DIONEX

Application Note 278

Now sold under the Thermo Scientific brand

Thermo SCIENTIFIC

UHPLC Determination of Sialic Acids with Fluorescence Detection

INTRODUCTION

Protein post-translational modification is important in biological activity. Among the many modification motifs, sialylation has been shown to be critical in determining glycoprotein bioavailability, function, stability, and metabolism.[1] *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are routinely determined in glycoprotein products, although over 50 natural sialic acids, including numerous *O*-acetylated sialic acids, have been identified.[2] Because humans do not generally produce Neu5Gc and have antibodies against it, the presence of this sialic acid in a therapeutic agent can potentially lead to an immune response.[3] Consequently, therapeutic protein efficacy, pharmacokinetics, and potential immunogenicity are impacted by the degree and identity of sialylation in a biological pharmaceutical product.

To determine total sialic acid content, sialic acids are first released from glycoproteins by acid hydrolysis or enzymatic digestion. Once liberated, there are many options for quantification. Numerous spectroscopic methods exist; however, interferences in these methods can overestimate the concentration of sialic acids in many samples. Furthermore, these methods do not differentiate between the form of sialic acid and, therefore, chromatographic methods that separate the individual analytes are preferred.[4]

Among the chromatographic methods there are direct detection methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD),[5] and those that require sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC). One common fluorescent labeling method, using 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) to label the sialic acids, was first published by Hara et al.[6-7] Although the fluorescent labeling method determines sialic acids indirectly, the chromatographic conditions are less likely to change *O*-acetylated sialic acids, allowing identification of a wider range of sialic acids.[8]

In this work, *N*-acetylated sialic acids are determined and *O*-acetylated sialic acids are identified by UHPLC with fluorescence detection following acid hydrolysis and DMB derivatization of five glycoprotein samples: calf fetuin, bovine apo-transferrin, human transferrin, sheep α_1-acid glycoprotein, and human α_1-acid glycoprotein. The described assay uses a short format Acclaim® RSLC 120 C18 column that allows fast run times (10 min) and requires less organic solvent compared to a commonly used 40 min method. The sensitivity of fluorescence detection easily allows determination of sialic acids that are present in the pmol range in glycoprotein hydrolyzates. This sensitivity allows for straightforward determination of Neu5Ac, Neu5Gc, and *O*-acetylated sialic acids in the derivatized samples.

EXPERIMENTAL

Dionex UltiMate® RSLC system including:

- SRD-3600 solvent rack (Dionex P/N 5035.9230)
- HPG-3400RS pump with a 350 µL mixer (Dionex P/N 5040.0046)
- WPS-3000TRS autosampler (Dionex P/N 5840.0020)
- Sample loop, 25 µL (Dionex P/N 6820.2415)
- TCC-3000RS column compartment (Dionex P/N 5730.0000)
- Pre-column heater (Dionex P/N 6722.0530)
- Viper™ capillary kit, RS system (Dionex P/N 6040.2301)
- FLD-3400RS fluorescence detector with dual PMT (Dionex P/N 5078.0025)

Chromeleon® 7.0 Chromatography Workstation

Polypropylene injection vials with caps and septa, 0.3 mL (Dionex P/N 055428)

Microcentrifuge tubes, 1.5 mL (Sarstedt P/N 72.692.005)

Dry block heater (VWR P/N 13259-005)

REAGENTS AND STANDARDS

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Acetonitrile (Honeywell P/N 015-4)

Methanol (Honeywell P/N 230-4)

N-Acetylneuraminic acid (Neu5Ac, NANA) Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA) Ferro Pfanstiehl

Glyko® Sialic Acid Reference Panel (ProZyme P/N GKRP-2503)

Glacial acetic acid (J.T. Baker P/N 9515-03)

2-Mercaptoethanol (Aldrich P/N M6250)

Sodium hydrosulfite (Sigma P/N 157953)

1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) (Sigma P/N D4784)

Micro BCA™ Protein Assay Kit (Thermo Scientific, P/N 23235)

