Process optimization of lyophilization for long term stability of Edaravone nanosuspension

Running title: Lyophilization of nanosuspension

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Abstract
Aim: The aim of present study is to optimize a lyophilization process of nanosuspension loaded edaravone for long term stability.
Methodology: The effect of type and concertation of cryoprotectant, freezing temperature, cooling rate and primary drying temperature on redispersibility and particle size is thoroughly studied. A six-month stability study was conducted to compare changes in the particle size and redispersibility of lyophilized nanocrystal with an aqueous suspension at either 4°C or 25°C/60% relative humidity (RH). The selected optimized formulation is furthers studied for its solid-state characterization, residual moisture content.
Results: Out of best three possible cryoprotectants tested, trehalose was shown to be the most efficient at achieving minimum particle growth and easy redispersibility. Low freezing temperatures, slower freezing rates, and longer secondary drying times were also shown to be beneficial. Aqueous saturation solubility enhancement was also observed in dried lyophilized product. During the stability study, insignificant particle growth was observed for lyophilized product at stored at 25°C/65% RH and 40 ± 2°C/75 ± 5% RH.
Conclusion: A lyophilization protocol was developed that allowed solid nanocrystals to be reconstituted with minimal changes in their physicochemical properties. During a six-month period, lyophilized product stored at 25°C exhibited the greatest stability, showing minimal change in the particle size and redispersibility was observed.
Key words: Lyophilization, cryoprotectant, cooling rate, nanosuspension, redispersibility, particle growth

Introduction
Nanoparticles are most studied and focused formulation aspects for the targeted drug delivery system. Due to smaller surface area, solubility and dissolution enhancement of poorly water-soluble drug specifically BCS class II drugs, is achieved through nanoparticulate drug delivery system. Nevertheless, the long-term stability is crucial criteria for the development of nanoparticles specially in nanosuspension available in aqueous form. Physical instability in terms of particle agglomeration due to Oswald ripening and chemical instability due to hydrolysis of stabilizers leads to drug leakage and drug degradation itself during storage [1].
Nevertheless, the newly constituted nanosuspensions are undergone the downstream process for solidification for most promising applications like oral dosage form or reconstituted solid particle for oral or parenteral administration. Various methods of solidification such as spray drying, freeze drying, fluid bed drying, different granulation methods have been reviewed and studied by various formulation scientists.
Major concern with downstream process is effect of induce stress on the physical properties such as retention of original particle size and narrow range distribution of nanosuspension (generally d50<1µm and d95<2.5µm) [2]. This means prevention of particle aggregation or fusion during induced thermal stress. Another aspect is conversion of

API's crystalline nature into different polymorphs or amorphous to crystalline form and vice versa. These impact on the drug stability, solubility and dissolution behavior of the drug candidates. Therefore, formulation and process parameters of solidification process must be studied and optimized for the preserving the originality in terms of particle size and solid-state characterization in order to improve the solubility and dissolution behavior and bioavailability of drug. Apart from this, some other considerations like redispersion time and residual moisture content are also important.

Of the above aforementioned solidification methods, freeze drying, also known as lyophilization, is most preferable and industrial process which includes removing of aqueous phase from the frozen sample by means of sublimation. Despite of its disadvantages like lengthy process and high energy consumption, it is more suitable method for the thermosensitive material as sublimation of solvent takes place under reduce pressure [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.

Freezing and subsequent drying in the lyophilization induce various thermal stress which imparts destabilization of nanocrystals. During freezing particles are aggregate due to confinement of nanocrystals into interstitial space of formed ice crystal. Upon drying, stearic hinderance of used stabilizer in the formulation leads to particle fusion. In addition to this, several processing factors may also affect the particle size and redispersibility of nanocrystals. Freezing stress can be determined by cooling rate and temperature. Moreover, freezing behavior depends on equipment use such as lyophilized shelf, quenching of liquid nitrogen, deep freezer etc. and freezing intensity i.e. vacuum, shelf temperature and time [4]. Apart from this, utility of cryoprotectant generally essential in preventing particle agglomeration. Type and its concentration have significantly affected the solid-state properties of final product [5].

