Journal of Chromatographic Science, Vol. 33, April 1995

Analysis of Fluorescently Labeled Sugars by Reversed-Phase Ion-Pairing High-Performance Liquid Chromatography

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Abstract

Reducing sugars, including monosaccharides, disaccharides, and a trisaccharide, are derivatized by reductive amination with 7-amino-1,3-naphthalene disulfonic acid. Reversed-phase ion-pairing high-performance liquid chromatography is then used to separate these visibly fluorescent, charged conjugates. Isocratic elution with triethylamine–acetic acid from a phenyl column, a C_{18} column, and C_{18} and phenyl columns in series gives good separations of a mixture of monosaccharides and a mixture of disaccharides and trisaccharides. Resolution of certain monosaccharides is enhanced by replacing triethylamine with a chiral amine and using gradient elution. Further enhancement of resolution is achieved by adding phenylboronic acid, an agent capable of complexing with the vicinal diol functionality present in many sugars. The trimethylamine–acetic acid eluant permits detection by either ultraviolet absorbance or fluorescence, and the addition of a chiral ion-pairing agent or a phenylboronic acid complexing agent necessitates fluorescence detection. A reversible Schiff base form of the fluorescent sugar conjugate is prepared; it is sufficiently stable to perform fractionations but sufficiently unstable to be converted to a fluorescent label and reducing sugar.

Introduction

High-performance liquid chromatography (HPLC) has been widely used for the analysis of sugars (1). Early studies relied on refractometry, although the introduction of high sensitivity, pulsed amperometric detectors (2) have largely replaced all other methods in the detection of underivatized sugars. Derivatization is not only important for detection of sugars but also for their separation. Because underivatized sugars have very little hydrophobicity and are often uncharged, there are limitations on the ways in which they can interact with HPLC columns. Hydrophobic derivatives of reducing sugars can be conveniently prepared by reductive amination with 2-aminopyridine (3). These derivatives are sufficiently hydrophobic to be separated by reversed-phase HPLC (4). Although the 2-aminopyridine derivatives are not visibly fluorescent, they can be easily detected using an on-line fluorescent detector. Pyridylaminated sugars, however, are often difficult to purify because of their hydrophobicity; for example, they interact with Sephadex (Pharmacia; Piscataway, NJ) and Bio-Gel (Bio-Rad; Hercules, CA) desalting columns making the removal of salts and excess reagents difficult (5).

Our laboratory focused its efforts on preparing sugar derivatives that are both visibly fluorescent and charged (6) for analysis and fractionation. Such derivatives are easily prepared by reductive amination with tags containing sulfonated aromatic amines such as 7-amino-1,3-naphthalenedisulfonic acid (AGA). Sugar–AGA conjugates can be separated by anion-exchange HPLC (6) as well as by other charge-based methods, such as polyacrylamide gel electrophoresis (PAGE) (6–8) lectin-affinity electrophoresis (8,9), and capillary electrophoresis (CE) (10–12). Removal of excess reagents and salts used in the preparation of these sugar fluorescent conjugates is easily performed using desalting columns because these charged conjugates do not interact with standard gel-based supports and can be easily detected using a hand-held ultraviolet lamp because of their bright visible fluorescence.

Ion-pairing, reversed-phase HPLC had been previously used by our laboratory to separate sulfated disaccharides derived from glycosaminoglycans (13). This study examines the use of ion-pairing HPLC to separate derivatized oligosaccharides containing sulfonate groups. This separation method can be used in both analytical and preparative modes. Reversible, fluorescent labeling of reducing sugar is also explored as a new approach for the fractionation and purification of sugars.

