Chapter VI

Stability Studies

Stability of a pharmaceutical product may be defined as the capability of a particular formulation, in a specific container/ closure system to remain within its physical, chemical, micro-biological, therapeutic and toxicological specifications over a predetermined period of time (shelf life of the product).

The importance of stability testing in the development of pharmaceutical dosage forms is well recognized in the pharmaceutical industry. 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 different environmental factors such as temperature, humidity and light. In addition, product related factors influence the stability, e.g. the chemical and physical properties of the active substance and the pharmaceutical excipients, the dosage form and its composition, the manufacturing process, the nature of the container-closure system, and the properties of the packaging materials. Also, the stability of excipients that may contain or form reactive degradation products, have to be considered (Fitzpatrick et al., 2002; Goskonda et al., 1998). As a result of stability testing, a re-test period for the active substance or a shelf life for the pharmaceutical product can be established, and storage conditions can be recommended (Draft guidance, Stability Testing of New Drug Substances and Products, ICH Guidelines, 2003).

Stability studies of the formulations play a pivotal role in the overall efficiency or performance of the delivery system. The storage stability studies are of prime concern in the development of pharmaceutically acceptable products as

they are essential for three main reasons; safety of the patient, legal requirements concerned with the identity, strength, purity and quality of the drug, and to prevent the economic repercussions of marketing an unsuitable product (Vadas, 1995). Hence, the stability prediction and assurance of therapeutic efficacy of a dosage form are desired for moral, legal, competitive and public health reasons. Shelf-life of a formulation is the time from the date of manufacture until chemical or biological activity of formulation is decreased upto 90% of labeled potency with no appreciable or deleterious change in physical characteristics.

The applications of certain physicochemical principles in the performance of stability studies have proved to be of considerable advantage in the development of stable dosage forms. For this purpose, accelerated stability testing at high temperature and humidity conditions are often employed to predict the shelf life of drug.

Matthews performed stability studies of nanoparticulate formulations to assess long term stability of developed products (Matthews, 1999). Different lyophilized nanoparticulate formulations were separately taken in amber colored glass vials sealed with aluminum caps. The studies were performed at 40 ± 2°C at 75 ± 5% RH for 6 months. At the end of the storage period, the formulations were analyzed for physical appearance, size, shape, surface morphology, drug content and *in vitro* drug release studies.

In the present work, although the instability of the nanoparticles in the dispersion was overcome by lyophilisation using cryoprotectants, the influence of the storage conditions like temperature and humidity on particle size and drug content are also important in maintaining the integrity of these delivery systems before use for the biological studies.

6.1 METHODOLOGY

The stability studies were carried out in accordance with the ICH guidelines for new drug products. The protocol for stability study consisted of exposing the optimized nanoparticulate formulations stored in closed containers to 5°C ± 2°C and 25°C ± 2°C at 60 ± 5% RH for upto 6 months. Drug loaded optimized nanoparticulate formulations were placed in amber colored glass vials and lyophilized. After lyophilisation, the vials were closed with rubber closures, sealed with aluminium caps and stored at 5°C ± 2°C and room temperature (25°C ± 2°C) at 60 ± 5% RH for a period of six months. The samples were analyzed initially and periodically after 2, 4 and 6 months for change in appearance (if any), particle size, zeta potential and residual drug content upon reconstitution with PBS (pH 7.4). The data obtained were used for analysis of any physical or chemical degradation at storage condition and for determining the precautions required for storage of developed formulations.

6.1.1 EFFECT OF STORAGE ON APPEARANCE

The appearance of turbidity in reconstituted formulations is an important criterion to establish the stability of parenteral colloidal suspensions. It was checked by visual observation and change in appearance or development of turbidity, if any, was recorded in **Table 6.1 & 6.2.**

6.1.2 EFFECT OF STORAGE ON PARTICLE SIZE & PDI

Particle size and particle size distribution (PDI) are important parameters to assess stability of colloidal formulations. Increased particle size indicates particle aggregation and increased PDI shows greater uneven particle size distribution of formulation on storage. The particle size and PDI of nanopartic-

ulate formulations after storage for 6 months at 5°C ± 2°C and 25°C ± 2°C at 60 ± 5% RH and upon reconstitution with PBS (pH 7.4) was determined by photon correlation spectroscopy using Malvern Zetasizer 3000 HS_A (Malvern Instruments Ltd, Worcestershire, UK). The observations were recorded in **Table 6.3** and shown graphically in **Fig. 6.1 - 6.4.**

