Application note | 000472

HPAE-PAD

HPAE-PAD method for determination of Hib capsular polysaccharide content

Authors

Sachin Patil and Jeffrey Rohrer, Thermo Fisher Scientific, Sunnyvale, CA, USA

Keywords

Haemophilus influenzae, ribitol, vaccine, Dionex CarboPac MA1 column, Dionex ICS-5000+ system, palladium hydrogen reference electrode, Dionex ICS-6000 system

Goal

Demonstrate an HPAE-PAD method for the determination of ribitol in Hib capsular polysaccharide acid hydrolysis samples

Introduction

Haemophilus influenzae type b (Hib) causes serious infections such as pneumonia and meningitis in young children. It also causes other serious invasive diseases, including epiglottitis, septic arthritis, osteomyelitis, and pericarditis. The capsular polysaccharide, consisting of a repeating unit of 5-D-ribitol-(1→1)-β-D-ribose-3-phosphate,[1] plays an important role in the virulence of the organism. After the initial vaccine, which was simply the purified polysaccharide (PRP), more immunogenic polysaccharide-protein conjugate Hib vaccines were developed. The conjugate vaccines have shown that they are effective in treating Hib infections in infants.[2] The conjugate vaccine consists of PRP oligosaccharides (oligo-Hib) covalently attached to a carrier protein.[3] Because vaccine stability is important for vaccine effectiveness, stability needs to be monitored in bulk conjugates as well as final production lots. The Hib vaccine production process also needs to be monitored during processing steps.[4] Different methods used for this purpose include colorimetric assay, ribose assay, phosphorus assay, NMR, and high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAE-PAD).[5]

HPAE is a well-established method for separating carbohydrates and employs specific interactions between the hydroxyl and carboxyl groups of carbohydrates and the stationary phase. The separation is based on charge, size, composition, and linkage isomerism. When hyphenated with PAD (HPAE-PAD), this approach offers high chromatographic resolution and high sensitivity for directly determining monosaccharides, sialic acids, and other carbohydrates. Thus, no sample derivatization is required. This saves analyst time and expense, while eliminating exposure to hazardous derivatization chemicals. PAD is a selective and sensitive detection technique. HPAE-PAD is routinely used for the analysis of Haemophilus influenzae type b (Hib) capsular polysaccharide-based vaccines after total acid or alkaline hydrolysis.[5–12]

This work describes an HPAE-PAD method for the determination of ribitol in Hib capsular polysaccharide acid hydrolysis samples.[5,11] Separation of ribitol from TFA acid hydrolysis products was achieved under isocratic elution conditions using a Thermo Scientific™ Dionex™ CarboPac™ MA1 column. The method proposed here was validated for performance with respect to linearity, precision, sensitivity, and accuracy. The acid hydrolysis samples were also tested using a palladium hydrogen reference electrode (PdH).

Experimental

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free™ Ion Chromatography system*

The Dionex ICS-5000+ system is an integrated ion chromatograph that includes:

  - DP Dual Pump (P/N 061712) with degas option
  - DC detector compartment (P/N 061767) with single-temperature zone
  - Electrochemical detector (P/N 061719) and cell (P/N 061757)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Palladium hydrogen reference electrode (P/N 072075)
  - Carbohydrate disposable Au working electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 066480)
  - 10 µL sample loop

- AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt P/N 72.692.005)
- Savant™ SpeedVac™ medium capacity vacuum concentrator (P/N SPD140DDA-230)

*Note: These analyses can also be run on a Dionex ICS-6000 system

Software

Thermo Scientific™ Chromeleon™ Chromatography Data system (CDS) version 7.2.10, or higher

Conditions

Columns	Dionex CarboPac MA1, 4 × 250 mm analytical column (P/N 044066), Dionex CarboPac MA1 4 × 50 mm guard column (P/N 044067)
Column temperature	30 °C
Compartment temperature	25 °C
Flow rate	0.5 mL/min
Eluent	400 mM Sodium hydroxide
Injection volume	10 µL (Full_Loop)
Typical backpressure	~1,600 psi* (100 psi = 698.5 kPa approximately)
Working electrode	Gold disposable on PTFE (P/N 066480)
Gasket (PTFE) for disposable electrode	2 mil (0.002, 1 mil = 25.4 µm) (P/N 060141)
Waveform	Carbohydrate 4-potential waveform for Ag/AgCl and PdH reference electrode (Table 1)

*Never run the Dionex CarboPac MA1 column above 2,000 psi.

