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應用手冊

Analysis of N-Linked Glycans from Coagulation Factor IX, Recombinant and Plasma Derived, Using HILIC UPLC/FLR/QTof MS

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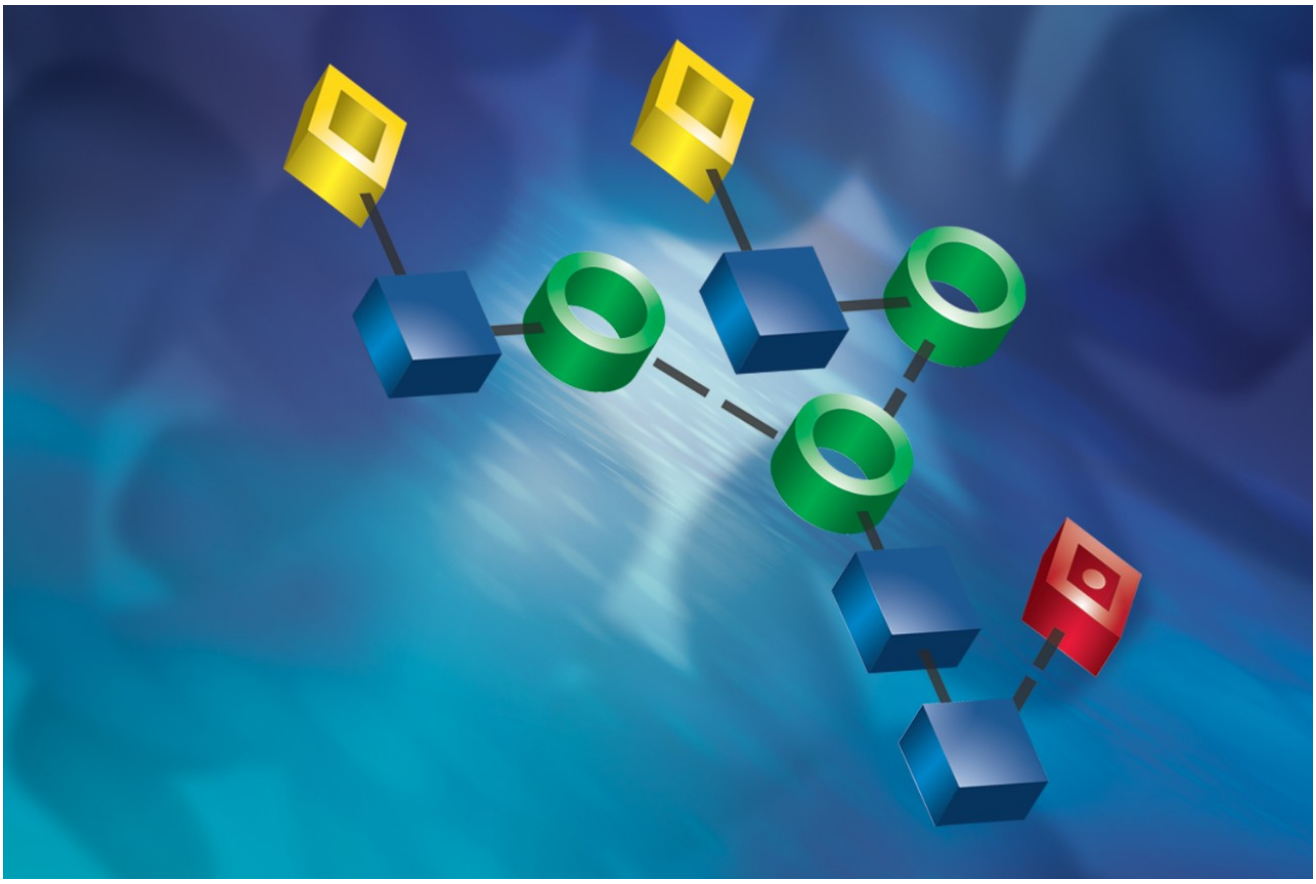


Abstract

HILIC UPLC/FLR/QTof MS is a powerful glycan characterization tool for characterization of complex glycans samples. Conventional HPLC method lacks the resolution power and sensitivity. Fractionation for MS analysis step is eliminated since the ACQUITY UPLC System is directly interfaced into a Xevo QTof MS. SimGlycan Software is part of the system solution, which interprets the collision fragmentation data, and offers a mean to elucidate glycan structures.

