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Reproducible and sensitive determination of charged oligosaccharides from haptoglobin by PNGase F digestion and HPAEC/PAD analysis: glycan composition varies with disease

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Many studies have reported changes in the carbohydrate structure of serum glycoproteins in disease, but this information is often of limited value for understanding disease mechanisms because it is obtained with simple and/or indirect methodologies that determine only one structural feature. On the other hand, more detailed carbohydrate methodologies are time-consuming and require a lot of purified material. Using haptoglobin (Hp) as a model protein, a new procedure was devised that determined the oligosaccharide composition of very small amounts of Hp in a relatively short time. The Hp was purified by batch affinity-chromatography, oligosaccharides were removed with PNGase F, and the oligosaccharide composition of charged species was determined using HPAEC/PAD (Dionex carbohydrate analyser). The method was applied to the analysis of Hp from eight healthy individuals and 37 patients with different inflammatory diseases or cancers. Twenty-seven oligosaccharides were consistently detected, but the majority could not be identified. However, by calculating retention times relative to the sialylated biantennary peak (Neu5Acα2-3/6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-3/6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc) it was possible to compare profiles quantitatively. Although no peak was identified as disease-specific, characteristic and reproducible profiles were obtained. Particularly striking were reductions in the major peaks in Crohn's disease, rheumatoid arthritis, stomach cancer, accompanied by increases in unidentified peaks. Previous studies suggested that many of the unknown peaks were due to increased sialylation and fucosylation. Only small changes in patterns were observed for breast and ovarian cancer. The new procedure will be very useful in the characterization of oligosaccharide composition of glycoproteins in clinical specimens.

***Keywords*: cancer, haptoglobin, inflammation, oligosaccharide**

Introduction

Many studies have reported carbohydrate changes in serum glycoproteins in disease [1–3]; notable among these findings are the degalactosylation of immunoglobulin (IgG) in rheumatoid arthritis, the changes in the branching of alpha-1-acid glycoprotein (AGP) in inflammation, the desialylation of transferrin (Tf) in alcoholism, and the increased fucosylation of haptoglobin (Hp) in cancer. Whether these changes, however, are passive markers of pathogenesis or contributors to the pathogenic process itself is unclear. For example, the increased expression of sialyl Lewis x (a terminal tetrasaccharide that contains *N*-acetylneuraminic acid (Neu5Ac) and fucose) on AGP [4] and Hp [5] in inflammation would suggest that these molecules could interfere in the sLe^x/selectin interaction between leukocytes and endothelial cells. Furthermore, it is well established that molecules like AGP [2] and Hp [6] can act as immunosuppressive agents. To resolve this problem, more detailed knowledge is needed on the carbohydrate structures that are found on the molecules that make-up the different glycoforms of these proteins. This type of information is becoming increasingly available for some of the serum glycoproteins, for example IgG and Tf, but for others much of the available information is mainly limited to studies of specific carbohydrate features using lectins or other simpler techniques.

Our previous studies of changes in the glycosylation of Hp in disease, using lectins and monosaccharide analysis [6–11], indicated that certain carbohydrate structures (very highly-fucosylated structures in ovarian cancer, highly-sialylated structures in Crohn's disease and highly branched

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structures in alcoholic liver disease) predominate in different diseases. It was also shown that different serum glycoproteins (Hp and anti-protease inhibitor) exhibited different changes in carbohydrate structure in the same disease [6]. These conclusions suggested, therefore, that the abnormal glycosylation changes are closely associated with the disease process itself, and for this reason alone they deserved further study.

Following on from our previous work, the aim of this study was to establish a procedure to routinely investigate the oligosaccharide content of serum glycoproteins in clinical specimens. This required a method that could reproducibly analyse small amounts of glycoprotein in a relatively short time. Hp was to be used as a model protein for this purpose and the method developed was evaluated by investigating the oligosaccharide composition of Hp isolated from healthy individuals and from patients with different diseases, using the Dionex carbohydrate analyser.

