Introduction of a Novel Labelling Strategy to Facilitate LC-MS Analysis of Released *N*-Glycans

Philip Widdowson[1], Zoltan Szabo[2], Shaheer Khan[2] and Jonathan Bones[3]

[1]Thermo Fisher Scientific, Runcorn, UK, [2]Thermo Fisher Scientific, San Francisco, USA, [3]National Institute for Bioprocessing Research and Training, Dublin, IRE

ABSTRACT

Purpose: The introduction of a novel labelling strategy for the analysis of released *N*-glycans has been developed and here we evaluate 3-aminobenzene sulfonic acid (3-ASA) performance and ability to facilitate enhanced identification and comprehensive *N*-glycan characterization

Methods: Industry relevant glycoprotein samples were prepared for analysis using the optimized 3-ASA labelling strategy. The 3-ASA labelled released glycan samples were analyzed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to Thermo Scientific™ Q Exactive ™ and Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometers in negative ionization mode. The performance of this novel labelling approach was benchmarked against the market leading commercially available glycan labels, *Rapi*Fluor-MS (Waters Corporation) and 2AB (purchased from Sigma). A ^{13}C-labelled isotopologue of 3-ASA was also utilized and the potential for isotopic quantification assessed.

Results: 3-ASA performance is highly comparable to the market leading glycan labels with respect to chromatographic separation and calculated relative glycan abundances of industry relevant glycoproteins. Mass spectrometry of 3-ASA labelled glycans negative ionization mode which proves advantageous when compared to positive mode ionization strategies. More structurally relevant diagnostic ions were observed in glycans labelled with 3-ASA compared to other commercially available labels which led to enhanced identification and characterization glycan structures. Utilizing ^{12}C and ^{13}C-isotopologues of 3-ASA permitted isotopic quantification of glycans and opens up the potential for comparability, isotopic quantification and comprehensive structural characterization all within a single analysis whilst also increasing the potential throughput by a factor of 2.

INTRODUCTION

Glycosylation assessment is a regulatory requirement during the manufacture of glycoprotein therapeutics. ICH[1] guidelines (ICH Q6B) outline the extent to which the attached carbohydrates should be characterized to ensure safety, efficacy and batch-to-batch consistency. Currently, released glycan analysis is primarily performed by derivatizing the released glycan with a fluorescent label. Most, but not all, fluorescent glycan labels are compatible with mass spectrometric analysis, which is a highly desirable tool for comprehensive glycan identification. The majority of glycan quantification is carried out using fluorescence detection and comparison of relative peak abundances between samples. There are drawbacks to this approach, however, as it is heavily reliant on chromatographic separation, making it near impossible to adequately quantify the relative abundances of co-eluting species without additional mass detection. In addition, the over-reliance on chromatographic separation means that high-throughput approaches using liquid chromatography (LC) separation remains a challenge.

A novel approach, using 3-aminobenzene sulfonic acid (3-ASA) has been developed and here we evaluate 3-ASA as a strategy for the labelling and subsequent LC-MS analysis of released N-glycans, facilitating enhanced identification and comprehensive glycan characterization. MS2 fragmentation in negative ion mode generated more structurally informative spectra than in positive ion mode, due to the increased propensity for cross-ring fragments to be formed in negative ion mode[2].

MATERIALS AND METHODS

Glycoprotein samples (hulgG and trastuzumab) were prepared using a modified version of the Applied Biosystems™ GlycanAssure™ HyPerformance kit incorporating the 3-ASA label. LC separation was performed using HILIC chromatography on a Thermo Scientific™ Vanquish™ Horizon UHPLC system. High resolution mass spectrometry (HRMS) was performed in negative ionization mode using both Q Exactive and Q Exactive Plus Orbitrap Mass Spectrometers controlled by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) and Thermo Scientific™ Xcalibur™ software.

