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Journal of Chromatography A, 847 (1999) 209–211

JOURNAL OF CHROMATOGRAPHY A

Short communication

Determination of the molecular size distribution of *Haemophilus influenzae* type b–tetanus toxoid conjugate vaccines by size-exclusion chromatography

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Abstract

The molecular size of *Haemophilus influenzae* (Hib) type b polysaccharide–protein conjugate vaccines is an important physico–chemical parameter which correlates with immunogenicity. This paper describes the experimental conditions for high-performance size-exclusion chromatography on a PL Aquagel-OH 60 column to determine the size distribution of Hib high-molecular-weight conjugate vaccines.

Keywords: Vaccines; Polysaccharides; Proteins

1. Introduction

A type of vaccine against *Haemophilus influenzae* type b (Hib) is composed of the purified high-molecular-weight capsular polysaccharide polyribosyl–ribitol phosphate that, after derivatization with adipic dihydrazide, is covalently linked to formalin-detoxified tetanus toxin (PRP-TT) [1]. So far, there is no animal model for the evaluation of the clinical potency of Hib conjugate vaccines and therefore various in vitro physico–chemical procedures are used as indicator of the vaccine's in vivo specificity and immunogenicity [2]. One critical parameter for which consistency in manufacturing and formulation needs to be shown is the molecular size distribution of the vaccine. International requirements for Hib conjugate vaccines were introduced in 1991 [3] and the assessment of molecular size is requested for batch release and established by the European Pharmacopoeia [4]. This feature of Hib PRP-TT conjugate vaccine has already been evaluated by gel filtration chromatography with soft gel such as Sepharose and recently by fast protein liquid chromatography (FPLC) and high-performance size-exclusion chromatography (HPSEC) [5–7]. However, the columns used either in FPLC or in high-performance liquid chromatography (HPLC) had a fractionating range not optimal for this high-molecular-weight compound, because part of the product eluted at the void volume.

The present study was undertaken to determine whether the recently introduced PL Aquagel-OH 60

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00287-3

HPSEC column would be suitable for the determination of the molecular size distribution of Hib PRP-TT vaccine.

2. Experimental

2.1. Materials

The vaccine used throughout this study was Act-HIB (Pasteur Mérieux MSD). A freeze–dried, single human dose, containing about 10 μg of conjugated polysaccharide and 40 mg of sucrose as stabilizer, was resuspended in 200 μl of high-quality deionized water (≥18 MΩ) and 50 μl was injected (polysaccharide concentration, 50 μg ml^{-1}). Four different lots were analysed and for each lot three vials were tested in triplicate.

2.2. HPSEC analysis

The apparatus routinely used was a Dionex system (Sunnyvale, CA, USA) consisting of a quaternary gradient pump (GMP II), an eluent degas module, a variable-wavelength detector and a Rheodyne sample injector (Model 9126) equipped with a 50-μl sample loop. Dionex chromatography software AI-450 was used to program runs and to perform data analysis.

A PL Aquagel-OH 60 column (Polymer Labs., Germany) (300 mm×7.5 mm, 8 μm particle size) serially equipped with a PL Aquagel-OH guard column (50 mm×7.5 mm) was used. PL Aquagel-OH is a rigid macroporous packing for aqueous SEC with an extremely hydrophilic polyhydroxyl surface. The molecular weight resolving range of the column, determined by polyethylene oxides, is $2\cdot10^5$–$1\cdot10^7$. Elution was performed at 1 ml min^{-1} with 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 7.0 and the absorbance recorded at 214 nm [7]. The column was kept at a constant temperature of 25°C.

2.3. Determination of the distribution coefficient K_D

Void volume (V_0) and total volume (V_t) of the column were determined respectively with high-molecular-weight DNA (λ-DNA, Boehringer Mannheim, Germany) and sodium azide (Sigma). The elution volume (V_e) of the Hib conjugate vaccine was calculated from the peak of the absorbance curve and the distribution coefficient (K_D) was calculated according to Acker's formula: $K_D=(V_e-V_0)/(V_t-V_0)$ [8].

The percentage of vaccine eluted before a given K_D was calculated by Dionex chromatography software AI-450, according to the following formula:

$$\frac{\text{Area of vaccine elution curve before a given } K_D}{\text{Total area of vaccine elution curve}} \cdot 100$$

3. Results and discussion

Under the experimental conditions used in this study, V_0 corresponded to 6.77 ml and V_t to 11.42 ml. The elution profile of the vaccine composed of native Hib-polysaccharide bound to tetanus toxoid chromatographed on the PL Aquagel-OH 60 column had a symmetric peak eluting at 8.29±0.01 ml to which a K_D of 0.32 corresponded. A minor peak, eluting close to V_t, corresponded to the stabilizer.

