Analysis of 2-Aminobenzoic Acid-Labeled Monosaccharides and Glycoprotein-Derived Oligosaccharides by Online Cleanup Liquid Chromatography in the Reversed-Phase and Hydrophilic Interaction Liquid Chromatography Modes

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Abstract
Quantitative analysis of monosaccharides or glycoprotein glycans by high-performance liquid chromatography (HPLC) often involves labeling of the saccharide aldehyde groups with fluorescent tags to enhance sensitivity and selectivity. However, the methods required to remove the large excess of labeling reagents from the reaction mixture are time-consuming. Furthermore, these methods often hinder the quantitative analysis of the labeled samples. Here, we developed an online sample cleanup procedure for HPLC analysis of 2-aminobenzoic acid (2-AA)-labeled monosaccharides or oligosaccharides using a ten-port valve and mini columns. An online purification system using a combination of short HLB columns with the valve was proposed for the analysis of 2-AA-labeled monosaccharides utilizing reversed-phase modes. In the analysis of 2-AA-labeled glycans derived from glycoproteins, a short CN column with the valve was proposed utilizing hydrophilic interaction liquid chromatography (HILIC) modes. Optimized conditions enabled the direct injection of the diluted labeling reaction mixture into the chromatographic system without any prior removal of the excess labeling reagents. These methods were successfully applied to the analysis of various monosaccharides and N-linked glycans released from specific glycoproteins.

Keywords: 2-Aminobenzoic acid; Monosaccharides; Glycoprotein-derived oligosaccharides; Online cleanup liquid chromatography

1. Introduction
Saccharides are one of the most abundant biological molecules that are widely distributed in nature. Most saccharides are neutral and highly hydrophilic, and do not possess chromophoric or fluorimetric properties [1-3]. Therefore, numerous fluorescent amines have been developed to derivatize saccharides by reductive amination and improve their sensitivity and resolution in both HPLC and capillary electrophoresis (CE) analyses [4-8]. Introduction of hydrophobic tags onto saccharides also enhances the sensitivity of LC-MS applications [9]. In these labeling reactions, a saccharide is quantitatively converted to a Schiff’s base in the presence of a large excess of an aromatic amine and a hydride reagent. However, the presence of excess reagents often impairs the reliable analysis of saccharides by LC or CE. Therefore, the excess reagents need to be removed from the reaction mixture before analysis. Solvent extraction is the most convenient way to remove excess reagents from a reaction mixture. However, this type of extraction is limited to hydrophobic tags, which often impairs the resolution on reversed-phase LC because of strong adsorption of fluorescent groups onto the stationary phases. Gel filtration is the most effective way to separate oligosaccharides labeled with hydrophilic tags from the reaction matrix [10]. Paper chromatography has been utilized for the purification of labeled glycans [11]. The paper chromatography technique has largely been superseded by solid phase extraction (SPE) using hydrophilic phases such as glass, cellulose, cyano-silica [12,13] and polyamide S6 or hydrophobic phases using nylon filter disks [14], carbon [15] and HLB cartridges [16]. All of these procedures are time-consuming and result in a partial loss of the saccharide derivatives.

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which impairs the quantitative analysis of low molecular weight saccharides. For example, Pabst et al. reported that the solid phase purification of aminobenzoic acid-labeled monosaccharides resulted in a 20 to 25% loss of the derivative during manual sample preparation [17]. These problems may be resolved by incorporation of preparation steps into the separation systems. 2-AA has been widely used to label N-linked oligosaccharides. These 2-AA-labeled N-linked oligosaccharides have been analyzed by several techniques including mass spectrometry, HPLC and CE. The N-linked oligosaccharides were separated based on their size or the degree of polymerization (d.p.) utilizing a HILIC mode of separation with an Amide column [18]. The fine separation was based on the differences between the linkages and the component monosaccharides of the glycans on an ODS column [19]. This fine separation of 2-AA-labeled oligosaccharides according to the differences in the glycan structures is likely due to the highly hydrophilic nature of 2-AA. However, the hydrophilicity of 2-AA makes its removal from the reaction mixture difficult. Excess 2-AA is often removed by a combination of extraction and chromatographic methods, but it took about 2-3 hours to remove excess reagents in these processes and may impair the quantitative recovery of derivatives.

