Optimization of the Lowry Method of Protein Precipitation from the *H. influenzae* Type b Conjugate Vaccine Using Deoxycholic Acid and Hydrochloric Acid

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Abstract The Lowry method was used in this study to measure protein in *Haemophilus influenzae* type b (Hib) conjugate vaccines (polyribosylibitol phosphate-tetanus toxoid; PRP-TT) using deoxycholic acid (DOC) to induce protein precipitation. Trichloroacetic acid (TCA) did not induce precipitation adequately from the Hib conjugate bulk and the freeze-dried Hib conjugate product. Its yield was approximately 50%. The matrix structure of Hib conjugate inhibits precipitation by TCA. Although the Lowry method can be carried out without precipitation in Hib conjugate bulk when no residual impurities (adipic acid dihydrazide [ADH], 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl [EDAC], phenol and cyanogens bromide [CNBr], *etc.*) are present, it cannot be used for Hib conjugate products that contain sucrose 8.5%, because 8.5% concentration of sucrose enhanced the protein concentration. DOC- and HCl-induced precipitation is an alternative method for evaluating the protein content of the Hib conjugate bulk and the Hib conjugate product. The precipitation was optimal with a final concentrate of 0.1% for DOC at 4°C and pH 2. This Lowry method, using DOC/HCl precipitation to induce protein precipitation, was confirmed a consistent, reproducible, and valid test for proteins in Hib conjugate bulk and its freeze-dried product.

Keywords: *Haemophilus influenzae* type b, conjugate vaccine, deoxycholic acid precipitation, method validation, Lowry method

INTRODUCTION

Haemophilus influenzae type b (Hib) is responsible for at least 96% of all cases of invasive *Haemophilus* infections [1-4] and is the leading cause of bacterial meningitis among infants and young children worldwide. This organism also causes other invasive infections, including epiglottitis, cellulitis, pneumonia, pericarditis, arthritis, bacteremia, empyema, and osteomyelitis [5,6]. US Public Health Service officials estimate that 1 in 2,000 children contact a Hib-induced disease before the age of 5 years in the United States [7]. Pittman [8] demonstrated that a Hib capsular polysaccharide is an important toxic substance and that an antibody to the type b capsule conferred type-specific protection against lethal Hib infection in experimental animals. The bacterial polysaccharide is the important substance that can be developed industrially [9]. The Hib capsular polysaccharide consists of repeating oligosaccharide units, which are relatively primitive antigenic units that elicit a weak immune response involving minimal T-cell activity [10]. The quest for a Hib vaccine that is immunogenic and protective in young infants has involved attempts at converting a capsular polysaccharide (polyribosylribitol phosphate [PRP]) antigen from being T-cell-independent to being T-cell-dependent antigen, using the carrier-hapten principles first defined by Landsteinder [11]. The introduction of more than one attachment site within the capsular polysaccharide chain significantly alters the characteristics and composition of the glycoconjugate products derived from a carrier protein. Originally, the conjugation technique used to detoxify the bacterial toxin [12]. When each reaction partner (carrier and hapten) has multiple points for covalent bond formation, conjugation will result in a cross-linked grid or lattice matrix. Regardless of the degree of cross-linking or solubility, a number of accessible sites will remain on the surface of any lattice matrix, ensuring some degree of antigenic activity. The rate and strength of antibody binding will provide some overall gauge for the accessibility of antigenic groups within the lattice of the conjugate product [13]. To maintain antigenicity, the structural components of an antigen must not be modified or moved; if this must occur, the changes must be kept to a minimum. Therefore, carbohydrate and protein analysis is needed for the quality control.

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Many assays are available to determine the total amount of protein in a mixture [14]. However, it can be challenging to select the most suitable method of analysis. A good strategy for selecting an appropriate assay is to compare the results of 2 methods, such as A_{280} measurements and one of the copper-based chromogenic methods that rely on different chemical properties. The results of total protein analytical methods commonly disagree with one another by as much as 5 to 20% even in the case of a protein that is not laden with interfering compounds for precipitation. In crude samples, such disagreements may be much greater. There are 2 general ways to deal with such discrepancies: (1) remove any interfering compounds that are likely to upset one or both assays or resort to a third method (*e.g.* the biuret analysis), if enough sample is available; or (2) conduct a total nitrogen analysis, *e.g.* using the Kjeldahl method or a modern version of it. It is a generally reliable practice to assume that all proteins and polypeptides contain close to 16.5% nitrogen by weight; by multiplying the weight of nitrogen determined using Kjeldahl analysis by a factor of 6, one should obtain a valid benchmark of the weight of protein in an ammonium salt free sample. Unfortunately, this analysis is both time-consuming and sample-consuming. For this reason, it is not used frequently in research laboratories. However, its use has increased in the biopharmaceutical industry, primarily as a method of validating an easier way to analyze the protein concentration.

