High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid

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Received on October 2, 1997; revised on January 16, 1998; accepted on January 16, 1998

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Facile labeling of oligosaccharides (acidic and neutral) in a nonselective manner was achieved with highly fluorescent anthranilic acid (AA, 2-aminobenzoic acid) (more than twice the intensity of 2-aminobenzamide, AB) for specific detection at very high sensitivity. Quantitative labeling in acetate-borate buffered methanol (pH 5.0) at 80°C for 60 min resulted in negligible or no desialylation of the oligosaccharides. A high resolution high performance liquid chromatographic method was developed for quantitative oligosaccharide mapping on a polymeric-NH2 bonded (Aster) column operating under normal phase and anion exchange (NP-HPAEC) conditions. For isolation of oligosaccharides from the map by simple evaporation, the chromatographic conditions developed use volatile acetic acid-triethylamine buffer (pH 4.0) systems. The mapping and characterization technology was developed using well characterized standard glycoproteins. The fluorescent oligosaccharide maps were similar to the maps obtained by the high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), except that the fluorescent maps contained more defined peaks. In the map, the oligosaccharides separated into groups based on charge, size, linkage, and overall structure in a manner similar to HPAEC-PAD with contribution of -COOH function from the label, anthranilic acid. However, selectivity of the column for sialic acid linkages was different. A second dimension normal phase HPLC (NP-HPLC) method was developed on an amide column (TSK Gel amide-80) for separation of the AA labeled neutral complex type and isomeric structures of high mannos type oligosaccharides. The oligosaccharides labeled with AA are compatible with biochemical and biophysical techniques, and use of matrix assisted laser desorption mass spectrometry for rapid determination of oligosaccharide mass map of glycoproteins is demonstrated. High resolution of NP-HPAEC and NP-HPLC methods combined with mass spectrometry (MALDI-TOF) can provide an effective technology for analyzing a wide repertoire of oligosaccharide structures and for determining the action of both transferases and glycosidases.

Key words: anthranilic acid/2-aminobenzoic acid/fluorescence labeling/oligosaccharide mapping(characterization)

Introduction

It has been well established that glycoproteins typically display an impressive number of carbohydrate structures, and this very nature is a challenge for separation and isolation of pure oligosaccharides for structure elucidation. The structural diversity in glycoproteins arises from the combined action of specific but incomplete biosynthetic and degradation events in the cell. Obviously, the knowledge of carbohydrate structures present in a glycoprotein is a prerequisite for understanding its glycobiology (for reviews, see Varki, 1993; Dwek, 1996). In the last decade, various approaches have been developed to achieve separation of oligosaccharides with higher resolution and greater sensitivity of detection (for a short review, see Davies and Hounsell, 1996). Among these techniques, separation by HPAEC-PAD has been popular due to its ability to analyze carbohydrates without derivatization (see Townsend, 1995, for a review). But this method is plagued by unstable baselines, loss of sensitivity due to noise, and use of high pH and high salt (Anumula and Taylor, 1991). Since the carbohydrate fractions must be desalted for isolation, the characterization of oligosaccharides which occur in low abundance is difficult with HPAEC-PAD methods. In addition, PAD is nonspecific for carbohydrates (Weitzhandler et al., 1996). All the HPAEC-PAD problems in the analysis of carbohydrates have been eliminated in the current technology with an increase in the resolution and the sensitivity.

Based on previous experience with anthranilic acid and its derivatives, anthranilic acid (AA, 2-aminobenzoic acid) was introduced as the second smallest preferred fluorescent label for carbohydrate analysis with very high sensitivity (Anumula, 1993, 1994). AA is highly fluorescent (more than twice as intense as 2-aminobenzamide (AB)), and is specific for labeling the reducing mono- and oligosaccharides. During these studies, two reports (Guile et al., 1996; Townsend et al., 1996) appeared in which AB was used as the fluorescent tag for labeling oligosaccharides. In contrast to the reported observations that AA is not suitable for analysis using ion-exchange, reversed phase and mass spectrometry (Bigge et al., 1995), we have found that indeed AA is the preferred label over the AB for carbohydrates and their characterization using variety of biochemical and spectroscopic techniques. In addition, it is being proposed that all the applications developed for characterization of carbohydrates using HPAEC-PAD (for review see Townsend, 1995) and HPLC methods using 2-aminopyridine (Hase, 1994) are directly applicable to the versatile AA labeled oligosaccharides, including the analysis by gel electrophoresis (Bigge et al., 1995). Obviously, current methods available for analysis of the reduced oligosaccharides are not directly applicable to this technology.

This report describes a rapid method for oligosaccharide mapping of glycoproteins with high resolution and very high sensitivity using polymeric-NH2 column and acidic volatile buffer systems (pH 4.0) which allow for isolation of the base...
labile oligosaccharides by simple evaporation. The fluorescent oligosaccharide maps obtained can be compared directly with those generated by the HPAEC-PAD method for facile interpretation by analogy. In addition, a method for characterization of the neutral oligosaccharides based on size and structure was developed using an amide column. Results presented here demonstrate a significant advancement in technology for oligosaccharide mapping of glycoproteins and for the oligosaccharide structure elucidation.

