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Development of a high performance anion exchange chromatography analysis for mapping of oligosaccharides

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ABSTRACT

In the present study a HPAEC-PAD method is described that was developed for monitoring the consistency of N-glycosylation during the production and purification of recombinant proteins and monoclonal antibodies. The method successfully separated 18 neutral and sialylated oligosaccharides. Results obtained were compared with MALDI-TOF MS and it was shown that both methods gave similar results. In addition, a method validation was performed showing that the HPAEC-PAD analysis was well suited for the mapping and characterization of oligosaccharides. The method was found to be robust and additionally the precision was significantly better compared to the MALDI-TOF MS method.

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1. Introduction

The biopharmaceutical market of therapeutic proteins is undergoing a rapid expansion, not least in the field of monoclonal antibodies (mAbs) where there are currently 20 therapeutic mAbs on the market and about 500 antibody based development programs in pipeline [1]. Characterization of protein therapeutics is naturally a vital part in drug development, where both the primary sequences and post-translational modifications such as glycosylation need to be studied closely. Protein glycosylation affects many different physicochemical properties, such as solubility, viscosity and through stabilizing protein conformation and protein folding, as well as biological properties such as modulating activity, participating in cell–cell interactions and determining circulation half-life [2–4]. Since proper glycosylation is often critical for the protein function, the production of therapeutic glycoproteins needs to be closely monitored, as both reactor conditions, improvements in cell productivity and downstream processing can affect the glycan heterogeneity [5–7]. Oligosaccharide analysis is thus important in the production of glycoprotein pharmaceuticals as well as in fundamental research.

Since introduced in the 1980s, high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) has become a valuable tool in glycoprotein characterization [8,9]. The advantage is that it can separate and detect oligosaccharides without any derivatization and yet offers excellent resolution and a high sensitivity, with a detection limit in the 300 fmol region [10]. Mass spectrometry based methods are also commonly used for both quantitative analysis using MALDI-TOF MS [11,12], LC–MS [13] and structural analysis using MS/MS based techniques [14]. Also, an interesting combination of MS and HPAEC-PAD has been suggested [15]. Other alternatives are the use of HPLC or capillary electrophoresis in combination with UV or fluorescence detection, where chromophores often are introduced to the sugars [10,13,16,17]. Previously, several studies have been published using HPAEC-PAD for the characterization of protein glycosylation [2,8,18–24], but none of these has been shown to separate all glycans found in recombinantly produced mAbs and simultaneously separate both sialylated and neutral oligosaccharides. The aim of the present study was thus to develop an HPAEC-PAD method that could separate neutral as well as sialylated oligosaccharides more efficiently than previously described methods, by optimizing both gradient and eluent composition, including pusher concentration and pH. The developed method

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Table 1
Oligosaccharide structures.

□	N-acetylglucosamine (GlcNAc)
◊	Fucose (Fuc)
○	Mannose (Man)
■	Galactose (Gal)
◁	Sialic acid (Neu5Ac)
1. Man 3	
2. Man-5	
3. Man-7	
4. Man-9	
5. G0 (NGA2)	
6. G2 (NA2)	
7. G0F (NGA2F)	
8. G1F (NA2G1F)	
9. G2F (NA2F)	
10. G2A1 (A1)	
11. G2FA1 (A1F)	
12. G2A2 (A2)	
13. G2FA2 (A2F)	

was validated and compared with an existing MALDI-TOF MS analysis.

2. Experimental

2.1. Chemicals

Neutral and acidic oligosaccharide standards were obtained from Dextra laboratories (Reading, UK), except for A2F (G2FA2) which was purchased from Sigma. A schematic view of all the oligosaccharides used is shown in Table 1. Peptide N-glycosidase F (PNGase F) was obtained from Roche Diognostics GmbH (Mannheim, Germany). All other chemicals used were of analytical grade if not stated otherwise.

2.2. Monoclonal antibodies

Two monoclonal antibodies were used throughout the study, denoted mAb 1 and 2. They were recombinantly expressed in CHO cells and recovered using different combinations of chromatography steps. Both mAb 1 and 2 were of the IgG1 type.

2.3. Enzymatic release of oligosaccharides

The N-glycans were released through enzymatic digestion using PNGaseF. About 200 μg of glycoprotein were mixed with 2 U (nmol/min) of PNGase F and the reaction was incubated at 37 °C over night. The glycans were collected and separated from the protein by 15 min centrifugation at 14,000 rpm using a Millipore microcon spin column with 30 kDa weight cut off.

