Chapter 6
Stability study

6.1. Introduction

Stability is defined as the capacity of a drug substance or drug product to remain within established specifications to maintain its identity, strength, quality, and purity throughout the retest or expiration dating periods (Draft guidance, Stability Testing of Drug Substances and Drug Products, FDA, 1998). The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions (Draft guidance, Stability Testing of New Drug Substances and Products, 2003). Physical, chemical, and microbiological data are generated as a function of time and storage conditions (e.g., temperature and relative humidity [RH]). It is a well-known fact that for drug delivery systems, stability of the formulation is one of the most critical parameters from the pharmaceutical aspect. The storage conditions are particularly important to define in order to start biological studies and to make sure that the drug doses used would be preserved. For this purpose, accelerated stability testing at high temperatures and humidity conditions are often employed to predict the shelf life of drugs.

Particulate delivery systems like microparticles and nanoparticles are widely used to deliver a wide range of drugs. The nanoparticles protect the drug from metabolizing enzymes, sustain the release, to be administered orally or injected locally, and target specific tissues by incorporating surface ligand moieties. Poly (lactide), poly(glycolide) and their copolymers approved by the U.S. Food and Drug Administration (FDA) represent a major class of synthetic biodegradable materials essentially useful for the preparation of microparticles and nanospheres. The factors that influence the chemical degradation of PLGA are well known and include polymer molecular weight, ratio of lactic to glycolic acid in the co-polymers, polymer-drug ratio, environmental temperature, pH, and geometry of the delivery system (Burcu Sayin et al., 2004 & Gasper, M.M. et al., 1998). The main mode of degradation for the PLGA polymer is purely through simple hydrolysis of the ester bonds and does not involve any enzymatic activity.(Mauduit, J. et al., 1996) In vivo it degrades into lactic acid and glycolic acid. Lactic acid enters the tricarboxylic acid cycle and is metabolized and subsequently eliminated from the body as

carbon dioxide and water. Glycolic acid is either excreted unchanged in the kidney or it enters the tricarboxylic acid cycle and is eventually eliminated as carbon dioxide and water (Burcu Sayin et al., 2004. It has been shown that PLGA nanospheres and microspheres have a shelf-life of more than 3 months (PLGA 50:50, 0.63 dL/g) (Feng, S. et al., 2001).

The liposomes are more susceptible to environmental conditions like temperature/humidity and were affected by oxidation/hydrolysis. Change in environment tends to aggregate and therefore leakage of the drug substance form the lipid vesicles. Liposomal aggregation, bilayer fusion and drug leakage are the main problems of physical stability encountered in any liposomal formulation which could greatly affect the shelf life of liposomes. Unfortunately, liposomal formulations do not meet the required standards for long term stability of pharmaceutical preparations if they are stored as aqueous dispersions (Fransen et al., 1986). The encapsulated drug tends to leak out of the bilayer and the liposomes might aggregate or fuse upon storage.

Here the liposomes were evaluated for the effect of temperature on the particle size and percent entrapment efficiency i.e. aggregation and/or leakage of the DC from the liposomes over the period of 3 months. All the formulations were stable when stored at 2-8°C and there were some aggregation and leakage seen when stored at 25°C.

Although, the instability of the nanoparticles in the dispersion is overcome by lyophilization using cryoprotectants, the influence of the storage conditions like temperature and humidity on the particle size and drug content are important in maintaining the integrity of these delivery systems before use for the biological studies.

6.2. Methodology

The stability studies were carried out in accordance with the ICH guidelines for new drug products. The stability studies were carried out for the nanoparticle formulations at 5°C ± 3°C for 6 months and (25°C ± 2°C/60 ± 5 % RH) up to 6 months. Three batches at optimized process and formulation conditions were prepared and subjected to stability studies. The nanoparticles were filled in glass vials, closed with rubber closures and sealed with aluminum caps.

The samples were withdrawn at predetermined levels and were examined visually for physical appearance. The contents of the vials were evaluated for the particle size, zeta potential and drug content.

Statistical Analysis and Data Interpretation

Three batches of each formulation was evaluated three times, data of nine experiments are expressed as Mean ± SD. The data were compared using ANOVA and student's t-test and difference larger than the value at $p<0.05$ were considered significant.

"Significant change" *was* considered under following conditions

- A 5 percent change in assay from its initial value
- Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test particle size and drug content may be expected under accelerated conditions.

The results of stability studies are recorded in Tables: 6.1 to Table: 6.3 and Figures: 6.1 to Figure: 6.2.