Results

Separation of AA labeled oligosaccharides by normal-phase high performance anion-exchange chromatography (NP-HPAEC)

With an objective of developing a simple, robust, highly sensitive and highly reproducible method for oligosaccharide mapping, a gradient system for oligosaccharide mapping was developed using volatile buffer systems on a durable polymeric-NH$_2$ column. An additional objective was to develop a method that would compare favorably to the current standard of mapping using the HPAEC-PAD. The NP-HPAEC gradient described for mapping was optimized initially for separation of standard fetuin oligosaccharides in a reasonable time with high resolution. These conditions were used to determine the oligosaccharide map of other glycoprotein standards and unknown glycoproteins. A typical oligosaccharide map of the fetuin obtained by this method and its comparison to the map obtained by the HPAEC-PAD using underivatized oligosaccharides is shown in Figure 1. The fetuin usually contains more than 23 distinct oligosaccharides (Green et al., 1988). The results indicate that the fluorescent map of fetuin has resolved into more peaks within each charge group than the HPAEC-PAD map. These results can be compared to the other fetuin maps reported using HPAEC-PAD (Townsend et al., 1989; Parekh and Patel, 1992). Since the HPAEC-PAD map of fetuin has been well characterized (Townsend et al., 1989), it serves as a standard against which all other methods must be compared. The comparative results demonstrate that indeed the fluorescent map has a higher resolution than the HPAEC-PAD map. Due to specific labeling of the reducing oligosaccharides by AA, the peaks corresponding to free sialic acid and other PAD reactive materials are not detected in the fluorescent map. Furthermore, relatively large intensities observed for the O-linked oligosaccharides in the HPAEC-PAD are not observed in the fluorescent map. Perhaps this is due to degradation or modification at the reducing terminal of the O-linked oligosaccharides obtained by the hydrazinolysis under N* or N+O conditions on Glycoprep 1000 or during the course of analysis in high pH conditions. The fluorescent oligosaccharide map of fetuin was highly reproducible from the derivatization reaction to the analysis. The relative intensity of the peaks compares favorably to that obtained by the HPAEC-PAD map.

For the purpose of discussion, structures for the oligosaccharides and their abbreviations are shown in Figure 2. In analogy of the fetuin HPAEC-PAD map and its established structures (Townsend et al., 1989) to the fluorescent map, it could be concluded that perhaps the elution order of peaks 3, 3a, 4, and 4a has been reversed. This observation was confirmed by isolating the peaks 3, 3a, 4, 4a separately from the HPAEC-PAD map as described previously (Anumula and Taylor, 1991) and analyzing the peaks under NP-HPAEC mapping conditions after labeling with AA. Presumably, this change in elution order is due to selectivity of the amine bonded column towards the sialic acid linkages (α2–3 vs. α2–6) (Figures 1 and 2). It has been confirmed independently with 3′-sialyl lactose and 6′-sialyl lactose model systems that the lactose with α2–3 linked sialic acid eluted earlier than the α2–6 linked sialic acid.

Another example of greater resolving power of this method compared to HPAEC-PAD method is shown by the map of a rIgG, which contains both complex and high mannose type of structures (Figure 3). Fine resolution obtained by this method is quite obvious. Although this antibody contains about 23% of high mannose type oligosaccharides, the HPAEC-PAD map obtained for this rIgG could be easily interpreted as a “normal/typical” profile containing mainly the 2ACG0, 2ACG1, and 2AC structures (Anumula, 1994; Weitzhandler et al., 1994).

Derivatization of oligosaccharides with anthranilic acid

Commercial fetuin oligosaccharide library was used to determine the optimum time for the derivatization reaction and the extent of desialylation during the reaction. Derivatization reactions were carried out as described in the Materials and methods and the results are shown in Figure 4. The conditions developed previously for quantitative labeling of monosaccharides (Anumula, 1994) were also nonselective for derivatization of both acidic and neutral oligosaccharides present in a mixture. The results obtained with fetuin oligosaccharides suggest that the
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Fig. 2. Carbohydrate structures and their abbreviations used in this paper. Isomeric tri-antennary complex structures are abbreviated as 3AC4/3 for the branch arising from the core α1–3 linked mannose via carbon-4 and 3AC6/6 for the branch arising from the core α1–6 linked mannose via carbon-6. Bisecting GlcNAc, Fuc, and sialic acid are abbreviated as B, F, and S in these structures. Structures identified with the prefix “Fet” correspond to the peaks in the fetuin map shown in Figure 1.

Fig. 3. Comparison of oligosaccharide maps of a recombinant IgG obtained with NP-HPAEC with fluorescence detection (AA labeled) and HPAEC-PAD (underivatized). Peak identified with an asterisk was resolved into two peaks in the NP-HPAEC analysis (data not shown). G0 and G1 are the fucosylated biantennary complex with 0 and 1 peripheral galactose residues respectively. M5 to M7 are the high mannose structures with the composition ranging from Man5GlcNAc2 to Man7GlcNAc2.

oligosaccharides reacted efficiently and nonselectively as indicated by the parallel lines obtained with various states of sialylation. Reaction was complete by the end of 45 min and after 75 min a slight decrease in the area of peaks containing two to four sialic acids was observed. Lack of increase in peak area of the neutral oligosaccharides during the course of the reaction combined with parallel lines obtained for the oligosaccharides containing 1–4 sialic acids (Figure 1, peaks 1 to 4) suggest that there is little or no desialylation during the reaction.

Similar experiments with AB as a fluorescence label suggested that it was able to specifically label only the neutral oligosaccharides in methanol-acetate-borate reaction medium. However, it was demonstrated that AB could be used for labeling both neutral and acidic oligosaccharides under the anhydrous conditions of DMSO-acetic acid mixture (7:3) (Bigge et al., 1995). The fetuin map obtained with AB label was similar to the map obtained with AA label except for the lower intensities of the peaks and the earlier elution of oligosaccharides in a frame shift manner. This is likely due to lack of an additional negative charge (−COÖ) from the AB label. Some of the fluorescent properties of the AA and AB labeled oligosaccharides, prepared using two different methods, are described in Table I. The fluorescence of the AA label itself was about 3× the intensity of the AB and when the fetuin oligosaccharides were labeled with AA and AB using their optimum conditions, the intensity of the AA fetuin peaks 3 and 4 was 3.5× and 3.2×, respectively, compared to the AB labeled peaks. Furthermore, when the derivatizations were
carried out in methanol-acetate-borate medium, an increase in intensity of more than 44% for peaks 3 and 4 was observed with AA over AB. Quantitative fluorescence yield of AA derivatives suggest that indeed the AA is more reactive than AB even in the presence of water.

