Note

Analysis by High-performance Anion-exchange Chromatography of Component Sugars as Their Fluorescent Pyridylamino Derivatives

Jun Suzuki,1 Akihiro Kondo,2 Ikunoshin Kato,* Sumihiro Hase†† and Tokui Ikenaka†††

Department of Chemistry, Osaka University College of Science, Toyonaka, Osaka 560, Japan
*Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., Otsu, Shiga 520–21, Japan

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Component sugar analysis is one of the most important procedures for the determination of sugar structures, and a highly sensitive method is needed. We have analyzed sugar components in glycoconjugates by high-performance liquid chromatography with fluorescence labeling.1 A component analysis can be done with a trace amount of a sample, but it takes time (two days) and involves laborious procedures, including a complex step of purification to remove the excess reagents. To overcome these problems, we describe here an improved method with simplified procedures.

2-Aminopyridine was purchased from Wako Pure Chemical Ind. Ltd. (Osaka) and recrystallized twice from n-hexane. Ganglioside (bovine brain) was from Bachem Fein Chemikalien AG (Bubendorf), and borane-dimethyamine complex was from Aldrich Chemical Co. (Milwaukee). Taka-amylose A was prepared as reported previously,23 and z1-acid glycoprotein was kindly presented by Dr. K. Schmid (Boston University).

A Shimadzu LC-6A liquid chromatograph equipped with a Rhodyne Model 7125 injector and a Hitachi Model F-1050 fluorescence spectrophotometer was used. The columns used were a TSKgel SAX column (4.6 × 75 mm, Tosoh Corp., Tokyo), a TSKgel Sugar AXI column (4.6 × 150 mm), and an HPIC AS6 column (4.6 × 250 mm, Dionex Corp., Sunnyvale, CA).

The neutral sugar components were treated with 1.5 M HCl-methanol at 90°C for 4 hr and then trimethylsilylation, before being assayed by gas-liquid chromatography (OV-17 column, 2 m).3,4 The amino sugars were assayed with an amino acid analyzer (Hitachi Model 835).

Procedure. A sample (0.01–10 nmol of sugars or a glycoconjugate) was placed in a glass tube tapered at the bottom (10 × 100 mm) and dried. To the residue was added 40 μl of 4 M trifluoroacetic acid, and the tube was sealed under vacuum. The tube was heated at 100°C for 3 hr, and ribose was added as an internal standard. The solution obtained was freeze-dried twice with added water. Free amino groups were acetylated by adding 55 μl of a mixture of methanol-pyridine-water (30:15:10) and 2 μl of acetic anhydride. The solution was left for 30 min at room temperature with occasional stirring. The solution was freeze-dried, and to the residue was added 7 μl of a coupling reagent prepared by mixing 1.00 g of 2-aminopyridine, 0.47 ml of acetic acid, and 0.60 ml of methanol. The tube was sealed and heated at 90°C for 15 min. The excess reagents were removed by evaporating under a stream of nitrogen gas at 60°C for 20 min. Then, 10 μl of a reducing reagent, prepared just before use by mixing 59 mg of borane-dimethyamine complex and 1 ml of acetic acid.

Fig. 1. Separation of the PA-Sugars by High-performance Anion-exchange Chromatography. A TSKgel Sugar AXI column (4.6 × 150 mm) was used with a borate buffer as described in the text. A mixture of sugars (1 nmol of each) was treated as described in the text and 1/100 was injected. 1, PA-N-acetylgalactosamine; 2, PA-2-deoxyribose; 3, PA-rhamnose, PA-N-acetylmannosamine; 4, PA-xylene; 5, PA-N-acetylgalactosamine; 6, PA-ribose; 7, PA-glucose; 8, PA-mannose; 9, PA-fucose; 10, PA-galactose.

Abbreviation: PA-, pyridylamino.

* Present address: Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., Otsu, Shiga 520–21, Japan.
†† To whom correspondence should be addressed.
††† Present address: Tezekayama Gakuen Junior College, Harumidai, Sakai, Osaka 590–01, Japan.


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was added. The tube was re-sealed and heated at 90°C for 30 min. The reaction mixture was dried twice under a stream of nitrogen gas with 30 μl of toluene at 40°C for 10 min each time to remove the excess reagents. The residue was dissolved in 500 μl of water, and a part (usually 5 μl) of the solution was analyzed by HPLC with a TSKgel Sugar AXI column. The buffer used was a mixture of nine parts of 0.7 M boric acid, adjusted to pH 9 with potassium hydroxide, and one part of acetonitrile. The flow rate was 0.3 ml/min and the column was operated at 65°C. For detecting the PA-sugars, an excitation wavelength of 310 nm and an emission wavelength of 380 nm were used.

The previous method required two days, and purification was needed after N-acetylation and again after pyridylation. To simplify the method, we used volatile reagents throughout the procedure. We have recently reported an improved procedure for pyridylation, in which most of the excess reagents can be removed by evaporation. This procedure was used in the analysis of component sugars, and the reaction time and the concentration of the reagents were modified. The coupling reagent was diluted with methanol, as the original reagent was viscous. The procedure for N-acetylation was also improved by the use of volatile reagents. The separation of the PA-sugars was also re-investigated because the reversed-phase column used in the previous method was affected by contaminating materials, and separation of PA-glucose and PA-mannose was unsatisfactory. We tested three anion-exchange columns, the TSKgel Sugar AXI column giving the best results.

Various amounts of standard sugars were pyridylaminated, and a part of the solution was analyzed by HPLC. The PA-sugars were well separated as shown in Fig. 1. A peak arising from a trace amount of 2-aminopyridine appeared at the elution time of 6 min. The relationship between the peak heights (and also the peak areas) and the concentration was linear in the range of 0.01 to 10 nmol (data not shown). Total recovery of the sugars as PA-sugars was 95% for N-acetylglucosamine and 90% for glucose, and the reproducibility was good (less than 3% error).

The usefulness of this method was tested by analyzing several glycoconjugates with ribose as the internal standard (Table I). The results by our new method were in good agreement with those obtained by gas-liquid chromatography or by the amino acid analyzer. No contaminating peaks due to polypeptide chains or the lipid moiety of glycoconjugates were found. By our method, 50 pmol of a glycoconjugate could be analyzed, and the time needed for preparing the PA-sugars was reduced from 2 days to 2 hr while the sensitivity remained the same.

Table 1. Sugar Composition of the Glycoconjugates

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<tr>
<th>Sample</th>
<th>GalNAc</th>
<th>GlcNAc</th>
<th>Glc</th>
<th>Man</th>
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<tr>
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</table>

a Determined by the present method (4 M trifluoroacetic acid at 100°C for 3 hr).
b Determined by gas-liquid chromatography (1.5 M HCl-methanol at 90°C for 4 hr).
c Determined by an amino acid analyzer (4 M methanesulfonic acid at 110°C for 24 hr).

References