

CONVENIENT SAMPLE PREPARATION FOR HUMAN SERUM N-GLYCAN PROFILING BY MASS SPECTROMETRY: APPLICATION TO CONGENITAL DISORDERS OF GLYCOSYLATION (CDG) TYPE I

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ABSTRACT

The development of a robust method suitable for medium to high throughput *N*-glycan profiling for a simple and rapid platform for human serum sample preparation. Congenital disorders of glycosylation (CDG) are multisystem disorders due to defects in *N*-glycosylation synthesis pathway. This ultimately produced abnormal *N*-glycan which affected their structure and metabolic function. The method to assess the diversity of the *N*-linked glycan released from human serum was developed using the filter aided *N*-glycan sample preparation (FASP) protocol. The samples were boiled in sodium dodecyl sulphate (SDS), removed by urea and exchanged for volatile buffer. Then, PNGase F treatment was applied in the upper chamber of a membrane spin filter to release *N*-glycan. The released glycan were recovered in the filtrate following centrifugation, and subsequently analysed using MALDI-TOF. Authentic standard glycoprotein is used to assess the sample processing protocol together with human serum and CDG type 1 patient. Non-derivatised *N*-glycan from glycoprotein standard and human serum were analysed using MALDI-TOF. Bi and tri-antennary sialylated glycans were observed (m/z 2792.5, 3241.8, 3603.0, 3965.7 [$M+Na$]⁺) from standard fetuin for validation of sample processing. Additionally, there are differences upon comparing *N*-glycan profiles between control and CDG type 1 serum. We show that the protocol is suitable and advantageous for *N*-glycan profiling of human serum since only a small volume of serum is required, which facilitates working with replicate samples. This method was found to work efficiently on different sample matrices and reduced sample preparation time before MALDI-TOF analysis.

KEYWORDS: Human Serum, *N*-glycan, Mass Spectrometry, Congenital Disorders of Glycosylation, MALDI-TOF

INTRODUCTION

Protein glycosylation is the process of adding monosaccharides/oligosaccharides (glycans) to proteins that synthesise glycoproteins in the biosynthesis pathway. It is a complex process without a template as a rule which results in an abundance of different glycan structures (1,2). Glycosylation is known to possess a vital purpose that can influence protein folding and stability, cell interactions and communication (3,4) which occur in two major compartments: the endoplasmic reticulum (ER) and the Golgi apparatus (5). This includes protein quality control, protein sorting from ER to Golgi apparatus as well as degradation (6,7).

Glycoprotein glycans are attached to proteins either through a nitrogen atom (*N*-linked) or an oxygen atom (*O*-linked). *N*-glycosylation occurs at a consensus sequence, in which glycosylation occurs at the asparagine (Asn, N) residue of the Asn-Xaa-Ser/Thr sequon where Xaa is any amino acid except proline, while *O*-glycosylation involves the addition of monosaccharides to a serine or threonine residue (8).

One of the disease groups associated with defective protein glycosylation is congenital disorders of glycosylation (CDG) discovered by the paediatrician Jaak Jaeken (9) previously known as carbohydrate-deficient glycoprotein disorders. They belong to metabolic diseases and are caused by defects in the protein *N*-glycosylation process. These disorders have a broad range of severities depending on the mutation and genetic background thus making clinical presentations lack uniformity. It can be from mild motor disorders to severe mental retardation and even death to the newborn (10,11,12). Currently, there are more than 125 different CDG known worldwide (13).

CDG can be caused by defects in glycosylation pathway due to enzyme or sugar transporter defects in the cytosol, ER and Golgi compartment (14,15). There are two types of CDG, where type I is caused by defects in the assembly of the lipid-linked oligosaccharide precursor and its transfer to the protein that results in the absence of complete glycan chains in glycoproteins within ER. Type 2, on the other hand, refers to the defects which occur during further processing of the protein-bound glycans in the Golgi apparatus that result in the formation of incomplete glycan chains as a final product (13,15).

Standard diagnostic screening for CDG patients is commonly performed by transferrin isoelectric focusing (TIEF) (16). In the normal TIEF

pattern, tetrasialotransferrin pattern is the main isoform present whereas in CDG type 1 tetrasialotransferrin density is reduced with a subsequent increase in asialo and diasialotransferrin. Otherwise, trisialo and monosialo density are increased in CDG type 2 while tetrasialotransferrin density is reduced or normal. Other method such as high pressure liquid chromatography and capillary electrophoresis are also applied to identify defects in *N*-glycosylation of CDG type 1 and CDG type 2 (17).

