WHITE PAPER

Determination of polyribosylribitol phosphate (PRP) in *Haemophilus influenzae* vaccine using ion chromatography with pulsed amperometric detection

Haemophilus influenzae type B (Hib) is a major cause of bacterial meningitis in children in the United States and in many other developed countries. The capsular polysaccharide (PS) of Hib plays an important role in the virulence of the organism. Many pathogenic bacteria possess a polysaccharide capsule that encloses the cell, modulates the flow of nutrients to the cell surface, and protects against dehydration. When a bacterium establishes an infection in a mammalian host, the polysaccharide capsule hides cell surface components from elements of the mammalian immune system, such as antibodies and complement proteins that otherwise would activate mechanisms to kill the pathogen.

Metrohm

Capsular polysaccharides are high molecular weight polymers that contain a strict repeat unit. This repeat unit can be a single monosaccharide unit or can be an oligosaccharide unit that contains as many as eight sugar residues. The repeat units can be linear or branched, and are sometimes linked together by phosphodiester bonds. Structural studies have revealed that the Hib PS is a polymer made of a repeating unit of 5-D-ribitol-(1-1)-β-D-ribose-3-phosphate **[1]**.

To prevent Hib disease, a purified PS vaccine has been developed and is effective in children over 18 months of age. Although polysaccharide capsules are immunogenic in children and adults, development of a protective immune response may be too slow to defend the host against disease. In many cases, antibodies directed against the capsular polysaccharide are protective, and the prior existence of these antibodies prevents establishment of the infection—this is the basis for their use as vaccines or components of conjugate vaccines.

Capsular polysaccharides are T-cell–independent type 2 immunogens. Because of the delayed development of the relevant segment of the human immune system, unconjugated polysaccharide vaccines usually only induce a poor immune response in infants (less than 18 months old) and thus are not used for this population group **[2]**. To protect children younger than 18 months who are at higher risk for the disease, the PS has been coupled with protein carriers in order to improve its immunogenicity.

Covalent attachment of a capsular polysaccharide (or an oligosaccharide derived from it) to a protein carrier creates a glycoconjugate vaccine. The immune response to the saccharide component of conjugate vaccines is T cell-dependent and is similar to the response for proteins. The conjugate vaccines have induced antibodies against the PS in babies as young as a few months old, possibly by switching the PS from a T-independent antigen to a T-dependent antigen. Clinical trials have shown that Hib conjugate vaccines are very effective in preventing the disease in infants and children. There are four licensed Hib conjugate vaccines in the US.

QUALITY PARAMETERS FOR GLYCOCONJUGATE VACCINES

As vaccines are medical products for human use, they require rigorous characterization and assays to ensure final product quality and consistency **[3]**. For glycoconjugate vaccines, it is important to measure both free and total PS to ensure its quality. The proportion of unconjugated PS must be monitored as the presence of large amount of unconjugated PS may suppress immunity to the antigen **[2]**. Additionally, the presence of free PS is a key indicator of process consistency and is an indirect measure of the covalent attachment to the carrier.

The polysaccharide concentration must be confirmed as it is directly related to the product quality. Currently, the PS content of the conjugate vaccine is measured colorimetrically as ribose using an orcinol assay method **[1, 4]**. If a vaccine contains a sugar stabilizer such as lactose, it is not possible to measure the PS content by the orcinol assay method due to severe interference by the sugar stabilizer **[1]**.

An alternative indirect nephelometric assay **[1]** based on the rate of antigen(PS)-antibody aggregation has been applied to measure the PS content of a conjugate vaccine containing lactose as a stabilizer. This method requires special reagents (e.g. the antibody to the PS) and the values obtained reflect only approximate amounts of the PS in the conjugate vaccine. These values are not precise and reliable.

ANALYSIS OF POLYSACCHARIDES BY HPAEC-PAD

High-performance anion-exchange chromatography (HPAEC) in alkaline conditions with pulsed amperometric detection (PAD) is now used to analyze monosaccharides, disaccharides, oligosaccharides, and other sugars **[1, 3–5]**. This technique is used to determine a range of carbohydrates in different sample matrices.