Materials and Methods

Blood specimens were obtained by venupuncture from eight healthy individuals (HI) (median age 40 y); six patients with rheumatoid arthritis (RA) (median age 57 y); eight patients with Crohn's disease (CR) (median age 42 y); eight patients with stomach cancer (ST) (median age 75 y); eight patients with breast cancer (BR) (median age 59 y); and seven patients with ovarian cancer (OV) (median age 58 y). Sera were obtained by low speed centrifugation. All the healthy individuals and patients were non-smokers and consumed low amounts of alcohol. None of the healthy individuals were taking medication. All the 'RA' patients fulfilled the ARA diagnostic criteria [12] and had moderate/severe disease activity. Most were receiving various anti-inflammatory therapies. The 'CR' patients had a median disease duration of 6 y and a median disease-activity score of 5 [13]; most of the patients were receiving prednisolone. All the 'ST' patients had progressive malignant disease and 5/10 were diagnosed as having liver metastasis; none were receiving cytotoxic chemotherapy. All the 'BR' patients had progressive disease and the majority were receiving mitoxanthrone treatment. All the 'OV' patients had stage III/IV disease (FIGO classification) and the majority were receiving carboplatin treatment.

Hp was purified from 250 µl serum using affinity chromatography with a rabbit anti-Hp antibody (Binding Site). Briefly, the antibody was coupled to CNBr-activated Sepharose 4B beads (Pharmacia Ltd) at a concentration of 5 mg Ab per ml beads. The serum was mixed with 500 µl of Ab coupled beads, incubated for 1.5 h at 25 °C and unbound proteins were removed by washing nine times with 2 ml of 25 mM Tris-HCl (pH 8.0) containing 140 mM NaCl, 1 mM $CaCl_2$, 0.5% (w/v) Nonidet P40 and 0.1% (v/v) phenyl methyl sulphonyl fluoride. To remove the salts, beads were quickly washed twice with 2 ml of deionized water. Bound Hp was eluted from the beads with 1 ml of 0.1 mM trifluoroacetic acid (TFA). Purity was checked by SDS/PAGE electrophoresis (8% gels) and a Laemmli buffer system. The methods used have been previously described [7–10]. All specimens gave very pure preparations and the average yield was 82 µg.

Oligosaccharides were removed from Hp using the following experimental procedure [14]. All reagents were obtained from Sigma unless otherwise stated. The method was slightly modified from that previously developed, in that the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphate (CHAPS) was used instead of NP40 (the former detergent was used because it gave much less background contamination on the chromatography profiles). Purified Hp (50 µg) in 50 mM ammonium formate buffer, pH 8.6, containing 0.4% (w/v) SDS was heated at 100 °C for 3 min and an equal volume of the buffer solution containing 2% (v/v) mercaptoethanol, 1.2% (w/v) CHAPS and 0.1 M EDTA was added and the mixture was heated at 100 °C for further 3 min. After cooling, 0.2 U PNGase F (Boehringer Mannheim Biochemica) was added and incubated at 37 °C for 20 h. Removal of all the oligosaccharides was checked by electrophoresis in a PHAST system (Pharmacia Biotech, Belgium) and staining for carbohydrate (Boehringer Mannheim, Biochemica). Finally, the sample was dialysed overnight at 4 °C in a microdialyser (Pierce), lyophilized, redissolved in 100 µl double-distilled deionized water, and stored at 20 °C until required for analysis.