Experimental

Materials

D-Glucose (Glc), D-mannose (Man), D-galactose (Gal), L-arabinose (Ara), L-fucose (Fuc), L-rhamnose (Rham), D-xylose (Xyl), *N*-acetyl-D-glucosamine (GlcNAc), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), gentiobiose (β-D-Glc(1→6)-D-Glc), maltose (α-D-Glc-(1→4)-D-Glc), lactose (β-D-Gal-(1→4)-D-Glc), cellobiose (β-D-Glc-(1→4)-D-Glc), melibiose

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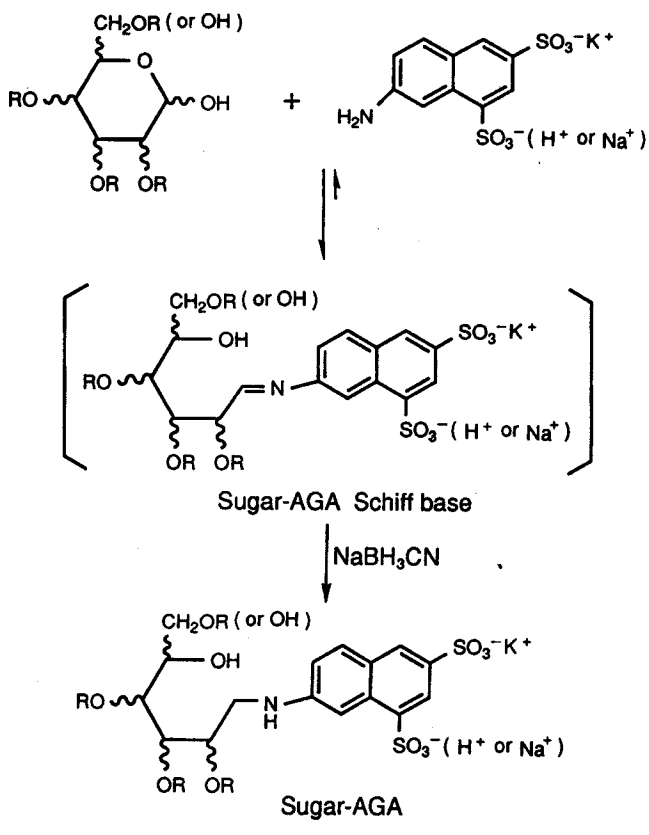
(α-D-Gal-(1→6)-D-Glc), and cellotriose (β-D-Glc-(1→4)β-D-Glc-(1→4)-D-Glc) were from Sigma Chemical (St. Louis, MO). Sodium cyanoborohydride, triethylamine, (*R*)-(+)-*N,N*-dimethyl-1-phenethylamine, phenylboronic acid, and 7-amino-1,3-naphthalenedisulfonic acid monopotassium salt (Amido-G-Acid, AGA), ^2H_2O (99.96 at. % ^2H), and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) (99 at. % ^2H) were from Aldrich (Milwaukee, WI). The monopotassium salt of AGA was used after recrystallization from distilled, deionized water. The 20- × 22-cm and 10- × 6-cm vertical slab gel units and Bio-Gel-P2 were from Bio-Rad (Hercules, CA). The TE70 semi-dry electrophoresis transfer unit and Nylon 66 plus (positively charged nylon membrane) were obtained from Hoefer Scientific Instruments (San Francisco, CA). Glycine, tris(hydroxymethyl)aminomethane (TRIS), boric acid, glycerol, *N,N,N',N'*-tetramethylethylenediamine (TEMED), *N,N'*-methylenebisacrylamide, and Sephadex G25 were from Sigma. All other reagents were HPLC or reagent grade.

Preparation of fluorescently labeled sugars by reductive amination

Monosaccharide or oligosaccharide (300 µg) dissolved in 15 µL water was combined with 30 µL of 1M sodium cyanoborohydride and 15 µL of 0.2M AGA (recrystallized from water [6]) in acetic acid–water (15:85). The reaction mixture was incubated at 37°C overnight. Sugar–AGA conjugates were desalted, and much of the excess AGA was removed using a 1.5- × 30-cm Bio-Gel P2 or Sephadex G25 column eluted with water. The products could then be analyzed without further purification despite the presence of small amounts of residual reactants. Unlabeled sugar could not be detected by fluorescence, and AGA eluted at a position different from the sugar–AGA conjugates (as can be confirmed through co-injection of sample with AGA) and thus usually did not interfere with this analysis. In cases where it was necessary to completely remove AGA, the sugar–AGA conjugate was purified by preparative gel electrophoresis or by strong anion-exchange HPLC (6).



Scheme 1. Synthesis of sugar–AGA Schiff base and reduced sugar–AGA conjugates.