6.1.3 EFFECT OF STORAGE ON ZETA POTENTIAL

The zeta potential of reconstituted formulations was measured by Laser Doppler anemometry-based multiple angle particle electrophoresis analyzer, Malvern Zetasizer 3000 HS_A (DTS Ver. 4.10, Malvern Instruments Ltd, Worcestershire, UK). The observations were recorded in **Table 6.4** and shown graphically in **Fig. 6.5 - 6.6.**

6.1.4 EFFECT OF STORAGE ON PERCENT RESIDUAL DRUG CONTENT

The residual drug content of formulations was determined periodically every two months during storage for 6 months. The percent residual drug content was determined by analyzing the amount of drug in the nanocarriers using UV spectrophotometeric method reported earlier (**Section 5.4.5**). The change in drug content in the formulations was used to analyze the effect of accelerated conditions of storage on the formulations (**Table 6.5, Fig. 6.7 - 6.8**).

Table 6.1: Effect on appearance of optimized formulations of Pioglitazone and Rosiglitazone at 5°C ± 2°C during 6 months storage

Time interval	Change in appearance after reconstitution in PBS pH 7.4			
	PIO-NP	Tf-PIO-NP	ROS-NP	Tf-ROS-NP
Initial	No Change			
2 Months				
4 Months				
6 Months				

\- Indicates no turbidity

\+ Indicates slightly turbid

++ Indicates considerable turbid

Values are mean ± s. d. (n =3)

Table 6.2: Effect on appearance of optimized formulations of Pioglitazone and Rosiglitazone at 25°C ± 2°C at 60 ± 5% RH during 6 months storage

Time interval	Change in appearance after reconstitution in PBS pH 7.4			
	PIO-NP	Tf-PIO-NP	ROS-NP	Tf-ROS-NP
Initial	Clear	Clear	Clear	Clear
2 Months	-	+	-	+
4 Months	+	++	+	++
6 Months	+	++	++	++

\- Indicates no turbidity

\+ Indicates slightly turbid

++ Indicates considerable turbid

Values are mean ± s. d. (n =3)

Table 6.3: Effect on particle size and PDI of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage

Particle size(nm) and PDI of nanoparticulate formulations during 6 months storage at 5°C ± 2°C								
Time interval	PIO-NP		Tf-PIO-NP		ROS-NP		Tf-ROS-NP	
	Size	PDI	Size	PDI	Size	PDI	Size	PDI
Initial	215 ± 3	0.122	394 ± 8	0.409	229 ± 5	0.265	389 ± 8	0.351
2 Months	219 ± 4	0.134	391 ± 6	0.413	231 ± 3	0.273	392 ± 6	0.355
4 Months	218 ± 5	0.129	397 ± 9	0.410	233 ± 2	0.269	388 ± 5	0.361
6 Months	221 ± 3	0.142	398 ± 5	0.429	239 ± 8	0.272	393 ± 7	0.381
Particle size(nm) & PDI of nanoparticulate formulations during 6m storage at 25°C ± 2°C & 60 ± 5% RH								
Initial	214 ± 4	0.132	396 ± 6	0.391	228 ± 4	0.255	385 ± 6	0.346
2 Months	289 ± 2	0.338	491 ± 4	0.432	308 ± 8	0.410	491 ± 4	0.463
4 Months	426 ± 8	0.411	614 ± 4	0.496	448 ± 2	0.521	625 ± 8	0.531
6 Months	734 ± 6	0.492	889 ± 6	0.532	689 ± 6	0.595	939 ± 8	0.599

Table 6.4: Effect on zeta potential of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage

Time interval	Zeta potential of nanoparticulate formulations during 6 months storage at 5°C± 2°C (mV)			
	PIO-NP	Tf-PIO-NP	ROS-NP	Tf-ROS-NP
Initial	- 21.6 ± 1.9	-4.8 ± 1.1	-20.5 ± 1.3	-2.1 ± 0.6
2 Months	- 20.3 ± 1.4	-4.3 ± 1.2	-19.4 ± 1.4	-2.2 ± 1.0
4 Months	- 19.9 ± 1.6	-3.9 ± 1.2	-19.0 ± 1.2	-1.9 ± 0.8
6 Months	- 19.5 ± 1.7	-3.4 ± 1.6	-18.2 ± 1.6	-1.6 ± 0.4
Zeta potential of nanoparticulate formulations during 6 months storage at 25°C ± 2°C at 60 ± 5% RH (mV)				
Initial	- 21.3 ± 1.8	-4.9 ± 1.1	-20.9 ± 1.6	-2.4 ± 0.4
2 Months	- 19.2 ± 1.9	-3.8 ± 1.2	-17.6 ± 1.4	-1.9 ± 0.2
4 Months	- 16.6 ± 1.4	-2.6 ± 1.4	-14.2 ± 1.8	-1.6 ± 0.6
6 Months	- 14.0 ± 1.6	-2.8 ± 1.1	-12.5 ± 1.3	-1.2 ± 0.4