Carbohydrates are weak acids with pK_a values in the range of 12–14. Consequently, at high pH values their hydroxyl groups are either partially or totally transformed into oxyanions, enabling this class of compounds to be selectively eluted as anions by HPAEC in a single run.

Under alkaline conditions, carbohydrates are readily separated by quaternary-ammonium-bonded anion-exchange columns, where the order of increasing retention is correlated with decreasing pK_a value. HPAEC-PAD easily separates and detects carbohydrates without the need of analyte derivatization.

The PS in the vaccine is first depolymerized in alkaline medium to its single repeating unit: ribitolribose phosphate. Then, the PS repeating unit in the alkali-treated vaccine is separated from a large amount of the sugar stabilizer (if present) by HPAEC, and then quantified using the amperometric detection method **[1, 3–5]**. The procedure is simple and the sensitivity is much better than with other assays.

PULSED AMPEROMETRIC DETECTION (PAD)

In amperometric detection, a potential is applied between the working and reference electrode, and the resulting current is then measured between the working and auxiliary electrode. In the pulsed amperometric detection (PAD) mode, three different potentials are applied (E1, E2, and E3) in three different steps (**Figure 1**). E1 corresponds to the working potential, while E2 and E3 are the cleaning potentials. PAD mode is recommended for the analysis of carbohydrates.

Working electrode (WE): Gold
Reference electrode (RE): Ag/AgCl
Auxiliary electrode (AE): Stainless steel

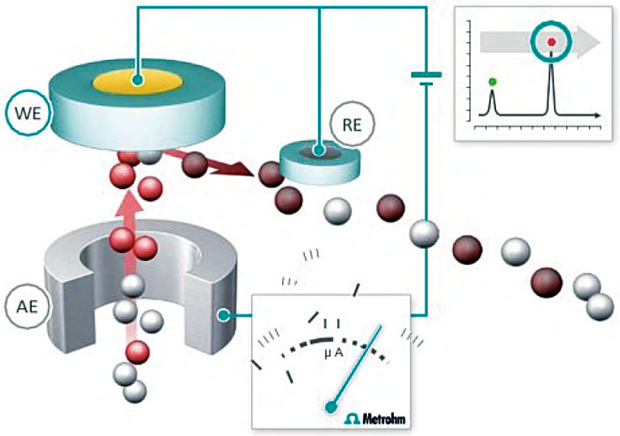


Figure 1. Pulsed Amperometric Detection (PAD)

INSTRUMENTS AND METHOD PARAMETERS

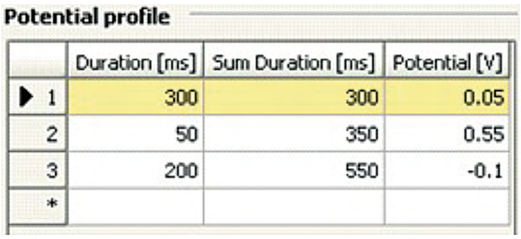
The general instrumentation setup (**Table 1**) consisted of a professional ion chromatograph (IC) equipped with a binary high pressure pump. A high capacity anion exchange column was used for separation and an amperometric detector was used for detection. The Metrohm 889 IC Sample Center was used for sample injection.

INSTRUMENT CONFIGURATION

Item	Article number
940 Professional IC Vario ONE/HPG	2.940.1140
IC Amperometric Detector	2.850.9110
889 IC Sample Center	2.889.0020
Thin-Layer cell	6.5337.210
Ag/AgCl reference electrode	6.1257.720
Separation column	Anion exchange column
MagIC Net 3.3 Professional	6.6059.332

Table 1. List of required items for the reported IC analysis using amperometric detection

Flow Rate	1.0 mL/min
Column temperature	Ambient
Sample volume	50 µL
Sampling Device	889 IC Sample Center
Recording time	20.0 minutes
Detector	Amperometric detector
Mode of analysis	PAD
Working electrode	Gold (Au)
Reference electrode	Ag/AgCl reference electrode



