P-218-T

ANALYSIS OF N-LINKED GLYCANS FROM RECOMBINANT AND HUMAN PLASMA DERIVED COAGULATION FACTOR IX, USING HILIC UPLC/FLR/QTOF MS

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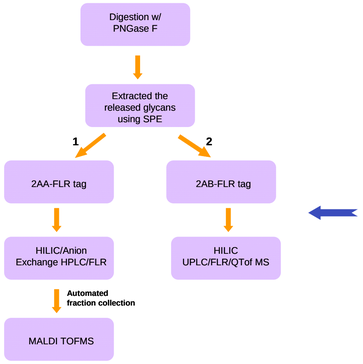
INTRODUCTION

- Glycosylation of therapeutic protein drugs is of particular importance because it plays vital roles in the clinical performance of these drugs.
- In this work, we studied 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.[1]
- Previous findings on their glycoforms were done using analytical techniques other than mass spectrometry (MS).
- Two analytical workflows were applied for FIX N-linked glycan profiling; one is HPLC(HILIC-Anion)/FLR with off line fractionation and MALDI TOF MS, and the other one uses a UPLC(HILIC)/FLR/QTof MS platform. Results from both workflows were shown here.

METHODS

Workflows:



RESULTS

•Results from workflow 1

HILIC/Anion Exchange HPLC

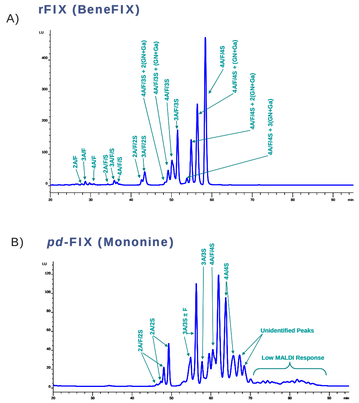


Figure 1. HPLC (HILIC-Anion)/FLR separation of 2AA-labeled glycans released from (A) recombinant factor IX (rFIX, BeneFIX) and (B) plasma derived factor IX (pd-FIX, Mononine). MALDI TOF MS identified glycans were labeled with abbreviations at their corresponding chromatogram peak top.

2AA: 2-aminobenzoic acid
2AB: 2-aminobenzamide
HILIC: Hydrophilic interaction liquid chromatography
FLR: Fluorescent

Workflow 1:

- HPLC — Agilent HP 1100 with fluorescent detector
- Column — Shodex Asahipak NH2P-50D column
  Mobile phase A: 2% acetic acid, 98% MeCN
  Mobile phase B: 92% Water, 3%TEA, 5% acetic acid
- *Amount of glycan injected: glycans released from 112 µg of protein*
- Fraction collection — 0.5 min. intervals (lyophilized)
- MALDI TOF — Voyager DE mass spectrometer (Neg. Ion) (Matrix: 2AA in 25% Ethanol)

Workflow 2:

- UPLC — Waters ACQUITY™ Ultra Pressure Liquid Chromatography with fluorescent detector
- Column — Waters ACQUITY™ UPLC® BEH Glycan column
  Mobile phase A: 100mM Ammonium Formate (pH 4.3)
  Mobile Phase B: 100% MeCN
  Column temperature: 60 °C
  Flow rate: 0.4 ml/min
  Gradient: 70 –50% B in 60 minutes

- *Amount of glycan injected: glycans released from 13 µg of protein*
- MS and MSMS — Waters Xevo™ QTof MS (Pos. Ion)

Premier Biosoft SimGlycan® was used for glycan structure assignment using MSMS data

•Results from workflow 2

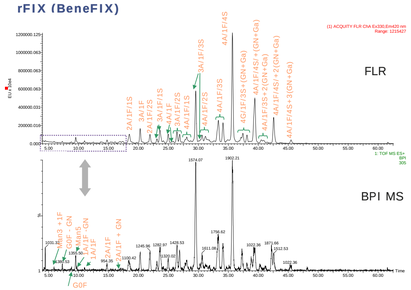


Figure 2. UPLC(HILIC)/FLR/MS of 2AB labeled rFIX (BeneFIX) glycans. Low abundant glycans in the low mass region was observed and confirmed by MS analysis. In addition, isomeric glycans (sialic acid positional isomers) were better resolved than the chromatogram generated in workflow 1.

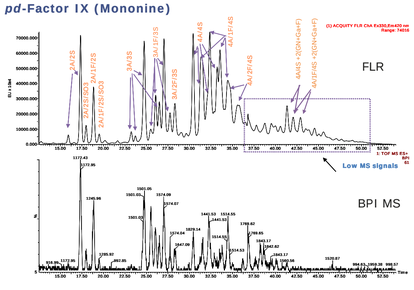


Figure 3. UPLC(HILIC)/FLR/MS of 2AB labeled pd-FIX (Mononine) glycans. More glycans were identified, such as sulfated glycans and multiply fucosylated glycans. The possibility of phosphorylation was excluded by alkaline phosphatase reaction (data not shown). In addition, MSMS fragmentation was used to further confirm their identification (see Figure 4. A-C).

