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# Comparative analysis of formulation buffers and storage temperatures on stability of a novel, recombinant multi-epitope peptide vaccine against COVID-19

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## ABSTRACT

**Background:** Formulation of therapeutic proteins is of utmost importance for their stability and biological activity. Protein-based vaccines also require optimal formulation for long term storage, transport and usage. Various analytical studies are performed to evaluate the stability of vaccines over a time period at different storage temperatures.

**Objectives:** The present study provides a comparative analysis of the stability of a novel multi-epitope COVID-19 vaccine in three different formulation buffers and two different storage temperatures.

**Methods:** Three different formulation buffers were prepared by addition of different adjuvants (FB01, FB02 and FB03). According to the International Conference on Harmonization (ICH- Q5C) guidelines, real-time and accelerated stability studies were carried out to determine the stability of the novel vaccine candidate at two storage temperatures – +2–8 ºC (real-time, up to 6 months) and +25 ºC (accelerated, up to 1 month).

**Results**: The stability assessment showed that the recombinant multi-epitope peptide vaccine was more stable at +2–8 ºC storage temperature in all three buffers tested as compared to when stored at +25 ºC.

**Conclusion**: The study reports that formulation buffer, FB01, is the most preferred buffer for maintaining the stability of the peptide vaccine at +2–8 ºC for 6 months.

**Keyword:** *Adjuvants, COVID-19; formulation; multi-epitope vaccine; stability studies.*

## 1. INTRODUCTION

Human immune system is an intricate network of T cells, B cells and antibodies involving the interplay of various proteins like cytokines and cellular receptors (1). Continuous research in the field of immunology has provided great insights into the functioning of the immune system. The immune system recognizes various pathogens like bacteria, fungi and viruses, but not every interaction is completely understood (1). Vaccines have been developed to prime our immune system against different pathogens, in order to protect from infections (2). The traditional classification of vaccines includes categorizing them into live attenuated and killed inactivated vaccines. The antigens in live attenuated vaccines comprise viable, weakened pathogens. In killed vaccines; heat or chemical inactivated pathogens function as antigens (2,3). Over years, with the progress in recombinant DNA technology, various protein- and peptide-based vaccines have been developed against different diseases apart from other biomolecules like DNA and mRNA.

Protein-based vaccines include subunit vaccines and conjugate vaccines. Highly antigenic portions of the viral or bacterial proteins form the basis of the subunit vaccines. Conjugate vaccines provide broad spectrum immunity by using bacterial lipopolysaccharides along with a carrier protein which serves as the antigen (2–4). However, the antigenic epitopes of protein-based vaccines are only weak stimulants of the immune system on their own. They are usually administered with adjuvants to increase their antigenicity and thus efficacy (5). The adjuvants used for various vaccines include traditional adjuvants and novel modern adjuvants (6). Traditional adjuvants are mineral salts like aluminium hydroxide (alum) and calcium phosphate, oil emulsion adjuvants like Freund's adjuvant (complete and incomplete) and montanide and immune-stimulatory complexes (ISCOMS), which are a complex antigen delivery system comprising a mixture of antigens and other molecules like cholesterol, saponins and phospholipids (6–8). Novel adjuvants comprise of liposomes and other

nanoparticles, bacterial derivatives like bacterial toxins, lipopolysaccharides, bacterial ghosts and other cell wall components, virus-like particles carrying empty capsids against the target pathogenic virus and oligonucleotides, mainly cytosine phosphor guanosine (CpG) dinucleotide motifs (6–8). Although adjuvants are important for increasing the efficacy of the vaccines, addition of adjuvants can lead to changes in structure of the protein antigen and thus decrease the efficacy. Adsorption of the protein onto alum or presence of oil-in-water emulsion or TLR-agonists can lead to changes in the protein conformation due to electrostatic interactions and denaturation (9). Furthermore, proteins are extremely labile to changes in the physical properties like pH, light, temperature and osmolarity, with high probability of unfolding and aggregation in the absence of optimal buffer conditions (10).