SAMPLES

Five glycoproteins were selected for analysis:

Calf fetuin (Sigma P/N F2379)

Bovine apo-transferrin (b. apo-transferrin) (Sigma P/N T1428)

Human transferrin (h. transferrin) (Sigma P/N T8158)

Sheep α_1-acid glycoprotein (s. AGP) (Sigma P/N G6401)

Human α_1-acid glycoprotein (h. AGP) (Sigma P/N G9885)

CONDITIONS

Column:	Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm
Mobile Phase:	9:7:84 Acetonitrile:methanol:DI water
Flow Rate:	0.45 mL/min
Temperature:	45 ºC (column compartment)
Inj. Volume:	1 µL
Detection:	Excitation λ, 373 nm Emission λ, 448 nm
Noise:	~2000 counts
System Backpressure:	~285 bar (~4130 psi)
Run Time:	10 min

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phase

Transfer 180 mL acetonitrile and 140 mL methanol into a 2 L glass volumetric flask. Fill the flask to the mark with DI water, cap the flask, and invert the flask to mix the mobile phase well.

Acetic Acid, 4 M

Transfer 22.5 mL glacial acetic acid to a polyethylene bottle containing 77.5 mL DI water.

Stock Standard Solutions

Dissolve 149.8 mg of Neu5Ac in 50 mL DI water and 41.0 mg Neu5Gc in 50 mL of DI water. This results in 9.68 mM and 2.52 mM stock solutions, respectively. Dilute 500 µL of 9.68 mM Neu5Ac and 130 µL of 2.52 mM Neu5Gc to 48.4 mL total with DI water. Aliquot this mixed stock of 0.10 mM Neu5Ac and 6.8 µM Neu5Gc into 1.5 mL cryogenic storage vials and store at -40 °C.

Standard Solutions

Both the stock solution described above and a combined sialic acids reference standard containing Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7,(8),9Ac3 were used to identify sialic acids.

Dissolve the contents of the combined reference standard vial in 25 µL DI water to prepare the standard for derivatization.

Prepare calibration standards by diluting the combined stock solution as shown in Table 1. For example: Pipet 100 µL combined stock solution into a 1.5 mL microcentrifuge tube. Pipet an additional 100 µL

Table 1. Sialic Acid Standards Preparation						
Combined Stock Sandard (µL)	Acetic Acid, 4 M (µL)	DI water (µL)	Neu5Ac (µM)	Neu5Gc (µM)	Neu5Ac in 1 µL Injection (pmol)	Neu5Gc in 1 µL Injection (pmol)
100	100	0	50	3.9	50	3.9
100	75	75	40	3.1	40	3.1
100	200	100	25	2.0	25	2.0
100	250	150	20	1.6	20	1.6
100	300	200	17	1.3	17	1.3
100	375	275	14	1.1	14	1.1
100	500	400	10	0.78	10	0.78
50	400	350	6.2	0.49	6.2	0.49
100	100	100	2.1	0.16	2.1	0.16

DI water and 200 µL of 4 M acetic acid to prepare a standard of 25 µM Neu5Ac and 2.0 µM Neu5Gc in 2 M acetic acid. It is critical that the standards are in the same matrix as the samples. If the standards are not prepared in the same acid concentration, the derivatization reaction efficiency will not be the same for both standards and samples, resulting in a potentially large systematic error in the analyte quantification.

Protein Stock Solutions, 4.0 mg/mL Nominal

Dissolve 3.44 mg of α_1-acid glycoprotein in 860 µL of DI water. Gently swirl to thoroughly mix the solution. Prepare 200 µL aliquots of the solution in microcentrifuge vials to minimize freeze/thaw cycles when the stock is needed. Store all protein solutions at -40 °C. Repeat this process for each glycoprotein.

Working Stock Protein Solutions

Pipet 250 µL of a protein stock solution into 1750 µL of DI water to prepare a ~0.5 mg/mL working stock. Aliquot 400 µL of the working stock into individual microcentrifuge tubes and store the working stock solutions at -40 °C. Measure the working stock protein concentrations before hydrolysis by using a colorimetric BCA protein assay kit. This assay is critical to determining the concentration of the working stock solutions.