Mass spectrometry (MS) in clinical application is gaining attention for glycan profiling and for the study of glycan structures (6,18,19). It has become a significant tool in glycoprotein structure-function studies, even though reliable medium-throughput methods for establishing the glycome are still lacking. Common methods for glycoprotein structural studies include the release of the glycan from the polypeptide chain for the following analysis. The most extensively accepted method for *N*-glycan release uses the enzyme peptide- N^4 -(acetyl- β -glucosaminyl)-asparagine amidase from *Elizabethkingia meningoseptica* (previously *Flavobacterium meningosepticum*) (PNGase F). This enzyme cleaves the amide bond linking glycan to the peptide of glycoproteins in solution, and releases intact *N*-glycan for analysis (20). In MS analysis, most detergents and involatile reagents are unsuitable to the signal. Nevertheless, in order to extract glycoprotein from membrane proteins, the use of detergents is vital for efficient membrane solubilisation, which is essentially removed prior to MS analysis.

This inherent reagent incompatibility presents a significant challenge in effectively solubilising and sampling all glycoproteins from serum, cell or tissue specimens while also processing them sufficiently for sensitive MS analysis. Here we will explore the development of a robust method suitable for medium to high throughput *N*-glycan profiling for a simple and rapid platform for human serum sample preparation (21,22). Our protocol makes use of very efficient sodium dodecyl sulphate (SDS) solubilisation of glycoproteins from serum lysates combined with membrane filter devices for sample handling and *N*-glycan release. This approach is ideal for the medium-throughput analysis of serum, since it only requires 50 μ L serum. Our method is believed to benefit routine MS *N*-glycan profiling and also allow for easy adaptation to other limited samples, such as human fibroblasts or small biopsies.

MATERIALS AND METHODS

Reagents

Bovine pancreatic ribonuclease B (RNase B), Fetuin and PNGase F, 2-mercaptoethanol, dithiothreitol (DTT), iodoacetamide (IAA), urea, sodium dodecyl sulphate (SDS), 2,5-dihydroxybenzoic acid (DHB), Amicon Ultra-0.5 (Ultracel-30 membrane, nominal mass cutoff 30 kDa, Millipore) were obtained from Sigma Aldrich. All solvents (LCMS-grade water, acetonitrile, methanol) were from Fisher Scientific.

Samples

Control serum was obtained from 3 healthy volunteers, staff from the Institute for Medical Research (IMR) and pooled together. One patient serum screened by TIEF for CDG type 1 was collected from archived samples at the Institute for Medical Research.

Serum Lysis

The processing of serum samples were performed according to the reported protocol [21]. Human serum (50 μ L) was mixed together with 200 μ L lysis buffer (4% (w/v) SDS, 100 mM Tris/HCL pH 7.6, 0.1 M dithiothreitol). The sample was heated to 95°C for 5 min, and the supernatant was collected and kept at -80°C.

Filter Aided *N*-glycan (FANG) Separation

The supernatant (200-300 μ L) collected after serum lysis was transferred into a 1.5 mL plastic microfuge tube. Urea solution was added (8 M urea in 100 mM Tris/HCL pH 8.5) to the sample in a ratio of 9:1 (urea solution to sample by volume). The solution (430 μ L) was transferred to an ultrafiltration device (nominal mass cut-off 30 k) and centrifuged at 15000 x *g* for 10 mins, when a further 430 μ L was transferred and centrifugation was repeated until all the sample had passed through the ultrafiltration device. The sample retained above the filter membrane was washed three times with 250 μ L of the 8 M urea solution and centrifuged each time at 15000 x *g* for 10 min.

The alkylating reagent iodoacetamide was added to prevent disulphide bond formation by forming S-carboxymethylcysteine. An iodoacetamide solution was prepared fresh and the reaction was conducted in the dark. 100 μ L 40 mM iodoacetamide in urea solution was added to the ultrafiltration device and vortexed at 500 rpm for 1 min. The ultrafiltration device was placed in the dark at room temperature for 15 min before being centrifuged at 5000 x *g* for 10 min. 250 μ L 10 M urea

solution was added to the ultrafiltration device and centrifuged at 5000 x *g* for 10 min. Then, 250 μ L of 50 mM ammonium bicarbonate (pH 7.5-8) was added to the ultrafiltration device and centrifuged at 5000 x *g* for 10 min. The last step was repeated two further times. The ultrafiltration device was transferred to a new collection tube.

