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Quantitation of low level unconjugated polysaccharide in tetanus toxoid-conjugate vaccine by HPAEC/PAD following rapid separation by deoxycholate/HCl☆

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Abstract

A simple and rapid acid precipitation method has been applied successfully for separating free capsular polysaccharide of *Haemophilus influenzae* type b (polyribosyl ribitol phosphate, PRP) from PRP–tetanus toxoid conjugate (PRP-T) in a final dosage amount of low-level materials. The unconjugated PRP was found to stay in the supernatant without precipitation, while conjugated PRP-T was fully precipitated. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been applied for analysis of the PRP content in the supernatant after the separation. This method requires minimum sample handling and is specific, sensitive and reproducible making it suitable for release and stability testing of PRP-T in final containers.

Keywords: Anion exchange chromatography; Polyribosyl ribitol phosphate; Polysaccharide conjugate vaccine; *Haemophilus influenzae*; Deoxycholate precipitation

1. Introduction

Haemophilus influenzae type b (Hib) purified capsular polysaccharide (PRP) vaccine has demonstrated efficacy in prevention of Hib disease in children over 18 months-of-age [1]. However, protection of children younger than 18 months, who are at higher risk for the disease, and whose immune systems are not mature enough to respond to the PRP vaccine, requires vaccines in which the PRP is conjugated to a protein carrier [2–5]. Consequently, vaccines containing Hib PRP (poly (→5-D-ribitol-(1-1)-β-D-ribose-3-phosphate) [5]) conjugated to a protein carrier such as tetanus toxoid (PRP-T [2]) are very important for prevention of Hib disease in infants.

Reliable, sensitive and specific methods are required for determination of the content of both the conjugated and unconjugated PRP in protein–polysaccharide conjugate vaccines. Recent advances in chromatographic technology

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have provided high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detectors (PAD) for separation and quantitation of saccharides at the subnanomole level [6,7]. We report here a successful method that couples HPAEC-PAD with an acid precipitation separation [8] capable of quantifying low-level free, unconjugated PRP in the PRP-T conjugate vaccine.

2. Experimental

2.1. Samples and materials

The purified high molecular weight PRP unconjugated polysaccharide powder and PRP-T conjugate liquid vaccine were produced by Pasteur Merieux Connaught by licensed vaccine production methods. The samples were dissolved in saline solution and the concentrations were adjusted based on phosphorus content determination [9]. Deoxycholic acid (DOC), DOC sodium salt, glucosamine-1-phosphate (used as an internal standard), hydrochloric acid, sodium hydroxide and sodium acetate were purchased from Sigma Chemical Co. (St. Louis, MO). PRP-T vaccine was used as reference standard for HPAEC-PAD.

2.2. Preparation of the 1% DOC solution and sample solutions

The 1% (w/v) DOC solution was prepared by first either dissolving DOC in pH $\geq$ 12 solution or by directly dissolving DOC sodium salt in water. The DOC solution was titrated with concentrated HCl to bring to the desired pH. Samples and control samples were prepared in 1 ml volumes at different concentrations.

2.3. Separation of PRP from PRP-T by DOC/HCl precipitation

In order to precipitate the PRP-T conjugates, 100 μl of prepared DOC solution was first mixed separately with 1 ml each of samples and control samples and incubated in a 0°C ice bath for 30 min. A 50 μl sample of 1 M HCl solution was then added to the mixture which was centrifuged in a Beckman Microfuge 11 at 10 000 rpm ($6000 \times g$) for 15 min. The supernatant was then removed and the sugars therein were hydrolyzed as below.

2.4. Hydrolysis of separated PRP

Hydrolysis solution contained 12.5 μg/ml internal standard (glucosamine-1-phosphate) and 1.5 N NaOH. Samples (unknown samples, control samples, standards and blank) were mixed with the hydrolysis solution at the volume ratio of 4:1 (samples:hydrolysis solution). Seven dilutions of the unconjugated PRP standard (20, 16, 8, 4, 2, 1 and 0.5 μg/ml) were made in saline for the standard curve. Saline solution was used as blank. After overnight hydrolysis at room temperature, the hydrolyzed sample was filtered through a 10 kDa cutoff membrane (Amicon, Beverly, MA) to remove protein residues from the samples. HPAEC-PAD experiments for each sample (including PRP and the blank) were carried out in triplicate. Reference standard samples were analyzed in doublet for the standard curve. Good standard curve linearity was observed ($r^2 \geq 0.990$).

