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Application Note 105

Glycosylation Analysis of Human Serum Transferrin Glycoforms Using Pellicular Anion-Exchange Chromatography

INTRODUCTION

Glycoforms and Protein Sialylation

Glycoproteins are proteins with a carbohydrate attached to the polypeptide backbones through one or more glycosylation sites. Oligosaccharides can be linked to a protein through a serine or a threonine as *O*-linked glycans, or through an asparagine as *N*-linked glycans. Glycoprotein glycoforms contain identical polypeptide backbones and differ from one another in the oligosaccharides attached to the glycosylation sites. To complicate the analytical task, the oligosaccharides attached to glycoproteins can also be sialylated at the nonreducing end of the glycans and the degree of sialylation can vary. Thus, the variation in glycoprotein sialylation can also create a collection of glycoforms.

Use of Pellicular Anion Exchange Columns for Fractionation of Glycoforms

The DNAPac™ PA-100 column, a pellicular anion-exchange column, has been successfully used to fractionate sialylated glycoprotein and sialylated glycopeptide glycoforms.[1,2] Separations of glycoforms are dependent upon the difference in the degree of sialylation of the glycoforms. The greater the degree of sialylation, the longer the glycoforms are retained on the column; thus, allowing the glycoprotein to be separated into different glycoform populations.

Examination of fetuin glycopeptide separations using the DNAPac PA-100 column indicated that glycopeptide glycoforms can also be fractionated based on the structure of the attached sialylated oligosaccharides.[2] The selectivity based upon structural differences in the carbohydrate moiety may allow separation of glycoforms with oligosaccharides that are sialylated on different oligosaccharide branches, but contain the same number of sialic acid residues.

The DNAPac PA-100 column is available in analytical (4 x 250 mm), semipreparative (9 x 250 mm), and preparative (22 x 250 mm) formats. The analytical column can be used to obtain a preliminary separation of glycoforms using a small amount of sample. The semi-preparative and the preparative columns can then be used to fractionate a larger quantity of the glycoprotein so that column fractions can be collected for further analysis.

Use of HPAE-PAD for Glycosylation Analysis of Fractionated Glycoforms

Once the DNAPac PA-100 fractions are obtained, PNGase F digestions can be performed. PNGase F, an amidase, removes *N*-linked oligosaccharides attached to glycoproteins. The released oligosaccharides from the different PNGase F digested fractions can be analyzed using HPAE-PAD (High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection) using a CarboPac™ PA-100 column.[3–5] Because the retention of sialylated oligosaccharides on the CarboPac PA-100 column is primarily based on the number of sialic acid residues attached to the glycan, the distribution of the sialylated species of a particular glycoform fraction can be estimated based on retention time and peak areas. Treatment of these digests with neuraminidase (an exoglycosidase that removes terminal sialic acid from sialylated oligosaccharides) and subsequent analysis of the digests with HPAE-PAD, confirms the presence of sialylated oligosaccharides in the glycoform fractions.

Human Serum Transferrin

Transferrin, an iron-binding glycoprotein, is found in serum and extravascular fluids in a variety of vertebrates.[6] Transferrin glycosylation varies depending on the species and on the tissues from which the protein is isolated. In addition, transferrin from the same tissue of the same species may exhibit variations in glycan structures, particularly with respect to the degree of sialylation.[7–9]

Human serum transferrin (HST), a serum β-globulin, has been shown to contain two *N*-linked glycosylation sites.[7] Prior studies found that HST contains 85% biantennary and 15% triantennary glycans.[10] One disialylated oligosaccharide and two different trisialylated oligosaccharides are believed to make up the majority of the biantennary and the triantennary species, respectively. In addition, 82% of HST has a biantennary oligosaccharide at both sites, 17% has a triantennary oligosaccharide at one site and a biantennary oligosaccharide at another site, and 0–1% has a triantennary oligosaccharide at each site.[11]