Ammonium bicarbonate solution (72 μ L 50 mM) was added to the ultrafiltration device, followed by 8 μ L of 500 unit/mL PNGase F solution. The ultrafiltration device was sealed with parafilm and incubated at 37°C for 16 h. After incubation, the device was centrifuged for 15 min and washed again with 100 μ L LCMS grade water (repeated once). The ultracentrifugation device was centrifuged again for 15 min. The released glycans are harvested from the collection tube

MALDI TOF/TOF

Matrix

DHB (2,5 dihydroxybenzoic acid 10 mg/mL) was dissolved in 80% acetonitrile: LCMS grade water and vortexed.

Sample spot

Matrix solution (1 μ L) and 1 μ L sample solution were spotted onto MALDI plate. The spot was dried at room temperature before placed into the sample compartment on Ultraflex III mass spectrometer.

Mass Spectrometry Analysis

TOF-MS: A Bruker Daltonics ultraflex III TOF/TOF mass spectrometer equipped with a Smartbeam™ laser was used in positive-ion mode. The mass spectrometer was calibrated externally using a Bruker Peptide Mix II MALDI standard preparation. Mass spectra were recorded over the *m/z* range 800-4000 using a total of 4000 shots in steps of 800 which were summed to give one spectrum. One spectrum was recorded from each sample spot. The laser power setting was varied over the range 40-60%. MS/MS spectra were obtained on precursor ions accelerated at 8 kV and selected in the time ion gate. The selected precursor was fragmented using collision induced dissociation (CID) in the LIFT cell, and separated using reflectron mode.

Method development for *N*-glycan release from human serum

A simple and rapid protocol has been developed for glycoprotein solubilisation from serum, followed by

N-glycan release and isolation in a high-molecular-weight cutoff (30 kDa) membrane filter device for MALDI-TOF MS analysis (Figure 1). The workflow consists of three stages: pre-processing, *N*-glycan release and preconcentrate in a single membrane filter.

Serum samples are boiled in SDS lysis buffer solution and the supernatant was transferred into membrane filter unit. SDS was removed and replaced with urea buffer solution, then disulphide bonds in the proteins were chemically reduced using iodoacetamide solution. Subsequently, urea solution was exchanged with ammonium bicarbonate buffer.

PNGase F was added to the membrane filter unit and incubated overnight to release *N*-glycans from serum glycoproteins. Previously, ultrafiltration membrane unit was used to collect peptides for proteomics analysis (22) and *N*-glycan from wild type Chinese Hamster Ovary cultured cells (21). The released *N*-glycans were collected in a new tube and spotted onto MALDI plate.

MALDI-TOF MS analysis of serum *N*-glycan samples display the prevalence of singly charged ion with sodium as an adduct in accordance to reported literature (23).