Introduction

Glycosylation of therapeutic protein drugs is of particular importance because it plays a vital role in the clinical performance of these drugs. In this work, we study the N-linked glycans from two Coagulation Factor IX biologics that are used for Hemophilia B treatment; one is recombinant (rFIX, BeneFIX) and the other one is derived from human plasma (pd-FIX, Mononine). Both Factor IX proteins are heavily glycosylated (Figure 1).[1] Previous findings on their glycoforms were done primary using orthogonal HPLC separation techniques, typically via Ion Exchange chromatography (IEX) and hydrophilic interaction (HILIC) modes, due to the complex nature of the Factor IX glycans. For mass profiling and structure characterization, mass spectrometry (MS) was typically used offline for LC fractions.

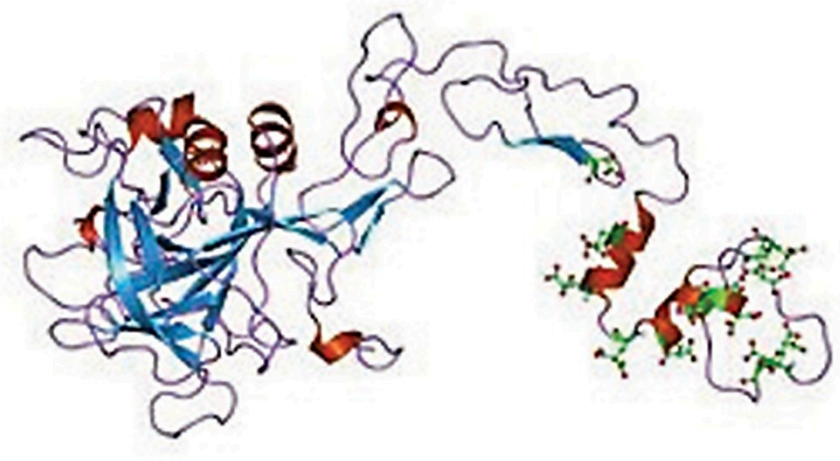


Figure 1. Coagulation factor IX structure.

Waters has developed a HILIC UPLC/FLR/QTof MS analytical platform for fluorescent-labeled glycan

characterization. Significant improvements can be made such as peak resolution, speed, sensitivity, and the ability to identify and quantify even the minor glycans. An ACQUITY UPLC System is directly interfaced to a Xevo QTof MS, eliminating the need for fractionation. Comparative analysis of rFIX and pd-FIX glycans using this platform is demonstrated.

Experimental

Sample

Figure 2 shows the basic glycan analysis workflow. FIX proteins are reduced and alkylated using DTT and IAM, followed by PNGase F enzymatic digestion overnight to release the glycans. The glycans are extracted using HILIC SPE device and labeled with 2-aminobenamide (2AB) dye, and the excess dye was removed by HILIC SPE again.[2]

