In-depth Analysis of Non-derivatized N-linked Glycans Using Ion Chromatography- Orbitrap Mass Spectrometry

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ABSTRACT

We report on a novel workflow that combines fast release of *N*-linked glycans and their analysis by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) coupled to a Q Exactive mass spectrometer. Ion chromatography supports simultaneous separation and detection of neutral and sialylated (charged) glycans without the need for derivatization. The chromatographic resolution of glycans is based on the number of sialic acid units, branch and positional isomers and the presence/absence of core or outer arm fucose. This work exemplifies the importance of denaturation prior to digestion. The reproducibility of peak area distribution of glycans released from denatured monoclonal antibody (mAb) was excellent and within 5% for glycans present at ≥1% of the total glycan pool. MS^2 spectra with diagnostic fragments allow for highly reliable annotation of glycan species. High resolution separation of hu-AGP glycans resulted in 53 peaks illustrating the excellent resolving power of HPAE for charged glycan species. The method described here supports highly informative glycan analysis without introducing labeling bias.

INTRODUCTION

The development of a reliable, high throughput workflow for analysis of *N*-linked glycans is required in the biopharmaceutical industry. Currently, the commonly used analytical approaches are based on glycan derivatization, which is known to cause issues such as the differential loss of sialic acid and incomplete labeling. Our new sample preparation method includes a novel, fast in-solution glycoprotein digestion. The glycans are quickly purified from the proteins, and from reducing agents and detergents using graphitized carbon and HILIC SPE materials. Applying this approach, the preparation of 96 samples can be completed in approximately 2 hours, thus supporting high throughput analysis. The new glycan sample preparation method is scalable, making it adaptable to ion chromatography and other analytical approaches. In our workflow, glycan analysis was performed using HPAE coupled to high resolution MS. This new approach enabled us to conduct bias-free, in-depth characterization of *N*-linked glycans released from glycoproteins. Therefore, the present method is ideally suited for biopharmaceutical quality control (QC) and MS analysis of antibody-derived glycans.

MATERIALS AND METHODS

Sample preparation

Glycoprotein solutions were prepared at 10 mg/mL concentration in PBS buffer. PNGase F (Prozyme, Hayward, CA) was added to the mixture followed by incubation at 50 ^{0}C for 15 min. Deglycosylation was followed by clean up using graphitized carbon and HILIC SPE materials (Thermo Fisher Scientific, Rockford, IL).

High Performance Anion Exchange Chromatography

A Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ dual IC system with Thermo Scientific™ Dionex™ AS-AP autosampler was used for glycan separations with PAD detector. The column used was a Thermo Scientific™ Dionex™ CarboPac™ PA200 BioLC™ Analytical Column (3 x 250 mm) at 0.5 mL/min flow rate. The system was equipped with a Thermo Scientific™ Dionex™ ERD 500 electrolytically regenerated desalter. Current applied on the desalter was 350 mA and the regenerant (water) flow rate was 2 mL/min. The column flow stream was split post column and 280 µL/min was diverted into the suppressor eluent channel and MS.

Mass spectrometry

The Thermo Scientific™ Q Exactive™ mass spectrometer was employed in negative mode ESI. Full mass scan: *m/z* 400-2000, resolution: 70,000 (FWHM) at *m/z* 200, AGC: 1x10^6, maximum IT: 120 ms. Data dependent MS^2 using Top 10. Data analysis was performed using SimGlycan software (Premier Biosoft) .

