DIONEX

Analysis of Sialic Acids Using High-Performance Anion-Exchange Chromatography

INTRODUCTION

Sialic acids comprise a large family of *N*- and *O*-substituted neuraminic acids. The amino group of neuraminic acid is linked to either an *N*-acetyl or *N*-glycolyl group, which yields *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), respectively. The hydroxyls of both sialic acids are often modified. The biosynthesis, function, and occurrence of sialic acids have been reviewed[1–2]. Sialic acids occupy terminal positions on many mammalian glycoprotein and glycolipid oligosaccharides. When a glycoprotein loses sialic acid residues, it has a reduced serum half-life, and in some cases reduced activity[3]. Therefore, it is important to know the sialic acid content of a glycoprotein when assaying its function or its efficacy as a pharmaceutical therapeutic. There have been a number of chromatographic methods described for sialic acid analysis[4]. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is an effective way to determine Neu5Ac and Neu5Gc without derivatization[4–9]. Many modifications of the Neu5Ac and Neu5Gc hydroxyls are base labile, and therefore cannot be analyzed using standard HPAE-PAD techniques. These substituted sialic acids can be separated at neutral pH and detected using PAD with the addition of postcolumn base[4].

Linearity, detection limits, reproducibility, and accuracy of HPAE-PAD analysis of Neu5Ac and Neu5Gc are evaluated in this Technical Note. A variety of ways to separate Neu5Ac, Neu5Gc, and KDN (3-deoxy-D-glycero-D-galacto-2-nonulosonic acid), an internal standard, using the CarboPac™ PA10 column as well as methods to release sialic acids from glycoproteins are included in this Note. The information presented here is intended to be useful to investigators interested in validating the performance of HPAE-PAD for sialic acid analysis.

EQUIPMENT

Dionex DX-500 Chromatography system consisting of:

- LC30 Chromatography Module
- GP40 Gradient Pump
- ED40 Electrochemical Detector (gold electrode and amperometry cell)
- AS3500 Autosampler (stainless steel needle)

PeakNet Chromatography Workstation

GP40 pump performance can be validated using the GP40 Validation Kit (Dionex P/N 50809). The ED40 Electrochemical Detector can be validated using the ED40 Validation Kit (Dionex P/N 40046).

REAGENTS AND STANDARDS

Sodium acetate (Fluka Chemika-BioChemika)

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

N-Acetylneuraminic acid (Neu5Ac) and *N*-Glycolylneuraminic acid (Neu5Gc) (Pfanstiehl Laboratories, Inc.)

6 N Hydrochloric acid (ABI-Perkin Elmer)

3-Deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), (Toronto Chemicals)

Bovine fetuin (GIBCO-BRL)

Bovine transferrin, apo (Calbiochem)

Human transferrin, partial Fe (Boehringer Mannheim)

Arthrobacter ureafaciens neuraminidase (Boehringer Mannheim)

Autosampler vials, caps, and septa (Sun Brokers, Wilmington, NC, part numbers 500–114, 200–288, 200–396)

CONDITIONS

Column: CarboPac PA10 Analytical Column with guard (P/N 46110)

Flow Rate: 1 mL/min

Detection: ED40 Electrochemical Detector (gold electrode)

Inj. Vol.: 25 µL

Waveform f

Time (sec)	Potential (V)	Integration
0.00	+ 0.05	
0.20	+ 0.05	Begin
0.40	+ 0.05	End
0.41	+ 0.75	
0.60	+ 0.75	
0.61	– 0.15	
1.00	– 0.15	

Eluent: A: 100 mM Sodium hydroxide
B: 100 mM Sodium hydroxide/ 1 M Sodium acetate

Method:

Time (min)	A (%)	B (%)
Initial	93	7
0.0	93	7
10.0	70	30
11.0	70	30
12.0	93	7

A new, gold (Au) working electrode was used for all of the experiments described below. To polish the working electrode, use the procedure described in Dionex Technical Note 40. Erase with a sulfur-free pencil eraser (P/N 49721) prior to initial use only.