Table 1. Carbohydrate 4-potential waveform for the electrochemical detector (ED)

Reference electrode type	Time (s)	Potential (V)	Gain	Ramp region	Integration
Ag/AgCl**	0	0.1	Off	On	Off
	0.2	0.1	On	On	On
	0.4	0.1	Off	On	Off
	0.41	-2	Off	On	Off
	0.42	-2	Off	On	Off
	0.43	0.6	Off	On	Off
	0.44	-0.1	Off	On	Off
	0.5	-0.1	Off	On	Off
PdH	0	1.07	Off	On	Off
	0.2	1.07	On	On	On
	0.4	1.07	Off	On	Off
	0.41	-1.03	Off	On	Off
	0.42	-1.03	Off	On	Off
	0.43	1.57	Off	On	Off
	0.44	0.87	Off	On	Off
	0.5	0.87	Off	On	Off

**Reference electrode used in Ag mode (Ag/AgCl reference)

Reagents

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Sodium hydroxide (NaOH), 50% w/w (Fisher Scientific P/N SS254-500)

Standards

- Ribose (Sigma, P/N R7500)
- Ribitol (Sigma P/N 02240)
- *Haemophilus Influenzae* Polysaccharide Polyribosyl Ribitol Phosphate (PRP), 2nd International Standard (NIBSC P/N 12/306)

Stock and calibration standards

Solid standards were dissolved in DI water at 10 g/L concentrations. These were further diluted with DI water to yield the desired mixture concentrations.

To determine the linear calibration range, a 10 g/L solution of ribitol was diluted 10-fold to a 1.0 g/L standard solution using DI water. The 1.0 g/L standard solution was further diluted 10-fold to a 100 mg/L solution. The 100 mg/L solution was diluted with DI water to ribitol concentrations ranging from 0.1 to 5 mg/L. All solutions were maintained at -20 °C until needed.

Acid hydrolysis

The PRP polysaccharides standard was dissolved in DI water to yield a 1 mg/mL solution. This solution was further diluted to yield a 10 µg/mL solution. Next, 250 µL of this solution (or DI water for negative control) was mixed with 250 µL of 4 N TFA and incubated at 100 °C for 2 h. At 2 h, all samples were dried in a vacuum concentrator at room temperature and resuspended in 500 µL DI water. All samples were further diluted 2- to 32-fold with DI water and injected directly.

Eluents

400 mM sodium hydroxide

It is essential to use high quality deionized (DI) water of high resistivity (18 MΩ·cm or better) containing as little dissolved carbon dioxide as possible. Biological contamination should be absent. Plastic tubing in the water system should be minimized, as it often supports microbial growth, which can be a source of carbohydrate contamination. It is imperative to minimize contamination with carbonate, a divalent anion at pH >12, because it binds strongly to the columns and interferes with carbohydrate chromatography, causing a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide. Dilute 20.8 mL of the 50% sodium hydroxide solution into 1 L of DI water to prepare a 0.4 M solution. After preparation, keep the eluent blanketed under UHP grade nitrogen at 34 to 55 kPa (5 to 8 psi) at all times. More details on eluent preparation for HPAE-PAD can be found in Technical Note 71.[12]

Results and discussion

Separation

Separation of ribitol from acid hydrolysis reactions was achieved using a Dionex CarboPac MA1 4 × 250 mm column under isocratic elution conditions using 400 mM sodium hydroxide. Figure 1 shows a standard containing 2 mg/L each of ribitol and ribose. Figure 2 shows the chromatogram of a 4-fold diluted acid hydrolysis reaction solution. The ribitol peak is well resolved from an adjacent peak. The resolution between the two peaks is 1.7. Total method run time is 35 min.