Clinical Significance of HST Sialylation

Variation of glycoprotein sialylation has significant clinical implications. For example, HST of cancer, rheumatoid arthritis, and haemochromatosis patients show an increased level of sialylation[12]; whereas patients with heavy alcohol consumption show a high incidence of asialo HST glycoforms.[13] Clearance of glycoproteins has also been shown to be related to sialylation. For example, catabolism of human serum transferrin in the liver has been found to be dependent on the interactions between the carbohydrate moiety and the asialoglycoprotein receptor. When the sialylation of HST is low, elimination from circulation is more rapid.[9]

Analytical Strategy

In this Application Note, procedures for glycoprotein sialylation analysis are described. HST is used as a model glycoprotein because of its clinical significance and its availability from commercial sources. The analytical strategy is summarized as follows:

1. Fractionation of HST into distinct populations of glycoforms according to the degree of sialylation.
2. Removal of *N*-linked oligosaccharides from the glycoform fractions.
3. Profiling of the removed glycans.
4. Identification of the sialylated glycans using neuraminidase.
5. Estimation of the relative distribution of the sialylated oligosaccharides.

EQUIPMENT

Dionex DX-500 BioLC® system consisting of:
GP50 Gradient Pump
AD20 Absorbance Detector
ED40 Electrochemical Detector
AS3500 Autosampler

PeakNet Chromatography Workstation

Savant Speed Vac® Concentrator, Model A290 (Savant Instruments-EC Apparatus, Inc.)

Spectra/Por® Membrane; molecular weight cut off: 1000; diameter: 29 mm, length: 5 m (Spectrum Medical Industries, Inc., available from VWR Scientific)

REAGENTS AND STANDARDS

Human serum transferrin, holo, iron saturated (Boehringer Mannheim)

Triton X-100, hydrogenated, protein grade, 10% (Calbiochem)

Anhydrous sodium acetate (Fluka Chemika-BioChemika)

Sodium hydroxide, 50% w/w (Fisher Scientific)

Acetonitrile, HPLC-grade (EM Science)

Ammonium acetate (EM Science)

Sodium phosphate, Na_2HPO_4 (Sigma)

β-Mercaptoethanol, approximately 98%, d = 1.11 g/mL (Sigma)

Trifluoroacetic acid (TFA), protein sequencing grade, 1-mL sealed ampules (Sigma)

Peptide-*N*-Glycosidase F (PNGase F), from *Flavobacterium meningosepticum*, recombinant in *E. coli* (Oxford Glycosciences)

Neuraminidase from *Vibrio cholerae* (Oxford Glycosciences)

N-Acetylneuraminic acid, NANA, P/N A9646 (Sigma)

Asialo biantennary, NA2, P/N C-024300 (Oxford Glycosciences)

CONDITIONS AND METHODS

Columns: CarboPac PA-100 Analytical Column, 4 x 250 mm, and Guard Column, 4 x 50 mm

DNAPac PA-100 Analytical Column, 4 x 250 mm, and Guard Column, 4 x 50 mm

DNAPac PA-100 Semi-Prep Column, 9 x 250 mm

Zorbax® RP300-C18 Analytical Column, 4.6 x 150 mm

Flow Rates: CarboPac PA-100 Analytical Column, 1 mL/min

DNAPac PA-100 Analytical Column, 1 mL/min

DNAPac PA-100 Semi-Prep Column, 5 mL/min

Zorbax RP300-C18 Analytical Column, 1 mL/min

Pulse Setting for ED40 Detector:

t (s)	E (V)	Integration
0.0	0.05	
0.2	0.05	Begin
0.4	0.05	End
0.41	0.75	
0.6	0.75	
0.61	–0.15	
1.0	–0.15	

Absorbance Detector: UV, 215 nm

Eluent A: 250 mM Sodium acetate

Eluent B: 500 mM Sodium hydroxide

Eluent C: Deionized water (DI H_2O), 17.8 MΩ-cm resistance or better

Eluent D: 500 mM Ammonium acetate

Eluent E: 0.1% TFA in deionized water

Eluent F: 0.085% TFA in 10% deionized water/90% acetonitrile

Methods:

Method	Column	Time (min)	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)
1	CarboPac PA-100	0	8	20	72	0	0	0
		5	8	20	72	0	0	0
		65	80	20	0	0	0	0
		65.05	8	20	72	0	0	0
		70	8	20	72	0	0	0
2	DNAPac PA-100 Analytical & Semi-Prep	0	0	0	95	5	0	0
		3	0	0	95	5	0	0
		33	0	0	80	20	0	0
		36	0	0	80	20	0	0
		36.05	0	0	95	5	0	0
		41	0	0	95	5	0	0
3	Zorbax RP300-C18	0	0	0	0	0	95	5
		5	0	0	0	0	95	5
		60	0	0	0	0	40	60
		61	0	0	0	0	20	80
		64	0	0	0	0	20	80
		65	0	0	0	0	95	5
		90	0	0	0	0	95	5

PREPARATION OF SAMPLES AND SOLUTIONS

Eluent A: 250 mM Sodium acetate

Dissolve 20.5 g of anhydrous sodium acetate into a final volume of 1.0 L of deionized water. Filter the eluent through a 0.2-µm filter, and then vacuum degas the eluent for 5 minutes before use.

Eluent B: 500 mM Sodium hydroxide

Filter 1.0 L of deionized water through a 0.2-µm filter. Then vacuum degas the deionized water for 5 minutes. Dilute 26 mL of 50% (w/w) sodium hydroxide to a final volume of 1.0 L with the degassed water.

Eluent C: Water

Filter 1.0 L of deionized water through a 0.2-µm filter. Then vacuum degas the deionized water for 5 minutes before use.

Eluent D: 500 mM Ammonium acetate

Dissolve 38.5 g of ammonium acetate in deionized water to make up a 1-L solution. Filter the ammonium acetate solution through a 0.2-µm filter before use.

Eluent E: 0.10% TFA in Deionized water

Dilute 500 µL of TFA to 500 mL with deionized water and vacuum degas.

Eluent F: 0.085% TFA in 10% Deionized water/90% Acetonitrile

Add 425 µL of TFA to 50 mL of deionized water. Dilute to 500 mL with acetonitrile and vacuum degas.

EXOGLYCOSIDASE AND AMIDASE PREPARATIONS

Neuraminidase from *Vibrio cholerae*

Dissolve 0.2 U of the enzyme in 40 µL of the 5X incubation buffer (50 mM sodium acetate with 4 mM calcium chloride, pH 5.5) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

PNGase F (from *Flavobacterium meningosepticum*, recombinant in *E. coli)*

Dissolve 20 U of the enzyme in 40 µL of the 5X incubation buffer (20 mM sodium phosphate with 50 mM EDTA, 0.02% sodium azide, pH 7.5) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

CARBOHYDRATE STANDARDS

N-Acetylneuraminic acid (NANA, supplied as 25 nmol dry powder, final concentration: 50 nmol/mL)

Add 500 µL of deionized water to 25 nmol of the carbohydrate as supplied.

NA2 (Asialo biantennary oligosaccharides)

To prepare each oligosaccharide stock, add 500 µL of water to 100 µg of the carbohydrate as supplied.

Fractionation

A quantity of 10 mg of HST was dissolved in 1 mL of 100 mM sodium phosphate, pH 7.2. An injection of 2 mg (200 µL) was then applied to the DNAPac PA-100 semi-prep column. Three column fractions designated F1, F2, and F3, as shown in Figure 2, were collected. F1 was collected between 18 and 20 minutes, F2 was collected between 22.5 and 24.5 minutes, F3 was collected between 28 and 30 minutes.