PREPARATION OF SAMPLES AND SOLUTIONS

Eluents

100 mM Sodium Hydroxide

It is essential to use high-quality water of high resistivity (18 MΩ-cm or better) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Borate, a water contaminant that can break through water purification cartridges (prior to any other indication of cartridge depletion), can be removed by placement of a BorateTrap™ cartridge (Dionex Corporation) between the pump and the injection valve. Plastic tubing in the water system should be minimized, as it often supports microbial growth, which can be a source of carbohydrate contamination. It is extremely important to minimize contamination with carbonate, a divalent anion at pH ≥12, because it binds strongly to the columns and interferes with carbohydrate chromatography, causing a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 2 L of water to prepare a 0.1 M sodium hydroxide solution. After preparation, keep the eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times.

100 mM Sodium Hydroxide/1 M Sodium Acetate

Measure approximately 800 mL of water into a 1-L graduated cylinder. Add a stir bar and begin stirring. Weigh out 82.0 g of anhydrous, crystalline sodium acetate. Add the solid sodium acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt dissolves, remove the stir bar with a magnetic retriever. Using a plastic pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide and add it to the acetate solution. Rinse the pipette by drawing up the acetate solution into the pipette and dispensing it back into the graduated cylinder several times. Add water to the solution to reach a final level of 1000 mL. Replace the stir bar and stir briefly to mix. Vacuum filter through a 0.2-µm nylon filter. This may be slow, as the filter may clog with insolubles from the sodium acetate. After preparation, keep this eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times.

Degassing the eluent solutions is not necessary, but can be performed as an extra precaution for carbon dioxide removal.

Standards

Dry Neu5Ac and Neu5Gc in a vacuum desiccator over sodium hydroxide pellets for 64 hours. Prepare primary solutions of 9.68 mM Neu5Ac (149.8 mg, 50 mL), 2.52 mM Neu5Gc (41.0 mg, 50 mL), and 3.58 mM KDN (4.8 mg, 5 mL). From these three primary solutions, prepare a mixture with a concentration of 0.4 mM of each (10,000 picomoles in a 25-µL injection). Dilute this mixture according to the specifications in Table 1 for linearity and minimum detection analyses (25-µL injections).

Table 1 Dilutions for linearity analysis		
pmol/25 µL	**Volume of 0.4 mM mixture (µL)**	**Volume of water (µL)**
5000	200	200
2000	80	320
1000	40	360
500	20	380
250	30	1170
pmol/25 µL	**Volume of 250 pmol/ 25 µL mixture (µL)**	**Volume of water (µL)**
100	160	240
50	80	320
25	40	360
10	16	384
5	16	784
pmol/25 µL	**Volume of 5 pmol/ 25 µL mixture (µL)**	**Volume of water (µL)**
2	100	150
1	50	200
0.5	25	225

Freeze 18 mL of a mixture of Neu5Ac and Neu5Gc (0.01 mM each) in 450-µL aliquots. Add 50 µL of a 0.1 mM KDN solution to each vial at the time of use. Use the resulting mixture for reproducibility studies and sialic acid analysis of glycoproteins.

Samples

Protein

Measure the protein concentrations of solutions for sialic acid analysis by using the BCA Protein Assay (Pierce Chemical).

Acid Hydrolysis

Dissolve each of the following proteins in 400 µL of 0.1 M hydrochloric acid: 51 µg of bovine fetuin, 83 µg of human transferrin, and 88 µg of bovine transferrin. Heat for 1 hour at 80 ˚C; dry in a SpeedVac (Savant); and redissolve each hydrolysis in 450 µL of deionized glass-distilled water and 50 µL of 0.1 mM KDN prior to analysis. These are general hydrolysis conditions. Because acid will destroy some of the sialic acid it releases, hydrolysis conditions should be optimized. To optimize acid hydrolysis conditions for a glycoprotein, design experiments similar to those described in Fan et al.[10]

Neuraminidase Digestion

Dissolve each of the following proteins in 200 µL of 0.1 M sodium acetate at pH 5 containing 1 mU neuraminidase: 51 µg of bovine fetuin, 83 µg of human transferrin, and 88 µg of bovine transferrin. Incubate the resulting solutions for 18 hours at 37 ˚C. Prior to analysis, add 250 µL of water and 50 µL of 0.1 mM KDN to each digest.