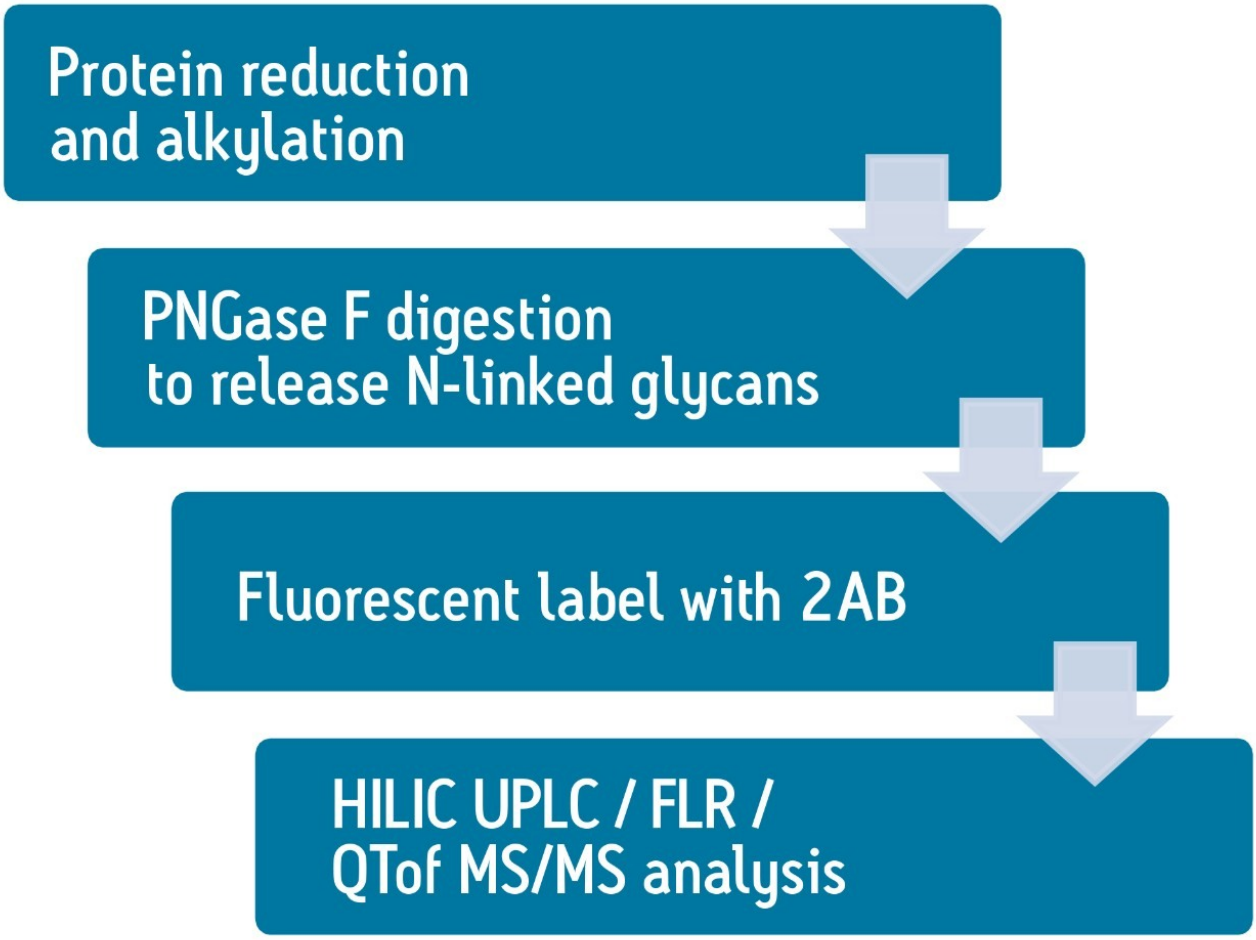


Figure 2. Glycan analysis workflow.

LC Conditions

LC System:	ACQUITY UPLC
Detection:	ACQUITY UPLC FLR
Column:	ACQUITY UPLC BEH Glycan Column (2.1 x 150 mm)
Column temp.:	60 °C
Sample temp.:	10 °C

Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	100 mM Ammonium Formate (~ pH 4.3)
Mobile phase B:	100% Acetonitrile (Fisher Optima)

Gradient table

Time (min)	Flow rate	%A	%B	Curve
Initial	0.400	39.0	70.0	-
60.00	0.400	50.0	50.0	6
60.10	0.250	95.0	5.0	6
63.00	0.250	95.0	5.0	6
64.00	0.300	30.0	70.0	6
65.00	0.400	30.0	70.0	6
72.00	0.400	30.0	70.0	6

MS conditions

MS system:	Xevo QTOF MS
Ionization mode:	ESI positive
Capillary voltage:	3200 V

Cone voltage:	30 V
Desolvation temp.:	350 °C
Desolvation gas:	800 L/Hr
Source temp:	100 °C
Acquisition range:	700 to 2000 *m/z*
Collision energies:	4 V
Lockmass:	Cesium Iodide (CSI): 1 µg/µL in 50% MeCN

Data Management

MassLynx 4.1

SimGlycan v. 2.9 (Premier Biosoft)

Results and Discussion

rFIX and pd-FIX has very different glycans profiles (Figure 3, 4). Glycans released from rFIX are mostly fucosylated bi-, tri-, and tetra-antennary complex type glycans. Additional lactosamine units were observed in some glycans. Man5 and a core N-glycans with an addition of fucose were also identified (Table 1). Glycans from pd-FIX are show more heterogeneity, especially for the larger glycans.

