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Process optimization of Lyophilization for long term stability of Edaravone nanosuspension

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Abstract
Downstream processing during nanosuspension formulation can impose stress on particles, potentially leading to Ostwald ripening. To maintain its integrity throughout storage, solidification is important. This study thoroughly investigated the effect of cryoprotectant type and concertation, freezing temperature, cooling rate and primary drying temperature on redispersibility and particle size along with a six-month stability study. Optimal results were achieved using trehalose, low freezing temperatures, slower freezing rates, and extended secondary drying times. Additionally, the lyophilized product demonstrated enhanced aqueous saturation solubility. Solid-state characterization and residual moisture content analysis were performed on the optimized formulation. A robust lyophilization protocol was successfully developed, allowing the reconstitution of solid nanocrystals with minimal changes to their physicochemical properties. Over the six-month period, formulation exhibited the greatest stability, with negligible changes in particle size and excellent redispersibility. Lyophilization can improve the efficiency of the prepared nanosuspension based drug delivery system.

Keywords: Cooling rate, Cryoprotectant, Lyophilization, Nanosuspension, Particle growth, Redispersibility

Introduction
Nanoparticles are among the most extensively studied drug delivery systems, especially the targeted drug delivery application. Due to smaller size and larger surface area nanoparticles significantly enhance the solubility and dissolution of poorly water-soluble drug specifically BCS class II drugs. However, the long-term stability of nanoparticles, especially when formulated as aqueous nanosuspensions, remains a major challenge. Physical instability in terms of particle agglomeration caused by Oswald ripening and chemical instability due to hydrolysis of stabilizers can results in drug leakage and degradation during storage [1].

To overcome these challenges and extend the application of nanosuspensions particularly for oral or parenteral delivery they are often subjected to downstream solidification processes. These include methods such as spray drying, freeze drying (lyophilization), fluid bed drying, and various granulation techniques.

While effective, these methods can exert physical and thermal stress on the nanosuspension, potentially affecting its original particle size distribution (typically d50 < 1 µm and d95 < 2.5 µm) [2]. These stresses can also lead to undesirable transformations in the solid-state properties of the active pharmaceutical ingredient (API), such as polymorphic changes or conversion between crystalline and amorphous forms, thereby impacting solubility, dissolution, and overall drug stability. Therefore, optimization of formulation and process parameters of solidification is essential to preserve the physical and chemical integrity of the nanoparticles after solidification. Apart from this, some other considerations like redispersion time and residual moisture content are also important to improve the solubility, dissolution behavior and bioavailability of drug.

Of the above aforementioned solidification methods, freeze drying, also known as lyophilization, is considered the most preferred industrialized method for thermosensitive materials. This process removes water through sublimation under reduced pressure, thereby avoiding high thermal exposure. Although freeze drying is energy-intensive and time-consuming, it is preferred in the pharmaceutical industry due to its ability to preserve heat-sensitive compounds [3].

On the contrary, various challenges have to be considered during the lyophilization of the nanocrystals such as maintaining original particle size because particle aggregates during the stress induced in the process.

Lyophilization introduces thermal stress during both the freezing and drying stages, which can lead to nanocrystal aggregation. During freezing, nanocrystals become confined within interstitial spaces of ice crystals, promoting aggregation. During drying, steric hindrance from stabilizers may cause particle fusion. Additional process parameters such as cooling rate, freezing temperature, drying shelf temperature, and duration also significantly influence redispersibility and final particle size. Equipment type (e.g., lyophilizer shelf, liquid nitrogen quenching, deep freezers) and freezing intensity (vacuum level, shelf temperature, and freezing duration) play crucial roles [4]. The inclusion of cryoprotectants is generally essential for minimizing aggregation during lyophilization. Both the type and concentration of cryoprotectant can significantly impact the physicochemical properties of the final dried product [5].