**Table I.** Comparison of fluorescence intensities obtained with various derivatives prepared using two different procedures

<table>
<thead>
<tr>
<th>Fluorescent derivative</th>
<th>AA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AB&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Tags only</td>
<td>310&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fetuin oligosaccharides&lt;sup&gt;d&lt;/sup&gt;</td>
<td>350&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 3</td>
<td>320&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 4</td>
<td>6730&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 4</td>
<td>4410&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 3</td>
<td>220&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 4</td>
<td>170&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>160&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
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<sup>a</sup>Derivatives prepared using equal amount of oligosaccharides.
<sup>b</sup>Fluorescence intensities of AA and AB were measured at their optimum excitation wavelengths of 360 nm and 350 nm, respectively.
<sup>c</sup>Relative intensities when AB values set equal to 100.
<sup>d</sup>Fetuin peaks correspond to the peaks identified in the Figure 1 and were analyzed without purification using Nylon syringe filters.
<sup>e</sup>Derivatization procedure uses dimethylsulfoxide-acetic acid reaction medium as described in Materials and methods.
<sup>f</sup>Derivatization procedure uses methanol-borate buffer system as described in Materials and methods.

Glycoproteins treated with PNGase F or endo H/F can be derivatized directly using the digest (i.e., without purification of the digest to remove the salts and detergents, etc.) since the oligosaccharides are purified following derivatization to remove the excess reagent for determining their maps (data not shown).

**Oligosaccharide mapping of glycoproteins**

The separation conditions developed for optimum resolution of the fetuin oligosaccharide were used for determining the fluorescent oligosaccharide map of various glycoprotein standards and the results are shown in Figure 5. The intent of this figure is to establish the relative elution times and the selectivity of this column for various oligosaccharide structures. Major oligosaccharides in the recombinant sCD4 (CHO cell derived) have 2AC structure, with and without fucose, and contain one to two sialic acids in α2–3 linkages only. The transferrin oligosaccharides have 2AC structures (Figure 2) with one to two sialic acids only in α2–6 linkages and without fucose (Spik et al., 1985). Comparison of these two maps suggests that the selectivity of this amine bonded column is different from that of the pellicular column (HPAEC-PAD) (Anumula and Taylor, 1991) and the oligosaccharides with α2–3 linkages eluted earlier than those with α2–6 linkages. In addition, minor 3AC isomeric structures containing two sialic acids from transferrin were eluted in front of the 2ACS2 structures. Therefore, the elution time of the oligosaccharides is more strongly influenced by the type of sialic acid linkages than the number of constituent monosaccharides. A slight resolution of isomeric 2ACS1 structures can be seen in the transferrin map and it could be improved using shallow gradients. Addition of a fucose residue to the 2AC structures with one to two sialic acids would make them more hydrophobic (sCD4) and elute earlier than those without fucose in their respective charge groups, which is similar to HPAEC-PAD analysis.

The oligosaccharide maps obtained for α1-AGP and porcine thyroglobulin have higher resolution (increase in number of peaks) than the HPAEC-PAD analysis (Anumula and Taylor, 1991) and perhaps have even better resolution than the separation of mixture of AB labeled RNase B and α1-AGP oligosaccharides reported recently (Townsend et al., 1996). The oligosaccharide maps were comparable to those obtained by the HPAEC-PAD method and suggest further that the labeling with AA is nonselective, i.e., high mannose vs. complex type oligosaccharides. Baseline resolution of the asialo complex and high mannose oligosaccharides was not achieved; however, each type of oligosaccharides separated quite well (data not shown). Interfering by the high mannose in the complex oligosaccharides can be removed by digestion with α-mannosidase prior to the analysis as described earlier (Anumula and Taylor, 1991).

**Table II.** Comparison of relative molar proportions of high-mannose oligosaccharides obtained from porcine thyroglobulin by different methods

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Percent distribution according to AA fluorescence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPAEC-PAD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HPLC UV&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Phenol/H2SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>ManGlcnAc&lt;sub&gt;2&lt;/sub&gt;</td>
<td>22</td>
<td>22</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>ManGlcnAc&lt;sub&gt;2&lt;/sub&gt;</td>
<td>24</td>
<td>15</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>ManGlcnAc&lt;sub&gt;2&lt;/sub&gt;</td>
<td>16</td>
<td>16</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>ManGlcnAc&lt;sub&gt;2&lt;/sub&gt;</td>
<td>18</td>
<td>23</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>ManGlcnAc&lt;sub&gt;2&lt;/sub&gt;</td>
<td>20</td>
<td>24</td>
<td>18</td>
<td>9</td>
</tr>
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</table>

<sup>a</sup>Molar responses were assumed equal for all the oligosaccharides.
<sup>b</sup>Values from Anumula and Taylor, 1991.
<sup>c</sup>Values from Daniel, 1987; perbenzoylation-UV method.
<sup>d</sup>Values from Tsuji et al., 1981.