2.5. HPAEC-PAD setup

A Dionex 500 system with a CarboPac PA-10 column and a guard column, an autosampler and Peaknet software (version 4.3) (Dionex Corp, Sunnyvale, CA) were used for the HPAEC-PAD experiments to measure the separated PRP contents. Sugars resulting from hydrolysis were detected by pulsed amperometry using a Dionex model ED40 electrochemical detector. Two solutions were used for equilibration, elution and column regeneration: Solution A, 25 mM NaOH/100 mM NaOAc and Solution B, 1 M NaOAc/250 mM NaOH. Isocratic elution for 25 min with solution A was used for separation, and 50% A and 50% B for 15 min for regenerating the column, followed by 10 min isocratic run with 100% solution A for equilibration.

3. Results and discussion

3.1. Optimization of separation conditions: pH of DOC solution

Separation of PRP from PRP-T requires complete precipitation of PRP-T without precipitating PRP. Optimization of pH conditions for DOC solutions was carried out first with separate unconjugated PRP and conjugated PRP-T samples. Solutions with 20 μg/ml PRP or 20 μg/ml (polysaccharide content) PRP-T were treated separately with 1% DOC solutions at various pH conditions.

As DOC does not dissolve in water, it has to be dissolved initially in high pH (~ 12–14) aqueous solution, followed by careful titration with HCl solution to bring the solution pH to the desired value. Deoxycholic sodium salt can be used as the alternative.

Seven pH conditions were tested (Table 1). A cloudy precipitation slurry was seen for conditions where the pH was < 7. A 100 μl sample of these prepared 1% DOC solutions with different pH values (a uniform slurry was used for lower pH situations) was mixed separately with 1 ml of samples of 20 μg/ml PRP and samples of 20 μg/ml PRP-T. The mixed solutions were kept in an ice bath for 30 min. Then, 50 μl of 1 M HCl was added into each mixture and centrifuged at 4°C for 15 min. It was observed that after adding 50 μl HCl into each mixture of sample/DOC, the solution pH changed to 2.5–3 for all mixtures irrespective of the original pH of the DOC-sample mixture (from 1.6 to 12.5). Thus it was decided to control the pH level during DOC preparation instead of monitoring the pH value after HCl was added.

Table 1
HPAEC-PAD analysis of PRP content in sample supernatant after DOC/HCl treatment at different pH values[a]

Sample/pH of DOC solution	PRP content (μg/ml)	% Recovery (sample/control × 100)
PRP-T control/no DOC	21.5	100
PRP-T/pH 1.6	2.8	13
PRP-T/pH 2.6	3.0	14
PRP-T/pH 5.1	9.2	43
PRP-T/pH 6.0	5.8	27
PRP-T/pH 6.4	1.6	7
PRP-T/pH 6.8	0	0
PRP-T/pH 7.4	1.4	6
PRP-T/pH 12.5	3.4	16
PRP control/no DOC	23.1	100
PRP/pH 1.6	21.3	92
PRP/pH 2.6	20.8	90
PRP/pH 5.1	17.3	75
PRP/pH 6.0	18.3	79
PRP/pH 6.4	21.7	94
PRP/pH 6.8	22.2	96
PRP/pH 7.4	19.0	82
PRP/pH 12.5	21.6	94

[a] Recovery values are expressed as percentages of the PRP content observed prior to DOC/HCl precipitation.

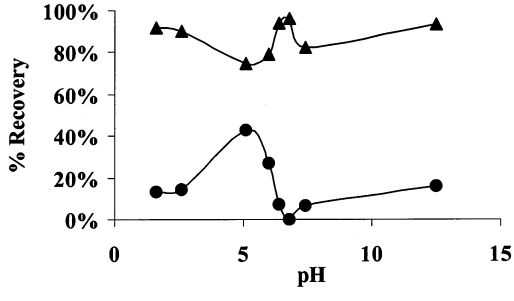

Fig. 1. PRP content (% recovery) of PRP (▲) or PRP-T (●) with 1% DOC solutions at different pH values.