Given that different drugs exhibit varying responses based on their hydrophobicity, dissolution characteristics, and aggregation tendencies, it is critical to optimize both formulation and process parameters specifically for each compound. Therefore, the objective of this work is to systemically evaluate the impact of lyophilization process on redispersed particle size and saturation solubility of EDR nanosuspension formulated by sono-precipitation method. Key variables investigated include the effect of type and concertation of cryoprotectant, freezing temperature, cooling rate and primary drying temperature. The optimized formulation was further assessed for its solid-state characterization and residual moisture content to ensure long-term stability and improved drug performance.

Materials and Methods

Materials

Edaravone was kindly gifted by Sun Pharmaceutical Ltd and Soluplus was gifted from the BASF, Mumbai. Tween 80 as a surfactant, methanol, mannitol, trehalose, lactose monohydrates, poly (vinyl alcohol) were purchased from commercial source. All used reagents were of analytical grades.

Methodology

Preparation of nanosuspension

EDR nanosuspension was formulated by precipitation-ultrasonication method by slight modification [6]. Methanolic solution of EDR acted as a solvent and aqueous solutions containing soluplus as a stabilizer and Tween 80 as a surfactant acted as antisolvent. Precisely, solvent was introduced into the anti-solvent system with continuous stirring for the evaporation of the organic phase about 30 minutes at room temperature. For further reduction in particle size, sonicate the prepared formulation using probe sonicator having a tip diameter of 8.0 mm at ultrasonic power inputs (600A). The ultrasound burst period was set to 2 seconds, with a 2-second pause between bursts, for a total of 5-7 cycles. The temperature was controlled using an ice bath to prevent loss of the nanoparticles and drug loss.

Freeze-thaw study

Based on literature review, sugars are recognized as effective cryoprotectant. This is attributed due to their favorable hydrogen bonding interactions, as explained by the water replacement hypothesis. These cryoprotectants may also interact synergistically with nanosuspension-stabilizing polymers, thereby offering enhanced protection during freezing and drying [7].

EDR loaded nanosuspension were prepared and three different sugar-based cryoprotectant such as trehalose, mannitol and lactose monohydrates were evaluated at 1:3 ratio (net solid content: cryoprotectant). 1 mL of each formulation was transferred Type 1 borosilicated glass vial. All the vials are sealed with bromo butyl slotted rubber and placed in the shelves of lyophilizer (Virtis Advantages Plus Lyophilizer). A formulation without cryoprotectant was used as a control. All the samples were subjected to a freeze-thaw cycle by freezing for 12hrs in deep freezer (-20 0C) followed by thawing at room temperature (280 C). After thawing, each sample was evaluated for the particle size and polydispersity index by zetasizer. Redispersibility of the final product was investigated by reconstituting the lyophilized cake with the same amount of water lost during the lyophilization process. The samples were vortexed for 30 seconds and visually observed for ease of dispersion and presence of undispersed particles.

The qualitative scale used for redispersibility evaluation was as follows:

- = fully dispersed within 30 s of vortexing
+ = remaining few undispersed visible agglomerates
++ = no dispersion of solid after 30 S of vortexing

Effect of concentration of cryoprotectant

Increase in cryoprotectant concentration found to be beneficial for redispersibility, however, protection may plateau or even decline when concentrations exceed an optimal threshold. Based on the initial freeze-thaw studies, trehalose and mannitol were chosen for further investigation due to their favorable performance. Based on observed decreasing particles size with higher concentrations, further four different concentrations were chosen for study. Starting with the ratio used in freeze thaw study and increasing up-to 2.5 fold (1:4, 1:6, 1:8, 1:10) along with control sample (without cryoprotectant). The conditions used for lyophilization process were: freezing up to - 400 °C for 2 hrs, primary drying -200 °C for 24 hrs and secondary drying 200 C for 16hrs were selected with vacuum of 120 mTorr. The final lyophilized products were analyzed for particle size and redispersibility to determine the optimal cryoprotectant concentration.