RESULTS

Scheme 1. Workflow of glycan preparation

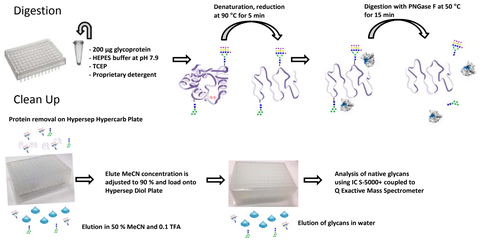
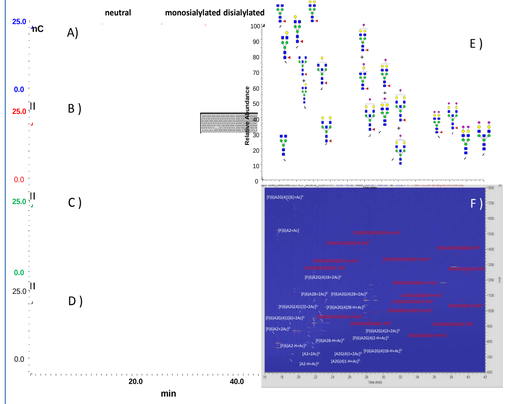


Figure 1. Effect of heat denaturation on deglycosylation and annotation of human IgG glycans



The workflow (Scheme 1) consists of a rapid release and clean up of the released glycans. Prior to releasing the glycans, the glycoproteins of interest must be heat denatured. This can be challenging since IgG type biosimilars are known to precipitate easily. In the presence of salts such as buffers, IgGs are even more prone to precipitation. The role of added detergent is to keep denatured glycoproteins, especially IgGs in solution.

Figure 1A shows the glycosylation profile of human polyclonal IgG (hu-IgG) dissolved in 1 X PBS buffer after heat denaturation and digestion. This is identical with Figure 1C where the IgG was dissolved in water and heat denaturation preceded the digestion. In the experiments shown in Figure 1B and 1D, the deglycosylation is not complete, as evidenced by the decreased amount of mono- and di-sialylated glycans. Glycan species occurring in hu-IgG were separated by a gradient developed for glycans released from mAb so some glycan species are not fully resolved here. Based on the resulting highly accurate mass values, accurate annotation can be readily accomplished (Figure 1E and 1F). As shown in the mass vs. time map (Figure 1F), neutral glycans form adducts with either one and two acetate ions, monosialylated glycans form adduct with one acetate ion.

Figure 2A and 2B reveal very good reproducibility for the new workflow (n=3 replicates of a therapeutic mAb) and indicate how extracted mass chromatograms (XIC) help identify minor glycan species such as the sialylated glycans in figure 2B. After baseline (blank) subtraction (important for reproducible glycan integration), "minor" peaks appear in the chromatogram. Figure 2B shows the base peak chromatogram of the mAb glycans. Annotation of sialylated peaks was performed by extracting the *m/z* values found in the time ranges depicted in the figure 2B insets (where sialylated glycans are expected to elute). Glycans identified (Figure 2C and Scheme 2) are consistent with those have been described in the literature [1].

Scheme 2. Glycans identified on mAb

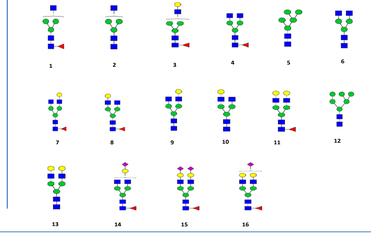
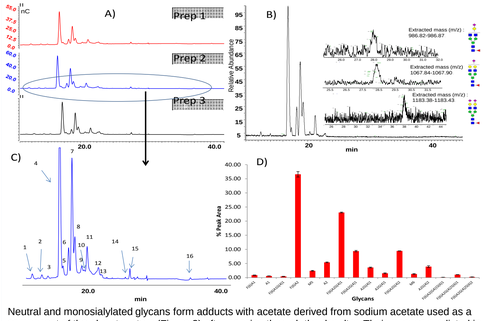


Figure 2. Reproducibility of sample preparation and annotation of minor peaks



Neutral and monosialylated glycans form adducts with acetate derived from sodium acetate used as a component of the eluent system (Figure 3) after passing through the desalter. Their masses are listed in Table I. For example, the adduct of F(6)A2G(4)1[6] formed with two acetate ions (*m/z 871.3084*) provides an information rich MS2 spectrum (Figure 4B) when compared to the adduct formed with one acetate (*m/z 841.2982*, Figure 4A). The fragment ion differences between the singly- and doubly- adducted acetate ions are listed in the Venn diagram (Figure 5). Notably, in negative ESI mode the precursor with two acetates generates considerably more crossring and glycosydic fragment ions, and is thus more important for annotation purposes.

