MULTIPLE REACTION-MONITORING ASSAYS FOR HIGH THROUGHPUT VALIDATION, MONITORING AND QUANTIFICATION OF SITE-SPECIFIC GLYCOSYLATION AND GLYCOFORMS IN THERAPEUTIC GLYCOPROTEINS

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Hongwei Xie, Ying Qing Yu, Joomi Ahn, and Scott J. Berger
Biopharmaceutical Sciences , Waters Corporation, Milford, MA USA

INTRODUCTION

- **Glycosylation is a critical product attribute for many biotherapeutic molecules, including monoclonal antibodies (mAbs), recombinant glycoproteins, and glycosylated subunit vaccine proteins.**
- **Site specific determination of major and minor glycoforms is often a challenging requirement for product characterization.**
- **A methodology combining hydrophilic interaction UPLC (HILIC) and multiple reaction-monitoring (MRM) mass detection for glycopeptides has been developed for the confirmation, monitoring, and quantification of site-specific glycosylation.**
- **We have demonstrated this approach to be useful for site-specific glycoform determinations, including cases of co-eluting glycopeptides and minor peptide glycovariants.**
- **The utility of the approach has been illustrated with the characterization of N-linked glycosylation for two recombinant mAbs and multiple recombinant influenza subunit vaccine proteins.**

EXPERIMENTAL

UPLC: Waters ACQUITY UPLC™ equipped with a Glycan Separations Technology 1.7 µm (2.1 x 150 mm, 60 °C, 0.2 ml/min) HILIC column. A complex gradient (81-68% MeCN in 10 mM NH4-formate over 10 min, then to 45% over 50 min) was applied.

MS: Xevo TQ MS operated at unit resolution with a 5 msec dwell time in the ESI+ MRM acquisition mode.

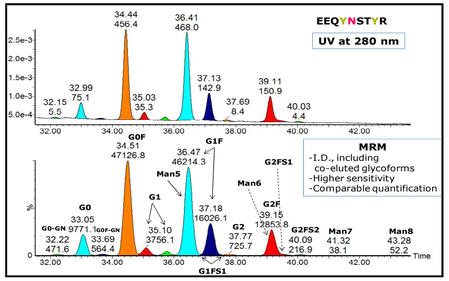
Samples: RapiGest-assisted 4h tryptic digests. ~3 µg (10 µl) of each sample were loaded for each analysis.

HILIC-UV-MRM-MS TRASTUZUMAB GLYCOPEPTIDE MAPPING EXPANDS THE QUANTIFICATION CAPACITY BEYOND OPTICAL UV DETECTION SENSITIVITY

Trastuzumab Glycoform	RT min	MRM Transitions Sum of Areas	HILIC LC-MRM Glycopeptide (%)	%RSD n = 6	HILIC LC-FLR Glycan (%)	%RSD n = 6
G0 - GN	32.15	1143.4 → 204.1 1143.4 → 366.1	0.85	6.9	0.74	1.7
G0	33.00	1245.2 → 204.1 1245.2 → 366.1	6.56	6.6	5.42	7.1
G0F - GN	33.63	1216.9 → 204.1 1216.9 → 366.1	1.42	6.8	1.21	13.0
G0F	34.41	1318.3 → 204.1 1318.3 → 366.1	43.95	2.4	43.37	3.6
G1a	35.01	1326.3 → 204.1 1326.3 → 366.1	1.59	7.4	1.47	9.0
G1b	35.73	1326.3 → 204.1 1326.3 → 366.1	0.69	14.5	0.80	2.8
Man5	36.24	1204.1 → 204.1 1204.1 → 366.1	1.49	3.7	1.62	1.0
G1Fa	36.50	1399.3 → 204.1 1399.3 → 366.1	26.56	2.4	28.21	2.7
G1Fb	37.12	1399.3 → 204.1 1399.3 → 366.1	10.49	5.9	9.68	4.4
G2	37.72	1407.3 → 204.1 1407.3 → 366.1	0.17	16.2	0.57	5.1
G2F	39.11	1480.4 → 204.1 1480.4 → 366.1	5.54	2.9	6.18	7.1
G2FS1	39.19	1626.5 → 204.1 1626.5 → 366.1	0.30	7.5	0.73	3.6
G1FS1a	36.71	1545.6 → 204.1 1545.6 → 366.1	0.17	14.4	ND	ND
G1FS1b	37.50	1545.6 → 204.1 1545.6 → 366.1	0.06	17.2	ND	ND
Man6	38.85	1285.2 → 204.1 1285.2 → 366.1	0.05	13.7	ND	ND
G2FS2	39.78	1777.7 → 204.1 1777.7 → 366.1	0.08	15.5	ND	ND
Man7	41.40	1366.3 → 204.1 1366.3 → 366.1	0.02	22.3	ND	ND
Man8	43.30	1447.4 → 204.1 1447.4 → 366.1	0.04	19.3	ND	ND
AVG (all)				10.3		5.1
AVG (common)				6.9		5.1

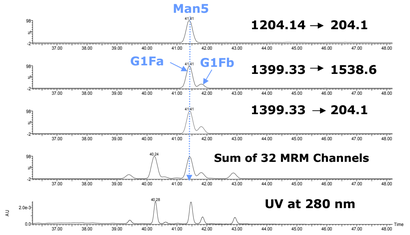
Data from 32 MRM Channels (2 per Glycoform) were used to determine the relative glycovariant composition for the heavy chain glycopeptide EEQYNSTYR.

HILIC MRM exhibited greater sensitivity for glycopeptide detection than 2AB labeled LC-FL studies of released glycans, while yielding comparable RSD (6.9% vs 5.1%) values for the common set of glycoform detections.