Quantitative aspects of this method were examined by comparison of the reported values for the high mannose type oligosaccharides from porcine thyroglobulin and the results are...
and overall structure. In addition, small mucin type (O-linked) and high mannose type oligosaccharides based on size, linkage separate method was developed for the analysis of asialo complex gradients (not described) generated from the mapping solvents, a characterization of AA labeled neutral oligosaccharides using shallow although the polymeric-\text{NH}_2 column could be used for characterization of AA labeled neutral oligosaccharides using shallow gradients (not described) generated from the mapping solvents, a separate method was developed for the analysis of asialo complex and high mannose type oligosaccharides based on size, linkage and overall structure. In addition, small mucin type (O-linked) oligosaccharides can be analyzed with this method (data not shown). Results obtained with sCD4, rIgGs, neuraminidase treated thyroglobulin (porcine), mild acid resistant \text{α}-\text{AGP}, ovalbumin and RNase B (endo H and PNGase released) are shown in Figure 6. Although these conditions are capable of separating oligosaccharides differing by a monosaccharide within a class, the mixture of complex and high mannose types are not separated completely from each other. Since the oligosaccharides are separated based on size, those with and without fucose (sCD4) and mono- vs. di- N-acetylglycosamines at the reducing end of the oligosaccharides (RNase B; EH vs. NG) were separated well with increase in size. It is interesting to note that the isomeric 2ACF structures with one galactose on either of the two arms were separated (rIgG) but they are not defined at the present time. Also, the isomeric structures of 3AC appear to be separated under these conditions (see minor peaks in sCD4). Furthermore, the oligosaccharides eluted close to the number of monosaccharides of the dextran ladder (for example 2ACF with 10 monosaccharides eluted as Glc$_9$ and Man$_9$Glc$_2$ as Glc$_7$,4) in this method unlike the recent report using the AB labeled oligosaccharides (Guile et al., 1996) in which these two species eluted as Glc$_7$,45 and Glc$_6$,13, respectively.

Calibration of the amide column with dextran ladder or with a series generated from both high mannose and complex type oligosaccharides was useful in determining the number of monosaccharides removed or added in a particular enzymatic reaction. This was particularly useful in the interpretation of an unusual type of glycosylation in rlg$_1$ type of molecules produced in CHO cells (data not shown). On the other hand, the sialylated oligosaccharides eluted much earlier than their desialylated counterparts in this NP-HPLC. This is perhaps due to the formation of sialic acid salt bridges with triethylamine rendering a more hydrophobic character to the oligosaccharides or the hydrophobic character of the sialic acid itself. This could be used advantageously for the analysis of polysialylated high molecular weight oligosaccharides.

**Separation of AA labeled neutral oligosaccharides**

Although the polymeric-\text{NH}_2 column could be used for characterization of AA labeled neutral oligosaccharides using shallow gradients (not described) generated from the mapping solvents, a separate method was developed for the analysis of asialo complex and high mannose type oligosaccharides based on size, linkage and overall structure. In addition, small mucin type (O-linked) oligosaccharides can be analyzed with this method (data not shown). Results obtained with sCD4, rIgGs, neuraminidase treated thyroglobulin (porcine), mild acid resistant \text{α}-\text{AGP}, ovalbumin and RNase B (endo H and PNGase released) are shown in Figure 6. Although these conditions are capable of separating oligosaccharides differing by a monosaccharide within a class, the mixture of complex and high mannose types are not separated completely from each other. Since the oligosaccharides are separated based on size, those with and without fucose (sCD4) and mono- vs. di- N-acetylglycosamines at the reducing end of the oligosaccharides (RNase B; EH vs. NG) were separated well with increase in size. It is interesting to note that the isomeric 2ACF structures with one galactose on either of the two arms were separated (rIgG) but they are not defined at the present time. Also, the isomeric structures of 3AC appear to be separated under these conditions (see minor peaks in sCD4). Furthermore, the oligosaccharides eluted close to the number of monosaccharides of the dextran ladder (for example 2ACF with 10 monosaccharides eluted as Glc$_9$ and Man$_9$Glc$_2$ as Glc$_7$,4) in this method unlike the recent report using the AB labeled oligosaccharides (Guile et al., 1996) in which these two species eluted as Glc$_7$,45 and Glc$_6$,13, respectively.

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**Oligosaccharide maps with MALDI-TOF mass spectrometry**

Oligosaccharides from various glycoproteins used in this study were also analyzed by MALDI-TOF mass spectrometry with and without AA derivatization. In contrast to the previous report (Bigge et al., 1996), the AA labeled oligosaccharides can be easily analyzed by MALDI-TOF MS. Results obtained with the AA labeled ovalbumin oligosaccharides (endo H released) compared to the underderivatized are shown in Figure 7. All the oligosaccharides yielded a set of triplet masses for each species and these were identified as mono-, di-sodium, and sodium-potassium adducts. The mono-sodium adduct had the highest intensity and the masses corresponding to M+H ions were practically negligible. The relative peak intensities in both MS maps (native and AA derivatized) compare favorably to each other, suggesting that molar responses do not change following derivatization with AA and peaks corresponding to the underderivatized species could not be detected in the AA MS map. The MS map of ovalbumin (endo H released) contained 15 components including the 8 major species described by Kobata (1984). It is of interest to note that the two different oligosaccharides have the same mass in the ovalbumin mixture and, therefore, it cannot be assumed that a single peak in MS is a pure species. In this regard, further identification by high resolution LC-MS and by their sensitivity to exoglycosidases is highly desirable. Similarly, MALDI-TOF MS analysis of the AA labeled porcine thyroglobulin...
Fig. 6. Separation of AA labeled neutral oligosaccharides from standard glycoproteins by NP-HPLC. The oligosaccharides used were previously released by endo H (EH) from ovalbumin, by endo H and PNGase (NG) from RNase B, and by hydrazinolysis from α1-acid glycoprotein, thyroglobulin (porcine), recombinant IgG, and sCD4. M5 to M9 are the peaks corresponding to the high mannose type oligosaccharides and the arrows indicate either the action of an endo H or an addition of a fucose residue. Vertical bars indicate the eluted position of AA labeled dextran ladder in glucose units. 2ACFG1 and 2ACFG0 are the fucosylated biantennary complex type oligosaccharides with and without a peripheral galactose residue. Structures were confirmed by exoglycosidase treatments and/or MALDI-TOF MS (data not shown).

