V.V. Agon and C. Goebel

Analysis of derivatised carbohydrates of natural and recombinant peptide hormones by LC/MS/MS

Australian Sports Drug Testing Laboratory (ASDTL), National Measurement Institute, Pymble, NSW, Australia

Introduction

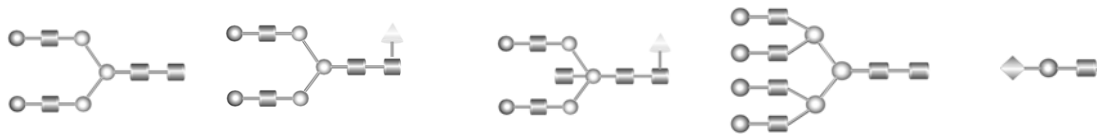
Peptide hormones such as hCG, LH and EPO and their recombinant analogues are on the WADA Prohibited List. hCG and LH are often misused by male athletes to stimulate the production of endogenous testosterone following administration of steroids. EPO is used to increase oxygen capacity for endurance sports. These hormones are naturally produced in the body and the amino acid sequences are indistinguishable from their recombinant analogues.

However, these peptide hormones undergo post-translational modification wherein carbohydrates are covalently linked to asparagine residues (N-linked sugars) or to serine/threonine residues (O-linked sugars) within the peptide backbone. Glycosylation, particularly in recombinant technology, is dependent on the species and the type of cell used, which has a bearing on the resulting isoform profile. The variation in the formation of isoforms is the basis of the current gel method for the discrimination between urinary and recombinant EPO.

Preliminary experiments were performed using commercially available glycan standards to evaluate labelling techniques using a variety of chemical derivatives. Initial experiments were also performed on N-glycans following deglycosylation of glycoprotein hormones such as human Chorionic Gonadotropin (hCG) and recombinant EPO. The labelled carbohydrates were analysed by LC/MS/MS and compared with unlabelled glycans and glycan standards to reveal structural information on the sugar groups as well as isoform composition.

Materials and Methods

Commercially available glycan standards (Figure 1) were used to examine the derivatisation of the chemical reagents.



NA2 NA2F NA2FB NA4 SLN

Figure 1. Carbohydrate standards used for derivatisation reactions. Symbol key: (●) hexose; (■) N-acetylhexosamines; (▲) fucose; (◆) sialic acid.

Derivatisation of Glycans: Reductive Amination using 2-picoline borane

Fresh solutions of 2-AB (2-aminobenzamide), 2-AMAC (2-aminomethylacridone) and 6-AQ (6-aminoquinoline) (48 mg/mL in 85% DMSO/15% acetic acid) and the reducing agent 2-picoline borane (1 M in DMSO) were prepared. The sample was reconstituted in 50 µL water and mixed with 25 µL of both the labelling solution and reducing agent (Figure 2). The mixture was incubated at 65°C for 2 hours (300 rpm). Glycans were desalted using PGC tips (Thermo Scientific) conditioned with 80% acetonitrile/0.1% TFA. The tips were washed with water followed by sample loading. The tips were washed with water and the glycans eluted with 25% acetonitrile/0.05% TFA. The sample was vacuum dried prior to LC/MS/MS analysis.

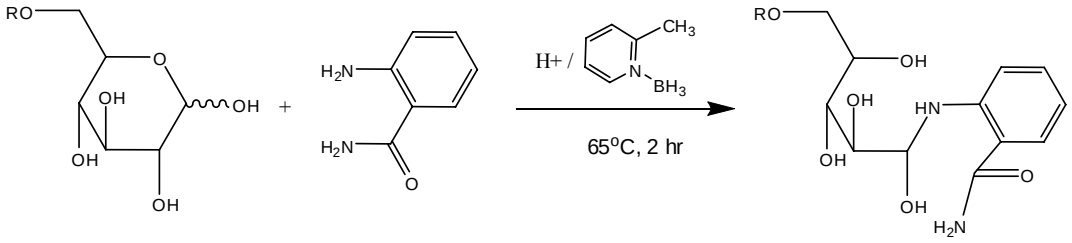


Figure 2. Derivatisation of glycans at the reducing end using aryl amine reagents such as 2-AB, 2-AMAC and 6-AQ with 2-picoline borane as the reducing agent. R = rest of the glycan.

Reductive Amination using Pyridine-Borane Complex

Solutions of 7-amino-4-methylcoumarin (AMC; 60 mM in DMF) and borane-pyridine complex (200 mM in acetic acid) were prepared. The dried glycan was dissolved in 20 µL of AMC and 20 µL of borane-pyridine complex solutions. The sample was heated at 70°C for 1 hour (300 rpm) and then the reaction was quenched with 60 µL milli-Q water and chilled in an ice bath (Figure 3). A SCX-type SPE (Silicycle) was conditioned with methanol.

