Chapter 9

STABILITY STUDIES

9.1 Introduction

Stability studies are primary tool to reveal the expiry date and ideal storage conditions for newly developed or existing pharmaceutical product [1, 2]. International Conference on Harmonization (ICH) has recommended the protocol for performing the stability testings. Stability studies are used to establish and assure the safety, quality and efficacy of a pharmaceutical product, and needs to be performed from early developmental stage till the lifecycle of the product. Novel drug carrier systems have been comprehensively explored for drug delivery, drug targeting and controlled release. However, instability of the formulation has been remained as a major limitation. Hence, if a novel product has to enter market, it must be stable throughout its storage period, retain the encapsulated drug with its characteristic particle size before reaching the targeted tissues to produce the desired therapeutic effect [3-5].

Physical stability of a formulation such as aggregation/flocculation and fusion/coalescence affects the shelf life of a vesicular system. This can result in loss of encapsulated drug and increase in particle size of the vesicles. Aggregation means formation of larger units where particles comes together and forms larger particles. Aggregation could result into coalescence where new colloidal structures are formed and entrapped drug is leached out, which is an irreversible process. These two instabilities affects encapsulation efficiency, drug release which are critical to gain desired therapeutic effect. Moreover, increase in size cririically affects the fate of the formulation in *in vivo* conditions [6]. Present investigation is focused on observing and studying the stability of formulation by assessing the parameters of formulation like visual appearance, % Entrapment efficiency (%EE), % drug loading (%w/w), particle size with polydispersity index (PDI), zeta potential and cumulative drug release.

9.2 Materials and Equipments

9.2.1 Materials

Dichloromethane, Methanol and Chloroform (A. R. grade) were purchased from S.D. Fine-chemicals limited (Vadodara, India). Triton X100 was purchased from Sigma Aldrich (St. Louis, MO, USA). Distilled water was prepared using in-house distillation assembly. 0.22µ membrane filter was purchased from Pall Life Sciences (Mumbai, India). All other reagents were purchased from S.D. Fine-chemicals limited, Baroda, India and were of analytical reagent grade.

9.2.2 Equipments

- Analytical Weighing Balance (ATX 224, Shimadzu, Japan)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- Particle Size Analyzer 3000 HS (Zeta Sizer Nano Series, Malvern Instruments, UK)
- UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan)
- Cooling Centrifuge (Remi Equipment, Mumbai, India)

9.3 Method

Short-term accelerated stability studies were performed according to the ICH Q1A (R2) guidelines for GCH loaded PEGylated and Non-PEGylated spherulites and VLB loaded PEGylated and Non-PEGylated spherulites. Spherulites formulation was kept in glass vials and stored at two different temperatures viz. 2-8 °C (refrigerated) and 25°C±2°C, 60±5 RH (room temperature) for 3 months. Initially all formulations were characterized for visual appearance, % Entrapment efficiency (%EE), % drug loading (%w/w), particle size with polydispersity index (PDI), zeta potential and cumulative drug release. At specific time intervals of 1, 2, and 3 months all samples were tested for the above mentioned charcteristics. All the characterization methods are described previously in Chapter 6 Characterization.

9.4 Results and Discussion

9.4.1 Stability study of GCH loaded Non-PEGylated spherulites

GCH loaded Non-PEGylated spherulites samples were analyzed for any instability issues over the period of 3 months. The samples were kept at two different temperatures viz. 2-8 °C (refrigerated) and 25°C±2°C, 60±5 RH (room temperature) and analyzed at predetermined time intervals. Table 9.1 shows the stability study of formulation stored at 2-8 °C (refrigerated) and Table 9.2 shows the stability study of formulation stored at 25°C±2°C, 60±5 RH (room temperature).

Table 9.1: Stability study of GCH loaded Non-PEGylated spherulites at Refrigerated condition (2-8°C). Data represents mean of three experimental values (n=3; mean±SD).

Storage condition		**Refrigerated condition (2-8°C)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	76.28±1.16	75.96±1.08	75.52±1.29	75.42±0.96
% Drug Loading (%w/w)	9.38±0.89	9.34±0.63	9.28±0.91	9.26±0.36
Particle size (nm) (PDI)	204.9±1.68 (0.43±0.02)	205.6±1.47 (0.51±0.01)	205.8±1.89 (0.24±0.01)	205.9±1.42 (0.37±0.03)
Zeta potential (mV)	-26.5±1.2	-26.3±0.8	-26.4±0.21	-25.9±0.18
Cumulative %Drug Release after 48 hours	93.09±1.21	94.39±1.07	93.67±1.67	93.29±1.41

*Experiment was done in triplicate (n=3).