Preparation of Samples for the Zorbax C18 Reversed-Phase Separations

1. DNAPac PA-100 fractions of HST: 2 mg of HST was injected onto the semi-prep DNAPac PA-100 column. Fractions were collected as described above. Each fraction (in ammonium acetate buffer) was dried in a SpeedVac, redissolved in 200 µL of distilled water, and then dried again using the SpeedVac. This procedure was repeated four times to remove the residual ammonium acetate. These dried samples were finally redissolved in 200 µL of deionized water.
2. Unfractionated HST: 1 mg of HST was dissolved in 1 mL of water.

PNGase F Digestion

1. DNAPac PA-100 fractions of HST: Each fraction (in ammonium acetate buffer) was dried in a SpeedVac, redissolved in 200 µL of distilled water, and then dried again using the SpeedVac. This procedure was repeated four times to remove the residual ammonium acetate. These dried samples were then redissolved in 100 µL, 100 mM sodium phosphate, pH 7.2 with 10 mM β-mercaptoethanol and 4 µL Triton X-100. PNGase F, 1 U, was added to each sample, and the samples were incubated at 25 °C for 24 hours.
2. Unfractionated HST: 500 µg of HST were dissolved in 250 µL, 100 mM sodium phosphate, pH 7.2 with 10 mM β-mercaptoethanol and 10 µL Triton X-100. PNGase F, 2 U, were then added, and the sample was incubated at 25 °C for 24 hours.

Dialysis of the PNGase F Digests

The PNGase F digests were transferred into individual Spectra/Por dialysis tubing, and were dialyzed in distilled water for 27 hours. The distilled water was replaced four times (at 2, 4, 6, and 24 hours after the beginning of dialysis). The dialyzed samples were analyzed by HPAE-PAD as described in the *Conditions and Methods* section. Each dialyzed sample, 50 µL, was also retained for treatment with neuraminidase as described below.

Neuraminidase Digestions

A quantity of 50 µL from each of the PNGase F treated, dialyzed samples was dried in a SpeedVac. Each of the dried samples was then dissolved in 50 µL of

100 mM sodium acetate, pH 5.5, and incubated with 5 mU of neuraminidase at 37 ˚C for 24 hours. Samples were analyzed by HPAE-PAD as described in the *Methods* section.

RESULTS AND DISCUSSION

Fractionation of HST Glycoforms

HST was analyzed using an analytical DNAPac PA-100 column as shown in Figure 1A; three major peaks can be identified from 15 to 28 minutes. Treatment of the HST sample with neuraminidase (an exoglycosidase that removes sialic acid residues from the nonreducing ends of the attached oligosaccharides) collapses all three peaks into a broad peak eluting close to the void volume of the column, as shown in Figure 1B.

The results indicate that upon removal of terminal sialic acids, the retention of all three HST populations is significantly reduced. Prior work has indicated that upon removal of sialic acids, HST elutes at the void volume. The neuraminidase digestion apparently did not remove all the sialic acids from the HST glycans as the treated HST peak is not eluted at the void volume as shown Figure 1B. The results, however, provide strong evidence that the existence of the three peaks from the untreated HST is due to the sialylation of the HST glycans.

HST was also separated using a semi-preparative DNAPac PA-100 column. The goal was to inject a larger amount of HST (in this example, milligrams), and to collect fractions corresponding to the three peaks in a single separation. As shown in Figure 2, the semi-preparative separation resembles the one using the analytical column. The three peaks were identified as F1, F2, and F3 (Fractions 1, 2, and 3 respectively), as shown in Figure 2, and the fractions were collected for further analysis.

Reversed-Phase HPLC of the DNAPac PA-100 Fractions

The DNAPac PA-100 fractions and an unfractionated HST were analyzed using a Zorbax C18 column. A single peak eluting at 44.2 minutes was obtained from a reversed-phase separation of the HST (before neuraminidase treatment), as shown in Figure 3A. A single peak with identical retention time is obtained from each of the three fractions, and these peaks also coelute with that of the unfractionated HST, as shown in Figures 3A–3D. The results confirm that all three DNAPac peaks originate from HST, and suggest that there is no detectable degradation of HST on the DNAPac column.