Autosampler Test

Prior to starting the analyses, the autosampler should be tested to ensure that it is functioning properly. To test the performance of a variable volume injector equipped with a 100-µL injection loop, analyze four injections each of 5, 10, 15, 25, 50, and 75 µL of a 10 µM solution of glucose. Use a 100 mM sodium hydroxide eluent at a flow rate of 1.5 mL/minute. Plot peak area against injection volume to determine linearity. The coefficient of determination (r^2) generated from linear regression should indicate linearity ($r^2 \geq 0.99$). Each replicate injection set should yield a peak area RSD of ≥ 1%. An RSD ≥ 2% indicates a need for maintenance (see the autosampler reference manual for troubleshooting guidance).

RESULTS AND DISCUSSION

Separation of Sialic Acids

As can be seen in Figure 1, Neu5Ac, KDN, and Neu5Gc are well resolved with this method. Eluting between Neu5Ac and Neu5Gc, KDN has an appropriate retention time to serve as an internal standard for analyzing these sialic acids. Because these three sugar acids are well resolved, there are many conditions that can be used to separate them (see Appendix). The separation used for the experiments described here was chosen because it is relatively fast (27 minutes between injections) and Neu5Ac is retained well beyond the void (t_R=5.8 min).

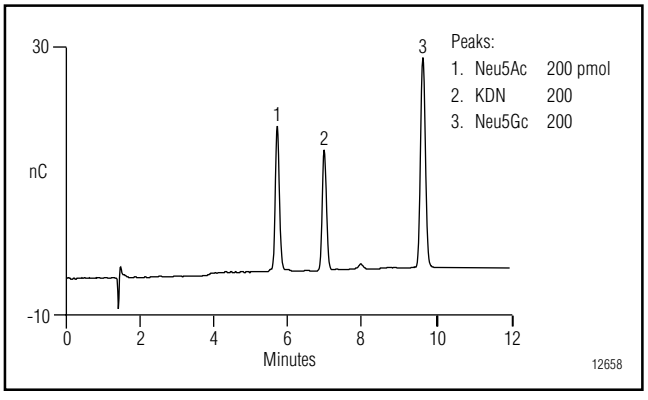

Figure 1 *HPAE/PAD separation of Neu5Ac (5.8 min), KDN (7.1 min), and Neu5Gc (9.8).*

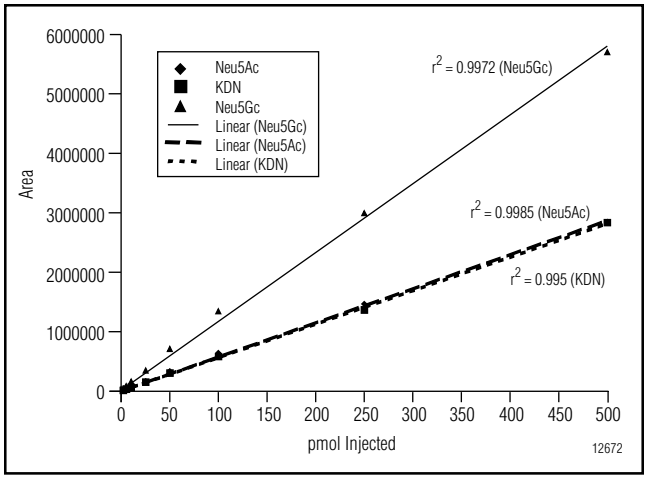

Figure 2 *Linearity analysis of sialic acids (2–500 pmol).*

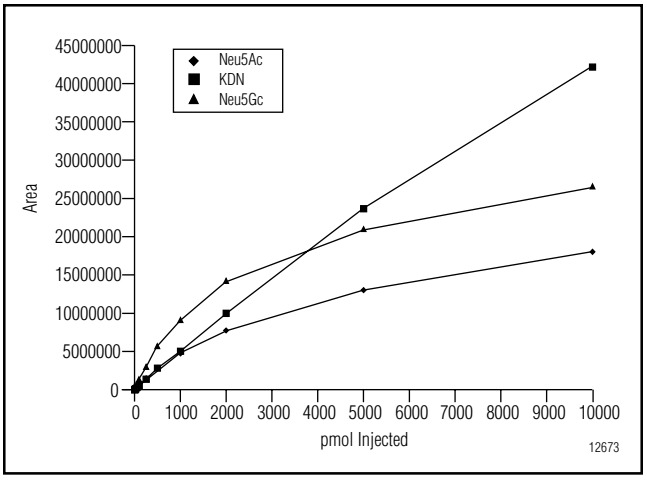

Figure 3 *Linearity analysis of sialic acids (2–10,000 pmol).*

Increased Neu5Ac retention may be beneficial if sialic acids are assayed in biological fluids or in the presence of nonionic detergents.

