CHAPTER 6

STABILITY STUDIES

6 STABILITY STUDIES

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to maintain its identity, strength, quality, and purity throughout the retest or expiration dating periods (Draft guidance, Stability Testing of Drug Substances and Drug Products, FDA, 1998).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a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions (Draft guidance, Stability Testing of New Drug Substances and Products, 2003).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 order to start biological studies and to make sure that the drug doses used would be preserved for scheduled shelf-life. 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 (NPs) are widely used to deliver a wide range of drugs. The NPs protect the drug from metabolizing enzymes, sustain the release, 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 which 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 and Sema Calis, 2004; Gasper MM 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 and Sema Calis, 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, Huang G, 2001).

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

6.2 Methods

6.2.1 Stability study of Nanoparticles

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

The samples were withdrawn at predetermined levels. For accelerated condition (i.e. 25 ± 2°C / 60 ± 5% RH) sampling was done at 1, 2, 3, 6 M and for 5 ± 3°C sampling was done at 3 and 6 M. The contents of the vials were evaluated at specific time interval for physical appearance, Particle size (PS), Zeta potential (ZP) and drug content as describe in chapter 5 and compared with initial data. The drug content in the initial sample was considered as 100 percent. Data are expressed as mean ± SD, n=3. Stability data for Tf-TMD-NPs, Lf-TMD-NPs, Tf-LTG-NPs and Lf-LTG-NPs were shown in Table 6.1, 6.2, 6.3, and 6.4 respectively. The comparison of PS, ZP data of initial with data at specific time period at different stability conditions (5 ± 3°C and 25 ± 2°C / 60 ± 5% RH) was done and plotted in graph. Comparison of PS data at initial and different stability conditions of formulations (Tf-TMD-NPs and Lf-TMD-NPs) was shown in Fig. 6.1 Comparison of ZP data at initial and different stability conditions of formulations (Tf-TMD-NPs and Lf-TMD-NPs) was shown in Fig. 6.2. Similarly comparison of PS data of Tf-LTG-NPs and Lf-LTG-NPs was shown in Fig. 6.5. Fig. 6.6 shows the comparison of ZP data of Tf-LTG-NPs and Lf-LTG-NPs.

The 6 M samples of conjugated NPs stored at 5 ± 3°C were evaluated for drug release. The results of comparative drug release after 6M with respect to initial at 5 ± 3°C for Tf-TMD-NPs, Lf-TMD-NPs, TF-LTG-NPs and Lf-LTG-NPs are shown in Fig. 6.3, 6.4, 6.7 and 6.8 respectively.

6.2.2 Stability study of Microemulsion and Nanoemulsion

ME and NE were evaluated for stability study by performing physical and chemical stability of the optimized formulation (Kantaria S et al., 1999). The optimized formulations have been subjected to accelerated stability study for the assessment of physical stability. Chemical stability of the formulation was assessed by long term stability study.

6.2.2.1 Accelerated physical stability study

Accelerated stability studies are the essential tools to study the thermodynamic stability of emulsions (Sheikh Shafiq and Faiyaz Shakeel, 2007; Nornoo AO and Chow DS, 2008).

1. The formulations were centrifuged for 30 minute at 10,000 rpm and observed for phase separation.
2. The systems were kept for freeze/ thaw cycles between - 21°C and 25°C for not less than 12 hours at each stage.
3. The systems were subjected to 6 cycles of heating / cooling cycle by keeping them at 4 °C and 45 °C for not less than 48 hours at each stage.

The formulations were observed for Globule size (GS), Zeta potential (ZP) and %Transmittance (%T) before and after the centrifugation, freeze thaw cycle and heating cooling cycle as describe in chapter 5. Data are expressed as mean ± SD, n=3. Physical observation for phase separation, if any, between the oil and aqueous phase, was carried out after completion of study. The data was recorded in Table 6.5

6.2.2.2 Long term stability study

In long term stability study, the emulsions were packed in the borosil screw capped vials and were kept at storage and accelerated conditions including temperature and humidity. MEs of both drugs were kept at 25 ± 2°C / 60 ± 5% RH (storage condition) and 40 ± 2°C / 75 ± 5% RH (accelerated condition) for period of 6 months. While, NEs of TMD and LTG were kept at refrigeration temperature, 5 ± 3°C (storage condition) and 25 ± 2°C / 60 ± 5% RH (accelerated condition) for 6 months. For accelerated condition sampling was done at 1, 2, 3, 6 M and for storage condition (5 ± 3°C) sampling was done at 3 and 6 M. Over the time period emulsion systems were assessed for their GS, ZP, physical stability, assay and pH as describe in chapter 5 and compared with initial data. The drug content in the initial sample was considered as 100 percent. Data are expressed as mean ± SD, n=3. The stability data for TME, LME, TNE and LNE was recorded in Table 6.6, 6.7, 6.8 and 6.9 respectively.

The comparison of GS and ZP data of initial with data at specific time period at different stability conditions (25 ± 2°C / 60 ± 5% RH and 40 ± 2°C / 75 ± 5% RH) for ME was done and plotted in graph. Comparison of GS data at initial and different stability conditions of formulations (TME and LME) was shown in Fig. 6.9. Fig. 6.10 shows the comparison of ZP data at initial and different stability conditions of formulations (TME and LME). Similarly comparison of GS, ZP data of initial with data at specific time period at different stability conditions (5 ± 3°C and 25 ± 2°C / 60 ± 5% RH) for NE was done and plotted in graph. Comparison of GS data at initial and different stability conditions of formulations (TNE and LNE) was shown in figure6.11. Comparison of ZP data of TNE and LNE was shown in Fig. 6.12.