The sample (in methanol) was loaded onto the cartridge and washed with methanol. The derivatised glycan was eluted with 30% methanol and vacuum dried. An Oasis HLB cartridge was conditioned with methanol then acetonitrile and finally with water. The sample (in water) was loaded onto the cartridge and washed with water. The labelled glycan was eluted with 30% acetonitrile, vacuum dried and analysed by LC/MS/MS.

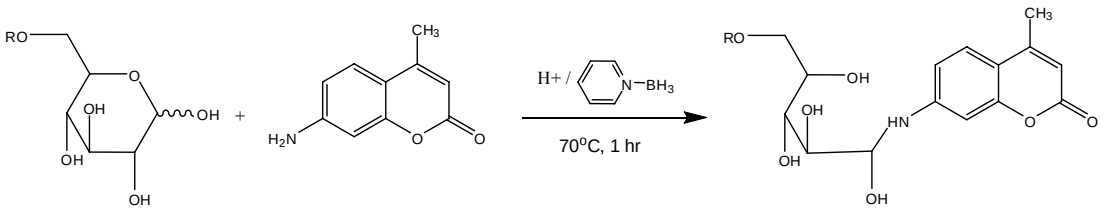


Figure 3. Derivatisation of glycans at the reducing end using aryl amine reagents such as AMC with picoline-borane as the reducing agent. R = rest of the glycan.

Non-reductive Hydrazide-Mediated Coupling

100 µL of 5 mM BACH (biotinamidocaproyl hydrazide) solution (in 30% acetonitrile) was added to the dried glycan and vacuum dried. To the dried mixture, 30 µL of 95% methanol was added and incubated at 90°C for 1 hour (300 rpm) (Figure 4). The sample was vacuum dried prior to LC/MS/MS.

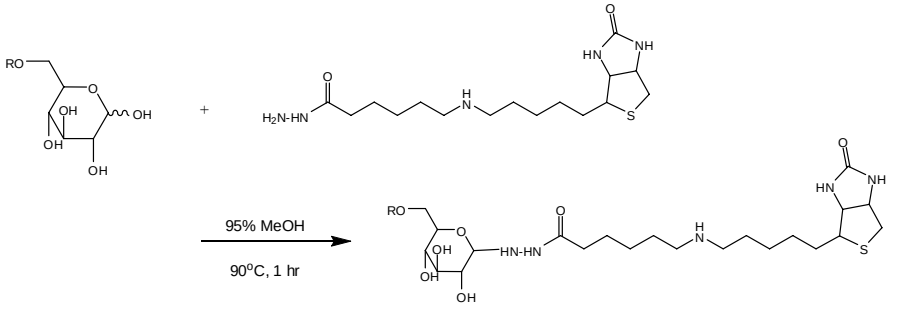


Figure 4. Reaction showing the non-reductive hydrazide-mediated coupling of a biotin derivative to a glycan. R = rest of glycan.

N-linked deglycosylation:

The glycoprotein was made up to 45 µL with 50 mM Tris-HCl buffer (pH 8). To this, 5 µL of denaturation solution was added (2% SDS and 1 M β-mercaptoethanol) and heated at 95°C for 5 minutes. The sample was cooled and 5 µL of 15% Nonidet P-40, 5 µL PNGase F and 2 µL neuraminidase were added. The mixture was incubated for 3 hours at 37°C (300 rpm). The released N-glycans were collected using a 10kDa filter (Vivaspin). The released N-linked glycans were desalted using PGC tips as described in reductive amination using the 2-picoline borane method. Samples were vacuum dried prior to derivatisation.

LC/MS/MS Analysis

All samples were separated on a TSKgel Amide-80 column on the Agilent 1100 LC coupled to ABSciex 4000 QTrap. Solvents used were 0.1% formic acid and 0.1% formic acid in acetonitrile (50 µL/min). Analysis was performed in positive ion mode using precursor ion scanning (diagnostics ions *m/z* 204 and *m/z* 366) using DP of 20 V and CE of 35 V (MS/MS).

Results and Discussion

Presented in Table 1 is the summary of all labelling techniques performed in this study using commercially available glycan standards.

Table 1. Summary of labelling techniques performed with carbohydrate standards.