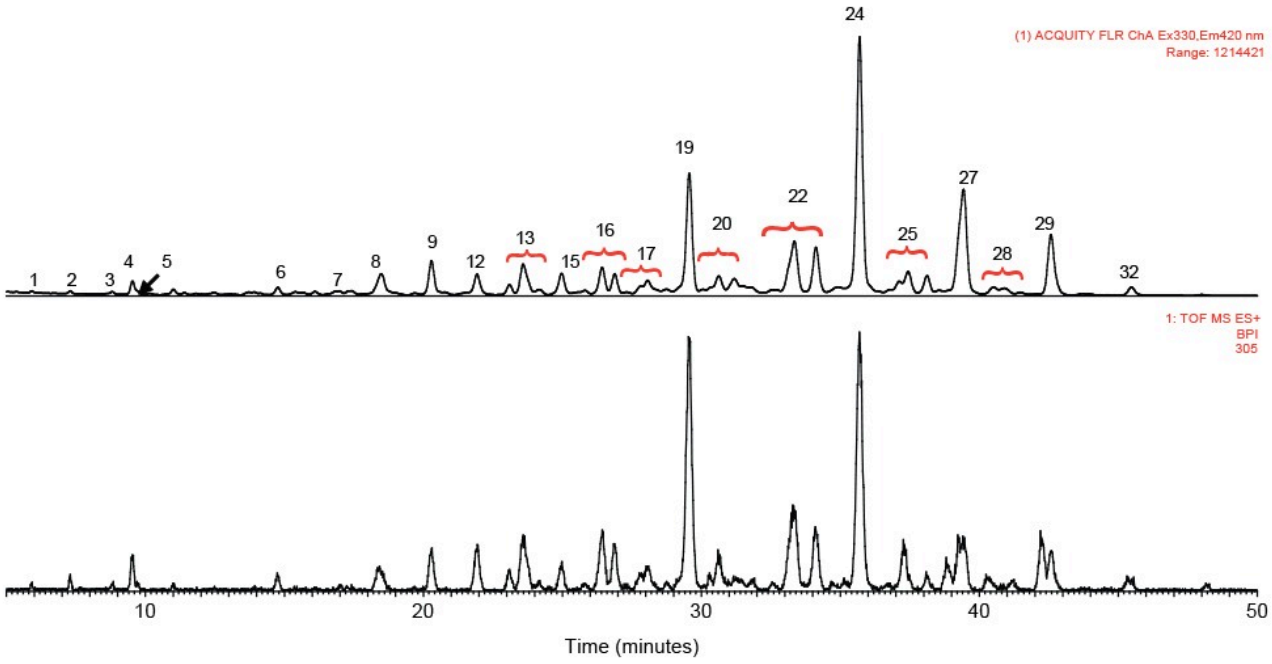


Figure 3. HILIC UPLC/FLR/QTof MS of 2AB labeled rFIX (BeneFIX) glycans. Low abundant glycans in the low mass region were observed and confirmed by MS analysis. In addition, isomeric glycans (sialic acid positional isomers) were well resolved. The peak number corresponds to the glycan listed in Table 1.