Acetic Acid Hydrolysis of Proteins

Add 14 µg, 20 µg, 25 µg, 13 µg, and 7 µg of fetuin, h. transferrin, b. transferrin, h. AGP, and s. APG, respectively, to individual 1.5 mL microcentrifuge vials with 200 µL of 2 M acetic acid. For example, pipet 50 µL of the working protein stock, 50 µL of DI water, and 100 µL of 4 M acetic acid to prepare the solution for hydrolysis. Hydrolyze the protein solutions for 2 h by the method of Varki et al.[9] to preserve *O*-acetylated sialic acids. For comparison, these samples were also analyzed by HPAE-PAD without derivatization.[10]

DMB Derivatization Reagent

Prepare the DMB reagent in the order that follows. Add 1.5 mL of DI water to a glass vial. To this solution add 172 µL of glacial acetic acid. Mix well. To this solution add 112 µL of 2-mercaptoethanol. Mix the solution well. Add 4.9 mg of sodium hydrosulfite to the solution and mix. The solution may become cloudy in appearance. Lastly, add 3.5 mg of DMB hydrochloride and 200 µL DI water and mix the solution well. The reagent is light sensitive and should be stored at -20 °C in the dark when not in use. Best results were obtained in this work with fresh derivatization reagent. Previous authors have reported stability of the derivatization reagent when it is stored at -20 °C in amber plastic vials.[11] However, for this work the reagent was used within 24 h of preparation.

Derivatization Conditions

Derivatize samples and standards by adding 50 µL of the derivatization reagent to 50 µL of sample in a 1.5 mL screw-cap microcentrifuge vial. Transfer the vials to a heating block and incubate for 2.5 h in the dark at 50 ± 2 °C. Samples, standards, and controls must be derivatized at the same time with the same preparation of derivatization reagent. After 2.5 h of incubation, freeze the solutions at -40 °C to slow the reaction.

Thaw the samples and transfer to 0.3 mL injection vials. Best results were obtained within 24 h of derivatization. Derivatized samples both continue to react with excess DMB and also degrade upon exposure to light and oxygen and should be analyzed as soon as possible.

Precautions

Derivatization reagent preparation, sample derivatization, and sample transfers to injection vials must be performed in a fume hood. Analyze samples promptly. Derivatized samples will degrade faster on exposure to light and it is strongly recommended that a temperature-controlled autosampler be set to 4 °C and the samples be kept in the dark by using amber vials or by keeping the autosampler cover closed. When filling low-volume conical vials, it is important to ensure that all air is removed from the cone of the vial. If bubbles are present, peak area precision will be poor.

As noted by Hara et al., the concentration of acid will affect the efficiency of the reaction.[6,7] It is important for the sample conditions to be matched in the standards that are derivatized to avoid systematic error due to different derivatization efficiency. For best accuracy, derivatize the standards in a matrix as similar to the samples as possible. Also, optimize the derivatization conditions to ensure full response from the sialic acids present in the sample. Take care to find conditions that derivatize as much of the free sialic acids in samples as possible, without degrading the standards that are derivatized in parallel to the samples. Such decomposition can lead to systematically high results. The best conditions can vary between different types of samples, so consider the conditions listed above as guidelines only.

RESULTS AND DISCUSSION

Figure 1 shows the separation of a sialic acid reference standard mixture on the Acclaim RSLC C18 column. As can be seen, Neu5Gc and Neu5Ac are well separated from one another. The *O*-acetylated sialic acids are also present in this standard with Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7(8),9Ac3 identified. In the case of Neu5,9Ac2, a reagent peak can co-elute, resulting in interference with the sialic acid peak, as depicted in Figure 2. The intensity of this reagent peak varied with the derivatization reagent preparation. The sialic acids of interest are separated in under 5 min, with an additional 5 min of run time to ensure that late-eluting peaks are removed from the column.