Effect of freezing temperature and cooling rate

To evaluate the influence of freezing conditions on lyophilization outcomes, EDR nanosuspensions with trehalose (1:4 ratio) were subjected to two different freezing temperatures: -40 °C (commonly used, where ~90% of water is frozen) and -70 °C (a more aggressive freezing condition). Both freezing processes were maintained for 2 hours. Cooling rate also influences the size of ice crystal formation affecting interstitial spacing and the degree of supercooling and nucleation. Slow cooling tends to produce larger ice crystals, while rapid cooling leads to smaller crystals, influencing redispersibility. Prior to freezing, the shelf temperature was ramped to 50C for 20 minutes for establishment of thermal equilibrium to minimize the super cooling effects on the subsequent lyophilization steps. Cooling rate was compared between 2 °C /min and 0.5 °C /min. The subsequent lyophilization parameters were as

follows: primary drying at -20 °C for 24hrs and secondary drying at 20 °C for 16hrs. A vacuum of 120 mTorr is applied during the drying process.

Effect of secondary drying time

To understand the impact of secondary drying time on the rate of nucleation and its behaviour and particle size, the lyophilization protocol was carried out with two drying time intervals: 8 hours and 16 hours at a constant temperature of 20°C. The remaining lyophilization process parameters were same as described above whereas the freezing temperature was set at – 40 °C for 2 hrs. This investigation aimed to determine whether extended secondary drying improves the removal of residual moisture and preserves nanoparticle characteristics post-lyophilization.

Solid state characterization

Nanosuspension was lyophilized using trehalose as a cryoprotectant. Lyophilized solid nanosuspension was subjected for DSC, XRD and SEM studies.

Differential scanning colorimetry [8]

Using an aluminum sealed pan and the DSC-8000 PerkinElmer Thermal Analysis instrument, DSC thermograms were produced. Under an inert nitrogen environment with a flow rate of 20 mL/min, the analysis was carried out at a scanning rate of 10 °C/min, covering a temperature range of 45-270 °C. Maintaining a steady nitrogen supply helps to reduce the DCS cell's heat gradient. Tests were conducted on lyophilized product, soluplus, trehalose, and pure EDR. As a guide, the same empty pan was utilized.

Powdered X-ray diffraction analysis [9]

The material's crystalline nature and dispersion were identified by XED. The samples of pure EDR, Soluplus, Trehaloes and lyophilized nanosuspension of EDR were analyzed by X-ray powder diffractometer (Bruker D8 AXS Advance, Germany). The analysis involved exposing the samples to a sealed X-ray tube operating at 40 kV voltage and 30 mA of current. Measurements were conducted in θ/2θ geometry on a flat plate, with 2θ ranging from 5 to 90 degrees, and a step width of 0.0500.

Scanning electron microscopy

The lyophilized dry powder of nanosuspension W89 was topographically characterized using a scanning electron microscope (Tecnai 20, Philips, Holland) running at electron beam conductivity operating at an acceleration voltage of 200 kV. The samples were coated with carbon to improve the conductivity of electron beam.

Short Term Stability

A short-term stability studies (4 weeks) of EDR NPs containing 1:4 ratio of net solid content to trehalose solution was subjected to stability studies as per ICH guidelines at 30±2 °C/65±5% RH and 40±2 °C/75±5% RH. Lyophilized samples in borosilicated amber colored glass vial with screw-top cap was kept in the stability chambers. The samples were withdrawn at the end of 30, 60, 90, and 180 days. Samples are reconstituted and evaluated for its particle size and redispersibility.

Result and Discussion

Freeze-thaw study

As lyophilization process is expensive, selection of an effective cryoprotectant is utmost important to preserve nanoparticle integrity. The freeze-thaw studies are commonly used to screen cryoprotectant by simulating the stress of freezing during lyophilization. If the cryoprotectant fails to protect the nanosuspension during this stage, it is likely to be ineffective in the complete lyophilization process. Three cryoprotectants trehalose, mannitol, and lactose monohydrate were selected based on literature evidence. In general, rapid freezing leads to lower particle size compared to slow freezing, as it restricts particle movement and prevents aggregation [10].

