Journal of Chromatography B 1081–1082 (2018) 33–40

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

New stationary phase for hydrophilic interaction chromatography to separate chito-oligosaccharides with degree of polymerization 2-6

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ARTICLE INFO

Keywords:
Chito-oligosaccharides
Hydrophilic interaction liquid chromatography (HILIC)
Stationary phase
3-aminophenylboronic acid

ABSTRACT

A new 3-aminophenylboronic acid-functionalized stationary phase based on silica for hydrophilic interaction liquid chromatography (HILIC) was developed and showed great HILIC characteristics on separation for chito-oligosaccharides. The material was synthesized by grafting 3-aminophenylboronic acid group to silica, and it was characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR), elemental analysis and thermal gravimetric analysis (TGA). Nucleobases and nucleosides were used to evaluate the retention property and to investigate retention mechanism by the models designed for description of partitioning and surface adsorption through adjusting ratio of water in the mobile phase. Parameters affecting chromatography behavior such as ionic strength, buffer pH and column temperature were also investigated. Results have indicated that the retention mechanism was a combination of partitioning and surface adsorption, and the hydrogen bond seemed to be the main force for the retention behavior. Finally, the new 3-aminophenylboronic acid-functionalized based on silica stationary phase was applied to separate chito-oligosaccharide samples with optimized mobile phase conditions and showed acceptable chromatograms.

1. Introduction

Recently, chito-oligosaccharides have gained more attention due to their biological functions [1],such as anti-inflammation [2], anti-obesity [3], immmunostimulating [4,37] and anti-tumor effect [5,38,39], as one of the most important steps in structural and biological studies, separation and purification of chito-oligosaccharides attracts wide concern. It is essential that the chito-oligosaccharides used in both cell biological and structural studies must be well separated and highly purified. But the separation of chito-oligosaccharides is challenging, owing to the fact that these analytes are polar, hydrophilic, and poorly retained on reversed-phase high-performance liquid chromatography (RP-HPLC) columns. Ion-exchange chromatography, coupled with electrochemical detection, has been widely employed for carbohydrate (including chito-oligosaccharides) analysis [6]. Though this analytical technique exhibits high resolution and sensitivity, the drawbacks, such as low capacity, high cost and general incompatibility with mass spectrometry (MS) cannot be neglected. In 1990, hydrophilic interaction liquid chromatography (HILIC) was defined since amino-silica stationary phase with aqueous eluents was used to analyze oligosaccharides [7]. HILIC is an alternative technique to RP-HPLC, where analytes are separated on a polar stationary phase and eluted by a binary mobile phase with the main component usually being 5–40% water in acetonitrile [7]. In the last few years, HILIC has attracted great interest and is employed for many applications of separating metabolites [8,9], pharmaceuticals [10,11], carbohydrates [12], and natural products [13]. It provides new strategy to chito-oligosaccharides separation. However, the naked silica and aminopropyl groups modified silica present poor stability in aqueous mobile phase [14]. Accordingly, a number of silica-based bonded polar phases, such as cyclodextrin

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https://doi.org/10.1016/j.jchromb.2018.02.024
Received 15 November 2017; Received in revised form 16 February 2018; Accepted 17 February 2018

[8,15], amide [16,17], and zwitterionic sulfobetaine ligands [18], have been developed to meet the saccharide separation needs, which proved to be effective in the separation of oligosaccharides including chito-oligosaccharides [19,20] The advantages of HILIC, such as good retention of polar compounds, high selectivity, and MS compatibility, make it be one of the most suitable chromatographic modes for chito-oligosaccharide separations [21].

The binding interaction of boric acid and *cis*-diol was first reported in 1949 [22], promoting the development of boronate affinity chromatography (BAC). The principle is based on reversible covalent complex formation/dissociation between boronic acids and cis-diols in an alkaline/acidic aqueous solution [23]. Boronate affinity chromatography is a unique affinity chromatographic mode that permits the specific isolation and separation of cis-diol containing compounds, such as glycoproteins, nucleosides and saccharides [23,24]. Because of the significant advantages of HILIC and BAC, it can be expected that the combination of HILIC and BAC will provide new promises for the separation of chito-oligosaccharides which contain cis-diol compounds.

In this study, we developed a general method to prepare 3-Aminophenylboronic acid silica (APBA-silica) and expected it acting as a potential HILIC stationary phase to separate chito-oligosaccharides. APBA is normally used in molecular imprinting as functional monomer [25], and to our knowledge, this is the first time that BAC was combined with HILIC to realize the separation of chito-oligosaccharides. The structure of the resulted APBA-silica was characterized and we evaluated the performance of this new stationary phase in the separation of nucleobases and nucleosides by HILIC mode and tried to demonstrate their retention mechanisms. Finally, we applied it to the separation of chito-oligosaccharide samples and the acceptable results were obtained. This work may shed light on a new perspective for the development of chito-oligosaccharides.