6.3. Results and Discussions

The stability studies of the formulations were performed in order to study the influence of varying environmental conditions on the parameters of the formulation influencing the therapeutic response. The stability studies were carried out in accordance with the ICH guidelines for drug substances intended to be stored in a refrigerator. The stability of the nanoparticles and liposomes were assessed for physical observation, particle size, zeta potential and the drug content (with respect to the initial) at 5°C ± 3°C for 6M and 25°C ± 2°C/60% RH ± 5% RH for 6 months. The drug content in the initial sample was considered as 100 percent. For accelerated condition (i.e. 25°C ± 2°C/60% ± 5 %RH) the sampling was done at 1, 2, 3, 6 months and for 5°C ± 3°C the sampling was done at 1, 3, 6 months.

The results for the stability studies are represented in table 6.1 and 6.2 for PLGA-DC-NP and PLGA-DC-RGD-NP respectively. The graphical representations are given in Figure: 6.1 and Figure: 6.2. The results for LP-DC and LP-DC-RGD are recorded in Table: 6.3.

Table: 6.1. Stability studies data of PLGA-DC-NP

Stability conditions	Description & Redispersibility	Particle size (nm)	Zeta potential (mV)	Drug content %
Initial	Free flowing white powder with easy redispersibility	210.3 ± 2.7	-38.4 ± 2.6	100.0 ± 2.4*
5°C ± 3°C				
1 M	Free flowing white powder with easy redispersibility	209.4 ± 3.6	-38.9 ± 1.5	98.3 ± 2.2
3 M	Free flowing white powder with easy redispersibility	211.7 ± 3.2	-39.2 ± 1.7	97.6 ± 2.2
6 M	Free flowing white powder with easy redispersibility	212.4 ± 2.6	-37.1 ± 2.2	97.4± 1.5
25°C ± 2°C/60% RH ± 5% RH				
1 M	Free flowing white powder with easy redispersibility	210.3 ± 4.5	-38.4 ± 2.8	98.6± 2.2
2 M	Free flowing white powder with easy redispersibility	220.4 ± 6.5	-36.2 ± 3.2	96.8± 2.8
3 M	White powder with poor flow and difficult redispersibility	532.3 ± 9.6	-26.8 ± 2.4	94.5± 2.5
6 M	White powder with poor flow and poor redispersibility	845.6 ± 6.7	-13.6 ± 1.4	93.6 ± 2.2

* Initial drug content was labeled as 100% and the drug content at different time points are with respect to the initial drug content

Table: 6.2. Stability data of PLGA-DC-RGD

Stability conditions	Description	Particle size (nm)	Zeta potential (mV)	Drug content %
Initial	Free flowing white powder with easy redispersibility	230.7 ± 2.3	-10.2 ± 2.8	100.0± 1.6
5°C ± 3°C				
1 M	Free flowing white powder with easy redispersibility	232.5 ± 5.6	-10.5 ± 1.4	99.2 ± 2.6
3 M	Free flowing white powder with easy redispersibility	230.4 ± 4.7	-11.3 ± 1.5	98.8± 2.5
6 M	Free flowing white powder with easy redispersibility	235.6 ± 8.5	-10.5 ± 2.3	98.1± 1.3
25°C ± 2°C/60% RH ± 5% RH				
1 M	Free flowing white powder with easy redispersibility	236.2 ± 6.3	-10.4 ± 1.5	98.2± 2.1
2 M	Free flowing white powder with easy redispersibility	239.4 ± 5.6	-9.2 ± 1.1	96.5± 2.2
3 M	White powder with poor flow and difficult redispersibility	514.8 ± 12.5	-8.4 ± 2.3	96.2± 2.4
6 M	Light pink powder with poor flow and poor redispersibility	861.5 ± 14.5	-4.2 ± 1.4	95.3 ± 2.6

Table: 6.3. Stability data of Docetaxel liposomes

(a) Stability data on % EE

Liposomes	Initial	2-8°C			25± 2 ° C			
		1 month	3 month	6 month	1 month	2 month	3month	6 months
Non-PEGylated	100±2.4	99.1±1.4	98.4±2.6	98.2±2.8	98.8±3.6	97.4±3.4	95.2±3.8	94.3±4.2
PEGylated	100±3.4	99.2±2.1	98.8±2.4	98.4±2.6	98.3±2.3	97.8±2.5	96.4±2.4	94.8±3.5
LP-DC-RGD	100±2.6	99.4±2.2	98.4±2.3	97.9±2.7	99.3±2.5	98.3±2.6	97.4±2.8	94.2±2.2

