7 Stability studies

A few decades ago, only a few options were available for vaccines: an attenuated or inactivated virus, killed bacteria, inactivated toxin and polysaccharide preparation. Today, vaccines are developed using not only these traditional approaches, but also exploring the recent advantages of modern biotechnology, including DNA plasmids, recombinant viral vectors and recombinant proteins and many more. Presently, vaccines represent the most diverse class candidates in the pharmaceutical industry, including live attenuated pathogens (viruses, bacteria or parasites); inactivated pathogens; whole cells expressing immunogens; proteins, peptides and polysaccharides either un-conjugated or conjugated to various carriers; recombinant viral vectors; and recombinant plasmid DNA (Vose 1999 and Struck 1997). This diversity is compounded by the inclusion of various chemical or biologic adjuvants, antigen combinations, cytokines, delivery methods, polymers for delivery systems and complex excipients. Each one of these approaches present different challenges with respect to manufacturability and production consistency, analytic characterization and safety and stability evaluation. Hence, the types of product characterization, quality assessment, safety evaluation and stability studies involved in the non- clinical and pre-clinical development of new vaccines are numerous and complex (Jacob 2006). The interaction of components with each other in the formulation, with containers and closures and with external environment alters shelf life of the product and before entering to pre-clinical studies, it is necessary to determine non-clinical (i.e. In-vitro) stability studies (Yoshioka 1994).

Protein and plasmid products are often particularly sensitive to environmental factors. Hence, Stability assays are used to demonstrate that the vaccine product is stable in the final container, and the results can be used to set a shelf-life under appropriate storage conditions. Stability-indicating tests generally include assays for potency, physical degradation, moisture (if the vaccine is lyophilized), pH (if appropriate), sterility and endotoxin, and viability of cells (if applicable) (Liska 2007).

The objective of a stability study is to evaluate the quality of a biopharmaceutical product under various environmental conditions over a specific timeframe. Suitable storage and shipment conditions for drug substance as well as for drug product can be recommended for such studies. Shelf life or a retest period can also be determined.

Typically, real-time stability studies are undertaken to demonstrate stability; however, accelerated stability studies at elevated temperatures may provide early indication of product stability or instability. Accelerated stability studies are designed to determine the rate of change of vaccine properties over time as a consequence of the exposure to temperatures higher than those recommended for storage. These studies may provide useful support data for establishing the shelf-life or release specifications but should not be used to forecast real time real condition stability of a vaccine. They could also provide preliminary information on the vaccine stability at early developmental stages and assist in assessing stability profile of a vaccine after manufacturing changes (Jacob 2006).

7.1 Protocol for Stability studies

The tripartite harmonized ICH guidelines for biotechnological/biological products deal with the particular aspects of stability test procedures needed to take account of the special characteristics of products in which the active components are typically proteins and/or polypeptides (information taken from: (http://private.ich.org/cache/compo/363-272-1.html#Q5C).

ICH and Centre for Biologics Evaluation and Regulations (CBER) guidelines were followed for the stability studies of the prepared formulations. The recommended temperature for the protein based vaccine preparation is 2-8°C as per the CBER guidelines for biotechnology based vaccine formulations. Studies at higher (temp~ 20°C) were also conducted to check the degradation profile of the formulation. For these studies, various individual batches were prepared for analysis for getting a clear idea. Each batch was prepared from 20 mg of B-PEG-PLA, 0.2 mg of DDAB and to this 30 μg of ESAT-6 and Ag-85B proteins individually was conjugated. They were suspended in 150 μL of PBS and 10% of 50% w/v glycerol was added as

cryoprotectant. Individual tubes were frozen in liquid nitrogen and they were the lyophilized overnight. After lyophlization individual tubes were sealed properly to prevent moisture absorption. All tubes were preserved in sealed outer container containing anhydrous calcium carbonate as desiccant. Then they were stored at respective temperatures (2-8 ^{0}C and -20 ^{0}C). Each tubes' content were then suspended just before analysis in 150 µL volume of PBS, 10 times dilution were made and MicroBCA® assay were performed in triplicate. The data were compared with freshly prepared batch and % protein content was calculated. The initial concentration of the conjugated protein was determined and tabulated as 0 day reading and subsequently the readings were taken at specified time periods.

For antigens in pure form, 1 mg/ mL solution was prepared in PBS and aliquot in 50 µL of solution, lyophilized similarly as formulation and all tubes were stored with formulation tubes. The stability of lyophilized pure protein (ESAT-6 and AG 85-B) were checked at -20°C, 2-8°C and at RT along with the formulation

7.2 Results and Discussions

Numerous peptides and proteins elicit desirable therapeutic effects, but frequently utilization in therapeutic approaches have met with limited success owing to possible toxicity in the systemic circulation, poor bioavailability, premature degradation, poor stability and an inability to achieve useful therapeutic measures. Report suggested that conjugation of proteins to nanoparticles with specific chemical bond can circumvent these problems, achieving greater stability and higher specificity in delivery to the site of disease (Wu 2005).

