Procainamide labelling as part of a flexible glycoprofiling system for monitoring of Gal-α1-3Gal related Glycosylation Critical Quality Attributes (GCQAs) of monoclonal antibody (mAb) therapeutics throughout the product life cycle.

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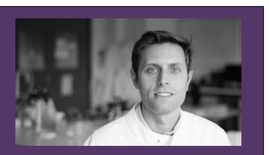

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Introduction

Galα1-3Gal is a disaccharide fragment found on the glycans of some glycoprotein therapeutics produced in mammalian expression systems - particularly those from mouse-derived cell lines. Galα1-3Gal is a non-human epitope that:

- Has been the causative agent for adverse clinical reactions (including IgE-mediated anaphylaxis) in patients with certain immunological profiles. [1]
- Could lead to diminished clinical efficacy via increased serum clearance by neutralising antibodies. [2]

Given this, Galα1-3Gal is a high priority Glycosylation Critical Quality Attribute (GCQA) and developers of biologic drugs should effectively optimise, measure and control the glycosylation of their products to limit Galα1-3Gal levels throughout the product life cycle. However, measurement of the amounts of Galα1-3Gal can be difficult as these epitopes are often hidden by the complexity of the glycan profiles.

Advances in glycoanalytical technologies have allowed us to detect and quantify the levels of Galα1-3Gal on both approved glycoprotein therapeutics as well as drugs in development. Here we explain our strategy for detection, identification and quantification of Galα1-3Gal bearing glycans. Our focus for this has been on:

- Producing a practical workflow suitable for QbD-based drug realisation and
- Compliance with emerging regulations from the FDA, EMA, KFDA and cFDA. [2-4]

1. Overall Strategy for detection and quantitation of Galα1-3Gal bearing glycans

Figure 1 outlines the workflows we use for quantitative measurement of Gala1,3Gal in mAb therapeutics. N-glycans are released from the glycoprotein using PNGAse F endoglycosidase then derivatised with one of two fluorescent labels - **2-aminobenzamide** (**2-AB**) which is widely used in the industry for biopharmaceutical glycan analysis and **procainamide** (**PROC**) [4]. Labelled glycans are run on two orthogonal analysis platforms - HILIC (Hydrophilic Interaction Liquid Chromatography) UHPLC and ESI-MS/MS - generally in hyphenated configuration.

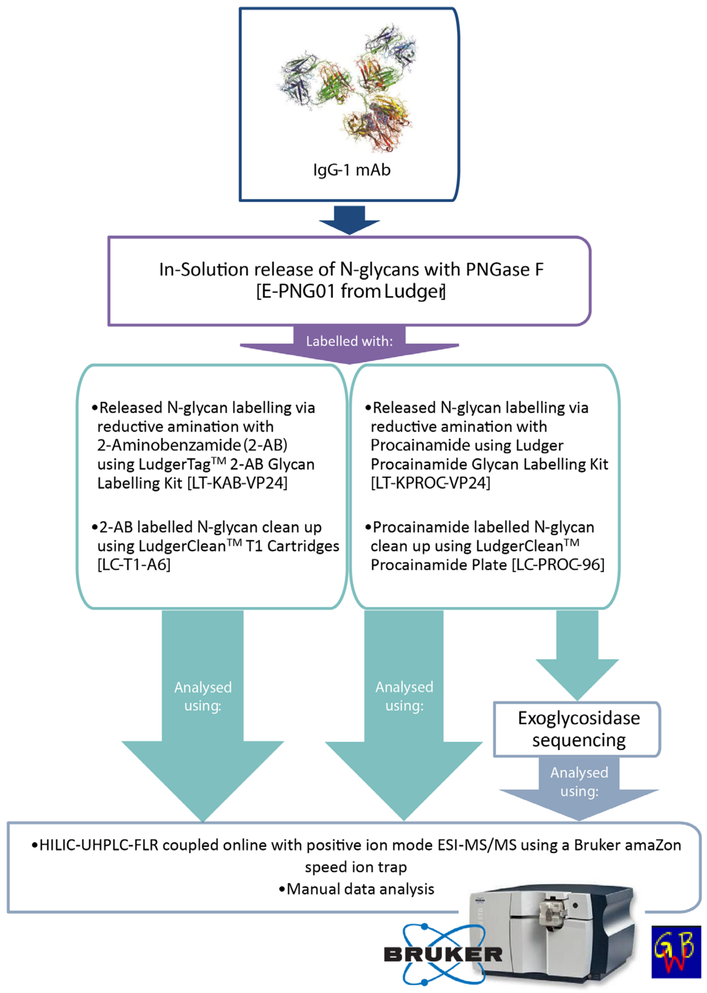

Figure 1: General glycoprofiling scheme for quantitative analysis of Galα1-3Gal containing N-glycans

2. Choosing between 2-AB and procainamide for measurement of Galα1-3Gal

We evaluated the performance of the two labelling systems in this scheme for their ability to furnish reliable structure information and quantitation of Galα1-3Gal bearing glycans in a standard IgG_1 mAb. The results are summarised in Figure 2 and Table 1.

