Unique Freeze-Drying approaches in Haemophilus influeza type b conjugate vaccines (Hib).

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Abstract:

Haemophilus type b conjugate is freeze sensitive and also labile to heat. Freeze sensitivity is one of the major causes for wastage of vaccines during transport and storage in health care centers or pharmacy store. Currently *Haemophilus type b* conjugate vaccine is available in both liquid and lyophilized forms. Lyophilized *Haemophilus type b* conjugate vaccine is reconstituted with sterile WFI (Water For Injection) before injection. Reconstituted vaccines will become freeze and heat sensitive, cannot be stored for longer periods, to be used within 6 hours after reconstitution. This research work aimed to develop freeze stable and heat stability improved in *Haemophilus type b* conjugate vaccine with varying Lyophilization cycle hours. Lyophilization is an excellent option to protect vaccine from freezing and heat effects. In general stability of the vaccines in lyophilized state is better compared to liquid state. Lyophilized vaccine of *Haemophilus type b* conjugate vaccine was prepared with Tetanus toxoid bulk antigen. The developed lyophilized vaccines were resistant to freezing and heat stability was enhanced substantially when compared to other liquid vaccines. This lyophilized vaccine can be extemporaneously reconstituted before administration using suitable diluent. The lyophilized cycle hours (23 hrs.) of *Haemophilus type b* conjugate vaccine also enhanced stability, and moisture content and stood good in all the recommended and required tests.

Key words: *Haemophilus type b* conjugate, Lyophilization cycle, Freeze stable, Stability, moisture content.

INTRODUCTION

Haemophilus influenzae type b (Hib) vaccine in low- and middle-income countries has been limited by cost and unsuitability of Hib conjugate vaccines for a long time storage. A new affordable and a non-infringing production process for a Hib conjugate vaccine is needed to promote its applicability in nations where *H.influenzae* problem is emerging.

Haemophilus type b (H. influenza) is an important cause of meningitis and pneumonia in children, causing an estimated three million cases of serious disease and hundreds of thousands of deaths annually

worldwide (Swingler, 2003). There is a need to determine the size of the effects of the vaccine, to enable cost-effectiveness comparisons with competing priorities in developing countries (Swingler er al., 2003).

In conjugate vaccines, the immunizing potential of bacterial cell surface polysaccharides (PS) has been enhanced by covalent coupling to protein. A large amount of knowledge of the special features associated with the novel vaccine type, combining properties of polysaccharide and protein antigens, have been obtained through the Hib model, i.e. clinical trials and subsequent large-scale use of the vaccine (Keith Redhead et al., 1994).

Invasive infections caused by *Haemophilus influenza* type b (Hib) one an important cause of morbidity and mortality among infants and young children in many countries. The most acceptable, and probably most efficient, way of administering this vaccine is to provide it concurrently with the existing diphtheria-tetanus-whole-cell pertussis (DTP) vaccine. The vaccines available differ from each other in the size of the polysaccharide chain and the identity of the protein conjugated to the PRP (World Health Organization, 1980).

In freeze-drying water is frozen in the vaccine and then subjected to a high vacuum condition. The lyophilized form of vaccine can be stored for a long time use.

MATERIALS AND METHODS

Material

Haemophilus type b conjugate was prepared by conjugating polysaccharide poly ribosyl ribitol phosphate (PRP) with tetanus toxoid in compliance with WHO TRS 897. The *Haemophilus influenzae type b* was grown in synthetic basal media and fermentation media. The fermented *Haemophilus type b* was allowed to undergo a next stage for inactivation and cell separation followed by Purified polysaccharide (PRP) preparation. Purified polysaccharide (PRP) was conjugated with Tetanus Toxoid bulk. All the chemicals used for testing were of analytical grade and purchased from commercial suppliers.

Methods

The *Haemophilus influenzae type b* was grown in synthetic basal media, which included general salts like monosodium glutamate, disodium hydrogen phosphate, sodium chloride, potassium chloride, ammonium chloride etc. Supplements included dextrose, magnesium chloride, cysteine; NAD, hemin and low molecular weight diafiltered permeate of yeast extract.