Table 6.5: Effect on drug content of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months of storage

Time interval	During 6 months storage at 5°C ± 3°C			
	PIO-NP	Tf-PIO-NP	ROS-NP	Tf-ROS-NP
Initial	100.0 ± 2.6 %	100.0 ± 2.0 %	100.0 ± 1.6 %	100.0 ± 2.2 %
2 Months	98.8 ± 2.2 %	99.3 ± 1.3 %	98.2 ± 1.4 %	99.2 ± 2.0 %
4 Months	98.0 ± 1.6 %	98.6 ± 1.8 %	97.4 ± 2.0 %	98.8 ± 1.6 %
6 Months	97.6 ± 2.4 %	98.1 ± 2.0 %	96.1 ± 1.2 %	97.8 ± 1.8 %
	During 6 months storage at 25°C ± 2°C at 60 ± 5% RH			
Initial	100.0 ± 2.4 %	100.0 ± 1.6 %	100.0 ± 2.0 %	100.0 ± 1.6 %
2 Months	97.2 ± 2.0 %	98.2 ± 2.0 %	97.9 ± 1.6 %	98.4 ± 1.4 %
4 Months	95.6 ± 1.4 %	96.7 ± 1.8 %	96.3 ± 1.2 %	96.5 ± 1.8 %
6 Months	93.5 ± 1.8 %	95.3 ± 1.2 %	94.4 ± 2.2 %	95.8 ± 2.6 %

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

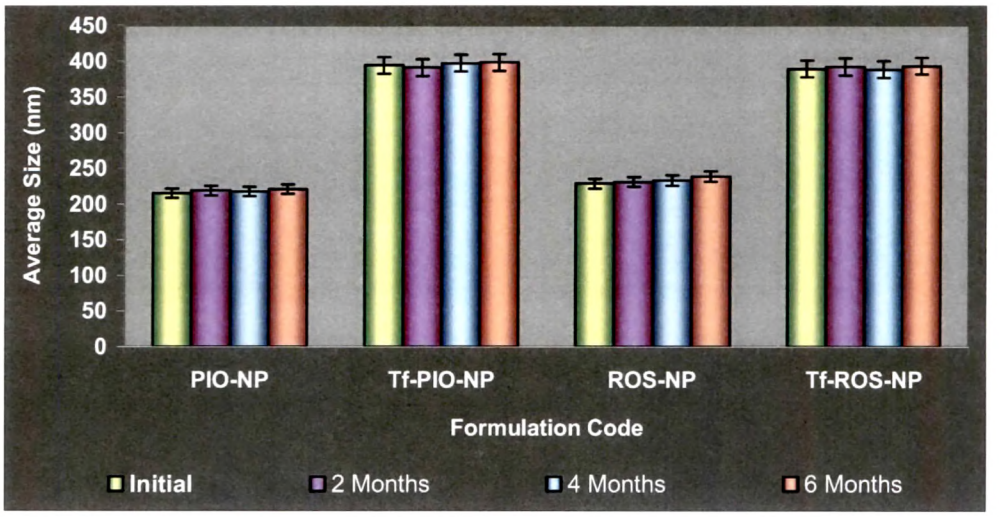

Figure 6.1: Effect on particle size of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 5°C ± 2°C