Preparation of fluorescently labeled oligosaccharide–AGA Schiff base conjugate

Oligosaccharide (17 µmol) and AGA (67 µmol of sodium, potassium salt) were dissolved in water. The reaction mixture was incubated at 50°C for 24 h. The product was purified on a semi-preparative SAX-HPLC column (1 × 25 cm) eluted at 4 mL/min with 1M sodium chloride in water. Fluorescence detection (excitation wavelength, 255 nm; emission wavelength, 450 nm) was used. The conjugate was desalted on a 1.5- × 30-cm Bio-Gel P-2 column eluted with distilled water.

HPLC analysis

HPLC was performed to analyze the sugar–AGA conjugates. Two systems were used, one equipped with an SP 8860 ternary HPLC pump (Spectra Physics; Santa Clara, CA), fixed-volume loop injector (No. 7125, Rheodyne; Cotati, CA), and Spectra 100 variable wavelength ultraviolet detector. The second system was equipped with two face programmable LC-7A pumps (Shimadzu; Tokyo, Japan) and a variable-wavelength RF-535 fluorescence detector (Shimadzu). In the studies performed using absorbance detection, the reaction mixture was injected on a Spheri-5 RP18 of dimension 4.6 mm × 22 cm or an Alltech phenyl column (Deerfield, IL) or both. The phenyl, C_{18}, and phenyl and C_{18} columns in series were eluted with 0.1M triethylamine–acetic acid (pH 4.0) at 1.0 mL/min (1.5 mL/min for the two-column system). The wavelength was fixed at 250 nm for detection. In the studies performed using fluorescence detection, C_{18} and phenyl columns Vydac; Hesparia, CA) of dimensions 4.6 mm × 22 cm (5-µm particle size) were used. The C_{18} column was used to analyze the oligosaccharide–AGA Schiff base conjugates. Isocratic elution was at 1 mL/min in 0.1% triethylamine in water adjusted to pH 3.5 with glacial acetic acid. The studies on the chiral amine ion-pairing reagent used a phenyl column eluted over 60 min at a flow rate of 1.0 mL/min with a 1–20% gradient in solvent B, where solvent A was 0.1% aqueous *R*-(+)-*N,N*-dimethyl-1-phenethylamine adjusted to pH 3.75 with acetic acid, and solvent B was 50% solvent A and 50% acetonitrile. The phenyl column was also eluted over 60 min at a flow rate of 1 mL/min with a 15–20% gradient in solvent B, where solvent A was 0.1% aqueous chiral amine and 0.1% phenylboronic acid adjusted to pH 3.75 with acetic

acid, and solvent B was 50% solvent A and 50% acetonitrile. All the HPLC separations using fluorescence detection were performed on a variable wavelength detector set at an excitation wavelength of 255 nm and an emission wavelength of 450 nm. Both ultraviolet absorbance and fluorescence detectors could be used in tandem when the ion-pairing reagent had no ultraviolet absorbance.

Individual components were co-injected with each sample mixture to confirm the assignment of each peak in a chromatogram.

Analysis of the oligosaccharide–AGA Schiff base conjugate by CE

A Dionex capillary electrophoresis system (Sunnyvale, CA) was used in these analyses. Separation and analysis were carried out in a fused-silica capillary tube (externally coated except where the tube passed the detector) (75-µm i.d., 375-µm o.d., and 68 cm long) from Dionex. The electrode buffer contained 20mM sodium phosphate and 50mM sodium dodecyl sulfate (SDS), pH 9.0. The voltage was constant at 15,000 V, and a fluorescent detector was used (excitation wavelength, 254 nm; emission wavelength, 455 nm).