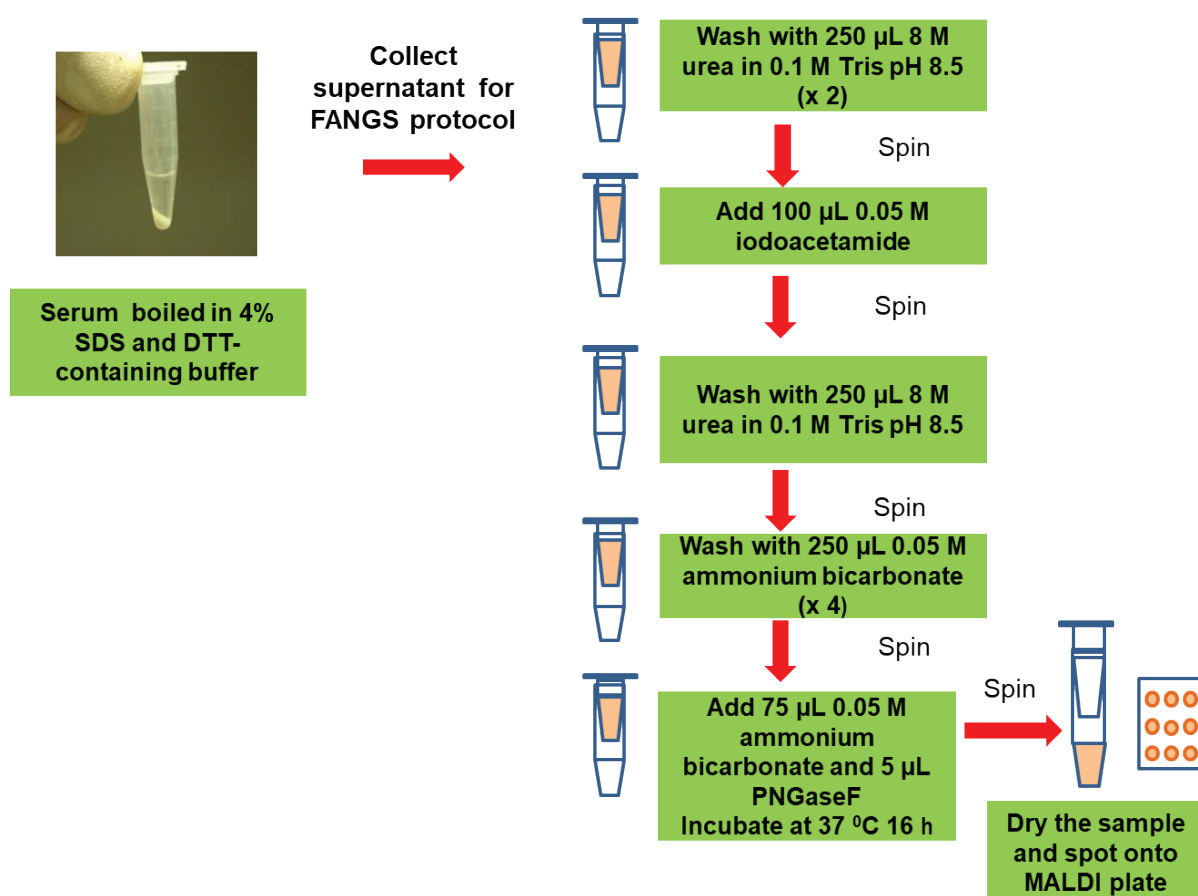


Figure 1. Diagram of serum *N*-glycan released using FANG protocol.

Validation of method

In order to validate the method, we analysed two standards (RNase B and fetuin) using this approach, where both *N*-glycan profiles have been published by others to evaluate the performance of our method.

The first standard, RNase B is a glycoprotein that contains Man₅ to Man₉ high mannose structures with multi antennary as shown in Figure 2. Strong [M+Na]⁺ signals were observed at *m/z* 1257.3, 1419.4, 1581.4, 1743.5 and 1905.6 for the expected RNase B

glycans: Man₅GlcNAc₂, Man₆GlcNAc₂, Man₇GlcNAc₂, Man₈GlcNAc₂ and Man₉GlcNAc₂. The spectrum is similar to that reported by Harvey et al. (24) for the analysis of RNase B using hydrazine- released oligosaccharides.

The second standard, fetuin was chosen because it has a complex bi- and tri-antennary glycans structure with sialylated glycans (acetylneuraminic acid residues, Neu5Ac). In the spectrum obtained from the glycans from fetuin, a well characterised series of bi- and tri-antennary monoisotopic sialylated glycan signals

at $[M+Na]^+$ m/z 2792.5 ($NeuAc_2Hex_5HexNAc_4$), 3241.8 ($NeuAc_2Hex_6HexNAc_5$), 3603.0 ($NeuAc_3Hex_6HexNAc_5$) and 3965.7 ($NeuAc_4Hex_6HexNAc_5$) were detected (Figure 3). The signal at m/z 3965.1 corresponds to a species incorporating two ^{13}C atoms – the monoisotopic signal for this species is weak in this spectrum due to the glycan's high molecular weight.

This is comparable to a published paper where they used fetuin as one of the standard glycoproteins to evaluate the performance of their solid phase permethylation of glycans for MS analysis (25). Kang et al. (25) also used fetuin as a standard glycoprotein in the development of their solid phase permethylation procedure and our spectra are similar to what was reported by them.

RESULTS

High mannose and complex type glycans with bi- and tri-antennary structures are found in the MALDI

mass spectrum of *N*-glycans from normal serum. The predicted structures were proposed according to structural information obtained from previous studies (26,27). High mannose $[M+Na]^+$ glycan signals at m/z 1257.7, 1419.8, 1581.9, 1744.0, 1906.1 were observed. Additionally, fucosylated $[M+Na]^+$ glycan signals at m/z 1485.9, 1648.0, 1689.0, 1810.1 and 2175.3 as well as sialylated glycan signals at m/z 1955.1, 2101.3 and 2246.3 were also observed (Figure 4A). CDG type 1 patient gave a complement of $[M+Na]^+$ signals to that from normal serum with signals at m/z 1257.8 and 1906.2. In addition, the $Hex_6HexNAc_2$, $Hex_7HexNAc_2$ and $Hex_8HexNAc_2$ (at m/z 1419.8, 1581.9 and 1744.0) that were detected in normal serum were not observed among the CDG type 1 patient (Figure 4B). Furthermore, fucosylated glycan signals at m/z 1485.9, 1648.1 and 1689.1 were less intense in CDG type 1 patient compared to signals in normal serum. Table 1 presents the *N*-glycan structures as representative of all glycans detected in MALDI-TOF MS.