Figure 3. Adducts of neutral and monosialylated glycans with acetate

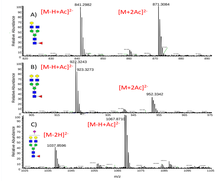


Table 1. Mass accuracy of the adducts

Glycan	Adduct structure	Adduct (theoretical)	Adduct (observed)	Mass accuracy (ppm)
G1F[6]	$[M+H+Ac]^{2-}$	841.3016	841.2982	-4
	$[M+2Ac]^{2-}$	871.3124	871.3084	-4.6
G2F	$[M+H+Ac]^{2-}$	922.3280	922.3243	-4
	$[M+2Ac]^{2-}$	952.3388	952.3342	-4.8
A1F	$[M-2H]^{2-}$	1037.8649	1037.8596	-5.1
	$[M+H+Ac]^{2-}$	1067.8757	1067.8710	-4.4

Figure 4. MS^2 spectra of the Acetate adducts of F(6)A2G(4)1 glycan

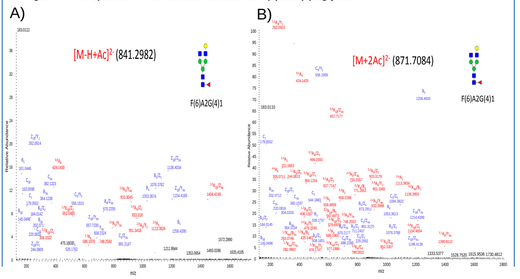


Figure 5. Venn diagrams showing unique and common fragment ions of adducts formed with acetate

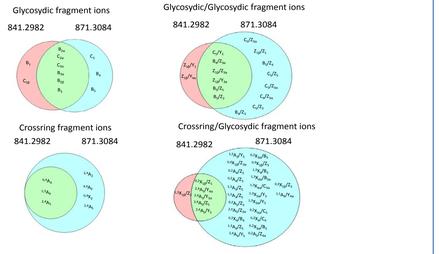
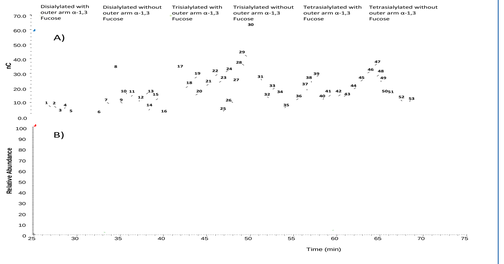


Figure 6 highlights the advantages of HPAE. Human α-1-Acid glycoprotein (AGP) has 5 glycosylation sites with glycans comprised of multiple sialic acid residues representing different charge states. Glycans terminated by either α-2,3 or α-2,6 sialic acid linkages can have many isomers which are well separated by HPAE (with glycans consisting of mostly α-2,3 linkages eluting later than those isomers dominated by α-2,6 linkages). MS^2 spectra of these isomers unfortunately don't quantify the number of different (α-2,3 vs. α-2,6) sialic acid linkages. However, a diagnostic ion at *m/z 306.119* ($^{0,4}A_2$-CO_2) corroborates the presence of α-2,6 linkages [2].

A further advantage of HPAE is that the presence of outer arm α-1,3 fucose reduces retention of glycans, eluting them significantly earlier. Thus, each charge state resolves into two subgroups of glycans (fucosylated and afucosylated), facilitating the identification. Glycans identified are listed in Table II and their structures are presented in Scheme 3. Glycans annotated in red were not recognized by the SimGlycan software, so these required manual annotation. The average mass accuracy of 53 peaks was -0.12 ppm showing excellent reliability of the data acquired by the Orbitrap mass analyzer.