Linearity of Sialic Acid Analysis with HPAE-PAD

To ascertain the linearity and minimum detection limits of Neu5Ac, KDN, and Neu5Gc, 5 injections (25 µL) of the 0.4 mM mixture and 5 injections (25 µL) of each of the dilutions were made (see Table 1). Figure 2 shows that Neu5Ac, KDN, and Neu5Gc have good linearity between 2 and 500 picomole injections (r^2 = 0.9985, 0.9995, 0.9972, respectively). Figure 3 shows that the linearity range for KDN extends well beyond the ranges for Neu5Ac and Neu5Gc. Because the electrochemical response for KDN is linear within the linear range of Neu5Ac and Neu5Gc, KDN is an appropriate internal standard.

Minimum Detection Limits

Figure 4 shows a chromatogram of 1 picomole each of Neu5Ac, KDN, and Neu5Gc. Neu5Ac and Neu5Gc have peak heights over three times greater than the noise measured between 4 and 5 minutes. The peak height for KDN is slightly less than three times the same noise measurement. The peak height for 500 femtomoles of Neu5Gc is greater than three times the noise.

Reproducibility

To determine reproducibility, 200 picomoles (20 µL) of the standard mixture prepared for reproducibility studies (see "Preparation of Standards") was analyzed repetitively for 48 hours (107 injections). 500 µL of the standard mix were put into each of six vials. Twenty injections were made from five of the vials and seven injections were made from the sixth vial. Figure 5 shows the results of 107 consecutive injections of a standard mixture of Neu5Ac, KDN, and Neu5Gc. The area RSDs for Neu5Ac, KDN, and Neu5Gc are 2.4, 2.2, and 2.8%, respectively. Sometimes an initial five to seven injections are required until there is a stable response, as shown in Figure 7. When the responses of Neu5Ac and Neu5Gc are corrected with the internal standard, their area RSDs are 1.3 and 1.1%, respectively (see Figure 6). We have found that KDN corrects for some injection-to-injection variability. Some statistics from this experiment are reported in Table 2. We have also found that temperature affects the electrochemical response of these compounds. Therefore, it may be possible to improve response reproducibility by maintaining the electrochemical cell at a constant temperature. A second system had area RSDs of 4.0, 2.9, and 1.7%, respectively. After correcting with the internal standard, the area RSDs for Neu5Ac and Neu5Gc were 2.6 and 1.9%. In this case, KDN does not improve an already low RSD for Neu5Gc. In general, we have found that KDN is a