Labelling reagent	Sugar std	Reducing agent	Clean-up method	Ionisation mode	m/z ions observed (derivatives)	Comments
2-AB (MW 136)	NA2	2-picoline borane	Porous graphitic carbon tips	+ve / -ve	$[M+Na+H]^{2+}$ 881.8 $[M+Na-2H]^{-}$ 1760.6	-Labelling worked. -Unlabelled glycan eluted later. -Higher intensity observed in +ve mode
	NA2F				$[M+Na+H]^{2+}$ 954.5 $[M+Na-2H]^{-}$ 1906.8	
2-AMAC (MW 210)	NA2	2-picoline borane	Porous graphitic carbon tips	+ve	none	-Labelling did not work. -Only unlabelled glycan observed
	NA2F				none	
6-AQ (MW 144)	NA2	2-picoline borane	Porous graphitic carbon tips	+ve	$[M+Na+H]^{2+}$ 886.3	Labelling worked but on a relatively lower intensity.
	NA2F				$[M+Na+H]^{2+}$ 959.5	
AMC (MW 175)	NA4	Pyridine-borane complex	a) SCX SPE b) Oasis HLB SPE	+ve/-ve	$[M+Na+2H]^{3+}$ 845.3 $[M+Na+H]^{2+}$ 1267.2	Labelling worked. Longer clean-up step with 2 overnight drying. Signals are observed better at +ve mode.
BACH (MW 372)	NA2		If required: C18 SPE	+ve	$[M+Na+2H]^{2+}$ 1000.1	Labelling worked for all glycan standards. This labelling method is the quickest so far. Clean-up is mostly unnecessary but maybe performed.
	NA2F				$[M+Na+2H]^{2+}$ 1072.8	
	NA2FB				$[M+Na+2H]^{2+}$ 1173.5	
	NA4				$[M+Na+2H]^{2+}$ 1363.5	
	3'-SLN				$[M+Na]^{+}$ 1031.7	
	6'-SLN				$[M+Na]^{+}$ 1030.6	
	Neu5Ac			+ve/-ve	$[M+H]^{+}$ 663.5	Labelling worked but need to optimise LC separation using reversed-phase columns
	Neu5Gc				$[M+H]^{+}$ 679.6	

Shown in Figures 5 and 6 are precursor ion scans of 2-AB labelled NA2 and BACH labelled NA2. The chromatogram of the 2-AB labelled glycan shows incomplete derivatisation of NA2 glycan compared to that of BACH labelled NA2, which shows almost complete labelling. The labelling efficiency is particularly important when dealing with low levels of glycans in biological medium.

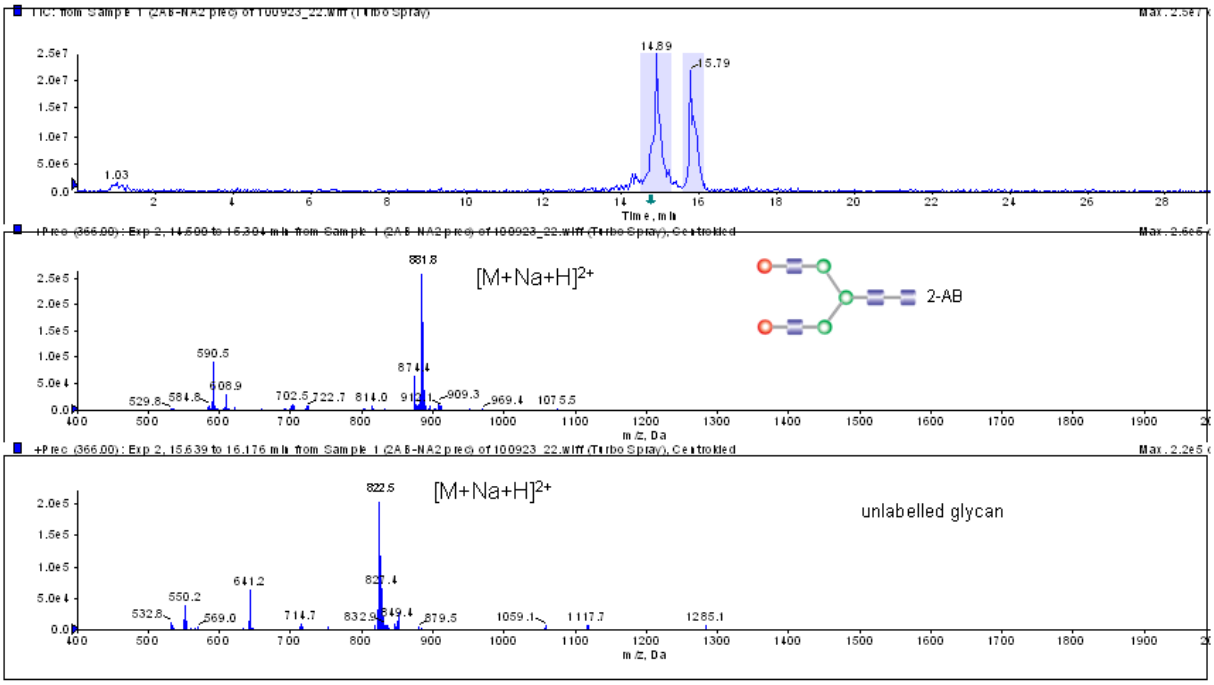


Figure 5. Precursor ion scan of 2-AB labelled NA2 glycan separated on a TSKgel amide-80 column coupled to ABSciex 4000 QTrap.