2.4. HPAEC-PAD

The HPAEC-PAD analysis was performed on a DIONEX ISC3000 system (Sunnyvale, CA, USA) including pump, detector, temperature controlled column and detector compartment and an autosampler. A CarboPac PA200 column with guard column was used at a flow rate of 0.5 ml/min and a column temperature of 30 °C. The injection volume was varied between 5 and 10 μl. The pulse setting for the amperometric detector was the carbohydrates standard quad, as named by Dionex.

Three eluents were prepared in plastic bottles that were carefully purged using helium. The eluents consisted of 250 mM sodium acetate (A), 191 mM NaOH containing 10 mM sodium acetate (B) and water (C). The oligosaccharides were eluted using the program given in Table 2.

2.5. MALDI-TOF

A MALDI-TOF (MALDI-DUAL) from Waters (Milford, MA, USA) was used in the positive mode using reflectron optics. 2,5-Dihydroxybenzoic acid was used as matrix and the glycans were detected as singly charged sodium adducts.

Table 2
Gradient program, A: 250 mM NaAc; B: 191 mM NaOH and 10 mM NaAc; C: water.

Time (min)	A (%)	B (%)	C (%)
0.0	0	29	71
12.0	0	29	71
24.0	10	29	61
42.0	48	29	23
42.1	0	29	71
50.0	0	29	71

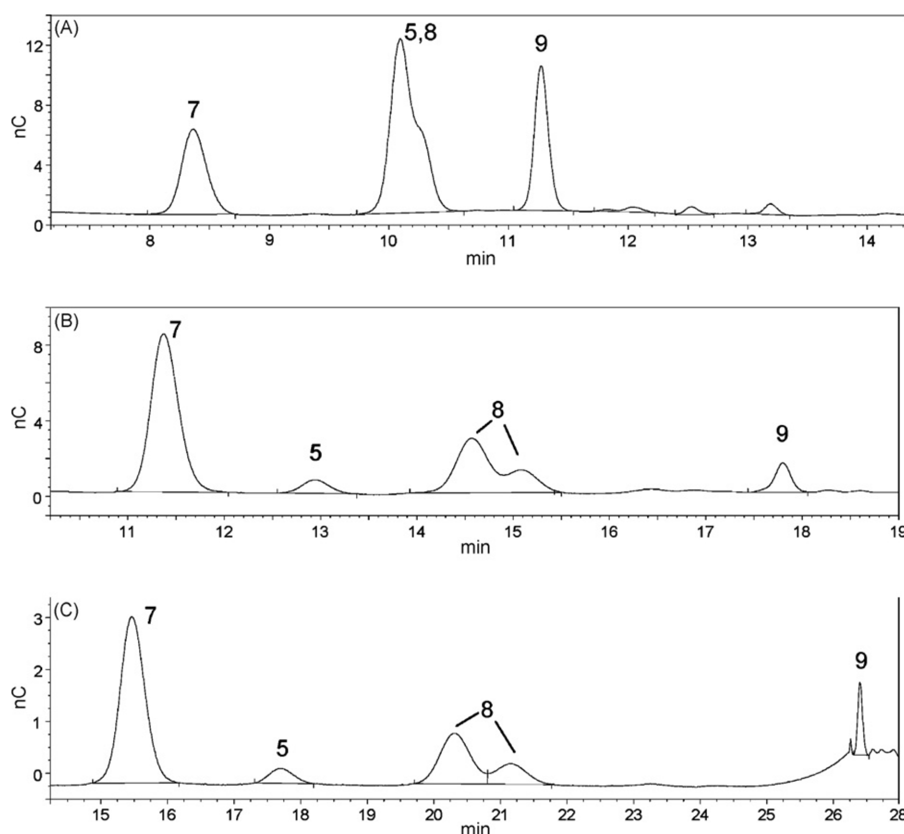

Fig. 1. Influence of NaOH concentration on glycans released from mAb 1. Isocratic conditions were used with: (A) 125 mM NaOH, (B) 100 mM NaOH, and (C) 75 mM NaOH.

3. Results and discussion

3.1. Effects of eluent composition on the elution of neutral glycans

The effects of sodium hydroxide (NaOH) and sodium acetate (NaAc) were first explored with neutral glycans, originating from mAb 1, using isocratic elution conditions. In Fig. 1 the NaOH concentration was decreased from 125 to 75 mM. The retention time increased with about a factor 2 and the two G1F isomers (see Table 1) separated better. The separation between these isomers has previously been shown to be quite difficult to accomplish using HPAEC [13]. All glycans, including isomers, were identified by their retention times and through comparison to commercial standards (Table 1) in addition to confirming the molecular weights using MALDI-TOF MS.

