Reversed-phase ion-pairing liquid chromatography/ion trap mass spectrometry for the analysis of negatively charged, derivatized glycans

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The significant complexity, similar polarity and lack of ionizable sites make the analysis of glycans an analytical challenge. These compounds are often derivatized and separated by normal-phase high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) followed by UV or fluorescence detection. Due to widespread use of reversed-phase chromatography coupled to electrospray mass spectrometry as an analytical tool, our laboratory has developed this methodology for the analysis of glycans derivatized with a negatively charged tag, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). It is possible to exploit the ability of this negatively charged tag to interact with a mobile phase ion-pairing reagent, allowing retention on a reversed-phase C₁₈ column for subsequent on-line UV or MS analysis. ANTS-derivatized samples, including a maltooligosaccharide ladder and glycans released from bovine ribonuclease B, bovine fetuin, and chicken ovalbumin, were analyzed using this method. In addition to reversed-phase retention, ribonuclease B and ovalbumin derivatives displayed highly desirable isomeric separation. With the use of mass spectrometric detection for glycan identity, this allowed relative quantitation of individual components. Copyright © 2003 John Wiley & Sons, Ltd.

With the growing popularity of proteomic and glycoprotein research, the development of simple and efficient separation techniques for glycan analysis is of significant interest. Glycans themselves present an interesting analytical challenge due to their complexity and similar polarity. Almost all modes of separation have been explored for glycan analysis, including normal-phase high-performance liquid chromatography (HPLC),¹⁻³ reversed-phase HPLC,⁴⁻⁷ anion-exchange HPLC,⁸⁻⁹ capillary electrophoresis,¹⁰⁻¹³ micellar capillary electrophoresis,¹⁴,¹⁵ gel electrophoresis,¹⁶,¹⁷ and others.¹⁸⁻²⁰ Due to the hydrophilic nature of sugars, normal-phase HPLC has become a popular choice. It provides structural information due to the fact that elution positions and mass can be directly correlated²¹ and that individual monosaccharide residues in specific linkages provide consistent retention increments. Unfortunately, most analytical laboratories employ reversed-phase chromatographic methods on a more regular basis and the above correlations do not apply²¹—even the misleading term ‘normal-phase’ is slowly being replaced by ‘hydrophilic interaction’.²² Although reversed-phase chromatography may present a more desirable approach, neutral underivatized glycans display no retention on the most popular C₁₈ stationary phases.

Another interesting area of research has included the use of ion-pairing chromatography, which is aimed at increasing the reversed-phase chromatographic retention of charged species. Most research employing this technique has been aimed at natively charged, underivatized glycans such as sulfated or N-acetylated glycans from heparin.²³⁻²⁵ Although researchers have been successful in this area, many glycans are neutral and not amenable to ion pairing in their native state. Although previous work has been performed on ion pairing of derivatized polysaccharides with LIF detection,²⁶ this area is relatively under explored. With the latter technique, a suitable tag is not necessarily hydrophobic, but charged. Glycans derivatized with such a tag would, therefore, be amenable to both normal- and reversed-phase chromatography, as well as electrophoretic methods. In addition, reversed-phase chromatography of charged species presents an ideal system for use with mass spectrometric detection.

Due to the complexity of many glycans released from glycoproteins, mass spectrometry has allowed the acquisition of detailed structural information,²⁷⁻²⁹ something not possible with UV or LIF detection. The ion trap mass spectrometer has been especially useful for obtaining structural information on glycans, owing to its ability for multiple-stage MS/MS analysis.³⁰⁻³² Although electrospray mass spectrometry is a desirable detection method, several requirements must be met: buffer additives must be volatile and/or present in low concentrations, and the analyte must have a readily ionizable or charged group. Reversed-phase

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ion-pairing chromatography of derivatized, charged glycans is well suited for this method.

In this paper we report on the development of a method which employs derivatization with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and ion-pairing reversed-phase chromatography coupled to ion trap mass spectrometric detection. For several glycans, this technique allowed the separation of isomeric species—a highly desirable aspect of any methodology aiming to profile glycan pools. ANTS derivatives were also shown to have increased sensitivity over native glycans, and easily interpretable MS fragmentation patterns. These properties provide a complementary technique to capillary electrophoresis\(^3\) and normal-phase chromatography\(^3\) for ANTS-derivatized glycans.