It is of interest to note that the oligosaccharide pools obtained by the automated hydrazinolysis (Glycoprep 1000, Oxford GlycoSystems) contained several artifacts in the MS. However, these artifacts were essentially eliminated when the AA labeled oligosaccharide pools were purified by the Nylon membrane filters prior to analysis.

Discussion

Based on our experience with 2-aminobenzoic acid and its derivatives which are compatible with various analytical methods of separation and characterization, anthranilic acid (AA) was introduced as the most preferred label for carbohydrate analysis with very high sensitivity (Anumula, 1993, 1994). Current studies
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demonstrate its utility in specific detection of reducing oligosaccharides in high resolution mapping, and characterization by HPLC and MS. Normal phase high performance anion exchange chromatographic (NP-HPAEC) method was developed for separation of the oligosaccharides with high resolution and very high sensitivity. For example, standard fetuin oligosaccharides (AA labeled) were separated into more peaks (Figure 1) than those obtained with HPAEC-PAD method using underivatized oligosaccharides (Townsend et al., 1989) and capillary electrophoresis (CE) (Guttman, 1996). In the case of CE separation, the oligosaccharides with increase in charge and in size separated at two extreme ends with poor resolution and, therefore, it may be suitable only for analysis of the neutral oligosaccharides. Relative molar ratios for N-linked oligosaccharide peaks were similar to those obtained with the HPAEC-PAD method. It is of interest to note that the relative molar ratios obtained for the O-linked oligosaccharides in the fluorescent fetuin map were lower than those obtained with the HPAEC-PAD. These observations suggest that the O-linked oligosaccharides obtained by hydrazinolysis had been modified at the reducing end and, therefore, could not be labeled with anthranilic acid. Obviously, this conclusion deserves an independent investigation.

Mapping conditions use acidic (pH ~4.0) solvent systems with volatile components (acetic acid and triethylamine). Therefore, the oligosaccharides containing base labile groups (e.g., acetyl) can be easily analyzed and the peaks collected from the map can be easily desalted by evaporation. Indeed, the major problems associated with the HPAEC-PAD analysis such as high pH, high salt, and noisy baseline are eliminated in the current method.

Conditions developed previously for the derivatization of monosaccharides (Anumula, 1994) were also suitable for preparing the stable AA labeled oligosaccharides in nearly quantitative yield. Although the extent of derivatization with AA was not determined using radioactive oligosaccharides, considering the sensitivity of this method and shape of the curves obtained with the time of reaction using fetuin library (Figure 4) indicates that perhaps the derivatization has gone to completion. In addition, when the oligosaccharide mixture was subjected to HPAEC-PAD analysis following derivatization, peaks corresponding to the underivatized oligosaccharides could not be detected. Furthermore, experience with quantitative monosaccharide analysis of recombinant glycoproteins since the introduction of AA label (Anumula, 1994) suggests that indeed the derivatization is nearly quantitative. On the other hand, fetuin oligosaccharides could not be labeled with anthranilamide (2-aminobenzamide, AB) under these conditions, but the neutral oligosaccharides could be easily labeled (Table 1). As demonstrated earlier by Bigge et al. (1995), anthranilamide could be used for labeling carbohydrates in nearly anhydrous DMSO–acetic acid mixture (7.3). Comparison of these two methods suggests that the anthranilic acid is more reactive even in the presence of water and, therefore, this method can be used for rapid and nonselective labeling of all types of reducing oligosaccharides. Yield of the fluorescent AA derivatives was >2x greater than the AB derivatives and these observations suggest that efficiency of labeling oligosaccharides with AA is far superior than the AB.

After derivatization, AA and AB labeled oligosaccharides can be purified under normal phase conditions. After a systematic evaluation of the matrices and conditions, amide based matrices were found to be more suitable than cellulose and acrylamide matrices. Three different types of amide formats described in the purification procedure (see Materials and methods) are suitable for different needs (for e.g., preparative vs. analytical). A method based on the convenient Nylon Acrodisc syringe filter was used consistently and reproducibly for more than a year. These filters have a capacity to adsorb ~20 nmol of oligosaccharides (determined using maltoheptase). Under the conditions described here for purification, these filters can not retain saccharides with less than four monosaccharide units (determined using dextran ladder).

Under the derivatization conditions, there was little or no desialylation of the sialylated oligosaccharides. These observations are supported by the results obtained with standard fetuin oligosaccharides containing one to four sialic acid residues derivatized in aqueous methanol solution buffered with acetate-borate (~pH 5.0) (Figure 4). Furthermore, the oligosaccharide maps obtained for the glycoprotein standards: porcine thyroglobulin (contains both high mannose and complex type oligosaccharides), transferrin, recombinant scCD4, and α1-acid glycoprotein are nearly identical to those obtained with HPAEC-PAD with the exception of fine resolution in the fluorescent mapping method (Figure 5).

Because of high sensitivity of the NP-HPAEC method, it has been determined that <1.0 µg of fetuin following PNGase F digestion can be easily analyzed. Therefore, protein bands from SDS–PAGE and electroblotted PVDF membranes can be easily analyzed by this fluorescent method (unpublished observations) similar to the monosaccharide analysis (Du and Anumula, 1997). Also, of interest is that AA labeled glucose ladder (partially hydrolyzed dextran) can be separated into >25 peaks, but its use as a column calibrant has no value in the mapping method using an amine column in an ion-exchange mode (data not shown). On the other hand, calibration of the column to establish the elution windows for oligosaccharides with different degrees of sialylation is useful and this can be conveniently achieved with the fetuin library.