The basal medium was sterilized by autoclaving at 121 ºC under 15 lbs pressure for 20 minutes. Non-autoclavable components were sterile filtered using 0.2 µ filter made up of PES. The final Purified polysaccharide (PRP) was prepared by the below mentioned sequence,

The culture was harvested and conjugated by Inactivation using 0.6 % v/v Formalin (Porter Anderson and David H Smith, 1977). The supernatant was collected and concentrated to 18-22 fold using 100 kDa cassette and performed Diafilter 5-6 times with WFI. Again Diafiltration of the concentrate using 3-5 volumes of PBS, was done and then the crude polysaccharide Cetavelon Precipitation was performed. After the

centrifugation, precipitation was done using 32% Ethanol at 2-8° C for 14-18 hrs. After that centrifugation was done at 4500±500 rpm. Then Diafiltered with 5 volumes WFI through 100 kDa at 2-8° C and 0.22µ sterile filtration. Finally, the Purified polysaccharide preparation was conjugated with Tetanus Toxoid bulk.

Formulation and Lyophilization process

Formulation was done using 20 µg of purified PRP-TT, 0.01 M Tris buffer pH 7.0±0.1 sucrose as excipient (42 mg/mL) and thiomerosal (0.005%) as a preservative for single dose. The final blended bulk was filled in USP type I glass vials with suitable rubber stoppers and lyophilized to form a dried cake.

The vial and the amount of product to be lyophilized directly affect freezing, ice crystal formation and sublimation. These changes in the vial for lyophilization can help to decrease the freeze-drying cycle.

Changes in the freeze-drying cycle

After filling the vials, the product was introduced in to the lyophilizer at a positive shelf temperature. Developing a freeze-drying cycles that operates that at milder or negative temperatures is crucial. The freeze-drying cycle starts by ramping down to the desired freezing temperature, followed by Hold period. In the vials, the final freezing temperature was lowered to reduce the length of this phase by exploiting the thermal properties of the lyophilizer and the type of vial. The pressure decreased in the freeze-drying chamber, primary drying began with an increase in temperature. There is a specific point in the cycle when the product no longer absorbs enough energy to sublimate the ice in the vials. After primary drying, the temperature was increased to accelerate desorption (secondary drying). The temperature for secondary drying was positive. At the end of the cycle, the vials were closed under nitrogen gas atmosphere conditions and are were collected from the lyophilizer. The vials were then inspected and sampled for analytical tests.

Experiment 1

The first experimental run was performed on a tray of product HIb formulated vaccine. In that case, around 1000 vials were lyophilized in a regular cycle (30:00 hrs.). This batch was numbered as HIB/001A/13. Initial freezing temperature was -45 C. The shelf temperature was adjusted to be a negative temperature in primary drying. This change made the vaccine to get lyophilized at a lower temperature without compromising quality requirements, specifically the residual humidity. Primary drying started from -35 degree to +40 degrees temperature and the control vacuum from 0.18 millibar to 0.15 at the step no.8. Secondary drying was with positive temperature of 42 degrees and the vacuum of 0.05 millibar. Smaller crystals are less likely to form at lower temperatures, which could threaten sublimation in the primary phase or lead to a no satisfactory residual humidity in the final product. This temperature change could also lead to a collapse of the product during secondary drying, thus increasing product loss or heterogeneous qualifications of the product in each vial, across different batches (Table 1). Temperature plays a key role in lyophilization of a vaccine because it can accelerate the dilapidation of potency of the final product, which must be confirmed in the potency test.

Experiment 2

The second experimental run was performed on a tray of product HIb formulated vaccine. Around 1000 vials were lyophilized in a modified cycle (26:00 hrs.). This batch was numbered as HIB/002A/13. No changes in initial freezing temperature of -45 C. Then changes were made in primary drying Step no.1, 5, 6 and 7. The time was reduced 60 mins. in every step and there were no changes in vacuum set points. This changes were made as the vaccine could be lyophilized at a lower temperature without compromising aspect and quality requirements, specifically the residual humidity. Primary drying start from -35 degree to +40 degrees temperature and the control vaccum from 0.18 millibar to 0.15 at the step no.8 (Table 2). Changes in the hold duration of primary drying, not in ramp duration, will help the product effective and maintain the required residual moisture contents (Le Meste et al., 1985). Secondary drying was done with positive temperature of 42 degrees and the vacuum of 0.05 millibar without changing any parameters. This temperature change could also lead to a collapse of the product during primary drying, hence a set vacuum of 0.15 was maintained as it will not leave the product cake to collapse. It maintains the integrity of the cake formation.

