8.1 Introduction

Stability is one of the critical aspects in ensuring safety and efficacy of drug products. The prepared formulation need to maintain its characteristics upon storage. The common stability issues associated with nanoparticles include particle agglomeration, PDI, particle surface charge and drug leaching, while for nanoemulsion additional problems associated includes creaming and sedimentation (1). In order to ensure the stability of prepared nanoparticles, they were stored at 25± 2ºC/ 60 ±5 % RH and 5± 3ºC for 6 months as per ICH guidelines. Various parameters that are likely to change upon storage including particle size, drug retained in the nanoparticles and zeta potential were estimated at different time intervals. For prepared nanoemulsion, the stability studies were conducted at 5± 3ºC for 6 months. Stability study was not carried at 25±2ºC/ 60±5 % RH since the nanoemulsion formulation was stable at this condition (25±2ºC/ 60±5 %) in 1 month as depicted from its short term stability results (section 4.4.3.2).

8.2 Methods

8.2.1 Optimized Darunavir loaded solid lipid nanoparticles and peptide grafted Darunavir loaded SLNs

Stability studies of optimized formulation of Darunavir loaded SLNs (Dar-SLN2) and peptide grafted Darunavir loaded SLNs (Pept-Dar-SLN) was carried out according to International Conference on Harmonization (ICH) Q1A (R2) Guidelines 2003 (2). Comparative stability studies were carried out at 25 ± 2ºC/ 60±5 % RH and at 5 ± 3ºC for 6 months. Freshly prepared lyophilized samples of Dar- SLN2 and Pept-Dar-SLN were filled into type-1 tubular glass vials and stored at above mentioned conditions. At each sampling time, different vial was used for the stability testing. The content of vials were tested for different parameters. Particle size, PDI and zeta potential were measured by Malvern Zetasizer as procedure mentioned in section 5.3.1. For performing assay of Darunavir, the reconstituted sample was diluted suitably and analyzed by developed UV method (chapter 3). The amount of drug present at 0 hr was taken as 100 % for the assay. % Drug retained was estimated after reconstitution as per procedure mentioned for entrapment efficiency in section 5.3.2. Residual water content was estimated using Karl-

Fischer titration method as mentioned in section 4.3.1.3. Assay of peptide and % peptide conjugated were determined after reconstitution by Lowry's assay method as described in section 5.3.3. For peptide assay determination, the amount of conjugated peptide before lyophilization at 0 hr was taken as 100 %.

8.2.2 Optimized Darunavir loaded Nanoemulsion (DNE-3)

Nanoemulsion containing Darunavir (DNE-3) was prepared as per procedure mentioned in section 4.3.3.2. Freshly prepared samples were filled into type-1 tubular glass vials and stored at 5±3ºC for 6 months. At each sampling time, different vial was used for the stability testing. The content of vials were tested for different parameters. Globule size, PDI, zeta potential, creaming volume and % drug retained (as per procedure for entrapement efficiency estimation) were determined as mentioned in section 4.3.3.3. Assay was estimated after suitable dilution and analysis by developed UV method (chapter 3). The amount of drug present at 0 hr was taken as 100 % for the assay.

8.2.3 Optimized Peptide grafted Atazanavir sulfate loaded SLNs (Pept-ATZ-SLN)

Freshly prepared lyophilized samples of Pept-ATZ-SLNs were filled into type-1 tubular glass vials and stored at 25 ± 2ºC/ 60±5 % RH and at 5 ± 3ºC for 6 months. At each sampling time, different vial was used for the stability testing. The content of vials were tested for different parameters. Particle size, PDI and zeta potential were measured by Malvern Zetasizer as procedure mentioned in section 5.3.1. For estimation of Atazanavir sulfate assay, the reconstituted sample was diluted suitably and analyzed by developed UV method (chapter 3). The amount of drug present at 0 hr was taken as 100 % for the assay. % Drug retained was estimated after reconstitution as per procedure mentioned for entrapment efficiency in section 5.6.2. Residual water content was estimated using Karl-Fischer titration method as mentioned in scetion 4.3.1.3. Assay of peptide and % peptide conjugated were determined after reconstitution by lowry's assay method as mentioned in section 5.6.3. For peptide assay determination, the amount of conjugated peptide before lyophilization at 0 hr was taken as 100 %.

