Detection of Genetic Disorders of Glycosylation by Mass Spectrometry

A Quantitative Targeted Approach Using a 4000 QTRAP® system

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Congenital disorders of glycosylation (CDG) form a group of autosomal recessive metabolic disorders arising from defects occurring during the biosynthesis of protein glycans¹. There have been extensive studies into N-linked glycan disorders in which transferrins are glycosylated to different degrees². There have been far fewer studies on the O-linked disorders which predominantly involve errors in glycosaminoglycan synthesis. In humans, the most common mucin O-glycosylation occurs in which an N-acetylgalactosamine is attached to the hydroxyl of either a serine or threonine and a single galactose is linked to this primary sugar. N-acetyleneuraminic acid (NeuAc) attaches to the galactose to give either the asialo-, monosialo- or disialo- forms or asialo- in the absence of NeuAc.

ApoC-III is a 79 amino acid protein with a single core-1 O-glycan attached to T74. Three different isoforms can be distinguished that differ in the number of NeuAc residues attached to the O-glycan core. When the biosynthesis of the core-1 O-glycan is disturbed (Figure 1), an abnormal ApoC-III ratio is observed. The usual disruption observed in O-glycan disorders results in a decrease in the disialo- form and an increase in the asialo- and/or monosialo- forms dependent on the site of the block in the biosynthetic pathway. Exceptions to this pattern are known in sialuria, where the decreased production of CMP-NeuAc prevents inhibition causing an up-regulation of production of the disialo- intermediate and increased levels of free NeuAc in the blood and urine³. Abnormal ApoC-III patterns have also been observed in patients with cutis laxa, characterized by a significant increase in monosialo- and decrease in disialo- and normal asialo-ApoC-III levels⁴. An increase in disialo compounds would also be expected to occur in disorders of lysosomal degradation of glycoproteins. These disorders include α-mannosidosis, β-mannosidosis, fucosidosis and sialidosis.

Apolipoprotein C-III has been investigated for unusual O-glycosylation by isoelectric focusing (IEF)⁵,⁶, however, this technique is limited in its qualitative and quantitative abilities. A more specific, quantitative mass spectrometry based assay is required for the detailed analysis of the glycoprotein isoforms. The 4000 QTRAP® system was a key element in the development of this methodology due to the unique combination of scanning functions available on a single MS platform. This paper reports the presence of increased disialo-ApoC-III isoform in a patient with a clinical diagnosis of β-mannosidosis. The patient presented with severe ataxia, intellectual impairment, cerebellar hypoplasia, dystonia, and blindness.
Materials and Methods

Preparation of Samples: A gel-purified ApoC-III sample was digested with trypsin and used for preliminary method development. Plasma and urine samples were collected from research subjects that exhibited normal ApoC-III profiles and from one research subject suspected to have a sialic acid disorder. A rapid plasma sample preparation protocol was developed, consisting of enzymatic digestion (trypsin, 2hr, 37°C), size-exclusion filtration, followed by LC/MS/MS analysis. Urine samples were prepared by simply diluting 10-fold in water and direct analysis by LC/MS/MS.

Chromatography: Using an Agilent 1100 LC system, the plasma samples (40 mL) were analyzed using a C18 column (0.5 mm x 15 cm, 300A, Vydac) with a 0-40% acetonitrile gradient (60 min, 30mL/min). The diluted urine samples (10 mL) were analyzed using a C18 column (4.6 mm x 15 cm, Zorbax SB-CN) with a gradient of 100% acetonitrile to 95% 50mM ammonium formate over 6 min (1mL/min).

Mass Spectrometry: Information Dependent Acquisition (IDA) was performed using either an Enhanced MS (EMS), Precursor Ion Scanning (Prec) or Multiple Reaction Monitoring (MRM) as the survey scan to trigger MS/MS data acquisition. All experiments were conducted on a 4000 QTRAP® system equipped with a Turbo V™ source. MRM data was processed using MultiQuant™ Software.

Glycopeptide Isoform Characterization

Initial full scan MS driven IDA experiments on the gel-purified ApoC-III sample showed a complex chromatographic profile (>30 peaks) in which it is difficult to distinguish between glycosylated and non-glycosylated peptides. A more targeted acquisition strategy was required to characterize the glycosylated peptides.

The diagnostic fragment ions for two sugar constituents were utilized for the precursor ion scanning experiments (Figure 2) by monitoring the product ions at m/z 204.1 (GalNAc) and m/z 366.2 (GalNAc+Gal). This resulted in a much simplified chromatographic profile (Figure 3, top). The MS/MS spectrum of Peak 2 (monosialo-ApoC-III) is shown (Figure 3, bottom).
MRM Assay Development

MIDAS™ Workflow acquisition methods (MRM triggered IDA) were built. In this targeted approach, the protein sequence of ApoC-III (peptides D59-A79 and F61-A79) was modified to include all expected glycoforms. The glycoproteins were digested in silico and a list of precursor masses and potential y-ion fragments was generated. These theoretical MRM transitions (for both non-glycosylated and all possible glycosylated peptides) were used as a survey scan in data dependent mode to detect specific peptides (Figure 4). These MRM transitions triggered the acquisition of high quality MS/MS spectra providing confirmation of the identities of the detected species, both the peptide sequence and the glycosylation structure. From the observed fragmentation pattern, specific and sensitive MRM transitions were optimized (2 MRM transitions per peptide) for three control peptides (non-glycosylated) and the asialo-, monosialo- and disialo- forms of ApoC-III isoforms to create the final MRM assay.