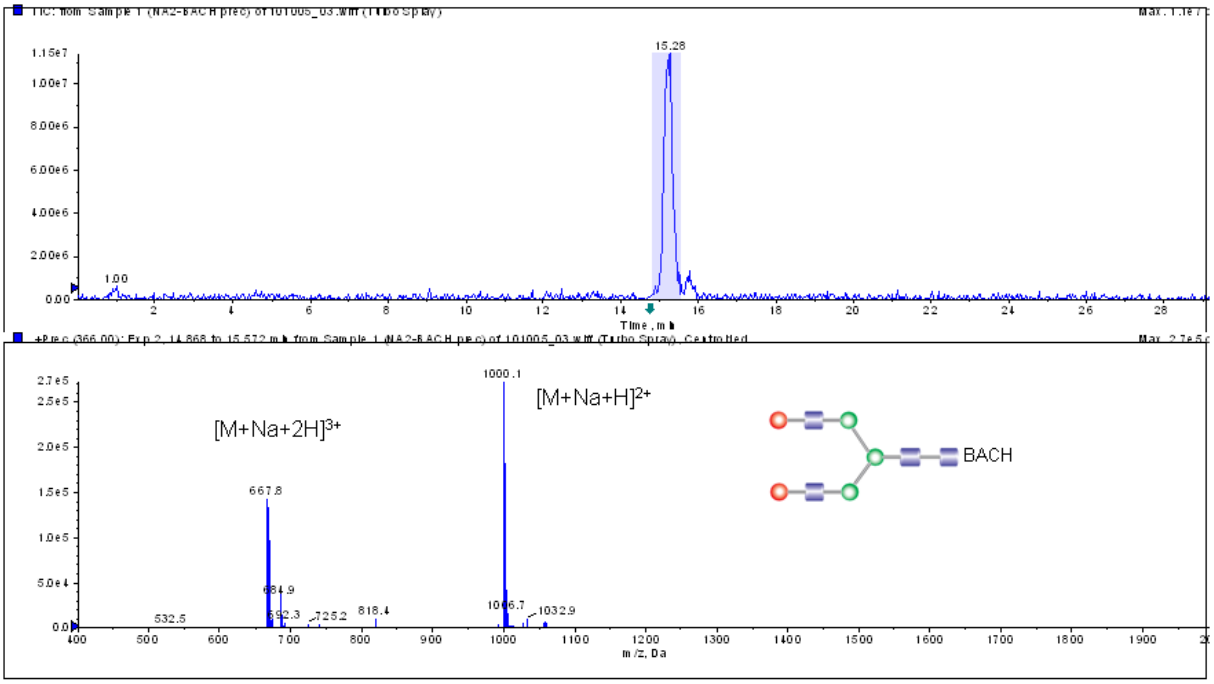


Figure 6. Precursor ion scan of BACH labelled NA2 glycan separated on a TSKgel amide-80 column coupled to ABSciex 4000 QTrap.

The derivatisation was also performed on a highly branched glycan standard NA4. Labelling of the glycan using 2-AB was not successful compared to BACH (Figures 7 and 8, respectively). Labelling of NA4 glycan with AMC was also successful (Figure 9) but the labelling method was more laborious than the other derivatisation techniques.

