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Development and validation of high-performance size exclusion chromatography methods to determine molecular size parameters of *Haemophilus influenzae* type b polysaccharides and conjugates

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ABSTRACT

Current vaccines against *Haemophilus influenzae* type b (Hib) consist of the polyribosyl ribitol phosphate (PRP) capsular polysaccharide chemically conjugated to a carrier protein. Among the various biological and physical analyses to be performed on these vaccines, the determination of the molecular size of the polysaccharide preparations throughout the conjugation process is particularly relevant. Comparison of results from high-performance size exclusion chromatography (HPSEC) with those routinely obtained using conventional gel permeation chromatography (CGPC) methods highlights the correlation between the two methods for determining the values of the chromatographic distribution coefficient (K_D) of native and activated polysaccharides. The resulting data showed that the K_D value is sufficient to characterize these polysaccharides using an HPSEC method. However, additional molecular size parameters (i.e., molar mass and hydrodynamic radius) are necessary for a reliable characterization of the tetanus conjugate (PRP-T), certainly due to the lattice-like structure of the conjugate. In practice, an absolute detection system in HPSEC composed of a low-angle light scattering detector, a viscometer, and a refractive index (RI) detector was used. As demonstrated, these HPSEC methods are rapid, accurate, and reproducible for the polysaccharides and their glycoconjugates and provide a relevant and more informative alternative to the current CGPC methods.

Haemophilus influenza type b (Hib)[1] is a major cause of bacterial meningitis in children under 5 years of age. Hib polysaccharide, a polymer of 5-D-ribitol-(1→1)-β-D-ribose-3-phosphate repeating unit (polyribosyl ribitol phosphate, PRP), is the protective immunogen used in commercially available anti-Hib vaccines [1].

Current Hib vaccines, based on polysaccharide conjugation to a protein (conjugate vaccine), are effective in infants, as demonstrated by the markedly reduced incidence of invasive Hib as these vaccines were introduced into routine infant immunization programs [2]. The tetanus conjugate vaccine is produced from native PRP chemically activated with adipic dihydrazide groups. During the activation process, in alkaline pH, the native PRP is partially depolymerized. This activated PRP (PRP-AH) is then covalently attached to the protein carrier tetanus toxoid to form conjugates (PRP-T).

The quality of a conjugate vaccine relies on specific immunochemical and physical properties of the polysaccharide preparations throughout the conjugation process [3]. Among the various biological and physical analyses to be performed, the determination of the molecular size is particularly relevant. Immunological properties and protective efficacy of vaccines have been shown to be directly correlated with molecular size and structural properties of polysaccharides and conjugates [4–7]. Moreover, this molecular size is critical for monitoring each step of the production process, as well as the stability of compounds, which involves verification of the depolymerization of the polysaccharide chains, proteolysis, or aggregation of the conjugates that may occur after storage at elevated temperature [8–11].

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[1] *Abbreviations used:* Hib, *Haemophilus influenza* type b; PRP, polyribosyl ribitol phosphate; PRP-AH, activated PRP; PRP-T, PRP tetanus toxoid; CGPC, conventional gel permeation chromatography; HPSEC, high-performance size exclusion chromatography; RI, refractive index; UV, ultraviolet; SDS, sodium dodecyl sulfate; LALS, low-angle light scattering; HPAEC, high-performance anion exchange chromatography.

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The molecular sizes of polysaccharide and conjugate preparations have been estimated by a variety of techniques, including conventional gel permeation chromatography (CGPC) with soft gels such as Sepharose CL-4B. In accordance with the current European Pharmacopoeia monograph for Hib conjugate vaccine [12], the percentage of polysaccharide eluted before a K_D value of 0.3 must not be less than 50% for PRP; for PRP-T, the percentage of polysaccharide eluted before a K_D value of 0.2 must not be less than 60% (Fig. 1).

Although appropriate, these CGPC methods are very time-consuming and require careful handling and large amounts of product, in contrast to high-performance size exclusion chromatography (HPSEC) methods that allow for quicker analyses and the use of small amounts of product and a large choice of detectors.

HPSEC has already been used to determine molecular size of bacterial polysaccharides [8,13–15] and conjugate vaccines [16–19].