Stability study was performed at refrigerated condition (2-8°C) and results shown in Table 9.1 revealed that Non-PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 72.62% and 208.9 nm, respectively.

Table 9.2: Stability study of GCH loaded Non-PEGylated spherulites at 25°C±2°C, 60±5 RH (room temperature). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Room condition (25°C±2°C, 60±5 RH)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	76.28±1.16	76.14±0.86	75.64±1.03	75.28±0.96
% Drug Loading (%w/w)	9.38±0.89	9.36±0.54	9.29±0.71	9.24±0.85
Particle size (nm) (PDI)	204.9±1.68 (0.43±0.02)	206.3±0.59 (0.41±0.02)	206.1±0.43 (0.21±0.02)	206.5±0.58 (0.37±0.03)
Zeta potential (mV)	-26.5±1.2	-26.1±0.45	-26.3±0.74	-26.8±0.61
Cumulative %Drug Release after 48 hours	93.09±1.21	93.24±1.21	92.10±0.85	93.91±0.68

*Experiment was done in triplicate (n=3).

Stability study was performed at 25°C±2°C, 60±5 RH (room temperature) and results shown in Table 9.2 revealed that Non-PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 72.16% and 210.78 nm, respectively.

Stability study of GCH loaded Non-PEGylated spherulites at two different temperatures revealed that formulation remained physically stable. All the critical quality attributes showed very negligible difference in values from initial to 3 months time period. However, it is recommended that the formulation should be stored at refrigerated condition (2-8°C) to avoid oxidation and hydrolysis of the amphiphillic lipids [7].

9.4.2 Stability study of GCH loaded PEGylated spherulites

GCH loaded PEGylated spherulites samples were analyzed for any instability issues over the period of 3 months. The samples were kept at two different temperatures viz. 2-8 °C (refrigerated) and 25°C±2°C, 60±5 RH (room temperature) and analyzed at predetermined time intervals. Table 9.3 shows the stability study of formulation stored at 2-8 °C (refrigerated) and Table 9.4 shows the stability study of formulation stored at 25°C±2°C, 60±5 RH (room temperature).

Table 9.3: Stability study of GCH loaded PEGylated spherulites at Refrigerated condition (2-8°C). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Refrigerated condition (2-8°C)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	77.42±0.69	77.38±0.72	77.15±0.57	76.89±0.49
% Drug Loading (%w/w)	9.02±0.33	9.01±0.86	8.98±0.65	8.94±0.34
Particle size (nm) (PDI)	209.2±1.4 (0.43±0.03)	208.7±0.56 (0.21±0.01)	208.2±0.75 (0.32±0.01)	206.2±1.84 (0.41±0.02)
Zeta potential (mV)	-33.3±1.8	-33.1±0.73	-34.2±0.52	-33.8±0.96
Cumulative %Drug Release after 48 hours	89.49±1.65	90.34±0.47	89.67±0.28	90.21±0.43

*Experiment was done in triplicate (n=3).

Stability study was performed at refrigerated condition (2-8°C) and results shown in Table 9.3 revealed that formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 75.29% and 198.1 nm, respectively.

Table 9.4: Stability study of GCH loaded PEGylated spherulites at 25°C±2°C, 60±5 RH (room temperature). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Room condition (25°C±2°C, 60 ±5 RH)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	77.42±0.69	77.29±0.77	77.06±0.38	76.73±0.91
% Drug Loading (%w/w)	9.02±0.33	9.00±0.78	8.97±0.70	8.93±0.83
Particle size (nm) (PDI)	209.2±1.4 (0.43±0.03)	208.8±0.89 (0.29±0.03)	208.5±0.58 (0.49±0.01)	208.2±0.60 (0.37±0.02)
Zeta potential (mV)	-33.3±1.8	-32.4±0.47	-33.6±0.65	-33.5±0.71
Cumulative %Drug Release after 48 hours	89.49±1.65	89.74±0.66	88.36±0.41	89.21±0.42

*Experiment was done in triplicate (n=3).