Oligosaccharides were separated on a Carbopac PA100 anion-exchange column using a sodium acetate gradient (20–200 mM) in 100 mM NaOH and the separated peaks were detected by pulsed amperometric detection (Dionex BioLC System). Under these elution conditions, neutral oligosaccharides were eluted very rapidly from the column as a single peak. Samples (40 µl) were injected on to the column using a Spectra System AS3500 Autosampler, data from the detector were collected by an ACI interface and all data were analysed by AI450 software (Dionex). An internal standard (50 ng) of glucose α1,6 diphosphate (GDP) was added to each sample, and with every batch of samples oligosaccharide standards ('DSB', Neu5Acα2-3/6Galβ1-4GlcNAcβ1-Manα1-6 (Neu5Acα2-3/6Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc, Cat. No. C-224300; 'MSB', 'DSB' with one Neu5Ac less, Cat. No. C-124300; and 'TST', Neu5Ac2-3/6Galβ1-4GlNAcβ1-4(Neu5Acα2-3/6Galβ1-4GlcNAcβ1-2)Manα1-3(Neu5Acα2-3/6Galβ1-4GlcNAc β1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc, Cat. No C-33530) were separated. The average retention times of 'MSB', 'DSB', 'TST' and GDP were 14.9, 26.3, 36.4 and 64 min respectively. With some batches of specimens a 'DSB' standard with an α1-6 fucose attached to the core GlcNAc (Cat. No C-224301) was separated on the column (retention time ~25 min). In preliminary experiments asialo 'DSB' (Cat. No C-024300), asialo 'DSB' (Cat. No C-035300), asialo 'DSB' with a β1-4 bisecting GlcNAc (Cat. No C-024310) and asialo α1-6 fucosylated 'DSB' (Cat. No C-024301) were separated under the same conditions as used for the

Table 1. Reproducibility of the separation of standard sialylated oligosaccharides on a PA100 anion-exchange column.

Separation	*DSB elution volume (ml)*	*Retention MSB relative to DSB*	*Retention TST relative to DSB*	*No. Observations*
I	26.2 ±0.2	0.57 ±0.01	1.38 ±0.005	(6)
II	27.2 ±0.1	0.59 ±0.03	1.37 ±0.005	(4)
III	25.3 ±0.1	0.58 ±0.004	1.39 ±0.005	(6)

The separations were carried out on different occasions over a period of 4 months.

sialylated oligosaccharides. The retention times for these latter components were between 7 and 8 min. Using the 'DSB' standard the relative retention times of unknown peaks were calculated, and separations between different samples and different diseases were compared. All oligosaccharide standards were obtained from Oxford Glycoscience and 50 ng was injected on to the column. Their structures were defined by the code number after each standard as stated by the suppliers. This is a modification of the nomenclature previously proposed [15].

Results

The reproducibility of the separated standard sialylated oligosaccharides on a PA100 column is shown in Table 1. The results show the elution volume for 'DSB' on three separate occasions, and the retention of 'MSB' and 'TST' on these occasions expressed relative to 'DSB'. It can be seen that there is a small variation in the elution volume of the 'DSB'; however, when the two other oligosaccharides were expressed relative to the 'DSB' the differences were very small.

A typical separation of oligosaccharides from Hp is shown in Figure 1. This example is taken from a separation of a specimen taken from a Crohn's disease patient; this was chosen because it clearly shows all the peaks that could be detected. In this case, twenty-seven peaks were detected, but the number of peaks in any particular specimen was sometimes less than this number (see Figures 2–7).

Figures 2–7 give the oligosaccharide profiles for specimens obtained from different healthy individuals and different patients in each of the diseases groups. 'MSB', 'DSB' and 'TST' were detected in all specimens. Four general points are worth noting: 1) the oligosaccharide profiles were very similar within each group; 2) the profiles were very different for some diseases from those of the healthy group (for example, in 'CR' and 'ST', and to lesser extent 'RA', there were increases in the number of peaks between the 'MSB' and 'DSB' peaks); 3) the majority of peaks were detected in all specimens; and 4) no peak was specific for any particular disease.

To quantitate the separated oligosaccharides, the area under each peak was calculated as a percentage of the total