Two methods of protein analysis are used for the Korean Minimum Requirement of Biological Products (KMRBP): the Kjeldahl method and the Lowry method [15]. Generally, the compendious method of Pharmacopoeia offers the advantage of being useful for registering and documenting the efficacy of a new drug. The Lowy method is dependent on both cupric and cuprous ion concentrations. Other examples of copper-based chromogenic methods are the biuret assay and the bicinchoninic acid (BCA) assay. Color development using such prochromogenic reagents as Folin-Ciocalteau, Folin and BCA, varies with the extent to which the cupric ions are reduced to cuprous ions [16]. The original Lowry method of total protein analysis [17] has been investigated many times to evaluate the effects of interfering compounds and the ability of detergents to solubilize otherwise insoluble proteins [18]. The Hartree-Lowry assay yields linear results over a wider range of protein concentrations compared with the original Lowry method; it also maintains its sensitivity to proteins and is superior to original method about preparation and stability of analytical reagents [19]. If the presence of non-protein compounds drastically interferes with the analysis of protein or if interfering compounds are present, the interfering compounds can be removed by precipitation, dialysis, or ultrafiltration. Precipitation with organic solvents or trichloroacetic acid (TCA) [20] has been used with pharmaceutical products because of the small quantity and low concentration of such compounds in those products.

In this study, we explored the efficiency of TCA- and deoxycholic acid (DOC)-induced protein precipitation for analyzing the protein content of the PRP-tetanus toxoid (PRP-TT) conjugate vaccine using tetanus toxoid as the carrier protein.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA; NIST, Cat. No. 927c) was purchased from the National Institute of Standards and Technology. Copper (II) sulfate-5-hydrate, sodium hydroxide (NaOH), sodium carbonate, sodium tartrate dihydrate, sodium deoxycholate, TCA, hydrochloric acid, and Folin-Ciocalteau's reagent (2 N) were purchased from Sigma (St. Louis, MO, USA). PRP, adipic acid dihydrazide-derived PRP (PRP-ADH), TT, Hib conjugate bulk (PRP-TT), and Hib conjugate product (the freeze-dried product) were prepared at the Vaccine R&D Center of the GreenCross Vaccine Corporation. TT and PRP-TT were used as the positive control and sample, respectively. The conjugate produced was diluted with water for injection (WFI) to PRP 100 μg per milliliter at the bulk stage, and the lot number of the conjugate vaccine used in this study was Hib7017, Hib7018 for bulk, and Hib8017, Hib8018 for product. The freeze-dried Hib conjugate product was formulated to PRP 20 μg per 1 mL of 8.5% sucrose solution.

Methods

Protein Assay

Three ampules of BSA (7% solution, 70 mg/mL) were pooled and mixed. The solution was diluted with distilled water to obtain standard dilutions of 25, 50, 75, and 100 μg/mL. A sodium hydroxide/sodium carbonate solution was prepared by dissolving sodium hydroxide 0.8 g and sodium carbonate 4 g in 100 mL of water. A 2 g quantity of copper (II) sulfate was dissolved in 100 mL of water to prepare a 2% copper solution.

A 4% sodium tartrate solution was prepared by dissolving sodium tartrate 4 g in 100 mL of water. An alkaline copper solution was made by mixing 0.5 mL of the sodium tartrate solution with 0.5 mL of the cupric sulfate solution and adding 50 mL of a sodium hydroxide/sodium carbonate solution into the mixture. A 1 N Folin-Ciocalteau phenol reagent was prepared by adding 5 mL of a 2 N Folin-Ciocalteau phenol solution to 5 mL of water. A 10% TCA solution was prepared by dissolving 10 g of TCA into 100 mL of water. A 5% TCA solution was prepared by mixing equal parts of the 10% TCA solution and purified water.