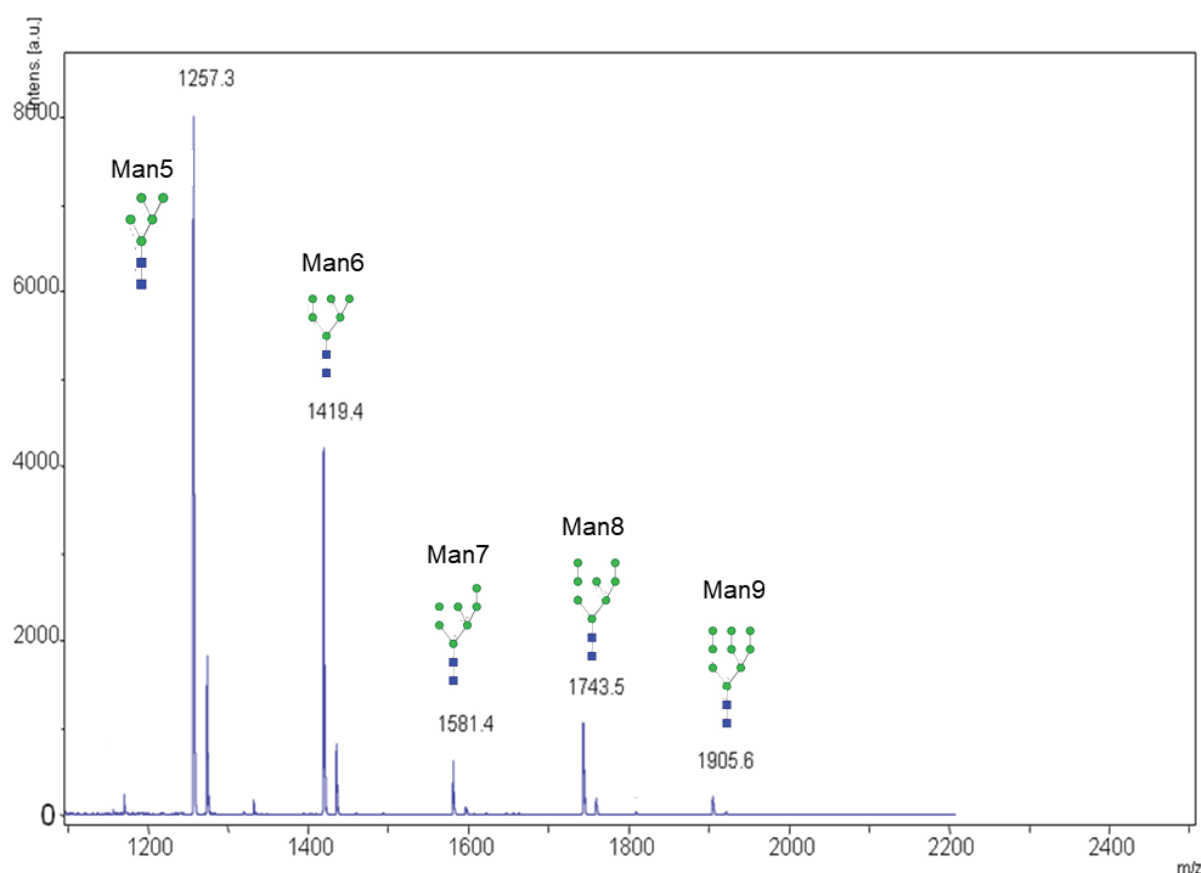


Figure 2. Spectrum of RNase B glycans after microcolumn recovery without permethylation