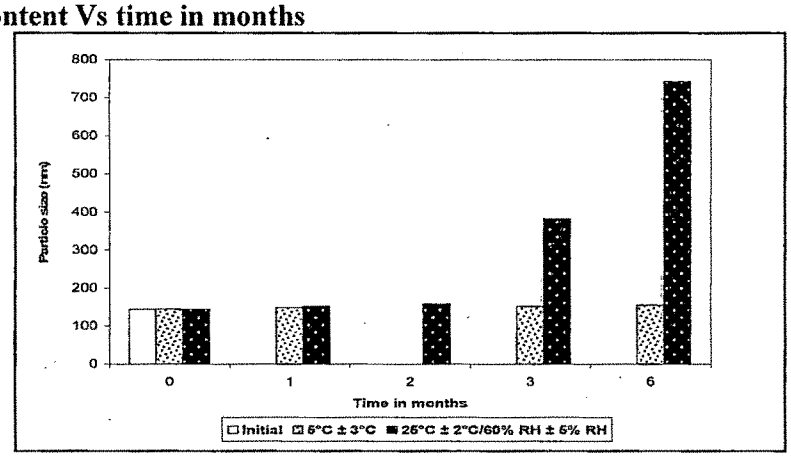
(b)Stability data on particle size

Liposomes	Initial	2-8°C			25± 2 ° C			
		1 month	3 month	6month	1 month	2 month	3month	6 months
Non-PEGylated	260.6± 2.4	263.2± 1.8	265.4± 4.6	269.2± 4.4	270.8± 5.8	279.4± 5.9	288.2± 7.6	585.6± 12.2
PEGylated	269.6± 3.4	270.2± 3.6	272.8± 4.8	275.4± 5.6	273.3± 6.7	274.8± 5.3	278.4± 3.2	492.2± 11.3
LP-DC-RGD	278.4± 5.2	279.2± 3.4	280.7± 3.4	279.4± 4.5	279.1± 5.3	283.4± 5.2	285.4± 3.4	467.4± 13.8

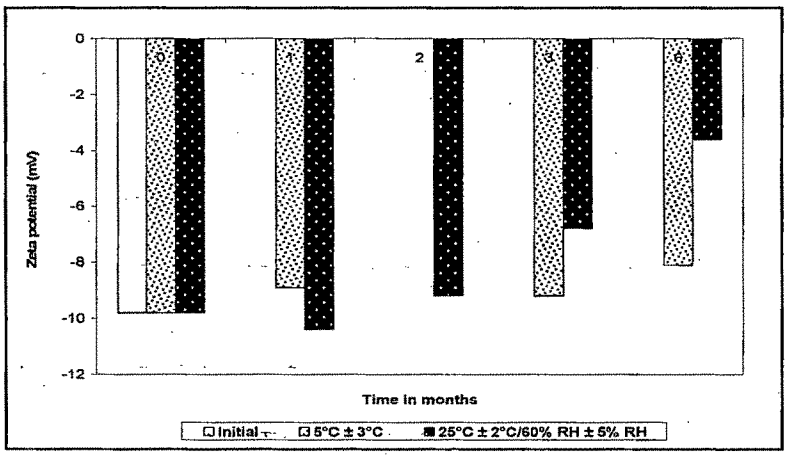
(c) Stability data on Zeta potential

Liposomes	Initial	2-8°C			25± 2 ° C			
		1 month	3 month	6month	1 month	2 month	3month	6 months
Non-PEGylated	-28.7 ± 1.3	-27.2 ± 2.7	-27.7 ± 1.6	-27.9 ± 1.5	-28.2 ± 2.4	-27.3 ± 1.7	-26.9 ± 2.1	-12.9 ± 2.5
PEGylated	-27.2± 1.8	-27.7 ± 1.8	-27.5 ± 1.4	-28.7 ± 2.2	-28.2 ± 2.3	-27.3 ± 1.5	-26.8 ± 2.9	-16.4 ± 3.1
LP-DC-RGD	-11.6± 1.2	-10.2± 1.4	-11.3± 1.4	-11.7± 1.7	-11.4± 1.5	-10.3 ±1.6	-10.9± 2.1	-18.9 ±2.8