Both TCA and DOC protein precipitation methods were evaluated as part of the Lowry protein assay [17]. Assays were carried out in disposable 1.5 mL microcentrifuge tubes. TCA precipitation was achieved when a 500 μL sample was mixed with a 500 μL portion of the 10% TCA solution. The resulting solution was centrifuged in a Bechman microfuge at 10,000 rpm (6000 × *g*) for 15 min. A pellet was analyzed for its protein content. To precipitate protein using DOC, we mixed 100 μL of the 10%

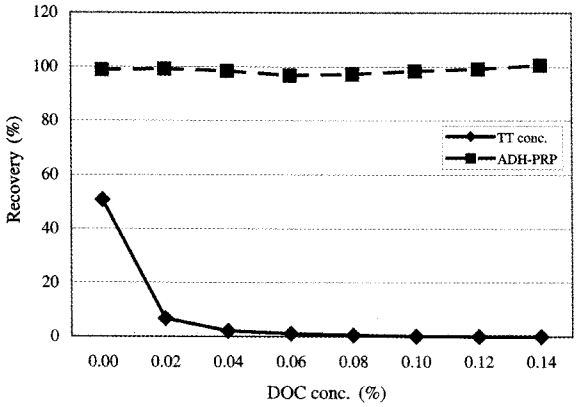

Fig. 1. Effect of deoxycholic acid (DOC) concentration on the precipitation of the mixture of tetanus toxoid and ADH-PRP at pH 2.

DOC solution separately with 1 mL samples and control samples and incubated them in a 0°C ice bath for 30 min. A 50 μL portion of a 1 M HCl solution was then added to the mixture, which was then centrifuged in a Bechman microfuge at 10,000 rpm (6,000 × g) for 15 min. The supernatant was removed and the pellet was solubilized by vortexing in 2.5 mL of alkaline copper solution for 30 sec. It was then left to stand for 10 min after being well shaken. A 2.5 mL portion of distilled water and 0.5 mL of 1 N Folin-Ciocalteau phenol reagent solution were added to the solution, which was then incubated for 30 min at 37°C. Absorbance was measured at 750 nm; a calibration curve was plotted based on the absorbance values obtained using standard solutions.

PRP Assay

After precipitation was complete, the supernatant was used for a free PRP assay [21,22]. The total PRP assay [21] for the product was performed after filtering it through a 50 kDa membrane (Amicon, Beverly, Mass, USA) in order to remove sucrose without inducing precipitation. The hydrolysis solution of the PRP assay used 12.5 μg/mL glucosamine-1-phosphate for the internal standard and 1.5 N NaOH. The unknown samples, standards, and blanks were mixed with the hydrolysis solution at a volume ratio of 4:1 (sample:hydrolysis solution, v/v). Each of 5 concentrations of the unconjugated PRP standard (Hib00304: 0.5, 1, 2, 3, and 4 μg/mL) was diluted in a saline solution to be read to create the standard curve. The blank consisted of the saline solution. After being incubated overnight at room temperature, the hydrolyzed sample was filtered through a 10 kDa membrane (Amicon) to remove protein residues from the samples. A high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis was carried out in triplicate with each sample. A Dionex 500 system consisting of a CarboPac PA-10 column and a guard column, an autosampler, and Peaknet software (Dionex Corp., Sunnyvale, CA, USA) was used in the HPAEC-PAD analyses to measure the PRP carbohydrate components. Sugars that were released as a result of the hydrolytic nature of this analysis were detected by pulsed amperometry using a Dionex model ED50 electrochemical detector. Two solutions were used for equilibrium, elution, and column regeneration: solution A, 25 mM NaOH/100 mM sodium acetate; and solution B, 1 M sodium acetate/250 mM NaOH. Solution A underwent isocratic elution for 25 min to achieve separation, and 50% of solution A and 50% of solution B underwent isocratic elution for 15 min to regenerate the column. This elution was followed by a 10 min isocratic run with full-strength solution A to achieve equilibration. The loading volume was 100 μL and the flow rate was 1 mL per minute.