The control sample (no cryoprotectant) failed to redisperse and could not be evaluated for particle size. All the samples with cryoprotectant exhibited improved redispersibility, though varying levels of particle size growth were observed. Lactose monohydrate showed significant increment in particle size, while trehalose demonstrated the least growth and a better polydispersity index compared to mannitol (Table 1). Thus, trehalose emerged as the most promising cryoprotectant.

Table 1: Freeze thaw studies

Type of cryoprotectant	Before lyophilization		After lyophilization			
(1:4)	Particle size (nm)	PDI	Particle size (nm)	PDI	Redispersibility	Cake appurtenance
Control	74.61	0.250	NA		+	No cake
Trehalose	76.28	0.276	77.90	0.305	-	more homogenous and porous
Mannitol	78.93	0.240	89.1	0.193	-	homogenous non-porous
Lactose monohydrates	71.49	0.178	108.5	0.354	-	Shrinkage

Effect of concentration of cryoprotectant

The level of stabilization also depends on the concentration of cryoprotectant. During freezing, the temperature below glass transition temperature (Tg′) of the cryoprotectant, a protective glassy coating around the nanoparticles, shielding them against the mechanical stress of ice crystals formation, thereby preventing aggregation.

According to the particle isolation hypothesis, the spatial separation of particles within the unfrozen fraction prevents aggregation but only when the cryoprotectant concentration and freezing rate are optimized. As freezing temperature below Tg′ of cryoprotectant has no effect on the glassy protective matrix of cryoprotectant formed around the nanoparticles [11]. Selection of its concentration is crucial and can be determined based on its diffusion rate and freezing rate. At higher concentration, rate of diffusion is rate limiting due to high viscosity and cryoprotectant remains in bulk frozen state. Although, at some critical concentration, the rate of diffusion is faster than freezing rate, results into particle aggregation. However, excessive cryoprotectant can increase viscosity and slow diffusion, potentially leading to particle aggregation due to incomplete coverage.

In mannitol-based formulations, a minor particle size increase (< 10 nm) was observed at the highest concentration, with negligible change in PDI. Trehalose did not show a consistent trend; however, its low concentration (1:4 ratio) was sufficient to provide effective protection with minimal particle growth (Table 2).

Table 2: Effect of concentration of cryoprotectant

Ratio	Type of cryoprotectant	Before lyophilization		After lyophilization		
		Particle size (nm)	PDI	Particle size (nm)	PDI	Redisersibility
Control		76.61	0.250	-	-	NA
1:4	Trehalose	54.47	0.083	56.70	0.115	-
	Mannitol	57.73	0.084	68.25	0.269	-
1:6	Trehalose	54.45	0.082	61.60	0.162	-
	Mannitol	59.22	0.108	76.28	0.276	-
1:8	Trehalose	63.85	0.141	67.60	0.162	-
	Mannitol	65.98	0.182	76.90	0.345	-
1:10	Trehalose	70.36	0.204	77.90	0.305	-
	Mannitol	75.96	0.209	77.63	0.273	-

Trehalose, due to its excellent glass-forming ability and capacity to form hydrogen bonds upon dehydration, proved superior in maintaining nanoparticle structure. Mannitol, though offering an elegant cake structure, has limitations due to eutectic formation and potential for shorter lyophilization cycles.

Effect of freezing temperature and cooling rate

The freezing procedures affects the crystalline structure and the properties of dried products. This process has significant effect on the subsequent sublimation stages because freezing sets the structures of the ice crystals and greatly influence the heat transfer and mass transfer process. Freezing temperatures influences the redispersion of dried product based on the separate aggregation processes during the sublimation stage. Two freezing temperatures were compared: -40 °C (commonly used, with ~90% water frozen) and -70°C (a more aggressive, rapid freezing condition). At same drying stage, samples frozen at -40°C exhibited adequate redispersibility compared to those frozen at -70 °C. This may be attributed to higher molecular mobility of solid material which allow more effective spatial arrangements and separation of nanocrystals.

