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HPAEC-PAD quantification of *Haemophilus influenzae* type b polysaccharide in upstream and downstream samples

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ABSTRACT

Due to the rapidly increasing introduction of *Haemophilus influenzae* type b (Hib) and other conjugate vaccines worldwide during the last decade, reliable and robust analytical methods are needed for the quantitative monitoring of intermediate samples generated during fermentation (upstream processing, USP) and purification (downstream processing, DSP) of polysaccharide vaccine components. This study describes the quantitative characterization of in-process control (IPC) samples generated during the fermentation and purification of the capsular polysaccharide (CPS), polyribosyl-ribitol-phosphate (PRP), derived from Hib. Reliable quantitative methods are necessary for all stages of production; otherwise accurate process monitoring and validation is not possible. Prior to the availability of high performance anion exchange chromatography methods, this polysaccharide was predominantly quantified either with immunochemical methods, or with the colorimetric orcinol method, which shows interference from fermentation medium components and reagents used during purification. Next to an improved high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) method, using a modified gradient elution, both the orcinol assay and high performance size exclusion chromatography (HPSEC) analyses were evaluated. For DSP samples, it was found that the correlation between the results obtained by HPAEC-PAD specific quantification of the PRP monomeric repeat unit released by alkaline hydrolysis, and those from the orcinol method was high ($R^2 = 0.8762$), and that it was lower between HPAEC-PAD and HPSEC results. Additionally, HPSEC analysis of USP samples yielded surprisingly comparable results to those obtained by HPAEC-PAD. In the early part of the fermentation, medium components interfered with the different types of analysis, but quantitative HPSEC data could still be obtained, although lacking the specificity of the HPAEC-PAD method. Thus, the HPAEC-PAD method has the advantage of giving a specific response compared to the orcinol assay and HPSEC, and does not show interference from various components that can be present in intermediate and purified PRP samples.

1. Introduction

Due to the need in recent years for a more rapid introduction of *Haemophilus influenzae* type b (Hib) conjugate vaccines worldwide [1–3], an improved production process, based on publicly

Abbreviations: CPS, capsular polysaccharide; DSP, downstream processing; ESTD, external standard; Glc, glucose; Glc-6-P, D-glucose-6-phosphate; Hib, *Haemophilus influenzae* type b; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; HPSEC, high performance size exclusion chromatography; IPC, in process control; kDa, kilo Dalton; μRIU, micro-refractive index unit; M_w, absolute weight-average molecular mass; MWCO, molecular weight cut-off; NA, nucleic acids; NaAc, sodium acetate; NaOH, sodium hydroxide; OD, optical density; PBS, phosphate buffered saline; PRP, polyribosyl-ribitol-phosphate (Hib CPS); Rib, D(−)-ribose; RI, refractive index; RIVM, National Institute for Public Health and the Environment; RU, repeating unit(s) of the polysaccharide; t_R, retention time; USP, upstream processing; WFI, water for injection; WHO, World Health Organization.

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[1] Intravacc is the new governmental Institute, working under The Dutch Ministry of Public Health, Welfare and Sport, responsible for the research and development activities in the field of translational vaccinology. It originates from the former Vaccinology Unit and the Laboratory Animal Facility of the National Institute of Public Health (RIVM) and the Netherlands Vaccine Institute (NVI).

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available data [4] has been developed at the National Institute for Public Health and the Environment (RIVM) of the Netherlands [5]. This process has been transferred to different vaccine producers belonging to the Developing Countries Vaccine Manufacturers Network (DCVMN) [6]. The RIVM and Intravacc1 in the Netherlands had already been involved in technology transfer of various other vaccine production processes for several decades [7].

The Hib bacterium was cultivated in a rich yeast-based medium, and the Hib capsular polysaccharide (CPS), poly-ribosylribitol phosphate (PRP) [8,9], was purified by differential precipitation using detergents and alcohol [5]. In order to evaluate the process steps during fermentation (upstream processing, USP) and purification (downstream processing, DSP), several quality control (QC) tests, both immunochemical (*e.g.* ELISA), physicochemical (*e.g.* high performance size exclusion chromatography (HPSEC) (both developed in-house) and colorimetric (orcinol), have originally been used. However, these tests can be influenced by matrix components (*e.g.* fermentation medium, detergents and alcohol) and are consequently not unconditionally applicable as in-process controls (IPC). Preferably, more specific methods should be used [10–14], fit for real-time monitoring. In order to meet these needs, a high performance anion exchange chromatography method with pulsed amperometric detection (HPAEC-PAD) has been developed [15], and evaluated, for its potential to overcome the aforementioned matrix effects. Correlation between the results obtained by HPAEC-PAD quantification of the PRP monomeric repeat unit (RU) released by alkaline hydrolysis [10–13,16], and those from the orcinol colorimetric assay (method adapted from Ashwell [17]) was expected to be high for DSP samples. USP samples which cannot be analyzed directly by the orcinol method due to interference by fermentation medium components (*e.g.* glucose), have so far been tested by a PRP-specific ELISA developed in-house. This ELISA has never been fully validated at RIVM, and therefore was not evaluated in this study. In addition, an HPSEC method that affords more complete information (*i.e.* multiple UV wavelength and refractive index detection [6]) than simple immunological and physicochemical tests has been used for the real-time monitoring of both USP and DSP. In this manuscript, these three different analytical methods (orcinol, HPAEC-PAD and HPSEC) have been evaluated for their potential to allow monitoring of the Hib vaccine production processes.