RESULTS

DOC: Effect on Protein Precipitation

DOC is insoluble under acidic conditions. This property was taken advantage of to precipitate the proteins of PRP-TT and TT from the Hib conjugate bulk and product. The supernatant contained a free PRP that did not interact with TT. A pellet contained TT and PRP-TT. Normally, a pellet would be analyzed for its protein content. In this study, however, the supernatant was used to confirm complete precipitation. To study the effect of DOC, several DOC concentrations were used to precipitate proteins. The model sample was designed to achieve the same concentration of Hib conjugate bulk with TT and PRP-ADH. PRP-ADH was a free PRP form at conjugation reaction. After DOC-induced precipitation was complete, it was found that 98% of the PRP-ADH remained in the supernatant, no matter what concentration of DOC was used. Up to 99% of TT precipitated out of solution with every DOC concentration exceeding 0.1% (Fig. 1).

Comparison of Two Precipitation Methods

The Hib conjugate vaccine uses PRP as the antigen and TT as the carrier protein. Fig. 2 shows a diagram of analysis. The Hib conjugate bulks and the freeze-dried products were treated using the same procedure. To remove interfering materials and measure the proteins accurately, we used two precipitation methods: TCA and DOC/HCl; the result of an assay without precipitation was used as a control. The general test department of the KMRBP is only required for TCA when it is used to induce protein precipitation. In the case of the registered conjugate vaccine, the Lowry method without precipitation was used [15]. The two precipitation methods had been used specifically to analyze the protein from Hib conjugate bulk and product. The results differed somewhat for each protein. The concentration of protein in the conjugate bulk was approximately 220 μg/mL without precipitation. When the TCA precipitation method was used, the protein content was 103.4 μg/mL. The yield from the TCA method was approximately half the amount obtained using the DOC method (Table 1). Because Hib8017 (the freeze-dried product) was formulated with

Table 1. Results of the protein assay of the Hib conjugate vaccine using various methods of precipitation prior to applying the Lowry assay

Kind of sample	Lot No.	Protein conc.[1] (μg/mL)			PRP (μg/mL)	TT/PRP[2]
		TCA	DOC	Original		
Conjugate	Hib7017	103.4	218.4	220.9	101.3	2.2 (2.2)[3]
	Hib7018	90.6	220.1	217.4	99.7	2.2 (2.2)
Product[4]	Hib8017[5]	20.7	47.5	51.8	21.6	2.4 (2.2)
	Hib8018	13.4	49.5	53.8	22.5	2.4 (2.2)

[1] Protein concentrations were determined by Lowry method at different precipitation methods. TCA (trichloroacetic acid), DOC (deoxycholate), Original (no precipitation treatment).
[2] The TT of PRP ratio was calculated with the original Lowry results.
[3] The figure in the parenthesis was calculated with the DOC treated Lowry results.
[4] These lyophilized products contained with 8.5% sucrose as the stabilizer.
[5] The Hib8017 was formulated with Hib7017 conjugate bulk.

Table 2. Comparison[1] of protein assay in conjugate bulk compared with product in Hib vaccine

	Conjugate bulk	Product
PRP conc.	NR[2]	16~24 μg/mL
TT/PRP ratio	1.8~3.0	2.0~4.0

[1] These criteria were referenced by Korea Minimum Requirements of Biological Products [14].
[2] NR; No requirement.

the Hib7017 conjugate bulk, the ratio of TT to PRP should have been the same in both samples. When no precipitation was induced, however, the ratio changed from 2.2 to 2.4 for the bulk and product, respectively. The DOC precipitation method resulted in the same ratio in both samples.

Table 2 shows the KMRBP specification for the TT/PRP ratio in the other registered Hib conjugate vaccine [15], which changed from 1.8:3.0 to 2.0:4.0. This indicates that a product contained material that interfered with the protein assay. To demonstrate these discrepancies, two studies were performed: one to demonstrate the influence of sucrose and another to demonstrate the disadvantages of the TCA precipitation method.

DOC: Protein Precipitation from Formulated Products

This study was designed to overcome the effect of sucrose on DOC precipitation. Table 3 shows the results in conjugate products (Hib8017 and Hib8018) and in formulated products (F1~F5). The formulated products F1 through F4 contained sucrose 8.5% and 2 substrates for the conjugation reaction (PRP-ADH and TT) as formulating materials (spiking materials). F5 was a diluted conjugate bulk without formulating materials. The target PRP concentration of these products was 20 μg/mL, except for the additional PRP-ADH concentration. F1 and F3 were formulated with PRP-ADH to increase the free PRP content. Data showed a recovery yield of more than 90%. The recovery yields for F1 and F4 formulated with

