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Measurement of Free Polysaccharide in Tetanus Toxoid-Conjugate Vaccine Using Antibody/Ammonium Sulfate Precipitation

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Abstract A method that effectively precipitates capsular polysaccharide of *Haemophilus influenzae* type b (polyribosylribitol phosphate, PRP) conjugated to tetanus toxoid (TT), PRP TT in a liquid vaccine has been developed to measure free PRP present in TT-conjugate vaccine. The method involves adding anti-TT antibody and ammonium sulfate to precipitate PRP-TT conjugate and measuring free PRP in the supernatant. This new method provides a complete precipitation of the total PRP-TT, and provides an accurate and reproducible measurements of free PRP. The accuracy of the assay was confirmed by spiking known amounts of unconjugated PRP to PRP-TT conjugate, and the new method was found to have no effect on free PRP while precipitating PRP-TT. The published acid precipitation method did not produce reproducible results due to incomplete precipitation of PRP-TT, especially when the vaccine is formulated in a salt-buffered solution.

Key words: *Haemophilus influenzae* type b, polyribosylribitol phosphate, polysaccharide conjugate vaccine, antibody/ammonium sulfate precipitation

Immunization with purified capsular polysaccharides (PRP, polyribosylribitol phosphate, →5-D-ribitol-(1-1)-β-D-ribose-3-phosphate) vaccine of *Haemophilus influenzae* type b (Hib) failed in children younger than 18 months, because of the T-cell independence that is an attribute of the PRP vaccine [15, 17]. In order to overcome the poor immunogenicity, PRP was chemically conjugated to the carrier proteins to convert the antigenic nature of PRP to a T-cell dependent one [4, 16, 18]. The conjugated PRP to the carrier proteins generates IgG antibodies and induces immunological memory. Consequently, these PRP conjugate vaccines have provided protection from Hib disease in infants [2, 3, 6, 11].

When considering antigenic characteristics of PRP and conjugated PRP to proteins, it is very important to control free PRP content in PRP conjugate vaccines. An earlier study [9] reported that a PRP-TT conjugate vaccine with more than 80% of free PRP did not elicit anti-PRP antibodies in mice even after undergoing two immunization processes, while a conjugate with less than 20% of the free PRP showed a primary antibody response which was boosted after the second injection. Therefore, PRP conjugate vaccines are tested for free PRP content [7, 12]. Currently, free PRP content of PRP-TT conjugate vaccines makes up less than 20% for the market release [12]. Consequently, reliable and accurate determination of a very small amount of free PRP in Hib conjugate vaccines is one of the important quality control parameters in development and production of theses vaccines. An acid precipitation method using deoxycholate(DOC)/HCl has been developed to quantify free polysaccharide, based on the differences between acid precipitation properties of polysaccharide-protein conjugate and polysaccharide alone, and it was applied to PRP-TT conjugate vaccines [9, 13] along with a meningococcal polysaccharide-diphtheria toxoid conjugate vaccine [14]. The DOC/HCl method was applied to measure free PRP of our conjugate vaccine. However, during the course of our experiments, free PRP contents measured were inconsistent depending on formulation conditions. Especially, higher free PRP content was obtained in the formulation vaccine in a salt-buffered solution than before formulation, when the DOC/HCl method was used, and this in fact could be an obstacle in developing the vaccine in a liquid form or combining it with other vaccines including salt components such as a DTaP-HepB vaccine [1]. In this study, we have developed a new method to

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separate the free PRP in the conjugate by using ammonium sulfate precipitation of PRP-TT which was treated with anti-TT antibody.

PRP, adipic acid dihydrazide-derivertized PRP (PRP-AH), TT and PRP-TT conjugate were prepared [4] at the Vaccine R&D Center of GreenCross Vaccine Corporation. The conjugate produced was diluted with water to 100 μg PRP/ml in a bulk stage, and the Lot number of the conjugate vaccine used in our study is known as Hib00503. The conjugate bulk (100 μg PRP/ml) was further diluted with either water or PBS (10 mM phosphate/0.15 M NaCl; pH 7.0) to 20 μg PRP/ml which corresponded to a general formulation concentration level of PRP-TT conjugate vaccines. The two formulated conjugate bulks were stored at 2- 8°C until used.

The acid precipitation method with a high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out according to Lei *et al*'s method [13], except for the sample volume that increased to 10 ml to adjust the pH to 2.5. The PRP-AH was used as a standard for HPAEC-PAD, and the PRP content was quantified by the bial assay [10]. The PRP concentration of standards was 0.5, 1.0, 1.5, 2.0, and 2.5 μg/ml and the correlation coefficients (R^2) of linear standard curves were greater than 0.99.

The experiment was repeated three times and the values shown in Table 1 are averages of the experimental results. There was a relatively large difference in the free PRP content measured between the two formulations. To find the reasons for this phenomenon, the protein in the supernatant after the DOC/HCl precipitation was measured by the BCA method. No protein was detected in the case of the formulated conjugate in water, but some protein were found in the case of the formulated conjugate in PBS (data not shown). These results indicate that the difference of measured free PRP content between the two formulated conjugates was due to incomplete precipitation of PRP-TT conjugate in the case of PBS formulation. Since the only difference between the two formulated conjugates was the presence of salt components, it appeared that salt components interfered with the process of PRP-TT precipitation by DOC/HCl.