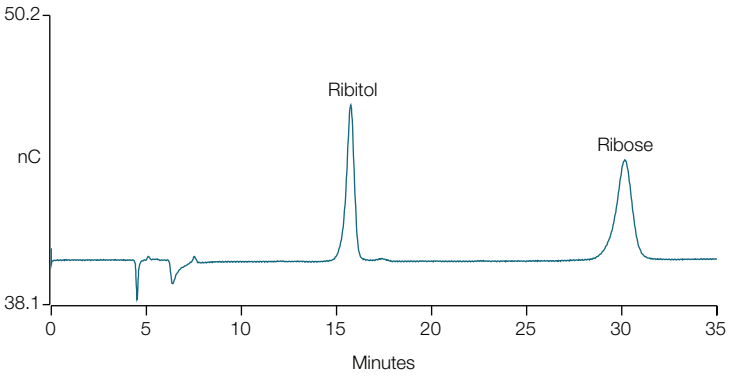

Figure 1. Analysis of a standard containing 2 mg/L ribitol and ribose on a Dionex CarboPac MA1 column

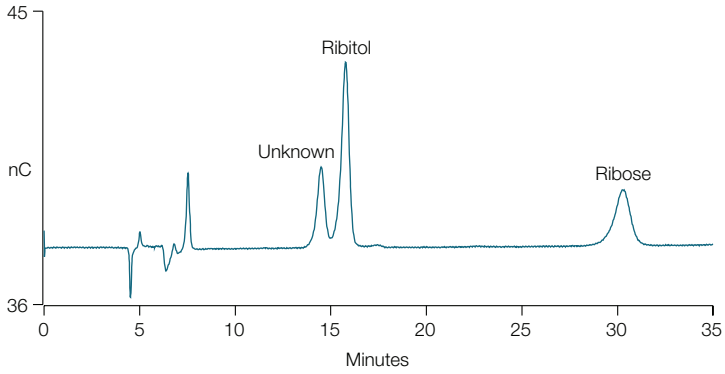

Figure 2. Analysis of 4-fold diluted acid hydrolysis sample on a Dionex CarboPac MA1 column

Linearity and precision

Linearity studies were conducted using calibration standards containing ribitol concentrations from 0.1 to 5 mg/L (9 levels) and making three injections of each concentration. The coefficient of determination value of 0.999 obtained using a linear curve fit indicates good method linearity (Figure 3).

Assay precision was evaluated by injecting three replicates at two ribitol concentration levels, 0.2 and 1 mg/L, and expressed as the RSDs of retention time and peak area from the series of measurements. The retention time RSDs were ≤ 0.05%, and the peak area RSDs were ≤ 2.93% (Table 2).

Table 2. Ribitol retention time and peak area precisions (n=5)

Concentration (mg/L)	RSD	
	RT	Peak area
0.2	0.03	2.92
1	0.05	2.3

Sensitivity

Method sensitivity was determined by analyzing ribitol standards and adjusting concentrations until S/N ratios of ~3 (LOD) and ~10 (LOQ) were obtained. To determine the LOD and LOQ, the baseline noise was first determined by measuring the peak-to-peak noise in a representative 1-min segment of the baseline where no peaks elute but close to the peak of interest. The signal was determined from the average peak height of three injections of ribitol. The LOD and LOQ values for ribitol were 0.05 mg/L and 0.017 mg/L, respectively. Figure 4 shows chromatograms obtained using 0.05 and 0.017 mg/L injections of ribitol.

Accuracy

Method accuracy was determined by spiking the acid hydrolyzed PRP samples. Unspiked sample had a PRP value of 4.6 mg as compared to theoretical value of 5 mg. This yield is consistent with that reported by NIBSC, which showed a similar PRP yield.[5] Spike recovery experiments were performed at two levels. Results included in Table 3 show good method accuracy.

Table 3. Ribitol spike recovery study (n=3)

Amount spiked (mg/L)	Amount recovered (mg/L)	Recovery (%)
0	0.46	—
0.2	0.65	94.0
1	1.42	95.9

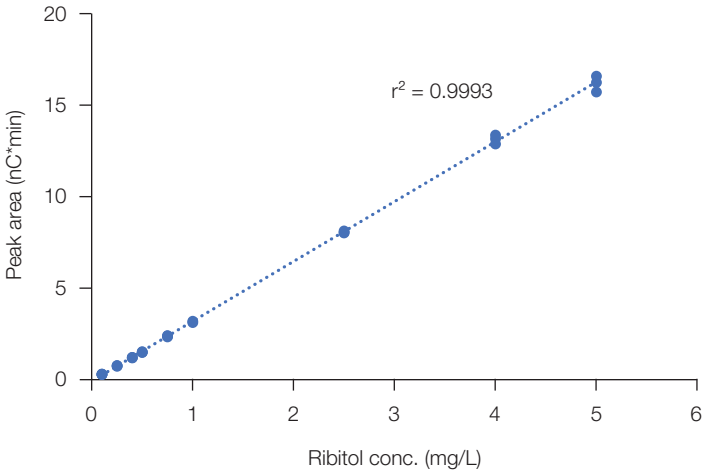

Figure 3. Linearity study (n=3)