An online purification system using a SPE column with a multi-port valve may be a more convenient way to remove excess reagents from the reaction mixture [20]. The analytes in a sample solution entrapped in an extraction column are delivered to an analytical column by changing valve positions and the analytes are quantitatively recovered by eluting with extractable solvent. Thus, band broadening of the elution process could be minimized by a back flush elution technique [21,22].

Here, we describe an approach for the entrapment of N-linked oligosaccharides and monosaccharides utilizing a HILIC mode analysis with an Amide column and reversed-phase HPLC, respectively. 2-AA-labeled oligosaccharides in the reaction mixture were separated from excess 2-AA by a CN column and entrapped on a HILIC column. In addition, 2-AA-labeled monosaccharides in the reaction mixture were separated from excess 2-AA by a HLB column and entrapped on an ODS column. This novel approach allows for more rapid removal of excess reagents and enables the quantitative profiling of various types of oligosaccharides and monosaccharides.

2. Experimental

2.1. Reagents and materials

2-AA was obtained from Tokyo Chemical Industry (Tokyo, Japan). Neuraminidase from Arthrobacter ureafaciens, galactose (Gal), glucose (Glc), rhamnose (Rha), arabinose (Ara) and fucose (Fuc) were obtained from Nacalai Tesque (Kyoto, Japan). The Inertsil CN-3 guard column (1.5 x 20 mm, 5 µm) was purchased from GL Sciences (Tokyo, Japan). The Oasis HLB online column (2.1 x 20 mm, 5 µm) was purchased from Nihon Waters K. K. (Tokyo, Japan). Human transferrin, N-acetylgalactosaminoglycan (GlcNAc), N-acetylgalactosamine (GalNAc) and mannose (Man) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Fetal calf serum fetuin was obtained from Gibco (Grand Island, NY, USA). Pyridine-borane, dimethylformamide, acetonitrile, and methanol were obtained from Wako Pure Chemical Industries (Osaka, Japan). Peptide-N 4-(acetyl β-glucosaminyl)-asparagine amidase F (PNGase F, EC 3.5.1.52) was purchased from F. Hoffmann-La Roche (Mannheim, Germany). Other reagents and solvents were of the highest commercially available grade. Water was purified using a Milli-Q device purchased from Millipore (Burlington, MA, USA).

2.2. PNGase F digestion

The PNGase F digestion procedure has been described previously [23]. 2-Mercaptoethanol (2.4 µL) and 10% sodium dodecyl sulfate (24 µL) were added to a 210 µL aqueous solution containing a glycoprotein (0.1 mg) and the mixture was heated at 100°C for 5 min. After the addition of 24 µL of 10% octyl phenoxypolyethoxylethanol (Nonidet P-40), 29 µL of 1 M sodium phosphate buffer (pH 7.5), and PNGase F (2 U/2 µL), the solution was incubated overnight at 37°C. Next, the solution was heated at 100°C for 5 min, and the proteins were removed as a precipitate by centrifugation at 20,000×g for 10 min followed by the addition of 695 µL of ethanol. The supernatant was evaporated to dryness using a centrifugal evaporator.

2.3. Labeling of oligosaccharides with 2-AA

The labeling procedure has been described previously [18]. The dried mixture was dissolved in a 2-AA (200 µL) solution containing the following reagents: 2-AA (30 mg), sodium cyanoborohydride (30 mg) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid. The mixture was then incubated at 80°C for 60 min. After the addition of distilled water (200 µL), the column switching mode analysis was initiated and a 10 µL aliquot was injected into the column.

As a reference procedure for manual sample preparation, the mixture was applied to a Sephadex LH-20 column (1.0 cm i.d., 30 cm length) equilibrated with 50% aqueous MeOH. The earlier eluted fluorescent fractions were collected and evaporated to dryness.