Cooling rate had a minimal effect on particle size; however, a rate of 2 °C/min slightly improved redispersibility compared to 0.5 °C/min (Table 3). Overall, moderate freezing conditions were more favorable.

Table 3: Effect of freezing temperature and cooling rate

Parameters	Before lyophilization		After lyophilization				
	Particle size (nm)	PDI	Particle size (nm)	PDI	% Moisture content	Cake appearance	Redispersibility
Freezing Temperature							
-40°C	54.47	0.083	56.70	0.115	-	Homogeneous, smooth, white & opaque	-
-70°C	54.47	0.083	61.60	0.162	-		+
Drying time							
8 hrs	54.47	0.083	57.66	0.327	2.19		
16 hrs	54.47	0.083	58.51	0.120	1.25		

Effect of drying time

The selection of drying temperature is depending on the glass transition temperature of cryoprotect, as it is directly involved in sublimation rather than undergoing melting point. The product temperature must not exceed the collapse temperature (for amorphous materials) or the eutectic temperature (for crystalline materials) of the product in order to avoid collapse or pore structure damage. The collapse temperature of mannitol is -32.8±0.8 °C and trehalose is -65 °C. Hence primary drying temperature was selected as -20 °C. Two secondary drying durations (8 and 16 hours at 20°C) were tested, with constant primary drying conditions. Particle size was not significantly affected by drying time, but a lower PDI and residual moisture content were recorded for the 16-hour drying cycle. Therefore, 16 hours was considered optimal.

Evaluation of optimized lyophilized product

Among all tested cryoprotectant, trehalose was found to be more satisfactory than mannitol. Once the temperature of the ice/freeze-concentrated trehalose-water system is taken below the Tg', a glassy matrix is formed which immobilizes and protects the nanoparticles from further interaction [12, 13]. Standard freezing conditions and extended drying cycles, favor adequate redispersion, minimum moisture retention, minimum particle growth with low PDI. These findings indicate that formulation variables (e.g., cryoprotectant type and concentration) have a more significant impact than operating parameters.

Morphology

The surface morphology of lyophilized nanocrystals and plain drug were observed by SEM (Figure 1). The lyophilized formulations presented spherical and uniform particle size with narrow distribution, which was in accordance with Zetasizer measurements. While plain drug showed acicular crystal habit. This change in morphology might be due to coating of soluplus which reduce the interfacial tension on the particle surface allows the excellent water-surfactant interaction and guaranteed reduction in particle size. Presence of Tween 80 responsible for the formation of micelles and this could be the reason of the spherical shape particles.