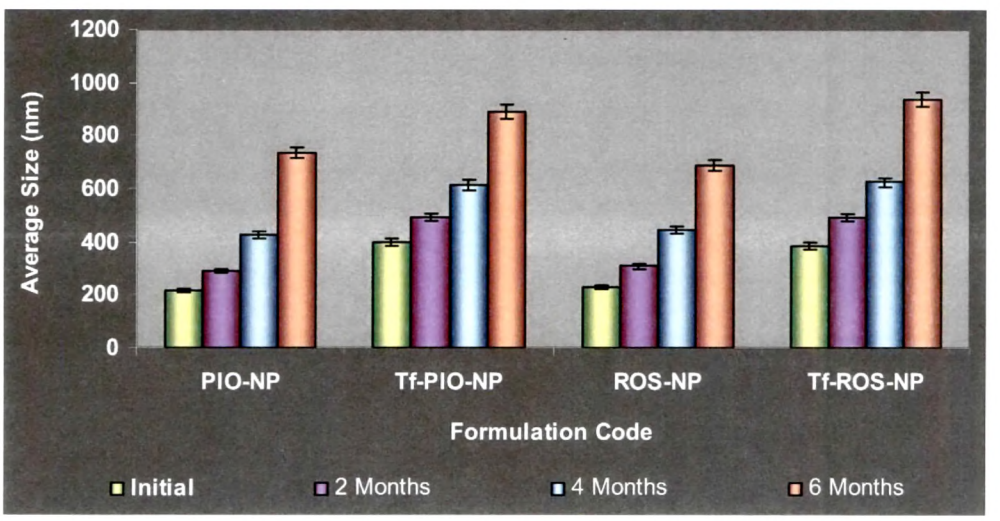

Figure 6.2: Effect on particle size of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 25°C ± 2°C & 60 ± 5% RH

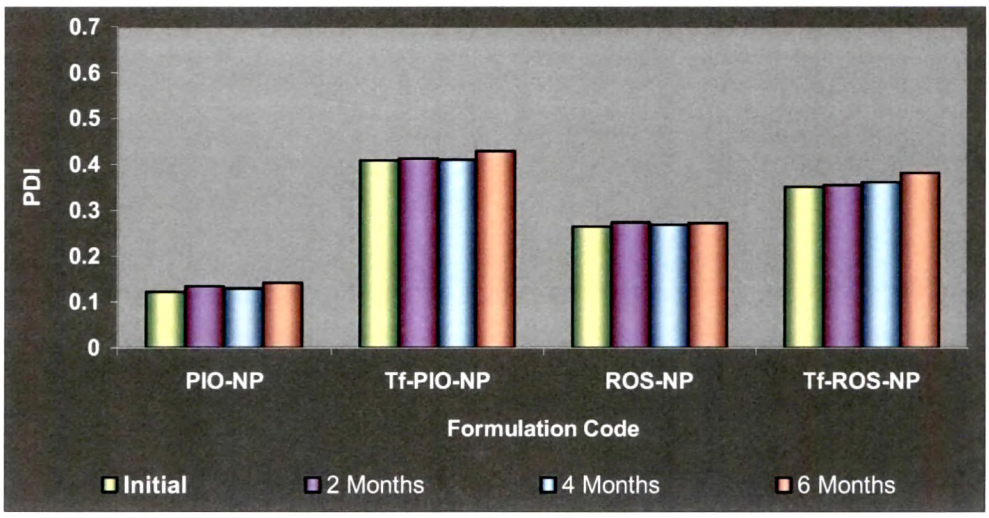

Figure 6.3: Effect on PDI of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 5°C ± 2°C