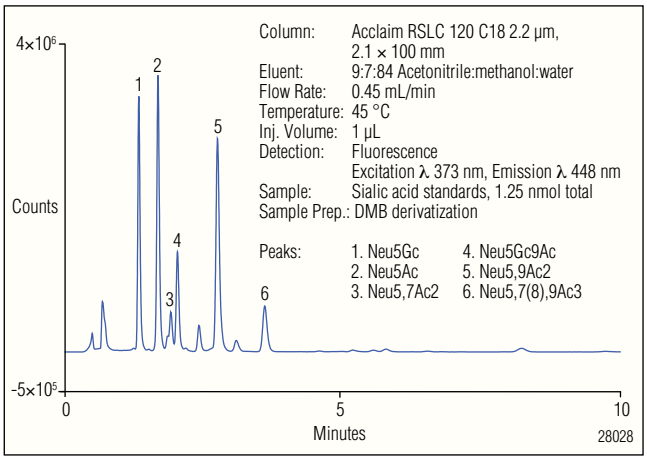

Figure 1. Separation of DMB derivatized sialic acid standards on the Acclaim RSLC 120 C18 column.

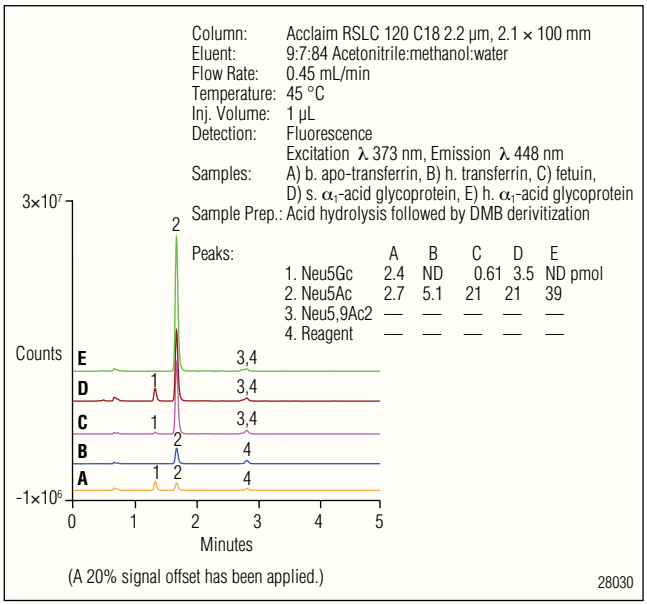

Figure 2. Separation of DMB derivatized sialic acids from five glycoprotein acid hydrolyzates on the Acclaim RSLC 120 C18 column.

Table 2. Linearity, LOD, LOQ, and Precision (n=5) for DMB Derivatized Sialic Acid Determination							
Standard	**Retention Time (min)**	**Retention Time Precision (RSD)**	**Peak Area Precision* (RSD)**	**Range (pmol)**	**Coeff. of Determination (r^2)**	**LOD (pmol)**	**LOQ (pmol)**
Neu5Ac	1.70	0.08	2.71	2.1–50	0.995	0.017	0.05
Neu5Gc	1.35	0.06	2.66	0.16–3.9	0.995	0.018	0.05

*Peak area precision is measured with a standard of 25 pmol Neu5Ac and 2.0 pmol Neu5Gc

Linear Range, Limit of Quantification, Limit of Detection, and Precision

Table 2 shows the calibration range, coefficients of determination, and precisions for injections of sialic acid standards. The efficiency of the derivatization reaction has significant impact on the peak area of standards from one preparation to the next. Preparing standards along with samples limits the effects of this variability; however, between-day peak areas were observed to vary by 17% and 19%, respectively, for Neu5Ac and Neu5Gc over 10 days of analysis. Similarly the limit of quantification (LOQ) and limit of detection (LOD) will vary by the efficiency of the reaction and may change between analysis days. Using the conditions described, the LOQ and LOD were determined to be 0.05 pmol and 0.017 pmol, respectively, for Neu5Ac. The LOQ and LOD for Neu5Gc were 0.05 and 0.018 pmol, respectively.