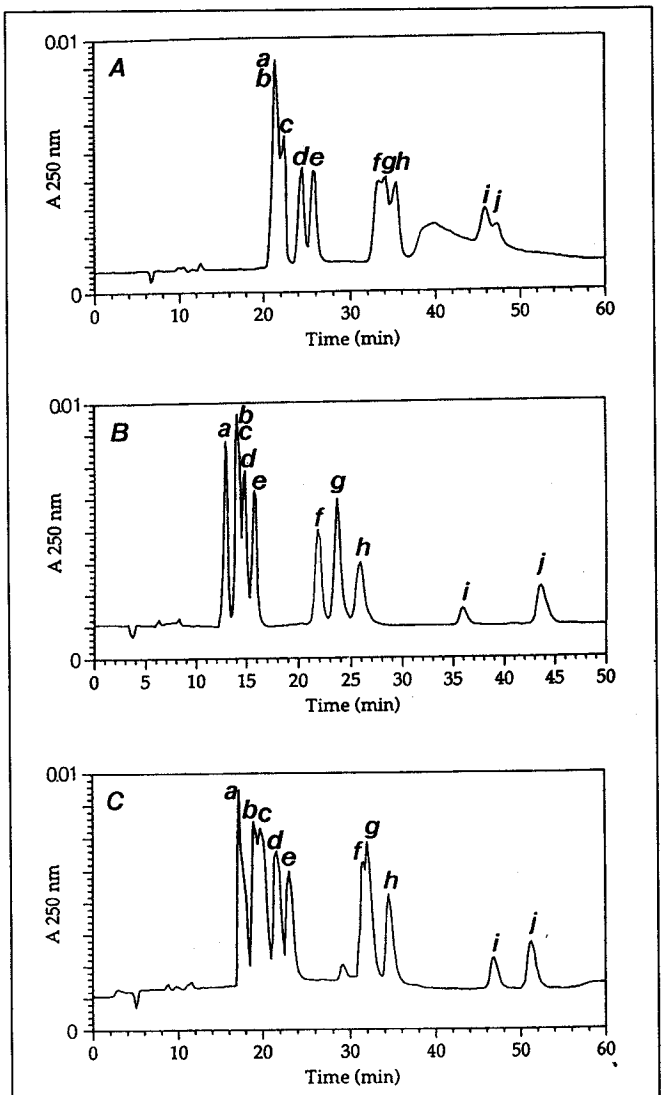


Figure 1. Isocratic separation of monosaccharide–AGA conjugates by ion-pairing HPLC on phenyl and C_{18} columns using triethylamine–acetic acid: A, a mixture of monosaccharide–AGA conjugates (20 ng each) applied to a phenyl column; B, the same mixture applied to a C_{18} column; C, the same mixture applied to C_{18}–phenyl columns in series. Peaks: a, Gal–AGA; b, Man–AGA; c, Glc–AGA; d, Ara–AGA; e, Xyl–AGA; f, Fuc–AGA; g, Rham–AGA; h, GlcNAc–AGA; i, GalA–AGA; and j, GlcA–AGA. The flow rates were 1.0 mL/min for A and B and 1.5 mL/min for C. Detection was by absorbance at 250 nm.

Characterization of the melibiose–AGA Schiff base conjugate by NMR

The Schiff base and reduced conjugates were dissolved in 500 µL of ^2H_2O (99.96 at. %) (1.5 mM) and freeze dried. This procedure was repeated two times to remove all the exchangeable hydrogens. The ^1H-NMR spectrum was acquired under ambient conditions at 600 MHz using a Bruker AMX-600 spectrometer with TSP as the internal standard.

Analytical polyacrylamide gel electrophoresis

The electrodes buffer contained 25mM TRIS adjusted to pH 9.0 with glycine. The resolving gel contained 19% (w/v) acrylamide and 1% (w/v) bisacrylamide in resolving buffer made from 325mM TRIS, adjusted to pH 9.5 with hydrochloric acid. The stacking gel contained 4.75% (w/v) acrylamide and 0.25% (w/v) bisacrylamide in stacking buffer made from 0.1M TRIS, adjusted to pH 8.0 with hydrochloric acid. The degassed resolving gel (5 mL), 30 µL freshly prepared 10% (w/v) ammonium persulfate, and 5 µL TEMED were mixed and poured vertically between 10- × 6-cm glass plates separated by 1.0-mm spacers. Polymerization of the resolving gel was completed in about 25 min. Then 2 mL degassed stacking gel, 35 µL 10% (w/v) ammonium persulfate, and 2 µL TEMED were mixed and poured on the top of polymerized resolving gel, and a well-forming comb was inserted. Polymerization of the stacking gel was completed in about 20 min. Samples were mixed with an equal volume of 50% (w/v) glycerol in water and loaded into the bottom of the wells. Electrophoresis was performed at 400 V (constant voltage) for 30 min at room temperature.