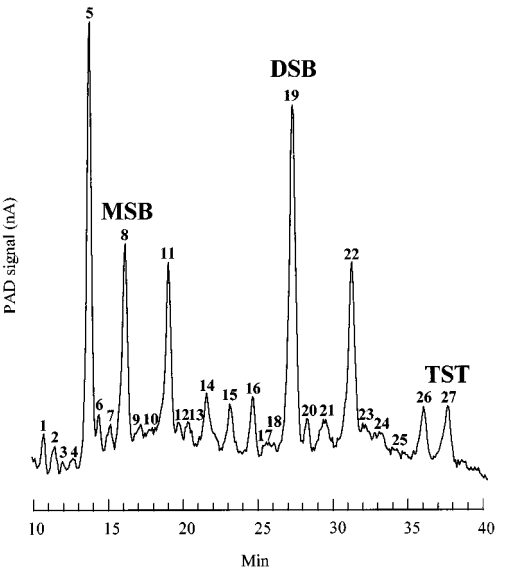

Figure 1. A typical separation of oligosaccharides from haptoglobin on a PA100 anion-exchange column. The specimen shown came from a patient with Crohn's disease. The carbohydrate species were eluted with 20–200 mM gradient of sodium acetate in 100 mM sodium hydroxide. Peaks for which standards were available are labelled 'MSB' (monosialylated biantennary), 'DSB' (disialylated biantennary), and 'TST' (trisialylated triantennary).

area. Separations were then divided into nine sections that were composed of either data for a single peak or pooled peaks. These data are given in Table 2. Statistical comparison of the different disease groups with the healthy group indicated that the data could be divided into two main categories. In first category, which was composed of the 'RA', 'CR' and 'ST' patients, there were significant and substantial reductions in the DSB peak. For the 'CR' and 'ST' patients there was also a significant reduction in the 'MSB' peak and for the 'CR' and 'RA' patients there was a significant reduction in the 'TST' peak. These changes were accompanied by significant and substantial increases in the peaks in sections '1–7' and '9–16'. For the 'CR' and the 'ST' there were changes in other peaks as well. In the second category, which was composed of

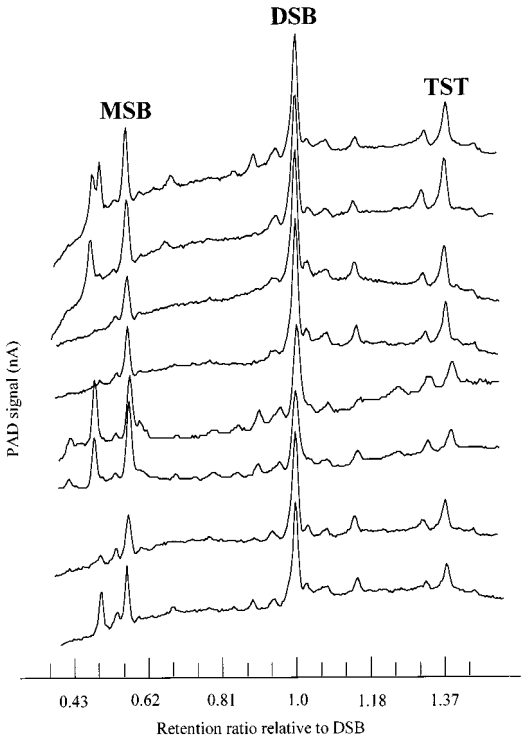

Figure 2. Chromatography profile of oligosaccharides of Hp from healthy individuals. In Figures 2–7 the elution position is given at a retention ratio obtained by dividing the elution volume by the elution volume for the 'DSB' peak.

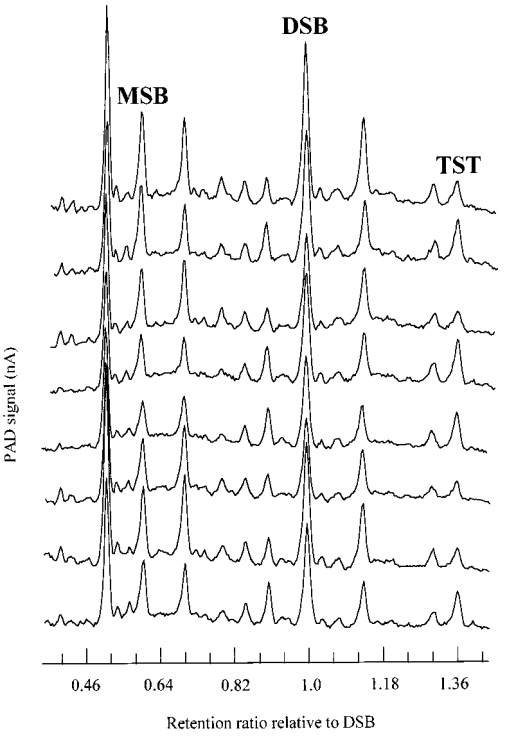

Figure 4. Chromatography profile of oligosaccharides of Hp from patients with Crohn's disease.