Reaction efficiency not only varies day-to-day, but also depends on the standard concentration. Greater variability in the peak area of high-concentration calibration standards was observed when injecting them throughout a sequence. The peak area increases as the derivatization reaction slowly continues. This increase in peak area is generally <5% for a sequence of 18 h; however, it should be noted that both further derivatization or decomposition can occur while the samples are in the autosampler. Peak area precisions listed in Table 2 are based on bracketing injections of a 25 pmol Neu5Ac and 2.0 pmol Neu5Gc combined standard. Sequential triplicate injections of the standard have peak area RSDs of 0.20 and 0.32 for Neu5Ac and Neu5Gc respectively.

Table 3. One Day of Triplicate Sample Analysis, Triplicate Injections per Sample						
Sample (replicate)	**Analyte**	**Amount (pmol)**	**Peak Area (counts*min)**	**Peak Area Precision (RSD)**	**RT (min)**	**RT Precision (RSD)**
fetuin (1)	Neu5Gc	0.6	9900	1.69	1.34	0.07
	Neu5Ac	25	465200	1.57	1.68	<0.01
fetuin (2)	Neu5Gc	0.7	12500	1.33	1.34	<0.01
	Neu5Ac	31	586400	2.12	1.68	<0.01
fetuin (3)	Neu5Gc	0.7	12200	0.51	1.34	0.01
	Neu5Ac	30	570100	0.31	1.68	0.01
h. transferrin (1)	Neu5Gc	ND				
	Neu5Ac	5.4	98700	0.96	1.68	0.06
h. transferrin (2)	Neu5Gc	ND				
	Neu5Ac	5.5	99900	0.67	1.68	0.01
h. transferrin (3)	Neu5Gc	ND				
	Neu5Ac	7.5	138200	0.65	1.68	<0.01
b. apo-transferrin (1)	Neu5Gc	2.8	50600	0.49	1.34	0.07
	Neu5Ac	2.8	48100	0.53	1.68	0.06
b. apo-transferrin (2)	Neu5Gc	3.2	57500	0.59	1.34	0.07
	Neu5Ac	3.1	54400	0.96	1.68	<0.01
b. apo-transferrin (3)	Neu5Gc	3.2	57400	0.88	1.34	<0.01
	Neu5Ac	3.1	54100	0.74	1.68	0.06
h. AGP (1)	Neu5Gc	ND				
	Neu5Ac	36	671200	0.22	1.69	<0.01
h. AGP (2)	Neu5Gc	ND				
	Neu5Ac	34	633700	1.73	1.69	0.06
h. AGP (3)	Neu5Gc	ND				
	Neu5Ac	46	867300	1.52	1.70	<0.01
s. AGP (1)	Neu5Gc	3.5	64900	0.81	1.35	0.01
	Neu5Ac	21	397500	0.95	1.69	<0.01
s. AGP (2)	Neu5Gc	3.6	66800	0.31	1.35	<0.01
	Neu5Ac	22	410000	0.42	1.69	0.07
s. AGP (3)	Neu5Gc	4.2	78800	1.26	1.35	<0.01
	Neu5Ac	26	486800	1.23	1.69	<0.01

ND = not detected

Determination of Sialic Acids

Figure 2 shows the separation of acid hydrolyzed and lyophilized protein samples. In each case, Neu5Ac and Neu5Gc are well separated from other components in the sample. In each case, Neu5Ac is present, and as expected, Neu5Gc is not detected in the human glycoproteins. Table 3 presents the results from one day of triplicate sample analysis.

Retention time precision was similar to that determined by injecting standards, with retention time RSDs ranging from <0.01 to 0.07. Peak area precision for triplicate injections, as measured by RSD, is generally good at <2.0. However, trending toward higher peak areas is observed, particularly in samples such as h. AGP and fetuin, which contain higher concentrations of Neu5Ac in the hydrolyzates compared to the other glycoproteins. Derivatization optimization for the individual samples may improve this variability.