6.2.3 Statistical analysis and data interpretation

Single batch of each formulation was evaluated three times; data are expressed as Mean ± SD. The data were compared using ANOVA and student's t-test and, difference larger than $p<0.05$ 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 PS and drug content may be expected under accelerated conditions.

6.3 Results and Discussion

6.3.1 Stability study of Nanoparticles

Table 6.1 Stability study of Tf-TMD NPs

Condition	Duration	Parameters evaluated			
		Redispersibility*	PS (nm)	ZP (mV)	%Drug content
--	Initial	0	157.5 ± 4.2	-11.06 ± 0.41	100.00
5 ± 3°C	3 M	0	159.6 ± 6.2	-11.16 ± 1.38	99.27 ± 1.06
	6 M	0	162.8 ± 5.6	-10.89 ± 0.49	99.14 ± 0.95
25 ± 2°C / 60 ±5% RH	1 M	0	161.4 ± 5.5	-11.02 ± 0.52	99.64 ± 0.93
	2 M	0	167.0 ± 6.8	-10.74 ± 0.63	98.39 ± 1.70
	3 M	0	172.2 ± 7.9	-10.59 ± 0.49	98.14 ± 1.28
	6 M	1	216.5 ± 10.6	-8.43 ± 0.76	96.18 ± 1.34

*Values are represented as mean ± SD, n=3; *0 easy redispersibility, 1 poor redispersibility*

Table 6.2 Stability study of Lf-TMD NPs

Condition	Duration	Parameters evaluated			
		Redispersibility*	PS (nm)	ZP (mV)	%Drug content
--	Initial	0	158.8 ± 3.9	-9.35 ± 0.29	100.00
5 ± 3°C	3 M	0	159.7 ± 4.6	-9.21 ± 0.41	99.73 ± 1.30
	6 M	0	163.2 ± 5.4	-9.39 ± 1.38	99.33 ± 1.21
	1 M	0	160.9 ± 5.0	-9.29 ± 0.33	99.52 ± 1.16
25 ± 2°C /	2 M	0	168.4 ± 5.2	-9.13 ± 0.51	99.42 ± 0.83
60 ±5% RH	3 M	0	176.2 ± 6.7	-8.93 ± 0.46	98.65 ± 1.05
	6M	1	231.7 ± 9.7	-7.34 ± 0.54	97.09 ± 1.23

*Values are represented as mean ± SD, n=3; *0 easy redispersibility, 1 poor redispersibility*

Figure 6.1 Comparison of PS at initial and different stability conditions of formulations (Tf-TMD-NPs and Lf-TMD-NPs)

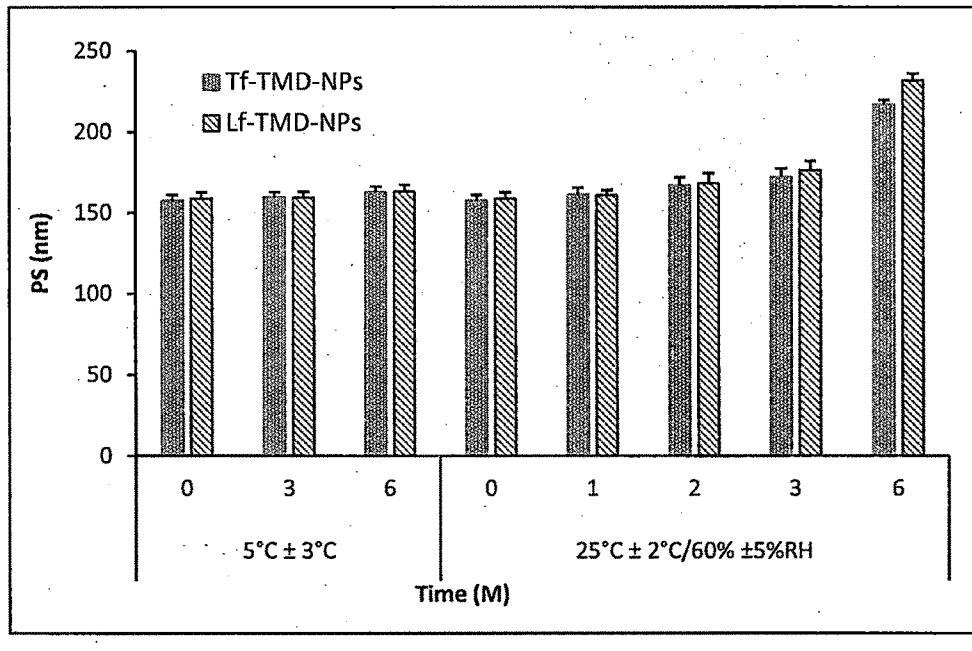


Figure 6.2 Comparison of ZP at initial and different stability conditions of formulations (Tf-TMD-NPs and Lf-TMD-NPs)