Relative Quantitation of ApoC-III Isoforms

Relative quantitation of the ApoC-III glycoforms in the plasma from two healthy and one diseased research subject was undertaken using the optimized MRM method. Duplicate samples were prepared and analyzed in triplicate. Peak areas were integrated for each of the ApoC-III isoforms: asialo-, monosialo- and disialo- and the average and standard deviation values were calculated and plotted as shown in Figure 5. The ApoC-III ratios for the two healthy subjects were very similar and as a result were averaged and plotted together for ease of comparison with the diseased research subject.

An abnormal ApoC-III ratio was observed for the diseased research subject with increased levels of disialo-ApoC-III and decreased levels of mono- and asialo-ApoC-III.
Quantitative Analysis of Free Neuraminic Acid in Urine

In the metabolic disorder sialuria, free neuraminic acid is found in elevated levels in the plasma and urine. A MRM method was developed to quantify the level of free neuraminic acid in urine. NeuAc standard was infused directly and a negative ion Enhanced Product Ion (EPI) scan was acquired (Figure 6, top) to determine that the most intense ions were the fragments at m/z -86.9 and -170.0. The collision energy, declustering potential and collision cell exit potential were optimized for the following MRM transitions:

\[ Q_1=-308.1 \rightarrow Q_3=-86.9 \] and \[ Q_1=-308.1 \rightarrow Q_3=-170.0 \]

The assay conditions consisted of a 10 mL injection of diluted urine onto a C18 column (4.6 mm x 15 cm, Zorbax SB-CN) on an Agilent 1100 capillary LC system. A gradient of 100% acetonitrile to 95% 50mM ammonium formate at 1mL/min over 6 min was used. Five sets of standard dilutions of NeuAc in urine were prepared and analyzed to generate calibration curves (Figure 6, bottom).

The level of NeuAc in the urine obtained from healthy and diseased subjects was assessed and found to be approximately equal. Five injections and two different dilutions were used. Urine from a healthy subject contained \( 23.5 + 2 \) mmol NeuAc/mol creatinine compared to urine collected from a diseased subject with \( 22.5 + 3 \) mmol NeuAc/mol creatinine\(^9\). The data suggested that the diseased subject was not afflicted with sialuria and as such additional genetic disorders were considered.

Discussion

The detailed biological characterization of these research subjects involved multiple targeted strategies on a single MS platform, the 4000 QTRAP® System. Selective precursor ion scanning triggering full scan MS/MS enabled detection and characterization of the glycopeptides isoforms. Quantitative MRM assays developed for the glycosylated protein isoforms showed an abnormal ApoC-III ratio with elevated levels of disialylated ApoC-III in the plasma from the diseased research subject compared to the healthy subjects' plasma. In addition, a method was developed for quantitation of free NeuAc levels in urine and it was found that there was no significant difference between the levels of NeuAc in the diseased sample compared to samples from the healthy research subjects.

Figure 6. Neuraminic Acid Assay in Urine. Negative ion enhanced product ion scan (EPI) for N-acetyl neuraminic acid (NeuAc) was performed to determine the best fragment ions to use for MRM analysis (top). A standard concentration curve for NeuAc in urine was acquired for quantitative analysis (bottom).
Biological Conclusions

The mass spectrometric approach used in this study allowed a complete evaluation of this glycosylation disorder by investigating both the protein glycan products of the biosynthetic pathway in plasma and the levels of free organic acid in urine. This study shows that the abnormal glycosylation patterns for ApoC-III observed are not solely characteristic of sialuria. The normal levels of NeuAc have precluded the diagnosis of sialuria and we are currently investigating additional CDG’s, including b-mannosidosis. Whilst O-glycan disorders such as sialuria and sialidosis are rare, this methodology can be applied to a range of glycoprotein metabolism disorders to provide more complete characterization of the disorder.

Key Features of the 4000 QTRAP® System for Complete Characterization Workflows

- High sensitivity, full scan MS/MS for fragmentation of glycosylated peptides and small organic acids.
- Precursor ion scanning for selection and identification of glycosylated peptides in complex biological mixtures.
- Multiple Reaction Monitoring (MRM) experiments for selective and sensitive quantification of specific glycopeptide isoforms or metabolites.
- Single MS platform for targeted identification, characterization and quantitation of proteins, applicable to any stable, post-translational or chemical modification of proteins.

References

7. Free trial version of MultiQuant™ Software is available at: www.absciex.com/Downloads/Software-Downloads
9. Creatinine levels in urine were measured with standard laboratory methods on a Vitros 5.1 FS analyzer (Ortho-Clinical Diagnostics) at the Mater Children’s Hospital and then used to normalize the free neuraminic acid concentrations in urine.