Figure 6. PAD (A) and MS base peak (B) chromatograms of hAGP glycans



Scheme 3. Annotated glycans on hAGP

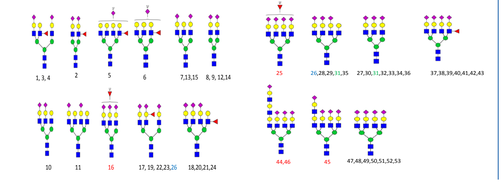


Table 2. Glycans identified from hAGP by HPAE-PAD/MS (X denotes unknown linkages of sialic acid)

	Peak No.	Retention time (min)	Observed mass	Charge state	Observed glycan mass	Theoretical glycan mass	Mass accuracy (ppm)	Glycan
Disialylated, outer arm fucose	1	27.15	910.3165	3	2733.9729	2733.9731	-0.07	A3F(3)1G(4)3S(X)2
	2	27.64	1183.4118	2	2368.8392	2368.8409	-0.72	A2F(3)1G(4)2S(X)2
	3	28.12	1365.9762	2	2733.9680	2733.9731	-1.87	A3F(3)1G(4)3S(X)2
	4	28.63	910.3166	3	2733.9732	2733.9731	0.04	A3F(3)1G(4)3S(X)2
	5	29.14	1032.0276	3	3099.1062	3099.1053	0.29	A4F(3)1G(4)4S(X)2
	6	32.53	1183.4121	2	2368.8398	2368.8409	-0.46	A2F(3)1G(4)2S(X)2
	7	33.84	1292.9484	2	2587.9124	2587.9152	-1.08	A3G(4)3S(X)2
	8	34.40	1110.3836	2	2222.7828	2222.7830	-0.09	A2G(4)2S(X)2
	9	35.37	1110.3836	2	2222.7828	2222.7830	-0.09	A2G(4)2S(X)2
	10	35.65	1292.9484	2	2587.9124	2587.9152	-1.08	A3G(4)3S(X)2
	11	36.15	1475.5149	2	2953.0454	2953.0473	-0.64	A4G(4)4S2(X)2
	12	37.07	1110.3834	2	2222.7824	2222.7830	-0.27	A2G(4)2S(X)2
	13	37.48	1292.9492	2	2587.9140	2587.9152	-0.46	A3G(4)3S(X)2
	14	38.27	1110.3836	2	2222.7828	2222.7830	-0.09	A2G(4)2S(X)2
	15	38.55	1292.9490	2	2587.9136	2587.9152	-0.62	A3G(4)3S(X)2
	16	40.08	1056.0351	3	3171.1287	3171.1264	0.73	A3F(3)2G(4)3S(X)3
Trisialylated, outer arm fucose	17	41.90	1007.3498	3	3025.0728	3025.0685	1.42	A3F(3)1G(4)3S(X)3
	18	42.60	1129.0591	3	3390.2007	3390.2007	0.00	A4F(3)1G(4)4S(X)3
	19	42.85	1007.3487	3	3025.0695	3025.0685	0.33	A3F(3)1G(4)3S(X)3
	20	43.27	1129.0590	3	3390.2004	3390.2007	-0.09	A4F(3)1G(4)4S(X)3
	21	44.34	1129.0591	3	3390.2007	3390.2007	0.00	A4F(3)1G(4)4S(X)3
	22	44.87	1007.3488	3	3025.0698	3025.0685	0.43	A3F(3)1G(4)3S(X)3
	23	45.86	1007.3495	3	3025.0719	3025.0685	1.12	A3F(3)1G(4)3S(X)3
	24	46.14	1129.0591	3	3390.2007	3390.2007	0.00	A4F(3)1G(4)4S(X)3
	25	46.85	1323.4594	3	3973.4016	3973.4120	-2.62	A4F(3)1G(4)4S(X)4
Trisialylated without outer arm fucose	26	48.00	1007.3486	3	3025.0692	3025.0685	0.23	A4F(3)1G(4)4S(X)3
	27	48.42	958.6630	3	2879.0124	2879.0106	0.63	A3G(4)3S(X)3
	28	49.36	1080.3734	3	3244.1436	3244.1428	0.25	A4G(4)4S(X)3
	29	49.76	1080.3728	3	3244.1418	3244.1428	-0.31	A4G(4)4S(X)3