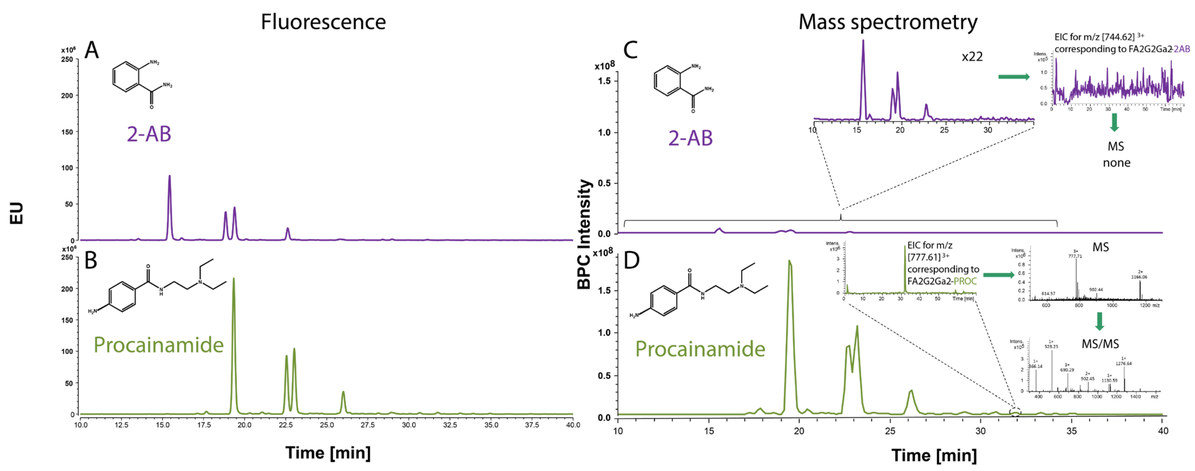

Figure 2: Comparison of 2-AB and Procainamide Labelled IgG1 mAb Glycans on HILIC-UHPLC and ESI-MS

A. HILIC-FLR of 2-AB labelled IgG_1 mAb N-glycans
B. HILIC-FLR of procainamide labelled IgG_1 mAb N-glycans
C. HILIC-ESI-MS BPC profile of 2-AB labelled IgG_1 mAb N-glycans
D. HILIC-ESI-MS BPC profile of procainamide labelled IgG_1 mAb N-glycans

UHPLC-FLR-ESI-MS/MS	2-AB	Procainamide
Fluorescence response	good	3x higher than 2-AB Detects minor peaks
HILIC-UHPLC separation	Comparable separation	
ESI-MS	low sensitivity	22x higher than 2-AB Detects minor peaks
ESI-MS/MS	low sensitivity	22x higher than 2-AB Good MS/MS for minor peaks

Table 1: Summary of Performance of 2-AB and Procainamide Labelled Glycans on HILIC-UHPLC and ESI-MS

This study was important for us as we had encountered Galα1-3Gal bearing glycans on drugs that biopharma clients had submitted to our glycoprofiling services labs. These were successfully identified and sequenced using HILIC-UHPLC and exoglycosidase sequencing of 2-AB labelled glycans. However, the quality of results from ESI-MS analysis of 2-AB glycans fell short of our requirements. This was mainly due to low signal-to-noise - particularly for MS/MS fragmentation experiments. This prompted us to search for alternatives - and preliminary experiments indicated that procainamide could give us better glycoanalysis results on both UHPLC and ESI-MS.

We spent two years developing the procainamide labelling and cleanup system and the resulting technology has now been incorporated as a standard glycoanalysis module for detailed, quantitative structure characterisation of drug glycans by HILIC-UHPLC-ESI-MS/MS (see Fig 4 and Table2). The system is flexible and can be tuned to provide accurate measurements of Galα1-3Gal levels using HILIC-UHPLC combined with exoglycosidase digests (see Fig 3). Together, the two workflows allow production of high resolution glycan maps that can be used to assess the risk of Galα1-3Gal induced safety issues of a particular product batch. The following section illustrates these workflows with the procainamide labelling system applied to analysis of Galα1-3Gal structures from an IgG_1 mAb.