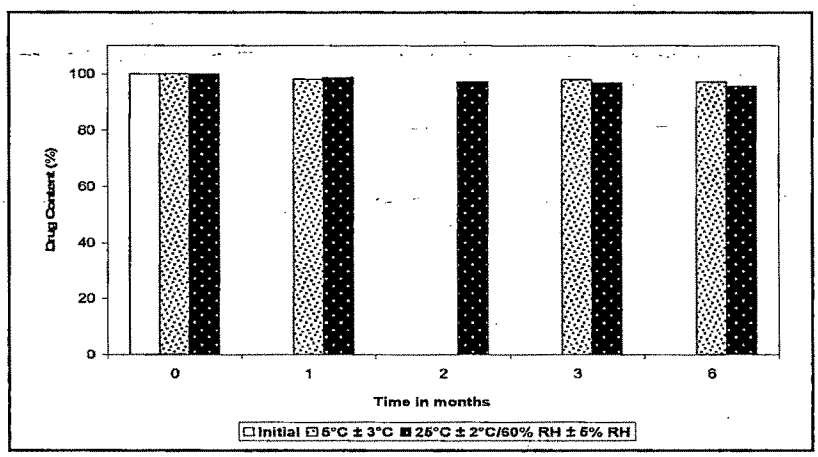
Figure: 6.1. Stability profiles-PLGA-DC-NP (a) particle size (b) zeta potential and (c) drug content Vs time in months



(a)

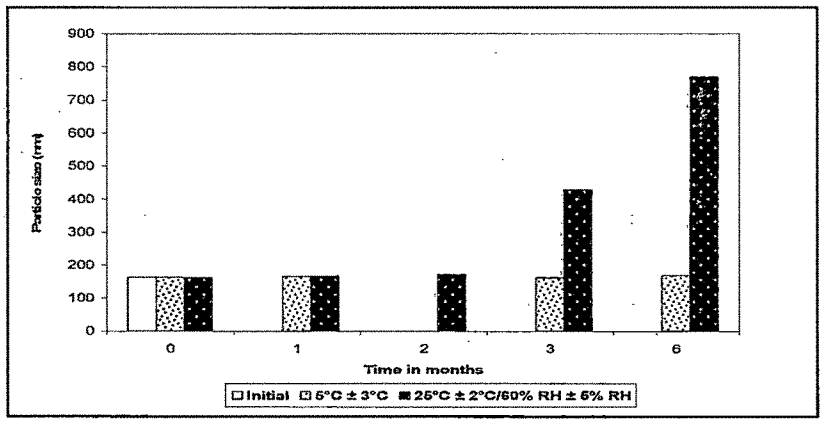


(b)

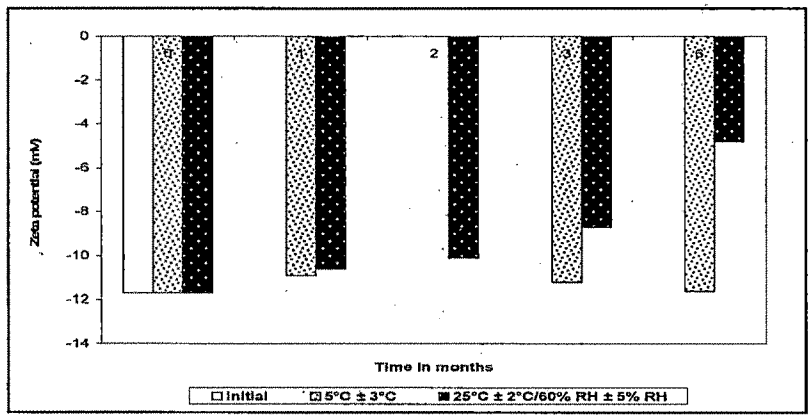


(c)

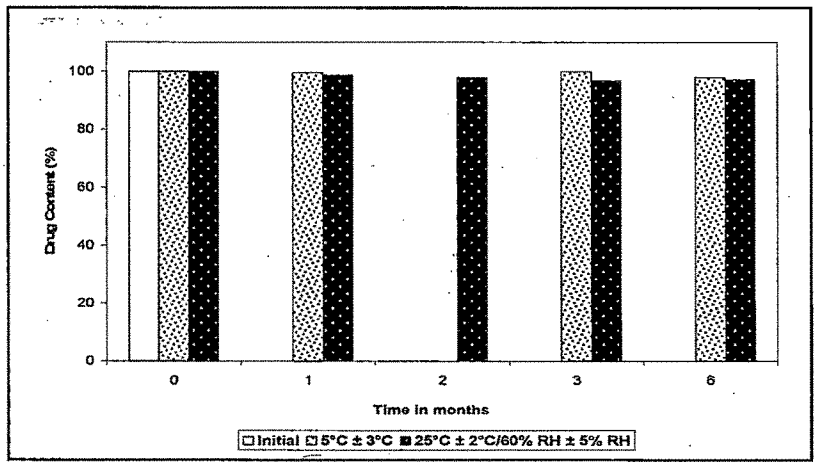
Figure: 6.2. Stability profiles- PLGA-DC-RGD-NP (a) particle size (b) zeta potential and (c) drug content Vs time in months



(a)



(b)



(c)

It was observed that unconjugated and conjugated nanoparticles of docetaxel there was no significant change (P>0.05) observed in particle size, zeta potential and drug content at 5°C ± 3°C for 6M.