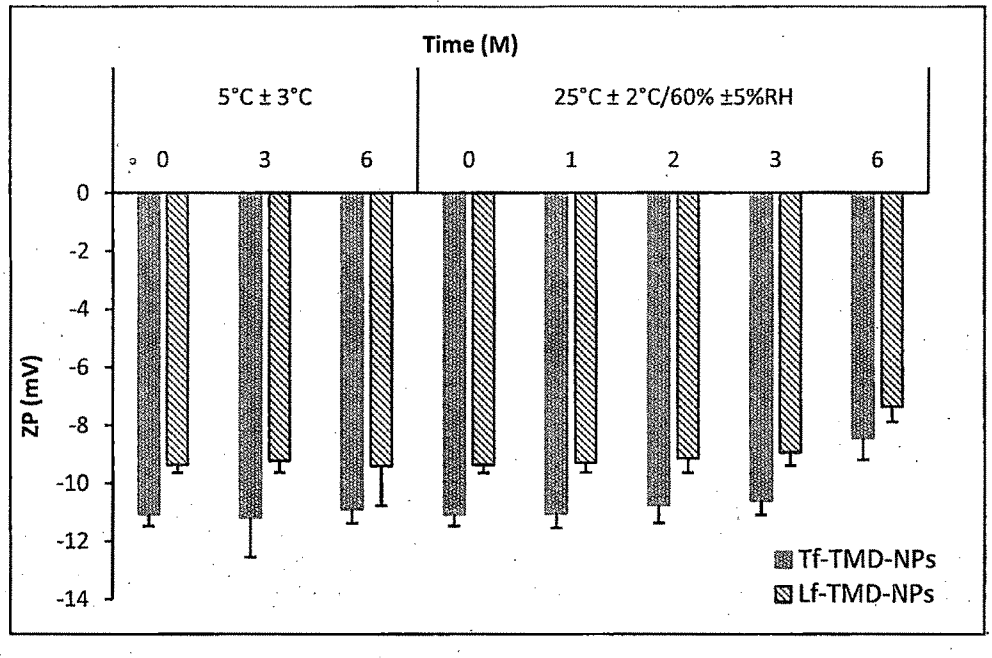


Figure 6.3 Comparative release profile of Tf-TMD-NPs after 6M at 5 ± 3°C

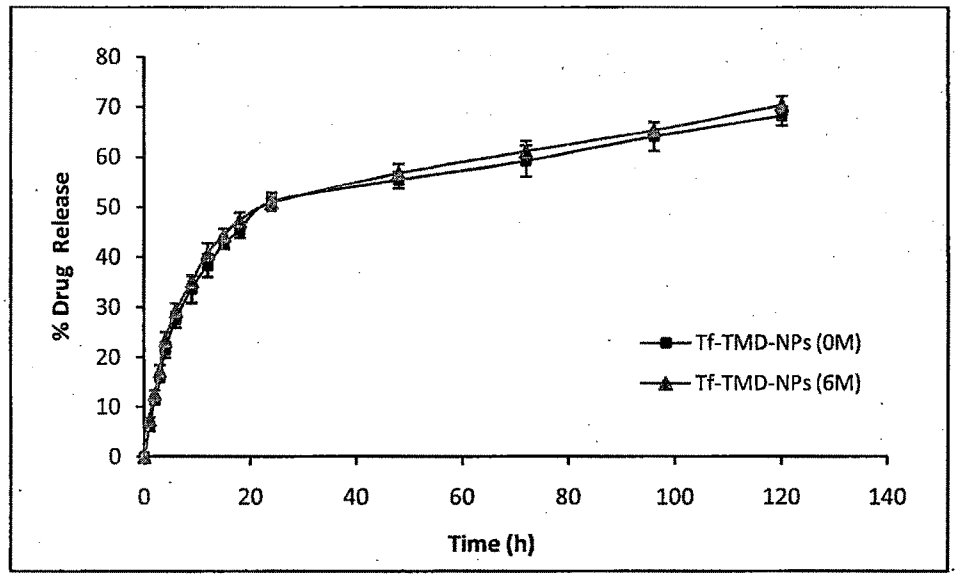


Figure 6.4 Comparative release profile of Lf-TMD-NPs after 6M at 5 ± 3°C

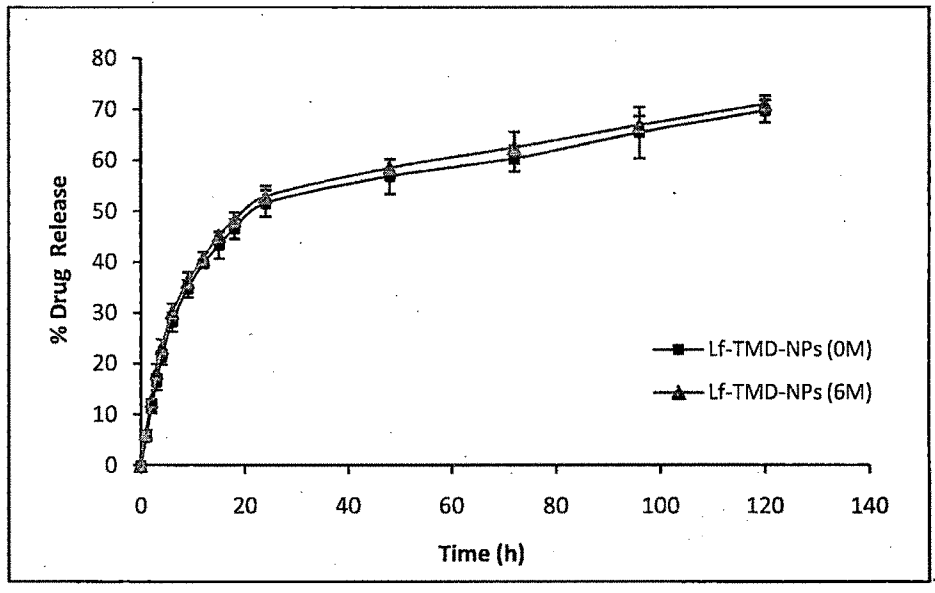


Table 6.3 Stability study of Tf-LTG NPs

Condition	Duration	Parameters evaluated			
		Redispersibility*	PS (nm)	ZP (mV)	%Drug content
--	Initial	0	151.0 ± 3.8	-12.88 ± 0.46	100.00
5 ± 3°C	3 M	0	152.1 ± 3.3	-12.65 ± 1.27	99.62 ± 1.01
	6 M	0	154.3 ± 3.5	-12.14 ± 0.36	99.21 ± 0.87
	1 M	0	155.6 ± 4.2	-11.89 ± 0.47	99.51 ± 0.85
25 ± 2°C /	2 M	0	161.4 ± 4.9	-11.37 ± 1.44	99.18 ± 1.34
60 ± 5% RH	3 M	0	170.8 ± 5.2	-10.78 ± 0.35	98.64 ± 0.95
	6 M	1	208.5 ± 3.2	-9.24 ± 0.56	97.56 ± 0.74