	30	50.20	958.6631	3	2879.0127	2879.0106	0.73	A3G(4)3S(X)3
	31	51.21	1080.3732	3	3244.1430	3244.1428	0.06	A4G(4)4S(X)3
	32	51.85	958.6631	3	2879.0127	2879.0106	0.73	A3G(4)3S(X)3
	33	52.46	958.6627	3	2879.0115	2879.0106	0.31	A3G(4)3S(X)3
	34	53.20	958.6630	3	2879.0124	2879.0106	0.63	A3G(4)3S(X)3
	35	53.87	958.6631	3	2879.0127	2879.0106	0.73	A3G(4)3S(X)3
	36	54.21	1080.3732	3	3244.1430	3244.1428	0.06	A4G(4)4S(X)3
	37	56.54	958.6629	3	2879.0121	2879.0106	0.52	A3G(4)3S(X)3
Tetrasialylated, outer arm fucose	38	57.05	1226.0905	3	3681.2961	3681.2940	-0.33	A4F(3)1G(4)4S(X)4
	39	57.55	1226.0902	3	3681.2940	3681.2940	-0.57	A4F(3)1G(4)4S(X)4
	40	58.30	1226.0902	3	3681.2940	3681.2961	-0.57	A4F(3)1G(4)4S(X)4
	41	58.74	1226.0901	3	3681.2937	3681.2961	-0.65	A4F(3)1G(4)4S(X)4
	42	60.00	1226.0898	3	3681.2928	3681.2961	-0.90	A4F(3)1G(4)4S(X)4
	43	60.53	1226.0902	3	3681.2940	3681.2961	-0.57	A4F(3)1G(4)4S(X)4
	44	61.65	1226.0902	3	3681.2940	3681.2961	-0.57	A4F(3)1G(4)4S(X)4
	44	62.19	1420.8255	3	4265.4999	4265.5026	-0.63	A4G(4)Lac2S(X)4
	45	62.91	1299.1149	3	3900.3681	3900.3704	-0.59	A4G(4)Lac1S(X)4
	46	63.51	1420.8260	3	4265.5014	4265.5026	-0.28	A4G(4)Lac2S(X)4
	47	64.20	1177.4047	3	3535.2375	3535.2382	-0.20	A4G(4)4S(X)4
	48	64.84	1177.4051	3	3535.2387	3535.2382	0.14	A4G(4)4S(X)4
	49	65.41	1177.4053	3	3535.2393	3535.2382	0.31	A4G(4)4S(X)4
	50	66.02	1177.4050	3	3535.2384	3535.2382	0.06	A4G(4)4S(X)4
	51	67.32	1177.4053	3	3535.2393	3535.2382	0.31	A4G(4)4S(X)4
	52	68.15	1177.4047	3	3535.2375	3535.2382	-0.20	A4G(4)4S(X)4
	53	68.30	1177.4048	3	3535.2378	3535.2382	-0.11	A4G(4)4S(X)4

CONCLUSIONS

- Separation by HPAE is based on charge, linkage and positional isomerism and fucosylation, resulting in excellent separation of many different glycan isomers
- Using our new workflow, 53 unique glycans were identified from hu-AGP
- The new glycan sample preparation workflow shown here provides excellent reproducibility
- Denaturation is required to achieve complete glycan release
- Adduct formation with acetate enhances fragmentation producing diagnostic ions useful for structural elucidation
- HPAE-PAD/MS is an excellent tool to profile and annotate complex mixture of native glycans released from different glycoproteins

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TRADEMARKS/LICENSING