■ GlcNAc, ● Mannose

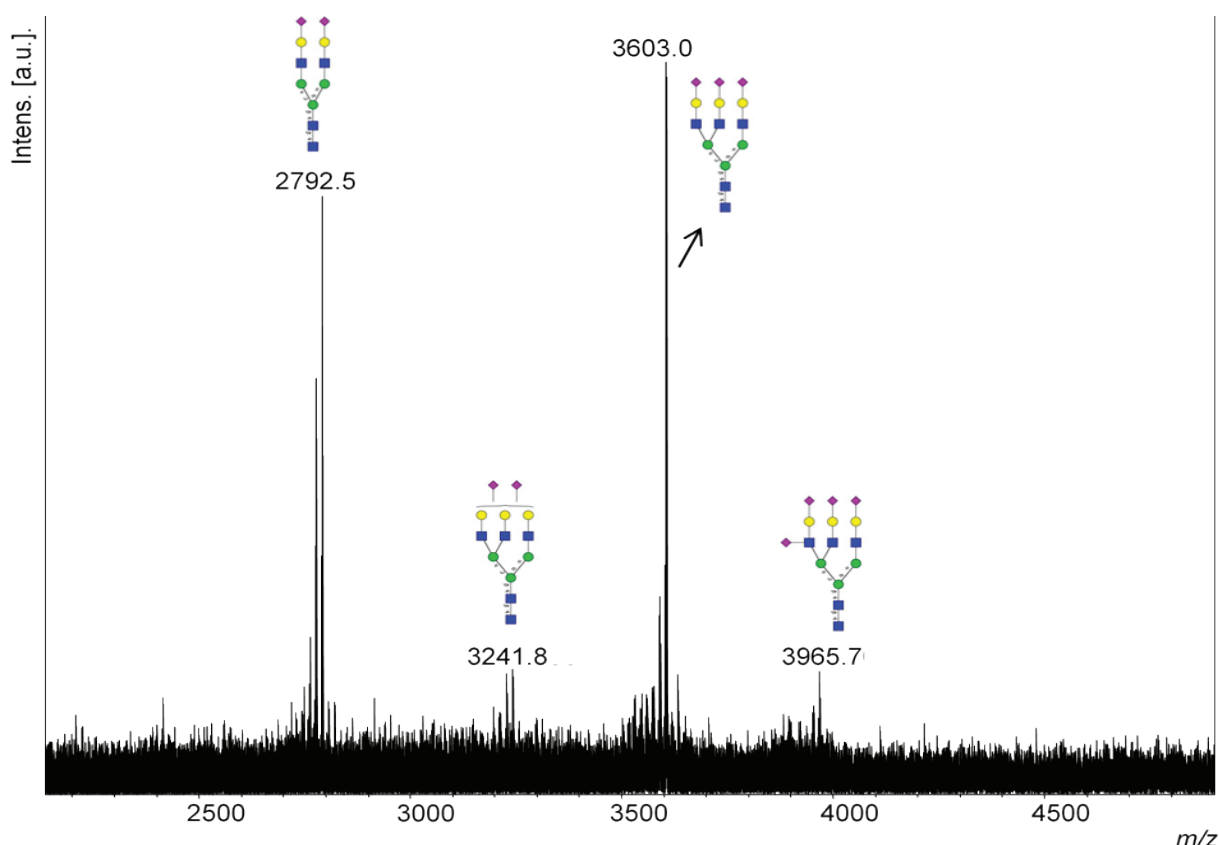


Figure 3. Spectrum of permethylated FANG released N-glycans from 2.5 µg fetuin (MALDI-TOF MS).

■ GlcNAc, ● Galactose, ● Mannose, ◆ Neu5Ac

DISCUSSIONS

In this study, the present work applies to human serum from normal control and CDG type 1. An ultrafiltration membrane unit was used as a mini reactor for the digestion, release and extraction of *N*-glycans. The glycans detected in MALDI-TOF MS analyses of RNase B and fetuin match with those reported in the literature (24,25). Next, we tested this protocol with samples from control human serum and CDG type 1 patient. With respect to normal control, high mannose glycans for Hex₅HexNAc₂-Hex₉HexNAc₂ were observed. This coincides with those reported in the literature for serum samples (28).

However, in CDG type 1 spectra, complete loss of *N*-glycan signals namely Hex₆HexNAc₂, Hex₇HexNAc₂ and Hex₈HexNAc₂ (at *m/z* 1419.8, 1581.9 and 1744.0) were observed. This is probably due to a defect in lipid-linked oligosaccharide before it is transferred to a nascent polypeptide in endoplasmic reticulum by oligosaccharyl transferase complex (10). Kondo et al. (42) reported missense mutations in the

phosphomannomutase 2 gene in two Japanese siblings with CDG type 1. Phosphomannomutase is an enzyme involved in synthesising lipid-linked oligosaccharides. This enzyme was found to be markedly reduced in liver, fibroblasts and leukocytes of these patients (42). Another study by Zhang et al. reported the presence of small *N*-tetrasaccharide that was thought to be due to reduced mannosylation of lipid-linked chitobiose in the endoplasmic reticulum (41).