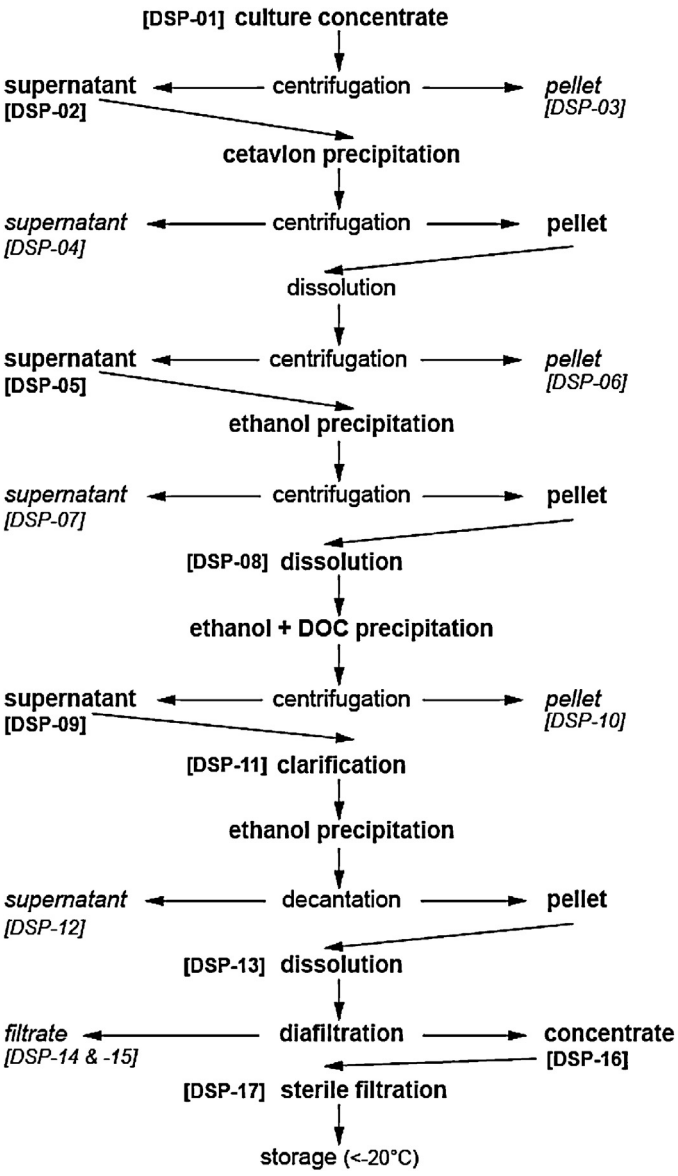

Fig. 1. Purification (DSP) of PRP by differential fractionation [5]. In bold: product fractions; in italic: waste fractions.

2. Materials and methods

2.1. Chemicals

All chemicals were of the highest possible grade: D-glucose-6-phosphate, sodium salt (Glc-6-P, M_W 282.12; Sigma nr. G 7879); D(−)-ribose (Rib, M_W 150.13; Acros Chimica nr. 13236); ferric chloride hexahydrate (Acros Organics nr. 21709); hexadecyltrimethyl-ammoniumbromide (Cetavlon, Merck nr. 8.14119); hydrochloric acid (Merck nr. 1.00317); orcinol (Acros Organics nr. 12955); sodium acetate (NaAc; Fluka nr. 71183); sodium deoxycholate (DOC, Merck nr. 1.06504); sodium hydroxide (NaOH, 50% solution; Baker nr. 61007067); Phosphate-buffered saline pH 7.2 (PBS, 10 mM sodium phosphate, 150 mM sodium chloride); PRP reference material (RIVM lot 00Hib350-1/2-7.1; 0.552 mg/mL (orcinol assay [17]) [5].

2.2. Hib fermentation

Batch fermentation took place in a 5 L bioreactor (Applikon, BL90) with a working volume of 3.5 L. The process as described before was followed [5]. Fermentation samples were collected hourly and more often at critical points (*i.e.* maximum OD, pH set point 7.5 and after cooling overnight). Optical density (OD_{590}) was measured using a standard 6300 (Jenway) spectrophotometer.

2.3. PRP purification

PRP purification took place at 100 mL scale. The process as described before was followed [5]. Diafiltration was performed using a standard Millipore, Pellicon XL filter on a Labscale (Millipore) system. All samples generated during each purification step (see Fig. 1) were collected and analyzed.

2.4. Orcinol assay

Performed as described by Ashwell [17]. Essentially PRP was quantified using D(−)-ribose (Rib) as the standard (5–60 nmol). All samples, and the PRP reference material (RIVM) used as positive control, were pre-diluted in triplicate to contain ~30 nmol of PRP (middle of the calibration curve for most accurate measurement). PRP concentrations were derived from the calculated mass of the

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