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Short communication

Analysis of pyridylaminated oligosaccharides using liquid chromatography–mass spectrometry with a monolithic capillary column

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ABSTRACT

We examined the utility of a monolithic capillary column in the analysis of pyridylaminated oligosaccharides. Fluorescence detection and mass spectrometry were used to monitor a series of oligosaccharides. Although the total-ion chromatogram appeared similar to that obtained with fluorescence detection, the sensitivity of this technique was limited, especially in the case of smaller oligosaccharides. This limitation was overcome by applying selected ion current monitoring. Further, the capillary column also exhibited good reproducibility. We showed that the retention times obtained by using the monolithic capillary column could be converted into the standard data to enable comparison of the experimental data with the existing data. Furthermore, our studies revealed an important difference in the separation profile, i.e., the monolithic capillary column could resolve smaller oligosaccharides to a greater extent.

1. Introduction

Glycan structures of glycoproteins, glycolipids, and polysaccharides are involved in many important biological functions [1–6]. There is a tremendous amount of heterogeneity in the structures because of the presence of isomers that arise due to differences in linkage positions, anomeric configurations and the branched structures [7]. These glycans are assembled by a template-independent process involving sequential reactions mediated by glyco-related enzymes at the Golgi apparatus. Glycans can be studied by a method that is capable of analyzing minute amounts of samples.

One of the most reliable techniques for detecting glycans in small amounts is the fluorescence detection often labeled by reductive amination with a fluorophore carrying an amino-functional group, e.g. 2-aminopyridine [8,9]. It has been reported that fluorescently labeled sugars can be separated by high-performance liquid chromatography (HPLC) with mass spectrometry (MS) [10]. Mapping of the two-dimensional HPLC profiles of pyridylaminated (PA)-sugars has been found to be a very useful in assessing the structure of oligosaccharides [11–15]. Takahashi and coworkers had developed a method, three-dimensional mapping of *N*-linked glycans using a reverse-phase (RP) column, a size fractionation column, and an anion exchange column [16]. The accumulated data of their work, which includes data for more than 400 *N*-glycans, are available on the internet (http://www.glycoanalysis.info/galaxy2/ENG/index.jsp). In this method, the retention times of the individual glycans were converted into glucose units (GU) using PA-glucose oligomers (PA-Glc$_n$) as a template; thus, the elution behavior of individual glycans can be compared in terms of a relative retention time. The obvious advantage of this method is that it permitted normalization of the retention time, thereby facilitating comparison of the data obtained in different conditions and those obtained from different columns [13–15]. A variety of PA-sugars are commercially available, have good ionization properties, and can be analyzed by MS [10]. Moreover, tandem MS (MS/MS) of the PA-sugars allows the identification of various glycans, including those containing isomers, which is extremely difficult in oligosaccharide analysis by MS [17–19]. Therefore, HPLC-based mapping of PA-sugars may become more powerful with the aid of MS/MS capability.

Capillary liquid chromatography (LC)–electrospray ionization (ESI)-MS and nano-LC–ESI-MS are useful in the analysis of minute amounts of analytes [20,21]. However, LC with a particulate stationary phase requires a relatively high pressure for good resolution, which is often an experimental limitation. A monolithic column is a single piece of a rigid, porous polymer that does not have any interstitial volume, but has internal porosity because of the presence of micro-, meso-, and macro-pores [22–25]. The absence of intraparticular volume forces the entire mobile phase to flow through the pores of the stationary phase. Theoretically, mass transport is enhanced by such convection; therefore, the resolution provided by this method is better than that obtained with micro-particles as the stationary phase. Moreover, the monolithic column is durable, it has no dead volume at the column inlet, and there are no channels in the column, thereby enabling a high flow rate. The stationary

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phase used in the monolithic column is immobilized on the capillary wall without a retaining frit; therefore, it is especially suitable for the fabrication of capillary columns [25,26].

Monolithic capillary columns with internal diameters in the size range of 10–500 μm have been used for high-resolution separation of biopolymers such as peptides, proteins, and nucleic acids [27]. In this report, we describe the utility of the monolithic capillary column in separating minute amounts of PA-sugars. Further, we also suggest a method to convert the obtained data into a format used in the database so as to facilitate high-throughput identification of oligosaccharides.

2. Materials and methods

2.1. Materials

PA-sugars were purchased from Takara Bio (Kyoto, Japan). Ammonium acetate was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were from Wako Pure Chemical Industries (Osaka, Japan). All solvents were either HPLC grade or LC/MS grade.

2.2. HPLC

The samples were separated by reversed-phase-HPLC (RP-HPLC) and were analyzed using a Waters 600E multisolvent delivery system equipped with a U6K injector (Waters Co., MA, USA). The analytes were detected by using the Shimadzu fluorescence detector RF-10AXL (Shimadzu Co., Kyoto, Japan). Data were analyzed by using the Smart Chrom software (KYA TECH Co., Tokyo, Japan).

The PA-sugars were separated on a Chromolith Performance RP-18e column (4.6 mm I.D. × 100 mm; Merck, Darmstadt, Germany) operated at room temperature. The mobile phase was composed of ammonium acetate (50 mM; pH 4.0; solvent A) and ammonium acetate (50 mM; pH 4.0) containing 2% *n*-butanol (solvent B). The proportion of solvent B over A was programmed to increase linearly from 3 to 20% in 50 min.