Table 4. Triplicate Sample Analysis Intraday and Between-Day Precision Over Three Days					
Sample	**Analyte**	**Average (One Day of Analysis) (pmol)**	**Average (mol analyte/mol protein)**	**Intraday Precision (RSD)**	**Between-Day Precision (RSD)**
fetuin	Neu5Gc	0.67	0.46	19	18
	Neu5Ac	29	20	18	19
h. transferrin	Neu5Gc	ND	ND	—	—
	Neu5Ac	6.1	4.8	2.7	12
b. apo-transferrin	Neu5Gc	3.1	1.9	2.4	13
	Neu5Ac	3.0	1.9	2.4	9.5
h. AGP	Neu5Gc	ND	ND	—	—
	Neu5Ac	39	25	6.8	13
s. AGP	Neu5Gc	3.7	4.0	6.1	11
	Neu5Ac	23	24	6.0	11

Precision and Accuracy of Determination

Table 4 shows the calculated results of sialic acid analysis for the proteins studied, intraday precision for one day of triplicate sample analysis, and between-day precision for three days of triplicate sample analysis, for a total of nine sample preparations for each glycoprotein. Variability between sample replicates can be large, with RSDs ranging from 2.4 to 19; therefore, optimization of the hydrolysis for individual glycoproteins is recommended. Between-day precisions, as RSD, range from 9.5 to 19. For the best accuracy, both the acid hydrolysis and subsequent DMB derivatization must be optimized with respect to one another and this combined optimization is highly recommended. Nonetheless, the amounts of sialic acids determined in the protein samples are consistent with literature results for the glycoproteins.[12-17]

Method accuracy was investigated by spiking protein acid hydozylates with known amounts of Neu5Ac and Neu5Gc equal to the determined amounts. For human glycoproteins, which did not contain Neu5Gc, 0.50 pmol of Neu5Gc was added. Recoveries for Neu5Ac ranged from 82–108% (Table 5). Recoveries for Neu5Gc were similar, ranging from 86–101%. These recoveries suggest that the method is accurate.

Table 5. Recoveries, Triplicate Hydrolyses		
Sample	**Analyte**	**Average Recovery (%)**
reagent blank	Neu5Gc	87.5
	Neu5Ac	90.7
fetuin	Neu5Gc	86.4
	Neu5Ac	81.6
h. transferrin	Neu5Gc	83.5
	Neu5Ac	102
b. apo-transferrin	Neu5Gc	97.9
	Neu5Ac	94.5
h. AGP	Neu5Gc	86.7
	Neu5Ac	86.6
s. AGP	Neu5Gc	101
	Neu5Ac	108

Table 6. Analysis of DMB Derivatized Samples Stored for 17 Days at -40 °C								
Sample (replicate)	**Analyte**	**Amount (pmol)**	**Peak Area Precision (RSD)**	**Peak Area (counts*min)**	**Amount After Storage (pmol)**	**Amount After Storage (pmol)**	**Peak Area (counts*min)**	**Change in Amount (change in peak area)**
					Freshly derivatized standards	Stored derivatized standards		
h. AGP (1)	Neu5Gc							
	Neu5Ac	49	0.70	887100	51	49	741700	4.9% (-16%)
h. AGP (2)	Neu5Gc							
	Neu5Ac	43	0.63	783100	52	50	761600	22% (-2.7%)
h. AGP (3)	Neu5Gc							
	Neu5Ac	44	1.01	797900	59	58	861400	35% (8.0%)
s. AGP (1)	Neu5Gc	4.1	1.03	70700	4.5	4.3	61700	9.7% (-13%)
	Neu5Ac	24	0.94	431300	26	25	373300	7.9% (-13%)
s. AGP (2)	Neu5Gc	3.7	1.21	63300	3.5	3.4	48500	-5.4% (-23%)
	Neu5Ac	21	0.58	385600	20	19	293300	-5.6% (-24%)
s. AGP (3)	Neu5Gc	4.1	1.36	70400	4.4	4.2	60600	7.3% (-14%)
	Neu5Ac	24	1.37	428000	25	24	366800	6.8% (-14%)

