9 STABILITY STUDIES

9.1 INTRODUCTION

Stability study of any formulation on storage is necessary as it reflects whether the desirable properties of the formulation are retained upon storage or not . These desirable properties include integrity of lipid-polymer vesicles and particle size distribution. Upon storage, hybrid vesicles are susceptible to many physical changes i.e., particles may undergo fusion as well as aggregation of particles may lead to increase in particle size of PLHNCs. Also loss of integrity of PLHNCs and subsequent leakage of Docetaxel and decomplexation of shRNA pDNA may take place (3). PLHNCs formulations are not stable enough in an aqueous dispersion form. So, to increase their stability the PLHNCs formulations were subjected to lyophilization. However, during lyophilization the lipid-polymeric hybrid formulations may undergo aforementioned physical changes. To avoid such changes different cryoprotectant i.e., sucrose, mannitol, glycerol, trehalose, povidone, dextran etc. can be used to maintain the PLHNCs tunability and integrity (5, 6). The physicochemical testing of such product should be carried out to check whether any changes take place in the PLHNCs in terms of its particle size and entrapment efficiency. So, as the ideal situation, after storage period the PLHNCs formulation (on rehydration) should retain the same characteristics it possessed before lyophilization. For hybrid vesicles an attention has been focused on two processes that affects the quality and therefore acceptability of PLHNCs (7). First leakage of entrapped drug from the hybrid vesicles may take place into the extra vesicular compartment (hydration medium). Secondly, there is a possibility of particles aggregation and/or fusion, which leads to formation of bigger sized particles (8-11). Although under dehydrated storage, there is least possibility of the formulation to encounter hydrolytic degradation. Another aspect that can be considered is oxidation of the outer lipid layer over storage (12).

As per the ICH guideline (for stability studies) Q1A (R2), stability studies should be performed on a drug product intended for storage in refrigerator. The stability study protocol was designed as per ICH guidelines (13) for countries falling under zone III (hot, dry) and zone IV (very hot, humid) (14); for 3 months storage period.

significant change in stability for a D-sh-PLHNCs is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
2. Any degradation product's exceeding its acceptance criterion;

3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation);
4. Failure to meet the acceptance criterion for pH;
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.

9.2 METHOD FOR STABILITY STUDY

Comparative stability studies were carried out on the potential PLHNCs formulations at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) and at long-term conditions (2-8°C) up to three months. Lyophilized PLHNCs formulations were filled in Type I tubular glass vials and sealed with chlorobutyl rubber stoppers, and finally sealed with aluminum seals. Sealed vials were stored at above mentioned conditions. At each sampling time, lyophilized powder from cake was reconstituted with Nuclease free water and checked for stability parameters shown in Table 9-1 later on.

Lyophilized PLHNCs dry powder formulations were stored in hard gelatin capsules kept in tightly closed HDPE container. Sealed containers were stored at above mentioned conditions (15-22). At each sampling point, different vials were used for the stability testing. The lyophilized PLHNCs dry powder formulations were examined visually for the evidence of discoloration. The content of the vials was tested for percentage shRNA pDNA complexation, Docetaxel assay, particle size, zeta-potential and water content and results are depicted in Table 9-2 later on.

9.3 SERUM STABILITY STUDY OF shRNA pDNA IN D-sh-PLHNCS

Assay of loaded shRNA pDNA is important parameter to determine any degradation in shRNA pDNA loaded PLHNCs during in-vivo application. Docetaxel-shRNA pDNA loaded PLHNCs were checked for integrity of complexed shRNA pDNA in presence of serum to evaluate chances of degradation during blood circulation as blood possess DNAse enzyme. Even during pulmonary administration, shRNA pDNA comes in contact with pulmonary fluids. Hence it is necessary to evaluate the stability of the shRNA pDNA PLHNCs in serum and simulated lung fluid.