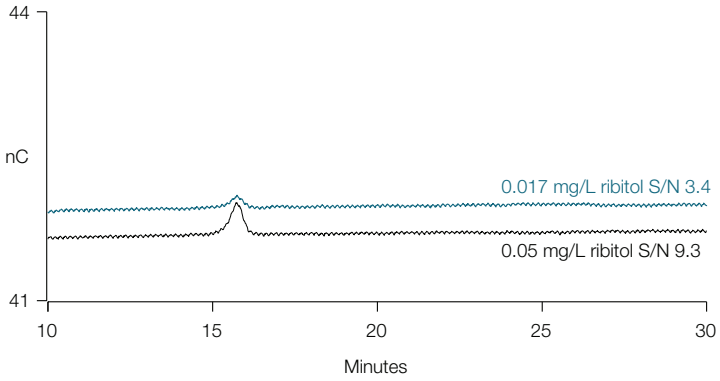

Figure 4. Analysis of standards containing 0.017 and 0.05 mg/L ribitol on a Dionex CarboPac MA1 column

Peak area response stability using a palladium hydrogen reference electrode

The acid hydrolysis samples were tested using a palladium hydrogen reference electrode (PdH). The PdH reference electrode is proposed to have a longer life span than the Ag/AgCl reference electrode. The use of a PdH reference electrode for this assay may offer significant cost savings. Performance of a PdH reference electrode has been previously shown to be comparable to that of an Ag/AgCl reference electrode using three HPAE-PAD applications.[13,14] Moreover, shorter- and longer-term stability studies showed good PdH reference electrode stability. The short-term stability study consisted of response measurements for 50 consecutive injections. In the long-term stability study, responses were measured six months apart.[13]

Here, for evaluating the stability of the PdH reference electrode, the Ag/AgCl reference electrode was replaced with the PdH reference electrode. A 4-fold diluted acid hydrolysis sample was injected in triplicate for seven consecutive days. The initial peak area responses generated on the PdH as well as the Ag/AgCl reference electrode are similar as shown in Figure 5 (Day 1, Ag/AgCl and PdH responses). Moreover, when using the PdH reference electrode, the peak area does not show significant change over seven days (Figure 5, Day 1 to 7, blue bars).

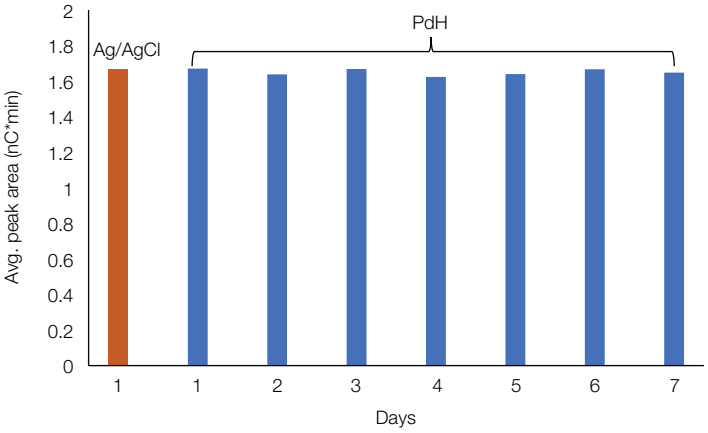

Figure 5. Stability of peak area response using a PdH reference electrode (n=3 measurement/day)

Conclusion

A method for the determination of PRP content in Hib capsular polysaccharide acid hydrolysis samples was developed using a Dionex CarboPac MA1 column. The ribitol peak was well resolved from an adjacent peak, and total run time was 35 min. The method demonstrated excellent precision and accuracy. The acid hydrolysis samples were also tested using a palladium hydrogen reference electrode. The palladium hydrogen reference electrode is proposed to have a longer life span than the Ag/AgCl reference electrode. The use of a palladium hydrogen reference electrode for this assay may improve method economics.

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