Table 1: 30 hrs. Lyophilization Cycle: (H1B/001A)

HIB LYO CYCLE 30 hrs.						
Step	**Step No.**	**Temp. (°C)**	**Vacuum (millibar)**	**Total Time (Mins.)**	**Ramp**	**Hold**
Freezing	1	-45	-	161	1	160
Evacuation			0.18	0		-
Primary drying	1	-35	0.18	150	30	120
	2	-30	0.18	210	30	180
	3	-25	0.18	120	30	90
	4	-20	0.18	90	30	60
	5	-10	0.18	210	30	180
	6	0	0.15	150	30	120
	7	20	0.15	300	60	240
	8	40	0.15	150	90	60
Secondary drying	1	42	0.05	235	5	230
Total Mins.				**1776**		
Total Hrs.				**29.60**		

Table 2: 26 hrs. Lyophilization Cycle: (H1B/002A)

HIB LYO CYCLE 26 hrs.						
Step	**Step No.**	**Temp. (°C)**	**Vacuum (millibar)**	**Total Time (Mins.)**	**Ramp**	**Hold**
Freezing	1	-45	-	161	1	160
Evacuation			0.18	0		-
Primary drying	1	-35	0.18	90	30	60
	2	-30	0.18	210	30	180
	3	-25	0.18	120	30	90
	4	-20	0.18	90	30	60
	5	-10	0.18	150	30	120
	6	0	0.15	90	30	60

	7	20	0.15	240	60	180
	8	40	0.15	150	90	60
Secondary drying	1	42	0.05	235	5	230
Total Mins.				**1536**		
Total Hrs.				**25.60**		

Experiment 3

The third experimental run was performed on a tray of product HIb formulated vaccine. Around 1100 vials were lyophilized in a modified cycle (23:00 hrs.). This batch was numbered as HIB/003A/. In this experiment no change was made in freezing stage of temperature at -45 C. Changes were made in the in the primary drying Step no.1 around 60 mins. Step 5 around 120 mins, step no 6 and 7 around 60 mins. Thus a reduction in hold periods and primary drying ramp duration was made. In step no.7 & 8 the duration was reduced to 30 mins. During this ramp duration the temperature was change from +20 to + 40 degress, hence the cake denature. In primary drying vacuum set points was also reduced from 0.15 millibar to 0.10 millibar for effective dryness and maintain the required moisture content of the product (Le Meste et al., 1985). Secondary drying was also reduced by 60 mins with positive temperature of 42 degrees and the vacuum of 0.05 millibar (Table 3).

Table 3: 23 hrs. Lyophilization Cycle: (H1B/0034)

HIB LYO CYCLE 18 hrs.						
Step	**Step No.**	**Temp. (°C)**	**Vacuum (millibar)**	**Total Time (Mins.)**	**Ramp**	**Hold**
Freezing	1	-45	-	161	1	160
Evacuation			0.18	0		-
Primary drying	1	-35	0.18	90	30	60
	2	-30	0.18	210	30	180
	3	-25	0.18	120	30	90
	4	-20	0.18	90	30	60
	5	-10	0.18	90	30	60
	6	0	0.10	90	30	60
	7	20	0.10	210	30	180
	8	40	0.10	120	60	60
Secondary drying	1	42	0.05	175	5	170
Total Mins.				**1356**		
Total Hrs.				**22.60**		

In the proves the sublimation time was higher than necessary because of the use of the present, commercial freeze-drying cycle for product with double the standard amount of doses. To prove that greater contact between the vial and the shelf contribute to a decreased freeze-drying cycle length. The pressure in the primary and secondary phases were the same as those used for commercial cycles. These pressures were kept constant

during the entire drying phase. With the modifications suggested, in the first cycle the freeze-drying process was decreased by 17 hours.

Testing methods

The lyophilized *Haemophilus type b* vaccine was tested for different quality parameters like visual inspection, moisture content, pH, PRP content and immunogenicity. Appearance was tested visually, moisture content was tested by Karl fisher titration method using Mettler auto titrator as per Indian Pharmacopoeia, pH was tested by potentiometric method using Thermo Orion pH meter having glass electrode at 20 to 25°C. Estimation of PRP in HIB-TT final lot by Orcinol Method, the PRP content range was Not less than 10 µg/dose (0.5 mL).