PNGase F Digestion of the DNAPac PA-100 Fractions

Treatment of the DNAPac PA-100 fractions with PNGase F released the *N*-linked oligosaccharides. The digestions were analyzed by HPAE-PAD to profile the released glycans, and to obtain a relative distribution of the sialylated oligosaccharides. The determination of the extent of sialylation of the oligosaccharides is possible because mono-, di-, and trisialylated oligosaccharides elute in the regions between 20–30, 30–40, and 40–48 minutes, respectively, using the method (Method 1) suggested in this note.[1-5] These retention time windows can be reconfirmed using available Oxford Glycosciences oligosaccharides (C-124300, C-224300, and C-335300).

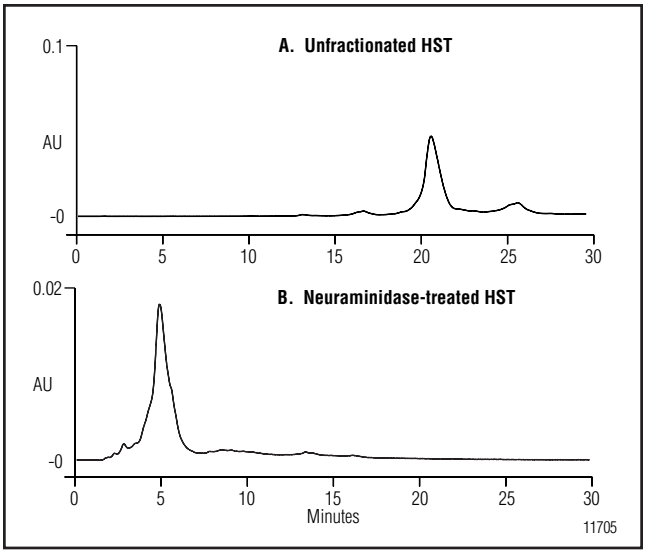

Figure 1 *(A) Separations of unfractionated HST. Column: DNAPac PA-100 analytical column. Sample injected: 600 µg; (B) Separation of Neuraminidase-treated HST. Column: DNAPac PA-100 analytical column. Sample injected: 50 µg.*

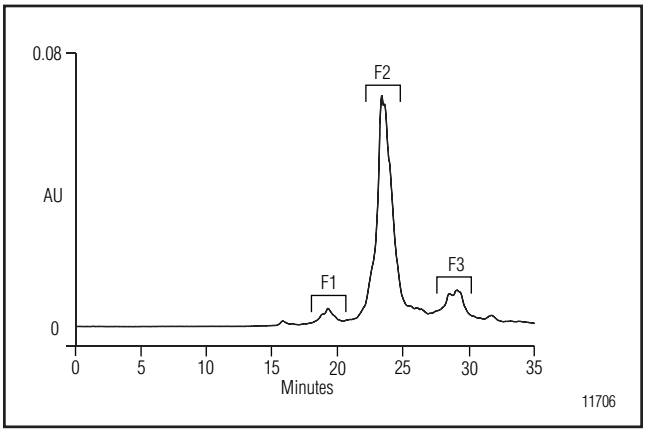

Figure 2 *Semi-prep DNAPac PA-100 separation of unfractionated HST. Column: DNAPac PA-100 semi-prep column. Sample injected: 2 mg.*

The relative distribution of the three classes of oligosaccharides was determined based on peak areas.

Figures 4A–4D show the separations of the PNGase F digestions of the unfractionated HST, F1, F2, and F3, respectively. For the separation of the unfractionated HST, a main peak (Peak 3) eluted at approximately 32 minutes, and three minor peaks (Peaks 4, 5, and 6) eluted between 32 and 36 minutes, as shown in Figure 4A. The retention times of these features suggested that the majority of glycans in the unfractionated HST are disialylated species. As shown in Figure 4B, Peaks 3, 4, and 5, corresponding to the disialylated species present in the unfractionated HST, are also observed in the separation of F1. In addition, two peaks (Peaks 1 and 2) eluted in the region between 21–24 minutes, indicating that there are monosialylated species present in F1. Similarly, the disialylated species (Peaks 3–5) observed in F1 and the unfractionated HST are also present in F2, as shown in Figure 4C. The mono-sialylated species found in F1, however, are absent in F2. Peaks 3–6, corresponding to the disialylated species of the unfractionated HST, are present in F3, as shown in Figure 4D. In addition, three peaks (Peaks 7–9) eluted at the region between 41–45 minutes, indicating that a population of trisialylated species is also present in F3.