8.2.4 Statistical Analysis

Statistical analysis of data was performed using Student-t test. GraphPad Prism (version 5, USA) was used for all analyses and P value < 0.05 was considered significant.

8.3 Results and discussion

8.3.1 Optimized Darunavir loaded solid lipid nanoparticles and peptide grafted Darunavir loaded SLNs

The stability testing of prepared Dar-SLN2 and Pept-Dar-SLN was performed at 25±2ºC / 60±5 % RH and 5 ± 3ºC for six months and the effect of various parameters was studied. As per the reported literatures, 25±2ºC / 60±5 % RH is considered to be an accelerated condition for stability testing (3, 4). The reason for this may be the low melting point of the lipids used for SLN preparation. The UV method developed was validated and found to be accurate, precise and sensitive and hence it was used for estimation of drug during stability studies. Results of stability studies are reported in Table 8.1 and Table 8.2. At both the storage conditions, Darunavir assay was found to be within the range (95-105 %) and the change was insignificant (P>0.05). For both formulations, there was insignificant change (P>0.05) in % Darunavir retained, particle size, zeta potential and water content after six months at both storage conditions. Peptide assay (for Pept-Dar-SLN) decreased significantly upon storage of Pept-Dar-SLN at 25ºC indicating its instability, while it remained stable at 5 ± 3ºC. Thus Darunavir loaded SLNs were stable at both conditions after six months while peptide grafted SLNs of Darunavir should be stored at 5 ± 3ºC.

Table 8.1 Stability data of Dar-SLN2

Sampling time (month)	Description/ visual appearance	Assay (%)	% drug retained	Water content (%)	Particle size (nm)	PDI	Zeta potential (mV)
Initial	White lyophilized cake	100	**% EE-** 91.27 ± 1.27	1.63 ± 0.11	188.43 ± 3.76	0.124 ± 0.015	- 50.23 ± 0.48
25±2ºC / 60±5 % RH							

1	White lyophilized cake	100.4 ± 1.1	91.13 ± 1.56	1.77 ± 0.15	190.3 ± 3.12	0.123 ± 0.021	-50.44 ± 0.71
2	White lyophilized cake	99.4 ± 0.9	90.58 ± 1.32	1.94 ± 0.17	192.06 ± 2.77	0.143 ± 0.021	-50.66 ± 1.11
3	White lyophilized cake	100.2 ± 1.0	90.35 ± 1.33	1.95 ± 0.18	194.03 ± 2.31	0.114 ± 0.014	-51.10 ± 1.25
6	White lyophilized cake	100.3 ± 2.1	89.36 ± 1.56	1.99 ± 0.15	195.06 ± 2.66	0.111 ± 0.012	-51.39 ± 0.76
5 ± 3ºC							
1	White lyophilized cake	100.12 ± 3.14	91.72 ± 1.24	1.71 ± 0.24	190.12 ± 2.81	0.111 ± 0.015	-50.23 ± 0.51
2	White lyophilized cake	100.67 ± 2.18	90.52 ± 1.12	1.71 ± 0.25	190.0 ± 2.40	0.216 ± 0.018	-51.16 ± 0.26
3	White lyophilized cake	100.43 ± 2.14	89.52 ± 1.48	1.76 ± 0.21	190.10 ± 2.5	0.146 ± 0.021	-51.10 ± 0.25
6	White lyophilized cake	100.06 ± 2.15	89.35 ± 2.3	1.82 ± 0.36	192.4 ± 3.16	0.231 ± 0.017	-50.88 ± 0.69