Experience with HPAEC-PAD suggested that an appropriate method for the separation of neutral oligosaccharides (e.g., high mannose and desialylated complex) differing by a single monosaccharide unit is highly desirable for characterization (Anumula and Taylor, 1991). The endo H (EH) cleaved oligosaccharide mixture from the ovalbumin provided a challenging problem for high resolution on the polymeric-NH2 column even in the shallow gradient systems generated from the mapping solvents or the modified mapping solvent systems. The ovalbumin mixture behaves as if they are composed of two groups of oligosaccharides and can not be resolved in a single run. However, in two separate runs with different gradients (not described) this mixture could be separated into more than 12 components. In order to improve the resolution further and to use it as a second dimension in separation, a method based on oligosaccharide size, linkage, and structure was developed using an amide column operating under normal phase conditions. Results obtained with neutral standard glycoprotein oligosaccharides (Figure 6) show that indeed the separation is better than that obtained with HPAEC-PAD method (Anumula and Taylor, 1991). In addition, the positional isomers of AA labeled high mannose oligosaccharides, Man9GlcNAc2, ManrGlcNac2, and Man9GlcNAc2 were separated to some extent on this amide column (Figure 6, RNase B: NG and EH). Therefore, NP-HPLC can be used as a second dimension in the separation of oligosaccharides and it is quite useful for characterization of not only the high mannose oligosaccharides but also the neutral complex oligosaccharides following various enzyme treatments. In NP-HPLC, high mannose type and complex type oligosaccharides without sialic acid were separated to some extent. Interference...
of the high mannose in complex can be removed by treatment with α-L-mannosidase. AA labeled dextran ladder could be used as a calibration mixture for this column and was useful in determining the number of monosaccharides removed in a particular glycosidase digestion. Furthermore, this method was useful in assessing the purity of the peaks obtained from the oligosaccharide map. It is of interest to note that the AA labeled oligosaccharides in NP-HPLC behave close to the number of their monosaccharide units (for example, 2ACF with 10 monosaccharides elutes as Glc9.1 and Man5GlcNAc2 as Glc7.4) compared to the recently reported AB method in which 2ACF eluted as Glc7.45 and Man5GlcNAc2 as Glc6.13 (Guile et al., 1996).

The ovalbumin (chicken) oligosaccharides released by endo H can be separated into eight peaks on an amide column (Figure 6) similar to HPAEC-PAD. However, MALDI-TOF analysis shows that it contains about 15 peaks (Figure 7) (more than 15 peaks in the PNGase released pool) and these mixtures can not be resolved in a single separation. On the other hand a single peak on MALDI-TOF may not represent a pure oligosaccharide species, since the hexoses and the N-acetylhexosamines have the same mass. In fact, this is the case in ovalbumin which contains Gln3Arg5Gln (GP-II-B) and Gln3Arg4Gln (GP-II-A) (Kobata, 1984) of the same mass and therefore gives rise to a single peak. It is of interest to note that in the AA mass map peaks corresponding to the underivatized oligosaccharides could not be detected, suggesting that perhaps the derivatization reaction has gone to completion under these conditions. Oligosaccharide map determination by MALDI-TOF (positive and negative modes) using an aliquot of the AA labeled mixture is a convenient way to harmonize the map obtained by either the polymeric-NH2 column or the amide column. Obviously, LC-MS (electrospray) analysis would be more appropriate for reconciliation of the fluorescent map.

In conclusion, two methods were developed for the analysis of oligosaccharides labeled with highly fluorescent anthranilic acid (1) normal phase anion exchange separation based on size, linkage and charge for mapping and (2) normal phase separation based on size, linkage, and overall structure for characterization. These methods provide specific and highly sensitive tools for the characterization of glycoproteins which may be available in limited amounts. Furthermore, the volatile solvent systems used in these methods were easy to remove from the collected fractions unlike the ammonium formate which was used in the recently reported methods (Guile et al., 1996; Townsend et al., 1996). Obviously, this is a desirable feature for characterization of oligosaccharides following separation by various techniques (e.g., treatment with enzymes, MS and NMR, etc.) without subjecting them to an additional desalting step. In contrast, the fractions collected from the HPAEC-PAD methods must be desalted for further characterization.

The mapping and the characterization technology described here has resolution which exceeds that obtained with the HPAEC-PAD methods. Furthermore, the problems associated with nonspecific detection, high pH, and high salt were eliminated. A large amount of information is obtained in a single run when the columns are calibrated with proper standards. In fact, well characterized standard glycoproteins used in this study for calibration of both the amine and the amide columns should facilitate in rapid interpretation/evaluation of the nature of glycosylation in unknown glycoproteins. The ability of these methods to separate the oligosaccharides differing by a single monosaccharide is useful in determining the loss of sugar residues upon treatment with glycosidases or the addition of sugars following transferase reactions. These novel technologies may be used for defining the glycosylation of proteins in a manner similar to the strategy proposed earlier (Anumula and Taylor, 1991).

Finally, the anthranilic acid is found to be a versatile label for studying glycosylation (monosaccharide composition (Anumula, 1997), oligosaccharide mapping and characterization) of proteins available in limited amounts. The derivatives are compatible with various biochemical, biophysical and separation techniques for structure determination. Furthermore, unlike the HPAEC-PAD, these methods operate under mild acid conditions (pH ~4.0) which are useful in isolation and characterization of the oligosaccharides modified with base labile groups (e.g., acetyl groups). Because the NP-HPAEC method separates structures on the basis of charge, size, linkage and overall structure, and the NP-HPLC method uses size, linkage, and overall structure, the combination of these two methods in addition to MALDI-TOF analysis affords a very powerful technology for analyzing a wide repertoire of oligosaccharide structures.