Stability study was performed at 25°C±2°C, 60±5 RH (room temperature) and results shown in Table 9.4 revealed that PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 74.71% and 205.21 nm, respectively.

Stability study of GCH loaded PEGylated spherulites at two different temperatures revealed that formulation remained physically stable. All the critical quality attributes showed very negligible difference in values from initial to 3 months' time period. However, it is recommended that the formulation should be stored at refrigerated condition (2-8°C) to avoid oxidation and hydrolysis of the amphiphillic lipids [3].

9.4.3 Stability study of VLB loaded Non-PEGylated spherulites

VLB loaded Non-PEGylated spherulites samples were analyzed for any instability issues over the period of 3 months. The samples were kept at two different temperatures viz. 2-8 °C (refrigerated) and 25°C±2°C, 60±5 RH (room temperature) and analyzed at predetermined time intervals. Table 9.5 shows the stability study of formulation stored at 2-8 °C (refrigerated) and Table 9.6 shows the stability study of formulation stored at 25°C±2°C, 60±5 RH (room temperature).

Table 9.5: Stability study of VLB loaded Non-PEGylated spherulites at Refrigerated condition (2-8°C). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Refrigerated condition (2-8°C)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	95.65±0.11	94.89±0.86	94.76±0.74	94.62±0.58
% Drug Loading (%w/w)	11.76±0.38	11.66±0.59	11.64±0.36	11.62±0.12
Particle size (nm) (PDI)	122.4±1.6 (0.24±0.01)	124.2±0.75 (0.19±0.03)	122.3±0.56 (0.32±0.02)	123.6±0.47 (0.25±0.03)
Zeta potential (mV)	-26.9±2.4	-26.7±0.41	-26.4±0.84	-25.8±0.74
Cumulative %Drug Release after 48 hours	90.84±1.25	90.65±0.97	89.28±0.53	90.22±0.89

*Experiment was done in triplicate (n=3).

Stability study was performed at refrigerated condition (2-8°C) and results shown in Table 9.5 revealed that Non-PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 91.59% and 124.9 nm, respectively.

Stability study was performed at 25°C±2°C, 60±5 RH (room temperature) and results shown in Table 9.6 revealed that PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 92.36% and 125.75 nm, respectively.

Table 9.6: Stability study of VLB loaded Non-PEGylated spherulites at 25°C±2°C, 60±5 RH (room temperature). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Room condition (25°C±2°C, 60 ±5 RH)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	95.65±0.11	95.36±0.65	95.19±0.53	94.79±0.51
% Drug Loading (%w/w)	11.76±0.38	11.72±0.76	11.69±0.87	11.64±0.76
Particle size (nm) (PDI)	122.4±1.6 (0.24±0.03)	123.4±0.61 (0.42±0.02)	123.5±0.74 (0.38±0.01)	123.2±0.92 (0.21±0.02)
Zeta potential (mV)	-26.9±2.4	-25.4±0.59	-26.2±0.58	-26.4±0.39
Cumulative %Drug Release after 48 hours	90.84±1.25	89.32±0.63	90.42±0.35	89.97±0.76

*Experiment was done in triplicate (n=3).

Stability study of VLB loaded Non-PEGylated spherulites at two different temperatures revealed that formulation remained physically stable. All the critical quality attributes showed very negligible difference in values from initial to 3 months time period. However, it is recommended that the formulation should be stored at refrigerated condition (2-8°C) to avoid oxidation and hydrolysis of the amphiphillic lipids [3].

9.4.4 Stability study of VLB loaded PEGylated spherulites

VLB loaded PEGylated spherulites samples were analyzed for any instability issues over the period of 3 months. The samples were kept at two different temperatures viz. 2-8 °C (refrigerated) and 25°C±2°C, 60±5 RH (room temperature) and analyzed at predetermined time intervals. Table 9.7 shows the stability study of formulation stored at 2-8 °C (refrigerated) and Table 9.8 shows the stability study of formulation stored at 25°C±2°C, 60±5 RH (room temperature).

Stability study was performed at refrigerated condition (2-8°C) and results shown in Table 9.7 revealed that PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 92.19% and 137.5 nm, respectively.