The prepared lyophilized vaccine reconstituted with water for injection prior to free polysaccharide separation from conjugated polysaccharide (Zielen et al., 1996). Alkaline hydrolysis of the standard and vaccine samples was performed using sodium hydroxide. C4 cartridge column was used for separating free from total polysaccharide prior to hydrolysis.

Stability studies

The lyophilized *Haemophilus type b* vaccine were tested for stability at accelerated and real time conditions as per ICH guidelines (ICH, 1995). Stress study was conducted for 12 months at 25 ± 2° C and real time stability study was conducted for 30 months at 2-8°C.

RESULTS

In all the three experiments the final lot of cake was neat and intact (Fig.1, 2, 3)

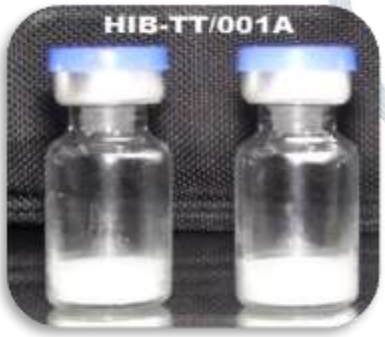


Figure 1

Figure 2

Figure 3

Post reconstitution solution appeared homogeneous. Vaccine was resuspended within 30 seconds and no clumps were observed for all the three experiments. (Fig.4).

Figure 4

The pH of all the final lots were within the limits. Moisture content was less than 3% in all the formulations as tested by Karlfisher titration.

PRP in HIB-TT final lot was performed by Orcinol Method and the results obtained were within the limit (Not less than 10 µg/dose). PRP content was not affected during different cycle hours of lyophilization process. Final blend PRP content was observed to be 24.2 µg/dose.

Stability studies

Post reconstitution appearance was good for all three formulations at both real time and stress conditions at all-time points. Results of key stability parameters like *haemophilus type b* conjugate Total PRP of all three formulations, at real time and accelerated conditions were shown in figure 5 and 6.

PRP reduction was totally less than 12% in 12 months at 25°C, in Batch No. HIB-TT/001A was 11.45%, Batch No. HIB-TT/002A was 11.58% and Batch No. HIB-TT/003A was 11.25% individually. In 24 months, in Batch No. HIB-TT/001A was 22.49%, Batch No. HIB-TT/002A was 22.11% and Batch No. HIB-TT/003A was 22.40% individually. In 36 months at 2-8°C PRP reduction was observed average of 22.33%