**EXPERIMENTAL**

**Materials**

Standard oligosaccharides, ANTS, sodium cyanoborohydride and triethylammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycans from bovine fetuin, bovine ribonuclease B and chicken ovalbumin were released from their native glycoproteins by large-scale manual hydrazinolysis followed by reacetylation.\(^3\) Low-volume dialysis cartridges were purchased from The Nest Group (Southborough, MA, USA).

**Sample preparation**

ANTS derivatization was performed in-house. This reaction methodology was adapted from a previously published procedure\(^3\) and ANTS was used in a 10-fold excess. Standard oligosaccharides were prepared as 0.01 M solutions in water. 100–300 \(\mu\)g of released glycans were prepared as 1–15 mg/mL solutions in water. After addition of ANTS and sodium cyanoborohydride, the solutions were held at 37\(^\circ\)C for 18–20 h. Due to the large excess of the ANTS reagent, products were placed directly in 1000 molecular-weight cutoff dialysis cartridges (The Nest Group) for removal of ANTS and other low molecular weight species. Cartridges were allowed to float overnight in water. Solutions were injected directly into the HPLC/MS system following dialysis.

**HPLC**

HPLC was performed using a HP 1100 system (Agilent Technologies, Wilmington, DE, USA) equipped with a diode array detector. A Symmetry C18 column (1.0 mm i.d. \(\times\) 15 cm length; Waters, Milford, MA, USA) was used at a flow rate of 0.050 mL/min. The mobile phases consisted of water (A) and methanol (B), both containing 10 mM triethylammonium acetate (pH 7), using a gradient of 1–5% B from 0–5 min (unless otherwise noted). The gradient was then held at 5% B for an additional 30 min. Following analysis, the column was re-equilibrated at 1% B for the next run. UV spectra were acquired at 220, 262 and 354 nm.

**Mass spectrometry**

On-line mass spectrometry was performed using an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with the standard electrospray source. All spectra were acquired in the negative ion mode.

The ion-pairing reagent triethylammonium acetate was chosen because of its volatile nature and compatibility with electrospray MS detection at low concentrations. An HPLC/UV chromatogram of the same sample as that shown in Fig. 1(a), analyzed with 10 mM triethylammonium acetate, is shown in Fig. 1(b). All reaction mixtures also contained an unknown hydrophilic polymeric species (as seen in Fig. 1(a)). The peak produced by this compound does not exhibit a shift in retention after the addition of triethylammonium acetate to the solvent system, providing a convenient reference compound for evaluation of separation profile. Periodically, this column was tested with a standard mixture (20 \(\mu\)L/mL acetone, 0.5 mg/mL acenaphthalene) using an isocratic acetonitrile/water mobile phase to ensure that the column integrity remained intact. It should be noted that the column should be re-equilibrated at 1% B for the next run. UV spectra were acquired at 220, 262 and 354 nm.

**RESULTS AND DISCUSSION**

**Method development**

Due to the high polarity of sugars and many derivatizing reagents used (including ANTS), reversed-phase HPLC without solvent additives is not a suitable method for the separation of these compounds.\(^3\) The retention behavior of an ANTS-labeled oligosaccharide without the use of an ion-pairing reagent is shown in Fig. 1(a), illustrating the lack of retention of these species. ANTS-derivatized compounds have three absorbance maxima at 220, 262 and 354 nm. Although these species have the highest absorbance at 220 nm, the detection of both 220 and 262 nm allows ANTS compounds to be distinguished from non-ANTS-containing species. Since ANTS is a natively charged species (above pH \(~\)2) and UV absorbent, it presents a promising candidate for ion-pairing reversed-phase HPLC with UV or MS detection. The ion-pairing reagent triethylammonium acetate was chosen because of its volatile nature and compatibility with electrospray MS detection at low concentrations. An HPLC/UV chromatogram of the same sample as that shown in Fig. 1(a), analyzed with 10 mM triethylammonium acetate, is shown in Fig. 1(b). All reaction mixtures also contained an unknown hydrophilic polymeric species (as seen in Fig. 1(a)). The peak produced by this compound does not exhibit a shift in retention after the addition of triethylammonium acetate to the solvent system, providing a convenient reference compound for evaluation of separation profile. Periodically, this column was tested with a standard mixture (20 \(\mu\)L/mL acetone, 0.5 mg/mL acenaphthalene) using an isocratic acetonitrile/water mobile phase to ensure that the column integrity remained intact. It should be noted that the column should be re-equilibrated at 1% B for the next run. UV spectra were acquired at 220, 262 and 354 nm.