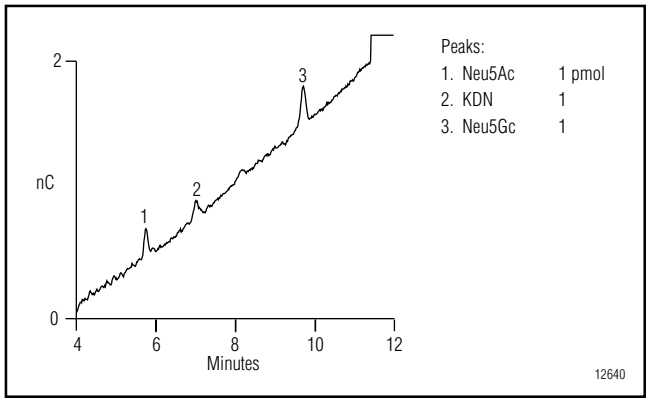

Figure 4 *HPAE-PAD of Neu5Ac, KDN, and Neu5Gc (1 pmol each).*

Table 2 Data and statistics from the repetitive sialic acid analyses

	48 h Analysis of Standards	48 h Analysis of Standards with Interspersed Samples
Average Neu5Ac Area (1 unit = 0.1 pC/sec)	1337469	1242470
Average KDN Area (1 unit = 0.1 pC/sec)	1330581	1182139
Average Neu5Gc Area (1 unit = 0.1 pC/sec)	2846095	2384343
Neu5Ac Area RSD (%)	2.4	4.1
KDN Area RSD (%)	2.3	4.5
Neu5Gc Area RSD (%)	2.8	4.0
Neu5Ac Area RSD (%) after Internal Standard Correction	1.3	2.5
Neu5Gc Area RSD (%) after Internal Standard Correction	1.1	2.3

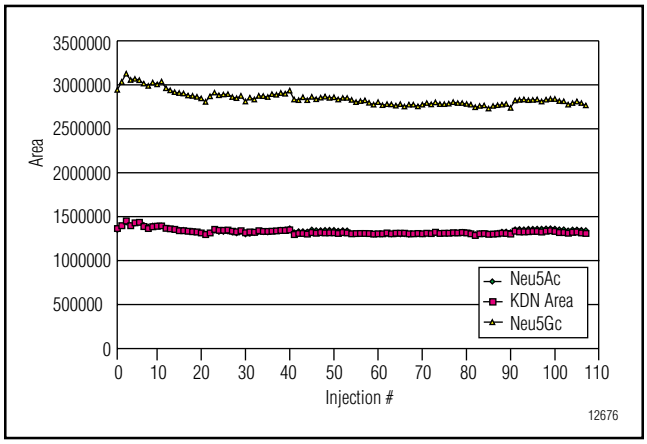

Figure 5 *48 hour repetitive analysis of sialic acids.*

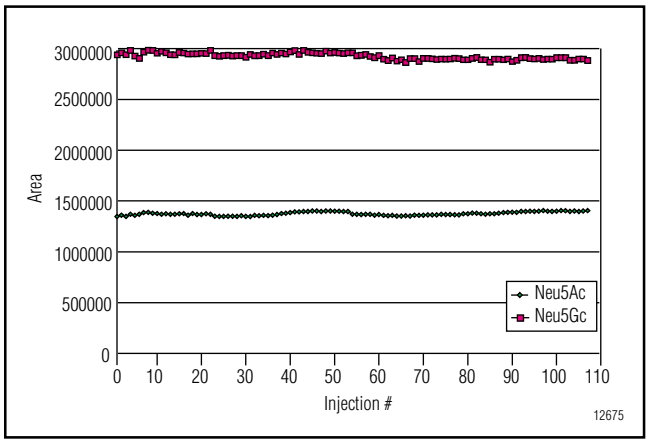

Figure 6 *Internal standard corrected.*

better internal standard for Neu5Ac than for Neu5Gc. The average retention times for Neu5Ac, KDN, and Neu5Gc were 5.78, 7.06, and 9.77 minutes, respectively. The retention time RSDs were 0.5, 0.4, and 0.3%, respectively. The second system had average retention times of 5.75, 7.05, and 9.79 minutes with RSDs of 0.7% each.

Reproducibility with Interspersed Samples

Under some circumstances, the presence of large amounts of protein has been observed to diminish the PAD response, presumably by fouling the electrode. The use of an internal standard usually compensates for this effect. To investigate whether the KDN internal standard provides adequate compensation under these analysis conditions, we made eight injections of the standard mixture (200 picomoles in 20 µL) followed by alternating four injection blocks of the sample (acid or neuraminidase digest) and the standard mixture. This experiment ran for 48 hours (108 injections). The samples were 0.1 N hydrochloric acid or neuraminidase digestions of bovine fetuin (2 µg in 20 µL), bovine transferrin (3.5 µg in 20 µL), or human transferrin (3.3 µg in 20 µL). During this experiment we also made two 20-µL injections of each digest blank (hydrochloric acid and neuraminidase). Figure 7 shows the effect of interspersing sample injections on the reproducibility of standard response. Some statistics from this experiment are reported in Table 2. Unlike the experiment in Figure 5, this experiment required seven injections to obtain a consistent response. Area RSDs, using every injection, were 4.1, 4.5, and 4.0%, respectively. After correcting with the internal standard, the area RSDs of Neu5Ac and Neu5Gc were 2.5 and 2.3%, respectively. A second system had area RSDs of 5.3, 5.3, and 6.2%, respectively. After correcting with internal standard, the area RSDs for Neu5Ac and Neu5Gc