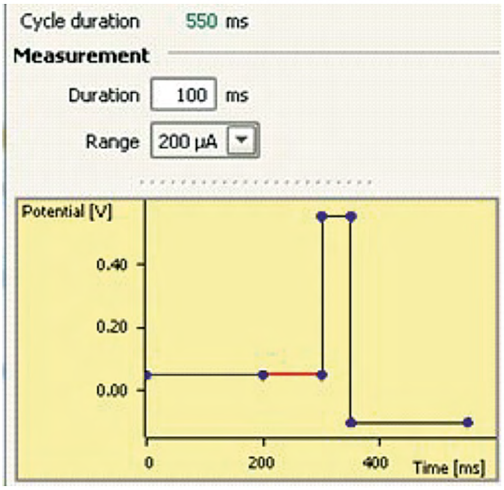


Table 2. Method parameters used for the analysis

SOLUTIONS

Chromatography	
Eluent A	300 mmol/L sodium acetate
Eluent B	100 mmol/L sodium hydroxide
Mobile phase composition	
Eluent A	50%
Eluent B	50%

HIB BUFFER SOLUTION

Hib buffer solution (0.2 mol/L NaCl and 10 mmol/L phosphate buffered at pH 6.0) was prepared by weighing 11.7 g sodium chloride, 0.54 g disodium hydrogen phosphate, and 1.33 g sodium dihydrogen phosphate into a 1000 mL volumetric flask, and then dissolving the solids by adding 800 mL ultrapure water (UPW). The pH was adjusted to 6.0 using HCl/NaOH and the content was diluted to 1000 mL with UPW.

CALIBRATION – PRP STANDARD

Calibration standards were prepared from the polyribosylribitol phosphate (PRP) stock solution (30 mg/L) by diluting with Hib buffer. All of the standards depolymerized in order to convert PRP to its monomeric form.

SAMPLE PREPARATION

Acid or base hydrolysis depolymerizes polysaccharides into oligosaccharides, monosaccharides, or smaller fragments that are polysaccharide-specific for the optimized hydrolysis conditions employed. Aggressive hydrolysis conditions can destroy some components of the polysaccharide. These fragments can be quantified directly by HPAEC-PAD.

PS Antigen	Acid Hydrolysis and HPAEC	Acid Hydrolysis, Fluorophore Labeling, and HPLC	HF Hydrolysis and HPAEC	Base Hydrolysis and HPAEC	Methanolysis and GC or HPAEC
PRP	Ribitol	–	Phosphate	PRP monomer	–

Chromatographic methods for the quantification of PRP in vaccine – USP general chapter 1234 **[2]**

TOTAL PRP ANALYSIS

A 0.4 mL aliquot of vaccine sample is diluted to 2 mL with Hib buffer. It is then depolymerized according to the procedure mentioned in the "**Depolymerization of PRP**" section below.

FREE PRP ANALYSIS

To quantify only free PRP, conjugated PRP has to be separated from the sample matrix. This can be done by solid phase extraction or chemical precipitation methods **[2]**. A 0.4 mL aliquot of vaccine sample is diluted to 2 mL with Hib buffer, then 1 mL of this diluted sample is passed through a C4 sample preparation cartridge to retain any conjugated vaccine. The C4 cartridge is then rinsed with 1 mL UPW (2-fold dilution). It is then depolymerized according to the procedure outlined in the "**Depolymerization of PRP**" section below.

DEPOLYMERIZATION OF PRP

A 2 mL portion of diluted sample/standard is added to a 2.5 mL vial. To this, 0.2 mL of 2 mol/L NaOH is added and the vial is closed and sealed. The vial content is mixed well by vortexing. It is then held at 55 °C ± 2 °C for approximately three hours in a temperature-controlled oven. After the depolymerization step, the vial is cooled to ambient room temperature and the content is filtered using 0.22 µm syringe filters.