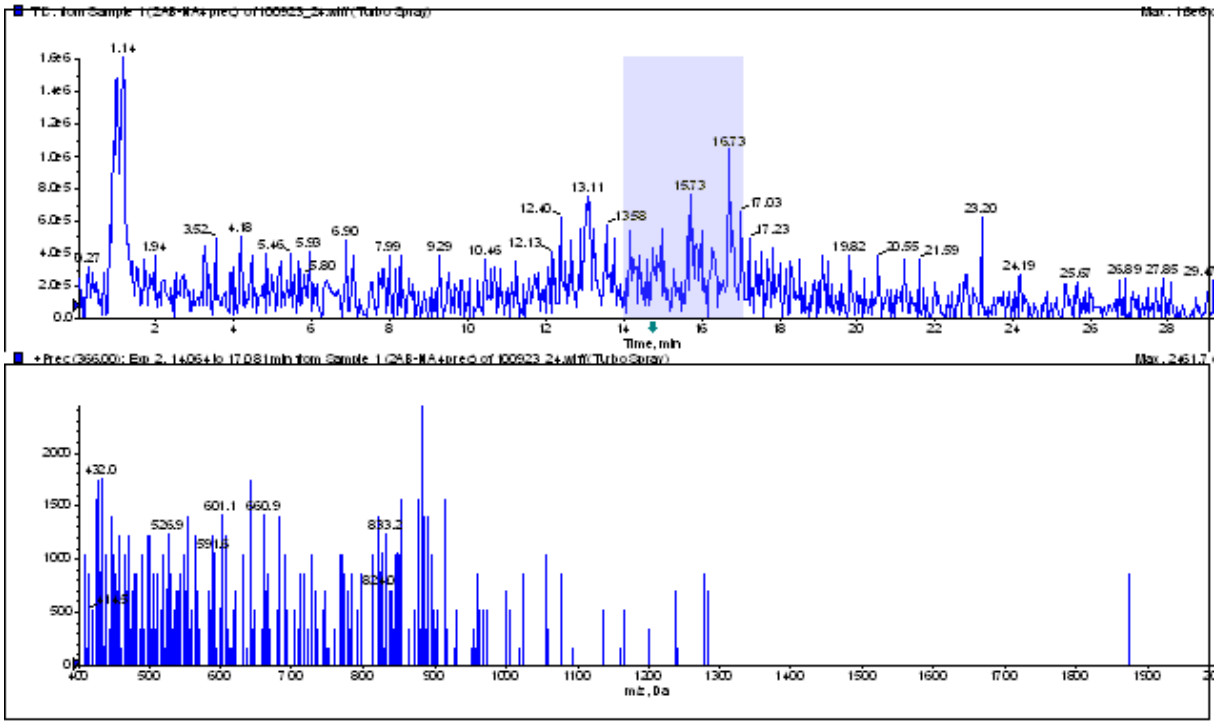


Figure 7. Precursor ion scan of 2-AB labelled NA4 glycan separated on a TSKgel amide-80 column coupled to ABSciex 4000 QTrap.

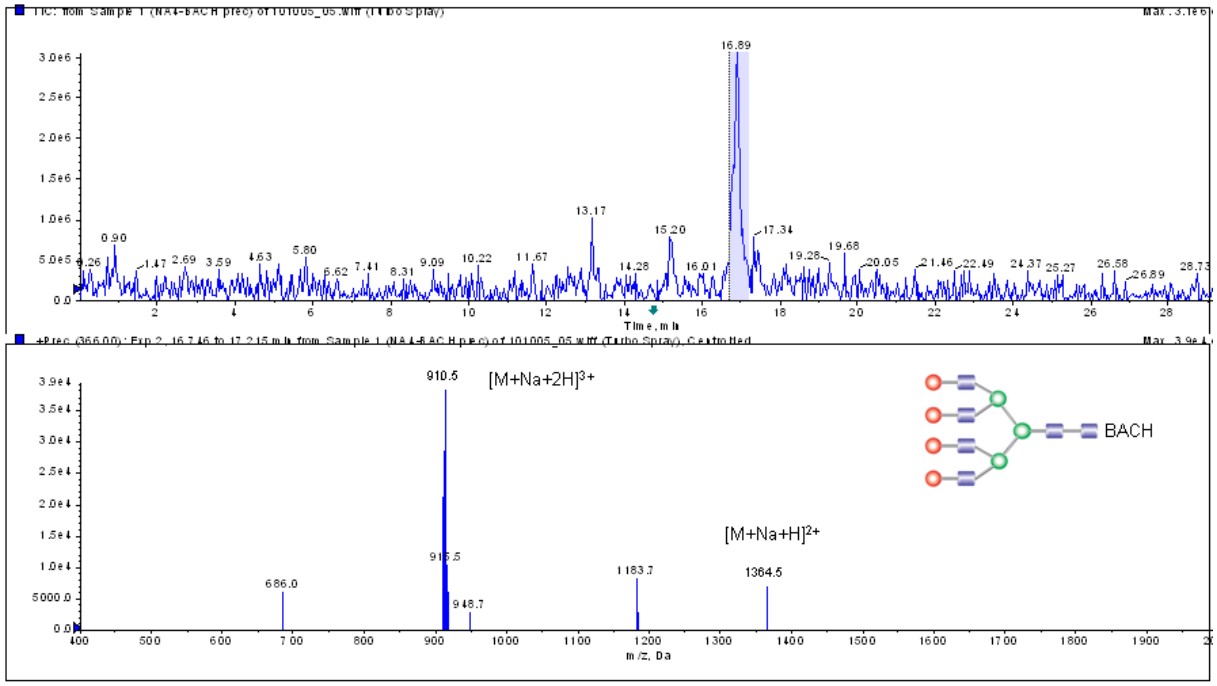


Figure 8. Precursor ion scan of 2-AB labelled NA4 glycan separated on a TSKgel amide-80 column coupled to ABSciex 4000 QTrap.