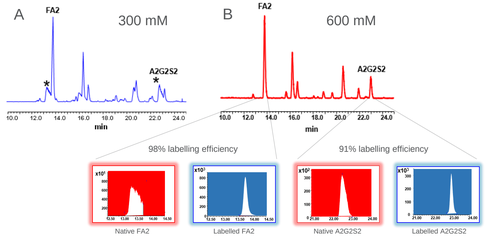
Figure 1. A: The GlycanAssure HyPerformance APTS labelling kit, B: 3-ASA. The design of the existing kit allows simple incorporation of the 3-ASA into the protocol without any significant changes to the final release and labelling procedure. 3-ASA is available as both ^{12}C- and ^{13}C-labelled isotopologues.

RESULTS

Development of 3-ASA for incorporation into GlycanAssure kit

The design and flexibility of the GlycanAssure APTS kit, allows effortless incorporation of alternative glycan labels into the workflow, without any significant alteration to the pre-labelling steps (denaturation and deglycosylation) or additional labour for the end-user. The denaturation and degylcosylation steps have already been previously optimized for commercialization of the GlycanAssure APTS kit[3] to ensure robust and reproducible protein unfolding and glycan release, therefore, the majority of further development has focused on optimizing the concentration of 3-ASA required to give most efficient labelling (Figure 3.)

Figure 3. Optimization of 3-ASA concentration for most efficient labelling. A) 300 mM and B) 600 mM final concentration were assessed. The * indicates significant levels of under-labelling. Inset: Extracted Ion Chromatograms (XICs) of fully labelled and native FA2 (left) and A2G2S2 (right) used to calculated labelling efficiency

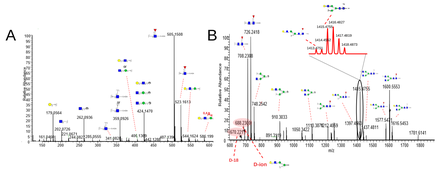


Significant under-labelling was observed across all glycan species (Figure 3A) at 300 mM 3-ASA When increased to 600 mM (Figure 3B), however, level of under-labelling is minimal, with labelling efficiencies calculated to be 98% for the fucosylated FA2 glycan and 91% for the biantennary, disialylated A2G2S2. 600 mM 3-ASA (in DMSO/acetic acid) was, therefore, used as the final concentration of label for further assessment and performance optimization studies.

Mass spectrometry performance in negative ionization mode

Negative ionization fragmentation of glycans provides significantly more structural information than is achieved using positive ionization labelling. 3-ASA carries a negative charge (Figure 1B), which permits this mode of fragmentation. The ability of 3-ASA labelled glycans to yield structurally informative data was assessed, using the FA2G1 glycan, commonly found as isomeric structures in monoclonal antibodies (mAbs), with the galactose residue found on either the 3- or 6-antenna. The fragmentation spectrum of one of these isomers was inspected for the presence of diagnostic fragment ions which could aid structural elucidation

Figure 4. Negative ion fragmentation of FA2G1 glycan labelled with 3-ASA. A: low mass fragmentation spectrum, B: high mass fragmentation spectrum. In both spectra, some key ions have been annotated including the structurally significant $^{0,4}A_3$ (m/z 586.2) and D-ion (with D-18) (m/z 688.2 and 670.2 respectively). These diagnostic fragments allow the galactose to be localized to the 6-antenna. Inset: the power of high resolution accurate mass spectrometry, permits the identification of this pair of fragment ions with highly similar m/z.



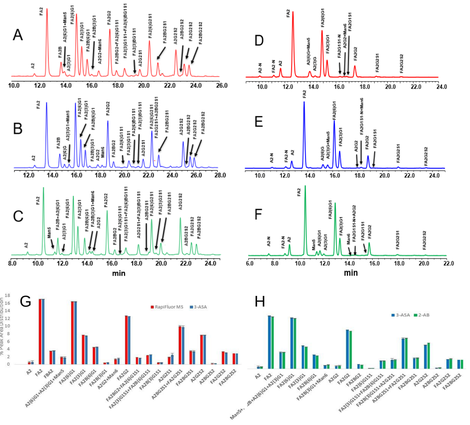
The presence of structurally informative ^{0,4}A and D-ions confirmed that the galactose was located on the 6-antenna[2], enabling this isomer to be unequivocally identified at FA2(6)G1. The ability to perform this level of structural characterization is a significant advantage of labelling with 3-ASA over other commercially available glycan labels.