3. Procainamide system for characterisation and quantification of Galα1-3Gal in drugs

We found that switching from 2-AB to procainamide labelling was very straightforward. The advantages were that we could use all of our standard glycan profiling protocols previously developed for 2-AB and, in addition, obtain useful LC-MS/MS fragmentation data. Our current workflow for detection and quantification of Galα1-3Gal bearing glycans uses a combination of our standard exoglycosidase sequencing protocols and LC-ESI-MS/MS of the procainamide labelled glycans.

Figures 3 and 4 and Table 2 summarise the results of this glycoprofiling scheme applied to quantitative characterisation of the N-glycans of an IgG_1 mAb that is particularly difficult to analyse by other methods. This drug contains a complex mixture of glycan structures, several of which co-elute on HILIC-LC and/or have the same mass composition. The relative proportion of glycans that carry the Galα1-3Gal epitope was determined by a matrix of HILIC-UHPLC analyses on procainamide labelled glycan samples treated with various exoglycosidase mixtures. Key to detection of Galα1-3Gal was differential treatment with alpha galactosidase which was included in some digests (Figure 3). In this system, the glycans are digested down until there are distinct peaks that only contain structures bearing the Galα1-3Gal epitope. This greatly simplified identification and quantitation of this GCQA.

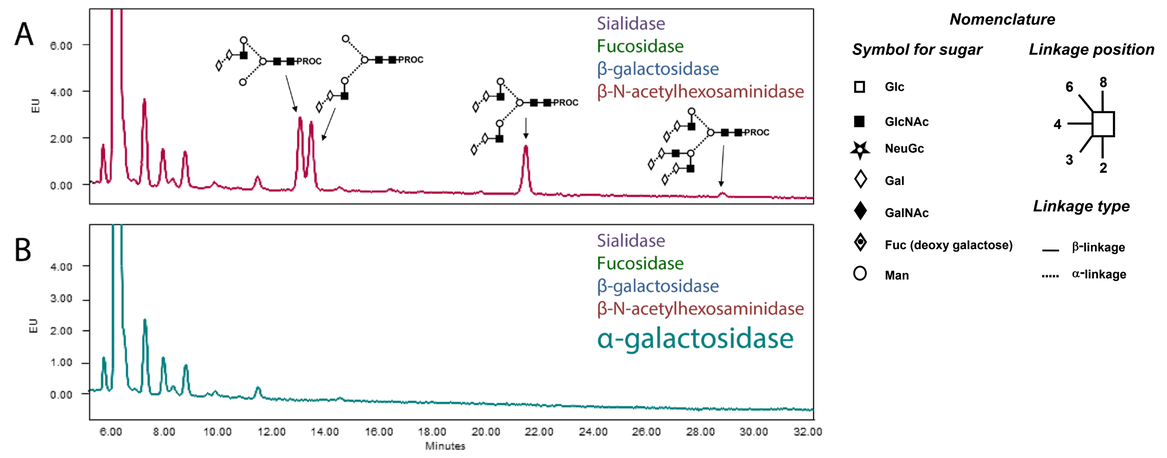

Figure 3: Exoglycosidase sequencing of procainamide labelled glycans

HILIC-UPLC profiles of the procainamide labelled N-glycans from IgG_1 mAb. (A) Profile after removal of sialic acids, fucoses, beta-galactoses, beta-N-acetylhexosamines - leaving glycans with Galα1-3Gal epitope. (B) Identities of Galα1-3Gal bearing glycans were confirmed after treatment with alpha-galactosidase (which causes all the peaks for the Galα1-3Gal glycans to move).