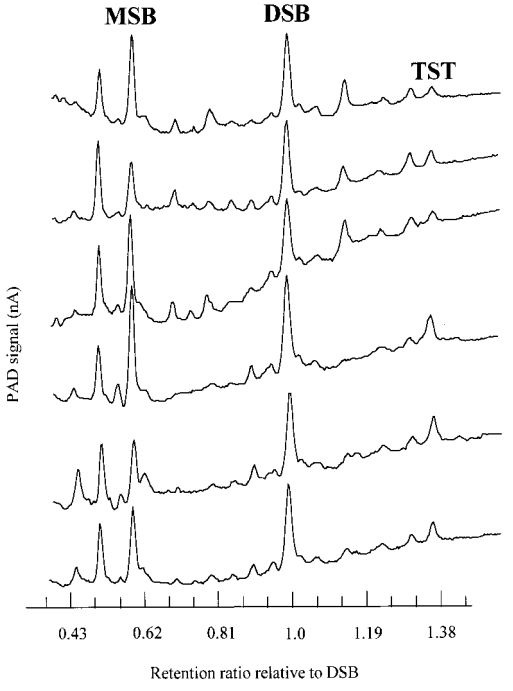

Figure 3. Chromatography profile of oligosaccharides of Hp from patients with rheumatoid arthritis.

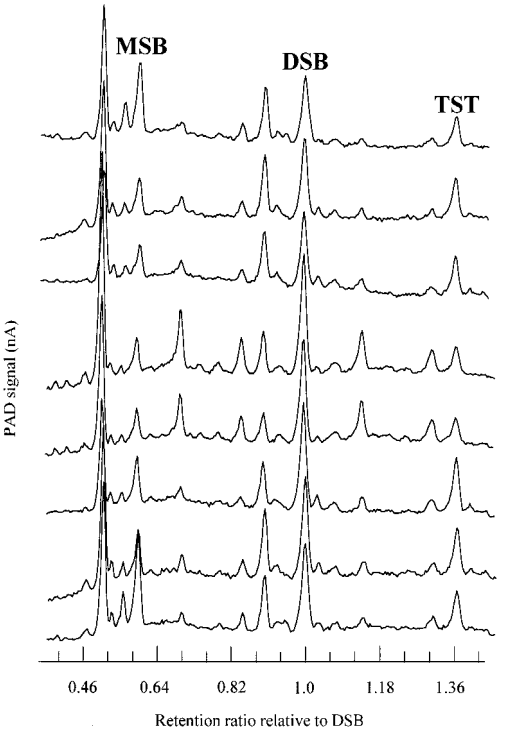

Figure 5. Chromatography profile of oligosaccharides of Hp from patients with stomach cancer.

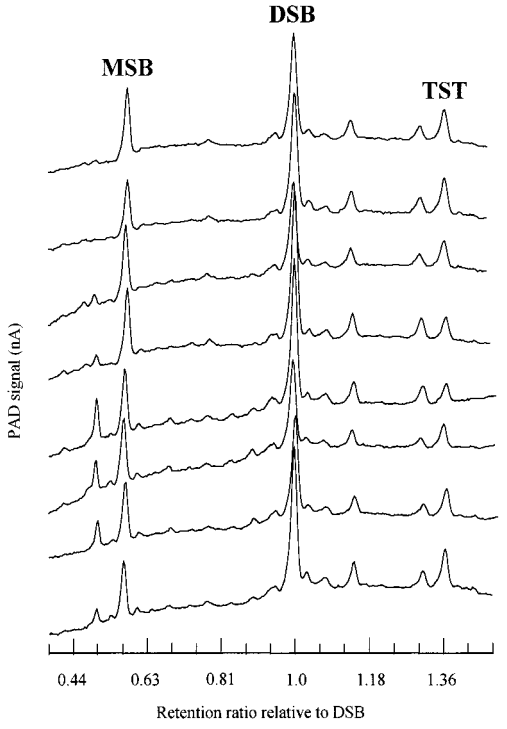

Figure 6. Chromatography profile of oligosaccharides of Hp from patients with breast cancer.