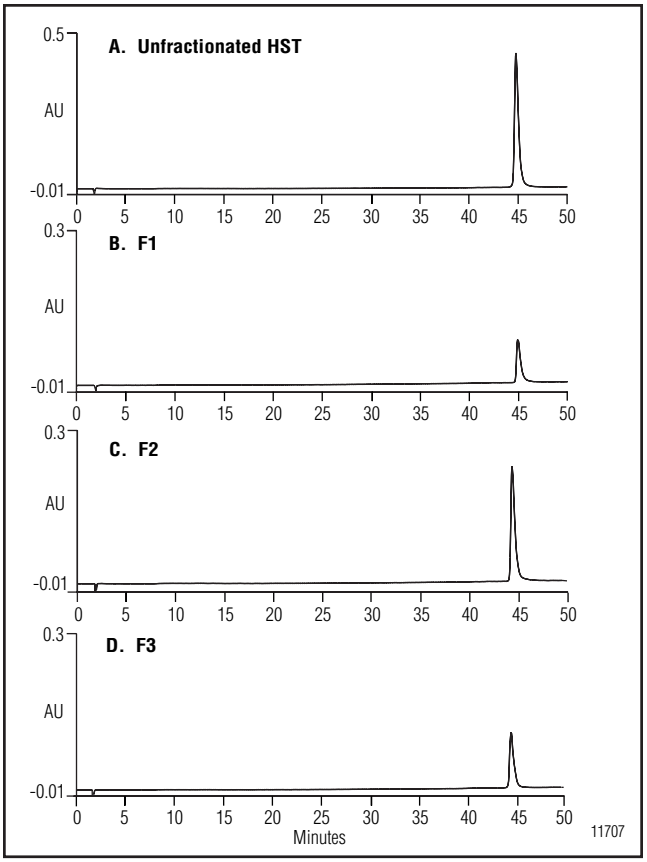

Figure 3 *Reversed-phase separation of unfractionated HST, F1, F2, & F3. (A) Unfractionated HST. Sample injected: 30 μg. (B) F1. Sample injected: 9 μL from the 200 μL reconstituted fraction. (C) F2. Sample injected: 9 μL from the 200 μL reconstituted fraction; (D) F3. Sample injected: 9 μL from the 200 μL reconstituted fraction. Column: Zorbax RP 300-C18 analytical column.*

Neuraminidase Treatment of the PNGase F Digestions

To confirm that the distribution of peaks observed in the separations of the different PNGase F digestions actually reflects oligosaccharide sialylation, neuraminidase digests were performed. Aliquots from each of the PNGase F digestions were treated with neuraminidase and then analyzed by HPAE-PAD. The retention times of the sialylated peaks observed in Figures 4 should be reduced significantly; whereas the nonsialylated oligosaccharides features should not be affected.

Separations of all three DNAPac fractions, the unfractionated HST, an asialo bi (NA2) standard, and a NANA standard, are shown in Figures 5A–5F. The sialylated oligosaccharides peaks (Peaks 1–9 of Figures 4A–4D) are no longer present in the four digestions shown in Figures 5A–5D, indicating that these peaks indeed represent sialylated oligosaccharides. The peaks eluting at approximately 13 minutes in Figures 5A–5D coelute with the NANA standard shown in Figure 5F. The peaks eluting at approximately 11.3 minutes from the unfractionated HST and F2 separations, as shown in Figures 5A and 5C, coelute with an asialo biantennary standard shown in Figure 5E. These results suggest that the main oligosaccharide species in HST is an *N*-linked, disialylated biantennary oligosaccharide. A broad peak eluting between 10 and 12 minutes is observed in F1 and F3, as shown in Figures 5B and 5D, respectively. Further investigation is needed to confirm the identities of these peaks.