Table 8.2 Stability data of Pept-Dar-SLN

Sampling time (month)	**Description/ visual appearance**	**Assay (%)**	**% drug retained**	**Peptide assay (%)**	**% Peptide conjugated**	**Water content (%)**	**Particle size (nm)**	**PDI**	**Zeta potential (mV)**
Initial	White lyophilized cake	100	% EE-91.37 ± 2.14	100	74.25 ± 1.73	1.83 ± 0.16	197.36 ± 2.15	0.167 ± 0.017	-34.31 ± 0.67
25±2ºC / 60±5 % RH									
1	White lyophilized cake	100.82 ± 1.67	91.10 ± 1.24	98.23 ± 2.68	73.24 ± 2.13	1.92 ± 0.14	199.11 ± 1.18	0.116 ± 0.014	-35.11 ± 0.32

2	White lyophilized cake	100.43 ± 2.14	90.22 ± 1.45	97.14 ± 2.72	72.33 ± 1.48	1.96 ± 0.32	201.23 ± 3.14	0.122 ± 0.016	-35.21 ± 0.69
3	White lyophilized cake	100.14 ± 1.15	90.35 ± 1.85	95.64 ± 2.89	70.48 ± 2.63	1.94 ± 0.23	202.36 ± 2.36	0.132 ± 0.018	-34.99 ± 0.71
6	White lyophilized cake	100.58 ± 2.14	89.22 ± 1.31	93.14 ± 2.56	68.29 ± 2.35	1.92 ± 0.26	203.14 ± 3.14	0.110 ± 0.021	-34.23 ± 0.23
5 ± 3ºC									
1	White lyophilized cake	100.36 ± 1.68	91.34 ± 1.34	100.14 ± 0.82	74.11 ± 1.63	1.97 ± 0.24	200.43 ± 2.32	0.131 ± 0.053	-33.12 ± 0.66
2	White lyophilized cake	100.72 ± 1.23	90.21 ± 1.26	100.23 ± 0.96	73.65 ± 2.01	1.94 ± 0.31	200.63 ± 2.66	0.156 ± 0.066	-35.43 ± 0.53
3	White lyophilized cake	100.16 ± 0.65	90.62 ± 1.80	99.54 ± 0.43	73.12 ± 2.11	1.96 ± 0.20	201.36 ± 1.54	0.178 ± 0.016	-34.67 ± 0.37
6	White lyophilized cake	100.18 ± 1.54	90.11 ± 1.74	99.12 ± 0.67	72.37 ± 2.34	2.00 ± 0.21	203.44 ± 1.67	0.146 ±0.012	-34.53 ± 0.68

8.3.2 Optimized Darunavir loaded nanoemulsion (DNE-3)

The results of stability study carried out for optimized batch of Darunavir loaded nanoemulsion (DNE-3) was shown in Table 8.3. The formulation remained stable with no significant change in its evaluated parameters ($P>0.05$) for a period of 6 months. This indicated that the prepared nanoemulsion is stable at 5 ± 3ºC.

Table 8.3 Stability data of Darunavir loaded nanoemulsion (DNE-3) at 5 ± 3ºC

Sampling time (month)	Assay (%)	% drug retained	Globule size (nm)	PDI	Zeta potential	Creaming volume (%)
Initial	100	% EE-94.27 ± 1.22	210.3 ± 1.36	0.121 ± 0.011	-39.98 ± 1.26	98.52 ± 0.2

1	100.80 ± 1.32	94.36 ± 1.78	212.4 ± 1.74	0.141 ± 0.034	-40.23 ± 0.52	98.26 ± 0.3
2	100.24 ± 1.42	94.44 ± 1.78	213.7 ± 2.12	0.101 ± 0.026	-41.26 ± 1.1	98.46 ± 0.3
3	100.72 ± 1.25	93.78 ± 1.54	213.8 ± 2.64	0.163 ± 0.029	-41.17 ± 1.2	97.21 ± 0.4
6	100.26 ± 2.10	92.36 ± 1.33	214.2 ± 2.13	0.119 ± 0.042	-41.26 ± 1.6	96.34 ± 0.5

8.3.3 Optimized Peptide grafted Atazanavir sulfate loaded SLNs

Results of stability are reported in Table 8.4. At both storage conditions, ATZ assay was found to be within the range (95-105 %) and the change was insignificant (P>0.05). There was insignificant change (P>0.05) in % drug retained, particle size, zeta potential and water content after six months. Peptide assay and conjugated peptide decreased significantly upon storage at 25ºC indicating its instability, while it remained stable at 5 ± 3ºC. Thus, formulated nanoparticles should be stored at 5 ± 3ºC.