**Materials and methods**

**Oligosaccharides from glycoproteins**

Oligosaccharides were obtained from commercial (Sigma) glycoproteins of highest purity fetuin (calf serum), RNase B (bovine pancreas), ovalbumin (chicken egg), thyroglobulin (porcine), α-1-acid glycoprotein (human serum), and recombinant sCD4 and IgGs produced in CHO cells (SmithKline Beecham) either by hydrazinolysis using Glycoprep 1000 (Oxford GlycoSciences), or by digestions with PNGase F, endo F, and endo H as described previously (Anumula and Taylor, 1991). Oligosaccharides were further characterized by exoglycosidase digestions (neuraminidase, α-L-fucosidase and β-galactosidase, etc.) as described previously (Anumula and Taylor, 1991). In addition, expected mass for various oligosaccharide structures was confirmed by MALDI-TOF. Bovine fetuin oligosaccharide library, asialo bi-, tri-, and tetra-antennary oligosaccharide standards and positional isomers of high mannose type oligosaccharides of M₄Gln₂ (Man₇GlcNAc₂) and M₃Gln₂ (Man₅GlcNAc₂) were purchased from Oxford Glycosystems. Structures identified in this article were based on coelution of peaks with standards, susceptibility to exoglycosidases, and determination of molecular weight by MALDI-TOF.

For hydrazinolysis, initially the glycoprotein samples were made salt and excipient free by organic solvent precipitation. Approximately 1–2 mg of glycoprotein in 0.5 ml of 2% ammonium bicarbonate was precipitated by adding four volumes of ethanol-ethylacetate (1:1 with 1% acetic acid) mixture. After mixing the samples, they were allowed to sit for 10–15 min at room temperature and the precipitate was collected by centrifugation. The pellet was resuspended in 0.5 ml of ammonium bicarbonate and mixed vigorously on a Vortex mixer to disperse. The sample was reprecipitated as above and the entire procedure was repeated for a total of three centrifugations. After draining the solvents completely, the final pellet was dried on a vacuum centrifuge for at least 2 h for release by hydrazinolysis using GlycoPrep 1000 (Oxford Glycosystems).

For enzymatic release, the proteins were digested initially with trypsin and then with PNGase F or endo F/H as described earlier (Anumula and Taylor, 1991). For glycoproteins available in small amounts, the PNGase F digestions were carried out in SDS and nonionic detergent (Nonidet P-40) containing buffers as described (Tarentino and Plummer, 1994). An aliquot of the digest (<50 μl) was used as such for derivatization with anthranilic acid.
(100 µl) and the excess label was separated prior to analysis by a sample cleanup procedure using Nylon syringe filter as described in the derivatization section.

Endo and exo glycosidase digestions were carried out as described for the oligosaccharides derivatized with and without AA as described previously (Anumula and Taylor, 1991). In digestion with AA labeled oligosaccharides, the proteins (enzyme and bovine serum albumin etc.) were precipitated with two volumes of ethanol-ethylacetate mixture as described above and an aliquot from the supernatant was injected for analysis. Also, enzyme digests were injected following dilution with solvent A and separation of the proteins, if precipitated, by centrifugation.

**Derivatization of oligosaccharides with anthranilic acid (AA)**

Derivatization of oligosaccharides with anthranilic acid (2-amino-benzoic acid) was carried out as described earlier (Anumula, 1994). A solution of 4% sodium acetate 3H2O (w/v) and 2% boric acid (w/v) in methanol was prepared first. This solution may be stored at room temperature for several months. The derivatization reagent was prepared fresh by dissolving 30 mg of anthranilic acid (Aldrich) and 20–30 mg of sodium cyanoborohydride (Aldrich) in 1.0 ml of the above methanol-sodium acetate-borate solution. Glycoprotein derived oligosaccharides (<3 nmol in 20–50 µl of water) were mixed with 0.1 ml of the anthranilic acid (AA) reagent solution in 1.6 ml polypropylene screw cap freeze vials with “O” rings (Sigma) and capped tightly. The vials were heated at 80°C in an oven or heating block (Reacti-Therm, Pierce) typically for <60 min. After cooling the vials to ambient temperature, the samples were diluted with 1.0 ml of 95% acetonitrile-water solution and mixed vigorously on a Vortex mixer.

Derivatized oligosaccharides were purified by normal phase chromatographic conditions using three different matrices or formats. In general, for sample cleanup, oligosaccharides were allowed to bind to the matrix in a 95% acetonitrile-water solution and then eluted with 10–20% acetonitrile in water. The derivatized oligosaccharides were purified using nylon syringe filter disks (0.45 µm, 25 mm dia., Nylon Acrodisc, Gelman Sciences). A 5 ml disposable plastic syringe was cut at the 2 ml mark to use it as a funnel. The nozzle was plugged tightly with glass wool in order to prevent air locks during filtration. The filter was primed with 95% acetonitrile-water using a syringe and then the pre-cut syringe was attached to the filter. The filter was rinsed with 2 × 1 ml of 95% acetonitrile using gravity flow method. Derivatized samples were diluted with 1 ml of 95% acetonitrile-water and mixed. The diluted reaction mixture was transferred as such without any centrifugations onto the filter and allowed to flow through. The filter was washed with 2 × 1 ml of 95% acetonitrile-water or 1–2 ml in small aliquots. Both flow through and washes were discarded, and the bound oligosaccharides were eluted with 2 × 0.5 ml (small aliquots) of 20% acetonitrile in water solution into a clean test tube or autosampler vial and mixed. An aliquot of 25–100 µl was used for analysis by normal phase high performance anion-exchange chromatography (NP-HPAEC) and normal phase high performance chromatography (NP-HPLC).