**Figure 1.** HPLC/UV chromatograms of an ANTS-(Glc)\(_7\) reaction mixture using (a) water/methanol and (b) water/methanol + 10 mM triethylammonium acetate.
be flushed thoroughly with an acetonitrile/water mix to remove ion-pairing reagent before being used for other applications.

On-line MS detection was achieved by connecting the UV detector to the electrospray source using a 75 μm inner diameter fused-silica capillary. Using ion-pairing HPLC, the MS response for ANTS-(Glc)7 was calculated to be approximately 20 × greater than underivatized (Glc)7 (based on reaction efficiency), and the MS detection limit (S/N = 3) for ANTS-(Glc)7 was approximately 300 pg (200 fmol) on-column. For quantitative purposes, it should be noted that distinct sugars react with ANTS at the same efficiency and exhibit very small differences (<8%) in relative ionization efficiency.

**HPLC/UV/MS of an ANTS-labeled maltoligosaccharide ladder**

A base peak chromatogram of a derivatized maltoligosaccharide ladder ([Glc]3–[Glc]10) is shown in Fig. 2, with a corresponding UV chromatogram displayed in the inset. The ladder was separated using a gradient of 1–5% B from 0–5 min (held at 5% B for the remainder of the analysis). Ladder components ([Glc]3–[Glc]10) eluted in a window from 21–28 min, and the peak produced by (Glc)7 corresponded to approx. 35,000 theoretical plates. Resolution between all components was >1.5. By comparison, underivatized ladder components eluted between 2 and 6 min using the identical conditions, exhibited poor resolution and less than 1000 theoretical plates. MS² data were collected in the data-dependent scanning mode of the mass spectrometer, wherein the instrument toggles between full-scan and MS/MS on the most predominant peak. During the analysis of the maltoligosaccharide ladder, the mass spectrometer was able to acquire fragmentation data on all components. MS/MS data acquired for the (Glc)7 ion at m/z 759.4 are shown in Fig. 3, wherein the Y-ion series predominates (Domon-Costello nomenclature). This spectrum is almost identical to the spectrum of ANTS-(Glc)7 acquired using capillary electrophoresis coupled to ion trap MS.

**HPLC/MS of ANTS-labeled bovine ribonuclease B glycans**

The ANTS ion-pairing method was tested next on a naturally derived sample, derivatized high-mannose glycans (GlcNAc)2(Man)5–9, designated here as Manₙ from ribonuclease B. These glycans, including isomers with different branching patterns, were separated with baseline resolution (Fig. 4). Approximately 290 μg total glycans (approx. 200 pmol) were injected on-column, and an HPLC gradient of 1–20% B from 0–40 min was used. Glycans contributing to over 1% of the total glycan pool were detected by MS, and consisted of Man₅, Man₆, three Man₇ isomers, two Man₈ isomers and Man₉. Published values for these glycans obtained by NMR spectroscopy are listed in Table 1.
Quantitative data obtained herein by LC/MS are remarkably consistent with these results. One Man$_8$ isomer (detected by NMR with a value of 0.4% of total glycans) was not detected by MS. In addition, a very small MS signal consistent with the structure of labeled Man$_4$ was found, co-eluting with that of Man$_5$. These data are consistent with results obtained by Townsend and co-workers, wherein two small Man$_4$ peaks and only two Man$_8$ isomers were detected by normal-phase HPLC using 2-AB-labeled glycans and fluorescence detection. MS$^n$ was also performed on these glycans in the data-dependent scanning mode. MS$^2$ and MS$^3$ spectra for Man$_5$ are shown in Figs. 5(a) and 5(b), and illustrate the ANTS-HexNAc core resulting from subsequent losses of all five hexoses and one HexNAc monomer. Additional peaks present in these spectra occurred reproducibly, and are suspected to be rearrangement products. Such rearrangements, termed 'internal residue losses', have been previously reported in the positive ion spectra of labeled carbohydrates. Although the identification of specific hexose monomers and linkage information were not possible, MS data provides valuable information on glycan components.