*Values are represented as mean ± SD, n=3; *0 easy redispersibility, 1 poor redispersibility*

Table 6.4 Stability study of Lf-LTG NPs

Condition	Duration	Parameters evaluated			
		Redispersibility*	PS (nm)	ZP (mV)	%Drug content
--	Initial	0	150.4 ± 4.0	-11.21 ± 0.35	100.00
5 ± 3°C	3 M	0	153.4 ± 3.5	-10.54 ± 1.26	99.82 ± 1.27
	6 M	0	156.2 ± 4.3	-10.22 ± 0.28	99.34 ± 1.19
	1 M	0	158.2 ± 3.2	-10.88 ± 0.57	99.68 ± 1.09
25 ± 2°C /	2 M	0	164.7 ± 6.1	-10.17 ± 1.38	99.29 ± 0.52
60 ± 5% RH	3 M	0	166.3 ± 5.8	-9.86 ± 0.42	98.77 ± 1.23
	6 M	1	221.2 ± 4.3	-8.05 ± 1.23	98.16 ± 0.78

*Values are represented as mean ± SD, n=3; *0 easy redispersibility, 1 poor redispersibility*

Figure 6.5 Comparison of PS at initial and different stability conditions of formulations (Tf-LTG-NPs and Lf-LTG-NPs)

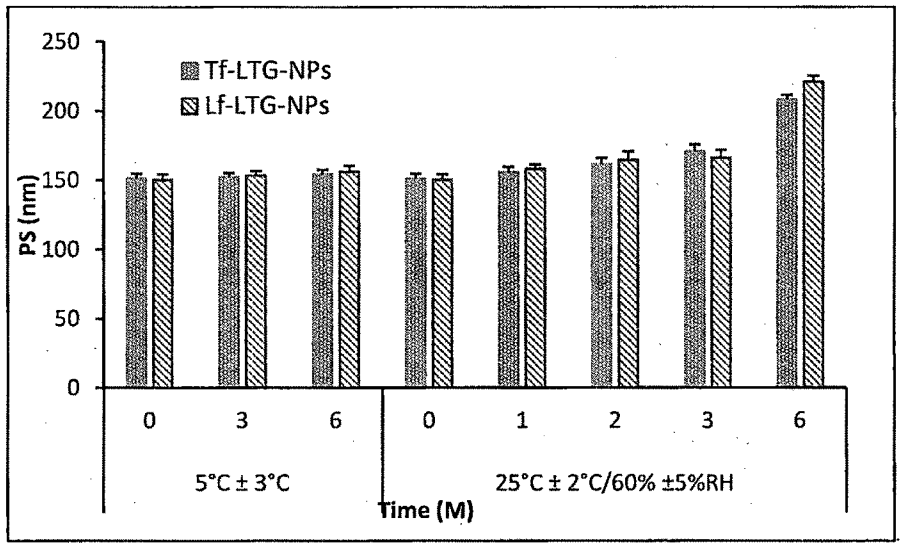


Figure 6.6 Comparison of ZP at initial and different stability conditions of formulations (Tf-LTG-NPs and Lf-LTG-NPs)