The above-mentioned variables exert varied influence on the different drug candidates specially hydrophobicity, dissolution behavior and stability upon aggregation during the lyophilization process. This is one of the reasons to optimize the freeze-drying process and formulation variables with respect to nanocrystal behavior.

Therefore, the aim of this work is to systemically study the effect of lyophilization process on redispersed particle size and saturation solubility of EDR nanosuspension formulated by sono-precipitation method. The effect of type and concertation of cryoprotectant, freezing temperature, cooling rate and primary drying temperature on redispersibility and particle size is thoroughly studied. The selected optimized formulation is furthers studied for its solid-state characterization, residual moisture content, invitro dissolution behavior of EDR.

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.

Method

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, it is found that sugars are good cryoprotectant. This is attributed to favorable hydrogen bonding interactions according to the water replacement hypothesis. It is also shown the potential interaction with nanosuspension- stabilizing polymers [7].

EDR loaded nanosuspension were prepared and then different cryoprotectant such as trehalose, mannitol, lactose monohydrates were tested at concentration 1:3. The solutions are added in the 1 ml Type 1 borosilicated glass vial prior to the lyophilization. All the vials are stoppered with bromo butyl slotted rubber and placed in the shelves of lyophilizer (Virtis Advantages Plus Lyophilizer). Any sample lacking cryoprotectant was used as a control. Keep all the solution under freezing for 12hrs in deep freezer (-20 ^{0}C) followed by thawing at room temperature (28^0 C) and evaluated for the particle size and polydispersity index by zetasizer. Redispersibility of the final product was investigated by replacing the same amount of water lost during the lyophilization process. Vortex the resultant sample for 30 seconds and visually observed for ease of dispersion and presence of undispersed particles.

- = 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 redispersibiity or may not improve the protection when used over a certain threshold amount. Based on the freeze thaw studies, trehalose and mannitol were chosen for further analysis. 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 - 40^0 C for 2 hrs, primary drying - 20^0 C for 24 hrs and secondary drying 20^0 C for 16hrs were selected with vacuum of 120 mTorr. Analyzed the final product for redispersibility and particle size.

Effect of freezing temperature and cooling rate

NPs were lyophilized using 1:4 ration of NPs:trehalose. For the evaluation of the effect of freezing temperature, different temperature -70^0C and -40^0C were used for 2 hrs. As - 40 ^{0}C is generally preferred temperature as at this temperature, most of the water converts into ice. Only 10% unfrozen water is represented. While - 70 is selected for comparison as this temperature shows more aggressive freezing variant. Cooling rate also influences the size of ice crystal and interstitial spaces. Also affect super-cooling and nucleation. Generally, formation of very large ice crystals when frozen slowly or formation of very small crystals due to rapid cooling.

In the beginning, the shelf temperature is ramp to 5^0C for 20 minutes for establishment of equilibrium to avoid the super cooling effects on the subsequent lyophilization steps. Cooling rate was compared between 2 ^{0}C/min and 0.5 ^{0}C/min. The rest protocol was as follows: primary drying at -20^0C for 24hrs and secondary drying at 20^0C for 16hrs. A vacuum of 120 mTorr is applied during the drying process.

Effect of drying time

For the understanding of effect of drying time on the rate of nucleation and particle size, the secondary drying at 20^0C for different time interval like 8 or 16 hrs were set. The other lyophilization process parameters were same as described above whereas the freezing temperature was set at - 40^0 C for 2 hrs.

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 10^0C/min, covering a temperature range of 45–270^0C. 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 redispersiblity.

Result and discussion

Freeze – thaw study

As lyophilization process is expensive, selection of an effective cryoprotectant is utmost important. Freeze thaw studies are used based on the principle that in the first step of lyophilization, if the cryoprotectant cannot protect the NPs against aggregation, will not be suitable for an effective lyophilizer product. Based on prior literature review three best cryoprotectant were selected and tested for suitable cryoprotectant. In general, rapid freeze condition yields lower

particle size compared to slow freezing. This might be due to nanoparticles would not get enough time to move around and aggregate [10].

Freeze thaw studies shows that the control sample was poorly redispersed and hence could not be measured particle size. All the lyophilized products containing different cryoprotectant were easily redispersed. All the samples showed considerable growth in particle size but lactose monohydrate displayed a significant increment in particle size. With comparison of trehalose with mannitol, trehalose shows better results in terms of particle size and polydispersibility indexes. The increment in particle size with trehalose was minimum compared to others (Table 1).