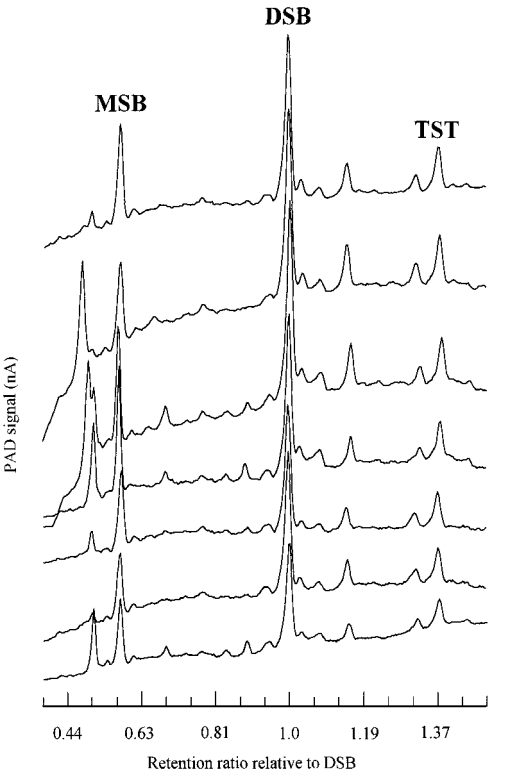

Figure 7. Chromatography profile of oligosaccharides of Hp from patients with ovarian cancer.

Table 2. The oligosaccharide content of Hp isolated from healthy individuals and patients with different diseases.

Group	*Oligosaccharide peak or group of peaks (% area)*								
	'1–7'	*'MSB'*	*'9–16'*	*'17'*	*'DSB'*	*'20'*	*'21'*	*'25'*	*'TST'*
Healthy (8)	8.7 ±6.3	16.1 ±2.4	3.1 ±2.7	3.4 ±0.5	43.1 ±5.9	3.5 ±0.7	4.6 ±1.9	4.5 ±1.5	12.1 ±3.7
RA (6)	20.2 ±4.4[b]	21.2 ±5.6	10.2 ±5.9[a]	3.0 ±0.8	30.4 ±3.5[b]	2.8 ±0.9	4.5 ±3.7	3.7 ±1.5	5.5 ±2.2[b]
CR (8)	23.6 ±4.3[c]	10.2 ±2.0[c]	18.9 ±4.4[c]	1.6 ±0.8[c]	22.0 ±2.7[c]	2.2 ±0.6[b]	10.3 ±1.4[c]	3.8 ±0.8	7.6 ±2.8[a]
ST (8)	28.2 ±3.0[c]	8.1 ±4.5[a]	22.8 ±6.5[b]	2.7 ±1.1	21.5 ±2.6[c]	2.3 ±0.8[a]	4.2 ±2.3	3.5 ±0.8	8.5 ±3.2
BR (8)	5.5 ±4.7	19.5 ±4.2	3.5 ±1.5	2.7 ±0.6	41.5 ±4.5	2.6 ±0.9[a]	7.5 ±1.6[b]	5.0 ±1.4	10.3 ±3.3
OV (7)	11.4 ±7.2	17.0 ±2.7	4.4 ±2.5	2.5 ±0.8	38.7 ±3.5	2.6 ±0.6[a]	6.9 ±1.0[a]	4.2 ±0.9	10.3 ±1.9

Oligosaccharide separations were analysed by calculating the area under each peak and expressing it as a percentage (mean ± SD) of the total area. The values given for '1–7' and '9–16' were obtained for data pooled from more than one peak, this was done because every peak was not measurable or present in every separation, and pooling the data allowed different groups to be compared. Further information on the notation and position of the different peaks is given in Figure 1. The number of specimens examined in each group is given in parentheses and each specimen was separated in duplicate. Differences between groups were analysed statistically by the Mann-Whitney test; [a]$0.05 > p > 0.01$; [b]$0.01 > p > 0.001$; [c]$0.001 > p > 0.0001$.

the breast and ovarian patients, the significant changes in oligosaccharide composition were very small and were restricted to decreases in peak '20' and increases in peak '21'.