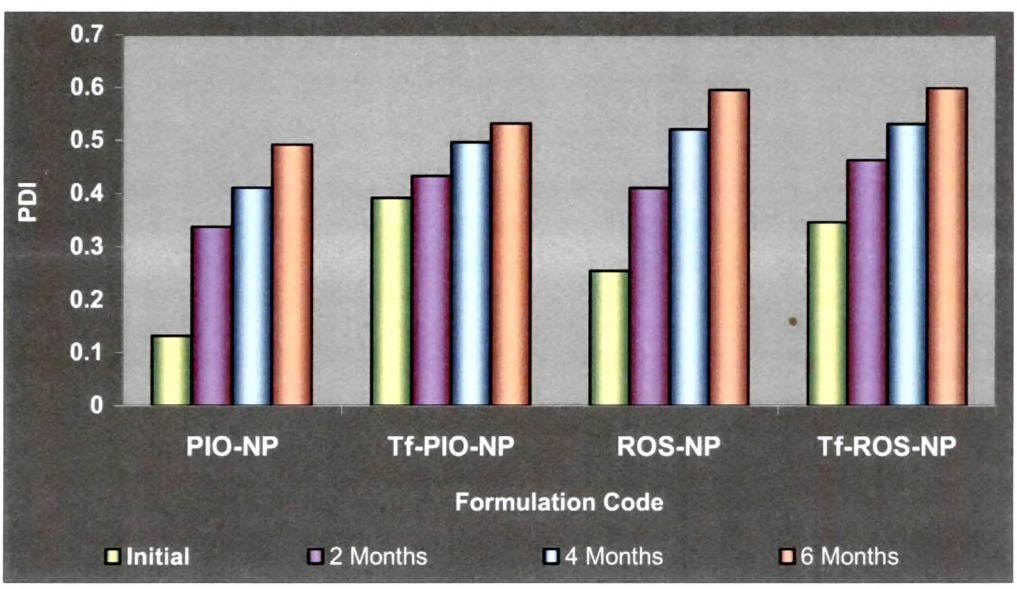

Figure 6.4: Effect on PDI of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 25°C ± 2°C & 60 ± 5% RH

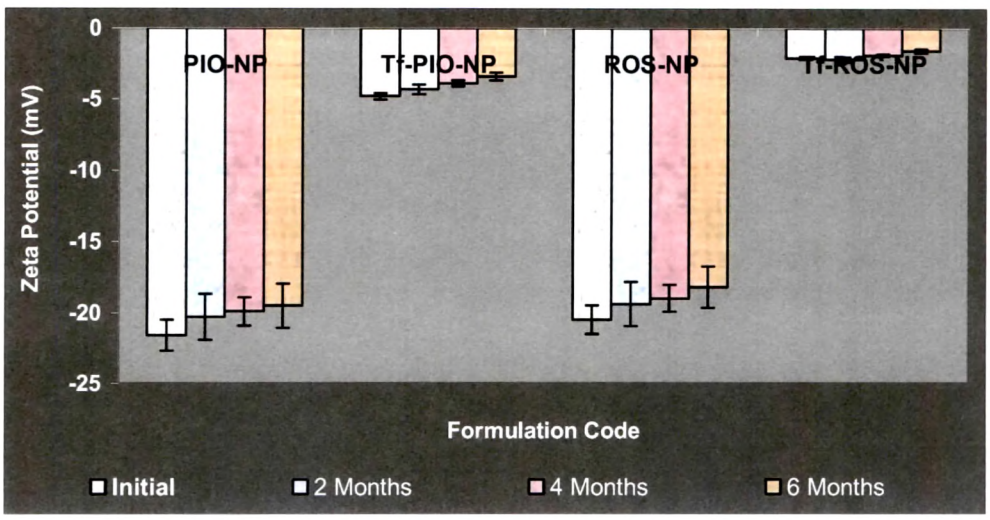

Figure 6.5: Effect on Zeta Potential of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 5°C ± 2°C

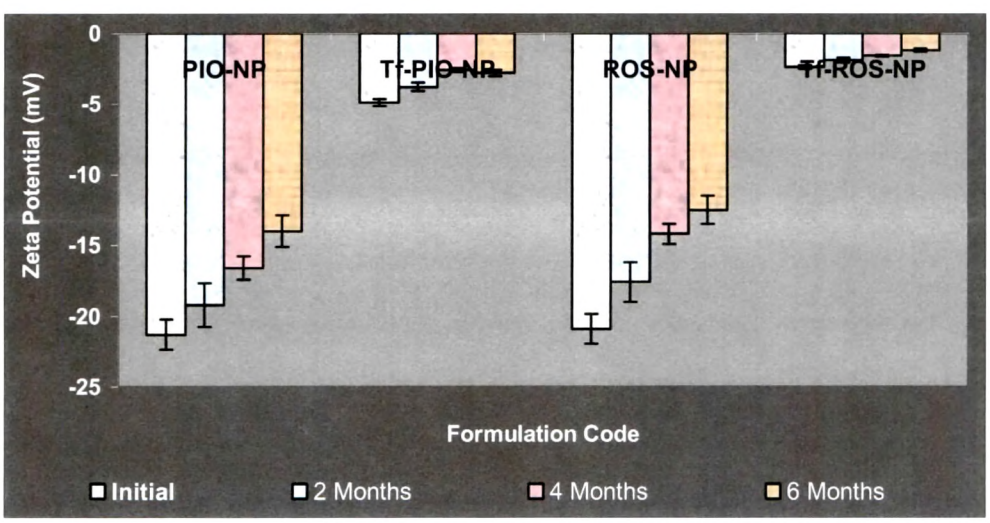

Figure 6.6: Effect on Zeta Potential of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 25°C ± 2°C & 60 ± 5% RH