Results and Discussion

The fluorescent conjugates of a variety of monosaccharides, disaccharides, and trisaccharides were prepared through reductive amination with 7-amino-1,3-naphthalenedisulfonic acid (AGA) (Scheme 1) (6). This derivatization utilized a large excess of the fluorescent amine, AGA, to obtain high yields of conjugate (6). These conjugates were generally analyzed after removing most of the excess AGA and salts by gel permeation chromatography on a Bio-Gel P-2 column. Two peaks were observed in the distilled water eluant by their visible fluorescence using a hand held ultraviolet light or by absorbance at 250 nm. The small peak, which eluted first, corresponded to the sugar–AGA conjugate, and the large peak, which eluted second, corresponded to excess AGA as was confirmed by PAGE analysis (not shown) (6).

Initial studies focused on the separation of a mixture of 10 fluorescent monosaccharide conjugates by isocratic elution from a phenyl column with triethylamine–acetic acid (Figure 1A). This analysis resulted in a separation into three groups of

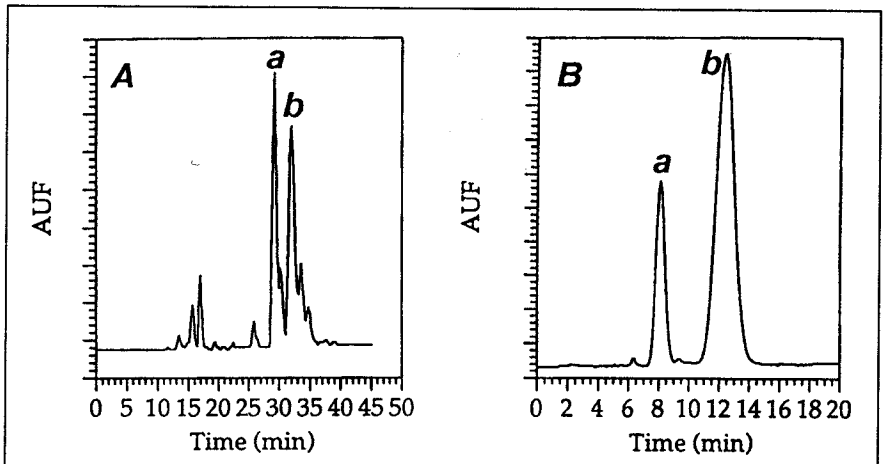


Figure 2. Gradient separation of Man–AGA from Glc–AGA on a phenyl column by ion-pairing HPLC: A, chiral amine was used as ion-pairing reagent; B, chiral amine was used in the presence of phenylboronic acid. The mixture contained approximately 1 ng of Man–AGA (a) and approximately 1 ng of Glc–AGA (b). Detection was by fluorescence (excitation wavelength, 255; emission wavelength, 450 nm).