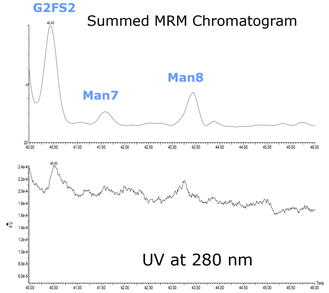
This humanized antibody heavy chain N-glycopeptide contains two UV adsorbing Tyr residues that produce a UV280 trace that is comparable to the summed MRM chromatogram for all 32 MRM Transitions.

MRM Analysis Facilitates Quantitation of Coeluting Peptide Glycovariants

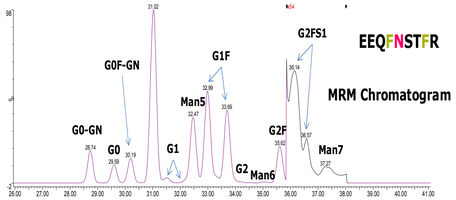


In this example MRM analysis permits independent quantitation of Man5 and G1 peptide glycoforms.

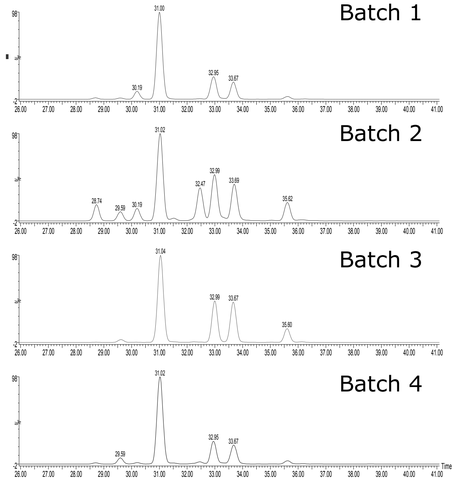
Relative Sensitivity: MRM > UV



COMPARISON (HILIC-UV-MRM-MS) OF MULTIPLE MURINE mAb BATCHES



Data from 32 MRM Channels (2 per Glycoform) were used to determine the relative glycoform composition of the murine heavy chain glycopeptide EEQYNSTYR. This peptide lacks UV selectivity of an A280/Tyr or A256/Trp, making UV quantitation at peptide bond (A210-220) susceptible to buffer/sample contaminant peaks.



Four batches of the purified murine IgG1 antibody were analyzed using the MRM glycopeptide assay. In higher throughput applications, a quick focused HILIC-MRM method could be employed to focus on this elution region.

HILIC-UV-MRM-MS OF SELECTED INFLUENZA VIRUS GLYCOPEPTIDES

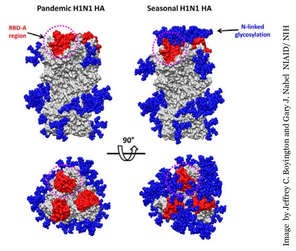
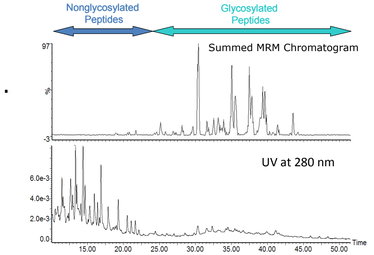


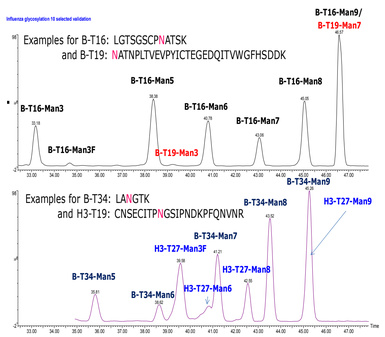
Image by Jeffrey C. Boyington and Gary J. Nabel NIAID/ NIH

Protein	N Site	Location	Motif
H1	N11	T1	NNSTD
H1	N54	T4	GNCSV
H1	N88	T5	ENGTC
H1	N125	T8	PNHTV
H1	N159	T10	PNLSK
H1	N286	T24	INSSL
H1	N480	T47	(K)NGTY
H3	N165	T13	LNVTM
H3	N246	T21	INSTG
H3	N285	T27	PNGSI
H3	N483	T52	(R)NGTY
B	N25	T3	VNVTG
B	N59	T8	LNCTD
B	N145	T16	PNATS
B	N167	T19	(K)NATN
B	N303	T30-31	LNKSK
B	N332	T34	ANGTK

A multi-subunit vaccine candidate containing three influenza hemagglutinins (H1, H3, B) was produced in an SF9 derived insect cell line. Each hemagglutinin is multiply glycosylated (e.g. TOP PANEL) N-Glycosylation was observed on multiple potential peptides by HILIC MRM analysis (TABLE).



The HILIC UPLC separation is effective for high resolution glycopeptide separations, and segregation of glycopeptides from the earlier eluting non-glycopeptides.



HILIC-Summed MRM chromatograms are shown for four identified N-glycopeptides (2 per panel). High mannose glycans dominate the observable glycovariant profiles. This is expected given the insect cell expression system.

CONCLUSIONS

- **HILIC UPLC analysis permits high resolution separations of peptide glycoforms, and serves to resolve glycopeptides from non-glycosylated peptides in the digest.**
- **HILIC-MRM analyses can be more selective and more sensitive for glycopeptide quantitation studies than UV based detection approaches, while reflecting comparable glycopeptide composition data.**
- **HILIC-MRM analyses can be more sensitive and executed far more rapidly than HILIC-FL analyses of 2-AB labeled released glycans, while generating comparable results.**
- **HILIC-MRM analysis is readily applicable to the study of site-specific glycosylation for more complex glycoproteins.**

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