All the analyses were performed at a flow rate of 1.0 mL/min. The excitation and emission wavelengths used for fluorescence detection were 320 and 400 nm, respectively.

2.3. Capillary-LC–MS

Solvent delivery on the monolithic capillary column was performed using GILSON 305 and 306 pumps with an 805 manometric module and an 811D dynamic mixer (Gilson Inc., WI, USA). The flow rate was reduced from 100 to 2.4 μL/min using an LC Packings Accurate^{TM} flow splitter (Dionex Co., CA, USA) equipped with a Cheminert CNW0070 injection valve (Valco Instruments Co. Inc., TX, USA) and a 200 nL injection loop. A make-up solvent, which was delivered by a syringe pump, was added to the solvent flow by using a Micro-Static Mixer (Upchurch Scientific, WA, USA) between the capillary column outlet and the ion spray. Mass detection was carried out by using an API Q-Star pulsar *i* (Applied Biosystems, CA, USA) system equipped with a microelectrospray ion source in the positive or negative mode, with a full scan between *m*/*z* 300 and 2000.

An Ex-Nano Mono Cap column (0.2 mm I.D. × 250 mm; GL Science, Japan) was operated at room temperature, and the mobile phase used for this column was composed of ammonium acetate (10 mM; pH 4.0; solvent C) and ammonium acetate (10 mM; pH 4.0) containing 2% *n*-butanol (solvent D). The proportion of solvent D over C was programmed to increase linearly from 0 to 50% in 40 min. Methanol at a flow rate of 10.0 μL/min was used as the make-up solvent. The MS spectra were acquired by using the Analyst Q software (Applied Biosystems, CA, USA).

3. Results and discussion

3.1. Effect of buffer concentrations on HPLC separation

The PA-sugars, which carry an imino group (pK_a 6.7), can be separated by RP-HPLC with a buffer as the mobile phase, however buffers may induce ion-suppression in MS analysis. In order to ensure successful detection, we examined the effects of different concentrations of ammonium acetate, which is volatile in nature and exerts minimal influences on MS analysis, for separation.

Firstly, we compared the PA-Glc$_n$ (PA-isomaltooligosaccharides; n = 5–15) retention times that corresponded to different concentrations of ammonium acetate (ranging from 10 to 200 mM; pH 4.0) by using an analytical HPLC column (4.6 mm I.D. × 150 mm) (Supplementary Fig. S1). While the difference between the retention times corresponding to 10 and 50 mM ammonium acetate was not significant, the retention times were found to be shorter at higher concentrations of ammonium acetate. Since the nature of the peaks in the chromatogram did not change on varying the concentration of the buffer (data not shown), we decided to use 10 mM ammonium acetate buffer as the mobile phase for capillary LC–MS.

3.2. Solvent for MS analysis

The separation of PA-sugars by using conventional HPLC is performed in relatively hydrophilic conditions due to the hydrophilic nature of compounds. This hydrophilic eluent, which contains a less volatile solvent such as water, causes less ionization of analyte. In order to improve ionization of the sample, methanol was used as a sheath liquid [20]. Methanol was added to a final ratio of 4:1 (v/v) after HPLC (flow rate into the MS: 12.4 μL/min). Fig. 1 shows the MS chromatogram of PA-Glc$_n$ in the negative mode with total-ion current (TIC, panel a) and ion extraction (panel b). In spite of methanol addition, the baseline obtained for TIC was not stable till the point of elution of PA-Glc$_6$; however, the peaks for the shorter Glc oligomers (PA-Glc$_{3-5}$) could be observed in the ion-extraction mode. The profiles of the chromatograms obtained using fluorescent detection (Supplementary Fig. S2) and MS detection (Fig. 1) were quite similar in the case of glycans with higher molecular weights than PA-Glc$_6$. In the MS detection, monovalent ions were observed in lower molecular weight than PA-Glc$_6$, whereas divalent ions were observed in higher molecular weight compounds (Supplementary Fig. S3). Thus the nature of ions formed by the capacity of charge versus the volume of the species under the given MS analysis conditions should be considered to use ion-extraction mode.

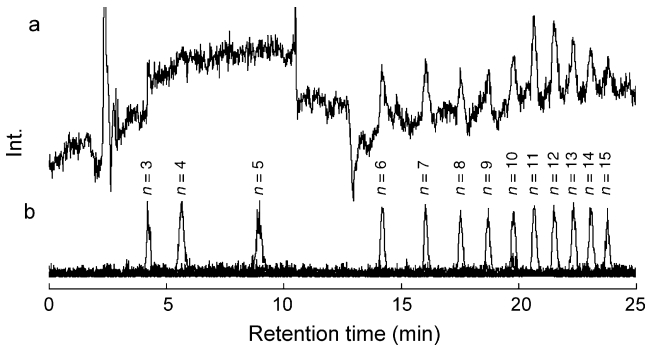

Fig. 1. Separation of PA-Glc oligomers on a monolithic capillary column using 10 mM ammonium acetate with *n*-butanol (flow rate, 2.4 μL/min) in the linear-gradient mode. Injection volume: 200 nL (0.4 pmol). (a) Total-ion chromatogram of PA-Glc$_n$ (n = 3–15) obtained using MS in the negative mode. (b) An overlaid chromatogram of the selected-ion monitoring chromatograms of ions with *m*/*z* values 583, 745, 907, 1069, 1231, 1393, 778, 859, 940, 1021, 1102, 1183, and 1264 in the range of ±3 amu.