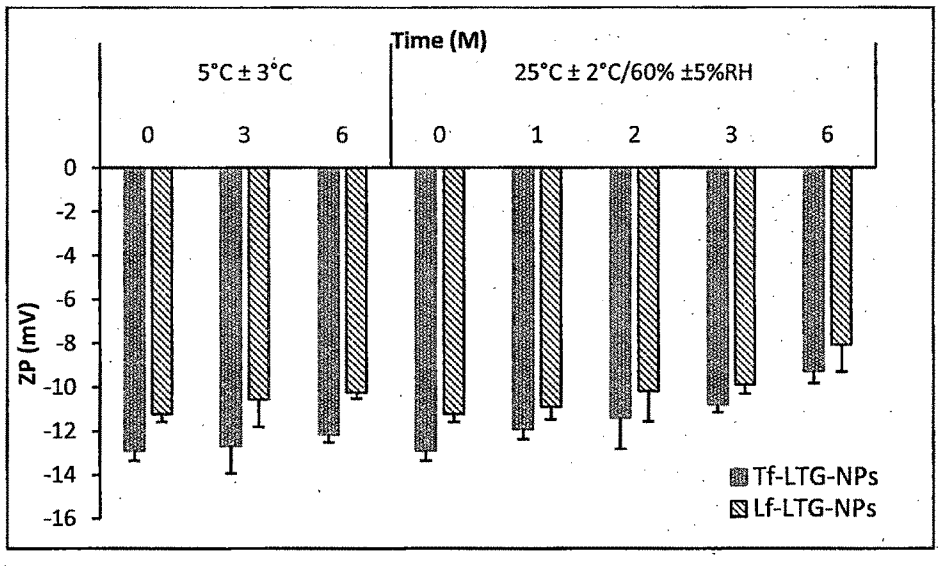


Figure 6.7 Comparative release profile of Tf-LTG-NPs after 6M at 5 ± 3°C

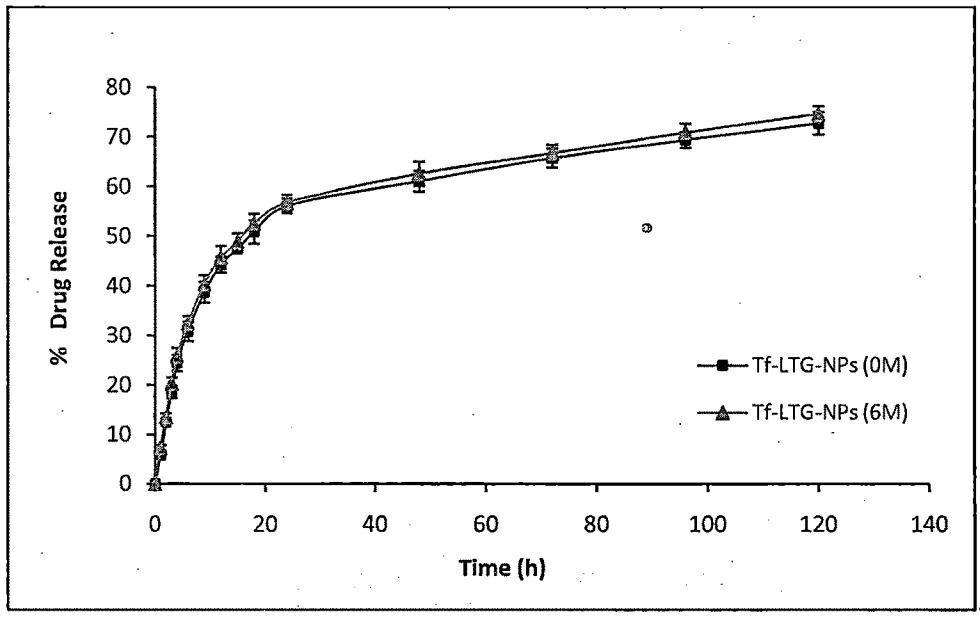
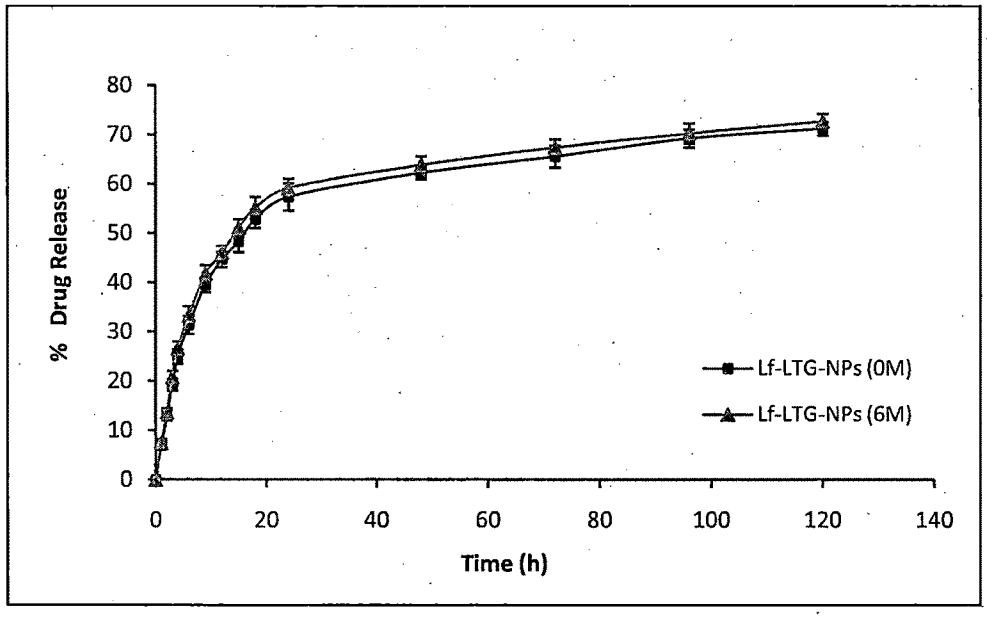


Figure 6.8 Comparative release profile of Lf-LTG-NPs after 6M at 5 ± 3°C



It was evident from the results that there was no significant change (P>0.05) observed in PS, ZP, drug content and redispersibility of protein conjugated NPs at 5 ± 3°C for 6M and 25 ± 2°C / 60 ± 5% RH for 3M.

At 25 ± 2°C / 60 ± 5% RH, the ZP of the NPs shifted towards the zero for conjugated NPs and after 6M increased significantaly towards 0 due to the degradation of PLGA. The lowered ZP values also might have contributed toward the aggregation of particles. In

addition, the NPs displayed poor redispersibility after 6 M. This may be due to the acidic conditions produced due to the degradation of PLGA into lactic and glycolic acid (Sahoo S et al., 2002; Betancourt T et al., 2007). Also, the Tf and Lf conjugated NPs demonstrated difference in the color than the initial powder after 6 months at 25 ± 2°C / 60 ± 5% RH. This could be indicative of the degradation of the surface Tf and Lf.

The drug content of the conjugated NPs was not altered up to 6M at 5 ± 3°C. However, the drug content was reduced after 6M storage at 25 ± 2°C / 60 ± 5% RH.

The release profile of the drug from the NPs was not affected upon storage at 5 ± 3°C. The similarity factor calculated between the initial and the 6M samples show values greater than 80, indicating high similarity between the initial and 6M release profile.

From the above study, we can demonstrate that the conjugated PLGA nanoparticles of TMD and LTG when stored at 25 ± 2°C / 60 ± 5% RH for 6M show instability reflected by change in physical appearance, increase in the PS, ZP and reduction in the drug content. Hence, we can conclusively specify that conjugated nanoparticles of TMD and LTG were stable and can be stored 5 ± 3°C for 6M retaining its original formulation characteristics.