Performance compared to other commercially available glycan labels

There are many commercially available labels for released glycan analysis on the market today. To show both utility and potential for competitiveness within the glycan analysis market, a direct comparison of 3-ASA with the market leading reagents has been carried out. Both a typical IgG1 molecule (trastuzumab – Figure 3. D-F), along with a more complex human IgG standard (Figure 3. A-C), were prepared and labelled with 3-ASA, *Rapi*Fluor-MS and 2AB.

Sialylated glycans can be difficult to analyse both due to their propensity to be lost during sample clean-up procedures and the lability of the sialic acid group. Incorporating hulgG into this comparison, allows the performance of 3-ASA to be benchmarked on a number of different glycan types (fucosylated, sialylated, bisecting GlcNAc).

Figure 4. FLD comparison of 3-ASA to other commercially available glycan labelling reagents. A, B and C: hulgG labelled with *Rapi*Fluor-MS, 3-ASA and 2AB respectively, D, E and F: Trastuzumab labelled with *Rapi*Fluor-MS, 3-ASA and 2AB respectively. G: Comparison of peak area distribution of identified glycans between *Rapi*Fluor-MS and 3-ASA, H: Comparison of peak area distribution of identified glycans between 2AB and 3-ASA.



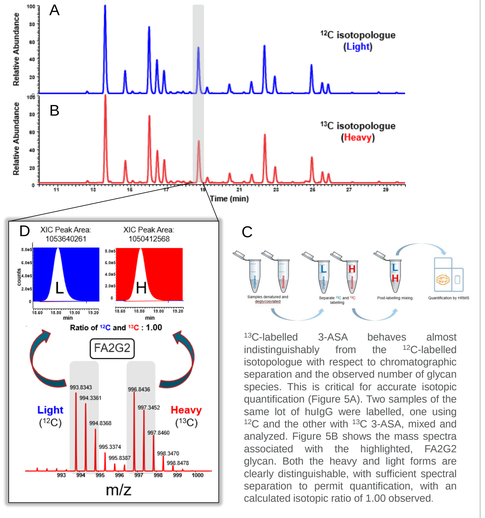
Aside from some minor differences in selectivity, the overall separation and relative abundance of detected glycan species in the 3-ASA labelled samples are highly comparable to both the *Rapi*Fluor-MS and 2AB labelled samples. Whilst there was a difference in the absolute intensities achieved between the different labels, in each case, the number of identified peaks was the same.

The graphical representation of peak area distribution is shown (Figures 3G and H), for the hulgG sample, for 3-ASA compared directly to *Rapi*Fluor-MS and 2AB respectively. In each case, comparability has been demonstrated for 3-ASA, with no significant differences between the observed relative abundances of any the identified glycan species. These data highlight both 3-ASA utility as a tool for released glycan analysis and its strong comparative performance relative to the market leading commercially available reagents.

Quantification of glycans using ^{12}C and ^{13}C-isotopologues

^{12}C and ^{13}C-labelled isotopologues of 3-ASA were prepared and utilized in the described labelling strategy. The availability of both ^{12}C and ^{13}C-labelled isotopologues facilitates a two-plexed analysis to be performed for high-throughput detection and quantification. Simply put, samples are labelled separately with ^{12}C and ^{13}C 3-ASA (Figure 5C), mixed and analyzed simultaneously. The differentially labelled samples are easily distinguished based on the 6 Da. mass difference between the ^{12}C and ^{13}C-labels with quantification performed using the relative isotopic ratio. This approach is of particular use for high-throughput comparability studies – such as clone screening or biosimilar comparability – as it permits the analysis of two samples simultaneously. This effective 2-fold increase in throughput with concurrent glycan characterization, relative quantification and comparability within a single run is a significant advantage of 3-ASA labelling over other approaches.

