JOURNAL OF DRUG DELIVERY RESEARCH

ISSN 2319-1074

RESEARCH ARTICLE

THE DOSE REDUCTION OF ROSUVASTATIN IN COMPARISON TO REFERENCE PRODUCT (CRESTOR TABLETS) USING NANOPARTICULATE FORMULATION APPROACH

Deepak Gupta, Vinod Mandowara, Shetul Patel and Pragna Shelat

K. B. Institute of Pharmaceutical Education and Research, Gujarat, India.

Corresponding author： Deepak Gupta,A-1322, Rajiv Park, Adinath Nagar, Odhav, Ahmedabad-382415, Gujarat, IndiaPh. No.: +91 81282 81695

ABSTRACT

Rosuvastatin, a HMG-CoA reductase inhibitor widely used in treatment of Hyper(dys)lipidemia causes myotoxicity and hepatotoxicity. These safety issues limit dose of Rosuvastatin, lead to additional monitoring of the patients as well as discontinuation of therapy. To alleviate the adverse effects and to improve efficacy and safety profile, Rosuvastatin was encapsulated in nanoparticulate formulation and compared with marketed reference formulation (Crestor tablets). The nanoparticles (NPs) were prepared using single emulsion diffusion method and optimized for particle size, PDI, zeta potential, encapsulation efficiency. The efficacy and safety of final formulation was evaluated in HFD induced hyperlipidemic albino rats. The results suggested that the NPs have significant improvement of efficacy and reduction of the toxicity in comparison to marketed reference formulation. By encapsulating the Rosuvastatin in the NPs, the 50% dose reduction can be achieved without compromising efficacy.

Keywords: Nanoparticles, Rosuvastatin, Dose reduction, HFD model, Myopathy, HMG CoA

INTRODUCTION

Hyper(dys)lipidemia is a condition which includes a decreased concentration of high density lipoprotein (HDL) cholesterol as well as qualitative changes in low density lipoprotein (LDL), notably the presence of small, dense LDL particles. Both abnormalities, together with raised triglycerides, are features of the metabolic syndrome, increasingly recognized as a harbinger of coronary heart disease (CHD)[1].

Statins are the treatment of choice for the management of hyperlipidemia because of their well proven efficacy. Statins inhibits the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase resulting in reduced cholesterol synthesis. Because of the associated risk of cardiovascular disorders with elevated lipid profiles, the statin market is increasing globally. Rosuvastatin is widely used statin for the maintenance of the Hyperlipidemia[2].

ISSN 2319-1074

Rosuvastatin falls in class III (high solubility, poor permeability) under BCS. The absolute bioavailability of Rosuvastatin was estimated as 20%. The volume of distribution of Rosuvastatin is approximately 134 L. Approximately 90% of Rosuvastatin is bound to plasma proteins, mainly to albumin. Metabolism of Rosuvastatin seems to be a minor route of elimination in humans, and Rosuvastatin is mainly excreted into bile as parent. In a human mass balanced study, 90% of the dose was recovered in feces, primarily (92%) as unchanged drug[3].

The most common and severe side effects of Rosuvastatin are myotoxicity (rhabdomyolysis[4] and myopathy[5]) and hepatotoxicity[6]. The incidence of statin-induced rhabdomyolysis is so severe that it causes discontinuation or dose reduction of the drug which again exposes the patients to the cardiovascular events[7]. The literature data suggest that exposure of the statin to the extrahepatic tissues followed by the inhibition of HMG-CoA reductase leads to the adverse effects of the drug[8].

The adverse effects of Rosuvastatin can be reduced by decreasing its exposure to extrahepatic sites and by decreasing plasma drug concentration. This can be achieved by entrapping the Rosuvastatin in NPs. The NPs circulates for longer time in plasma with controlled release of the drug that will reduce the side effects of the drug as well as improve the efficacy.

The NPs were manufactured using single emulsion diffusion method. The NPs were optimized for the particle size, PDI, Zeta potential and entrapment efficiency. The final formulation of NPs in different doses was administered to the animals with elevated levels of Lipids and their effect was measured in comparison to the marketed reference formulation (Crestor tablets).