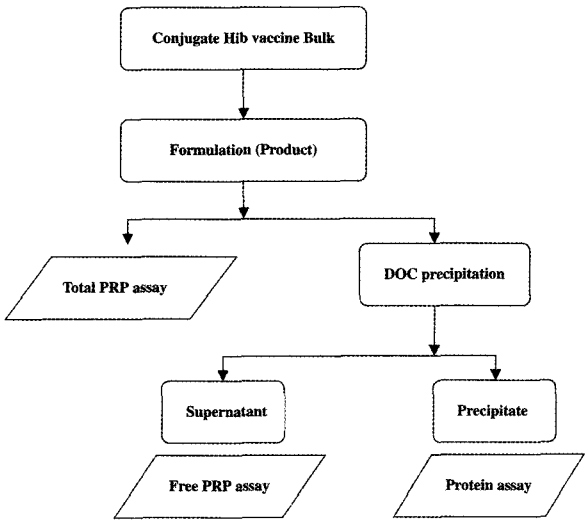

Fig. 2. Flow chart of the analysis of Hib conjugate vaccine.

TT exceeded 95%. These results showed that the DOC method of protein precipitation was not compromised by presence of sucrose.

Hydrolysis of Hib Conjugate Bulk

In order to solve the problems encountered with TCA-induced protein precipitation, Hib conjugate bulk was hydrolyzed at regular time intervals. Fig. 3 indicates that the 50 mL of Hib7017 was hydrolyzed at 37°C and pH 2. During hydrolysis, a 2 mL of the hydrolysate was taken out at 30-min intervals. This solution was neutralized using 0.1 N NaOH to prevent further hydrolysis. Each neutralized solution was treated with TCA and DOC to induce protein precipitation. After TCA-induced precipitation, the supernatant was analyzed for TCA-TT and TCA-PRP levels. After DOC-induced precipitation, the supernatant was analyzed for DOC-TT, only. The protein of the supernatant using TCA before hydrolysis was approximately 50% compared with total protein of DOC-induced precipitation (Table 1), indicating that the pro-

Table 3. Effect of deoxycholic acid on the precipitation of proteins from various product formulations

Sample	Spiking material			Test results (μg/mL)			TT/PRP-	Free PRP (%)
	ADH-PRP (μg)	TT (μg)	Sucrose (%)	Protein[1]	Total PRP[2]	Free PRP		
F 1[3]	5	10	8.5	56.7	25.2	7.3	2.3	29.0
F 2	–	10	8.5	56.4	19.8	2.3	2.8	11.6
F 3	5	–	8.5	44.8	25.8	7.5	1.7	29.1
F 4	–	–	8.5	45.2	21.2	2.4	2.1	11.3
F 5	–	–	–	46.6	21.6	2.3	2.2	11.9
Hib8017	–	–	8.5	47.3	21.6	2.1	2.2	9.7
Hib8018	–	–	8.5	49.5	22.5	2.2	2.2	9.8

[1] The protein concentrations were determined by Lowry method using the DOC precipitation treatment.
[2] The total PRP were analyzed before the DOC precipitation.
[3] The formulated samples (F1~F5) were prepared by using the Hib7017 at lab scale for this study.

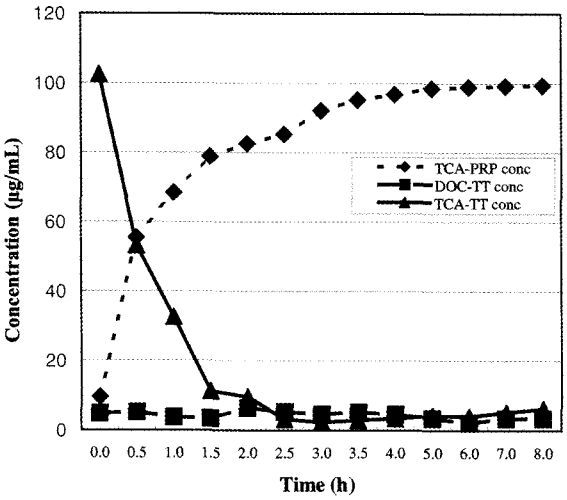

Fig. 3. Profile of tetanus toxoid and PRP concentrations in the supernatant following acid hydrolysis. After TCA-induced protein precipitation, the supernatant was analyzed for protein (TCA-TT) and free PRP (TCA-PRP). After DOC-induced precipitation, the supernatant was analyzed for protein (DOC-TT), only.

tein concentration in the supernatant was approximately 100 μg/mL (half the concentration in Hib conjugate bulk). With TCA precipitation, the protein concentration of supernatant was decreased with the duration of hydrolysis. When DOC was used to induce precipitation, the protein concentration in the supernatant did not change throughout hydrolysis time. Hydrolysis of PRP was completed in 4 h at 37°C and pH 2.