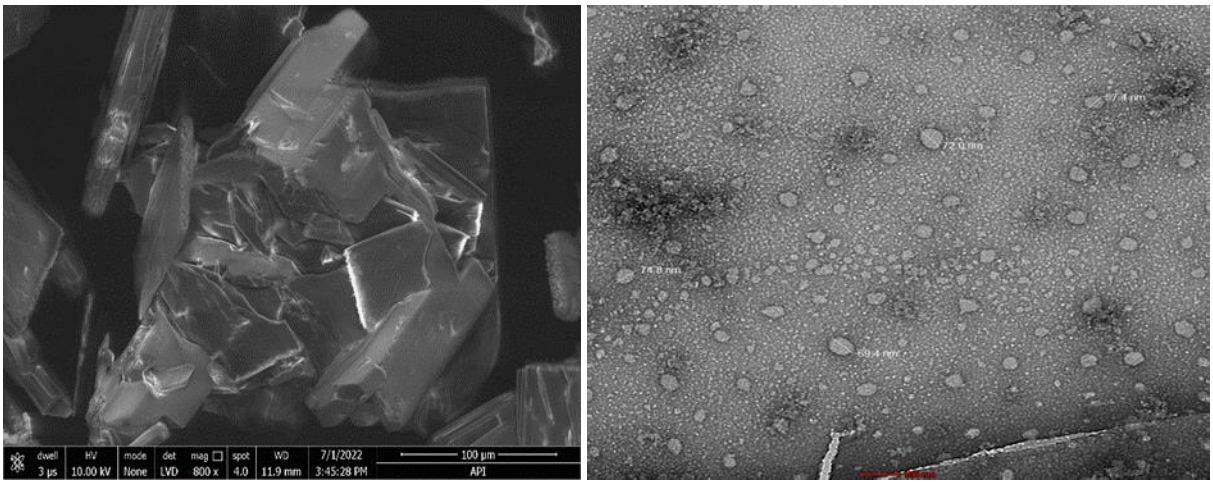

Fig 1: Surface morphology of the pure drug and lyophilized product

Crystalline state determination

Crystalline nature influences the physical stability, dissolution behavior and systemic performance. DSC and XRD are the tool for establishing the crystalline-amorphous state as well as the probable interaction with the excipient. In DSC thermogram (Figure 2), pure EDR shows a main sharp exothermic peak at 130 °C which correlates with the drug melting point and confirms its crystalline state. In the lyophilized product there is absence of the characteristic peak. This may be due to coverage of EDR particles surface by Soluplus. Moreover, the cleavage of internal crystal lattice due to high pressure homoginazation might be the reason for reduction in the crystallinity of lyophilized product.