Table 9.7: Stability study of VLB loaded PEGylated spherulites at Refrigerated condition (2-8°C). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Refrigerated condition (2-8°C)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	94.25±1.24	93.89±0.28	93.94±0.48	93.68±0.57
% Drug Loading (%w/w)	11.04±0.71	10.99±0.96	11.00±0.20	10.96±0.82
Particle size (nm) (PDI)	131.6±1.90 (0.32±0.01)	131.8±1.27 (0.27±0.01)	132.4±0.98 (0.19±0.03)	133.1±0.32 (0.42±0.02)
Zeta potential (mV)	-37.8±2.10	-36.4±0.90	-37.2±0.58	-36.9±0.93
Cumulative %Drug Release after 48 hours	86.85±1.47	87.21±0.59	86.35±0.69	88.29±0.86

*Experiment was done in triplicate (n=3).

Stability study was performed at 25°C±2°C, 60±5 RH (room temperature) and results shown in Table 9.8 revealed that PEGylated formulation was stable physically over the period of 3 months with negligible difference between the initial and final values of characterization. Moreover, a 12 month values of critical quality attributes like %EE and size of spherulites were calculated by extrapolating the mean values obtained at 3 months. It was found that at 12 months stability testing %EE and size of spherulites would be 91.24% and 137.4 nm, respectively.

Table 9.8: Stability study of VLB loaded PEGylated spherulites at 25°C±2°C, 60±5 RH (room temperature). Data represents mean of three experimental values (n=3; mean±SD).

Storage conditions		**Room condition (25°C±2°C, 60 ±5 RH)**		
Sampling period (months)	0	1	2	3
Visual observation	Milky suspension	Milky suspension	Milky suspension	Milky suspension
%EE	94.25±1.24	94.02±0.86	93.73±0.42	93.51±0.64
% Drug Loading (%w/w)	11.04±0.71	11.01±0.64	10.97±0.78	10.94±0.55
Particle size (nm) (PDI)	131.6±1.90 (0.32±0.01)	132.1±0.79 (0.11±0.03)	132.5±0.65 (0.36±0.03)	133.1±0.34 (0.24±0.02)
Zeta potential (mV)	-37.8±2.10	-37.5±0.96	-37.2±0.84	-36.7±0.65
Cumulative %Drug Release after 48 hours	86.85±1.47	88.47±0.76	87.56±0.93	88.12±59

*Experiment was done in triplicate (n=3).

Stability study of VLB loaded PEGylated spherulites at two different temperatures revealed that formulation remained physically stable. All the critical quality attributes showed very negligible difference in values from initial to 3 months' time period. However, it is recommended that the formulation should be stored at refrigerated condition (2-8°C) to avoid oxidation and hydrolysis of the amphiphillic lipids [7].

9.5 References

1. ICH, F. (1997). Q1C Stability Testing for New Dosage Forms. Guidance for Industry. US Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research. *Center for Biologics Evaluation and Research, ICH*.
2. Guidance for Industry. Q1A(R2) Stability Testing of New Drug Substances and Products (2003). U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Biologics Evaluation and Research (CBER). Available online. https://www.fda.gov/downloads/drugs/guidances/ucm073369.pdf. (Accessed on July 5, 2018).
3. Yadav, A., Murthy, M. S., Shete, A. S., & Sakhare, S. (2011). Stability aspects of liposomes. Indian Journal Of Pharmaceutical Education And Research, 45(4), 402-413.
4. Cacela, C., & Hincha, D. K. (2006). Low amounts of sucrose are sufficient to depress the phase transition temperature of dry phosphatidylcholine, but not for lyoprotection of liposomes. Biophysical journal, 90(8), 2831-2842.
5. Liposome Drug Products Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation. Guidance for Industry. Available online. https://www.fda.gov/downloads/drugs/guidances/ucm070570.pdf. (Accessed on July 5, 2018).
6. Armengol, X., & Estelrich, J. (1995). Physical stability of different liposome compositions obtained by extrusion method. Journal of microencapsulation, 12(5), 525-535.
7. Dhande, R., Tyagi, A., Sharma, R. K., & Thakkar, H. (2017). Biodistribution study of ^{99m}Tc-gemcitabine-loaded spherulites in Sprague–Dawley rats by gamma scintigraphy to investigate its lung targeting potential. Journal of microencapsulation, 34(7), 623-634.