Sample Stability after Derivatization

A set of DMB derivatized glycoprotein hydrolyzates was re-analyzed after 17 days of storage at -40 °C. The analysis was completed with both a freshly derivatized set of standards and the original standards prepared and stored with the samples. The comparative results of these stored samples are shown in Table 6. Values across replicates can be more variable after storage, with determined amounts being as much as 35% higher than the original quantification. This can be due to both continued derivatization of the samples, as well as degradation of the standards. Because the peak areas drop an average of 12%, it is likely that decomposition of the standards dominate the differences in quantification. Day-to-day variability in derivatization of standards can make comparison of samples with different standard curves difficult to interpret. Therefore, prompt analysis of samples with standards derivatized in parallel is recommended.

Sample Preparation Comparison to HPAE-PAD Analysis

Additional work illustrates the application of HPAE-PAD in the analysis of these samples.[10] Some comparisons can be made to this work. HPAE-PAD is a direct method that does not require derivatization; however, the strong base elution conditions do not allow for determination of *O*-acetylated sialic acids. If only the total amount of Neu5Ac and Neu5Gc are of interest, both HPLC and HPAE-PAD methods are appropriate because the *O*-acetylated sialic acids will hydrolyze in the basic elution conditions used for HPAE-PAD to the parent Neu5Ac or Neu5Gc. The time required to prepare samples for the two methods are dramatically different. Both methods require an optimized acid hydrolysis for consistent sample analysis. These steps will take approximately 3 h in total for a set of three triplicate samples and controls (12 digestions total). In addition to the time required for acid hydrolysis, DMB derivatization will require 2.5 h for the reaction with an additional 1 h to stop the reaction and prepare the samples for injection after the derivatization is complete.

CONCLUSION

In this work, glycoprotein acid hydrolyzates are analyzed to determine *N*-acetylated sialic acids and identify *O*-acetylated sialic acids by DMB derivatization followed by UHPLC and fluorescence detection. An isocratic method was developed with a mobile phase consisting of 9:7:84 acetonitrile:methanol:DI water and a short format Acclaim RSLC 120 C18 column that allows high resolution of the sialic acids in a 10 min analysis time and requires less organic solvent than other published methods. The sensitivity of fluorescence detection easily allows straightforward determination of sialic acids in the pmol range.

LIST OF SUPPLIERS

VWR, 1310 Goshen Parkway, West Chester, PA 19380 U.S.A. Tel: 800-932-5000. www.vwr.com

Fisher Scientific, One Liberty Lane, Hampton, NH 03842 U.S.A. Tel: 800-766-7000. www.fishersci.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178 U.S.A. Tel: 800-325-3010. www.sigma-aldrich.com

Ferro Pfanstiehl, 1219 Glen Rock Avenue, Waukegan, IL, 60085, U.S.A. Tel: 800-383-0126. www.ferro.com

ProZyme, 1933 Davis Street, Suite 207, San Leandro, CA 94577, U.S.A. Tel.800-457-9444. www.prozyme.com