6.3.2 Stability study of Microemulsion and Nanoemulsion

Table 6.5 Accelerated physical stability studies of TME, TNE, LME and LNE

Formulation		GS (nm)	ZP (mV)	%T
TME	Before ASS	16.69 ± 3.01	-8.97 ± 0.53	99.09 ± 0.42
	After Centrifugation	18.32 ± 2.84	-8.27 ± 0.45	99.12 ± 0.56
	After Freeze thaw cycle	17.76 ± 2.52	-7.64 ± 0.78	98.75 ± 0.37
	After Heating cooling cycle	17.54 ± 3.32	-8.58 ± 1.82	98.27 ± 0.46
TNE	Before ASS	136.2 ± 4.3	-17.28 ± 0.32	--
	After Centrifugation	142.4 ± 5.3	-16.76 ± 1.12	--
	After Heating cooling cycle	148.2 ± 7.1	-16.32 ± 0.58	--
LME	Before ASS	14.25 ± 2.73	-9.42 ± 0.82	99.74 ± 0.53
	After Centrifugation	17.59 ± 1.34	-8.86 ± 0.98	99.57 ± 0.45
	After Freeze thaw cycle	18.07 ± 2.16	-8.33 ± 1.27	98.86 ± 0.38
	After Heating cooling cycle	17.76 ± 1.45	-7.74 ± 0.66	98.12 ± 0.28
LNE	Before ASS	129.6 ± 4.1	-19.87 ± 0.88	--
	After Centrifugation	137.2 ± 5.6	-18.56 ± 1.23	--
	After Heating cooling cycle	131.9 ± 3.7	-19.43 ± 0.62	--

Values are represented as mean ± SD, n=3.

- **Precipitation of drug-** No precipitation of drug was observed in MEs and NEs during storage period.
- **Phase separation-** Phase separation was not observed during storage time period.
- **Centrifugation test-** The formulations of ME and NE were found to be stable and no phase separation was observed.

Table 6.6 Stability study of TME

Condition	Duration	Parameters evaluated				
		GS (nm)	ZP (mV)	%Assay	%T	pH
--	Initial	16.69 ± 3.21	-8.97 ± 1.53	98.67 ± 0.43	99.09 ± 0.42	6.2 ± 0.1
25 ± 2°C / 60 ± 5% RH	3 M	16.92 ± 2.15	-8.48 ± 0.88	98.32 ± 1.17	99.01 ± 0.34	6.2 ± 0.1
	6 M	17.45 ± 1.52	-8.12 ± 1.07	97.75 ± 1.70	98.72 ± 1.67	6.3 ± 0.1
40 ± 2°C / 75 ± 5% RH	1 M	17.32 ± 1.63	-8.36 ± 1.47	98.18 ± 1.42	98.52 ± 0.73	6.3 ± 0.0
	2 M	18.86 ± 2.21	-7.83 ± 0.73	97.63 ± 0.57	99.05 ± 1.56	6.1 ± 0.1
	3 M	19.57 ± 1.74	-7.28 ± 1.52	96.89 ± 1.09	98.33 ± 1.08	6.2 ± 0.1

Values are represented as mean ± SD, n=3.

Table 6.7 Stability study of LME

Condition	Duration	Parameters evaluated				
		GS (nm)	ZP (mV)	%Assay	%T	pH
--	Initial	14.25 ± 2.73	-9.42 ± 0.42	99.12 ± 0.52	99.74 ± 0.53	5.8 ± 0.1
25 ± 2°C / 60 ± 5% RH	3 M	14.89 ± 1.34	-9.27 ± 1.54	99.08 ± 1.23	99.57 ± 1.43	5.7 ± 0.1
	6 M	15.26 ± 1.73	-8.86 ± 0.68	98.58 ± 0.45	99.06 ± 1.64	5.7 ± 0.0
40 ± 2°C / 75 ± 5% RH	1 M	15.74 ± 1.65	-8.74 ± 0.72	98.75 ± 1.36	98.29 ± 1.44	5.6 ± 0.0
	2 M	16.48 ± 1.49	-8.42 ± 1.56	98.46 ± 0.76	98.78 ± 0.89	5.8 ± 0.1
	3 M	16.92 ± 2.13	-8.13 ± 0.63	98.24 ± 1.62	98.41 ± 1.27	5.7 ± 0.1

Values are represented as mean ± SD, n=3.

Figure 6.9 Comparison of GS at initial and different stability conditions of formulations (TME and LME)