### Table 1. Comparison of published and experimental values for percent of total glycans in bovine ribonuclease B

<table>
<thead>
<tr>
<th>m/z detected</th>
<th>Avg. m/z calculated for [M–2H]$^+$ ion</th>
<th>Identification based on m/z$^a$</th>
<th>Published value$^b$</th>
<th>Experimental value$^c$</th>
<th>Standard deviation$^d$</th>
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<td>800.4</td>
<td>800.2</td>
<td>GlcNAc$_2$Man$_5$</td>
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<td>51.9</td>
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<td>GlcNAc$_2$Man$_6$</td>
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<td>32.1</td>
<td>2.4</td>
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<td>962.5</td>
<td>962.4</td>
<td>GlcNAc$_2$Man$_7$ (1)</td>
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<td>2.3</td>
<td>0.5</td>
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<td>962.4</td>
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<td>0.4</td>
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<td>0.5</td>
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<td>GlcNAc$_2$Man$_8$ (3)</td>
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<td>0.5</td>
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<td>GlcNAc$_2$Man$_9$</td>
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<td>1.6</td>
<td>0.1</td>
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</table>

$^a$Identifications include ANTS, parentheses indicate a specific isomer.
$^b$NMR data obtained from Ref. 37.
$^c$Values based on the area under extracted mass MS peaks for specific isomers.
$^d$Standard deviation of experimental results, n = 3.
ND = not detected.

HPLC/MS of ANTS-labeled chicken ovalbumin glycans

Glycans cleaved from ovalbumin glycoproteins consist of over 25 distinct, neutral carbohydrate species. The base peak total ion chromatogram (TIC) of these glycans is illustrated in Fig. 6 and masses obtained correlate with structures previously reported to exist in the ovalbumin glycan pool (note: this sample may contain some glycans from other chicken egg white glycoproteins). Although MS/MS data on selected ions yielded a Y-ion series, this information did not give insight into specific hexose monomers. Relative abundances for various glycans can be seen in the base peak chromatogram, and can be quantified (relative to % of each component in the mixture) using the extracted ion chromatograms for each mass. Extracted ion chromatograms for selected ovalbumin components are shown in Fig. 7, wherein isomeric separation is achieved for several components. The peak at 32.04 min represents an unidentified compound with m/z 426. Although it is reproducible upon repeat analysis, it does not appear to be a carbohydrate. The relative quantitation values for ovalbumin glycans (the values represent the sum of all isomers detected) obtained herein by LC/MS are listed in Table 2. However, we have been unable to obtain corresponding NMR data to assess the general applicability of these comparisons.

HPLC/MS of ANTS-labeled bovine fetuin glycans

N-Linked glycans from bovine fetuin present a more complex mixture than ribonuclease B glycans, due to larger molecular
weight species, more isomeric components, and the presence of sialylated glycans. NMR analyses have shown at least 23 distinct biantennary (B) and triantennary (T) glycans, all containing at least one sialic acid residue (S). The large amount of isomeric species present is due to differences in sialic acid and galactose linkage, as well as the presence of sialic acid on different antennae. Although the underivatized form of these species will be retained on a reversed-phase column using triethylammonium acetate, it is important for a method used for glycan profiling to separate and detect both neutral and natively charged species.

Fetuin glycans were derivatized with ANTS, and the HPLC/MS base peak chromatogram is shown in Fig. 8. Approximately 1.4 μg total glycans were injected on-column, and an HPLC gradient of 1–20% B from 0–40 min was used.