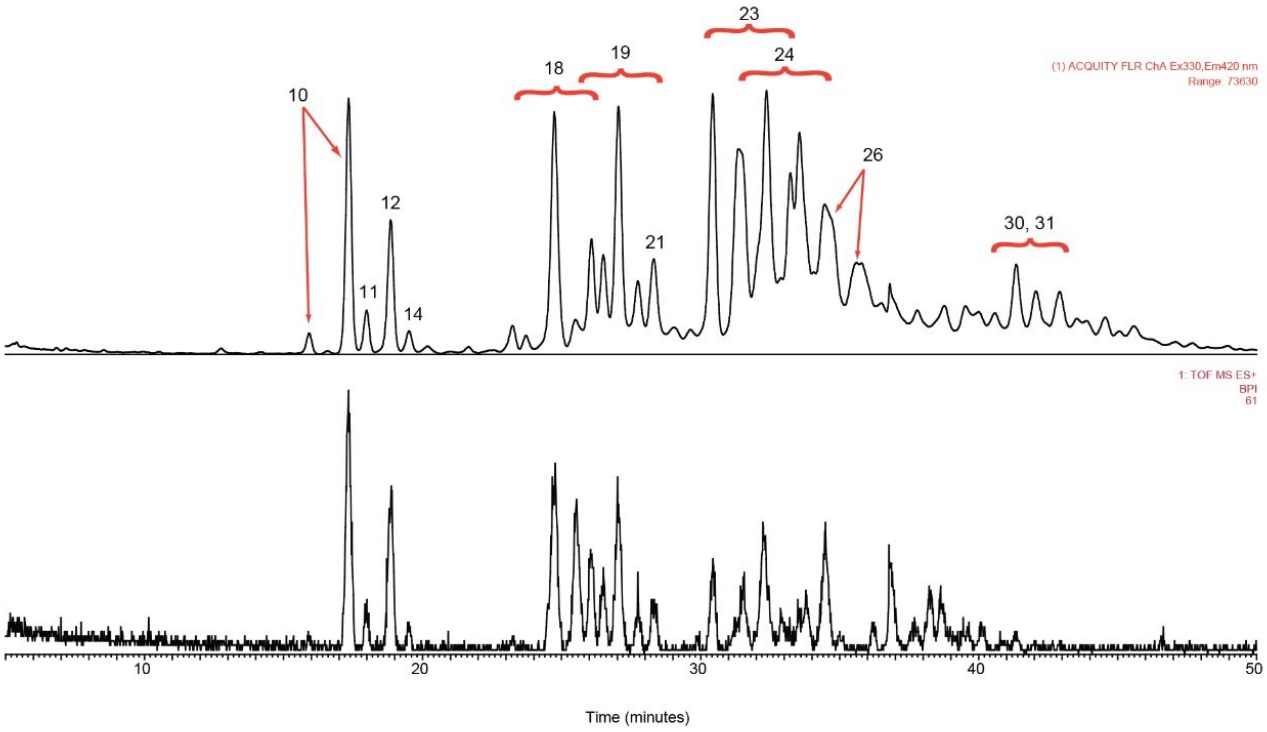
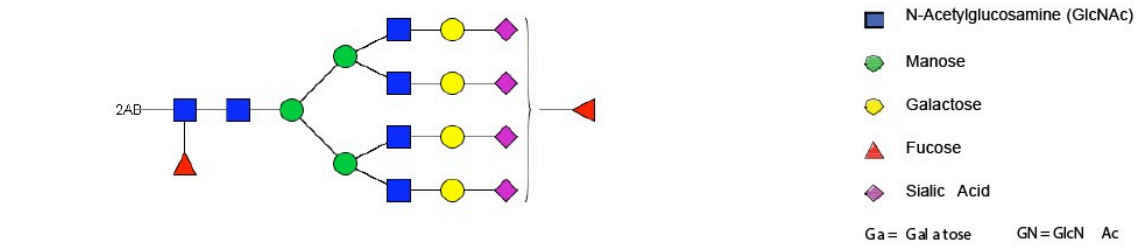


Figure 4. HILIC UPLC/FLR/MS of 2AB labeled pd-FIX (Mononine) glycans. More glycans were identified, such as sulfated glycans and multiply fucosylated glycans. The possibility of phosphorylation was excluded by alkaline phosphatase reaction (data not shown). In addition, MSMS fragmentation was used to further confirm their identification (see Figure 5 A-C).

Peak No.	Glycan	rFIX (BeneFIX)	pd-FIX (Mononine)	Observed MW	Theoretical MW
1	Man3 + 1F	+	—	1176.456	1176.455
2	GOF - GN	+	—	1379.534	1379.534
3	GOF	+	—	1582.612	1582.674
4	Man5	+	—	1354.511	1354.502
5	1A/1F - GN	+	—	1541.587	1541.587
6	2A/1F	+	—	1906.710	1906.719
7	2A/1F + GN	+	—	2109.760	2109.798
8	2A/1F/1S	+	—	2197.816	2197.814
9	3A/1F	+	—	2271.840	2271.850
10	2A/2S	—	+	2342.880	2342.851
11	2A/2S/SO3	—	+	2422.814	2422.808
12	2A/1F/2S	+	+	2488.912	2488.909
13	3A/1F/1S	+	—	2562.924	2563.945
14	2A/1F/2S/SO3	—	+	2568.868	2568.866
15	4A/1F	+	—	2636.962	2637.982
16	3A/1F/2S	+	—	2854.042	2854.042
17	4A/1F/1S	+	—	2928.066	2928.07
18	3A/3S	—	+	2999.112	2999.079
19	3A/1F/3S	+	+	3145.112	3145.137
20	4A/1F/2S	+	—	3219.134	3219.174
21	3A/2F/3S	—	+	3291.188	3291.195
22	4A/1F/3S	+	—	3510.224	3510.269
23	4A/4S	—	+	3655.277	3655.306
24	4A/1F/4S	+	+	3801.358	3801.365
25	4A/1F/3S +(GN+Ga)	+	—	3875.401	3875.402
26	4A/2F/4S	—	+	3947.373	3947.423
27	4A/1F/4S +(GN+Ga)	+	—	4166.418	4166.497
28	4A/1F/3S +2(GN+Ga)	+	—	4240.533	4240.533
29	4A/1F/4S +2(GN+Ga)	+	—	4531.617	4531.629
30	4A/2F/2S + 2(GN+Ga)	—	+	4677.610	4677.687
31	4A/3F/4S + 2(GN+Ga)	—	+	4823.694	4823.745
32	4A/1F/4S + 3(GN+Ga)	+	—	4896.783	4896.761