The sugars in the supernatant from each sample were hydrolyzed and then analyzed by Dionex HPAEC-PAD. The recovery of PRP content analyzed by Dionex assay after DOC/HCl precipitation treatment is summarized in Table 1. PRP concentrations of the control and test samples were calculated by interpolation using the standard curve and the percentage recovery of PRP content against the control sample amount (without precipitation treatment) has been calculated. The pH dependent PRP percentage recovery after DOC/HCl precipitation treatment is shown in Fig. 1.

At different pH levels, PRP-T precipitation efficiency varied from 60% to 100%, while the retention of the PRP polysaccharide in the supernatant from 80% to 96%. As summarized in Table 1 and shown in Fig. 1, DOC solution with pH 6.8 gives

Table 2
Observed PRP content in samples of PRP, PRP-T, or mixed PRP and PRP-T, before and after treatment with DOC solution (pH 6.8)/HCl

Sample	Total PRP (conjugated + unconjugated) before DOC treatment (μg/ml)	Supernatant PRP (after DOC treatment) (μg/ml)	% Recovery
10 μg PRP-T/ml	10.3	0	0
10 μg PRP/ml	10.7	10.3	96
10 μg PRP-T/ml +10 μg PRP/ml	20.7	9.8	47
1 μg PRP/ml	1.0	1.0	100
10 μg PRP-T/ml +1 μg PRP/ml	12.0	0.9	8

the best precipitation of PRP-T (100% precipitation) and best recovery of PRP (96%).

3.2. HPAEC-PAD analysis of unconjugated PRP after separation

Three sets of experiments were performed for the DOC precipitation test under the optimum separation condition (i.e. DOC was prepared with pH 6.8) for different samples: pure unconjugated PRP polysaccharide sample (1 and 10 μg/ml), conjugated PRP-T sample (10 μg/ml), and mixed PRP and PRP-T sample for spiking test (1 and 10 μg/ml PRP in 10 μg/ml PRP-T) (Table 2). A spike of 1 μg/ml represents an amount of PRP that would be just above the assay detection limit in vaccine final containers. A total of 10 μg/ml PRP-T is approximately the amount of material that would be present after preparation of final containers of vaccine for HPAEC testing. The recoveries of pure PRP in the supernatant were 96 and 100%, respectively, at initial concentrations of 10 and 1 μg/ml.

Precipitation efficiency of conjugated material was tested with a 10 μg/ml pure PRP-T sample, and the results showed 100% (i.e. no detectable PRP remained in the supernatant).

Separation of PRP and PRP-T was tested with two mixed PRP/PRP-T samples, 10 μg/ml PRP spiked into 10 μg/ml PRP-T, and 1 μg/ml PRP spiked into 10 μg/ml PRP-T. The results showed 100% PRP-T precipitation efficiency as tested earlier for the PRP-T only control sample, while the 10 μg/ml PRP spiked sample showed 92% recovery of PRP originally present in the mixture (i.e. $9.8/10.7 \times 100$). The 1 μg/ml PRP spiked sample showed 93% recovery of PRP originally present in the mixture (i.e. $0.93/1.0 \times 100$).

From this investigation, it has been demonstrated that pH control of the 1% DOC solution is the critical step for optimum separation of PRP from PRP-T. With the optimized separation condition, i.e. 1% DOC solution at pH 6.8, low level free polysaccharides can be successfully separated and analyzed. Furthermore we demonstrate that DOC does not interfere with the HPAEC-PAD analysis for sugar contents after hydrolysis of the separated PRP by comparing blank solution and control samples with and without DOC treatment.

In summary, Hib PRP conjugated to tetanus toxoid was completely precipitated, and recovery of unconjugated PRP recovery was better than 90%, for both high level spiked PRP (such as 10 μg/ml) and low level spiked PRP (such as 1 μg/ml). This method achieves good separation with a simple sample preparation and handling procedure, and sensitive detection with the HPAEC-PAD. It can be further applied for the stability test of the PRP-T conjugate vaccines to analyze the small amount of free PRP breakdown.

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