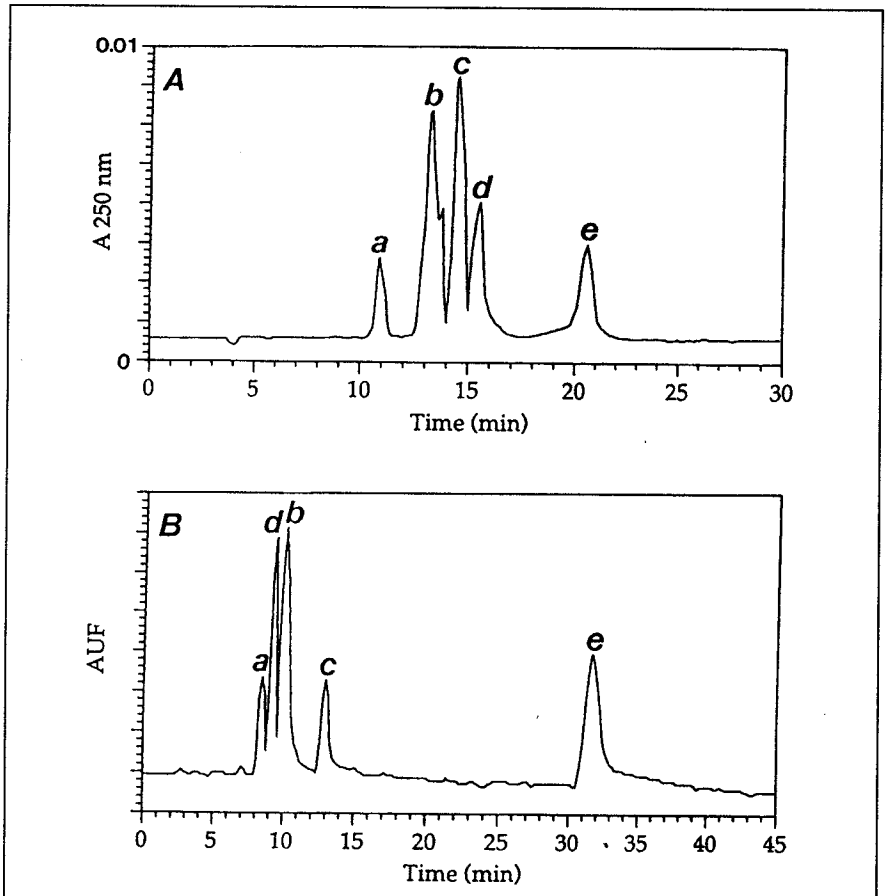


Figure 3. Isocratic separation of disaccharide–AGA and trisaccharide–AGA reduced and Schiff base conjugates by ion-pairing reversed-phase HPLC using trimethylamine–acetic acid. Labeled peaks correspond to the AGA conjugates of lactose (a), maltose (b), cellobiose (c), gentibiose (d), and cellotriose (e). In A, a mixture containing approximately 20 ng of each reduced form of sugar–AGA conjugate was applied to a phenyl column and eluted at a flow rate of 1.0 mL/min. Detection was by absorbance at 250 nm. In B, a mixture of approximately 1 ng of each Schiff base form of sugar–AGA conjugate was applied to a C_{18} column and eluted at a flow rate of 1 mL/min. Detection was by fluorescence (emission wavelength, 255; excitation wavelength, 450 nm).

monosaccharides: neutral hexoses and pentoses, deoxyhexoses, and uronic acids. This is consistent with a longer retention time for more hydrophobic sugars and for acidic sugars capable of ion-pairing with an additional triethyl ammonium. Within the neutral hexoses and pentoses, poor resolution was obtained between Gal–AGA, Man–AGA, and Glc–AGA. Similarly, in the deoxyhexoses Fuc–AGA failed to separate from Rham–AGA and in the uronic acids, GalA–AGA and GlcA–AGA were not completely resolved. The separation was considerably improved by replacing the phenyl column with a C_{18} column, resulting in the separation of 9 of the 10 monosaccharides under isocratic elution conditions (Figure 1B). Glc–AGA and Man–AGA were still unresolved on the C_{18} column. A combination of both C_{18} and phenyl columns using the same eluant clearly showed all 10 components of the mixture but did not give a baseline separation of Man–AGA from Glc–AGA and Fuc–AGA from Rham–AGA (Figure 1C).

These results, while promising, suggested that it might be useful to examine other ion-pairing reagents in combination with gradient elution to perform difficult separations, such as the separation of Man–AGA from Glc–AGA. A commercially available chiral amine, *R*-(+)-*N*,*N*-dimethyl-1-phenethylamine, was first examined under isocratic conditions using a phenyl column. Fluorescence detection was required because this ion-pairing reagent absorbed in the ultraviolet range. This method is capable of detecting 1 pmol of fluorescently labeled sugar. A slight enhancement was obtained in the resolution of a mixture of Man–AGA and Glc–AGA (not shown) when compared with the separation that relied on triethylamine. The application of a gradient resulted in a baseline separation of Man–AGA from Glc–AGA (Figure 2A). The large number of minor peaks observed using fluorescent detection probably arise from impurities in either the monosaccharide starting material or side products in the reductive amination reaction. Further studies using fluorescent detection relied on AGA–monosaccharide conjugates that were first purified by preparative PAGE or preparative SAX-HPLC (6). When phenylboronic acid (0.1%) was added to the eluant containing chiral amine, a further improvement in the separation of Man–AGA and Glc–AGA was observed. The baseline resolution of these purified fluorescent conjugates is shown in Figure 2B.