However, by nature the proteins did not find stable at 37°C for more than a day so, we did not proceed for hour to hour stability profile at 37°C. At 20°C, proteins were stable only for 3 days and stability profile could not be developed for 20°C also. Hence, here we have given data for 2-8°C and -20°C for both the lyophilized formulation and pure antigens. The evaluation was based of on protein content in both.

Stability studies

The % of proteins present per sample tubes were calculated with respect to protein concentration determined in freshly prepared batches.

Table 7.1 shows stability study data for ESAT-6 when conjugated with NPs and in pure form at 2-8^{0}C and -20 ^{0}C. Figure 7.2 shows stability studies comparison for ESAT-6 (in pure form and in formulation) when stored at 2-8 ^{0}C and -20 ^{0}C.

ESAT-6 stability profile					
Sr. No	Days	Stability study When stored at 2-8 ^{0}C/		Stability study When stored at -20 ^{0}C	
		ESAT-6 % protein when conjugated with NPs	ESAT-6 in pure form	ESAT-6 % protein when conjugated with NPs	ESAT-6 in pure form
1	00	100	100	100	100
2	03	99.18 ± 0.38	99.37 ± 0.35	99.88 ± 0.09	100
3	07	98.37 ± 0.27	98.85 ± 0.38	98.77 ± 0.11	99.88 ± 0.21
4	10	97.89 ± 0.26	98.11 ± 0.45	98.18 ± 0.19	99.45 ± 0.12
5	15	96.94 ± 0.45	97.79 ± 0.28	97.89 ± 0.22	99.30 ± 0.15
6	18	96.34 ± 0.62	96.35 ± 0.36	97.28 ± 0.28	99.25 ± 0.09
7	21	96.21 ± 0.65	96.22 ± 0.65	96.21 ± 0.35	99.25 ± 0.11
8	25	95.35 ± 0.54	93.45 ± 0.58	95.85 ± 0.48	99.15 ± 0.10
9	30	93.75 ± 0.45	91.05 ± 0.48	94.75 ± 0.51	99.10 ± 0.15
10	35	88.90 ± 0.33	89.45 ± 0.36	92.90 ± 0. 98	99.35 ± 0.16
11	39	85.36 ± 0.38	87.95 ± 0.31	89.52 ± 1.62	99.4 ± 0.17
12	45	79.98 ± 0.42	82.75 ± 0.51	88.98 ± 1.98	99.52 ± 0.11

Table 7.1 Stability studies at 2-8 ^{0}C and -20 ^{0}Cfor ESAT-6 (pure and in formulation)

Stability studies

Table 7.2 shows stability study data for Ag 85B when conjugated with NPs and in pure form at 2-8^{0}C and -20 ^{0}C. Figure 7.2 shows stability studies comparison for Ag 85 B (in pure form and in formulation) when stored at 2-8 ^{0}C and -20 ^{0}C.

Ag 85B stability profile					
Sr. No	Days	Stability study When stored at 2-8 ^{0}C		Stability study When stored at -20 ^{0}C	
		Ag85B protein when conjugated with NPs	% Ag85B in pure form	Ag85B protein when conjugated with NPs	% Ag85B in pure form
1	00	100	100	100	100
2	03	99.57 ± 0.089	99.55 ± 0.08	99.57 ± 0.09	100
3	07	99.17 ± 0.11	98.21 ± 0.19	99.17 ± 0.1	99.98 ± 0.02
4	10	98.89 ± 0.13	97.91 ± 0.29	98.89 ± 0.12	99.5 ± 0.03
5	15	97.14 ± 0.98	97.19 ± 0.89	97.14 ± 0.32	99.37 ± 0.03
6	18	96.86 ± 1.28	96.85 ± 1.98	96.86 ± 0.92	99.65 ± 0.02
7	21	95.21 ± 2.25	96.22 ± 1.89	96.21 ± 1.92	99.25 ± 0.08
8	25	94.75 ± 2.35	95.45 ± 1.75	95.75 ± 1.3	99.15 ± 0.07
9	30	92.85 ± 2.87	95.15 ± 1.22	94.85 ± 1.98	99.10 ± 0.07
10	35	88.81 ± 2.23	89.45 ± 2.1	93.90 ± 1.89	99.15 ± 0.06
11	39	84.36 ± 2.36	85.95 ± 1.75	93.36 ± 2.01	99.12 ± 0.06
12	45	82.98 ± 2.89	82.75 ± 2.25	92.98 ± 1.78	99.12 ± 0.06