Since mass vaccination against various diseases has become a global trend, stability of the vaccines during transport is also crucial. Thus, formulation of protein-based vaccines is of utmost importance in order to obtain long-term stability, efficacy and safety of the vaccines. The stability of protein-based therapeutics and vaccines in different formulation buffers and adjuvants is studied using real-time stability and accelerated stability protocols according to the International Conference on Harmonization (ICH) guidelines (Q5C – Stability testing of biotechnological/biological products) (11–13). While real-time stability studies are carried out for a prolonged timeline (six months to one year) at the actual storage temperature (+2–8 ºC or -20 ºC or -80 ºC), accelerated stability studies are carried out by storing the vaccine or protein-based therapeutic in question at room temperature (+23–25 ºC) for a short period of time (up to 1 to 3 months) (14–16). Acquiring data from real-time stability studies is largely time-consuming and thus, forms a bottle-neck for a product. On the other hand, data from the accelerated stability studies can be extrapolated to provide a shelf-life for the vaccines, albeit marred with inaccuracies (14–16).

An earlier study reported the expression and purification of a recombinant, multi-epitope peptide vaccine against severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) (17). The present study provides insights into the stability of the peptide vaccine in three different formulation buffers (FB01, FB02 and FB03) with adjuvants in real-time (6 months at the actual storage temperature, +2-8 ºC) and through accelerated stress study (1 month at +25 ºC). The study makes available data regarding the osmolarity, pH, aggregation and protein concentration in the samples at various time points. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the protein samples along with the data of protein concentration and aggregation indicate that formulation buffer 3 (FB03) provided the most optimal conditions for stability of the vaccine. The study also indicates that in the formulation buffers tested, storage at +25 ºC is not possible and cold-chain supply is required for storage of the vaccine

## 2. MATERIALS AND METHODS

### *Formulation of purified vaccine drug substance*

The vaccine drug substance obtained after ultrafiltration-diafiltration through 100 kDa amicon device (17) was used for formulation of vaccine. Three different adjuvants were used, *viz.* Saponin Vaccine Adjuvant (VAdv-Ly0009, Creative Biolabs, USA), Freund's Complete Adjuvant (FCA, Solution, VAdv-Ly0006, Creative Biolabs, USA) and Alum Adjuvant (VAdv-Ly0002, Creative Biolabs, USA) to prepare three different formulation buffers, FB01, FB02 and FB03, by adding adjuvant (10% [w/v] saponin, 10% [v/v] Freund's Complete Adjuvant and 10% alum [w/v], respectively) to the final buffer (50 mM Tris, 10% [w/v] sucrose, 0.01% [v/v] Pluronic F68 and 250 mM arginine, pH 7.2, conductivity 6 mS/cm) of the vaccine drug substance. Vaccine drug substance (1 mL) was mixed with 1 mL of each of the formulation buffers and stored in round bottom glass vials (2 mL, Avantor, USA).

### *Stability studies of purified vaccine drug substance*

For stability studies, 150 µL of the formulated vaccine product was aliquoted into various 300 µL round bottom glass vials (Avantor, USA). This was done for each formulation studied. A total of approximately 13 aliquots were made for each formulation. Eight of the 13 vials were stored at +2-8 ºC for 6 months (180 days) for real-time stability analysis and the remaining five were stored at +25 ºC for accelerated stability analysis over a time period of 30 days. Table 1 provides the sampling time points.

### *Analytical tests for stability studies*

Various analytical tests were performed for studying the stability of the vaccine product in different formulation buffers and temperatures. Table 2 depicts the analytical tests performed and their acceptance limits.

*SDS-PAGE analysis and protein estimation using bicinchoninic acid (BCA) assay*

Samples (30 µL) were withdrawn at the indicated time points and prepared for SDS-PAGE analysis and protein estimation. Formulated vaccine samples (~3 µg) were loaded onto 12% pre-casted SDS-PAGE gel (Bio-Rad) with 4% stacking gel along with 2.5 µL of PageRuler™ Prestained Protein Ladder (10–180 kDa, Thermo Scientific, USA). Protein bands were visualized using Coomassie Brilliant Blue as described earlier (17). The samples were tested for the total protein content by the Pierce™ BCA protein assay kit (Thermo Scientific, USA).