2.4. Online cleanup HPLC for reversed-phase analysis of 2-AA-labeled monosaccharides

An online HPLC system was constructed from the following equipment: (1) three JASCO 890PU pumps for
gradient elution and delivery of the conditioning solvent; (2) a Rheodyne 7125 injector with a 10 μL loop; (3) a VALCO ten-port valve, with ports 9 and 10 connected thus functioning as an eight-port valve; and (4) a Shimadzu RF-50 fluorimetric detector set at 325 nm (excitation)/405 nm (emission). Line connections and valve settings for online cleanup of 2-AA-labeled monosaccharides are shown in Fig. 1. For reverse-phase mode analysis, the following two solvents were prepared: water/AcOH (2:98, v/v) as eluent A and phosphoric acid/hexadecyltrimethylammonium hydroxide/water (1:200:18000, v/v) as eluent B. Eluents A and B were also used as the conditioning solvents for the HLB column (Oasis HLB 5 μm, 4.6 mm I.D. x 20 mm). The flow rate was set at 0.6 mL/min for gradient elution. The columns were pre-equilibrated with 15% of eluent A (85% B) with the valve positioned to connect the analytical column, a fluorometric detector, an injector, and the HLB column in this order (“Sample loading” in Fig. 1). The 2-AA labeling reaction mixture from the injector was delivered to the HLB column with 15% A and the effluent containing salts was discarded for 4 min. Next, in order to deliver the 2-AA-labeled monosaccharides, the valve was changed to connect the outlet of the HLB column to the analytical column (“Analysis”). The valve was then switched to “Sample loading” position again to wash off the excess 2-AA strongly adsorbed onto the HLB column with the solvent delivered from the HLB column.

2.5. Online cleanup HPLC for HILIC mode analysis of 2-AA-labeled oligosaccharides

All the equipment was identical to that used for monosaccharide analysis, without using a pump for delivering the conditioning fluid. For HILIC mode analysis, the following two solvents were prepared: AcOH/acetonitrile (2:98, v/v) as eluent A and AcOH/trimethylamine/water (5:3:95, v/v) as eluent B. Eluents A and B were also used as the conditioning solvents for the CN-type entrapping column originally prepared as a guard column (Inertsil CN-3, 1.5 mm I.D. x 10 mm). The flow rate was set at 0.6 mL/min for gradient elution and conditioning. Sample entrapping was performed by the “Sample loading” configuration shown in Fig. 2. The sample injected into port 1 was delivered to an entrapping column (port 2). Excess 2-AA eluted from the column from port 8 was monitored by a fluorimetric detector connected to port 7. The effluent fluorescence disappeared after 5 min. Next, the valve setting was changed to the “Analysis” configuration shown in Fig. 2 to connect the inlet of the entrapping column (port 2) to an analytical column (Tosoh TSKgel Amide80 5 μm 4.6 mm I.D. x 250 mm; port 3) and to initiate the gradient elution. The entrapped 2-AA-labeled oligosaccharides were back flushed and separated on the Amide column by a gradient program (30% B to 95% B for 136 min), and the effluent from the column through port 6 was monitored using a fluorimetric detector connected to port 7.

2.6. Neuraminidase digestion of 2-AA-labeled oligosaccharides in solution

α2-3,6,8,9-Neuraminidase (25 mU, 2 mL) was added to 5
mL of 2-AA-labeled oligosaccharides F, and the reaction mixture was incubated for 12 h at 37°C.

3. Results and discussion

3.1. Optimization of reverse-mode separation for the analysis of 2-AA-labeled monosaccharides

When HLB is utilized as a reverse-phase sorbent, the salts present in the 2-AA labeling reaction mixture under water-rich conditions are not retained and the 2-AA-labeled saccharides and 2-AA are entrapped. The amount of 2-AA in the reaction mixture reached a maximum of 11 µmol. 2-AA is more strongly retained on the phase compared with 2-AA-labeled saccharides. Therefore, a heart-cut procedure was adopted for extracting 2-AA-labeled monosaccharides from unretained contaminants and strongly adsorbed 2-AA.

Fig. 1 shows the setup for reverse-phase separation of 2-AA-labeled monosaccharides. This configuration makes it possible to reverse the connection between the injector and the entrapping column, and between the analytical column and the fluorimetric detector. In the heart-cut mode, unretained contaminants passed through the HLB column and the valve was switched to connect the HLB column to the analytical column in order to deliver the 2-AA-labeled monosaccharides. After the 2-AA-labeled monosaccharides were introduced into the analytical column, the valve was switched to the original position again. In this position, the HLB column connected to the outlet of the analytical column enables elution of a large amount of 2-AA reagent, which is strongly retained on the HLB column. The elution of 2-AA was achieved by gradually increasing the acetonitrile in the solvent delivered from the analytical column.