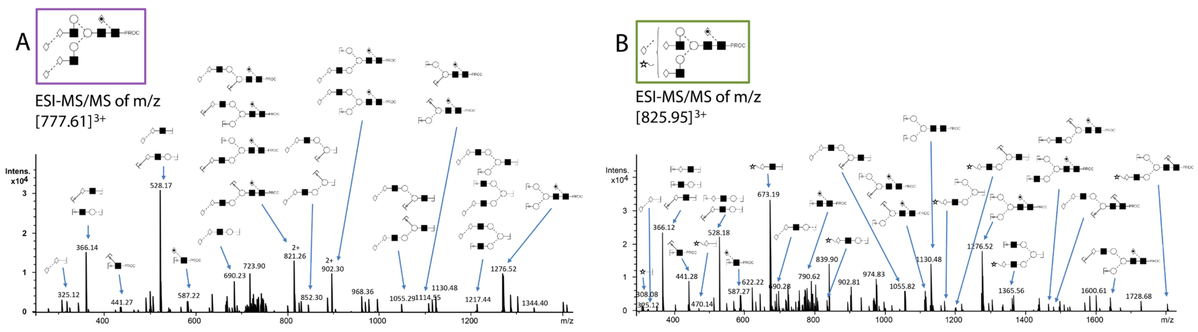

Figure 4: Example of MS/MS fragmentation patterns of procainamide labelled N-glycans released from IgG_1 mAb.

These show the high quality of MS/MS fragmentation data obtained for the procainamide labelled glycans. This greatly improves the ease of obtaining accurate, detailed glycan structures compared to equivalent work with 2-AB.

Structure	GU	% Area	ESI-MS/MS Composition Hex (H)	HexNAc (N)	Fucose (F)	NeuGc (Sg)	Mass found	Mass Calculated	Degree of Certainty GU	Exoglycosidase digestions	m/z at GU	MS/MS at GU
A1[6]	4.72	0.09	3	3	0	0	[667.28]$^{2+}$	[667.30]$^{2+}$	Y	Y	Y	Y
FA1	5.10	0.21	3	3	1	0	[740.30]$^{2+}$	[740.33]$^{2+}$	Y	Y	Y	Y
A2	5.20	0.82	3	4	0	0	[768.80]$^{2+}$	[768.84]$^{2+}$	Y	Y	Y	Y
FA2	5.59	39.7	3	4	1	0	[841.81]$^{2+}$	[841.87]$^{2+}$	Y	Y	Y	Y
Man5	5.97	0.27	5	2	0	0	[727.78]$^{3+}$	[727.81]$^{3+}$	Y	Y	Y	Y
FA3			3	5	1	0	[943.36]$^{2+}$	[943.41]$^{2+}$	Y	Y	Y	Y
A2[6]G1	6.05	0.56	4	4	0	0	[849.81]$^{2+}$	[849.86]$^{2+}$	Y	Y	Y	Y
FA1G1			4	3	1	0	[821.35]$^{2+}$	[821.35]$^{2+}$	Y	Y	Y	Y
A2[3]G1	6.14	0.29	4	4	0	0	[849.32]$^{2+}$	[849.86]$^{2+}$	Y	Y	Y	Y
FA2[6]G1	6.43	18.8	4	4	1	0	[922.85]$^{2+}$	[922.89]$^{2+}$	Y	Y	Y	Y
FA2[3]G1	6.55	21.7	4	4	1	0	[922.84]$^{2+}$	[922.84]$^{2+}$	Y	Y	Y	Y
FA3G1	6.66	0.56	4	5	1	0	[1024.37]$^{2+}$	[1024.43]$^{2+}$	Y	Y	Y	Y
FA1[6]G1Ga1	6.80	0.51	5	3	1	0	[902.33]$^{2+}$	[902.38]$^{2+}$	Y	Y	Y	Y
Man 6			6	2	0	0	[808.84]$^{2+}$	[808.78]$^{2+}$	Y	Y	Y	-
A2G1Ga1	6.91	0.37	5	4	0	0	[930.84]$^{2+}$	[930.83]$^{2+}$	Y	Y		
A2G2			5	4	0	0	[930.84]$^{2+}$	[930.89]$^{2+}$	Y	Y	Y	Y
FA1[3]G1Ga1	7.00	0.24	5	3	1	0	[902.33]$^{2+}$	[902.38]$^{2+}$	Y	Y	Y	Y
FA2G2	7.34	8.89	5	4	1	0	[669.61]$^{3+}$	[669.61]$^{3+}$	Y	Y	Y	Y