*Measurement of pH, osmolarity, turbidity and visual inspection*

Remaining 120 µL of sample volume was used for pH, osmolarity and turbidity measurements. Microprobes were used for the measurement of pH (Ross Prep HecT micro combination pH electrode, ThermoFisher Scientific, USA) and osmolarity (OsmoPRO® Multi-Sample Micro-Osmometer, Advanced Instruments, USA). Samples were also visually inspected for any observable aggregation and turbidity. Further, changes in turbidity were quantified by a turbidimeter (2100AN, HACH, Malaysia) at various time points after diluting the samples up to 3.0 mL (25 times dilution for all samples). All the samples fitting into the acceptance criteria (Table 2) were then used for SDS-PAGE analysis and protein estimation as described above.

*Dynamic light scattering (DLS)*

For all the formulated samples, DLS was performed to assess the particle size, and aggregate formation (day 180 post formulation for +2-8 ºC storage and day 7 post formulation for +25 ºC storage) using the Malvern Zetasizer (Malvern Panalytical Ltd., Worcestershire, UK) in triplicates.

## 3. RESULTS

***Stability analysis by pH, protein content, turbidity, osmolarity and visual inspection***

The stability assessment using the three formulation buffers, FB01, FB02 and FB03, showed that the recombinant multi-epitope protein vaccine against COVID-19 was more stable at +2–8 ºC storage in all the buffers (Table 3, Fig. 1) as compared to when stored at +25 ºC (Table 4, Fig. 2). Precipitation and heavy turbidity were observed by day 7 post formulation when stored at +25 ºC. No such precipitation was observed until day 180 in storage at +2–8 ºC. Results from all the analytical tests further showed that the vaccine was most stable in FB03 (containing 10% [w/v] alum as the adjuvant) (Fig. 1). As observed from readings for osmolarity, turbidity, pH and total protein content, the protein subunit vaccine formulated in FB03 showed values similar to day 0 even on day 180 (Table 3, Fig. 1). In case of FB02, differences were observed in osmolarity and turbidity after day 90 post formulation and storage at +2-8 ºC (Table 3, Fig. 1).

***SDS-PAGE analysis for protein stability***

SDS-PAGE analysis of all the samples for all the time points at +2–8 ºC showed a single prominent band at ~35 kDa (Fig. 3 A–F). All the samples showed negligible amounts of higher molecular weight bands. However, no degradation products were observed. Since precipitation was observed in samples stored at +25 ºC day 15 onwards, SDS-PAGE analysis was done only up to day 7 (Fig. 1A and B). As observed from the gels, a single intact band was observed for samples stored at +25 ºC util day 7 of storage.

***Aggregate analysis by DLS***

Dynamic light scattering (DLS) technique helps in assessing the particle size and zeta potential of the samples(18). Although the differences observed for FB01 with storage at +2–8 ºC were minor, the DLS data showed higher order aggregate peaks (Fig. 2). Similar pattern was observed along with low molecular weight protein in FB02, upon storage at +2-8ºC. Vaccine in FB03 when stored at +2–8 ºC showed a single peak at ~20 nm, as reported earlier (19) and was devoid of any higher order aggregate peak (Fig. 4). DLS data showed aggregates for all the samples stored at +25 ºC when tested at day 7 (data not shown) corroborating with the observation of precipitation visually and with turbidimeter.

## 4. DISCUSSION

This study presents the analytical data for the formulation and stability of the novel, multi-epitope vaccine candidate developed against COVID-19. This is one of the few studies to show stability of peptide vaccine in three different formulation buffers, encompassing different classes of adjuvants, i.e., ISCOMS (saponin in FB01), water-in-oil emulsion-based adjuvant (FCA in FB02) and aluminium salt adjuvant (alum in FB03) (20,21).