The derivatized oligosaccharides can also be purified using Oasis HLB cartridges (1 ml, Waters). The cartridge was placed in a suitable test tube or holder and rinsed with 2 × 1 ml of 95% acetonitrile in water using gravity flow method. The reaction mixture was diluted with 1.0 ml of 95% acetonitrile in water and mixed. Purification was carried out as described above. Similarly, prepacked 1-ml Spe-ed Amide-2 columns (Applied Separations, Allentown, PA) were used. The column matrices (Spe-ed Amide-2 and Polyamid 6 S, Riedel-de Haen AG, Germany) are available as bulk powders and therefore can be packed into columns for large scale preparations.

**Normal phase high performance anion-exchange chromatography (NP-HPAEC) for oligosaccharide mapping**

Purified AA labeled oligosaccharides were separated on an amine (NH2) bonded polymeric column (Polymer-NH2, 5 µm, 0.46 × 25 cm, cat. # S6403, PVA copolymer based, Advanced Separation Technologies (Astec), Whippany, NJ. Asahipak-NH2P (Shodex, Phenomenex, Torrance, CA) can be equally substituted in these separations. Separations were carried out at 50°C using a flow rate of 1.0 ml/min. Solvent system A consisted of 2% acetic acid and 1% tetrahydrofuran (inhibited) in acetonitrile and solvent system B consisted of 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran (inhibited) in water. The following gradient for mapping was used for optimum resolution of the oligosaccharides present in bovine fetuin: 30% B isotropic for 2 min followed by a linear increase to 95% B over 80 min and was held at 95% B for additional 15 min. The column was equilibrated with initial conditions for 15 min prior to the next injection.

For separation of neutral oligosaccharide (high mannose and complex) mixtures, a second gradient was developed which consisted of initial 30% B increased linearly to 50% B over 60 min. Column was washed with 95% B for 15 min and equilibrated with initial conditions for 15 min before the next injection.

HPLC systems consisted of either System Gold 127 S (Beckman) or HP1090 system (Hewlett Packard). The column was heated using a column heater (LC-22 temperature controller, Bioanalytcal systems). A precolumn filter with 0.2-µm stainless steel frit was installed between the autosampler and the column. The anthranilic acid derivatives were detected using 230 nm excitation and 425 nm emission wavelengths with either HP 1046A (Hewlett Packard) (Anumula, 1994) or 360 nm and 425 nm with Waters 474 or Jasco FP 920 HPLC fluorescence detectors. The following settings were used on the Waters/Jasco detector: gain, 100 (1000 max); band width 40; and response, slow. Under these conditions the Jasco/Waters detector was more sensitive with less baseline drifts and less noisy than the Hewlett Packard detector. Most of the data presented here was generated using the HP fluorescence detector. Data were collected on a Waters 860 Expert Ease chromatographic data management system for Vax network version 3.2.

**Normal phase high performance liquid chromatography (NP-HPLC) for neutral oligosaccharide characterization**

For separation of the neutral (asialo complex and high mannose type) oligosaccharides, a TSK gel-Amide-80 (0.46 × 25 cm) column was used. Separations were carried out at ambient temperature using 1.0 ml/min. Solvent A consisted of 0.1% acetic acid in acetonitrile and solvent B consisted of 0.2% acetic acid-0.2% triethylamine in water. Separation of the AA labeled oligosaccharides was carried out using 28% B isotropic for 20 min followed by a linear increase to 45% B at 80 min. Column was washed for 10 min with 95% B and equilibrated with 28% B for 15 min prior to next injection. Samples containing excess water were adjusted to >50% acetonitrile prior to injection of >50 µl aliquots.
Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS)

An aliquot of native and derivatized oligosaccharides (10–100 μl) were mixed with 1–4 μl of 2,5-dihydroxybenzoic acid (DHB, 0.1 M in methanol–water (9:1), Hewlett Packard) solution and dried using a vacuum centrifuge. Samples were dissolved in 3 μl of 80% methanol in water and 0.7 μl were applied onto a gold target. After drying the samples on the targets at room temperature, they were analyzed using a LD-TOF system (G2025, Hewlett Packard) with nitrogen laser (337 nm) operating in either positive or negative ion mode at an accelerating potential of 28 kV. Data was collected at a setting of 80–120 (180 maximum) for the laser power (3–9 μJ) and about 20–50 shots were averaged. Peptide standard mixture (G2025A, Hewlett Packard) consisting of oxytocin (1007.2 Da), arginine-8-vasopressin (1084.25 Da), angiotensin 1 (1281.49 Da), somatostatin (1637.9 Da), ANP (3160.66 Da), recombinant insulin (5807.7 Da), and recombinant hirudin (6963.52 Da) was used for external calibration.

Acknowledgments

We thank Ms. Mary Beth Ebert for critical reading of the manuscript.

Abbreviations

NP-HPAEC, normal phase high performance anion exchange chromatography; NP-HPLC, normal phase high performance liquid chromatography; AA, anthranilic acid or 2-amino benzoic acid; AB, 2-amino benzenamide; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; MALDI-TOF MS, matrix-assisted laser desorption time of flight mass spectrometry; PNGase F, peptide N-glycosidase from Flavobacterium meningosepticum; Endo H/F, endo-β-N-acetyl glucosaminidase H/F; α1-AGP, α1-acid glycoprotein.

References