2. Experimental

2.1. Chemicals and materials

Silica (particle size: 5 μm) was from Meigao Group Co. Ltd. (Qingdao, China). Ammonium formate, 3-chloropropyl triethoxy silane and 3-aminophenylboronic acid (APBA) were from Aladdin Reagent Co. Ltd. (Shanghai, China). Uracil, cytosine, amidopurine, and thymine were from J&K scientific Ltd. (Beijing, China). Chitobiose hydrochloride, chitotriose hydrochloride, chitotetraose hydrochloride, chitopentaose hydrochloride, chitohexaose hydrochloride were from BZ Oligo Biotech Co. Ltd. (Qingdao, China). Chito-oligosaccharide-I and chito-oligosaccharide-II were provided by Aikang Biotech Co. Ltd. (Dezhou, China). Acetonitrile (ACN), absolute ethanol, *N*, *N*-dimethyl formamide (DMF), trimethylamine were from Tianli Chemical Reagents Ltd. (Tianjin, China). Glucosamine, acrylic acid (AA), acrylamide (AM), methacrylic acid (MAA) were from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China).

2.2. Synthesis of 3-aminophenylboronic acid-functionalized silica

The 3-aminophenylboronic acid-functionalized silica (APBA-silica) was prepared according to the procedure as shown in Scheme. 1. Briefly, dry activation silica (200 mg) and 0.6 mL of γ-3-chloropropyl triethoxy silane were dispersed in 80 mL of DMF with magnetic stirring under nitrogen protection at 105 °C for 24 h to obtain SiO_2@Cl. The product was washed with DMF and ethanol and dried at 40 °C for 6 h. Next, the SiO_2@Cl (200 mg) and 3-aminophenylboronic acid (200 mg), which was chosen as functional monomer for capturing target molecules, were dispersed in 80 mL of chloroform by ultrasound for 10 min, and then 0.05 mL of pyridine was added as the deacid reagent. The reaction was carried out at room temperature for 6 h. The product was centrifuged, washed with chloroform to remove the unreacted reagent, and dried under vacuum for 6 h to obtain SiO_2@APBA. Because boronic acid can form diesters with all glycans that contain *cis*-diol groups [25], the chito-oligosaccharides can be separated when the SiO_2@APBA was used as stationary phase with appropriate mobile phase.

2.3. Material characterization

A TU-1900 Double-beam UV–vis spectrophotometer (Persee, China) was used to determine the absorbance of glucosamine and functional monomers. Scanning electron microscopy (SEM) images were obtained on a Quanta 200FEG (FEI, U.S.) to determine the size and morphology of the silica. Fourier transform infrared spectra (FT-IR) were recorded on an Avatar 360 (Nicolet, U.S.) instrument. The thermogravimetric analysis (TGA) was performed for powder samples (~10 mg) with a heating rate of 10 °C min^{-1} using a Netzsch STA 449C (Germany) thermogravimetric analyzer under a nitrogen flow up to 800 °C. F-Sorb 2400 (Bejing, China) and Vario EL (Germany) were used to measure BET (Brunauer, Emmett and Teller) and analyze elemental content of the samples. The surface concentration of bonded species on the silica was calculated according to the general equation proposed by Unger et al. [26].

$$Coverage\ (\mu mol/m^2) = \frac{\%X \times 10^6}{A_M n100(1 - \%X(M_W))/A_M n100)S} \quad (1)$$

where %X is the percent carbon or nitrogen increases in the bonded support as determined by elemental analysis, AM is the atomic mass of carbon or nitrogen, MW is the molecular weight of the species bonded to the silica surface, n is the number of carbon or nitrogen atoms present in the bonded species, and S is the specific surface area of the silica support in meters squared per gram.

2.4. Chromatographic conditions

The prepared stationary phase was slurry-packed into stainless steel column (150 mm length × 4.6 mm id.) with isopropanol as slurry and methanol as propulsion solvent under a pressure of 38 MPa. Before using, the column was rinsed for 2 h by ACN and water (90:10, v/v) at a flow rate of 1.0 mL/min.

All chromatographic experiments were performed on an Agilent Technology 1100 high performance liquid chromatography (HPLC) connected to a refractive index detector (RID). Mobile phase was prepared by mixing ACN and 200 mM of stock ammonium formate ($HCOONH_4$) to reach the desired ACN content (v/v). Stock solutions of $HCOONH_4$ were prepared in water and adjusted to the required pH with formic acid. The pH value of mobile phase was referred to the pH of the buffer before mixing with the organic solvent.

3. Results and discussion

3.1. Preparation

3.1.1. Functional monomer

The functional monomer should be determined before synthesis of modified silica to make strong bonding force between target molecules and functional monomer, benefitting the adsorption of target molecules. The Job's plot method [27] was used to measure the optimal proportion of functional monomer and template molecules. We chose four kinds of commonly used functional monomers and used nine different proportions, respectively. Meanwhile, glucosamine, which is the monomer of chito-oligosaccharide, was used as template molecules. The results were shown in Fig. 1. All the fitting curves were presenting parabolic shape, indicating that only when the ratio of glucosamine and functional monomer are consistent with the composition of inclusion compound, can we get the highest concentration of inclusion, leading to