Journal of Chromatography A, 1323 (2014) 97–103

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Size exclusion chromatography of polysaccharides with reverse phase liquid chromatography

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ARTICLE INFO

Article history:
Received 9 October 2013
Received in revised form 4 November 2013
Accepted 5 November 2013
Available online 13 November 2013

Keywords:
Polysaccharide
Reverse phase liquid chromatography
Size exclusion chromatography
Void volume

ABSTRACT

This work describes the use of a reverse phase (RP) column for size-based separation of hydrophilic polysaccharides. The entire separation window (before and after void volume) is used to provide unique separation of polysaccharides from other components in a glycol-conjugate vaccine or complex media of fermentation broth. The technique has also been applied to the separation of polysaccharides of different sizes. The effect of chromatographic parameters including type of packing material (CN, C8 and C18), pore size (80 and 300 Å) of stationary phase, concentration of organic solvent on the separation was investigated. In addition, characterization of size-based separation was evaluated by coupling of RP column with multi-angle laser light scattering (MALLs) detector.

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1. Introduction

There are different modes of separation in HPLC like normal phase, reversed phase, ion exchange, size exclusion and affinity chromatography [1–3]. In general, polarity is the primary characteristic of compounds that dictate reverse phase retention whereas size is the major attribute of compounds that dictate separation in size exclusion chromatography. All components in a mixture have unique behavioral characteristics related to their molecular structure. They can be described as being polar or non-polar, with a range of polarities between the most polar and most non-polar. They can also be described as small and large with wide range of hydrodynamic size.

Reversed-phase liquid chromatography (RPLC) is the most commonly used separation technique in HPLC [3–5]. It is estimated that about 80–90% of all HPLC separations are carried out under reversed-phase conditions [4,5]. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle wide range of compounds with different polarity and mass. Size exclusion chromatography (SEC) is rarely used for separation of small molecules, but is frequently used for the separation of macromolecules [5–7].

In biochemical analysis, RPLC has been widely used to separate a diverse array of compounds including lipids [6], amino acids [7], peptides [8], proteins [9], DNA, oligonucleotides [10] and oligosaccharides [11]. RPLC is usually not compatible with highly polar oligosaccharides or polysaccharides due to their lack of hydrophobic interaction with nonpolar stationary phase. Some research groups have reported the use of RPLC to separate derivatized or ion-paired oligosaccharides based on their increased hydrophobic interaction with stationary phase [12–14]. However, for the analysis of large polysaccharides (e.g. dextran, pullulan, agar, chitosan, cellulose, starch, pectin, etc.), SEC has been the major method used [15–18]. There has been no report on the use of RPLC for the separation of polysaccharides based on their size. In addition, separations described in the literature using RPLC have been carried out after the void volume. The separation window before the void volume is generally ignored and has not been explored.

In this work, we explored the unused separation window in RPLC for size separation of polysaccharides. The unique separation capability of this SEC-embedded RPLC was demonstrated in two applications: (1) separation of polysaccharides from other components in complex sample matrix; (2) separation of polysaccharide of different sizes.

2. Experimental

2.1. Materials

All chemicals were ACS grade or better unless otherwise indicated. Formic acid, sodium phosphate, sodium sulfate and dextrans were obtained from Sigma–Aldrich (Saint Louis, MO, USA).

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http://dx.doi.org/10.1016/j.chroma.2013.11.010

Table 1
Gradient profile of condition 1.

Time (min)	0	3	3.5	10	15	15.1	36
Flow rate (ml/min)	1	1	1	1	1	1	1
MP-A %	100	100	0	0	0	100	100
MP-B %	0	0	60	60	10	0	0
MP-C %	0	0	40	40	90	0	0

Ammonium hydroxide was purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile and isopropanol were purchased from EMD Chemicals (Billerica, MA, USA). Triflouracetic acid was purchased from Thermo Scientific (Rockford, IL, USA). De-ionized water was purified from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Polysaccharide–protein conjugate and ionic polysaccharide were produced in-house at Pfizer (Saint Louis, MO, USA). *Escherichia coli* RNA was purchased from Roche Diagnostics.

CN (PN:883995-905), C8 (300 Å, PN:883995-906), C8 (80 Å, PN:7995108-595) and C18 (PN:883995-902) columns were obtained from Agilent (Palo Alto, CA, USA). These columns have dimensions of 150 mm × 4.6 mm i.d, 5 μm particle size. The TSK-Gel G3000SWxl (PN: 7.5 mm × 30 cm, 5 μm) and GMPWxl (PN:08025, 7.5 mm × 30 cm, 10 μm) SEC columns were from Tosho Bioscience (San Francisco, CA, USA).

2.2. Instruments

Chromatographic experiments in this work were performed on an Agilent HP-1200 series HPLC system (Palo Alto, CA, USA). Components of the chromatograph include online degasser, quaternary gradient pumps, autosampler, a photodiode array UV detector, RI detector and an evaporative light scattering detector (ELSD). Instrument control, data acquisition and analysis were performed using Waters Empower chromatographic data system. For the SEC-MALLs and RPLC-MALLs experiments, an Agilent HPLC 1200 system with UV detector was coupled with an Optilab rEX Refractive index (RI) detector and a miniDawn multiple light scattering detector (MALLs) from Wyatt Technology (Santa Barbara, CA, USA). Determination of polysaccharide molecular weight and hydrodynamic radius (R_h) was performed by the ASTRA software.