OVERLAY OF LINEARITY STANDARDS

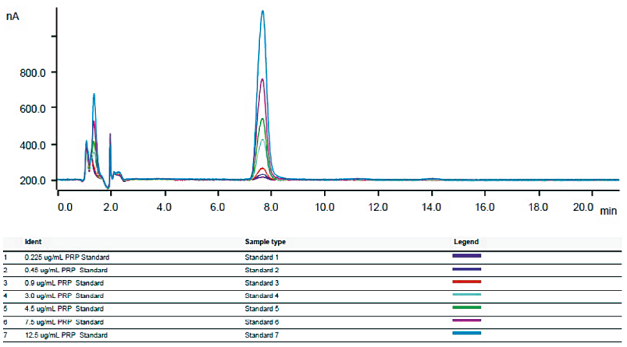
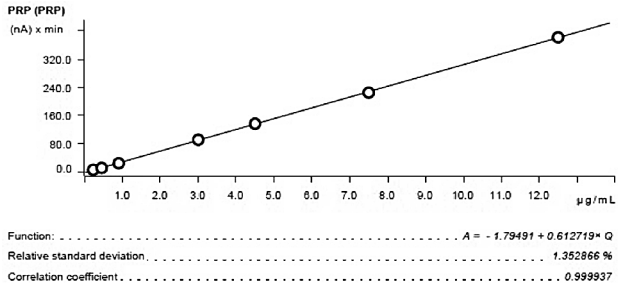


Figure 2. Overlay of linearity standards ranging from 0.225 mg/L to 12.5 mg/L

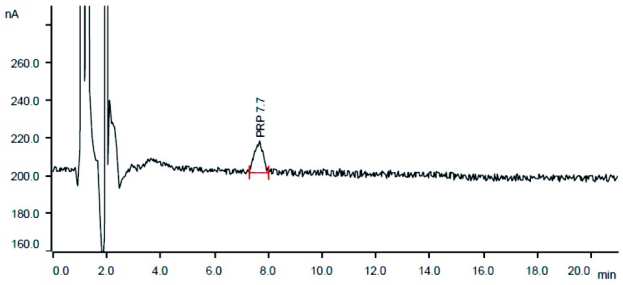
CALIBRATION CURVE



Function: . . . . . A = - 1.79491 + 0.612719* Q
Relative standard deviation . . . . . 1.352866 %
Correlation coefficient 0.999937

Sample type	Conc.	Area
Standard 1	0.225	6.236
Standard 2	0.450	11.429
Standard 3	0.900	24.631
Standard 4	3.000	91.465
Standard 5	4.500	136.955
Standard 6	7.500	225.208
Standard 7	12.500	382.252

Figure 3. Calibration curve of 0.225 mg/L to 12.5 mg/L PRP standard

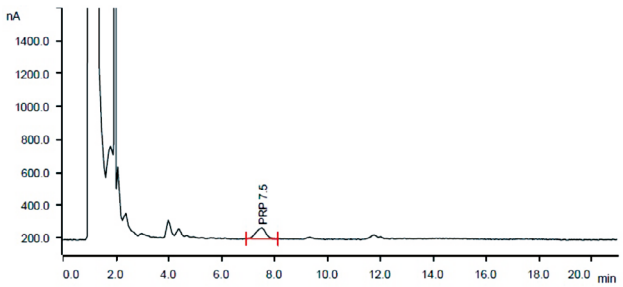


Peak number	Retention time min	Area (nA) x min	Height nA	Concentration µg/mL	Component name
1	7.7	6.2356	15.957	0.225	PRP