Discussion

This study has established a simple, reproducible and rapid procedure for investigating the oligosaccharide composition

of Hp in disease. Most of the species detected were probably sialylated oligosaccharides, because under the elution conditions used neutral oligosaccharides were not separated. Some of the peaks may have been due to sulphation, but this seems unlikely, because there is no evidence for the presence of this grouping on Hp; whereas Neu5Ac is known to be present in large amounts on Hp [11].

The procedure developed in this study was based upon a recently described method that completely removed oligosaccharides from small amounts of glycoproteins using PNGase F [14]. When this method was combined with a rapid affinity chromatography method for preparing the purified glycoprotein and an automated ion-exchange method for separating the oligosaccharides, 10 specimens could be easily processed in 1 week.

From the observed oligosaccharide profiles, it was impossible to identify all the minor peaks obtained because the appropriate sialylated standards were not available. A single exception to this was the α1-6 core fucosylated disialylated standard and this was tentatively identified as peak '17'. However, there was very little change in the latter component in all the groups, except in the 'CR' patients, where it was reduced.

It was possible to standardize the position of each peak in a separation, and compare the same peak between different separations, by assigning a retention-value relative to the 'DSB' peak. Initially, retention was defined relative to 'GDP' but early in the study it was found unnecessary to do this, as the 'DSB' peak was easily identified in all separations; therefore, GDP was used as an internal control to check oligosaccharide recovery after lyophilization.

An unexpected finding in this study was the large reduction in the content of the 'MSB', 'DSB' and 'TST' chains in 'RA', 'CR' and 'ST' and the appearance of other components adjacent-to and in-between these major species. There could be many reasons for this change. An obvious reason could be loss of Neu5Ac, which would result in lower retention on the column. However, previous monosaccharide analysis of Hp in 'CR', 'ST' and 'RA' did not detect any reduction in Neu5Ac content compared to healthy individuals; in fact, it was shown to increase in 'RA' and 'CR' [11]. It is more likely that our findings are explained by an increase in sialylated glycoforms rather than a decrease.

Another reason for this change could be the addition of fucose to the carbohydrate chain. This type of modification can occur as an α1-6 linkage attached to a *N*-acetylglucosamine (GlcNAc) residue in the core region or as α1-2 or α1-3 linkages attached to galactose (Gal) or GlcNAc residues respectively at the ends of the oligosaccharide chains. Previous monosaccharide studies have found that the fucose content of Hp can increase by up to two- to three-fold in 'RA', 'CR' and 'ST' [11]. Furthermore, it has been shown that the addition of fucose to sialylated oligosaccharides causes them to elute earlier from a Dionex ion-exchange column [15, 16]. Fucose in different numbers and in different positions on the carbohydrate chain could, therefore, have caused the observed changes in these diseases. A recent study of AGP has reported significant increases in fucosylated biantennary and triantennary oligosaccharides in 'RA' [17].

It seems probable that other structural changes were also contributing to the observed profiles in the 'RA', 'CR' and 'ST' groups, because the fucose content was previously shown to be increased in the 'BR' and 'OV' groups [11], but in the current analyses, the changes in oligosaccharide profile for these groups were small and limited to two peaks. A previous study using the PA100 anion-exchange column found that many structural changes can alter the elution properties of oligosaccharides [16]. The presence of Neu5Ac linked α2-6 to Gal rather than α2-3 decreased retention; addition of a bisecting GlcNAc on biantennary chains increased retention; and removal of Gal and GlcNAc decreased retention. Hence, many of the additional minor components detected in 'RA', 'CR' and 'ST', probably occurred through more than one structural change in the major 'MSB', 'DSB', or 'TST' glycoforms.

It is concluded, therefore, that the approach developed in this study gave very reproducible oligosaccharide profiles for different diseases. Although no peak was identified that was specific for any particular disease, the profiles for most of the diseases were very characteristic. This suggsts that the HPAEC/PAD method could in the future be very useful for mapping glycosylation changes in multiple clinical specimens.

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