Distribution of the PNGase F-Released, Sialylated Oligosaccharides

Peak area analysis of the different sialylated oligosaccharide peaks for the unfractionated HST and the HST fractions is shown in Table 1. Both the unfractionated HST and F2 contain more than 95% of disialylated oligosaccharides. F1, the fraction eluted before the main peak from the DNAPac PA-100 separation, contains approximately 30% and 70% of monosialylated and disialylated oligosaccharides, respectively. F3, the frac-

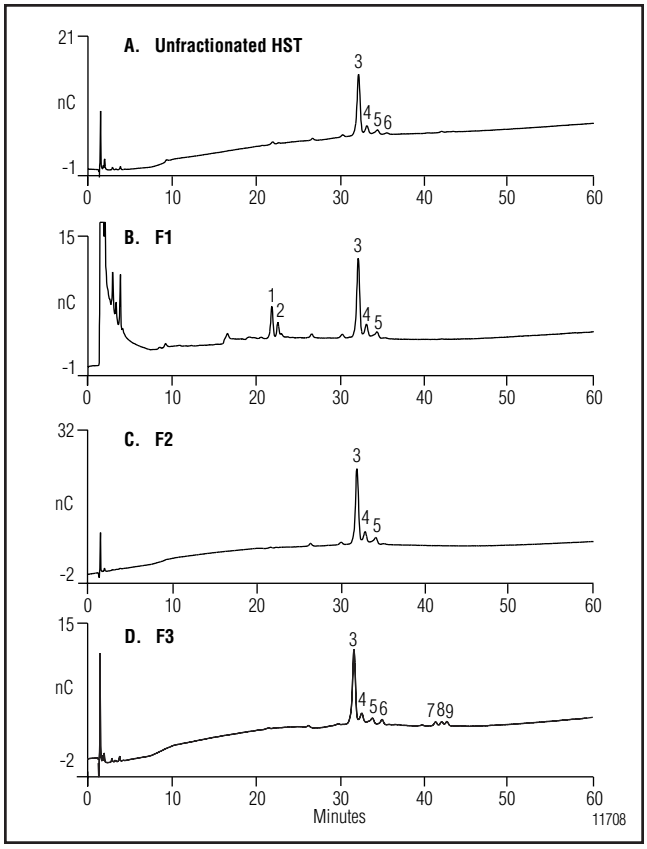

Figure 4 *Separations of PNGase F digestions of unfractionated HST, F1, F2, & F3. (A) Unfractionated HST. Sample injected: 25 µL from the PNGase F digest; (B) F1. Sample injected: 25 µL from the PNGase F digest; (C) F2. Sample injected: 10 µL from the PNGase F digest; (D) F3. Sample injected: 20 µL from the PNGase F digest. Column: CarboPac PA-100 column.*