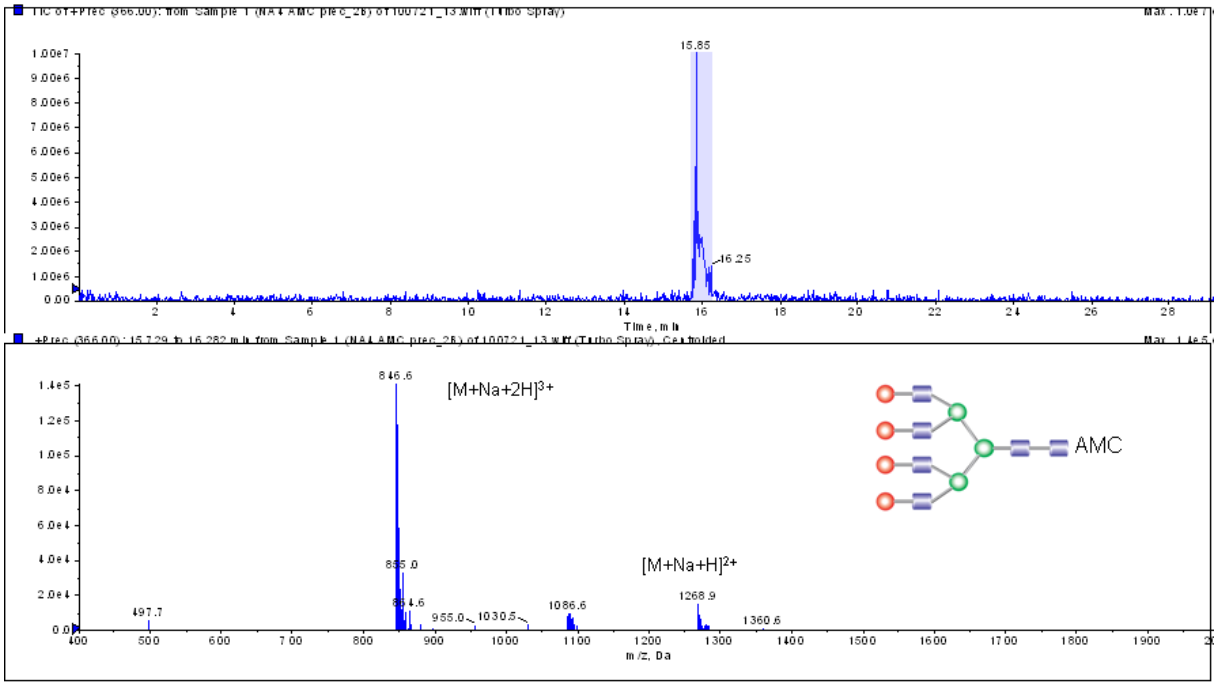


Figure 9. Precursor ion scan of AMC labelled NA4 glycan separated on a TSKgel amide-80 column coupled to ABSciex 4000 QTrap.

Among the derivatives examined so far, BACH was considered the most efficient in labelling glycans. The reaction is based on a non-reductive hydrazide mediated coupling so it does not require a reducing end. It also does not need any further purification if analysis is performed in a normal LCMS system, which makes the method simple and fast.

Deglycosylation reactions were performed on natural (Pregnyl) and recombinant (Ovidrel) hCG. Initial experiments were performed using only PNGase F for removing sugars on denatured glycoproteins as well as glycoproteins that underwent enzymatic digestion first (overnight) followed by deglycosylation with PNGase F. It was observed that the released N-glycans can be recovered from denatured hCG just using the PNGase F alone.

The precursor ion scans of free and derivatised N-linked glycans of Pregnyl and Ovidrel are shown in Figures 10-12. Only the BACH-labelled scans are shown here as the other labelling reagents, 2-AB and AMC, did not work. It was also observed that when the sialic acids are not removed, the sensitivity in the detection of the derivatised glycans was better than their unlabelled counterpart.

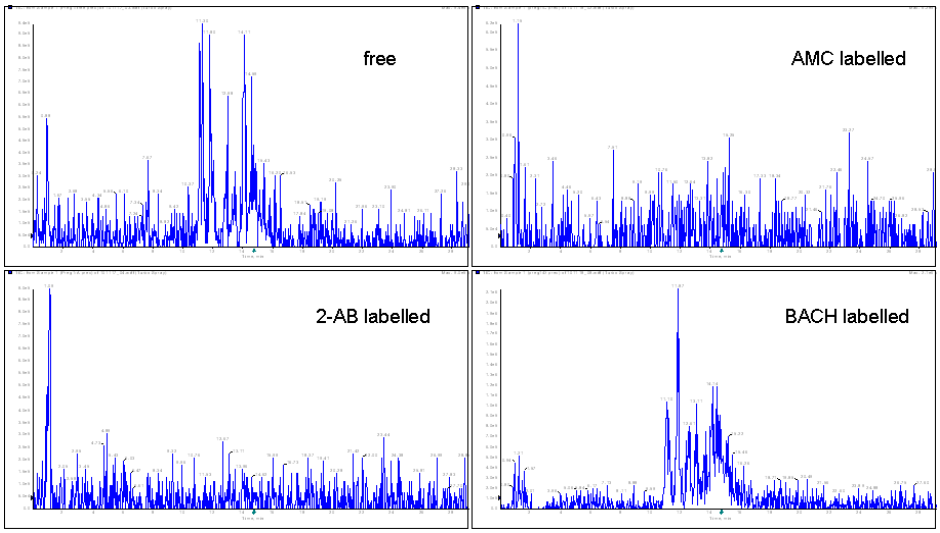


Figure 10. Precursor ion scans of N-linked glycans of Pregnyl that were free, AMC-, 2-AB- and BACH-derivatised. The sialic acids on these glycans are intact.