Validation of the Lowry Method Using DOC/HCl to Induce Protein Precipitation

The Lowry method was validated according to the guidelines of the International Conference of Harmonization (ICH) [24,25]. The samples used for validation purposes included the Hib conjugate bulk (~200 μg TT/mL), and the diluted Hib conjugate bulk, and the fomulated bulk. Acceptance criteria were determined by statistical analysis of accumulated test results. The validation parameters were accuracy, repeatability, intermediate precision, linearity, specificity, range, limit of quantitation (LOQ), and robustness. The results of this validation procedure are summarized in Table 4. Accuracy was calculated based on the recovery of samples formulated with BSA; the recovery range was 85 to 112%. Specificity was determined by calculating the amount of hydrolyzed TT in the formulated samples; this was determined to be 106.18%. Intermediate precision and robustness were determined by *t*-test analysis with 95% confidence intervals and were found to be acceptable. Linearity was indicated by a correlation coefficient of more than 0.9992. The LOQ was 2.11 μg/mL. Thus, all validation parameters were considered acceptable according to predetermined criteria.

DISCUSSION

The Lowry method is a copper-based chromogenic method of protein analysis. It is sensitive and reproducible for various kinds of protein. Unfortunately, many materials can impede the performance of this technique. Total protein results will vary for crude samples that are contaminated with such materials. The principle concern, then, is often not how sensitive a particular protein assay may be, but rather the extent to which the assay is affected by other substances in the material being analyzed. To avoid the effect of such substances, powerful protein precipitating agents, such as TCA, may be useful [26,27]. The Hib conjugate vaccine is a complex molecule in which PRP and TT form covalent bonds (Fig. 4), creating a matrix. Normally, a conjugation reaction changes molecular structures and epitopes. This type of change can interfere with the outcome of the protein assay. As shown in Table 1, the amount of protein released by TCA precipitation is only half of total protein concentration in the sample. TCA causes protein to precipitate out of solution by denaturing it to make it insoluble. It is difficult for Hib conjugate bulk to be denatured by TCA because of its matrix structure. For this reason, the TCA method of

Table 4. Summary of the validation of the Lowry method as a protein assay in Hib conjugate bulk

Parameter	Samples[1]	Test run	Analysis tool	Acceptance criteria	Validation results
Accuracy	Low, Medium, High, Low + spike[2], Medium + spike, High + spike	6	Spike recovery	80~120%	85~112%
Repeatability	Low, Medium, High	6	CV %[3]	<5%	2.51%
Intermediate precision	Low, Medium, High	6	*t*-test	$p>\alpha$ (0.05)	Low 0.67 Medium 0.23 High 0.20
Linearity	0, 25, 50, 75, 100 μg/mL	6	Correlation coefficient	r>0.999	>0.9992
Specificity	TT bulk + H_2O_2[4]	5	Recovery	80~120%	106.18%
Range	Low, Medium, High	5	Accuracy, Precision, Linieariy	Pass	Pass within standard range
LOQ[5]	Blank	6	LOQ	=(10*SD[6])/slope	2.11 μg/mL
Robustness[7]	Medium	12	CV %[3]	<5%	4.39% (30 min) 2.36% (40 min)

[1] The concentration of low, medium and high sample was about 100, 200, and 300 μg/mL, respectively.
[2] Spike solution was the bovine serum albumin solution (100 μg/mL).
[3] CV means coefficient of variation.
[4] TT bulk + H_2O_2 means the hydrolyzed tetanus toxoid bulk with hydrogen peroxide used as spike solution for specificity test.
[5] LOQ means limit of quantitation.
[6] SD means standard deviation.
[7] The robustness was analyzed by results acquired at two reaction times (30 and 40 min at 37°C).