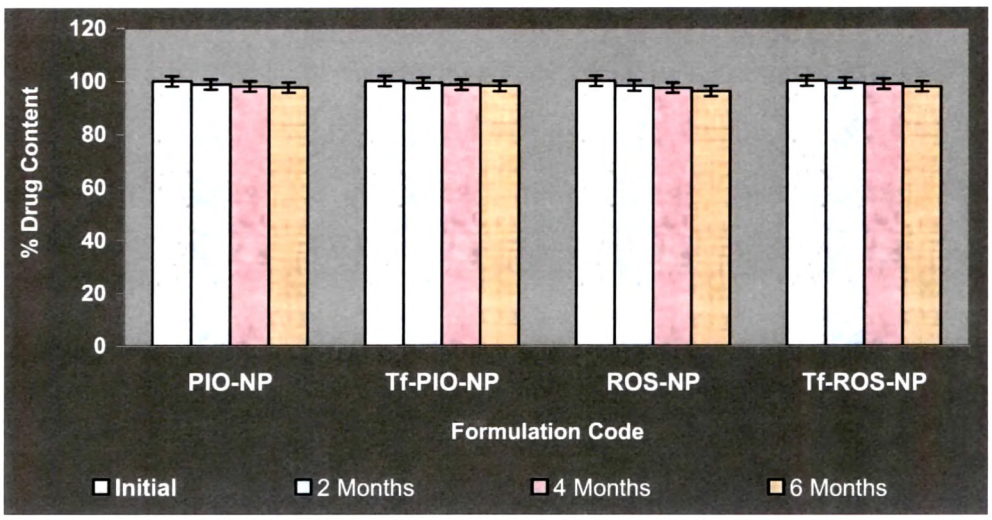

Figure 6.7: Effect on drug content of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 5°C ± 2°C

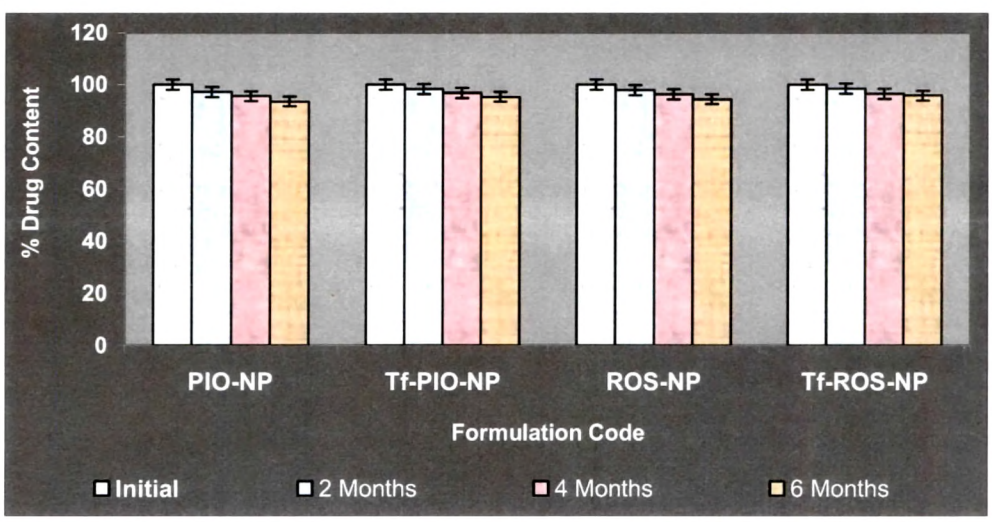

Figure 6.8: Effect on drug content of optimized nanoparticulate formulations of Pioglitazone and Rosiglitazone during 6 months storage at 25°C ± 2°C & 60 ± 5% RH

The nanoparticulate formulations stored at 25°C ± 2°C at 60 ± 5% RH showed smaller turbidity in case of unconjugated nanoparticulate formulations and considerable turbidity in case of conjugated nanoparticulate formulations after 4 months and further after 6 months of storage due to higher storage temperature and humidity (**Table 6.1** and **6.2**). The storage at 25°C ± 2°C at 60 ± 5% RH might have induced polymerization tendency of free carboxylic acid groups by degeneration of structure, which resulted in the appearance of precipitation and turbidity (Bhadra et al., 2005). Peptides and proteins have a tendency to coagulate at room temperature. Coagulation leads to aggregate formation of nanoparticles which causes turbidity in the colloidal suspension upon reconstitution.