Phenylboronic acid is known to complex with sugars containing vicinal diols as are found in both fluorescent conjugates. The stronger this interaction, the more hydrophobic the complex and the greater the retention time on the phenyl column.

The separation of a mixture of disaccharide–AGA and trisaccharide–AGA conjugates was next attempted using a phenyl column eluted isocratically with triethylamine–acetic acid (Figure 3A). Disaccharide–AGA conjugates eluted earliest followed by trisaccharide–AGA conjugates, suggesting that an increase in saccharide units increases retention time. This separation required no additional optimization and gave highly reproducible chromatograms when the eluant was carefully prepared. The separation of sugars using reversed-phase ion-pairing HPLC is comparable with that achieved using high pH ion chromatography with pulsed amperometric detection (2), and the use of a fluorescent label permits equally sensitive detection. One benefit of reversed-phase ion-pairing HPLC is that the elution conditions are mild and do not expose the sugar analyte to the highly basic conditions (pH of approximately 13) used in ion chromatography (2). In addition, unlike the pulsed amperometric detector, ultraviolet and fluorescence detectors are widely available and routinely used in most analytical laboratories.

To make this separation method useful for both analytical and preparative applications, the use of a reversible fluorescent label was explored. Melibiose, a disaccharide, was initially selected for this experiment, but the approach is applicable to any reducing sugar. Melibiose was combined with the sodium potassium salt of AGA under neutral conditions that favor the formation of the Schiff base. Excess AGA was removed, and the Schiff base form of the conjugate was purified by SAX-HPLC and desalted using Bio-Gel P-2 chromatography. Analysis of the purified conjugate by CE (11) showed that the melibiose–AGA Schiff base conjugate was pure (not shown). Both Mel–AGA Schiff base and Mel–AGA (prepared by reducing the Shiff base with sodium cyanoborohydride) migrated at 10 min and could not be resolved under the conditions examined. Gradient PAGE analysis (6) of the Mel–AGA conjugate and the Mel–AGA Schiff base conjugate also indicated that these compounds were highly pure and had slightly different migration positions, with the Schiff base form of the conjugate migrating ahead of the reduced conjugate (not shown). The separation of the Mel–AGA Schiff base conjugate from both the Mel–AGA conjugate and AGA was easily achieved by ion-pairing HPLC on a phenyl column (Figure 4). The Mel–AGA Shiff base conjugate was found to be stable on brief exposure to the conditions used for CE, gradient PAGE, and reversed-phase ion-pairing HPLC analysis. These conjugates were also examined by ^{1}H-NMR spectroscopy at 600 MHz. The spectra of the Mel–AGA Schiff base, Mel–AGA conjugate, and Mel are completely assigned in Table I.

The reversibility of the Mel–AGA Shiff base conjugate was examined in more detail using reversed-phase ion-pairing HPLC analysis. A mixture of the Mel–AGA Schiff base, AGA, and the Mel–AGA reduced form were easily resolved on a C_{18} column using isocratic elution with triethylamine adjusted to pH 3.5 with acetic acid (Figure 4). Although the Mel–AGA Schiff base conjugate was stable under these separation conditions, treatment with 0.1M hydrochloric acid for 12 h at 5°C resulted in its breakdown to Mel and free AGA. The decomposition of the Schiff base form could be further accelerated by the addition of a competing aldehyde (i.e., glucose). The reversibility of the Schiff base form of the Mel–AGA conjugate was confirmed using CE (11) and gradient PAGE (6) analysis. The Schiff base was stable in distilled water at neutral or basic pH when stored at 25°C for 3 days, showing less than 10% decomposition to AGA and Mel. Under the mildly acidic conditions (i.e., pH 3.5) used in SAX-HPLC chromatography, the Schiff base form was relatively stable, permitting its purification at room temperature over the course of a 1 h separation. These results demonstrate that reducing sugars may be reversibly labeled with the fluorescent amine AGA, purified or fractionated, and then recovered by mild acid treatment.