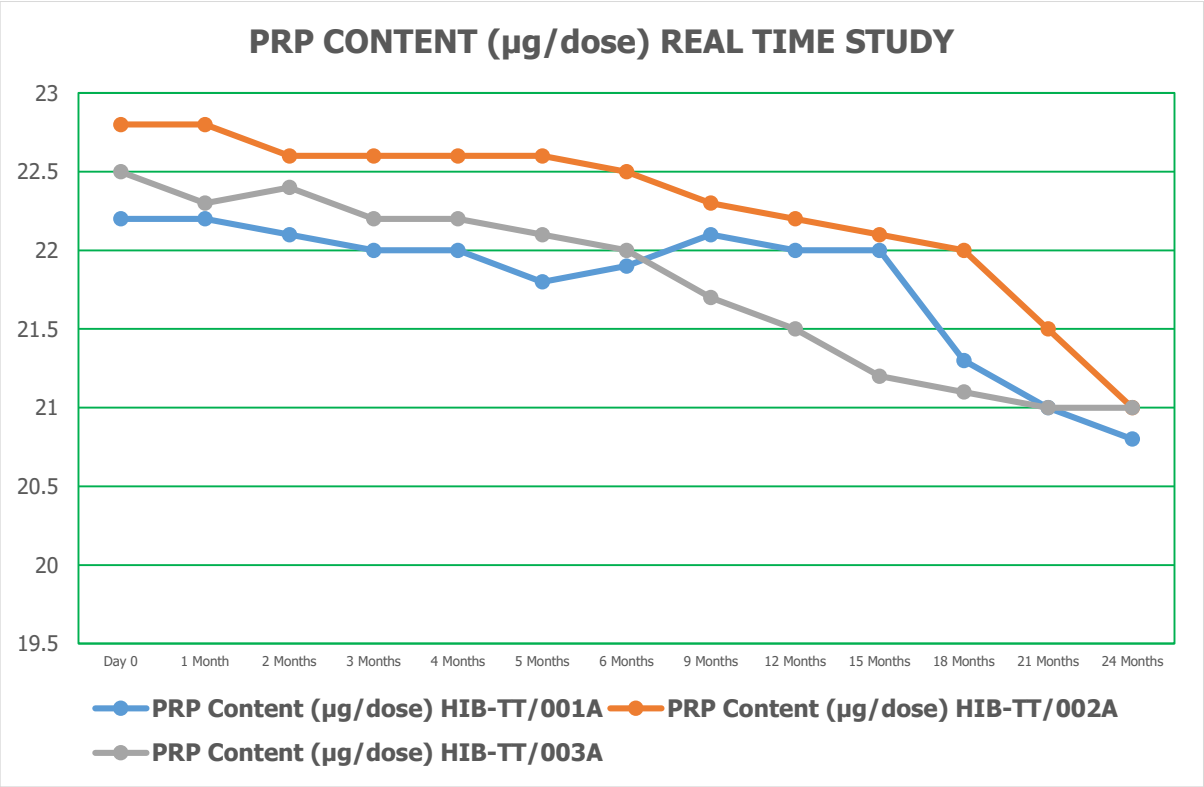
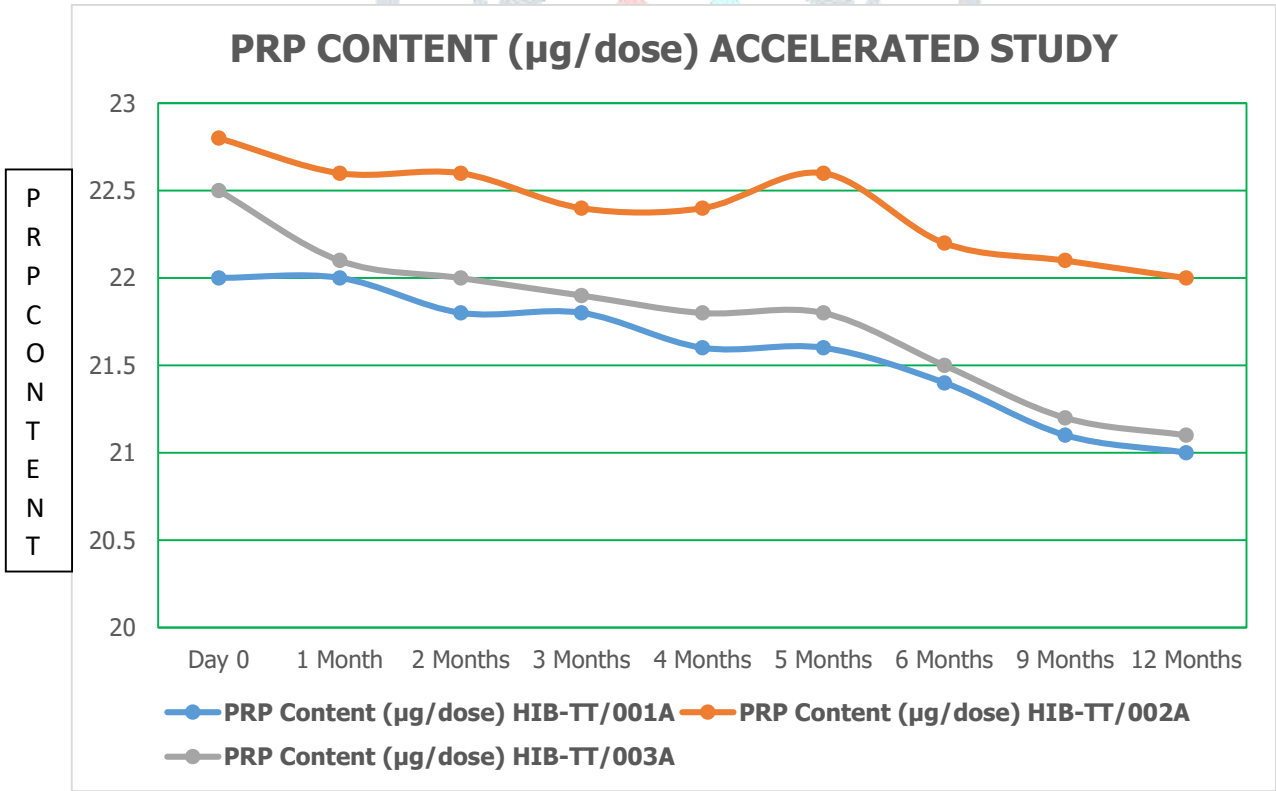