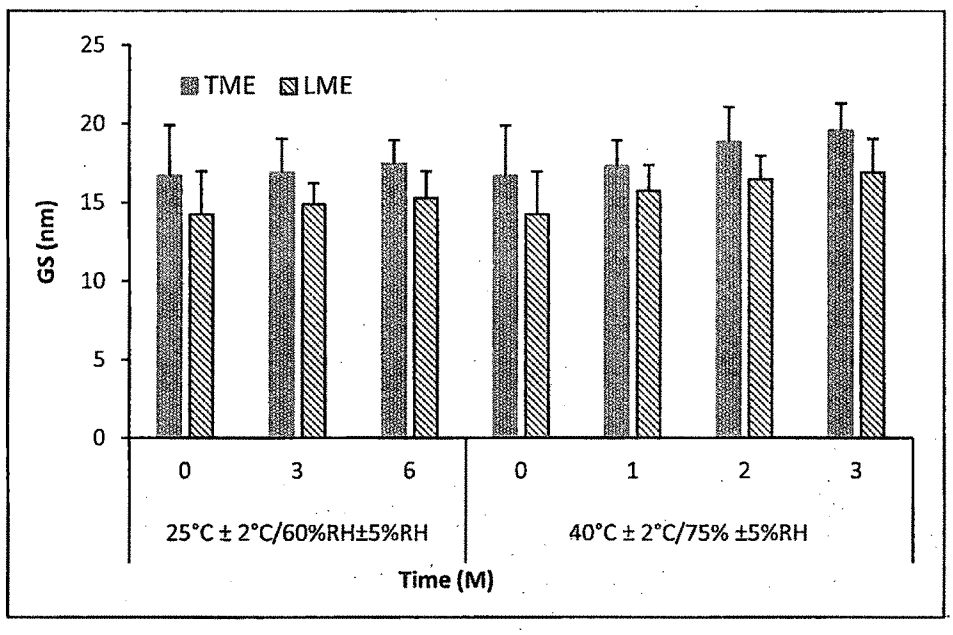


Figure 6.10 Comparison of ZP at initial and different stability conditions of formulations (TME and LME)

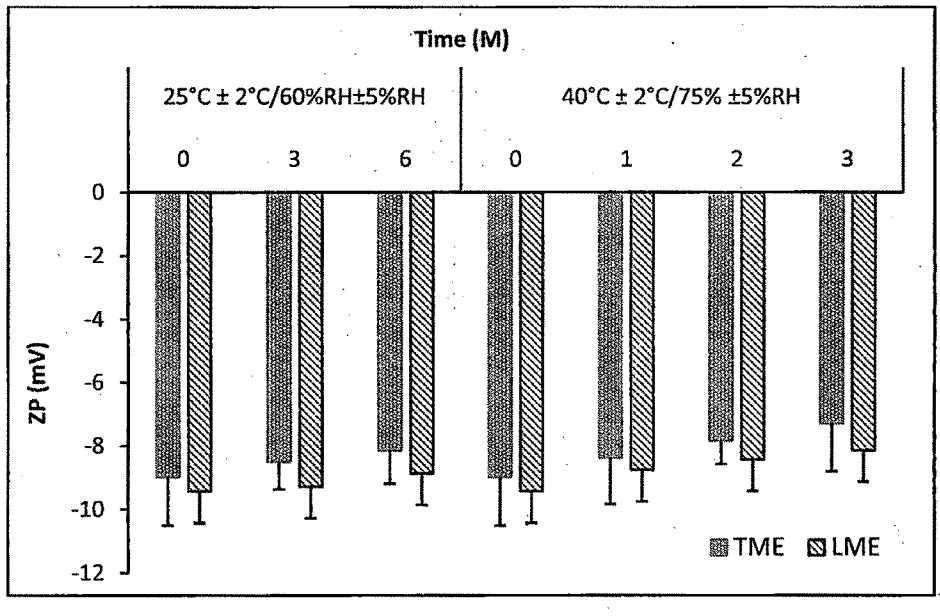


Table 6.8 Stability study of TNE

Condition	Duration	Parameters evaluated			
		GS (nm)	ZP (mV)	%Assay	pH
--	Initial	136.2 ± 4.3	-17.28 ± 0.32	99.54 ± 0.61	5.6 ± 0.1
5 ± 3°C	3 M	141.3 ± 5.1	-17.13 ± 0.47	99.36 ± 1.32	5.5 ± 0.0
	6 M	146.5 ± 4.7	-16.83 ± 0.36	99.13 ± 1.56	5.7 ± 0.1
25 ± 2°C / 60 ± 5% RH	1 M	145.7 ± 4.5	-16.93 ± 1.13	99.27 ± 1.28	5.6 ± 0.1
	2 M	150.6 ± 6.4	-16.48 ± 0.63	98.64 ± 0.74	5.4 ± 0.0
	3 M	157.4 ± 2.2	-15.79 ± 1.67	98.25 ± 0.88	5.5 ± 0.1

Values are represented as mean ± SD, n=3.

Table 6.9 Stability study of LNE

Condition	Duration	Parameters evaluated			
		GS (nm)	ZP (mV)	%Assay	pH
--	Initial	129.6 ± 4.1	-19.87 ± 0.88	98.83 ± 0.38	5.5 ± 0.0
5 ± 3°C	3 M	136.2 ± 5.2	-19.38 ± 1.25	98.56 ± 1.54	5.6 ± 0.0
	6 M	139.7 ± 4.3	-19.07 ± 0.43	98.26 ± 1.38	5.5 ± 0.1
25 ± 2°C / 60 ± 5% RH	1 M	137.6 ± 6.6	-19.29 ± 1.21	98.44 ± 1.84	5.4 ± 0.1
	2 M	141.3 ± 4.4	-18.75 ± 0.52	97.76 ± 2.03	5.6 ± 0.1
	3 M	148.5 ± 5.8	-18.34 ± 1.29	97.82 ± 1.57	5.3 ± 0.0

Values are represented as mean ± SD, n=3.

Figure 6.11 Comparison of GS at initial and different stability conditions of formulations (TNE and LNE)