Table 1. A complete list of 2AB labeled glycans observed from rFIX and pd-FIX. Complex type glycan with various degree of sialylation along with Man5 and Man3/1F were observed. The average mass error is less than 5 ppm.



Only the complex type glycans with polylactosamine units were observed from pd-FIX; the level of fucosylation is from 0 to 2 (Table 1). Two sulfated bi-antennary glycans were observed, which was not reported in the literature; these sulfated glycans were well resolved from their non-sulfated counterpart (Figure 4). The possibility of phosphorylation was excluded by alkaline phosphatase reaction (data not shown). In addition, MS/MS fragmentation was used to further confirm their identification (see Figure 5).

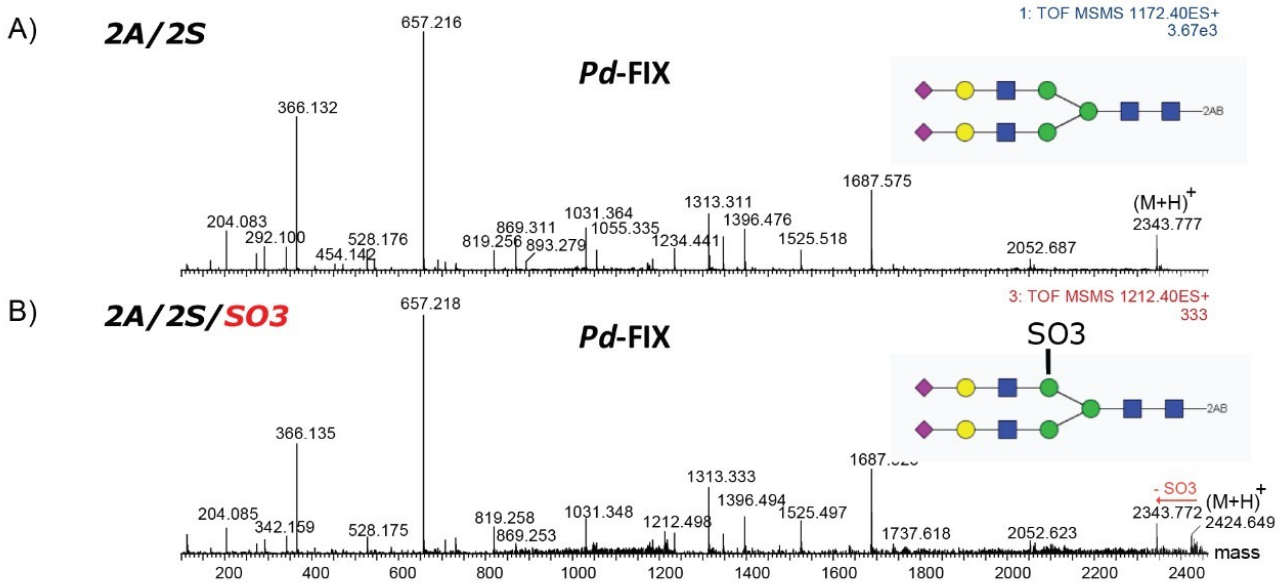


Figure 5. MS/MS fragmentation of sulfated glycans such as 2A/2S/SO3 were compared with its non-sulfated counterpart, 2A/2S. Facile loss of SO3 (-80 Da) was observed. The MS/MS data, after MaxEnt3 mass deconvolution, was submitted to SimGlycan software for structure assignment; and SimGlycan validated the proposed structures.