The study shows that the vaccine is most stable in FB03 than the other two formulation buffers tested (FB01 and FB02). The preferred temperature for storage was found to be +2–8ºC, indicating the compulsive need for cold chain supply of the vaccine. While acceptance limits for the parameters tested were set, the osmolarity of the FB02 was higher at the beginning due to the addition of FCA. Higher osmolarity (~400 mOsm/kg) was observed in serum of mice injected with FCA as compared to normal saline (300 mOsm/kg) (22). Presence of oil emulsion based adjuvant in FB02 further increased the protein aggregation, which was observed as slight increase in turbidity measurements and high molecular weight aggregates in DLS, coinciding with earlier observations that oil emulsion adjuvants may lead to protein aggregation (23). Use of saponin as adjuvant also led to increased aggregation, as observed by DLS data.

Measurement of pH through the tie period of stability analysis is equally important as changes in pH can lead to physical and chemical changes in the stored protein (24). pH similar to the isoelectric pH (pI) of the protein can lead to aggregation. Further, changes in pH to the acidic range can increase the chances of peptide bind hydrolysis, methionine oxidation and deamidation of the amino group of protein. On the other hand, basic pH can also lead to certain chemical changes like

cysteine oxidation resulting in disulphide bond formation, changes in disulphide linkages and deamidation through cyclic imine intermediate (24). Aggregation of peptide vaccine may be attributable to the decrease in pH when stored at +25 °C (Table 4). Similar results have been observed in earlier studies (25).

Free amino acids like arginine and glutamic acid in formulation buffer help in reducing protein-protein interaction, thus preventing aggregation (26). Presence of arginine in the formulation buffer cannot rescue the protein degradation and aggregation caused due to both CFA and saponin. This could be due to the micelle forming property of the adjuvants, which may affect the protein conformation. Alum is one of the most commonly used adjuvants, especially in the recently developed vaccines against SARS-CoV2 (27). Also, very few vaccines approved for human use have saponin or CFA as adjuvants due to their possible toxicity issues (27,28). This can be further explained by increase in the osmolarity of the samples which could lead to aggregate formation (29).

The study reports that alum is the most preferred adjuvant in maintaining the stability of the protein vaccine at +2–8ºC for 6 months. This coincides with other studies pertaining to stability and immunogenicity of vaccine candidates against COVID-19 with aluminium salts as the adjuvant (30,31). In context of a multi-epitope recombinant protein vaccine candidate presented here, another study showed that alum is a good adjuvant for a combinatorial three-protein subunit vaccine (32), supporting the observation of high stability of the vaccine in FB03.

## 5. CONCLUSION

The current study reports the optimal formulation buffer and storage condition of a novel, multi-epitope peptide-based vaccine for COVID-19. The real-time and accelerated stability studies demonstrated that the vaccine is most stable in formulation buffer adjuvanted with alum and stored at +2–8 °C, indicating low cost in terms of formulation and adjuvant requirement but a necessary clod chain requirement for the storage and transport. While the study provides data on the physical characterization of the peptide vaccine, its ability to comment about vaccine efficacy is limited by the lack of *in vitro* and *in vivo* studies.

**Table 1. Strategy for stability analysis of the formulated vaccine drug product**

| Sr. No. | Sampling time point | Real time stability (+2-8ºC) | Accelerated stability (+25ºC) |
|---|---|---|---|
| 1 | Day 0 | √ | √ |
| 2 | Day 7 | √ | √ |
| 3 | Day 15 | √ | √ |
| 4 | Day 30 | √ | √ |
| 5 | Day 90 | √ | × |
| 6 | Day 180 | √ | × |

**Table 2. Analytical tests and acceptance limits for stability of formulated vaccine product**

| Sr. No. | Tests | Acceptance limit |
|---|---|---|
| 1. | pH | 7.4±0.3 |
| | Osmolarity (should be isotonic to plasma) | 300±50 mOsm/kg |
| 2. | SDS-PAGE (Purity) | ~35 kDa |
| | Turbidity by Nephelometric Turbidity Unit (NTU) | 15±05 NTU |
| | Appearance (Visual observation) | Clear solution |
| 3. | Protein concentration | 450±30 (µg/mL) |