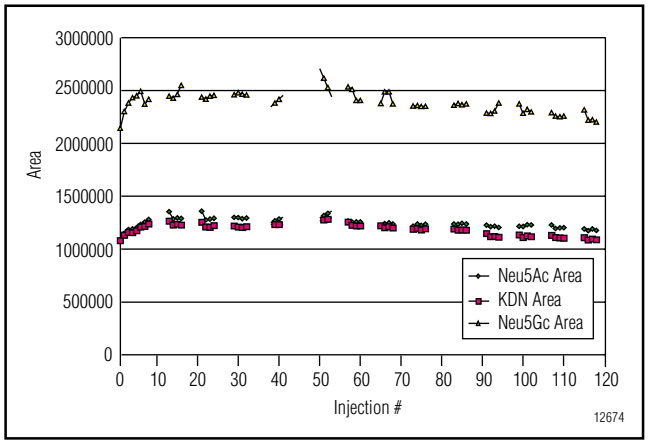

Figure 7 *48 hour sialic acid analysis with interspersed samples.*

were 2.3 and 1.8%. Though these experiments have higher area RSDs than the experiments without interspersed samples, the increased area RSDs are unlikely to be due to the samples, because four other 48-hour reproducibility experiments (two with standards, and two without standards) using an older working electrode (unpolished after > 1 year of operation) gave equivalent area RSDs that were all under 3%. The average retention times for Neu5Ac, KDN, and Neu5Gc were 5.79, 7.07, and 9.79 minutes, respectively. The retention time RSDs were 0.4% each. The second system had average retention times of 5.77, 7.04, and 9.77 minutes with RSDs of 0.3, 0.2, and 0.2%, respectively.

Sialic Acid Analysis of Glycoproteins

Table 3 shows the amount of Neu5Ac and Neu5Gc found in the three glycoproteins analyzed during the 48-hour experiment with interspersed samples. These values represent the data from four injections of duplicate samples of each preparation. The molecular weights for bovine fetuin, bovine transferrin, and human transferrin were assumed to be 48, 78, and 80 kDa, respectively. The sialic acid values determined for each glycoprotein are consistent with the known values[11–14]. Figure 8 shows chromatograms of 0.1 N hydrochloric acid (A) and neuraminidase digestions of bovine transferrin (B). Each chromatogram represents a 3.5-µg injection of bovine transferrin and the 200 picomoles of KDN that were added after sample digestion and subsequent drying. The response of KDN is less in the hydrochloric acid hydrolyses than in the neuraminidase digests. The response of KDN in the neuraminidase digests is the same as in the standard. Therefore, the response of KDN is reduced in the hydrochloric acid hydrolyses. The areas for Neu5Ac and Neu5Gc in Table 3 have been adjusted to account for this reduction, which is believed to be due in part to residual chloride from the hydrochloric acid (data not shown). We have found that when glycoproteins are hydrolyzed in 2 N acetic acid for 3 hours at 80 ˚C and analyzed by the method described here, KDN response is not inhibited. If comparison of neuraminidase and acid digestions indicates that neuraminidase completely releases the sialic acids from the glycoprotein (neuraminidase values are greater than or equal to hydrochloric acid values), we recommend using neuraminidase for the most accurate and precise results.