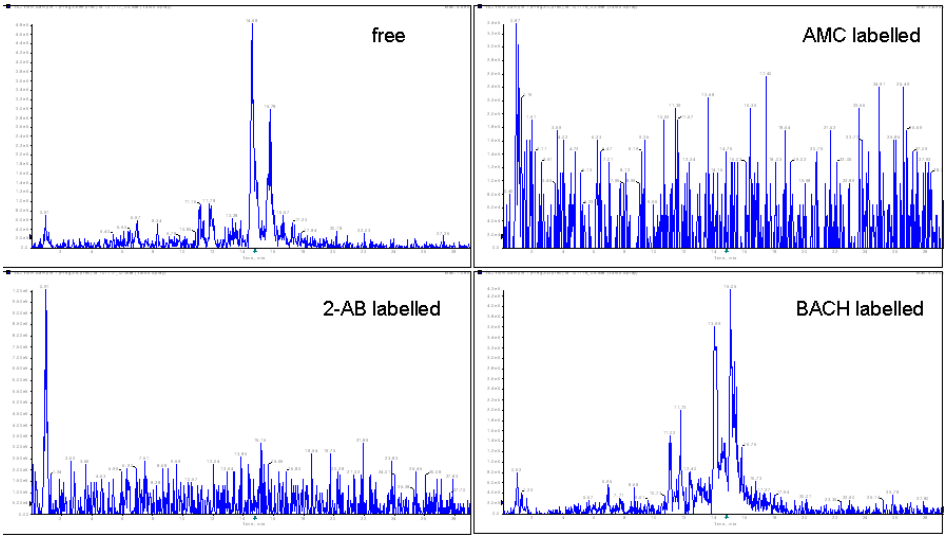


Figure 11. Precursor ion scans of N-linked glycans of Pregnyl that were free, AMC-, 2-AB- and BACH-derivatised. The sialic acids on these glycans were removed.

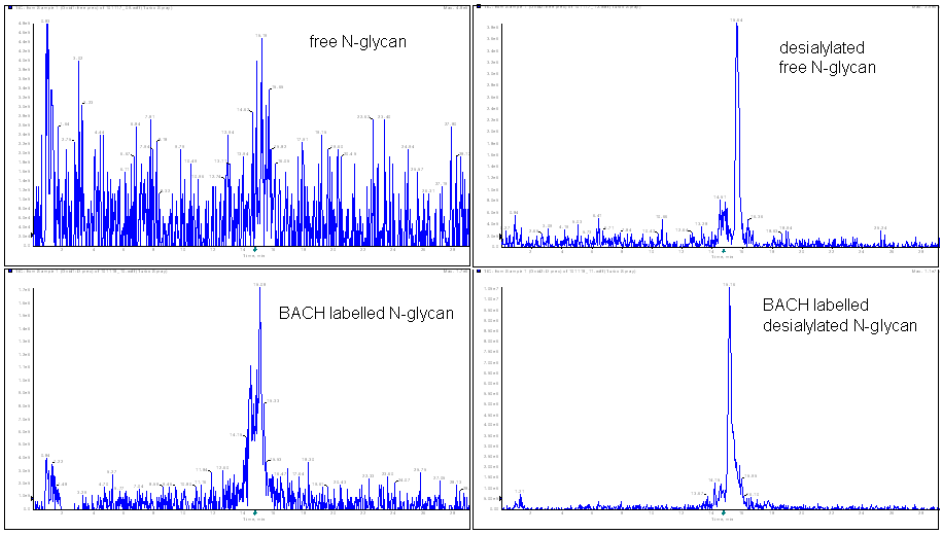


Figure 12. Precursor ion scans of N-linked glycans of Ovidrel that were free and BACH-derivatised. The sialic acids on these glycans are intact (left) and removed (right).

The increased sensitivity of the BACH-labelled N-linked glycans also showed the isoform distribution in Pregnyl and Ovidrel (Figure 13). It was observed that there were more isoforms present in the natural hCG (Pregnyl) compared to the recombinant form (Ovidrel) where the most dominant form was the biantennary glycan (maybe sialylated).

