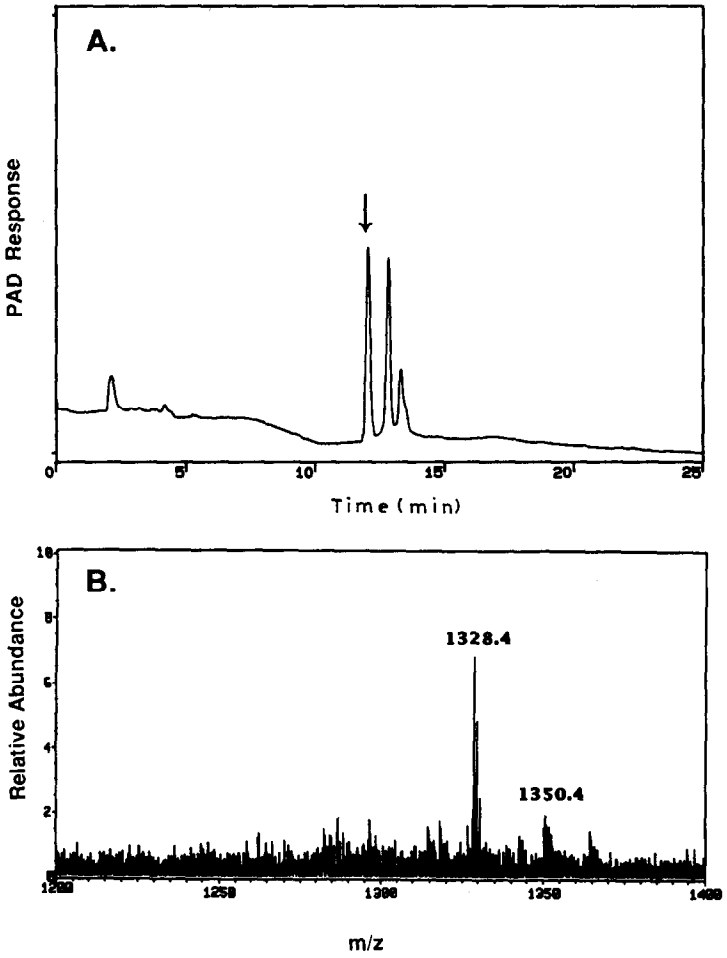


Fig. 9. FAB-MS analysis of the permethylated derivative of a high-mannose oligosaccharide after HPAEC. (A) Separation of a mixture of high-mannose oligosaccharides by HPAEC using gradient program 1. The column effluent was passed through an anionic micromembrane suppressor cartridge for on-line desalting. The collected oligosaccharide (arrow) was recovered by lyophilization, permethylated and analyzed by FAB-MS. (B) The FAB-MS spectrum of the derivatized oligosaccharide. The ions at *m/z* 1328.4 and *m/z* 1350.4 correspond to $[M+H]^+$ and $[M+Na]^+$, respectively, for reduced and permethylated $Man_5GlcNAc$ (compound **1**, Table I).

variety of high-mannose and desialylated complex oligosaccharides[8]. There are, however, several disadvantages to using permethylated derivatives for this application: (1) sample preparation is rather laborious and time-consuming; (2) the presence of even fairly low levels of salt can interfere with the permethylation chemistry, resulting in undermethylation[20]; and (3) the permethylated derivatives lack a good UV chromophore, which would be useful for monitoring the reversed-phase purification.

To overcome the limitations of the permethyl derivatives, we examined a different derivatization method: reductive coupling of *p*-aminobenzoic acid ethyl ester

(ABEE) to reducing oligosaccharides collected after HPAEC and ion suppression. ABEE derivatives[14,15] and derivatives with longer-chain alkyl esters of *p*-aminobenzoic acid[21] have been demonstrated to have desirable properties for FAB-MS. This type of derivatization chemistry offers several advantages over permethylation for use after HPAEC: (1) the derivatization should not be affected by residual sodium acetate resulting from incomplete ion suppression; (2) the derivatization introduces a UV chromophore, which simplifies subsequent reversed-phase HPLC purification; and (3) the longer-chain alkyl esters of *p*-aminobenzoic acid have been demonstrated to yield derivatives that ionize extremely efficiently, making sub-nanomole sensitivity feasible[21].

To test the suitability of ABEE derivatives for FAB-MS analysis after HPAEC separations, an aliquot of an asialo diantennary oligosaccharide (compound **10**, Table I) was collected after HPAEC using gradient program 1 with ion suppression. The sample was derivatized with ABEE and the derivative isolated by reversed-phase HPLC, as described in Experimental. The FAB mass spectrum of the isolated ABEE derivative is shown in Fig. 10 and contains a prominent $[M+H]^+$ ion (*m/z* 1587.5). Thus it appears to be quite feasible to analyze ABEE derivatives of oligosaccharides after HPAEC separations. As has been demonstrated by Poulter *et al.*[21], the use of longer-chain alkyl esters of *p*-aminobenzoic acid results in significantly improved sensitivity over ABEE derivatives and, therefore, will probably prove to be the reagents of choice for this application.

In conclusion, the results presented here demonstrate that HPAEC is a versatile method for separating both neutral and acidic oligosaccharides of the types commonly found as N-linked substituents of glycoproteins. The resolution and reproducibility of the separations make the technique suitable for oligosaccharide profiling. When used in conjunction with on-line ion suppression, HPAEC can be a "semipreparative"

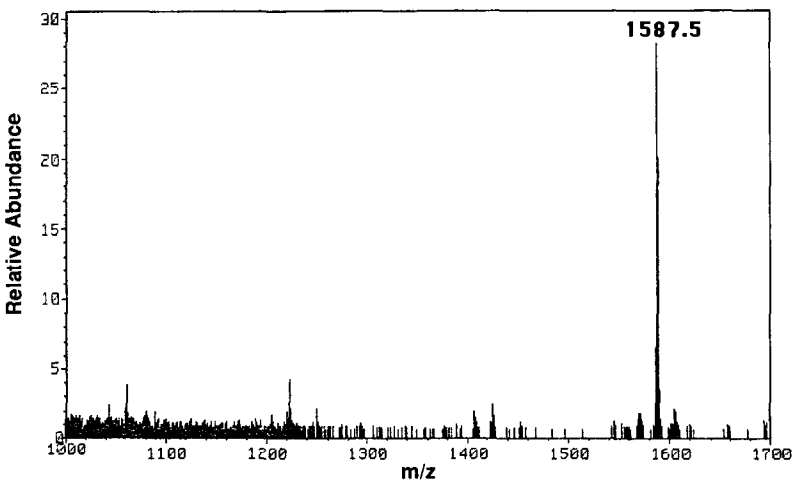


Fig. 10. FAB-MS analysis of the ABEE derivative of compound **10**. Compound **10** was subjected to HPAEC and collected as described in the legend to Fig. 9. The collected oligosaccharide was derivatized with ABEE as described in Experimental, and analyzed by FAB-MS. The ion at *m/z* 1587.5 corresponds to $[M+H]^+$ for the ABEE derivative of compound **10**.

technique, permitting identification of collected oligosaccharides by conventional chemical and spectroscopic methods. It should be noted that, although pulsed amperometry is a destructive method of detection, only a small percentage of the column effluent ever comes into contact with the detection electrode. Therefore, in our semi-preparative work it has been possible to collect oligosaccharides downstream of the detector without stream splitting. We have not observed significant degradation products in NMR spectra, in mass spectra, or by rechromatography of collected oligosaccharides.

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