The glycan profile from both rFIX and pd-FIX is very complex. HPLC-based methods lack the resolution needed to identify and quantify various glycan forms. The ACQUITY UPLC System coupled with the ACQUITY UPLC BEH Glycans Column alone is able to achieve baseline separation of glycans that are different in mass and degree of

sialylation; terminal sialic acid isomers are also well separated.

The accurate mass measurement from the Xevo QTof MS offers confident assignment of the glycans. MS/MS fragmentation along with SimGlycan Software gives further confirmation on glycan structure. Figure 6 shows that glycan 4A/1F/4S was observed from both rFIX and pd-FIX; the LC retention time is the same for the isobaric tetra-antennary glycans. However, the CID fragmentation showed distinct difference, which resulted from the fucose location on the glycans (Figure 5B). Figure 7 shows another example on how CID was used to differentiate isobaric doubly fucosylated glycans from both rFIX and pd-FIX glycans, again distinct fragment ions were used to differentiate the location of fucose. Biological influence caused by the location of fucose was documented in the literature.

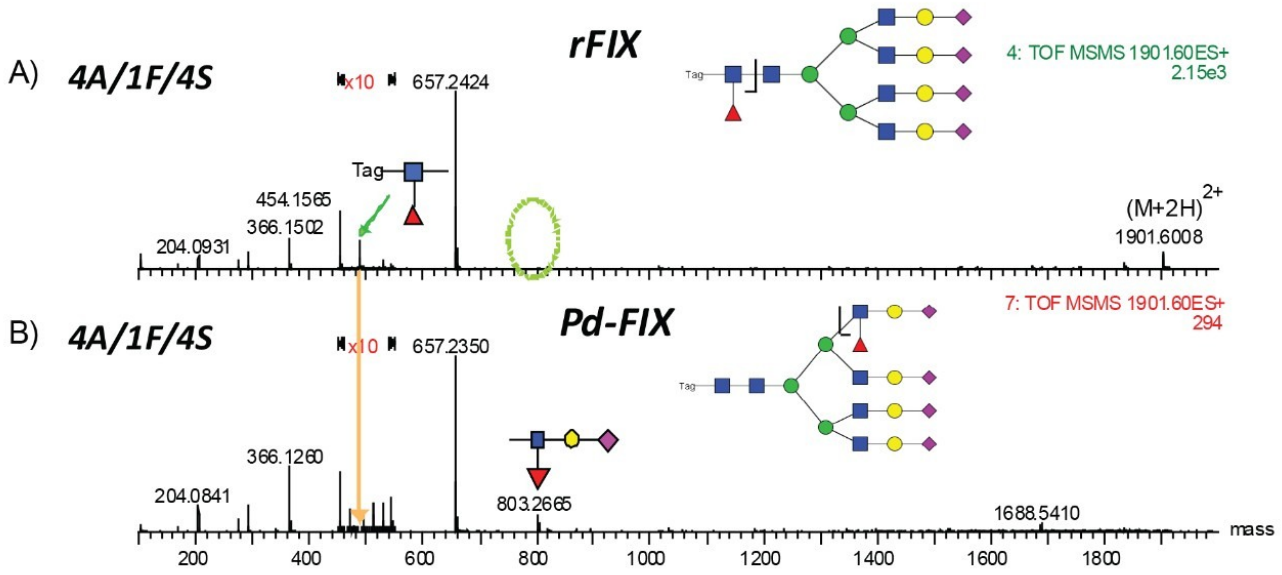


Figure 6. Differences in fucosylation were observed for rFIX and pd-FIX. Fucose from rFIX was located at the first GlcNAc residue in the core structure; while the majority of the fucosylation site for singly fucosylated pd-FIX glycans was located at the antenna. Fragment ions at m/z 488 and 803 were the diagnostic ions for probing the fucosylation sites.