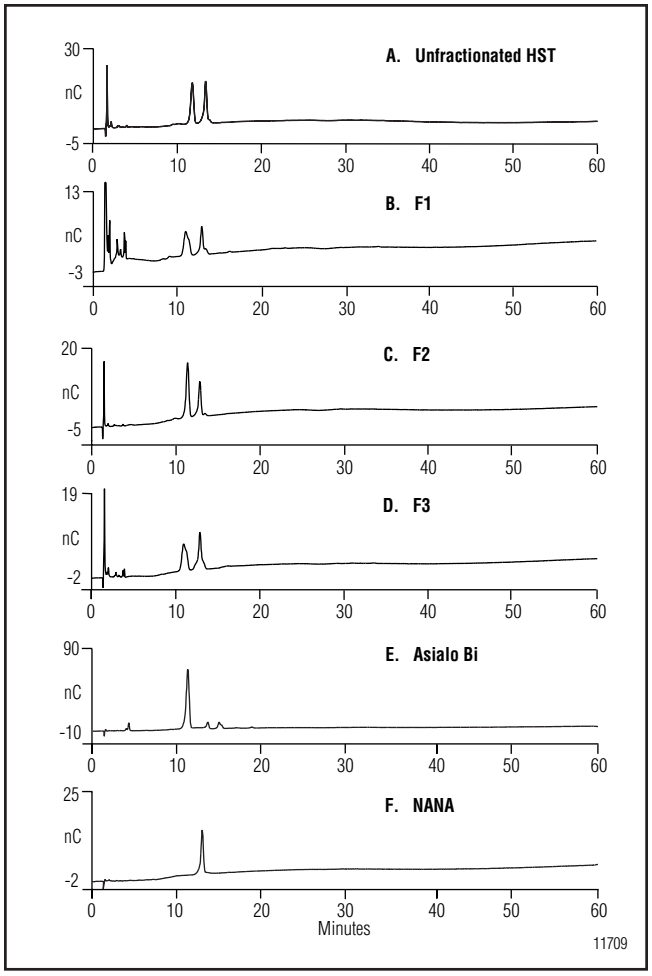

Figure 5 *Separations of neuraminidase-treated PNGase F digestions of unfractionated HST, F1, F2, & F3. (A) Unfractionated HST. 10 µL from the neuraminidase + PNGase F digest; (B) F1. sample injected: 20 µL from the neuraminidase + PNGase F digest; (C) F2. sample injected: 10 µL from the neuraminidase + PNGase F digest; (D) F3. 20 µL from the neuraminidase + PNGase F digest; (E) Asialo Biantennary Standard (PI 01); (F)* N-*Acetyl neuraminic acid. Column: CarboPac PA-100 column.*

tion eluted after the main peak from the DNAPac PA-100 separation, contained approximately 90% and 10% of disialylated and trisialylated oligosaccharides.

It has been reported that the relative molar electrochemical responses for di-, tri, tetra, and pentasialylated oligosaccharides are similar (4.8 ± 14% relative to glucose).[14] This peak area analysis thus provides a good approximation of the relative distribution of the sialylated glycans released form HST.

The peak area percentage of the disialylated species is higher compared to that of the monosialylated species, and to that of the trisialylated species in F1 (70% to 30%) and F3 (90% to 10%), respectively. These ratios indicate that there may be partially sialylated oligosaccharides present in the fractions. The glycoforms containing these partially sialylated oligosaccharides apparently were retained differently on the DNAPac column, compared to those with fully sialylated oligosaccharides and an identical number of sialic acids. Further investigation is needed to confirm the presence of these species in the two fractions.

Table 1 Peak area of sialylated oligosaccharide peaks

Sample	Peak Area (%)		
	Mono-sialylated	Di-sialylated	Tri-sialylated
Unfractionated HST	3	97	Below Detection Limit
F1	30	70	0
F2	1	99	0
F3	1	89	10

The results support earlier observations that structural differences in the carbohydrate moiety also contribute to separation of the glycoforms on the DNAPac column.[2]

SUMMARY

- The DNAPac PA-100 column fractionates sialylated glycoforms based on the degree of sialylation and other structural features. Glyco-forms with a higher degree of sialylation are retained longer in the column.
- The analytical DNAPac PA-100 column permits analytical scale separation of sialylated glycoforms. The semi-prep DNAPac PA-100 column allows fractionation of larger quantity of proteins (milligrams, in this example) suitable for post-separation analysis.
- Minor glycoform populations can be separated by the semi-prep DNAPac PA-100 column in sufficient quantity for subsequent analysis.
- HPAE-PAD, with a CarboPac PA-100 column, can be used to analyze the released oligosaccha-rides and characterize the distribution of sialylated oligosaccharides from the different glycoform populations.

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