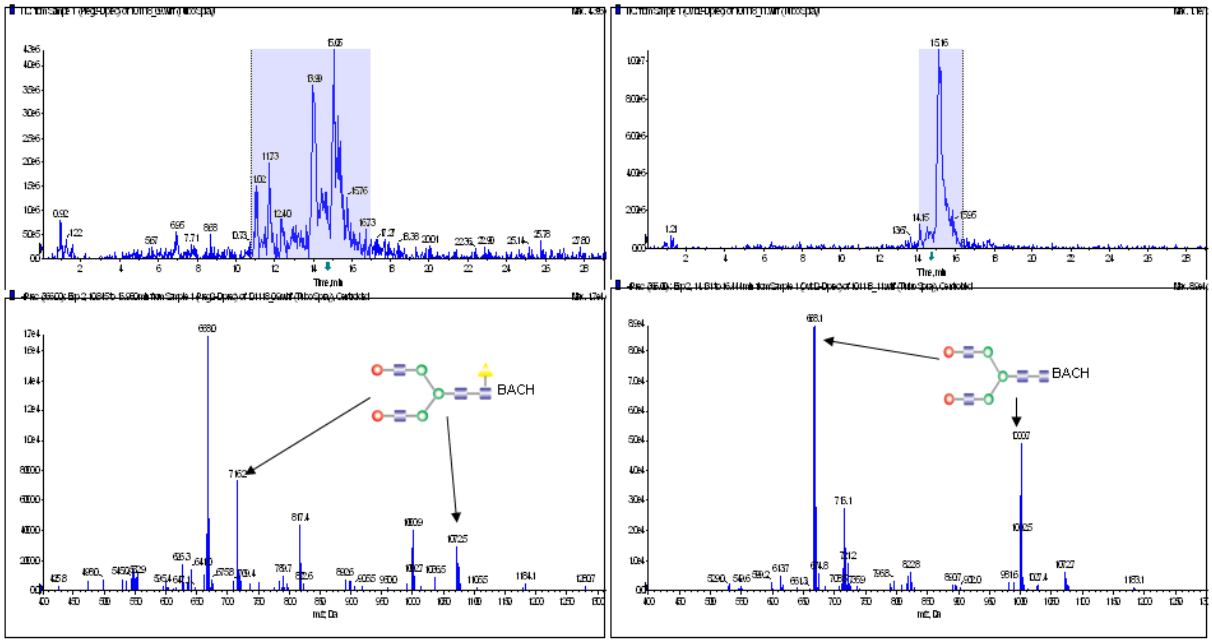


Figure 13. Precursor ion scans of BACH-labelled desialylated N-linked glycans of Pregnyl (left) and Ovidrel (right).

The differences between the O-linked glycans of natural and recombinant hCG were also investigated. At present, there is no optimal enzyme that can remove O-linked glycans as efficiently as PNGase F does for N-linked glycans. In this study, O-linked glycans were removed through β-elimination reactions to avoid peeling of the sugars and ensuring an intact reducing end for labelling reactions. This method was a β-elimination kit from Sigma, which contains a proprietary reagent and sodium hydroxide. The precursor ion scans for this method is shown in Figure 14. The reaction worked but the sensitivity was slightly increased after BACH derivatisation. Further experiments are on the way to analyse the chromatograms. Another method of removing O-linked is underway to compare recovery efficiency.

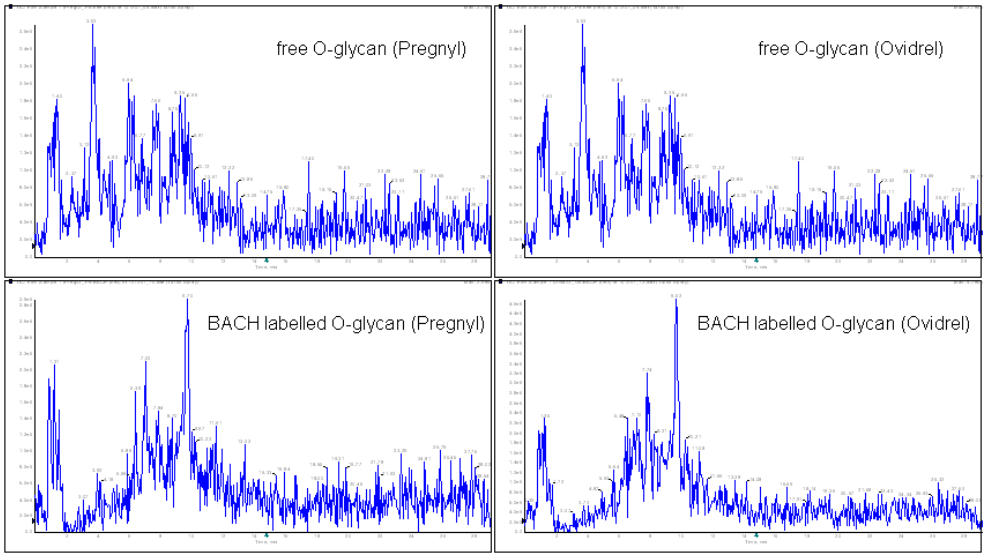


Figure 14. Precursor ion scans of free and BACH-labelled O-linked glycans from Pregnyl (left) and Ovidrel (right). The O-linked glycans were released from the glycoprotein (10 ug each) using the β-elimination kit from Sigma.

Experiments are currently underway in characterizing the carbohydrate groups of recombinant and natural glycoprotein of EPO. Due to the high heterogeneity of EPO, enzymatic digestion of the glycoprotein is required prior to N-linked deglycosylation. Once a method has been developed for both hCG and EPO, the limit of detection will be examined as well as application of the technique to purified glycoprotein from urine.

Acknowledgements

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• ASDTL (National Measurement Institute Australia)

References

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