Table 1. A complete list of 2AB labeled glycans observed from rFIX and pd-FIX. Proposed structures shown here were supported by MSMS data and SimGlycan software. Glycans that do not have proposed structures only have the MW confirmation from MS chromatogram.

Glycan	Proposed structure	rFIX (BeneFIX)	pd-FIX (Mononine)	Observed MW	Theoretical MW
Man3 + 1F		+	—	1176.456	1176.455
G0F - GN		+	—	1379.534	1379.534
Man5		+	—	1354.511	1354.502
1A/1F - GN		+	—	1541.587	1541.587
G0F		+	—	1582.612	1582.674
2A/1F		+	—	1906.710	1906.719
2A/1F + GN		+	—	2109.760	2109.798
2A/1F/1S		+	—	2197.816	2197.814
3A/1F		+	—	2271.840	2271.850
2A/2S		—	+	2342.880	2342.851
2A/2S/SO3	+ SO3	—	+	2422.814	2422.808
2A/1F/2S		+	+	2488.912	2488.909
3A/1F/1S		+	—	2562.924	2563.945
2A/1F/2S/SO3	+ SO3	—	+	2568.868	2568.866
4A/1F		+	—	2636.962	2637.982
3A/1F/2S		+	—	2854.042	2854.042
4A/1F/1S		+	—	2928.066	2928.07
3A/3S		—	+	2999.112	2999.079

Glycan	Proposed structure	rFIX (BeneFIX)	pd-FIX (Mononine)	Observed MW	Theoretical MW
3A/1F/3S		+	+	3145.112	3145.137
4A/1F/2S		+	—	3219.134	3219.174
3A/2F/3S		—	+	3291.188	3291.195
4A/1F/3S		+	—	3510.224	3510.269
4A/4S		—	+	3655.277	3655.306
4A/1F/4S		+	+	3801.358	3801.365
4A/1F/3S +(GN+Ga)		+	—	3875.401	3875.402
4A/2F/4S		—	+	3947.373	3947.423
4A/1F/4S +(GN+Ga)		+	—	4166.418	4166.497
4A/1F/3S +2(GN+Ga)		+	—	4240.533	4240.533
4A/1F/4S +2(GN+Ga)		+	—	4531.617	4531.629
4A/4S + 2(GN+Ga+F)		—	+	4677.610	4677.687
4A/1F/4S + 2(GN+Ga+F)		—	+	4823.694	4823.745
4A/1F/4S + 3(GN+Ga)		+	—	4896.783	4896.761

- N-Acetylglucosamine (GlcNAc)
- Mannose
- Galactose
- Fucose
- Sialic Acid

Ga = Galatose GN = GlcNAc

•MSMS fragmentation for structure confirmation

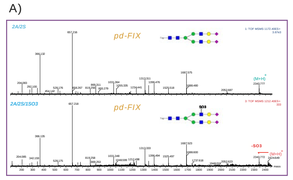
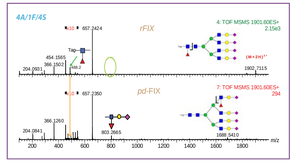
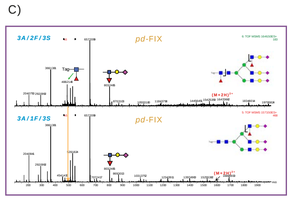


Figure 4A. MSMS fragmentation of sulfated glycans such as 2A/2S/SO3 were compared with its non-sulfated counterpart, 2A/2S. Facile loss of SO3 (-80 Da) was observed. The MSMS data, after MaxEnt1 mass deconvolution, was submitted to SimGlycan software for structure assignment; and SimGlycan validated the proposed structures.

Figure 4B. Differences in fucosylation was 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 4C. 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

- **N-linked glycan profiling using UPLC-HILIC/FLR/QTof MS was performed for two coagulation factor IX protein drugs. More glycans were observed comparing to HPLC(HILIC-Anion)/FLR/ Fraction Collection/MALDI TOF MS method, and other conventional HPLC methods.**
- **UPLC (HILIC) glycan separation improved the peak resolution, and enhanced the separation of isomeric glycans. For example, positional sialic acid isomer separation was achieved, also the separation of sulfated and sialyated glycans were observed.**
- **MSMS fragmentation and database search using SimGlycan software helped the glycan structure elucidation. Adding more confidence to the glycan structure assignment.**

References

1. "Structure Analysis of N-linked Sugar Chains of Human Blood Clotting Factor IX", *J. BioChem.* **128**; 175-180 (2000).

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