Materials and Methods

Materials

Rosuvastatin was obtained as a gift sample from Cadila Pharmaceuticals Limited. The other chemicals of the Laboratory grade were procured from the corresponding companies.

Analytical method

The UV spectroscopic method was used for analysis since its simple and widely used. As Rosuvastatin is poorly soluble in water, all the standard solutions were prepared in methanol. The scan of standard solution was taken from 190 to 370 nm wavelength at the scan speed 50 nm/s and the measurement of all standard solutions were taken at the absorbance maxima.

Drug-polymer Compatibility Studies

The drug-polymer compatibility study was performed to check the possible interaction between Rosuvastatin and polymer. The samples of drug, polymer and drug-polymer mixture (ration 1:1) were filled in separate vials. The samples were stored at 25ºC±2ºC/65%±5%RH and 40ºC±2ºC/75%±5%RH (test samples) and in refrigerator (control sample) for 28 days. The samples were withdrawn from the vials at the interval of 7, 14 and 28 days and were evaluated for the description and Assay.

ISSN 2319-1074

Preparation of NPs

Based on the initial trial and error experiments, the following method was used for the preparation of NPs.

Rosuvastatin and PLGA were dissolved in organic solvent to form the internal (organic) phase of emulsion. The surfactant was dissolved in water with stirring using magnetic stirrer to form the external (aqueous) phase of emulsion. The organic phase was transferred into the aqueous phase with stirring to make the emulsion. This emulsion was then homogenized using shaft homogenizer to convert into nanoemulsion. This nanoemulsion was added in water. The preparation was kept for stirring to allow sufficient time for solvent evaporation. This causes the solidification of the nanodroplets to form NPs suspended in aqueous phase.

To remove the excess surfactants from the surface of NPs, the washing of NPs was performed. The nanoparticle suspension was centrifuged and the supernant was discarded. The cake was resuspended in water. The same procedure was repeated one more time to get the cake which was finally resuspended in water.

The material as well as process related parameters were varied to check their effect on the particle size, PDI, zeta potential and entrapment efficiency. The following studies were performed as a part of optimization studies.

Screening of organic solvent/phase

Optimization of organic solvent/phase volume

Screening of different grades of PLGA

Screening and Optimization of surfactant

Optimization of drug loading

Optimization of Homogenization speed

Optimization of final dilution volume

Optimization of final mixing time

Freeze Drying Study

The freeze drying of NPs was performed to improve the stability of formulation. 2 ml of NPs suspension with different lyoprotectants (sucrose, dextrose, trehalose and mannitol with concentration 6 %w/v) was freeze dried. The reconstitution time of the freeze dried cake was checked to evaluate the suitability of freeze drying process. Further, the particle size, PDI and entrapment efficiency of the NPs were checked before and after freeze drying to evaluate the impact and suitability of freeze drying process[9].

In the next step, the concentration of Trehalose was varied from 4%-10% to optimize its concentration for freeze drying.

ISSN 2319-1074

In vitro Drug Release Study

The *in vitro* release of Rosuvastatin from the NPs was determined by dialysis membrane method[10]. NPs corresponding to 1 mg of drug entrapped were dispersed in 0.5 ml of phosphate buffer (pH 7.4, ionic strength 0.2) in dialysis bags (Sigma) with a molecular mass cut-off of 12000 Da. The bags were suspended in a vial containing 4.5 ml of phosphate buffer (pH 7.4, ionic strength 0.2) at 37±0.5ºC and kept in shaking water bath at 50 rpm for 7 days. The release medium was taken out and completely replaced with the fresh phosphate buffer medium at 0.5h, 1.0h, 1.5h, 2.0h, 2.5h, 3.0h, 3.5h, 4.0h, 5.0h, 6.0h, 8.0h, 10.0h, 12.0h, 18h, 24.0h, 48.0h, 72.0h, 96.0h,120.0h, 144.0h, 168h. The samples were stored in the refrigerator to prevent any possible drug degradation. The drug released at each time points were evaluated using the validated method.