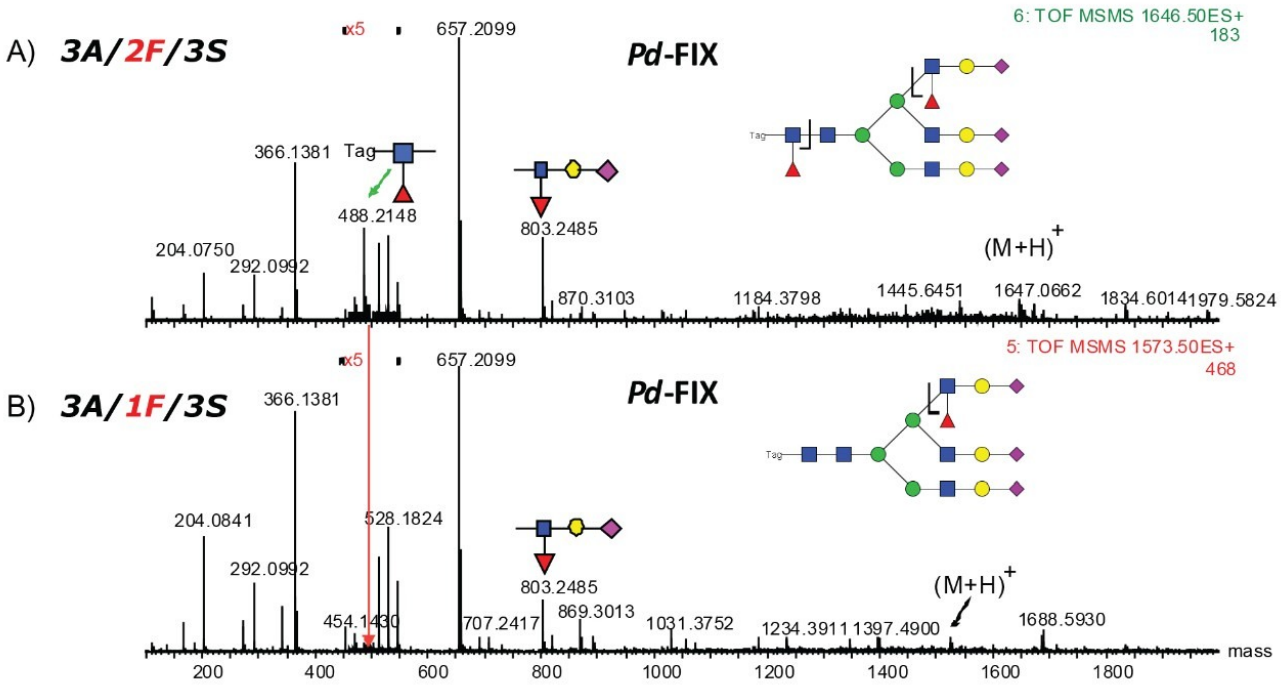


Figure 7. Some doubly fucosylated glycans were observed for pd-FIX sample. The two diagnostic ions at m/z 488 and 803 were observed, which indicated that one fucose was at the first GlcNAc residue (core structure) and the other one was located at the antenna.

Conclusion

Blood protein glycan characterization is known to be very challenging for scientists working in the biopharmaceutical field, since the glycans that attach to the protein backbone are highly heterogeneous and complex. Our solution for complex glycan separation and characterization is the UPLC/FLR/QTof MS analytical platform.

In addition to shortened run time enabled by ACQUITY UPLC technology, HILIC UPLC also offers significant improvement in peak resolution compares to conventional HPLC method; for example, positional sialic acid isomer separation is achieved, also the separation of sulfated and sialyated glycans were observed.

MS/MS fragmentation and database search using SimGlycan Software helped the glycan structure elucidation, adding more confidence to glycan structure assignment.

The ACQUITY UPLC/FLR/Xevo QTof MS analytical platform along with SimGlycan Software is a powerful and versatile tool for complex glycan characterization. Glycan profiling, mass confirmation, and structure elucidation

can all be done in a single LC/MS system. High quality data are generated with shorter analysis and data interpretation time. This is a valuable tool for researchers working with glycoprotein drugs.

References

1. Yasushi Makino, Kaoru Omichi, Nahoki Kuraya, Hideyuki Ogawa, Hitoshi Nishimura, Sadaaki Iwanaga and Sumihiro Hase. *J. Biochem*. 128, 175-180 (2000).
2. Ying Qing Yu, Martin Gilar, Jennifer Kaska and John C. Gebler. 2. Deglycosylation and Sample Cleanup Method for Mass Spectrometry Analysis of N-linked Glycan. Waters Application Note. 2007. 720001146EN.

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