Table 1. Free PRP content in the PRP-TT conjugates bulks formulated in water and in PBS determined by the DOC/HCl precipitation method.

Sample	Total PRP (μg/ml)[1]	Free PRP (μg/ml)[2]	Free PRP (%)
PRP-TT conjugate in water[3]	19.8	1.59 (±0.143)[4]	8.03
PRP-TT conjugate in PBS[3]	20.4	4.90 (±0.244)[4]	24.0

[1]PRP concentration in the formulated bulk before DOC treatment.
[2]These values were calculated in the way that the measured PRP concentration in the supernatant was divided by a dilution factor, 0.804.
[3]The lot number of conjugate used in this experiment is Hib00503.
[4]The experiments were repeated three times and the values in the parentheses mean standard deviation.

Although there was no exact information on the formulation conditions in the publications of Guo *et al.* [9] and Lei *et al.* [13], it appears from the published results that their formulated conjugate bulks contained salts. Contrary to our study, their results indicate that there were no effects of the salts on the performance of the DOC/HCl precipitation. This apparent contradiction in the results might have been due to the differences of TT or the conjugation method. Therefore, we have developed an alternative assay to quantify free PRP in a conjugate vaccine by using an antibody/ammonium sulfate precipitation method.

The ammonium sulfate precipitation method has been widely used in salting out proteins [5]. In addition, it has been employed in immunological analyses for measuring the amount of antibody such as radioimmunoassay [8]. In this experiment, the ammonium sulfate precipitation was used for precipitating protein that was conjugated to polysaccharide, and the antibody against the protein was added before the precipitation reaction to increase the specificity of the assay method.

Nine-tenth ml of PRP-TT conjugate was mixed with 0.1 ml of anti-TT antibody (250 IU/ml, Changchun Institute of Biological products, Changchun, Jilin, China), and incubated at 37°C for 2 h. Then, saturated ammonium sulfate solution was added to the mixture and incubated at overnight 4°C. Then, the mixture was centrifuged at 30,000 ×g (Beckman Avanti J-20) at 4°C for 30 min. The supernatant was collected and diafiltrated with ultrafiltration membrane filter (NANOSEP, molecular weight cut-off: 5,000, Pall Gelman Laboratory) against 10 volumes of water by centrifugation at 5,000 ×g (Beckman Avanti J-20) at 20°C for 10- 15 min to remove ammonium sulfate which might interfere with HPAEC-PAD. Then, after removing nearly all liquid from the filter by centrifugation under the same condition as the diafiltration, 0.3 N NaOH solution was added to the filter to hydrolyze the polysaccharide. After overnight hydrolysis at room temperature, the hydrolyzed polysaccharide was quantified by HPAEC-PAD.

The various ammonium sulfate concentrations were applied to precipitate PRP-AH, PRP-TT conjugate bulk, and anti-TT antibody-treated PRP-TT (Fig. 1), and all PRP-TT conjugate samples used in the experiments were diluted with water. When the ammonium sulfate concentration was above 60%, PRP-AH started to precipitate and nearly 80% of PRP-AH were precipitated at 80% ammonium sulfate. Thus, 60% was regarded as the highest ammonium sulfate concentration where PRP-ADH did not precipitate. In the case of the conjugate bulk, approximately 27% of PRP remained in the supernatant at 60% ammonium sulfate concentration, which was, as mentioned above, the highest ammonium sulfate concentration where PRP-AH did not precipitate. To check whether or not PRP-TT

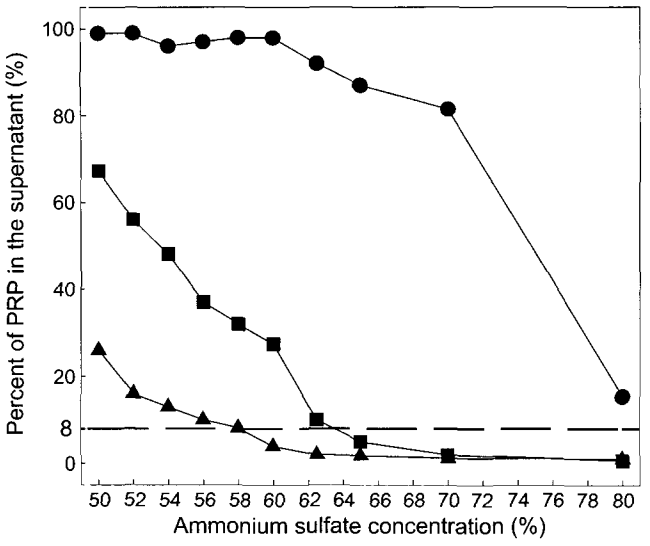

Fig. 1. Effect of ammonium sulfate concentration on the precipitation of PRP-AH (●), PRP-TT conjugate (◆), and anti-TT antibody-treated PRP-TT conjugate (▲).
The HPAEC-PAD was employed for quantifying the amount of PRP in the supernatant.