Figure 5



Batch No	HIBTT001A	HIBTT/002A	HIBTT/003A

Moisture content of *Haemophilus type b* conjugate of all three formulations, at real time and accelerated conditions were shown in figure 7 and 8

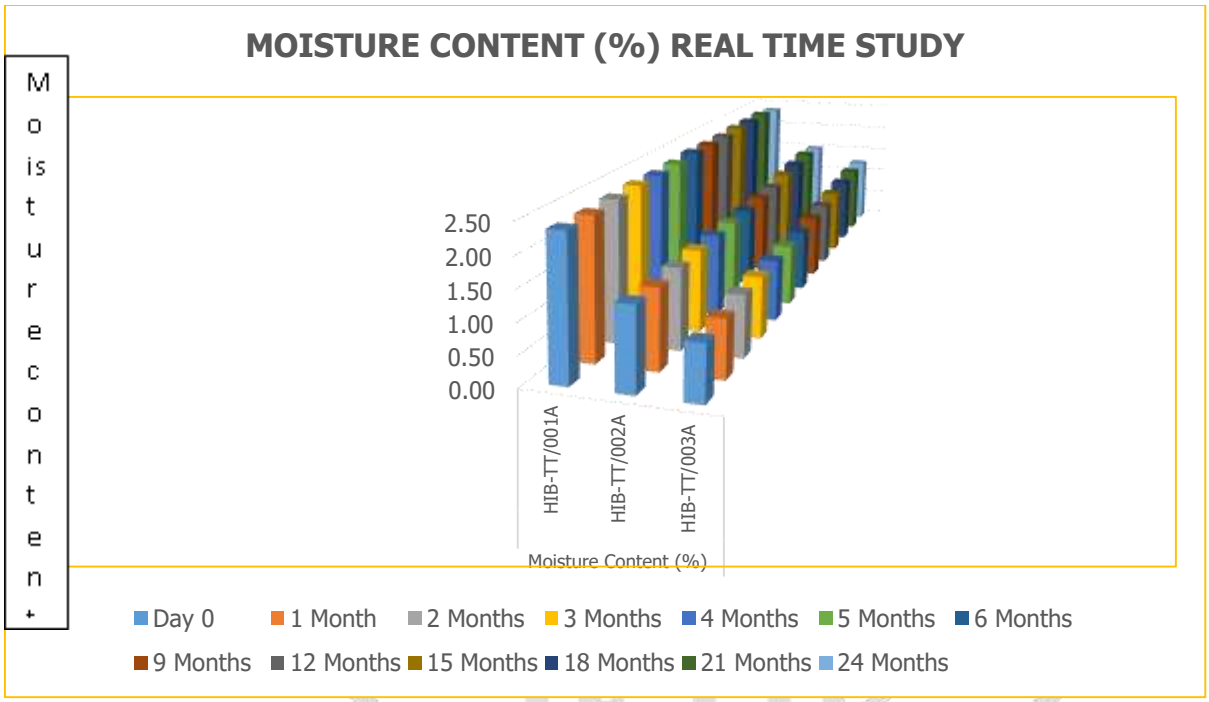


Figure 7

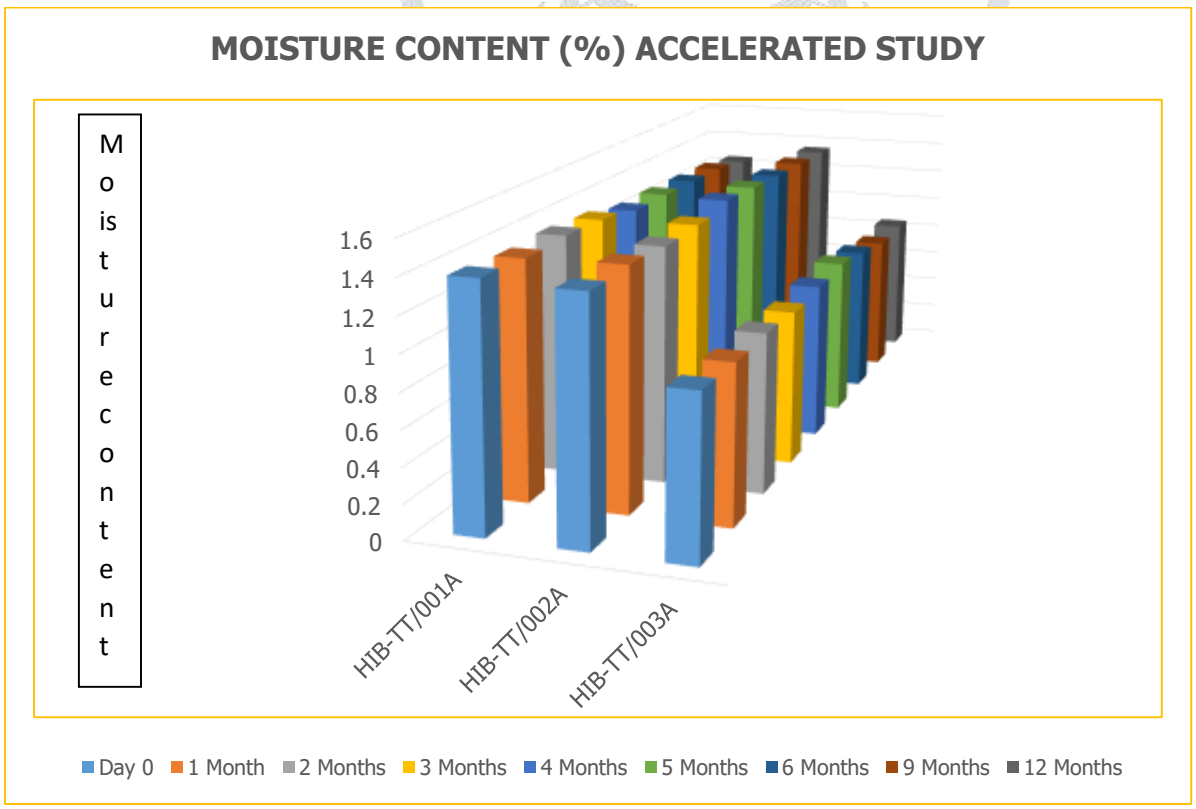


Figure 8

DISCUSSION

The lyophilized vaccine was good and devoid of aggregation of dried product. Shortening of Lyophlization cycle was done using reduction in primary and secondary drying stages (Kadam et al., 2005). Reduced cycle times reduce the cost of lyophilization as well. The lyophilized *haemophilus type b* conjugate

vaccine test results meets the requirement for individual components defined in Indian Pharmacopoeia, WHO TRS 897 and WHO TRS 941. In both real time and stress stability studies no major impact in PRP content ("Fig 5", "Fig 6".) was observed in the formulations studied (Australian Immunization Handbook, 2003). PRP reduction was totally less than 12% in 12 months at 25°C, in Batch No. HIB-TT/001A was 11.45%, Batch No. HIB-TT/002A was 11.58% and Batch No. HIB-TT/003A was 11.25% individually. In 36 months at 2-8°C PRP reduction was observed average of 22.33% in 24 months, in Batch No. HIB-TT/001A was 22.49%, Batch No. HIB-TT/002A was 22.11% and Batch No. HIB-TT/003A was 22.40% individually. The developed lyophilized *haemophilus type b* conjugate vaccine can be reconstituted before administration with suitable diluent like water for injection.

CONCLUSIONS

Improvement of the stability and increasing the shelf life of the *haemophilus type b* conjugate vaccines was achieved by the development of *Haemophilus type b* conjugate vaccine. Long shelf life (minimum 3 years) of lyophilized *Haemophilus type b* conjugate vaccine is suitable for stock piling for mass immunizations. The vaccine in lyophilized form was resistant to freezing. The lyophilized *haemophilus type b* conjugate vaccine can be used for immunization of children or adults who have not received haemophilus vaccination. By changing the temperature and pressure, the freeze-drying cycle of a vaccine was markedly decreased. The results obtained after the changes were in accordance to the international regulations, namely moisture content, aspect, accelerated thermostability and potency. Accordingly, the international regulations were also met after decreasing the freeze-drying cycle by 30 hrs to 22 hrs. The new freeze-drying cycle increased the production capacity of the vaccine and reduced the cost of the vaccine to meet the world immunization remarkably.

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