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A practical approach to optimization and validation of a HPLC assay for analysis of polyribosyl-ribitol phosphate in complex combination vaccines

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Abstract

The use of multi-factor statistical experimental design methodology minimized the vaccine material and laboratory resources required for optimization and validation of an HPLC assay for quantitation of deploymerized and total PRP. Components of the assay selected for optimization were adjuvant dissolution, ultracentrifuge conditions including ultracentrifuge model, sample diluent, mobile phase and column oven temperature. Previous experience has shown these components of the assay to be most troublesome and therefore required optimization prior to validation. Specificity, linearity, precision, accuracy and ruggedness were confirmed through a validation of the optimized assay. The validation also established the assay to be stability indicating, by showing that changes to the integrity of the PRP-OMPC conjugate could be detected.

Keywords: PRP; Assay validation; Assay optimization; Combination vaccine; HPLC; Design of experiment

1. Introduction

Polyribosyl-ribitol phosphate (PRP) conjugate vaccines protect against *Haemophilus influenzae* type b infection, a causative agent of bacterial meningitis and other serious systemic bacterial diseases in young children worldwide. It has been documented in the literature that PRP needs to be conjugated to a protein carrier in order to be immunogenic in infants [1]. In a multi-valent combination vaccine containing aluminum adjuvant, different species of PRP can be found: unconjugated PRP (also referred to as free-PRP), depolymerized-PRP (smaller chain-lengths of PRP conjugate), and full chain-length of PRP-conjugate. Depolymerized (d-PRP) and free-PRP are created by hydrolysis of the phosphodiester bond between the PRP monomers releasing PRP fragments at a rate that is affected by temperature and by interactions with the adjuvant or divalent cations present in the vaccine [2–4]. As the amount of d-PRP and free-PRP increases in the vaccine, the product is thought to be less immunogenic and consequently, these species become an important stability attribute to monitor [5–7]. The measurement of d-PRP and free-PRP is challenging and becomes increasingly so with the addition of other antigens, such as recombinant hepatitis B surface antigen, acellular pertussis components, diphtheria and tetanus toxoids, and polio antigens. A modified high performance anion exchange chromatography method with pulse amperometric detection (HPAEC-PAD) was developed [8] and coupled with ultracentrifugation was used for quantitation of d-PRP and free-PRP. This method has been successfully established for monovalent Liquid PedvaxHIB® and bivalent COMVAX® vaccines. In combination vaccines, components are either adsorbed to the aluminum adjuvant or unadsorbed. Unadsorbed components are found, to some extent, in the vaccine supernatant even after ultracentrifugation. These components must be separated from PRP in a selective and reproducible manner. Thus, to reduce the time and resources necessary to perform the optimization, statistical multi-factor experimental designs were used to evaluate many aspects of the HPAEC-PAD application. The results of the assay validation confirmed specificity, accuracy, linearity and precision. Further, ruggedness to different operators and to the ultracentrifuge model was verified. The

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optimized assay was, therefore, established as a valid method for measuring total and unconjugated PRP content in a combination vaccine. The unconjugated assay can be used to monitor stability of the final product since it meets the FDA guidelines on stability indicating assays. "A validated quantitative analytical procedure must be able to detect changes with time of the pertinent properties of the drug substance and drug product". This was evidenced through the validation experiments whereby a wide range of depolymerized PRP concentrations were accurately and precisely quantitated by the method and verified with real time stability data (data not shown).

2. Methods and materials

2.1. Materials

The multi-valent vaccine consists of PRP-OMPC, Polio, Pertussis, Diphtheria, Tetanus and Hepatitis B (heretofore to be referred to as the "combination vaccine"). The vaccine matrix without PRP-OMPC was provided by the Merck/sanofi-pasteur collaboration. The surrogate marker for d-PRP and free-PRP was native PRP (raw material used for the production of PRP-OMPC). PRP-OMPC was also used as the reference standard starting at 15 μg PRP/mL and serially diluting to 0.94 μg PRP/mL. The internal standard used in assay is α-D-glucosamine 1-phosphate purchased from Sigma-Aldrich.

2.2. HPAEC-PAD

Measurement of PRP content was performed using a high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method. The system consisted of a Dionex GP40 or GP50 gradient pump connected to a Thermal Separations AS3500 autosampler. A borate trap between the pump and autosampler was used to remove any interfering borate from the eluents. The autosampler was connected to a Dionex ED40 electrochemical detector equipped with an integrated pulse amperometry option through a Dionex PA10 guard and analytical columns. The columns were held in a Dionex LC30 chromatography oven at ambient room temperature then 30 °C after optimization of the oven temperature. The mobile phase used for separation was initially 28 mM sodium hydroxide/100 mM sodium acetate then optimized to 32 mM sodium hydroxide/120 mM sodium acetate. Separation of the analyte was completed by running the mobile phase for 23 min followed by 2.5 min gradient change to the regeneration phase of 250 mM sodium hydroxide/1M sodium acetate which ran 10 min and followed by a 2.5 min gradient change back to the mobile phase then re-equilibration of the columns for 10 min prior to the next sample injection. The flow rate used for all analysis was 1.2 mL/min.

2.3. Ultracentrifugation

Separation of d-PRP and free-PRP from conjugated PRP was performed using either a Beckman Optima TLX ultracentrifuge with a 120.2 rotor with initial settings of 600,000 × *g* at 5 °C for 30 min. To convert revolutions per minute (RPM or "speed") to a gravitational force (×*g*) Eq. (1) was used:

$$\text{Gravitational force}\,(\times g) = 1.12 \times \text{Radius} \times \left(\frac{\text{RPM}}{1000}\right)^2, \quad \text{Radius} = 38.9\,\text{mm} \tag{1}$$

A Sorvall Kendro Discovery M150 micro ultracentrifuge was also used but conversion from *g* force to RPM was not required on this instrument.

2.4. Experimental strategy for separation and quantification of PRP components

Percent d-PRP and free-PRP is a ratio between these species to the total amount of PRP. A sample is first subjected to dissolution of the adjuvant followed by ultracentrifugation to separate d-PRP and free-PRP from PRP-conjugate. The ultracentrifuged supernatant containing the d-PRP and free-PRP and the non-centrifuged total PRP sample are then hydrolyzed in 0.3 M sodium hydroxide to yield the constituent disaccharide (ribosyl-ribitol-phosphate). The hydrolysate is passed through a 10,000 MWCO microfilter to remove protein. The filtrate is quantitated in parallel with the whole sample by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The reference standard was treated the same as the vaccine samples with sample concentrations obtained through interpolation from the standard curve.

2.5. Adjuvant dissolution optimization

The various species of PRP found in combination vaccines passively adsorb to the aluminum adjuvant. To quantitate PRP components accurately, the adjuvant must first be dissolved. This is accomplished through the use of a dissolution buffer consisting of sodium hydroxide and sodium citrate. To achieve an optimum mix of the dissolution reagents, a four-parameter central composite experimental design (CCD) [9,10] was performed using sodium hydroxide concentration, sodium citrate concentration, incubation time, and incubation temperature as the parameters. Table 1 lists the parameters and ranges used in the design.

The reagents were combined in water to make a concentrated dissolution buffer, which was mixed with the vaccine sample to achieve the final concentration of hydroxide and citrate specified by the experimental design. Absolute dissolution bias was

Table 1
Parameters for adjuvant dissolution optimization and associated ranges

Parameter	Range
NaOH (mM)	0, 50, 100, 150, 200
Citrate (mM)	0, 25, 50, 75, 100
Time (min)	0, 10, 20, 30, 40
Temperature (°C)	11, 24, 37, 50, 63