The effect of NaAc was investigated by increasing its concentration from 2.5 to 10 mM using four even lower concentrations of NaOH: 71, 55, 38 and 25 mM. It was found that the retention times, which were quite long at these NaOH concentrations, were strongly reduced with increasing NaAc concentration and the resolution of the glycans were severely hampered above 2.5 mM NaAc (data not shown). Furthermore, it was shown that G0F had the same retention time using 38 and 21 mM NaOH (with 2.5 mM NaAc), but the separation between G0/G1F being much worse at 21 mM (Fig. 2). The best resolution obtained for these two glycans were between 55 and 71 mM NaOH, using 2.5 and 0 mM NaAc respectively (Figs. 1C and 2). However, it was decided to use a combination of 55 mM NaOH and 2.9 mM NaAc. The reason for choosing the lower NaOH concentration was the possibility to separate G0 and Man-5, which is an undesired glycosylation that is sometimes

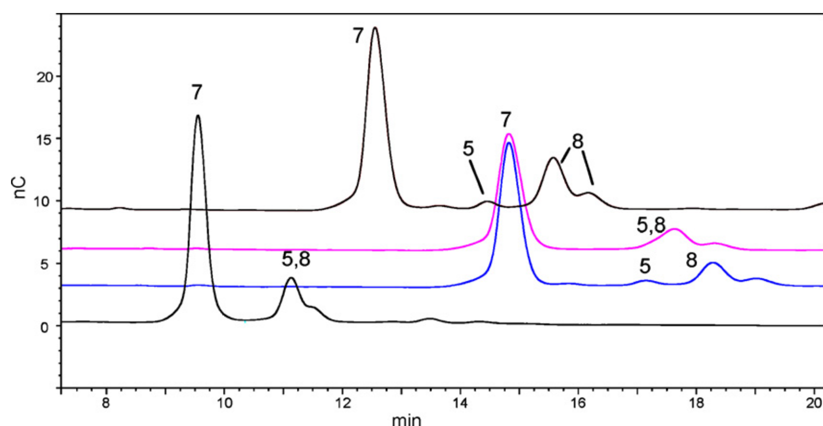

Fig. 2. Comparison of retention and selectivity of glycans released from mAb 1. Isocratic conditions were used with 2.5 mM NaAc and from top to bottom 55, 21, 38 and 71 mM NaOH added.

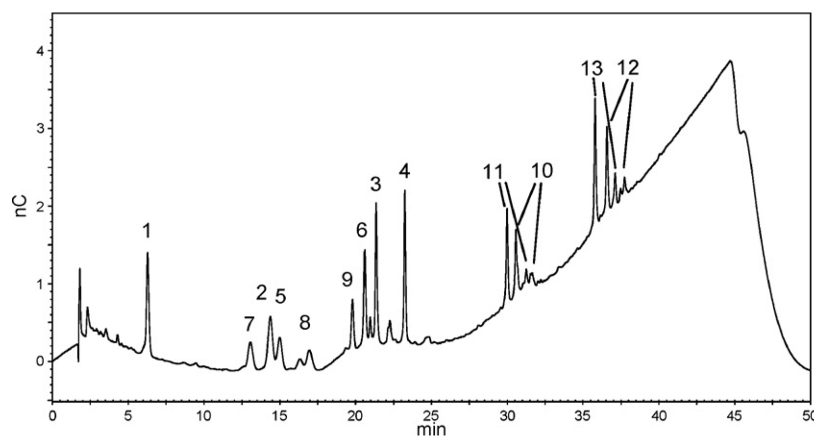

Fig. 3. Oligosaccharide standards with 4.5 pmol of each standard injected and separated using gradient elution as described in Table 2.

observed (data not shown). The NaAc concentration was slightly increased to compensate for the increased retention times at 55 mM NaOH. A disadvantage when lowering the NaOH concentration is that the detector response is decreased. For most glycans a reduction of about 30% in the area response was observed when the NaOH concentration was lowered from 71 to 38 mM, which is not critical for the quality of the results obtained, unless diluted solutions close to the detection limit are to be analyzed.