Table 8.4 Stability data of Pept-ATZ-SLN

Sampling time (month)	**Description/ visual appearance**	**Assay (%)**	**% ATZ retained**	**Peptide assay (%)**	**% Peptide conjugated**	**Water content (%)**	**Particle size (nm)**	**PDI**	**Zeta potential (mV)**
Initial	White lyophilized cake	100	% EE-94.59 ± 2.44	100	77.53 ± 2.03	1.32 ± 0.26	194.13 ± 2.65	0.122 ± 0.012	-33.45 ± 2.61
	25±2ºC / 60±5 % RH								
1	White lyophilized cake	101.78 ± 1.02	93.21 ± 1.34	98.74 ± 2.27	75.76 ± 2.44	1.69 ± 0.23	195.37 ± 2.47	0.138 ± 0.011	-35.86 ± 0.64
2	White lyophilized cake	100.27 ± 2.11	92.61 ± 2.23	98.48 ± 2.12	74.15 ± 2.14	1.93 ± 0.77	196.23 ± 2.58	0.120 ± 0.021	-35.56 ± 0.45
3	White lyophilized cake	100.55 ± 1.82	92.54 ± 2.87	95.90 ± 2.32	70.22 ± 2.35	1.96 ± 0.58	198.78 ± 2.45	0.116 ± 0.014	-35.23 ± 0.31

6	White lyophilized cake	100.26 ± 1.90	91.27 ± 2.31	94.28 ± 2.10	69.15 ± 2.41	2.12 ± 1.02	199.20 ± 2.35	0.115 ± 0.016	-36.32 ± 0.12
	5 ± 3ºC								
1	White lyophilized cake	100.26 ± 2.90	93.20 ± 2.10	100.12 ± 0.24	77.26 ± 1.25	1.43 ± 0.43	195.24 ± 1.58	0.111 ± 0.032	-34.14 ± 0.15
2	White lyophilized cake	100.35 ± 1.08	93.61 ± 1.67	100.32 ± 0.56	76.34 ± 2.23	1.60 ± 0.55	197.32 ± 1.67	0.116 ± 0.045	-34.63 ± 0.15
3	White lyophilized cake	100.75 ± 1.35	92.32 ± 2.67	99.78 ± 1.21	75.87 ± 2.41	1.65 ± 0.78	198.78 ± 1.15	0.126 ± 0.021	-35.23 ± 0.14
6	White lyophilized cake	100.62 ± 2.07	91.24 ± 2.43	99.55 ± 1.45	75.26 ± 2.32	1.76 ± 0.45	198.78 ± 1.25	0.116 ±0.010	-35.16 ± 0.32

8.4 References

1. Wu L, Zhang J, Watanabe W. Physical and chemical stability of drug nanoparticles. Advanced drug delivery reviews. 2011;63(6):456-69.
2. ICH Q1A(R2). Stability testing of new drug substances and products.: International conference on harmonization of technical requirements for registration of pharmaceuticals for Human use. 2003
3. Aji Alex MR, Chacko AJ, Jose S, Souto EB. Lopinavir loaded solid lipid nanoparticles (SLN) for intestinal lymphatic targeting. European journal of pharmaceutical sciences. 2011;42(1-2):11-8.
4. Mishra A, Vuddanda PR, Singh S. Intestinal Lymphatic Delivery of Praziquantel by Solid Lipid Nanoparticles: Formulation Design, In Vitro and In Vivo Studies. Journal of Nanotechnology. 2014;2014.