The Schiff base conjugates of the five disaccharides and trisaccharides analyzed in Figure 3A were prepared, purified by SAX-HPLC (6), desalted on a Bio-Gel P-2 column, and then analyzed on a C_{18} column using isocratic elution with triethylamine adjusted to pH 3.5 with acetic acid (Figure 3B). Although the separation of the sugar–AGA conjugate's Schiff base and reduced forms were comparable, their elution order differed.

Conclusion

Reducing sugars can be conjugated with a charged, intensely visible fluorescent label. Substantial purification can be

Table I. ^{1}H NMR Assignments of Melibiose–AGA Conjugate, Melibiose–AGA Schiff Base, and Melibiose*

	Galactose							Glucose						
Compound	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	H-1	H-2	H-3	H-4	H-5	H-6	H-6′
Melibiose–AGA	4.87	3.83	3.90	4.01	3.98	3.73	3.72	3.22	3.33	3.50	3.56	3.57	3.70	3.67
Melibiose–AGA† Schiff base	4.88	3.83	3.86	3.91	3.90	3.75	3.72	5.06	3.33	3.50	3.55	3.58	3.67	3.70
α–Melibiose‡	4.97	3.81	3.89	4.00	3.74	3.77	3.74	5.23	3.54	3.73	3.51	3.99	3.89	3.71
β–Melibiose	4.99	3.83	3.87	4.00	3.74	3.77	3.74	4.67	3.25	3.47	3.53	3.64	3.97	3.77

* 1D NMR was performed in ^2H_2O at 600 MHz using TSP as an internal standard.
† The Melibiose–AGA Schiff base also contains signals assigned to the substituted naphthalene aromatic ring system. Protons at positions 2,4,5,6, and 8 resonate at 8.36 (d), 8.33 (d), 8.02 (d), 7.35 (dd) and 7.93 (d), respectively. The substituted naphthalene ring system of the Melibiose–AGA reduced form has nearly identical chemical shifts.
‡ 1D NMR showed that Melibiose is a 40:60 mixture of α to β anomers.

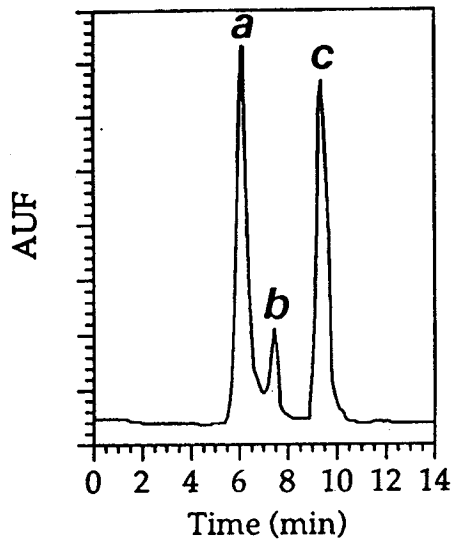


Figure 4. Isocratic separation of the reduced and Schiff base forms of Mel–AGA by ion-pairing HPLC on a C_{18} column. A mixture of Mel–AGA Schiff base (a), AGA (b), and Mel–AGA reduced form (c) (approximately 1 ng of each component) were applied and eluted at a flow rate of 1.0 mL/min with triethylamine–acetic acid (pH 3.5). Detection was by fluorescence (emission wavelength, 255; excitation wavelength, 450 nm).

achieved using a simple desalting column, and a high purity conjugate can be obtained by preparative gel electrophoresis or SAX-HPLC. These labeled sugars can be analyzed by ion-pairing HPLC on phenyl or C_{18} columns or both. The separation is dependent on the type of ion-pairing agent used and can be enhanced by the addition of phenylboronic acid complexing agent. These labels can also be introduced as a relatively stable Schiff base, and the formation of this Schiff base can be reversed after fractionation under mildly acidic conditions to recover the starting sugar.

Acknowledgment

Support from the National Institutes of Health Grant GM38060 and Korean Science and Engineering Foundation Grant (913-0301-01401) are gratefully acknowledged.

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Manuscript accepted September 8, 1994.