Table 3 Sialic acid determination of glycoproteins

Sample	Avg Mole Neu5Ac/M Protein	Avg Mole Neu5Gc/M Protein
Bovine Fetuin HCl	17 ± < 1	0.54 ± 0.05
Bovine Fetuin Neuraminidase	17 ± < 1	0.59 ± 0.04
Bovine Transferrin HCl	1.1 ± 0.1	1.9 ± < 0.1
Bovine Transferrin Neuraminidase	1.2 ± < 0.1	2.2 ± < 0.1
Human Transferrin HCl	3.8 ± < 0.1	not detected
Human Transferrin Neuraminidase	3.7 ± < 0.1	not detected

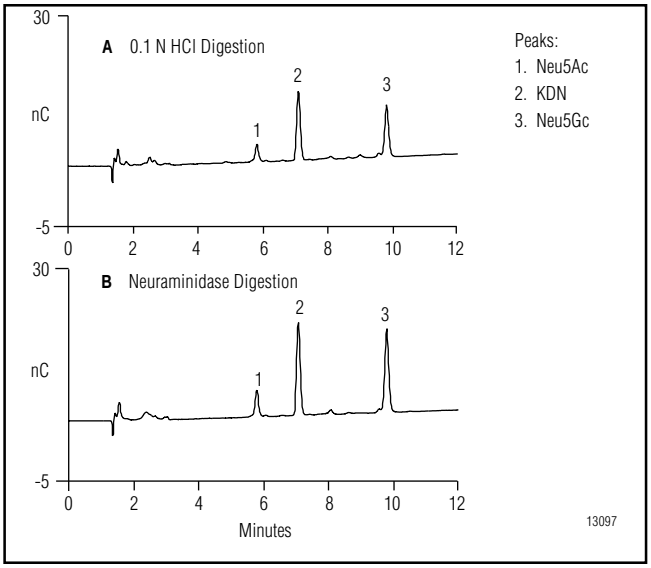

Figure 8 *Sialic acid analysis of bovine transferrin.*

ADVICE AND TROUBLESHOOTING

System Shutdown

After the experiment has finished, turn the electrochemical cell off and flow 100 mM sodium hydroxide through the cell at 0.1 mL/min. Install water and 200 mM sodium hydroxide in the other two eluent positions and proportion these to a ratio of 50:50. Turning off the cell minimizes working electrode wear.

Autosampler

Reproducibility is adversely affected by poor autosampler performance. When injection reproducibility is poor, first check that the flush line, sample syringe, and sample vials are free of air bubbles. If no bubbles are present, the likely cause is a worn rotor seal. The manufacturer recommends replacing the rotor seal at least every six

months. If there are missed or extremely low injections, it is possible that the injection needle is fully or partially obstructed. If the needle cannot be cleaned, replace it. The manufacturer recommends replacing the sample needle at least every six months. If the first injection from a vial is missed or very low, it is an early sign that the rotor seal is failing and should be replaced. Additional autosampler troubleshooting information is available in the AS3500 manual.

Working Electrode and Electrochemical Response

The electrochemical response for sialic acids is related to the state of the working electrode. The longer a working electrode is used without polishing, the lower its sensitivity. Table 2 shows a reduction in responses for each of the sialic acids between the two experiments. The experiment with interspersed samples was conducted two weeks after the first experiment. It was found that the response for sialic acids of a new, freshly polished working electrode was twice that of an electrode that had not been polished in over a year. This old electrode, an extreme case, exhibited MDLs (3x noise) of 2, 5, and 2 picomoles for Neu5Ac, KDN, and Neu5Gc, respectively. The old electrode had a linear range of 10 to 2000 picomoles rather than the 2 to 500 picomoles observed for the new electrode. The two-day reproducibilities of the two working electrodes were equivalent. Equivalent results were obtained when glycoprotein samples were analyzed as described above with the KDN internal standard using the new working electrode and then using the old working electrode. Electrodes should be polished as described in the ED40 manual and then erased with a sulfur-free pencil eraser prior to initial use only. If the electrode is not "erased" after polishing, response for sialic acids will steadily increase for at least 48 hours. With "erasing", response stabilizes after eight to ten injections. After 500 hours of operation using the separation conditions presented here, it was found that the working electrode was not worn enough to warrant polishing. This conclusion was reached after observation under a microscope.

Internal Standards

This document highlights the importance of using an internal standard. 3-deoxy-D-manno-2-octulosonic acid (KDO) has also been used as an internal standard for sialic acid analysis using HPAE-PAD[9]. Using the conditions presented here, KDO and KDN are not resolved. KDN was chosen because of its greater structural similarity to Neu5Ac and Neu5Gc. Glucuronic acid elutes between Neu5Ac and Neu5Gc, but its response does not trend in the same direction as the responses of both Neu5Ac and Neu5Gc, and using it as an internal standard can increase the area RSDs for Neu5Ac and Neu5Gc. *N*-acetylmuramic acid may be an effective internal standard, but it elutes after Neu5Gc, which is not ideal for an internal standard and would increase analysis time. At least one sample should be analyzed without KDN to ensure that there is no naturally occuring KDN in the sample or no unknown peak that will interfere with quantification.