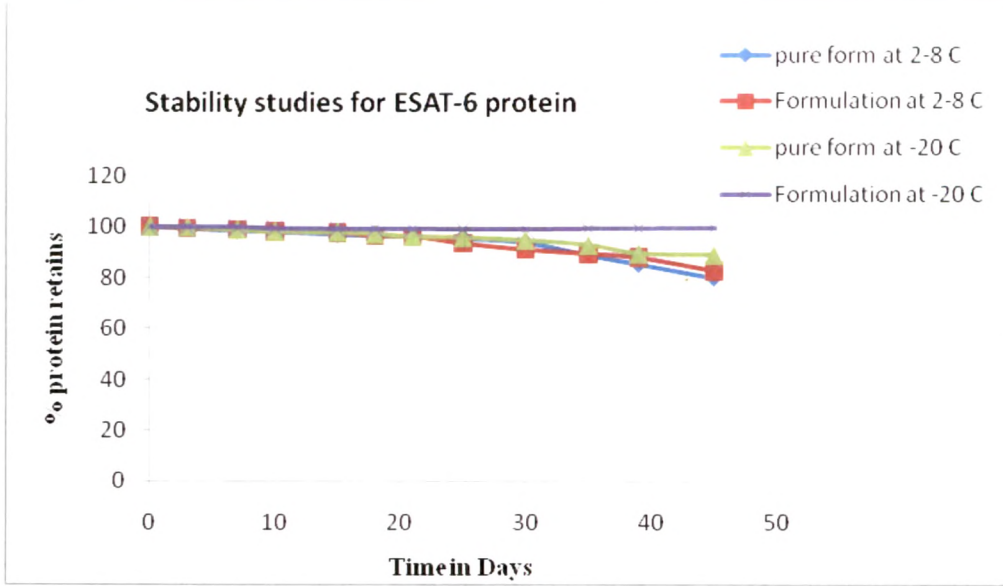

Figure 7.1 Stability studies of ESAT-6 at 2-8 ^{0}C and -20 ^{0}C

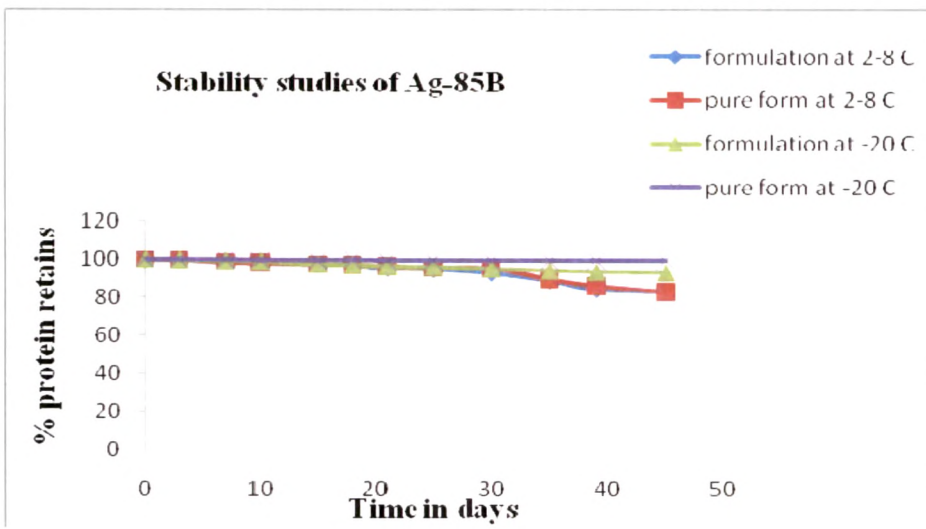

Figure 7.2 Stability studies of Ag-85B at 2-8 ^{0}C and -20 ^{0}C

At the end of Day 45, as shown in table 7.1 that at 2-8 ^{0}C, the concentration of ESAT-6 in B-PEG-PLA conjugated form was found 79.98% when compared with the ESAT-6 present in freshly prepared batches of ESAT-6 conjugated to B-PEG-PLA NPs. Hence, we can say, at the end of 45 days, about 20% of the protein either degraded or folded and in both the cases, the antigenic power of ESAT-6 alters. At 2-8^{0}C, on 45th day, the % ESAT-6 protein was 82.75% when compared with the freshly prepared ESAT-6 solution. Such estimation was done for the ESAT-6 in formulation and in pure form, stored at -20 ^{0}C; % concentration of protein was found 88.98 and 99.52 respectively. It indicates that there is slower degradation or folding of ESAT-6 at lower temperature.

For Ag85B, as shown in table 7.2, % protein concentration at Day 45, in the formulation and in the pure form, was 88.98 and 82.75 respectively. The protein concentration in both the case found was almost similar. When similar studies conducted at -20 ^{0}C, at the end of Day 45, the % concentration of Ag85 in formulation and in the pure form, it was 92.98 and 99.12 respectively. Similar conclusion we can make here that, at lower temperature, there is slower degradation or folding of protein.

If we consider, ±5% of the initial content as acceptable limit for the formulation, we can conclude that, the formulation was found stable for 30 days when formulation was stored at 2-8^{0}C and -20^{0}C, with storage at -20 C being more advisable.

7.3 References

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Reviewer's Comments

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