Figure 4. Results of analysis of a 0.225 mg/L PRP standard

SAMPLE ANALYSIS

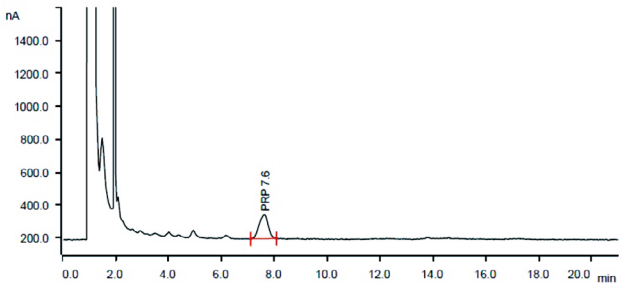
FREE PRP ANALYSIS



Peak number	Retention time min	Area (nA) x min	Height nA	Concentration µg/mL	Component name
1	7.5	29.3073	66.485	2.030	PRP

Figure 5. Example chromatogram of free PRP analysis

TOTAL PRP ANALYSIS



Peak number	Retention time min	Area (nA) x min	Height nA	Concentration µg/mL	Component name
1	7.6	57.7102	146.848	19.423	PRP

Figure 6. Sample chromatogram of total PRP analysis

RESULT

SAMPLE: HIB CONJUGATE VACCINE

Analyte	Concentration (mg/L)	Specification (mg/L)
Total PRP	19.42	16–24
Free PRP	2.03	< 20% of total PRP

SPECIFICATIONS

Total PRP should be within 80 to 120% of label claim, which is listed as 20 mg/L. Free PRP should be less than 20% of total PRP quantified. From the result table above, it is clear that both free and total PRP results are well within the required specifications.

SUMMARY

The Hib vaccine is used to prevent *Haemophilus influenzae* type B infections.

Unconjugated polysaccharide vaccines usually only induce a poor immune response in children under the age of 18 months and thus are not used for this population group. To protect infants younger than 18 months who are at higher risk for the disease, the PS has been coupled with protein carriers in order to improve its immunogenicity, forming a glycoconjugate vaccine.

As vaccines are medical products for human use, they require rigorous characterization and assays to ensure final product quality and consistency. For a glycoconjugate vaccine, it is important to measure both free and total PS to ensure its quality.

The PS in the vaccine is first depolymerized in alkaline medium to its single repeating unit, ribitolribose phosphate. Then, the PS repeating unit in the alkali-treated vaccine is separated from a large amount of the sugar stabilizer (if present) by HPAEC and quantified using an amperometric detection method. The Metrohm amperometric detector has superior sensitivity which results in excellent detection limits. Running costs are also significantly reduced, since disposable electrodes are not used.

All HPAEC-PAD results for the vaccine analysis are well within the required specification limits.

MagIC Net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Anion Eluent
IC Conductivity Detector
IC Conductivity Detector
MCS
A
B
Standard
Eluent Degasser
Sample Degasser
Standard
Eluent Degasser
Sample Degasser

References

[1] Tsai, C.; Gu, X.; Byrd, R.A. Quantification of polysaccharide in *Haemophilus influenzae* type b conjugate and polysaccharide vaccines by high-performance anion-exchange chromatography with pulsed amperometric detection. *Vaccine* **1994**, *12*(8), 701–706. **DOI:10.1016/0264-410X(94)90219-4**

[2] USP <1234> Vaccines for Human Use—Polysaccharide and Glycoconjugate Vaccines. In: USP 37–NF 32, Second Supplement. Rockville, MD: USP; 2014.

[3] Jeffrey S. Rohrer, J. S. Vaccine Quality Ensured by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. *SLAS Technology* **2019**, *25*(4), 1–9. **DOI:10.1177/2472630319890309**

[4] de Haan, A.; van der Put, R.; Beurret, M. HPAEC-PAD method for the analysis of alkaline hydrolyzates of *Haemophilus influenzae* type b capsular polysaccharide. *Biomed Chromatogr.* **2013**, *27*(9), 1137–1142. **DOI:10.1002/bmc.2918**

[5] van der Put, R.; de Haan, A.; van den Ijssel, J.; et al. HPAEC-PAD quantification of *Haemophilus influenzae* type b polysaccharide in upstream and downstream samples. *Vaccine* **2015**, *33*(48), 6908–6913. **DOI:10.1016/j.vaccine.2014.07.028**

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