**Table 3. pH, osmolarity, protein content and turbidity for all formulated samples stored at +2–8 °C**

| Time point | Test | FB01 | FB02 | FB03 |
|---|---|---|---|---|
| **Day 0** | pH | 7.29 | 7.54 | 7.42 |
| | Osmolarity (mOsm/kg) | 310 | 398^ | 298 |
| | Turbidity (NTU) | 13 | 11 | 10 |
| | Protein Concentration (µg/mL) | 540 | 560 | 557 |
| | Visual Observation | Clear | Clear | Clear |
| **Day 7** | pH | 7.27 | 7.59 | 7.43 |
| | Osmolarity (mOsm/kg) | 312 | 410 | 300 |
| | Turbidity (NTU) | 15 | 13 | 12 |
| | Protein Concentration (µg/mL) | 538 | 555 | 553 |
| | Visual Observation | Clear | Clear | Clear |
| **Day 15** | pH | 7.27 | 7.51 | 7.48 |
| | Osmolarity (mOsm/kg) | 315 | 412 | 305 |
| | Turbidity (NTU) | 17 | 16 | 13 |
| | Protein Concentration (µg/mL) | 540 | 549 | 557 |
| | Visual Observation | Clear | Clear | Clear |
| **Day 30** | pH | 7.32 | 7.57 | 7.45 |
| | Osmolarity (mOsm/kg) | 310 | 410 | 308 |
| | Turbidity (NTU) | 15 | 14 | 12 |
| | Protein Concentration (µg/mL) | 545 | 555 | 560 |
| | Visual Observation | Clear | Clear | Clear |
| **Day 90** | pH | 7.25 | 7.65 | 7.51 |
| | Osmolarity (mOsm/kg) | 320 | 672 | 315 |
| | Turbidity (NTU) | 19 | 18 | 13 |
| | Protein Concentration (µg/mL) | 510 | 580 | 562 |
| | Visual Observation | Clear | Clear | Clear |
| **Day 180** | pH | 6.92 | 7.78 | 7.25 |
| | Osmolarity (mOsm/kg) | 327 | 699 | 313 |
| | Turbidity (NTU) | 19 | 25 | 13 |
| | Protein Concentration (µg/mL) | 475 | 596 | 560 |
| | Visual Observation | Clear | Clear | Clear |

^The osmolarity was higher than the acceptable limits due addition of FCA (22).

**Table 4. pH, osmolarity, protein content and turbidity for all formulated samples stored at +25 °C**

| Time point | Test | FB01 | FB02 | FB03 |
|---|---|---|---|---|
| **Day 0** | pH | 7.29 | 7.54 | 7.42 |
| | Osmolarity (mOsm/kg) | 310 | 398^ | 299 |
| | Turbidity (NTU) | 13 | 11 | 10 |
| | Protein Concentration (µg/mL) | 542 | 557 | 557 |
| | Visual Observation | Clear | Clear | Clear |
| **Day 7** | pH | 6.51 | 6.83 | 7.32 |
| | Osmolarity (mOsm/kg) | 210 | 215 | 270 |
| | Turbidity (NTU) | 35 | 38 | 26 |
| | Protein Concentration (µg/mL) | 590 | 598 | 576 |
| | Visual Observation | PPT[#] | PPT[#] | Milky |
| **Day 15** | pH | 6.2 | 6.22 | 6.12 |
| | Osmolarity (mOsm/kg) | 70 | 80 | 78 |
| | Turbidity (NTU) | 49 | 51 | 55 |
| | Protein Concentration (µg/mL) | 610 | 615 | 598 |
| | Visual Observation | PPT[#] | PPT[#] | PPT[#] |
| **Day 30** | pH | ND* | | |
| | Osmolarity (mOsm/kg) | | | |
| | Turbidity (NTU) | | | |
| | Protein Concentration (µg/mL) | | | |
| | Visual Observation | PPT[#] | PPT[#] | PPT[#] |

*ND = Not determined. [#]PPT = precipitation. ^The osmolarity was higher than the acceptable limits due addition of FCA (22).