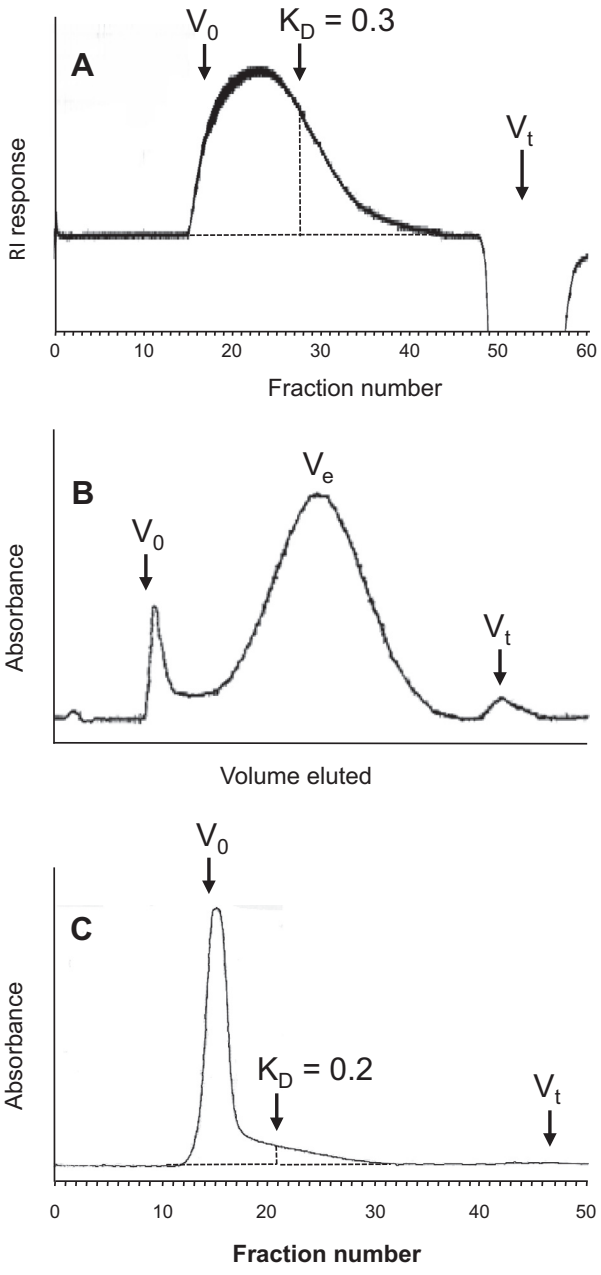

Fig.1. CGPC profiles for native PRP with RI detection (A), activated polysaccharide with UV detection at 206 nm (B), and conjugated polysaccharide consisting of Hib polysaccharide linked to tetanus toxoid with UV detection at 280 nm (C).

HPSEC with refractive index (RI) and ultraviolet (UV) detection have been used to determine the distribution coefficient (K_D) of the polysaccharides. These methods have been shown to be rapid and reproducible [8,16].

Static laser scattering and viscometric detection have been used to directly determine the distribution of molecular masses, hydrodynamic volume, and intrinsic viscosity for PRP and pneumococcal polysaccharide preparations [14]. Hennessey and coworkers [13] and D'Ambra and coworkers [15] demonstrated that molar mass estimation of native depolymerized PRP by secondary calibration using dextrans was not appropriate due to differences in hydrodynamic properties between dextrans (polysaccharide made with only glucose molecules) and other polysaccharides. Moreover, Jumel and coworkers [18] demonstrated that it is preferable to use an absolute detection system, such as static light scattering, for the determination of the molecular sizes of glycopolymers.

This article describes the development and evaluation of the performance of these methods for molecular size determination of PRP, PRP-AH, and PRP-T. A comparison of results from HPSEC with those routinely obtained using CGPC is discussed.

The HPSEC method offers the ability to determine additional molecular size parameters (i.e., molar mass and hydrodynamic radius), which allow for a reliable characterization of the PRP-T conjugates.

These new methods have been demonstrated to be a relevant alternative to the current CGPC assays for quality control of PRP, PRP-AH, and PRP-T due to their simplicity and reproducibility.

Materials and methods

PRP preparations for HPSEC analysis

All polysaccharides (native, activated, and conjugated) were obtained from Sanofi Pasteur Manufacturing Division (Marcy l'Etoile, France) and were representative of the current manufacturing process.

The total saccharide content for native, activated, and conjugated PRP was determined by the phosphorus method of Chen and coworkers [20]. For the PRP-T conjugate vaccine, the total protein content was determined by the Lowry method [21].

PRP samples were diluted in deionized water and adjusted to a final concentration of 250 μg/ml in sodium dodecyl sulfate (SDS, 0.1%, w/w).

PRP-AH samples were diluted to a final concentration of 250 μg/ml with phosphate buffer (0.2 M, pH 6.9).

PRP-T samples were adjusted in phosphate buffer (0.2 M, pH 6.9) to a final PRP concentration between 400 and 800 μg/ml.

Preparation of depolymerized polysaccharides

To establish whether the chromatographic conditions are sensitive enough to detect molecular size distribution modifications, the polysaccharides were subjected to a depolymerization process, either a basic hydrolysis or storage at 5 °C for a prolonged period of time:

- Three batches of native PRP were partially depolymerized with NaOH (10 mM) at room temperature for 5 min; six other batches were partially depolymerized with NaOH (10 mM) for either 5 or 15 min at 5 °C. Samples were neutralized by the addition of hydrochloric acid (100 mM).
- Ten batches of PRP-AH were partially depolymerized with NaOH (20 mM) at 10 °C for 5, 10, 15, or 30 min. Samples were neutralized by hydrochloric acid (100 mM).