HPSEC analyses were performed at a variety of sample concentrations to ensure that viscosity-related artifacts did not impact the results and sample recovery was complete. Upon variation of the nominal amount of Hib-conjugated polysaccharide injected onto the column from 10 to 50 μg ml^{-1}, the elution volumes or the peak shapes did not change,

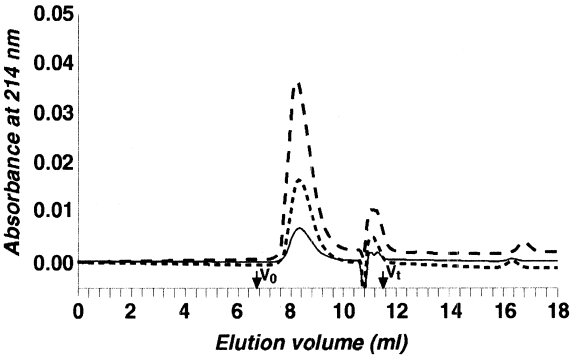

Fig. 1. Hib–PRP-TT conjugate eluted on the PL Aquagel-OH 60 column with 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 7.0 (flow-rate 1 ml min^{-1}). The vaccine was injected at the nominal value of 10 (—), 25 (---), 50 μg ml^{-1} (– – –) of Hib conjugated polysaccharide using a loop of 50 μl.

Table 1
Characteristic of *H. influenzae* type b vaccine after forced degradation at 37°C[a]

Days of treatment	K_D	Vaccine eluting with a $K_D \leq 0.5$ (%)
0	0.32±0.001	93.9±0.79
20	0.40±0.001	79.8±0.16
30	0.43+0.002	72.8±0.07

[a] Data are the mean and SD of multiple runs (three) of three vials for one lot.

while the peak heights and areas were proportional to the quantity of compound assayed (Fig. 1).

In order to establish also whether the chromatographic conditions were sensitive enough to detect conjugate molecular size distribution modifications, the vaccine, solubilized in water, was deliberately degraded. The elution profile of the vaccine did not change when the latter had been stored at 4°C during the shelf life, while, when it was stored at 37°C for different periods of time, the peak shifted in elution volume and the absorbance value varied (Fig. 2). The increasing K_D values as well as the decreasing percentage of product that eluted over time with a $K_D \leq 0.5$ demonstrated that the chromatographic conditions were sensitive in revealing progressive degradation of the compound (Table 1, Fig. 2). The $K_D \leq 0.5$ was selected on the basis that analysing four different lots of vaccine, 92.4% (SD±1.41) of the compound eluted before this value of K_D.

The same lots of PRP-TT vaccine examined in this study by PL Aquagel-OH 60 were in a previous work chromatographed using the TSKgel G5000PW$_{XL}$ column [7]. The same experimental conditions could be used for the two columns (flow-rate, mobile phase), but the fractionating range of PL Aquagel-OH 60 was better for this high-molecular-weight vaccine as it is not partially eluted in V_0. Thus, HPSEC can be successfully utilized to check batch to batch consistency of the size distribution of Hib high-molecular-weight conjugate vaccine. Furthermore, as the method can be used to evaluate single human doses of Hib vaccines, it might be particularly useful during studies on vaccine stability.

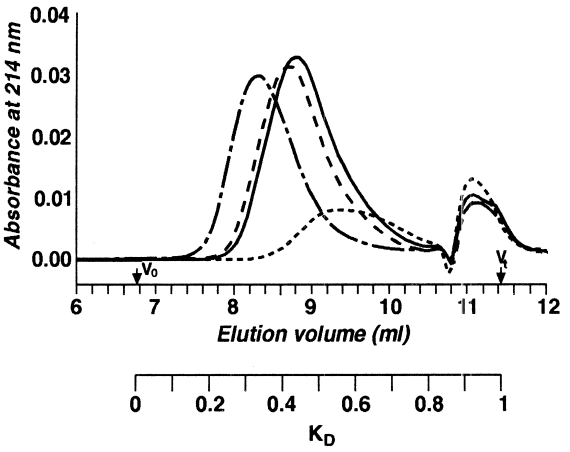

Fig. 2. HPSEC profiles for Hib–PRP-TT conjugate vaccine stored at 4°C (- – -) and after degradation induced by storage at 37°C for 20 (– – –), 30 (—) and 120 (---) days. K_D range (0→1) is represented by the second *x*-axis. The vaccine was injected at the nominal value of 50 μg ml^{-1} of Hib conjugated polysaccharide using a loop of 50 μl.

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