**Figures**

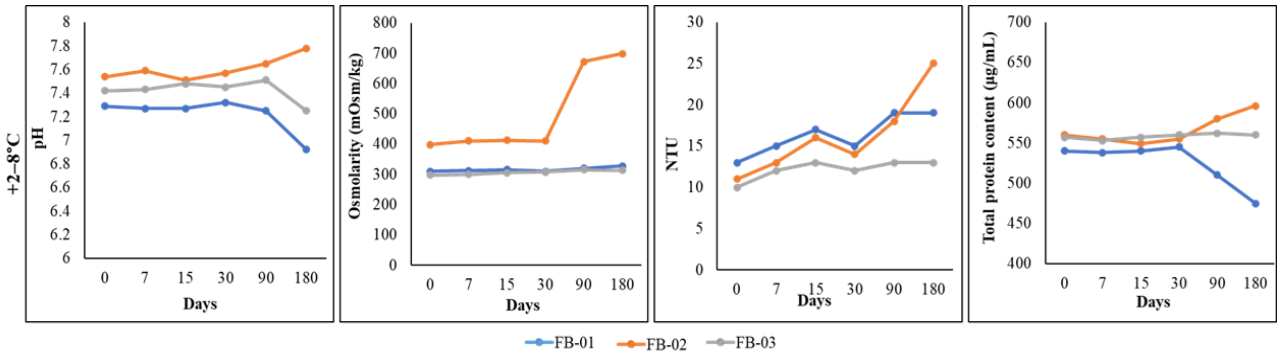


**Fig. 1. Trends in parameters tested during real-time stability study**

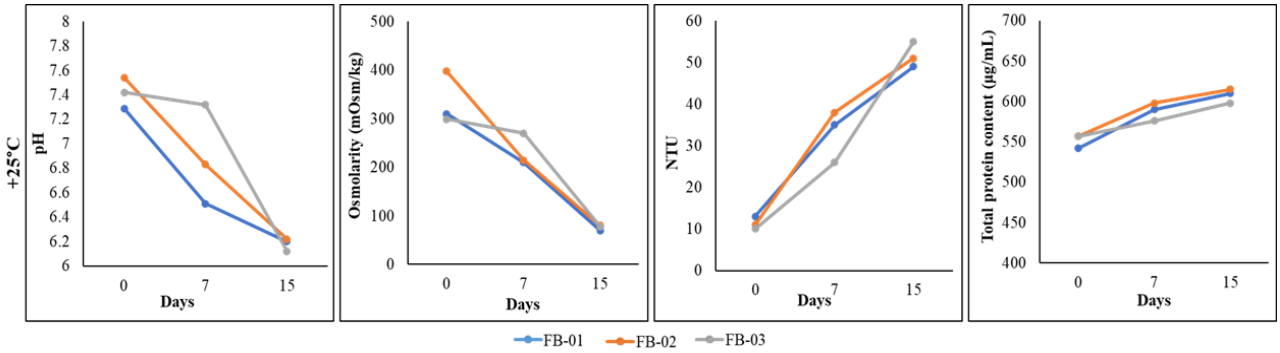


**Fig. 2. Trends in parameters tested during accelerated stability study**

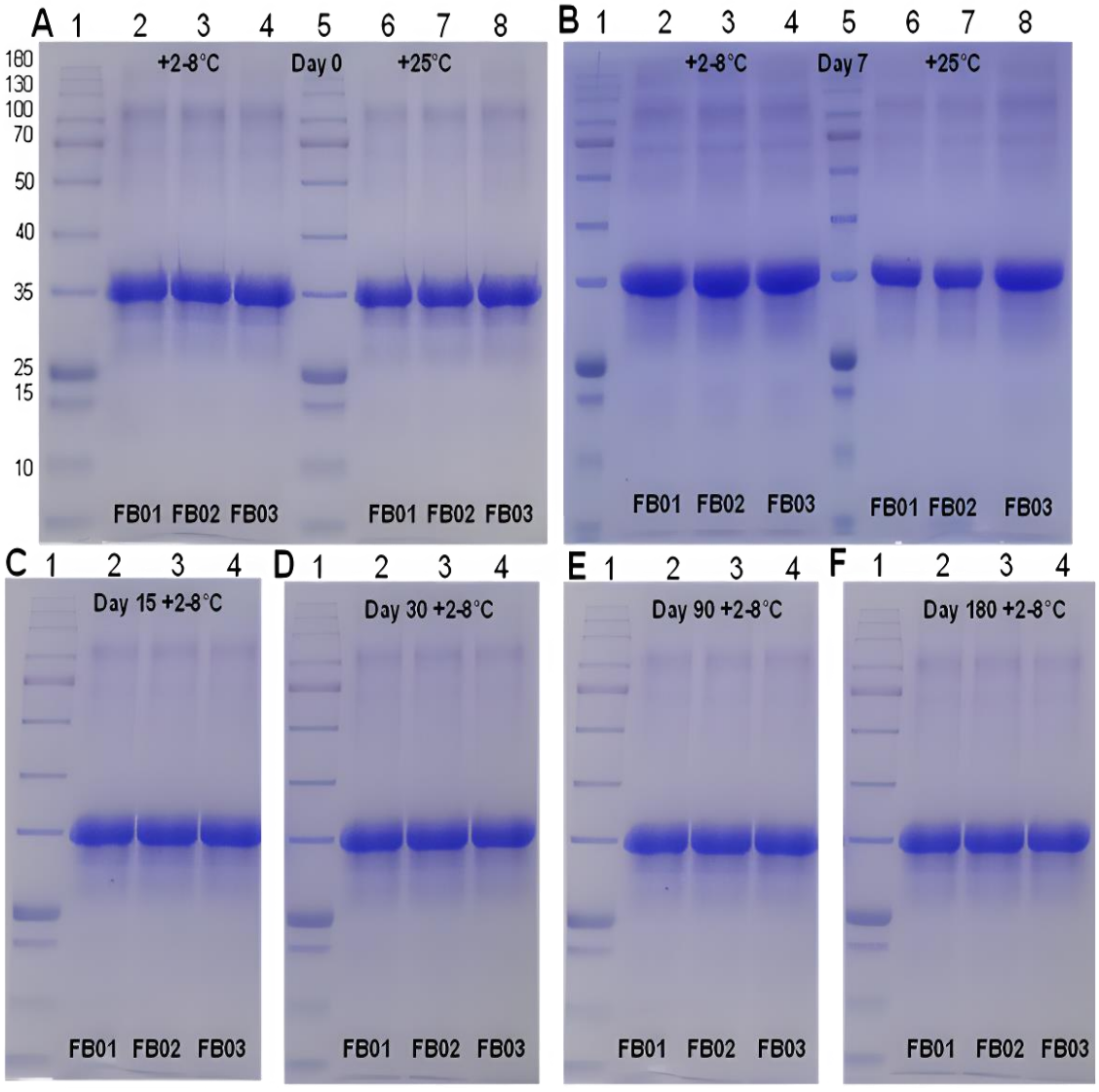


**Fig. 3. SDS-PAGE analyses of the samples withdrawn at different time points.**

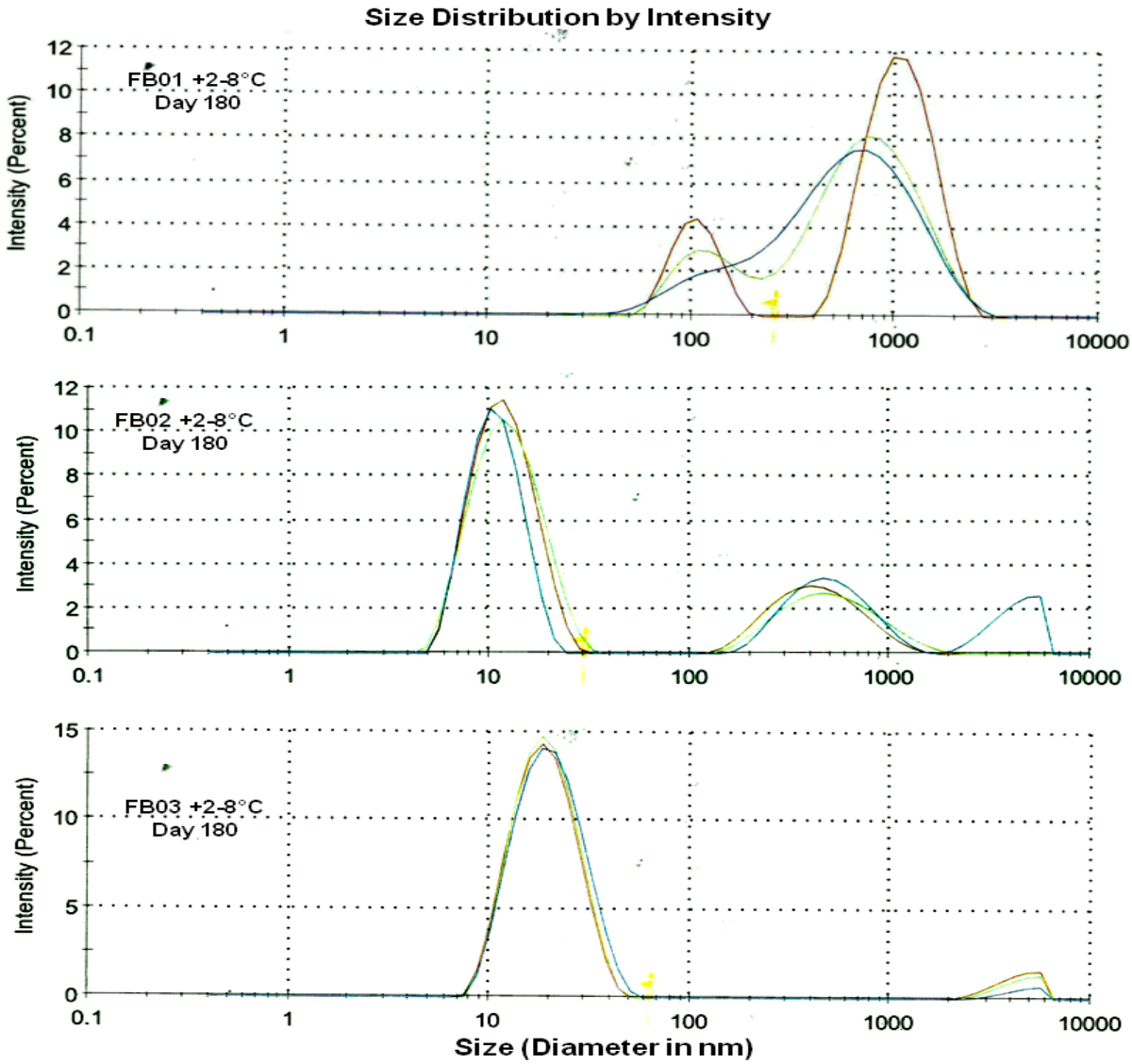


**Fig. 4. DLS distribution curves for triplicate runs of all formulated samples stored at +2–8 °C.**

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**Conflict of Interest Disclosure:**

The authors declare that they have no conflicts of interest regarding the publication of this research article.

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**Author's Contribution:**

Alok Singh contributed to the designing and execution of experiments, analysis of data and writing and compiling the manuscript. Prevesh Kumar and Diksha carried out the proof-reading and editing of the manuscript. Navneet Verma approved the data and manuscript for publication.

**Data Availability Statement:**

The manuscript incorporates all datasets produced or examined throughout this research study.

**Ethics Approval Statement:**

Not Applicable

**Informed Consent Statement:**

Not Applicable

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