Effect of concentration of cryoprotectant

The level of stabilization also depends on the concentration of cryoprotectant. During freezing, the temperature below glass transition temperature of the cryoprotectant, it forms a glassy coating around the nanoparticles protecting them against stresses like mechanical stress of ice crystals, thereby preventing aggregation. According to the particle isolation hypothesis, the spatial separation of particles within the unfrozen fraction results in insufficient cryoprotectant at higher concentration of nanoparticles, leading to aggregation. Particle isolation hypothesis suggests that the separation of individual particles within the unfrozen fraction prevents aggregation during freezing. Freezing temperature below Tg′ of cryoprotectant has no effect on the glassy protective matrix of cryoprotectant formed around the nanoparticles. Generally, with increasing the concentration of excipients results in smaller increase in particle size [11]. As, high cryoprotectant concentration is not advantageous in the lyophilization process. But selection of its concentration is utmost important and determined based on diffusion rate of cryoprotectant 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.

In case of mannitol, at highest concentration observed increment in particle size were <10 nm and change in PDI were negligible. However, for trehalose, such trend was not observed. With low concentration of trehalose, minimum increment in PS was observed (Table 2).

Trehalose has good glass forming properties and form hydrogen bond upon dehydration during lyophilization process. Hence it can be prevented the structure of NPs during the lyophilization process. Slower lyophilization improves its efficiency to maintain the particle size as well as redispersibility of final product. While mannitol is a bulky agent and promotes efficient lyophilization with elegant appearance of dried products. Formation of eutectic mixture, it requires shorter lyophilization cycle.

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 has 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. At same drying stage, samples frozen at -40^{0}C shows adequate redispersion compared to -70^{0}C. This may be due to higher molecular mobility of solid material which allow more effective spatial arrangements and separation of nanocrystals. There is negligible effect of cooling rate was observed but 2 ^{0}C/min shows better redispersibility.

As the temperature reduces, the particle size and PDI both become smaller but not significant difference was observed. (Table 3)

Effect of drying time

Below glass transition temperature of cryoprotect can be selected 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 a loss of pore structure. The collapse temperature of mannitol is -32.8±0.8 ^{0}C and trehalose is -65 ^{0}C. Hence primary drying temperature was selected as -20 ^{0}C. The effect of drying time was shown in the table. There was no observed difference in the particle size with 8 hrs and 16 hrs of drying time. But the smallest PDI were obtained for the 16 hrs drying time. Moreover, desired least residual moisture content was observed for 16 hrs drying time and hence 16 hrs would be selected as an optimized drying time.

Evaluation of optimized lyophilized product

Trehalose is found to be more satisfactory than mannitol as a cryoprotectant. 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].

Default freezing temperature shows adequate redispersion and longer drying time favor the sublimation and moisture retention was also minimum. Hence, from these results, it can be concluded that the formulation shows greater impact than operating parameters

Morphology

The surface morphologies of lyophilized nanocrystals and plain drug were observed by SEM (Fig.1). The lyophilized formulations presented spherical and uniform particle size, which was in accordance with Zetasizer measurements. They also showed narrow distribution. 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.

Crystalline state determination

Crystalline nature influences the physical stability, dissolution behavior and systemic performance. DSC and the XRD are the tool for establishing the crystalline-amorphous state as well as the probable interaction with the excipient. Fig. 2 shows the thermograms of EDR, soluplus, cryoprotectant and lyophilized product. Pure EDR shows a main sharp exothermic peak at 130 °C which correlates with the drug melting point and 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.

The X-ray diffractograms of EDR, Soluplus® and freeze dried nanocrystals are shown in Fig.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.

3.2. Short Term Stability

Table 4 shows the results of the short-term stability studies. From this, it is confirmed that the lyophilized product is stable.