The storage of the unconjugated and conjugated nanoparticles of docetaxel at 25°C ± 2°C/60% ± 5% RH, led to increase in the particle size. The increase in the particle size was not significant during the first month, however became significant and more prominent after 2, 3 and 6 months. During our analysis of samples, the polydispersity index of the nanoparticles stored at 25°C ± 2°C/60% ± 5% RH was found to increase as compared to the initial. The increase in the particle size may be due to the absorption of the moisture by the nanoparticles resulting in the coalescence of the small nanoparticles forming particles larger in size.

The nanoparticles were also observed for physical appearance. After 3 and 6 months the physical appearance was also changed, with loss of the free flowing property followed by the difficulty in redispersibility. Also, the RGD conjugated nanoparticles demonstrated difference in the color than the initial powder. At 6 months the color of the powder was light pink. This could be indicative of the degradation of the surface RGD.

At 25°C ± 2°C/60% ± 5% RH, the zeta potential of the nanoparticles shifted towards the zero for both unconjugated and conjugated nanoparticles. This may be due to the acidic conditions produced due to the degradation of PLGA into lactic and glycolic acid (Sanjeeb, K. et al., 2002). The lowered zeta potential values also might have contributed toward the aggregation of particles.

The drug content of the unconjugated and conjugated nanoparticles was not altered up to 6M at 5°C ± 3°C. However, the drug content was reduced after 6M storage at 25°C ± 2°C/60% RH ± 5% RH. This impact could be due to the moisture absorbed by the nanoparticles upon storage at 25°C ± 2°C/60% RH ± 5% RH, possibly resulting in the degradation of the drug.

The initial drug entrapment was found to be 72.6±2.4% and 69.6±3.4% and initial particle size 260.6±2.4 nm, 260.6±2.4 nm for Non-PEGylated and PEGylated liposomes respectively. Reduction in the entrapment after 3 months, were observed 69.2±2.8% (2-8°C) and 61.2±3.8% (25°C) while particle size was 269.2±4.4 nm (2-8°C) and 288.2±7.6

nm (25°C) for Non-PEGylated. In case of PEGylated liposomes, minor reduction in the drug entrapment was seen (68.2±2.1% and 61.2±3.8%) at 2-8°C and 25°C respectively and increase in the particles size was in the range of 8-12 nm from the initial (Table: 6.3). The release profile of the drug from the nanoparticles was not affected upon storage. The similarity factor calculated for the between the initial and the 6M samples show values greater than 80, indicating high similarity between the initial and 6M.

6.4. Conclusions

From the above study, we can conclude that the unconjugated and RGD-conjugated PLGA nanoparticles and liposomes of docetaxel when stored at 25°C ± 2°C/60% RH ± 5% RH for 6M show instability reflected by change in physical appearance, increase in the particle size, zeta potential and reduction in the drug content. Hence, we can conclusively specify that both unconjugated and conjugated nanoconstructs were stable and can be stored 5°C ± 3°C for 6M retaining its original formulation characteristics. Further, long term stability should be carried our further to assess the influence of the increasing time on the stability of the prepared nanoconstructs at 5°C ± 3°C.

6.5. References

Burcu Sayin et al., 2004, Influence of Accelerated Storage Conditions on The Stability Of Vancomycin-Loaded Poly(D,L-Lactide-Coglycolide) Microspheres, FABAD. J. Pharm. Sci., **29**, 111-116.

Feng, S. et al., 2001, Effects of emulsifiers on the controlled release of paclitaxel (Taxol) from nanospheres of biodegradable polymers, J. Control. Release., **71**, 53-69.

Gasper, M.M. et al., 1998, Formulation of Lasparaginase-loaded poly(lactide-co-glycolide) nanoparticles: influence of polymer properties on enzyme loading, activity and in vitro release, J. Control. Release., **52,** 53-62.

ICH guidelines (www.ich.org)

Mauduit, J. et al., M. Hydrolytic degradation of films prepared from blends of high and low molecular weight poly(DL-lactic acid)s, J. Biomed. Mater. Res., **30**, 201-207.

Sanjeeb K. et al., Residual polyvinyl alcohol associated with poly (lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake, Journal of Controlled Release, 105-114.

www.fda.gov