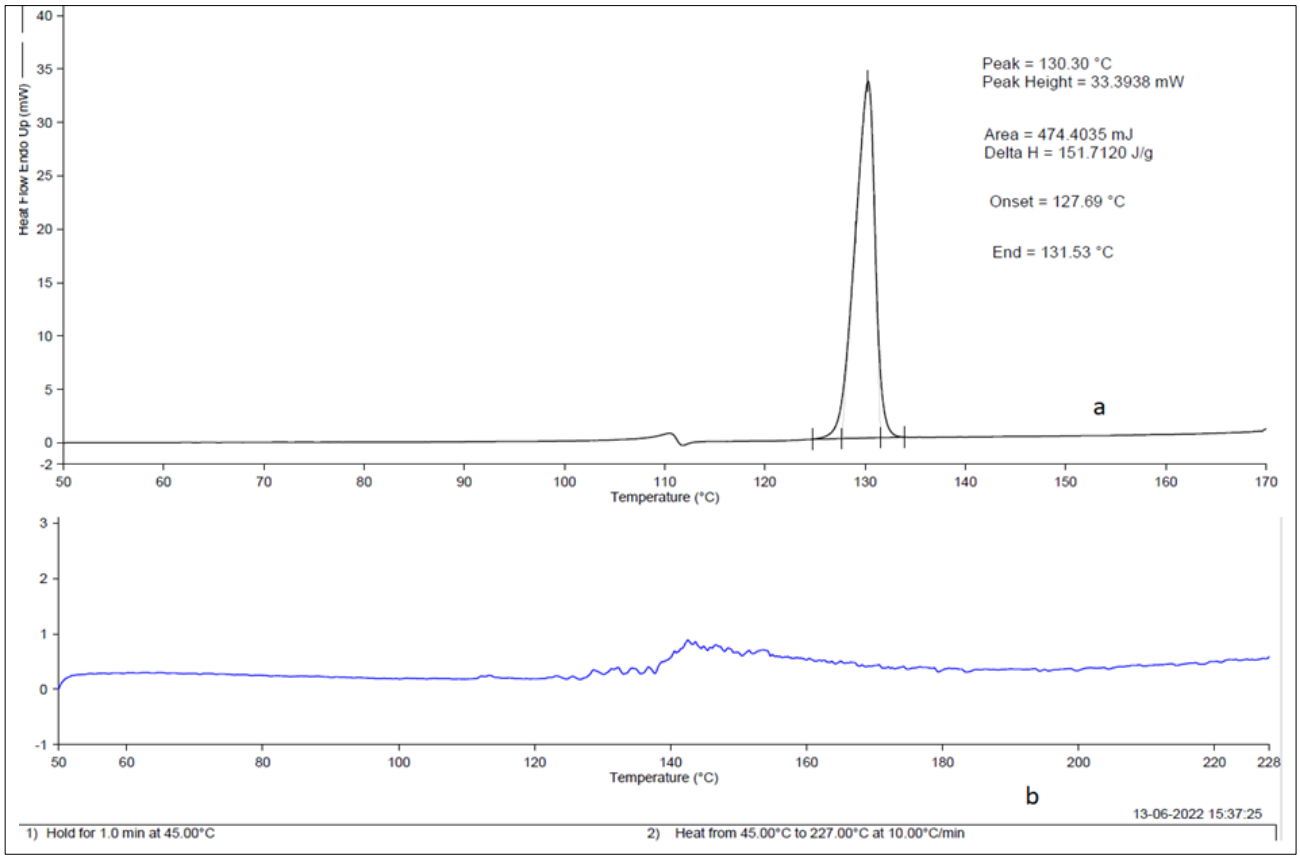

Fig 2: DSC thermogram: (a) Pure drug; (b) Lyophilized Product

The X-ray diffractograms of EDR, Soluplus® and freeze dried nanocrystals are shown in Figure 3. XRD spectra of EDR is showing distinctive peaks which reflect its crystalline nature while X-ray pattern of Soluplus® exhibit a halo pattern which indicate its amorphous nature. Characteristic peaks of the EDR are absent in the lyophilized product which represents reduction int the crystallinity. The reason for reduction of crystallinity of EDR could be the result of destabilization of the crystals during the prob sonication.

Short Term Stability

Stability studies confirmed the robustness of the optimized formulation. The lyophilized product remained physically stable over the testing period, with minimal changes in particle size and redispersibility (Table 4). This suggests the

freeze-drying process, when optimized with trehalose and appropriate lyophilization parameters, results in a stable, reconstitute nanosuspension suitable for long-term storage.