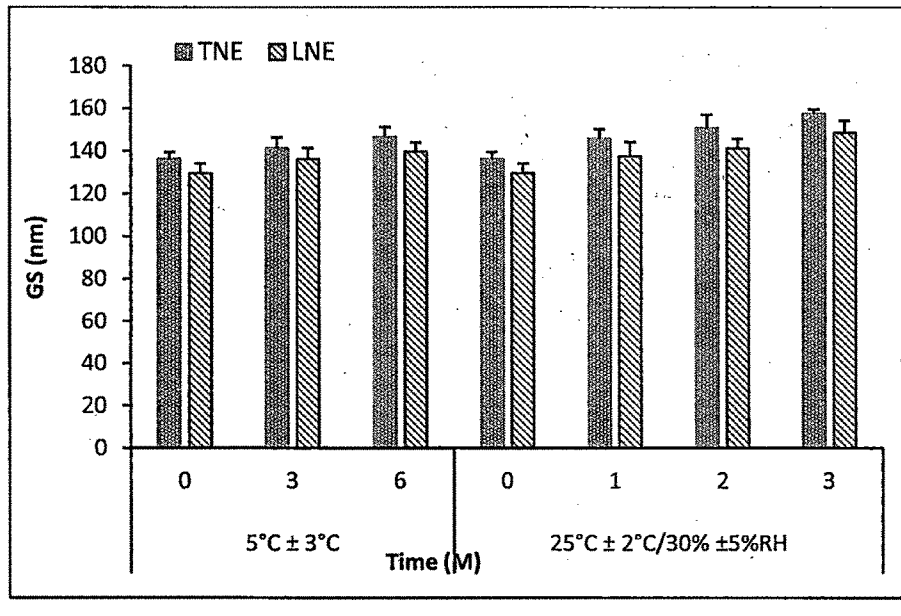
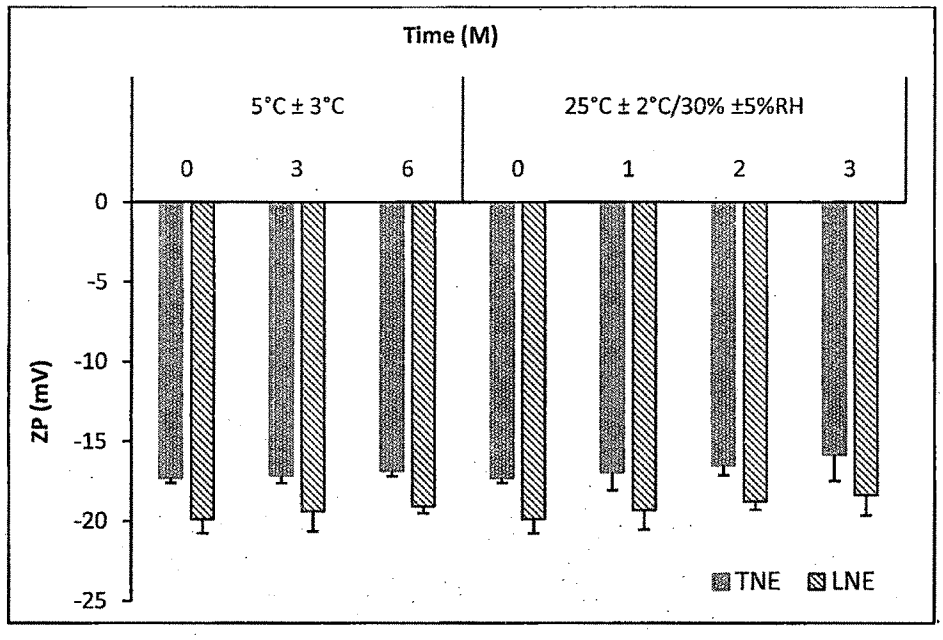


Figure 6.12 Comparison of ZP at initial and different stability conditions of formulations (TNE and LNE)



In long term stability study, the MEs and NEs containing TMD and LTG were packed in the borosil screw capped vials. MEs were kept at room temperature (25°C / 60% RH) and accelerated temperature (40°C / 75% RH) while NEs were kept at refrigeration temperature (5°C) and room temperature (25°C / 60% RH) conditions. During the storage period, ME and NE systems were assessed for their GS, ZP, assay, pH and %T (in case of ME) as shown in result. Over the time period there was an increment in GS and ZP and change in assay, %T, pH. However, the changes observed were non-significant when no visual indications of physical instability of MEs and NEs of TMD and LTG were seen. Irrespective of the storage conditions, the ME and NE system remained stable for 3 months duration at 40°C / 75% RH and 25°C / 60% RH respectively.

In order to assess the thermodynamic stability, the accelerated stability studies were done by subjecting the formulations for centrifugation, freeze-thaw cycle and heating cooling cycle. Before and after each treatment, GS, ZP and %T (in case of ME) of the formulations were determined and recorded (Table 6.5). The parameters after accelerated stability conditions were found to be nonsignificant which clearly indicates that the prepared MEs and NEs (TME, LME, TNE, and LNE) systems were thermodymically stable.

6.4 Conclusion

From the above study, we can conclude that the unconjugated and conjugated PLGA NPs of TMD and LTG when stored at 25 ± 2°C / 60 ± 5% RH for 6M show instability reflected by change in physical appearance, increase in the PS, ZP and reduction in the drug content. Hence, we can conclusively specify that both unconjugated and conjugated NPs of TMD and LTG were stable and can be stored 5 ± 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 NPs at 5 ± 3°C.

It was concluded from the results of the accelerated stability studies, done by subjecting the formulations for centrifugation, freeze-thaw cycle and heating cooling cycle that the prepared MEs and NEs (TME, LME, TNE, LNE) systems were thermodymically stable.

From the result of stability study, we can conclude that the MEs of TMD and LTG when stored at 40 ± 2°C / 75 ± 5% RH for 3M show instability reflected by changes, such, increase in the GS, ZP, reduction in the %assay and %T. Hence, we can conclusively specify that MEs of TMD and LTG were stable and can be stored 25 ± 2°C / 60 ± 5% RH for 6M retaining its original formulation characteristics. Similarly NEs of TMD and LTG when stored at 25 ± 2°C / 60 ± 5% RH for 3M show instability reflected by changes, such, increase in the GS, ZP, reduction in the %assay and %T. Hence, we can conclusively specify that NEs of TMD and LTG were stable and can be stored 5 ± 3°C for 6M retaining its original formulation characteristics.

6.5 References

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