Accelerated Stability Study[11]

The accelerated stability study of NPs was performed as per ICH guidelines for the drug products to be stored in refrigerator. The accelerated stability study was performed at 25±2ºC/60%±5%RH. The samples were kept within the vials with cap and without cap (as a control). The samples were withdrawn after 1, 2, 3 and 6 months and the different parameters like particle size, PDI and the entrapment of drug in NPs were measured to determine any change during the accelerated stability study.

pH Dependent Stability of Nanoparticles in Simulated Fluids[12]

The polymeric NPs are targeted to be administered through oral route of administration. After oral administration, the NPs will pass through different gastrointestinal regions with different pH and enzymatic conditions. This exposure can influence the physicochemical properties of NPs which is critical for the absorption. Further the different pH condition may also cause to the destruction of the NPs and the release of the drug from them which may result in decrease in drug entrapment and hence bioavailability. To test this hypothesis, Rosuvastatin loaded NPs were exposed to different pH media and the change in their physico-chemical properties was elucidated. pH dependent stability studies were carried out in test media with 0.1 N HCL (pH 1.2), pH 4.5 acetate buffer, pH 6.8 phosphate buffer and pH 7.4 phosphate buffer which simulates the different pH of GI tract. The detailed procedure is as stated below.

9 ml of test media was added to 1 ml of Polymeric NPs suspension. The samples in 0.1N HCl were investigated after 2 h and in pH 4.5 acetate buffer, pH 6.8 phosphate buffer and pH 7.4 phosphate were investigated after 6 h. The selection of time interval is based on expected formulation residence time in stomach and intestine. The Particle size, PDI and entrapment of the drug were determined on the preset time periods.

ISSN 2319-1074

In vivo Study

To evaluate the efficacy and safety of Rosuvastatin in NPs, the *in vivo* study was planned using high fat diet (HFD) model of albino rats[13]. The Male albino rats with average weight of 200g–400g were used for evaluation. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India were followed and prior permission was sought from the institutional animal ethics committee for conducting the study.

The detail of the study design is provided in **Table 1**.

Table 1 *In Vivo* Study Design

Treatment	**Diet**	**Dose**	**Blood Sampling point**	**Bio-chemical parameters**
Control group	NPD	-	Initial, 4, 6 and 8 weeks	**Efficacy parameters:** Plasma triglyceride, Plasma total cholesterol, LDL and HDL **Safety parameters:** SGOT, LDH and Glucose
No treatment	HFD	-		
Blank NP	HFD	a		
Crestor tablets	HFD	2 mg/kg/day		
Rosuvastatin NPS	HFD	2 mg/kg/day		
Rosuvastatin NPS	HFD	2 mg/kg/2day		

a: the dose of blank NPs administered was corresponded to the drug loaded NPs

The rats were divided into six groups of n = 6. One group received NPD and all other groups received HFD. Groups receiving HFD were again divided into no treatment, and treatment groups. The animals of treatment group were treated from 4 weeks-6 weeks with Rosuvastatin loaded NPs, Crestor tablets (reference product for Rosuvastatin) and blank nanoparticles. The dose of Crestor tablets (reference product for Rosuvastatin) was kept as 2mg/kg/day. The dose of the drug loaded NPs was same as the reference product in one group (2mg/kg/day) while half dose (2mg/kg/2day) to the reference product was administered in other group. Blood sampling was performed initially and after four, six and eight weeks. The efficacy and safety profile of Rosuvastatin in individual animals were evaluated using biochemical parameters.

Plasma total cholesterol levels (PTC), plasma triglyceride levels (PTG), high-density lipoprotein cholesterol (HDLC), plasma glucose levels (PGL), were estimated using commercially available diagnostic kits (Accurex kits). Plasma enzyme activities of lactate dehydrogenase (LDH) and aspartate transaminase (AST) were estimated using commercially available diagnostic kits (Pointe Scientific Inc., USA and Crest Biosystems, India, respectively). Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald formula.

ISSN 2319-1074

RESULTS AND DISCUSSION

Analytical Method Development and Validation

A new UV method was developed to quantitate Rosuvastatin in NPs. To check the specificity of method, the UV spectra of PLGA and Rosuvastatin were compared. No significant absorbance of PLGA was found at absorbance maxima of Rosuvastatin (244nm).