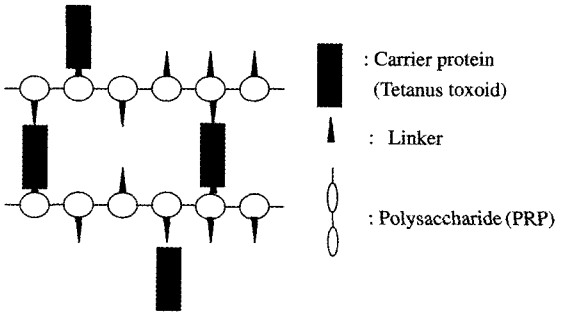

Fig. 4. The molecular structure of the Hib conjugate vaccine.

protein precipitation is not applicable to the analysis of Hib conjugate bulk and its freeze-dried product. Although no protein precipitation method has been successful in analyzing the protein of Hib conjugate bulk under little amount of residual impurities (ADH, EDAC, phenol, CNBr, *etc.*), the same method cannot be said about the Hib conjugate product because it contains 8.5% sucrose, which causes to overestimate its protein concentration. It has been reported that the Lowry method is compromised when the sample to be analyzed contains more than a 10 mM concentration of sucrose [20]. DOC also influences the outcome of the Lowry method [17]. The highest concentration it can detect reliably is 0.0625% w/v. In the Hib conjugate bulk, the protein concentration is approximately 200 μg/mL. Because of its high concentration of protein, it must be diluted to place its protein concentration in standard range. This dilution can overcome the barrier to detecting protein by DOC-induced precipitation in Hib conjugate bulk. In the case of the Hib conjugate product, a 2-fold dilution is necessary to overcome the influence of DOC. Because DOC-induced precipitation shows consistent results in both the Hib conjugate bulk and the freeze-dried product, this protein precipitation method overcomes the limitations in TCA efficacy due to the Hib conjugate's matrix structure. DOC is a small molecule (molecular weight: 392) that contains a carboxyl group and a hydrophobic ring as functional groups that can interact with the functional group of protein through ionic and hydrophobic bonds, respectively. A structure containing either type of bond can be precipitated out of solution using DOC under acidic conditions. DOC-induced precipitation has already been used to analyze free PRP levels in Hib conjugate bulk [23,26,27]. This method has 2 advantages: (1) it can be used to precipitate proteins out of matrices, such as the Hib conjugate vaccine; and (2) it is not affected by the sucrose content of the freeze-dried product. Although DOC/TCA precipitation had been studied previously [18], DOC concentrations of 0.015 and 0.75% were not enough to cause Hib conjugate vaccine proteins to precipitate out of solution completely. The DOC/TCA method was influenced very much by sucrose. To overcome this influence, sodium dodecyl sulphate (SDS) was added to an alkaline copper reagent solution [28]. When

the Hib conjugate was hydrolyzed under acidic conditions, the TCA protein precipitation method was fully applicable to Hib conjugate bulk. Because residual TCA can influence testing results, however, it must be removed using acetone. Originally, the sample should be minimized any change in its physicochemical properties before analysis. Based on this fact, DOC is more efficient than TCA for precipitating proteins from the Hib conjugate vaccine. The validation procedure indicated that the Lowry method using DOC/HCl to induce precipitation was a consistent, reproducible and valid test for Hib conjugate bulk and its freeze-dried product (data not shown). A validated physicochemical test may be more important than an animal efficacy test [29] for Hib conjugate vaccines. A current trend in product development is to switch from freeze-dried to liquid formulations. These liquid formulations use an aluminum gel as an adsorption agent or a buffer system as a stabilizer. Another protein precipitation method may be necessary to evaluate the protein content of liquid formulations of Hib conjugate [30].

CONCLUSION

In this study, it was found that TCA precipitation was not applicable to the analysis of protein in Hib conjugate bulk and its freeze-dried product. The DOC/HCl protein precipitation method was adapted to analyze the protein content of these substances under various conditions. In particular, it was adapted to overcome the influence of the high sucrose concentration in the freeze-dried formulation of the Hib conjugate product. It offered several advantages: (1) applicability of one procedure to both the Hib conjugate bulk and product; (2) minimization of the effect of other materials; and (3) failure of residual HCl to influence the outcome of the analysis during the neutralization process, which uses NaOH in an alkaline copper reagent solution.

The DOC/HCl precipitation method was applicable to Hib conjugate bulk and its freeze-dried product. Validation of this method based on ICH guidelines confirmed the Lowry method using DOC/HCl to induce protein precipitation as a consistent, reproducible, and valid test for the Hib conjugate bulk and its freeze-dried product.

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