Eluent Preparation

When preparing sodium hydroxide-containing eluents, the bottom few centimeters of the 50% (w/w) sodium hydroxide solution should not be used. This contains a large amount of carbonate ion, which causes a large baseline upset between 4 and 5.5 minutes in the chromatogram.

CONCLUSION

We have characterized the minimum detection limits, response linearity, and reproducibility for a sialic acid analysis method using HPAE-PAD. This document also shows that KDN is an effective internal standard that should be used for accurate sample quantification. These results will be helpful in determining "expected performance" with respect to validating sialic acid analysis methods using HPAE-PAD.

APPENDIX

Table 4 lists some of the other separation conditions we have investigated. If faster separations are desired, the flow rate can be increased to 1.5 mL/min or isocratic conditions can be used. Isocratic separations suffer from poor retention of Neu5Ac, which may be problematic for some samples.

LIST OF SUPPLIERS

Fluka Chemika-BioChemika, Fluka Chemie AG
Industriestrasse 25, CH-9471 Buchs, Switzerland.
Tel.: 81 755 25 11.

Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania, 15219-4785, U.S.A. Tel.: 800-766-7000.

Pfanstiehl Laboratories, Inc., 1219 Glen Rock Ave.
Waukegan, Illinois, 60085-0439, U.S.A.
Tel.: 800-383-0126.

Table 4 Separation conditions and retention times for sialic acids using the CarboPac PA10 (Guard and Separator)*

Separation Type	Gradient Time (min)	[NaOH] (mM)	Initial [NaOAc] (mM)	Final [NaOAc] (mM)	Neu5Ac Retention Time (min)	KDN Retention Time (min)	Neu5Gc Retention Time (min)
Gradient	20	100	20	180	11.5	14.3	20.8
Gradient	20	100	50	180	8.8	11.8	19.2
Gradient	15	100	50	180	8.3	10.8	16.8
Gradient	10	100	50	180	7.8	9.7	14.4
Gradient	15	100	70	200	7.0	9.0	14.8
Gradient	15	100	70	250	6.8	8.7	13.0
Gradient	15	100	70	300	6.2	8.1	11.7
Gradient	15	100	70	350	6.1	7.8	10.8
Gradient	10	100	70	300	5.9	7.3	10.0
Gradient	15	100	100	200	5.5	7.2	13.0
Gradient	15	100	100	250	5.2	7.1	11.5
Isocratic	—	50	150	150	3.7	4.7	8.6
Isocratic	—	100	100	100	5.8	8.5	22.2
Isocratic	—	100	120	120	4.8	6.8	15.7
Isocratic	—	100	150	150	3.7	5.1	10.1
Isocratic	—	100	200	200	3.0	3.7	6.1

*The flow rate for all separations is 1 mL/min.

Applied Biosystems, Inc./Perkin Elmer, 850 Lincoln Centre Drive, Foster City, California, U.S.A. Tel.: 415-570-6667.

Toronto Research Chemicals, Inc., 2 Brisbane Rd. North York, Ontario, Canada, M3J 2J8. Tel.: 800-727-9240.

Gibco BRL, Life Technologies, Inc., P.O. Box 6009 8717 Grovemont Circle, Gaithersburg, Maryland, 20884-9980, U.S.A. Tel.: 800-828-6686.

Calbiochem, P.O. Box 12087, La Jolla, California, 92039-2087, U.S.A. Tel.: 800-854-3417.

Boehringer Mannheim Corporation, 9115 Hague Rd. Indianapolis, Indiana, 46250-0414, U.S.A. Tel.: 800-262-1640.

Pierce Chemical Company, 3747 N. Meridian Rd. P.O. Box 117, Rockford, Illinois, 61105, U.S.A. Tel.: 800-874-3723.

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