Figure 5. Comparison of ^{12}C (light) and ^{13}C (heavy) isotopologues of 3-ASA plus the method of isotopic quantification. A: hulgG labelled with the ^{12}C isotopologue, B: hulgG labelled with the ^{13}C isotopologue, C: Schematic workflow of sample preparation of isotopic quantification (for example for quantification of biosimilar glycans relative to the originator) D: Example of isotopic quantification of the FA2G2 glycan. The same sample was labelled with both ^{12}C and ^{13}C isotopologues (as described in panel C). Following HRMS analysis, the isotopic ratio was determined to be 1.00 for this glycan.

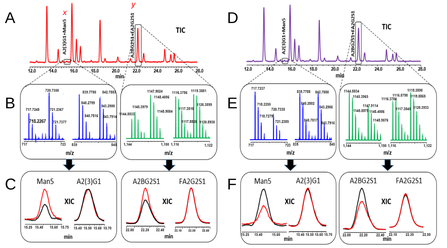


A further example of the power of isotopic quantification using 3-ASA is observed in Figure 6. In this example, two further lots of hulgG were prepared and the ^{12}C/^{13}C isotopic pairs quantified. The ability of this method to decipher subtle differences in glycosylation between sample can be observed. It is clear that in peak *x*, the amount of A2(3)G1 is consistent between the two samples, yet there is a difference in the levels of Man5. Similarly in peak y, the amount of FA2G2S1 remains consistent between the samples whilst the A2BG2S1 glycan levels are different.

^{13}C-labelled 3-ASA behaves almost indistinguishably from the ^{12}C-labelled isotopologue with respect to chromatographic separation and the observed number of glycan species. This is critical for accurate isotopic quantification (Figure 5A). Two samples of the same lot of hulgG were labelled, one using ^{12}C and the other with ^{13}C 3-ASA, mixed and analyzed. Figure 5B shows the mass spectra associated with the highlighted, FA2G2 glycan. Both the heavy and light forms are clearly distinguishable, with sufficient spectral separation to permit quantification, with an calculated isotopic ratio of 1.00 observed.

Such a determination would be impossible using a fluorescence-only method. Even when a standard mass spectrometry based approach is employed, identifying and quantifying this change would be difficult due to: a) the subtle nature of the change, b) in the case of the A2(3)G1/Man5 pair, the relatively low abundance of these species, c) the requirement to perform multiple injections to achieve the same result. For increased confidence, the experiment was repeated, except the samples were labelled with the opposite label (heavy or light) to the previous experiment – yielding highly comparable results (Figure 6D-F)

Figure 6. Comparison of two lots of hulgG with isotopic quantification. A: Combined TIC of ^{12}C labelled lot 1 and ^{13}C labelled lot 2, B: The isotopic ratios of the Man5/A2(3)G1 and A2BG2S1/FA2G2S1 co-eluting pairs, C: XICs of the heavy and light versions of each of the glycans highlighting differences, which can be quantified, in Man 5 and A2BG2S1 glycans. D-F: The same as for A-C with ^{12}C labelled lot 2 and ^{13}C labelled lot 1 hulgG



CONCLUSIONS

- A novel strategy, using 3-ASA, for released *N*-glycan labelling has been developed and optimized
- Fragmentation in negative ionization mode permits more structurally informative glycan characterization than is achieved when using positive ionization mode
- 3-ASA compares strongly against market leading glycan labelling reagents (*Rapi*Fluor-MS and 2AB)
- ^{12}C and ^{13}C isotopologues allow for isotopic quantification and higher throughput analyses which are advantageous, for example, for originator/biosimilar comparability and clone screening activities
- Enhanced structural characterization of released *N*-glycans using a novel fluorescent label in a manner accessible to the biopharma industry.

REFERENCES

[1] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6B

[2]Harvey, D. J. (2005), *JASMS*, **16**(5), 622-630

[3]Eckard, A. D *et al.*, (2017), *Am. Pharm. Rev*, Posted 22 December 2017

ACKNOWLEDGEMENTS

We would like to thank Dr. Dietmar Reusch (Roche, Penzberg, Germany) for a kind provision of samples used in this work. We would also like to thank our customers and colleagues in the BioProduction Division, Bio/Pharma and Product Marketing teams for continued support.

TRADEMARKS/LICENSING

PO65518-EN0519S

ThermoFisher SCIENTIFIC