The results obtained clearly show that improvements in separation as well as in retention times could be achieved by decreasing the NaOH concentration from the more commonly used 100 mM to about 50 mM. The resulting longer retention times were compensated by increasing the NaAc concentration essentially without affecting the resolution. Similar results with better separation at lower pH have previously been observed by McGuire et al. who showed that the optimal separation for monosaccharides was achieved at 30 mM NaOH, which is somewhat lower than the optimal concentration found for oligosaccharides in the present study [25]. In contrast, Cooper et al. came to the opposite conclusion that an increase in pH (250 mM NaOH) improved the separation of neutral oligosaccharides using a PA-100 column [20]. However, they used the same NaAc gradient in all experiments and better separation could most likely been achieved if a less aggressive gradient had been applied in the case of the lower NaOH concentration (100 mM). Further, a dual effect of NaOH could be seen in Fig. 2 at 38 and 21 mM. NaOH both sets the pH and therefore the ionization state of the analytes and it also acts as a pushing agent. At 21 mM the resolution of the peaks were reduced and the retention times were not increased anymore, indicating that the reduced amount of pushing ions (OH^-) was compensated by a decrease in the amount of analytes being in the anionic form, which are retained on the column.

3.2. Addition of a NaAc gradient for elution of acidic glycans

To improve the method further and including the possibility of analyzing acidic glycans, which are more strongly retained on the column, it was necessary to add a gradient step. Four acidic glycan standards were used, two containing one sialylated glycan (G2A1 and G2FA1) and two containing two (G2A2 and G2FA2). As for the neutral glycans, the fucosylated oligosaccharides eluted earlier [8]. Each of these standard glycans contains (at least) two isomeric forms, α(2,6)- and α(2,3)-linked Neu5Ac (N-acetyl neuraminic acid), where α(2,6) elutes first [21]. The approach was to start the gradient after the last eluting neutral glycan (Man-9). The best compromise between speed and separation was found in a gradient starting at 2.9 mM NaAc that was increased to 123 mM in 18 min. A chromatogram showing the separation of all the tested oligosaccharide standards is shown in Fig. 3 and the retention times obtained for the individual glycans are shown in Table 3. The only standards found to co-elute were the G2F and the high mannose species Man-6 (not shown).

3.3. Method validation

A method validation was performed, including parameters such as analytical range, robustness, repeatability and intermediate precision. The LOD and LOQ, defined as S/N ratio of three and nine, were estimated to be at least 0.3 and 0.9 pmol respectively using G0F and G1F, the glycan standards showing the lowest peak height response (Table 3 and Fig. 3). The response was linear in the entire investigated range (0–120 pmol). The response factors varied slightly between the different oligosaccharides. The high mannose glycans (Man-3 to Man-9) showed a response factor that was approximately twice as high as the neutral glycans containing a fucose, whereas the rest of the neutral glycans and all the acidic ones had response factors between those (data not shown). The response factors were found to be consistent when the experiments were repeated several times at different occasions. However, they are based on the manufacturers' information about the quantity of the oligosaccharides. These were purchased in either 10 or 20 μg as qualitative standards, and the accuracy of these amounts are unknown and of course crucial for the calculation of a correct response factor.

Table 3
Oligosaccharide standards 4.5 pmol injected, separated using gradient elution as described in Table 2.

Oligosaccharide	Retention time (min)	Peak height (nC)
1. Man-3	6.3	1.310
7. G0F	13.1	0.327
2. Man-5	14.4	0.654
5. G0	15.0	0.398
8. G1Fa	16.3	0.097
8. G1Fb	16.9	0.219
9. G2F	19.8	0.683
6. G2	20.6	1.269
3. Man-7	21.3	1.826
4. Man-9	23.2	1.940
11. G2FA1 α(2,6)	30.0	1.229
10. G2A1 α(2,6)	30.6	0.839
11. G2FA1 α(2,3)	31.2	0.191
10. G2A1 α(2,3)	31.6	0.131
13. G2FA2 α(2,6)	35.8	1.625
12. G2A2 α(2,6)	36.6	1.070
13. G2FA2 α(2,3)	37.1	0.395
12. G2A2 α(2,3)	37.7	0.192

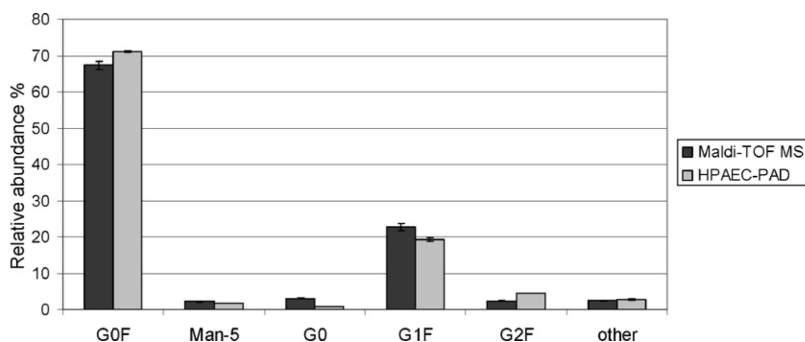

Fig. 4. Comparison of oligosaccharide mapping results obtained for mAb 2 using MALDI-TOF MS and HPAEC-PAD. Each result is an average of four samples and the error bars indicate the standard deviation.