Conclusion

Nanosuspension was prepared by sono-precipitate method. This is a down stream process which induce various stress on the nanoparticles. This contributes in the increment of surface net charge and surface free energy leads to Ostwald ripening. To stabilize the prepared nanosuspension lyophilization was selected as a method of choice. During lyophilization, freezing and subsequent sublimation influence the final quality of the lyophilized product. Treahalose an excellent cryoprotectant showed the minimum increment of particle size and maintenance of particle size distribution in the ration of 1:4 (drug:trehalose) in performed freeze thaw studies. Process parameters like primary freezing temperature were found to be -40^0C with 16 hours of secondary drying time. The lyophilized products showed the spherical morphology, by forming amorphous nature improves the water solubility of the nanosuspension. Prepared lyophilization product could be incorporated into different vehicle for drug delivery system.

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Tables

Table 1: Freeze thaw studies

Type of cryoprotectant	**Before lyophilization**		**After lyophilization**			
(1:4)	**Particle size (nm)**	**PDI**	**Particle size (nm)**	**PDI**	**Redisersibility**	**Cake appreance**
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

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	-

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^{0}C	54.47	0.083	56.70	0.115	-	Homogeneous, smooth, white & opaque	-
-70^{0}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		

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

Figures:

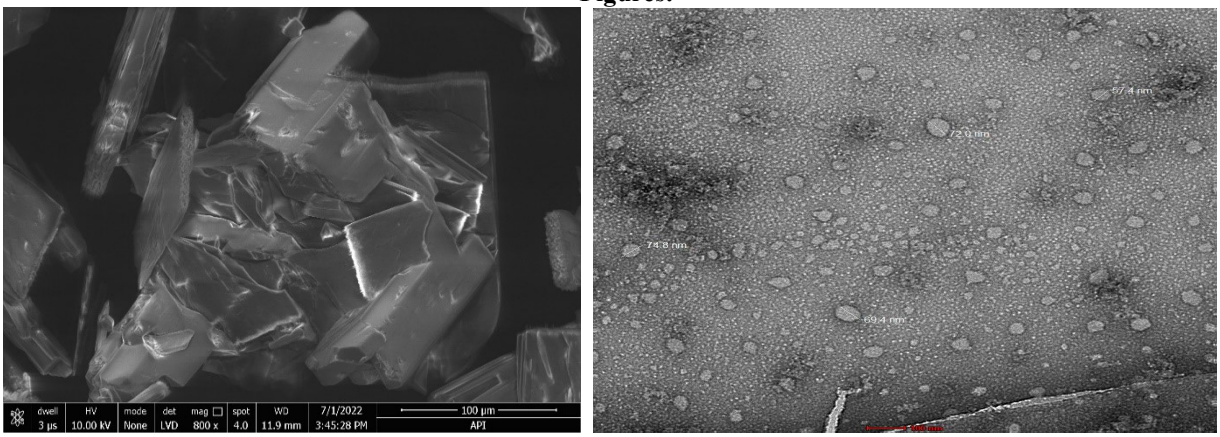

Fig.1: Surface morphology of the pure drug and lyophilized product

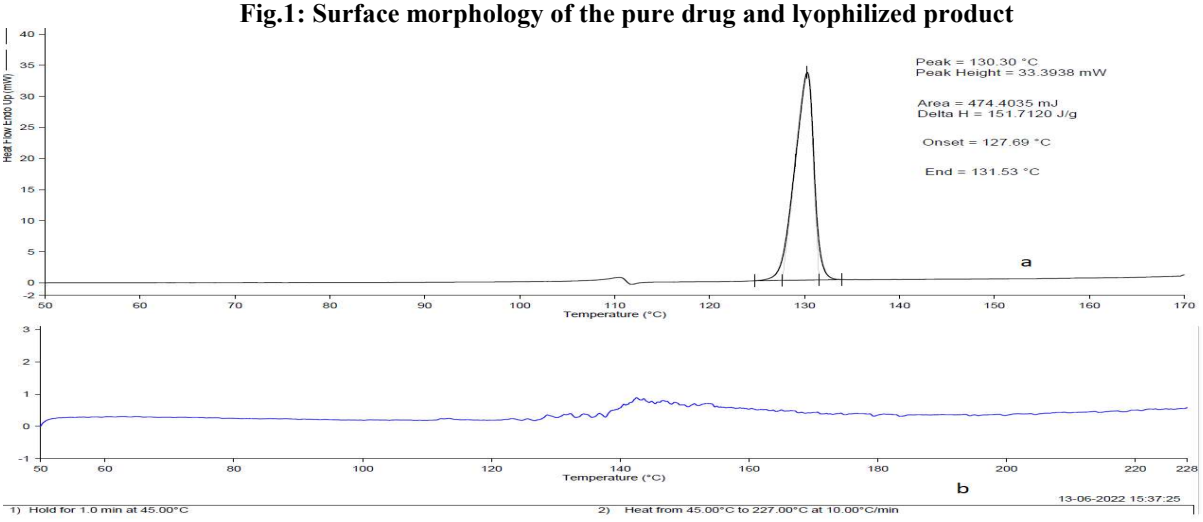

Fig.2: DSC thermogram: (a) Pure drug; (b) Lyophilized Product

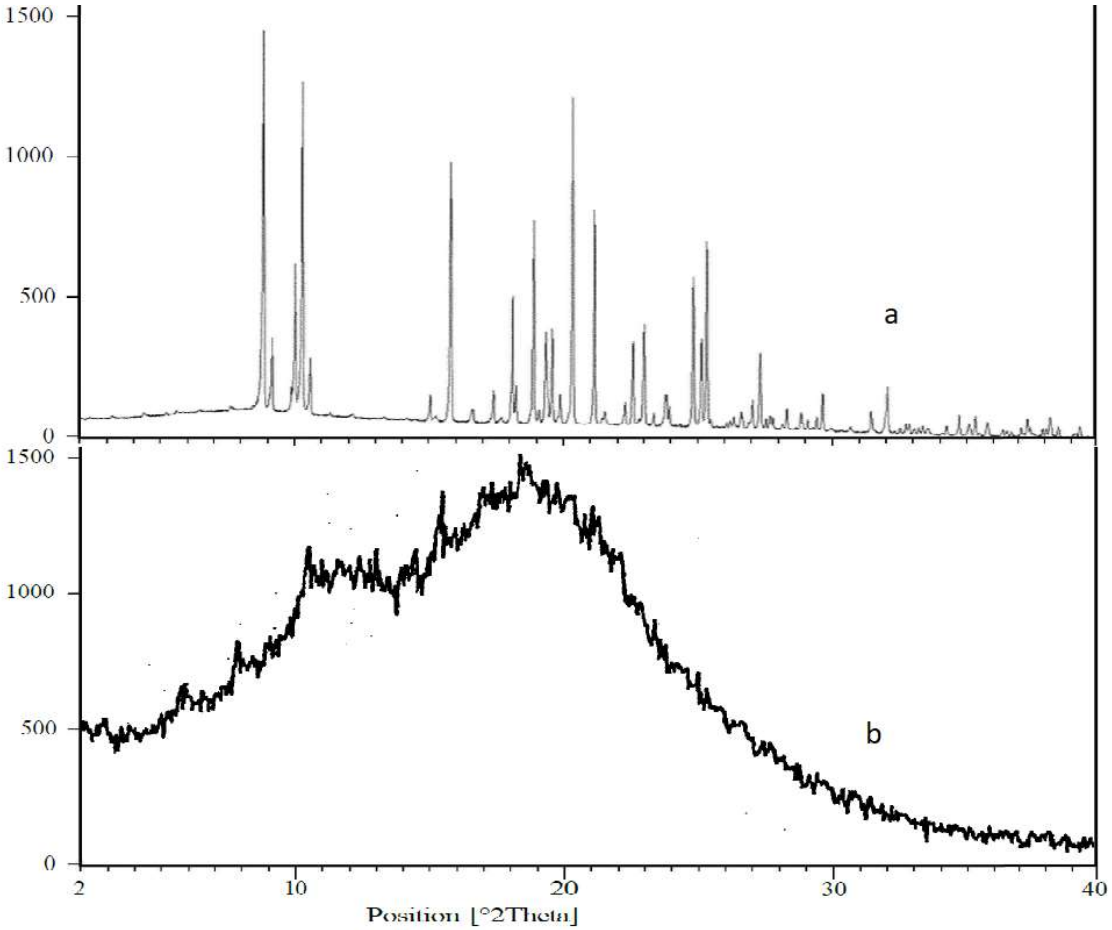

Fig.3: XRD diffraction pattern: (a) Pure drug; (b) Lyophilized Product