In the heart-cut procedure, the time setting may affect the recovery of 2-AA-labeled monosaccharides and the removal of excess reagents. The void volume of the HLB slurry packed column is 0.2 mL and 2-AA-labeled monosaccharides eluted at 4.5–12 min; therefore, the connection time of the HLB column to the analytical ODS column was set between 4 min and 12 min.
of 2-AA-labeled N-linked oligosaccharides derived from bovine fetuin and human transferrin. Their online cleanup and manual sample preparation with HPLC on HILIC columns are shown in Fig. 4 and Fig. 5, respectively. Bovine fetuin contains a series of di-, tri-, and tetrasialylated triantennary glycans. The tetrasialylated glycans reportedly contain an unusual linkage sequence of Galβ1→3GlcNAc [25,26]. 2-AA-labeled oligosaccharides from fetuin appeared from 43 to 52.5 min in the online cleanup HILIC mode. The manual sample preparation is shown in Fig. 4a. We detected a high excess reagent peak at 6 min despite purification. On the other hand, as shown in Fig. 4b, the excess reagent peak was not detected and the glycan peak was relatively high.

Human transferrin mainly contains disialyl diantennary glycans and a small amount of mono- and difucosylated oligosaccharides [27]. 2-AA-labeled oligosaccharides from transferrin appeared from 44 to 48.5 min in the online cleanup HILIC mode. The manual sample preparation is shown in Fig. 5a. We detected a high excess reagent peak at 6 min despite purification. On the other hand, as shown in Fig. 5b, the excess reagent peak was not detected and the glycan peak was relatively high. The major diantennary glycan appeared at 45 min in the online cleanup HILIC mode. Next, we applied this online cleanup method for the analysis of asialo fetuin and transferrin obtained by neuraminidase digestion. Fig. 6 shows the separation of 2-AA-labeled asialo glycans derived from transferrin and fetuin. The major diantennary asialo glycan of transferrin appeared at 45 min in the online cleanup HILIC mode (Fig. 6a). Fig. 6b shows the chromatogram of asialo glycans derived from fetuin. The terminal N-acetylneuraminic acid was digested with neuraminidase and generated two major

3. Results and discussion

3.2. Online cleanup LC analysis of 2-AA-labeled monosaccharides

We applied the online cleanup procedure to the analyses of 2-AA-labeled monosaccharides. We chose Glc, Man, Gal, Rha, Ara, Fuc, GlcNAc and GalNAc, which are glycan constituents. Their online cleanup and manual sample preparation with HPLC on ODS columns are shown in Fig. 3. The monosaccharides were eluted in the order of GlcNAc, GalNAc, Glc, Man, Ara, Fuc and Rha on the ODS column. The result of the manual sample preparation is shown in Fig. 3b. The detected excess reagent peak was high despite purification and the monosaccharide peaks were relatively low. In addition, despite mixing 10 nmol of each monosaccharide, the peak area varied and the number of theoretical plates was also low. On the other hand, as shown in Fig. 3a, the monosaccharides at a concentration of 10 nmol were detected without the interference of contaminants. We found that online cleanup procedure improved the theoretical plate number of each peak. The monosaccharide resolution exhibited complementary results for this separation mode.

3.3. Online cleanup LC analysis of 2-AA-labeled oligosaccharides derived from acidic glycoproteins

We applied the online cleanup procedure to the analyses of 2-AA-labeled N-linked oligosaccharides derived from bovine fetuin and human transferrin. The analytical conditions were identical to those described for Fig. 4.
peaks. These results demonstrated the utility of online cleanup in the HILIC analysis mode.

4. Conclusions
An online purification system using a combination of short HLB columns with F valve was proposed for the analysis of 2-AA-labeled monosaccharides utilizing reversed-phase modes. In the analysis of 2-AA-labeled glycans derived from glycoproteins, a short CN column with the valve was proposed utilizing HILIC modes. The optimized conditions were applied to the direct injection analysis of the 2-AA labeling reaction mixture of glycoprotein glycans and monosaccharides. The described methods remove tedious solid phase extractions and are applicable to the analysis of various types of glycans. This method may also be applicable to other types of labeling reactions such as those utilizing 2-aminobenzamide, etc. The application of this methodology to other types of labeling reactions will be reported in the near future.

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