2.3. Procedure for separation of polysaccharide from other components in polysaccharide–protein conjugate

Sample of polysaccharide–protein conjugate consists of polysaccharide–protein conjugate, unconjugated polysaccharide, unconjugated protein and histidine. Sample was directly injected if concentration of polysaccharide–protein is higher than 1.0 mg/ml, or diluted with 10 mM of histidine buffer (pH 7.0) to have 1.0 mg/ml polysaccharide–protein prior to injection. For SEC-embedded RPLC, loading of total polysaccharide (conjugated and unconjugated) was about 7 μg.

Separation was performed on a CN column, 150 mm × 4.6 mm i.d. 5 μm (Agilent, Palo Alto, CA, USA). Two different mobile phases and gradients were used. For condition one, mobile phase A (MP-A): 50 mM ammonium formate, pH 4.5, acetonitrile 20%; mobile phase B (MP-B): 0.1% TFA in water; mobile phase C (MP-C): 0.1% TFA in acetonitrile; gradient: see Table 1. For condition two, MP-A: 0.2% TFA in water; MP-B: 0.15% TFA in isopropanol; gradient, see Table 2. UV detection at 220 nm.

For conventional SEC, loading of total polysaccharide was 20 μg. Separation was performed on a Tosoh G3000SWxl, 300 mm × 7.8 mm i.d. 5 μm with flow rate 1 ml/min; mobile phase: 20 mM sodium phosphate, 50 mM sodium sulfate, pH 6.8 and detection with UV 220 nm.

2.4. Procedure for separation of polysaccharide from other components in fermentation broth

Samples from *E. coli* fermentation broth were centrifuged at 10,000 g for 5 min to pellet cell debris. Supernatant was transferred to an HPLC vial for analysis. For SEC-embedded RPLC, separation was performed with CN column, 150 mm × 4.6 mm i.d. 5 μm with RI detection. The detection cell temperature was 30 °C. Other conditions were the same as those for the polysaccharide-conjugate (Table 1). For conventional SEC, separation was performed with a GMPWxl, 300 mm × 7.8 mm i.d. 10 μm. Flow rate is 0.8 ml/min. Mobile phase is 0.1 M sodium phosphate, 150 mM sodium chloride, 0.1 mM EDTA, pH 6.7. Detection is RI with detection cell temperature at 30 °C.

2.5. Procedure for RPLC-MALLs of polysaccharide

Polysaccharide samples were prepared at 1 mg/ml in water. 15 μg of sample was loaded onto the column. The flow rate was 0.5 ml/min. Isocratic elution for 10 min with MP: 50 mM ammonium formate, pH 4.5, 20% acetonitrile. 24 kDa dextran was used for peak alignment, bandbroadening correction and normalization of MALLs detectors. Refraction index and dn/dc of the polysaccharide in RPLC mobile phase was measured with an Optilab rEX RI detector. RI detector temperature was 30 °C.

3. Results and discussion

3.1. Separation of polysaccharides, small molecules, polysaccharides–protein conjugates, protein and DNA

Polysaccharide–protein conjugates have been widely developed as vaccines against different infectious diseases. Quantitation of unconjugated or free polysaccharide and protein is needed to ensure consistency in efficacy and safety of the vaccine product. Existing methods for free polysaccharide based on high performance anion exchange chromatography (HPAEC) involve extensive sample preparation to precipitate conjugate and protein prior to analysis [19]. The CE method described by Nunnally can separate free polysaccharide from conjugate and protein, but suffers from poor reproducibility and low robustness [20]. Our exploratory work started with the evaluation of a simple RPLC method for the separation of free polysaccharide from polysaccharide–protein conjugates. The polysaccharide is capsular polysaccharide from bacteria [21]. Initially, it was thought free polysaccharides would elute at the void volume or slightly after the void volume since they are extremely polar and should have no retention or very weak retention on RP stationary phase. *To our surprise, it was observed that polysaccharide eluted* before histidine. As it is highly polar small molecule, histidine is hardly retained on hydrophobic stationary phase. Similar to elution volume of other highly polar small molecules including urea, thiourea, phloroglucinol [22,23], elution volume of histidine can be considered as an estimate of void volume (Fig. 1) in reverse phase column. In RPLC separation, the focus has been solely on peaks that elute after void volume, and rarely to peaks that elute before void volume. The question became why did the polysaccharide peak elute before the void volume. The RP column used in this investigation has a CN stationary phase with 300 Å pore size in 5 μm particles. The 300 Å pore size allows large molecules to diffuse into pores for both hydrophobic interaction and steric inclusion. For highly polar polysaccharides under the conditions used in this work (MP contains 50 mM ammonium formate, pH 4.5, 20% acetonitrile), hydrophobic interaction was minimized and size exclusion became the predominant mechanism. Free protein and polysaccharide–protein conjugate are much

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