The absorbance of Rosuvastatin was measured using the standard concentrations from 0.25-20 µg/ml. The results showed a very good linearity of the curve at the concentration range of 1-15 µg/ml. The results are summarized in **Table 2.** The calibration curve with line equation and R^2 value for Rosuvastatin is provided in the Error! Reference source not found.**1**.

The method was further validated for interday and intraday precision and the validation results are provided in **Table 3.** The results confirm that UV method can be used for quantification of Rosuvastatin in PLGA NPs.

Table 2 Validation Parameters for Rosuvastatin Analysis using UV Method

Parameters	Values
Linearity (µg/ml)	1-15
Linearity (R^2)	0.9998±0.0001
Slope	0.0578±0.0019
Intercept	-0.0029±0.0036

Table 3 Intraday and Interday Variability of Rosuvastatin Analysis using UV Method

Conc (µg/ml)	Intraday[a]		Interday[b]	
	Accuracy	Precision	Accuracy	Precision
1	93.91	2.259	94.24	1.773
8	101.06	0.743	100.81	1.110
15	103.34	0.487	102.33	0.683

[a] For intraday variability triplicates were analyzed three times on a single day.
[b] For interday variability triplicates of the concentrations specified were analyzed on three consecutive days.
Precision and accuracy represented as % RSD and % recovered respectively.

ISSN 2319-1074

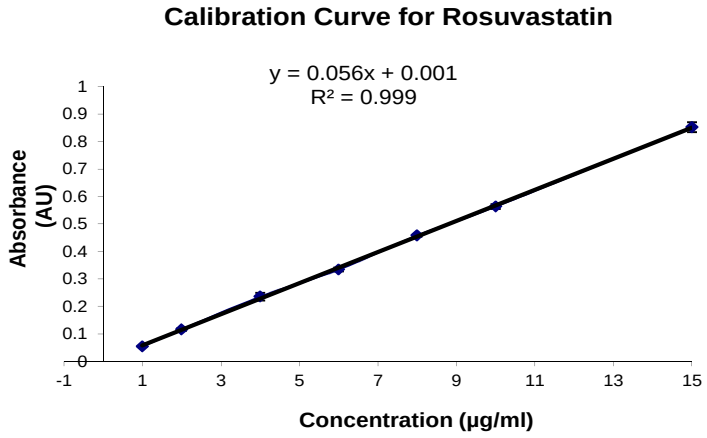

Fig. 1 Calibration curve for Rosuvastatin

Drug-polymer compatibility studies

The Results of drug-polymer compatibility study at different conditions are shown in **Table 4.**

The results show that Rosuvastatin is having good stability. No change in description and additional loss of the potency of Rosuvastatin was observed when combined with excipient. Further, the loss in potency of Rosuvastatin was more at high temperature and humidity. However, these values were not significant indicating the minimal effect of temperature and humidity on degradation of Rosuvastatin. The results confirm the compatibility of Rosuvastatin with polymer.

Table 4 Drug-polymer Compatibility Study

Condition	Sample	Description			Assay			
		7d	14d	28d	0d	7d	14d	28d
40±2ºC/75%±5%RH	PLGA	*	*	*	-	-	-	-
	Rosuvastatin	*	*	*	100.1%	99.8	99.9	99.4
	PLGA+Rosuvastatin	*	*	*	100.1%	99.6	99.8	99.4
25±2ºC/65%±5%RH	PLGA	*	*	*	-	-	-	-
	Rosuvastatin	*	*	*	100.1%	100.3	99.6	99.8
	PLGA+Rosuvastatin	*	*	*	100.1%	100.0	99.7	99.6
Refrigerator	PLGA	*	*	*	-	-	-	-
	Rosuvastatin	*	*	*	100.1%	100.2	100.2	99.9
	PLGA+Rosuvastatin	*	*	*	100.1%	99.9	99.8	99.9

* white powder

ISSN 2319-1074

Optimization of Formulation and Process Parameters

Effect of Organic Solvent/Phase

The organic solvent is used as internal phase to solubilize drug and polymer. The change in organic solvent might lead to change in solubility of drug, solubility of polymer and osmotic pressure of the internal phase. This can cause change in the particle size as well as entrapment efficiency. The results of organic solvent screening study are summarized in **Table 5.**

The smaller particles and less entrapment of the Rosuvastatin were observed with ethyl acetate in comparison to Acetone. Considering the critical role of particle size in effect of NPs and smaller dose of Rosuvastatin, it was decided to compromise the entrapment of the drug. Hence, ethyl acetate was finalized for further experimentation.