Complex glycan (at *m/z* 1501.9), fucosylated glycan (at *m/z* 1485.9, 1648.1, 1689.0, 1810.1, 1851.1 and 2013.2) and sialylated glycan signal (at *m/z* 1955.1, 2101.3 and 2246.4) were less intense in CDG type 1 patient compared to control. This is possibly due to incomplete glycan structure at *N*-glycosylation site before further glycans processing in the Golgi, as reported in previous studies (19,29). These findings are in agreement with a paper explaining that CDG type 1 happens in endoplasmic reticulum which produce incomplete or complete loss of glycan chain attached to the proteins (14,19,30).

In the case of fucosylated *N*-glycan, the

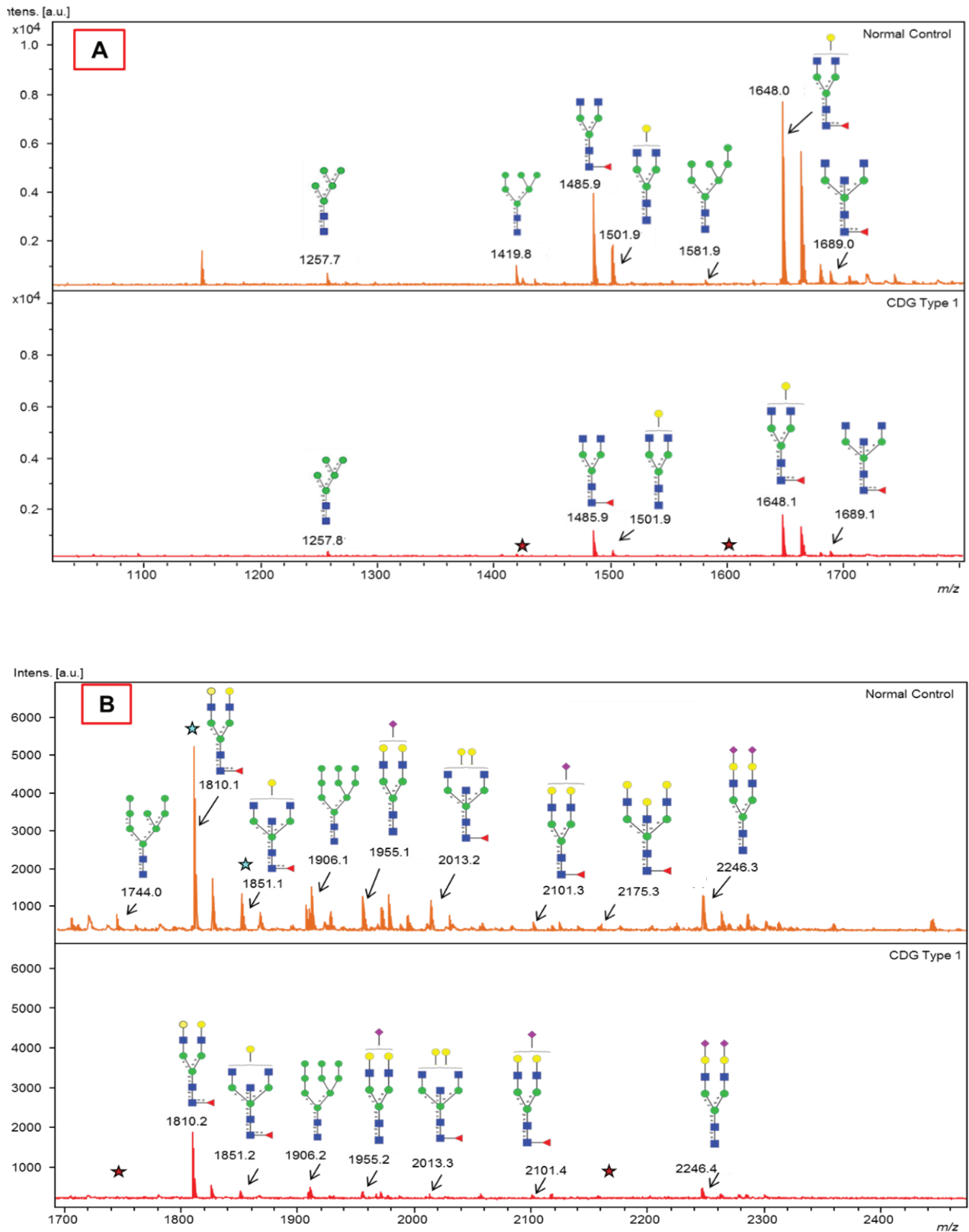
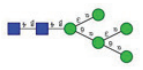
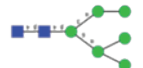
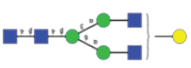
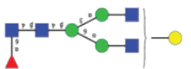
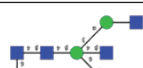
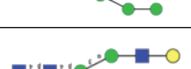
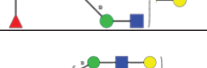