The robustness of the method, relative abundance (area %) and absolute area of the glycans, and the robustness of the retention times were investigated in two ways: varying the mobile phase composition ± 5% of NaOH and NaAc, and comparison of a freshly made mobile phase with an aged mobile phase (1–7 days). Oligosaccharides from a recombinant glycoprotein (not a mAb) expressed in CHO cells, containing both neutral as well as acidic glycans, were used throughout the validation. It was found that the relative abundance as well as the absolute area were unaffected by the change in mobile phase composition. The age of the mobile phase did not have a large impact on the results either, the relative abundance was not changed significantly but the peak area was found to vary slightly from day to day, although the trend was not clear. As expected the retention times varied a lot when the mobile phase composition was changed. A change in the NaOH concentration mostly influenced the retention of the neutral glycans eluting in the isocratic part of the program, while a change in the NaAc concentration affected the sialylated oligosaccharides more. The retention time decreased with the age of the mobile phase for all the glycans. When 1-week-old mobile phase was compared with freshly made, the retention times for the neutral oligosaccharides were only changed with a few tenths of a second, while the later eluting sialylated glycans were eluting more than a minute faster. The most likely reason for the faster elution with older mobile phase is that carbon dioxide in the air dissolves in the eluents. This would cause a slight drop in the OH^- concentration and hence a slower elution. But this effect is compensated for by the fact that carbon dioxide exists almost solely as the divalent carbonate ion at high pH, which is a much stronger pushing agent than OH^-.

The repeatability was found to be good, with especially the standard deviations for the relative abundances of the different glycans being low, ranging from 0.11% to 0.25% with an average of about 0.2%. The intermediate precision, *i.e.* the same analysis performed on different occasions, were as expected found to give a considerable higher standard deviation, varying from 0.9% to 2.4% with an average of about 1.5%.

The developed HPAEC-PAD method thus proved both to be quite robust and to give very consistent results with good precision. However, the retention times of the glycans were rather sensitive to small changes in composition and the age of the mobile phase. Consequently standards need to be run on a daily basis to assure a correct identification of the oligosaccharides.

3.4. Comparison of MALDI-TOF and HPAEC-PAD

The developed HPAEC-PAD method was put to the test by analyzing eight different batches of mAb 2 and comparing it with the results obtained when the same material was analyzed with MALDI-TOF MS. In both methods the results were calculated as the relative abundance (%) of the different oligosaccharides found. A comparison of the methods for one of the batches is shown in Fig. 4. Although these techniques are based on completely different detection principles the results were similar. The differences between the methods in all of the batches were in most cases relatively small, often less than 2.5% for the individual oligosaccharides. Thus, the relative response for the glycans was quite similar in both methods. The largest difference was observed in the G0 results, which stood out from the rest. The estimated abundance for this glycan was about a twice as high using the MALDI-TOF MS method. These findings are in agreement with Roberts et al. and Field et al., who also got similar results when comparing these two analytical methods [18,26].

The precision of the methods was estimated by combining all the results from the eight batches, in which each batch was digested twice and each digest was analyzed twice (both HPAEC-PAD and MALDI-TOF). It was found that the average oligosaccharide precision of the HPAEC-PAD method was significantly better ($p < 0.01$, *F*-test), having a standard deviation of 0.13% compared to 0.5% for the MALDI-TOF MS method.

3.5. Concluding remarks

The described HPAEC-PAD method proved to be well suited for monitoring batch to batch variability of the oligosaccharide composition in the production of monoclonal antibodies. Furthermore, it managed to separate the oligosaccharides commonly found in mAbs better than previously reported. It has also been successfully used in the characterization of two other N-glycosylated recombinant proteins (results not included), where it proved to be valuable due to the high occurrence of sialylated glycans in these proteins. Compared with MALDI-TOF MS, the major alternative technique, the HPAEC-PAD method gave comparable results but had some distinct advantages. These include a higher precision, easier automatization (with LC-autosampler), less cumbersome sample handling and the ability to analyze both neutral as well as sialylated oligosaccharides simultaneously. Also, many structural isomers can be separated with HPAEC-PAD, which is not possible with MALDI-TOF MS. In addition, the HPAEC-PAD technique is better quantitatively, since it would be possible to quantify in absolute terms using external calibration, if a validated standard could be obtained. On the other hand MALDI-TOF MS offers certain advantages too, as there is no need for oligosaccharide standards and the time spent on analysis is substantially shorter compared to HPAEC-PAD.

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