remained in the supernatant at this ammonium sulfate concentration (60%), protein in the supernatant was tested by several methods, but it failed because ammonium sulfate concentration was too high. In other words, it was impossible to confirm whether PRP-TT was precipitated completely under these conditions. Therefore, we had to check the precipitation of PRP-TT by another criteria, and the free PRP content (8.03%) measured by the DOC/HCl precipitation method (Table 1) was considered as a standard value for the free PRP content in this conjugate bulk. As shown in Fig. 1, the measured PRP content in the supernatant was similar to the standard value, when the ammonium sulfate concentration was between 62.5% and 65%, but PRP-AH precipitated at this ammonium sulfate concentration. Consequently, direct removal of the PRP-TT conjugate with ammonium sulfate precipitation alone appeared to be unsuitable for our study.

Table 2. Free PRP content in the PRP-TT conjugates bulks formulated in water and in PBS determined by the antibody/ammonium sulfate precipitation method.

Sample	Total PRP (μg/ml)[1]	Free PRP (μg/ml)[2]	Free PRP (%)
PRP-TT conjugate in water[3]	19.8	1.77 (±0.218)[4]	8.95
PRP-TT conjugate in PBS[3]	20.4	1.85 (±0.194)[4]	9.07

[1]PRP concentration in the formulated bulk before antibody/ammonium sulfate precipitation.
[2]These values was calculated in the way that the measured PRP concentration in the supernatant was divided by a dilution factor, 0.378.
[3]The lot number of conjugate used in this experiment is Hib00503.
[4]The experiments were repeated five times and the values in the parentheses mean standard deviation.

The ammonium sulfate precipitation was applied after reacting the conjugate with anti-TT antibody. As shown in Fig. 1, the PRP content in the supernatant was 8.2%, which was the closest value to the standard (8.03%) at 58% ammonium sulfate concentration where PRP-AH did not precipitate as mentioned above. When the concentration of anti-TT antibody was 25 IU/ml before the ammonium sulfate precipitation, it was found that the amount of antibody did not interfere with the performance of the assay method (data not shown). Therefore, 25 IU/ml anti-TT antibody treatment followed by 58% ammonium sulfate precipitation was chosen for further study.

The antibody/ammonium sulfate precipitation method was applied to the two formulated samples in water or in PBS (Table 2). The free PRP contents measured were similar for both formulated conjugate bulks, suggesting that the antibody/ammonium sulfate precipitation method could be used for our conjugate vaccine that was formulated in PBS. In addition, our results demonstrated reproducibility of the assay method through repeated

Table 3. Effectiveness of the antibody/ammonium sulfate precipitation method by spiking various amounts of PRP-AH in the two formulated conjugate bulks.

Spiked PRP-AH (μg)	Conjugate formulated in water[1]		Conjugate formulated in PBS[1]	
	PRP (μg) in the supernatant[2]	Spike recovery (%)[3]	PRP (μg) in the supernatant[2]	Spike recovery (%)[3]
1.0	2.48	89.6	2.51	85.4
2.0	3.46	93.6	3.53	93.7
3.0	4.88	109.9	5.11	115.1
5.0	6.78	103.9	6.36	94.1
7.0	8.30	95.9	9.15	107.1
10.0	12.58	110.0	10.86	92.0

[1]The lot number of conjugate used in this experiment is Hib00503.
[2]These values were calculated in the way that the measured PRP concentration in the supernatant was divided by the dilution factor (0.378) in order to convert it to the absolute one.
[3]The recovery percentage of spiked PRP-AH was calculated using the following formula.

$$\text{Spike recovery (\%)} \times \frac{\text{PRP (μg) in the supernatant (Table 3)} - \text{free PRP (μg) in the formulated conjugate in 1 ml (Table 2)} \times 0.9}{\text{Added PRP (μg) in the form of PRP-AH}} \times 100\ (\%)$$

experiments, showing each with a little standard deviation (Table 2).

In order to evaluate effectiveness of this new method, different levels of PRP-AH were spiked to the formulated conjugate samples (Table 3). The spike recovery of added PRP-AH ranged between 85% and 110%, which are considered to be within an acceptable range for the spike recovery. In addition, there was no difference in the spike recovery according to the amount of added PRP-AH.

In conclusion, it is shown in the present study that the antibody/ammonium sulfate precipitation method can very well be used to quantify free polysaccharide in TT-conjugate vaccines. The assay method was applied to our PRP-TT conjugates and able to resolve the limitation that was observed when the DOC/HCl precipitation method was applied to the formulated conjugate in a solution containing salt. Currently, attempts are being made to find the reasons of why incomplete precipitation of PRP-TT occurred in the acid precipitation method, and also to apply the newly developed assay method to other formulated conjugates such as aluminum gel adsorbed vaccine.

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