Figure 3. Spectrum of N-glycans released from normal control and CDG type 1 at (A) Low m/z range (B) High m/z range ■ GlcNAc, ● Galactose, ● Mannose, ▲ Fucose, ◆ Neu5Ac, ★ Not detected

Table 1. Identification of N-linked glycans from normal control and CDG type 1 patient

m/z [M+Na] ⁺ observed		Compositions				Normal control	CDG Type 1
		Hex	HexNAc	Fuc	NeuAc		
1257.7		5	2	0	0	✓	✓
1419.8		6	2	0	0	✓	ND
1485.9		3	4	1	0	✓	✓
1501.9		4	4	0	0	✓	✓
1581.9		7	2	0	0	✓	ND
1648.1		4	4	1	0	✓	✓
1689.0		3	5	1	0	✓	✓
1744.0		8	2	0	0	✓	ND
1810.1		5	4	1	0	✓	✓
1851.1		4	5	1	0	✓	✓
1906.1		9	2	0	0	✓	✓
1955.1		3	4	1	0	✓	✓
2013.2		4	4	0	0	✓	✓
2101.3		7	2	0	0	✓	✓
2175.3		4	4	1	0	✓	ND
2246.4		3	5	1	0	✓	✓

= observed, ND= not detected

N-glycan's signals were also reduced in CDG type 1 patient compared to control. Similar findings were reported in identification of *N*-glycan in wild type and gene knock-out pig fibroblasts study (31). Another report on breast cancer cell lines found that 2 biantennary fucosylated *N*-glycans were significantly decreased when they analysed the data using SPSS10.0 software (32). Recently, Barbosa et al. mentioned that the increased concentration of truncated *N*-glycans species were increased in CDG type 2 patient, which revealed the defective enzymes took place in the Golgi Apparatus (18).

According to the spectrum in Figure 4(A) and (B), the changes of *N*-glycan observed in normal and CDG type 1 can be due to complete loss of the *N*-glycan structure and changes in relative levels, implying the need for *N*-glycan quantification in future research. As for quantification of *N*-glycans which can be cumbersome for low abundance species and cause overlapping of peaks after chromatographic separation, isotope labelling could be an option for absolute quantification (33,34). Various studies have reported that there were changes in *N*-glycan structures in wild type and α -1,3-galactosyltransferase gene knock-out in pig fibroblast (31), cancer cells (35,36,37) and liver disease patients (38,39,40).

These findings showed that the application of the FANG protocol to human serum samples has enabled *N*-glycan profiling for CDG type 1 disorder, affirming the potential of using this protocol for further *N*-glycan quantifications. Further understanding of glycan structure and its relationship with the biosynthesis pathway will be crucial for better understanding and to facilitate the development of *N*-glycan based therapies.

CONCLUSION

We have developed a convenient and rapid protocol to allow solubilisation, release and isolation of *N*-glycans from glycoproteins in serum in a small, miniaturised approach for medium-throughput analysis of serum. It has been shown to be compatible with MS analysis for preparing samples for *N*-glycan profiling of normal serum and CDG type 1 patient. Thus, it allows comparison of differences in *N*-glycan from both samples. The protocol is simple, efficient and reduces time in sample preparation by generating released *N*-glycan for MS glycan profiling. This method should be invaluable for identifying differences in *N*-glycan profiles, thereby allowing it to be used as an alternative tool to screen

patients with CDG type 1 disease. The limitation of the study is that we only used one sample from a CDG type 1 patient. Thus, to make the results better in terms of intensity, one should pool samples from a few positive patients.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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