6.2.2 EFFECT ON PARTICLE SIZE AND PDI

It was observed from the results that particle size and PDI were not substantially affected in formulations stored at 5°C ± 2°C and the increase in average particle size and PDI was not significant ($P>0.05$).

However, the average particle size and PDI were found to increase significantly when formulations were stored at 25°C ± 2°C at 60 ± 5% RH (**Table 6.3**). The increase in particle size was more prominent after 4 months of storage and still more prominent after 6 months storage as compared to storage after 2 months and this might be due to aggregation of nanoparticles. The nanoparticles might have aggregated due to absorption of moisture by nanoparticles resulting in coalescence of nanoparticles to form larger particles.

Upon storage of optimized nanoparticulate formulations at 25°C ± 2°C at 60 ± 5% RH, transferrin conjugated nanocarriers exhibited proportionately

less increase in PDI as compared to unconjugated nanocarriers which may have occured due to the presence of transferrin molecules on the surface of nanocarriers that may have caused homogeneous aggregation of particles due to coagulation of peptide and thereby leading to uniform increase in size and thus less increase in PDI. The increase in particle size may also be attributed to lowering of zeta potential contributing towards the aggregation of nanoparticles.

6.2.3 EFFECT ON ZETA POTENTIAL

It is evident from the results that after 6 months storage period, zeta potential was slightly affected in formulations stored at 5°C ± 2°C (**Table 6.4**).

When formulations were stored at 25°C ± 2°C at 60 ± 5% RH, the zeta potential of the nanoparticles shifted towards the zero value for both conjugated and unconjugated nanoparticles. This might be due to the acidic conditions produced by the degradation of PLGA into lactic and glycolic acid moieties (Sahoo et al. 2002). The reduction of zeta potential at higher temperatures may also possibly be due to aggregation of nanoparticles at higher temperature with a subsequent reduction in surface area and hence surface charge.

6.2.4 EFFECT ON PERCENT RESIDUAL DRUG CONTENT

The formulations were found to be stable during 6 months stability studies when stored at 5°C ± 2°C as the difference in drug content was statistically insignificant ($P>0.05$) in case of all the formulations as per ICH guidelines. However the drug content was reduced significantly and maximum decrease in percent residual drug content was found for formulations stored at 25°C ± 2°C for 6 months.

More than 95% drug was retained for 6 months in all the nanoparticles when stored at 5°C ± 2°C, while less than 95% drug was found to be retained in unconjugated nanoparticles after 6 months when stored at 25°C ± 2°C **(Table 6.5, Fig. 6.7-6.8)**. The drug content of unconjugated pioglitazone and rosiglitazone nanoparticles was found to be reducing upto 93% and 94%, respectively after 6 months storage at 25°C ± 2°C. This could be due to the moisture absorbed by the nanoparticles at higher humidity and temperature possibly resulting in instability and degradation of PLGA leading to generation of acidic moieties which degraded entrapped drug and hence the nanoparticles showed a lesser drug content after storage period. The percent residual drug content was found to be more in case of conjugated nanoparticles as compared to unconjugated nanoparticles which may be due to less degradation of acidic groups due to their absence in conjugated nanoparticles and hence, less degradation of drug.

6.3 CONCLUSION

From the above study, it may be concluded that the transferrin conjugated and unconjugated nanoparticles of pioglitazone and rosiglitazone when stored at 25°C ± 2°C at 60 ± 5% RH for 6 months showed instability as reflected by appearance of turbidity, increase in particle size and PDI, change in zeta potential and reduction in drug content upon reconstitution with PBS (pH 7.4). However, both transferrin conjugated and unconjugated nanoparticles of pioglitazone and rosiglitazone were found to be stable when stored at 5°C ± 2°C for 6 months and retaining their original formulation characteristics i.e. appearance, particle size, PDI, zeta potential and residual drug content.

Hence, after analyzing all the data of the stability studies, it can be concluded that transferrin conjugated and unconjugated formulations of

pioglitazone and rosiglitazone are stable at 5°C ± 2°C and such formulations should be stored at refrigerated conditions (5°C ± 2°C) for better efficacy. Hence, ***in vivo*** biodistribution and ***ex vivo*** studies should be conducted on them to check the efficacy of optimized formulations.

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