Structure	GU	% Area	ESI-MS/MS Composition Hex (H)	HexNAc (N)	Fucose (F)	NeuGc (Sg)	Mass found	Mass Calculated	Degree of Certainty GU	Exoglycosidase digestions	m/z at GU	MS/MS at GU
FA2[3]G1Ga1	7.46	0.56	5	4	1	0	[669.61]$^{3+}$	[669.62]$^{3+}$	Y	Y	Y	Y
FA3G2	7.61	0.18	5	5	1	0	[732.22]$^{3+}$	[731.31]$^{3+}$	Y	Y	Y	Y
FA3G2	7.69	0.26	5	5	1	0	[737.31]$^{3+}$	[737.22]$^{3+}$	Y	Y	Y	Y
FA3G1Ga1	7.72	0.21	5	5	1	0	[737.31]$^{3+}$	[737.22]$^{3+}$	Y	Y	Y	Y
FA2[6]G1Sg1	7.97	0.10	4	3	1	0	[717.94]$^{3+}$	[717.96]$^{3+}$	Y	Y	Y	Y
FA2[3]G1Sg1	8.10	0.34	5	4	1	1	[771.98]$^{3+}$	[771.93]$^{3+}$	Y	Y	Y	Y
FA2G2[6]Ga1	8.22	1.09	6	4	1	0	[723.60]$^{3+}$	[723.63]$^{3+}$	Y	Y	Y	Y
FA2G2[3]Ga1	8.45	0.10	6	4	1	0	[723.60]$^{3+}$	[723.63]$^{3+}$	Y	Y	Y	Y
FA3G3	8.57	0.28	6	5	1	0	[791.28]$^{3+}$	[791.33]$^{3+}$	Y	Y	Y	Y
FA2G2Sg1	8.89	0.50	5	4	1	1	[791.28]$^{3+}$	[791.33]$^{3+}$	Y	Y	Y	Y
FA2G2Ga2	9.12	0.78	7	4	1	0	[771.93]$^{3+}$	[771.98]$^{3+}$	Y	Y	Y	Y
FA2G2Ga1Sg1	9.39	0.16	6	4	1	1	[825.94]$^{3+}$	[826.00]$^{3+}$	Y	Y	Y	Y
FA3G2Ga2 and FA3G3Ga1			7	4	1	0	[845.64]$^{3+}$	[845.33]$^{3+}$	Y	Y	Y	Y
FA2G2Ga1Sg1	9.73	0.43	6	4	1	0	[825.95]$^{3+}$	[826.00]$^{3+}$	Y	Y	Y	Y
A2G2Sg2	10.11	0.26	5	4	0	2	[825.62]$^{3+}$	[826.00]$^{3+}$	Y	Y	Y	Y
FA2G2Sg2	10.50	0.32	5	4	0	2	[874.29]$^{3+}$	[874.68]$^{3+}$	Y	Y	Y	Y
FA3G3Ga3	11.17	0.14	9	5	1	0	[953.38]$^{3+}$	[953.30]$^{3+}$	Y	Y	Y	Y

Table 2: Identification of Galα1-3Gal epitope on IgG_1 mAb - results from UHPLC-ESI-MS/MS analyses of procainamide glycans

Summary of GU, % Area, ESI-MS/MS and digestion data from IgG_1 mAb procainamide labelled N-glycans. Glycans identified as bearing the Galα1-3Gal epitope are highlighted in red.

Procainamide labelling system for monitoring of Galα1-3Gal Strategy to assess RISK for IgG_1 mAb	
Can you see it?	✓
Is it really Gal α1-3Gal?	✓
How much?	3.88%

Table 3: Analysis of Galα1-3Gal in the test IgG_1 mAb

Procainamide labelling, coupled with HILIC-UHPLC-ESI-MS/MS and exoglycosidases sequencing is a powerful workflow for accurate identification and quantitation of Galα1-3Gal bearing glycans.

References

1. Platts-Mills TA, Schuyler AJ, Tripathi A, Commins SP. (2015) '*Anaphylaxis to the carbohydrate side chain alpha-gal*'. Immunol Allergy Clin North Am **35**:247-260.
2. Fernandes, DL (2012) '*A Systematic Approach to Optimisation and Comparability of Biopharmaceutical Glycosylation Throughout the Drug Life Cycle*' in Subramanian, G (Ed) Biopharmaceutical Production Technology' published by Wiley-VCH Verlag GmbH & Co KGaA
3. Fernandes, DL (2012) '*Assessing Comparability of Glycosylation between a Biosimilar Antibody and the Innovator Product*', Antibodies Asia 2012, Shanghai, China
4. Tharmalingam T, Adamczyk B, Doherty MA, Royle L, Rudd PM. (2013) '*Strategies for the profiling, characterisation and detailed structural analysis of N-linked oligosaccharides*' Glycoconj J. **30**:137-146
5. Kozak RP, Tortosa CB, Fernandes DL, Spencer DI. (2015) '*Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization-mass spectrometry*' Anal Biochem **486**:38-40

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