Published values obtained by NMR indicate that fetuin glycans consist of three BS₁ isomers, four BS₂ isomers, three T₁ isomers, six TS₂ isomers, five TS₃ isomers and two TS₄ isomers, where B = biantennary glycan, T = triantennary glycan and S = sialic acid. Although full-scan MS data are not able to distinguish isomeric species (although certain HPLC peaks containing multiple isomeric species were observed to be quite broad and/or containing ‘shoulders’), the molecular weight allows identification of the glycan type. Glycans identified by LC/MS and experimental values for the % of each glycan in the total glycan pool (based on the area

### Table 2. Experimental values for percent of total glycans in chicken ovalbumin

| m/z detected | Avg. m/z calculated for [M–2H]⁺ ion | Identification based on m/z | Experimental value% of glycan pool | Standard deviation%
<table>
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<td>H₉N₉</td>
<td>3.5</td>
<td>0.60</td>
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</table>

*Identifications include ANTS.

This work was supported by NIH Grant No. GM54682. Published values obtained by NMR indicate that fetuin glycans consist of three BS₁ isomers, four BS₂ isomers, three T₁ isomers, six TS₂ isomers, five TS₃ isomers and two TS₄ isomers, where B = biantennary glycan, T = triantennary glycan and S = sialic acid. Although full-scan MS data are not able to distinguish isomeric species (although certain HPLC peaks containing multiple isomeric species were observed to be quite broad and/or containing ‘shoulders’), the molecular weight allows identification of the glycan type. Glycans identified by LC/MS and experimental values for the % of each glycan in the total glycan pool (based on the area

under the extracted mass peaks) are listed in Table 3. As in the case of ribonuclease B glycans, quantitative results are generally consistent with NMR data.

Further structural information can then be obtained by performing additional MS/MS scans. MS³ and MS⁵ data obtained from the TS₁ glycans eluting at approximately 28 min (Fig. 8) are shown in Figs. 9(a) and 9(b). Multiple-stage mass spectrometric analyses were performed on fetuin glycans using predetermined m/z values. It should be noted that multiple stages of MS must be performed on sialylated glycans in order to obtain structural information because the predominant fragmentation is due to non-specific losses of

**Table 3.** Comparison of published and experimental values for percent of total glycans in bovine fetuin

<table>
<thead>
<tr>
<th>m/z detected</th>
<th>Charge state</th>
<th>Avg. m/z calculated</th>
<th>Identification based on m/z</th>
<th>Published valueb</th>
<th>% of glycan pool</th>
<th>Experimental valuec</th>
<th>% of glycan pool</th>
<th>Standard deviationd</th>
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<tbody>
<tr>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>BS₁</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>1294.6</td>
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<tr>
<td>887.8</td>
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<td>—</td>
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aIdentifications include ANTS.
bNMR data obtained from Ref. 45.
cValues based on the area under extracted mass MS peaks for all isomers.
dStandard deviation of experimental results, n = 3.
ND = not detected, B = biantennary, T = triantennary, S = sialic acid.

**Figure 8.** HPLC/MS base peak chromatogram of ANTS-derivatized fetuin glycans. Gradient: 1–20% B from 0–40 min.

**Figure 9.** (a) HPLC/MS³ spectrum of ANTS-derivatized fetuin TS₁ glycans (m/z 887.6 → 790.6) and (b) HPLC/MS⁵ spectrum of ANTS-derivatized fetuin TS₁ glycans (m/z 887.6 → 790.6 → 736.6 → 682.6). Hex: hexose, Neu5Ac: sialic acid. Identifications are based on losses from the ion fragmented in the last stage of MS (shown in boxed insets). Labeled fragments are triply charged. Inset: structure of ANTS TS₁ fragment. □ = GlcNAc, ● = mannose, ○ = galactose.
the sialic acid residues. For example, MS/MS data on TS₁ glycans simply exhibit sialic acid loss (one predominant peak at m/z 791, triply charged), and MS³ data on this ion are required to obtain information on the core structure.

CONCLUSIONS

ANTS-derivatized glycans have previously been separated using both normal-phase HPLC/MS³ and CE/MS;33,46 reversed-phase HPLC/MS detection provides a complementary method to these analyses. Derivatization with ANTS is a relatively simple, fast, and inexpensive procedure and reversed-phase C₁₈ columns are standard in many analytical laboratories. Isomeric separation of ribonuclease B and ovalbumin glycans was achieved herein by reversed-phase methodology. Although this technique can be used with UV or LIF detection, mass spectrometry provides valuable information on glycan identification and the potential for quantitative evaluation of glycan pools.

REFERENCES