REFERENCES

1. Byrne, B.; Donohoe, G.G.; O'Kennedy, R. Sialic Acids: Carbohydrate Moieties that Influence the Biological and Physical Properties of Biopharmaceutical Proteins and Living Cells. *Drug Discovery Today* **2007**, *12*, 319–326.
2. Angata, T.; Varki, A. Chemical Diversity in the Sialic Acids and Related α-Keto Acids: An Evolutionary Perspective. *Chem Rev.* **2004**, *102*, 439–469.
3. Padler-Karavani, V; Yu, H.; Cao, H.; Chokhawala, H.; Karp, F.; Varki, N.; Chen, Xi.; Varki, A. Diversity in Specificity, Abundance, and Composition of Anti-Neu5Gc Antibodies in Normal Humans: Potential Implications for Disease. *Glycobiology* **2008**, *18*, 818–830.
4. Lacombe, R.; Salcedo, J.; Algeria, A.; Lagarda, M.J.; Barbera, R.; Mantencio, E. Determination of Sialic Acid and Gangloisides in Biological Samples and Dairy Products. *J. Pharm. Biomed. Anal.* **2010**, *51*, 346–357.
5. Rohrer, J.S. Analyzing Sialic Acids Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **2000**, *283*, 3–9.
6. Hara, S.; Yamaguchi, M.; Takemori, Y.; Nakamura, M. Highly Sensitive Determination of *N*-Acetyl- and *N*-Glycolylneuraminic Acids in Human Serum and Urine and Rat Serum by Reversed-Phase Liquid Chromatography with Fluorescence Detection, *J. Chromatogr., B: Biomed.* **1986**, *377*, 111–119.
7. Hara, S.; Yamaguchi, M.; Takemori, Y.; Furuhata, K.; Ogura, H.; Nakamura, M.; Determination of Mono-*O*-Acetylated *N*-Acetylneuraminic Acids in Human and Rat Sera by Fluorometric High-Performance Liquid Chromatography, *Anal. Biochem.*, **1989**, *179*, 162–166.
8. Klein, A.; Diaz, S.; Ferreira, I.; Lamblin, G.; Roussel, P.; Manzi, A.E. New Sialic Acids from Biological Sources Identified by a Comprehensive and Sensitive Approach: Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) of SIA Quinoxalinones. *Glycobiology* **1997**, *7*, 421–432.
9. Varki, A.; Diaz, S. The Release and Purification of Sialic Acids from Glycoconjugates: Methods to Minimize the Loss and Migration of *O*-Acetyl Groups. *Anal. Biochem.* **1984**, *137*, 236–247.
10. Dionex Corporation, *Direct Determination of Sialic Acids in Glycoprotein Hydrolyzates by HPAE-PAD.* Application Update 180, LPN 2831, **2011**, Sunnyvale, CA 2011.
11. Spichtig, V.; Michaud, J.; Austin, S. Determination of Sialic Acids in Milks and Milk-Based Products. *Anal. Biochem.* **2010**, *405*, 28–40.
12. Spiro, R. G.; Bhoyroo, V. D. Structure of the *O*-Glycosidically Linked Carbohydrate Units of Fetuin. *J. Biol. Chem.* **1974**, *249*, 5704–5717.

13. Townsend, R. R.; Hardy, M. R.; Cumming, D. A.; Carver, J. P.; Bendiak, B. Separation of Branched Sialylated Oligosaccharides Using High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **1989**, *182*, 1–8.

14. Richardson, N. E.; Buttress, N.; Feinstein, A.; Stratil, A.; Spooner, R. L. Structural Studies on Individual Components of Bovine Transferrin. *Biochem. J.* **1973**, *135*, 87–92.

15. Spik, G.; Bayard, B.; Fournet, B.; Strecker, G.; Bouquelet, S.; Montreuil, J. Studies on Glycoconjugates. LXIV. Complete Structure of Two Carbohydrate Units of Human Serotransferrin. *FEBS Lett.* **1975**, *50*, 296–299.

16. Friedrich, M.; Berger, E.G. Effect of Neuraminidase Treatment of IgG and Alpha 1-Acid Glycoprotein on Their Intestinal Transport in Suckling Rats in Vivo, *J. Pediatr. Gastroent. Nutr.* **1982**, *1*, 395–399.

17. Kuster, B.; Hunter, A.P.; Wheeler, S.F.; Dwek, R.A.; Harvey, D.J. Structural Determination of *N*-Linked Carbohydrates by Matrix-Assisted Laser Desorption/ Ionization-Mass Spectrometry Following Enzymatic Release Within Sodium Dodecyl Sulphate-Polyacrylamide Electrophoresis Gels: Application to Species-Specific Glycosylation of α_1-Acid Glycoprotein. *Electrophoresis* **1998,** *19,* 1950–1959.

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S./Canada (847) 295-7500

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

www.dionex.com

LPN 2817-02 PDF 08/16