Naked shRNA pDNA and sh-PLHNCs were incubated with 50 µl non-heat inactivated FBS or simulated lung fluid at 37℃ for different time periods. After that, formulation was diluted with Diethylpyrocarbonate (DEPC; Sigma)-treated H_20 to give a final volume of 100 µl. The samples were then vortexed with 200 µl of phenol: chloroform (1:1 v/v) and were subsequently spun at 14,000 rpm at 4°C for 10 min. From these centrifuged samples, aqueous

layer was separated out and mixed with DEPC treated water up to 1 mL and absorbance was taken at 260 nm using NanoDrop system.

9.4 RESULTS AND DISCUSSION

9.4.1 Stability of PLHNC formulation as per ICH guidelines

PLHNCs formulations must show physical stability in order to produce a commercially viable product (23). Preferable stability of formulation up to 1 to 2 years at storage conditions i.e., room temperature condition or at refrigerated condition is required for a pharmaceutically acceptable hybrid vesicle product. In order to make the formulation survive these long stability periods on shelf, lyophilization becomes a primary resort for stabilizing the developed PLHNCs product. However, this doesn't eliminate the requirements for the real-time stability monitoring as the physicochemical properties of the formulations are still prone to change on storage. To evaluate the stability of the Docetaxel and pDNA containing PLHNCs hybrid formulations, lyophilized PLHNC formulations has been evaluated for changes in particle size, zeta potential, Docetaxel assay (by combining free as well as encapsulate drug) , pDNA assay, Docetaxel encapsulation, and complexation efficiency has been determined. Results of the study are tabulated in Table 9-1 and Table 9-2.

During stability monitoring, no significant differences ($p > 0.05$) were found in all above-mentioned parameters at refrigerated condition. Lyophilized PLHNC formulations maintained their physical integrity and were observed as white porous cakes. Assay of the formulations stored at both conditions at each time point was between the range of 95-105% of initial levels which was acceptable. There was no significant change ($p<0.05$) in particle size and zeta potential after storage period at both conditions. Water content of the lyophilized cakes was not affected during the storage period ($p< 0.05$). Integrity of the shRNA plasmid remained within range during the stability studies. Even % complexation of ~95% was achieved after 3 months which is desirable. Stability studies at accelerated and refrigerated conditions demonstrate that the product was stable at both conditions for a period of 3 months and suggest that the product will be stable for longer periods at refrigerated conditions.

Table 9-1 Stability Testing Data of lyophilized Docetaxel and shRNA pDNA loaded PLHNCs

Storage conditions	Time	Visual compatibility	Docetaxel assay	Docetaxel encapsulation efficiency	shRNA pDNA Integrity	shRNA pDNA % complexation	water content	particle size	PDI	Zeta potential
Target specification	-	White crystalline powder	90-110 %	Maximum	90-110 %	Maximum	< 3.0 %	< 200 nm	< 0.200	> 10 mV
Before lyophilizatiion	0	Transluescent liquid	99.9 ± 1.6	91.6 ± 1.1	100.1 ± 1.5	96.4 ± 1.8	-	128.4 ± 2.4	0.124 ± 0.12	23.5 ± 2.4
After lyophilization	0	White porous cake	99.5 ± 1.2	92.6 ± 1.8	98.1 ± 1.2	95.2 ± 1.6	1.8 ± 0.4	129.6 ± 3.1	0.119 ± 0.14	23.9 ± 2.1
2-8°C	1	White crystalline powder	98.9 ± 1.2	91.8 ± 1.5	97.8 ± 1.4	95.6 ± 1.1	1.6 ± 0.7	122.9 ± 4.6	0.112 ± 0.14	22.8 ± 2.5
2-8°C	2	White crystalline powder	97.8 ± 1.6	92.4 ± 1.2	96.1 ± 1.6	96.1 ± 1.2	1.6 ± 0.5	124.9 ± 3.8	0.09 ± 0.11	25.6 ± 2.0
2-8°C	3	White crystalline powder	98.6 ± 1.4	91.6 ± 1.3	95.3 ± 2.1	95.7 ± 1.6	1.8 ± 0.6	119.6 ± 4.8	0.104 ± 0.08	27.3 ± 2.3
25°C/60% RH	1	White crystalline powder	96.1 ± 2.1	92.5 ± 1.4	94.3 ± 1.9	96.1 ± 1.8	1.8 ± 0.9	126.7 ± 3.8	0.128 ± 0.12	24.6 ± 1.8
25°C/60% RH	2	White crystalline powder	96.5 ± 1.8	93.1 ± 1.2	95.8 ± 1.2	94.9 ± 1.5	2.0 ± 0.4	124.6 ± 4.1	0.132 ± 0.14	24.9 ± 2.6
25°C/60% RH	3	White crystalline powder	95.9 ± 1.6	92.7 ± 1.8	94.8 ± 2.3	95.9 ± 2.1	1.9 ± 0.7	128.5 ± 3.7	0.148 ± 0.21	29.6 ± 1.2