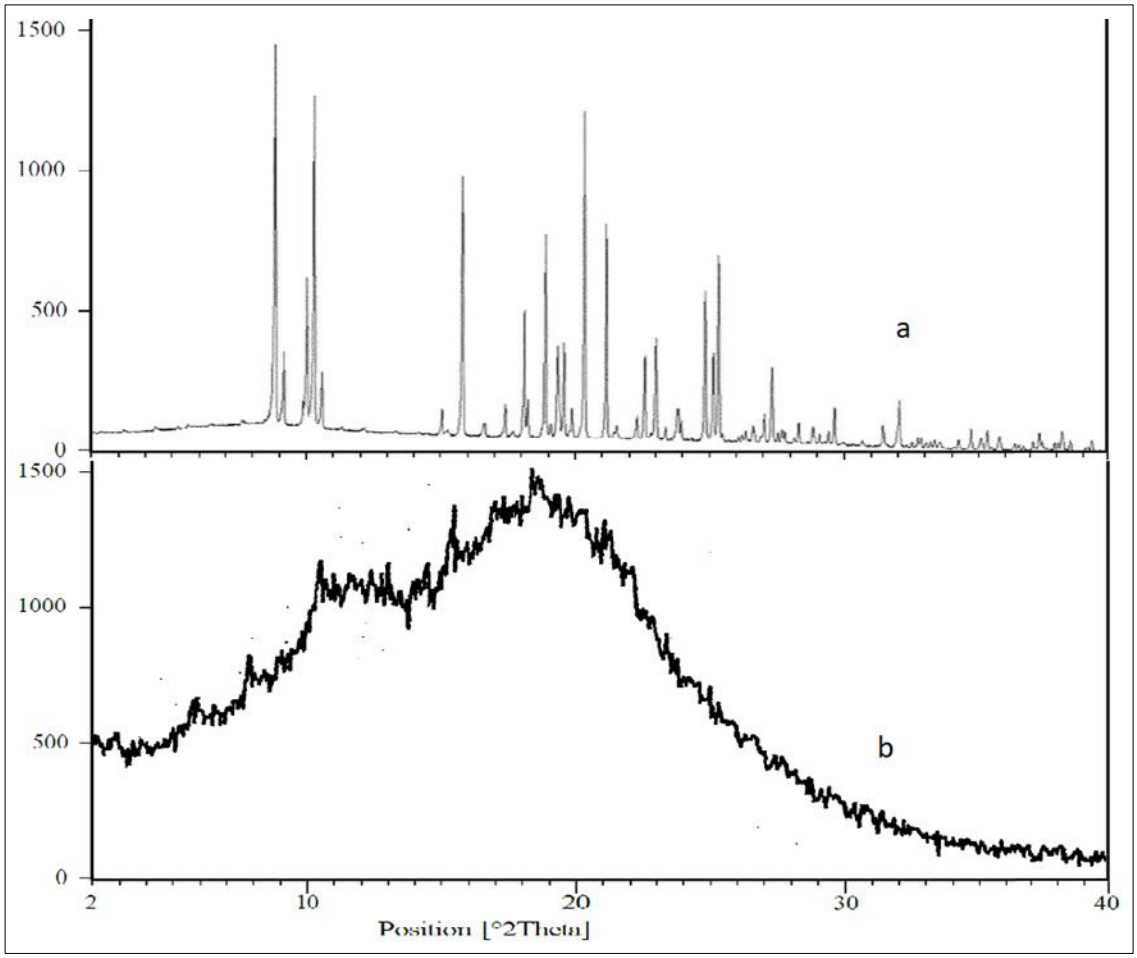

Fig 3: XRD diffraction pattern: (a) Pure drug; (b) Lyophilized Product

Table 4: Stability evaluations

Time (day)	Accelerated conditions (As per ICH guidelines)					
	25±2 °C/65±5% RH			40±2 °C/75±5% RH		
	Particle Size (nm)	Re-dispersibility	Appearance	Particle Size (nm)	Redispersibility	Appearance
0	56.70	+	White, free flowing	56.70	+	White free flowing
30	58.51	+	White, free flowing	61.60	+	White free flowing
60	60.67	+	White free flowing	63.21	+	White free flowing
90	61.34	+	White free flowing	65.89	+	White free flowing
180	61.71	+	White, cake	67.45	+	White, cake

Conclusion

Nanosuspension of EDR were sucessfully prepared by sono-precipitate method. This is a down-stream process which induce mechanical and thermodynamic stress on the nanoparticles. These stress contribute in the increment of net surface charge and surface free energy, potentially leading to Ostwald ripening and instability. To enhance the stability of the nanosuspension, lyophilization was employed. This technique effectively preserves nanoparticle properties by removing water through controlled freezing and sublimation. Among the tested cryoprotectants, trehalose demonstrated superior performance, with minimal particle size growth and maintenance of a narrow size distribution during freeze-thaw cycles. An optimal drug-to-trehalose ratio of 1:4 was established. Key process parameters were identified to achieve the desired lyophilized product quality: a freezing temperature of -40 °C and a secondary drying time of 16 hours. The lyophilized formulations exhibited spherical morphology, amorphous characteristics, and enhanced redispersibility, all of which contribute to improved aqueous solubility. Overall, the optimized lyophilized nanosuspension holds significant promise for incorporation into various drug delivery systems, offering improved stability and potential bioavailability enhancement for poorly water-soluble drugs.

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Statements and Declarations

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