Table 9-2 Stability Testing Data of lyophilized Docetaxel and shRNA pDNA loaded PLHNCs Dry powder formulation

Storage conditions	Time	Visual compatibility	Docetaxel assay	Docetaxel encapsulation efficiency	shRNA pDNA Integrity	shRNA pDNA % complexation	water content	Particle size (D_{90})
Target specification	-	White free flowing powder	90-110 %	Maximum	90-110 %	Maximum	< 3.0 %	< 5 µm
Before Dry powder formulation	0	White crystalline powder	99.5 ± 1.2	92.6 ± 1.8	98.1 ± 1.2	95.2 ± 1.6	1.8 ± 0.4	-
After dry powder formulation	0	White free flowing powder	98.1 ± 1.6	92.8 ± 1.2	97.9 ± 1.5	95.3 ± 1.2	1.2 ± 0.8	2.98 ± 1.31
2-8°C	1	White free flowing powder	97.5 ± 1.7	93.1 ± 2.1	98.2 ± 1.6	94.8 ± 1.3	1.4 ± 0.2	2.81 ± 1.29
2-8°C	2	White free flowing powder	96.4 ± 1.3	93.6 ± 1.4	97.5 ± 1.3	95.1 ± 1.9	1.3 ± 0.3	2.76 ± 1.69
2-8°C	3	White free flowing powder	96.8 ± 2.1	91.8 ± 1.3	96.7 ± 2.1	94.6 ± 1.8	1.6 ± 0.7	2.88 ± 1.21
25°C/60% R	1	White free flowing powder	96.5 ± 1.2	92.2 ± 2.8	97.1 ± 1.8	95.9 ± 2.1	1.8 ± 0.3	3.12 ± 1.22
25°C/60% R	2	White free flowing powder	95.3 ± 1.6	92.1 ± 1.2	96.9 ± 1.1	94.6 ± 1.2	1.9 ± 0.2	3.25 ± 1.12
25°C/60% R	3	White free flowing powder	96.1 ± 2.2	90.6 ± 3.1	95.9 ± 1.2	93.4 ± 1.9	1.7 ± 1.1	2.49 ± 1.14

9.4.2 Stability of shRNA pDNA in serum and simulated lung fluid

As shown in Table 9-3, degradation of naked shRNA pDNA occurred during 4 hrs and only 20 % of shRNA pDNA remained intact after 4 hrs. In case of sh-PLHNCs, shRNA pDNA remained intact even after 24 hrs of incubation time due to protective nature of lipid coat on the surface of PLHNCs. Moreover, outer PEGylated layer of DSPE-PEG_{2000} also provides steric shielding effect which can keep away serum enzymes from shRNA pDNA.

Table 9-3 Stability of shRNA pDNA

Treatment	Time	% recovery
standard	0 hr	100
	4	20.3
	8	4.6
50 µl fetal bovine serum	4	98.9
	8	96.8
	12	94.7
	28	92.7
Stimulated lung fluid	4	98.4
	8	97.9
	12	93.2
	24	91.1

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