The difference in particle size and entrapment of the NPs due to change in solvent can be correlated with the diffusion of the organic solvent into external phase. It was found from the literature that the diffusion of the acetone (diffusion rate= $1.14e^{-005}$) will be more than ethyl acetate (diffusion rate= $9.7e^{-006}$) in water. This leads to faster solidification of the NPs with acetone. Since the particle breakage in the liquid state can be much more in comparison to the solid state, smaller particle size was observed with ethyl acetate in comparison to acetone. Further the faster diffusion of acetone leads to very short time for the partition of the drug in external phase leads to high entrapment of Rosuvastatin with acetone.

Table 5 Effect of Organic Solvent/Phase on Characteristics of NPs

Solvent	Particle Size (nm)	PDI	Zeta Potential	Entrapment Efficiency (%)
Ethyl acetate	342.54±2.06	0.204±0.031	14.57±0.85	35.07±0.90
Acetone	506.64±3.61	0.229±0.082	13.44±1.05	48.39±0.32

Data shown as mean ± SD (n =3)

Effect of Organic Solvent/Phase Volume

The results of organic solvent volume optimization study are summarized in **Table 6.** Considering the ration of O:W allowed for the preparation of stable o/w type of emulsion, the maximum 4 ml of the internal phase was evaluated (80% of O:W amount).

The entrapment of Rosuvastatin and particle size of NPs was decreased with increase in internal phase volume with no linear correlation observed. There was no significant difference in zeta potential and PDI with different amount of internal phase. The 2 ml volume of organic phase was finalized for further experimentation.

ISSN 2319-1074

The difference in the entrapment and particle size can be correlated with the difference in the concentration of the solid phase with change in the organic phase volume. The solid phase concentration is much higher (high solid and less solvent) with less amount of organic phase. Due to less amount of organic phase per nanodroplet, the amount of organic solvent diffused to the external phase will be less and solidification will be much faster. This will lead to high particle size and entrapment of the drug.

Table 6 Effect of Internal Phase Volume on Characteristics of NPs

Internal Phase Volume	Particle Size (nm)	PDI	Zeta Potential	Entrapment Efficiency (%)
2 ml	342.54±2.06	0.204±0.031	14.57±0.85	35.07±0.90
3 ml	298.42±4.21	0.176±0.07	15.67±1.57	28±1.4
4 ml	271.88±10.59	0.186±0.05	14.82±0.90	25±1.0

Data shown as mean ± SD (n =3)

Effect of Grades of PLGA

The NPs were prepared using the 3 grades of PLGA polymer with L:G ratio i.e. 70:30, 50:50 and 30:70 and the results are summarized in **Table 7.**

The entrapment of Rosuvastatin and particle size of NPs was increased with increase in the L:G ratio in PLGA. There was no significant change in the zeta potential and PDI. To balance the particle size as well as the entrapment of Rosuvastatin, PLGA 50:50 was finalized for further study.

PLGA is a copolymer of lactic acid and glycolic acid. PLGA with high L:G ratio is more hydrophobic since lactic acid is more hydrophobic in comparison to Glycolic acid. This leads to high adhesive force within the polymer structure and affinity of the polymer to the organic solvent. Due to high adhesive force, the particle size reduction will be difficult. The organic solvent diffusion will also decrease due to increase in solvent polymer interaction. This leads to increase in particle size of NPs. Reduction in particle breakage and solvent diffusion will decrease drug diffusion from NPs.

Table 7 Effect of Polymer Grade on Characteristics of NPs

Polymer	L:G Ratio*	Particle Size (nm)	PDI	Zeta Potential	Entrapment Efficiency (%)
PLGA	70:30	333.33±3.345	0.176±9.387	18.633±4.869	38.810±1.190
	50:50	286.33±2.245	0.137±8.791	19.266±4.794	34.048±0.8123
	30:70	265.33±3.868	0.127±8.615	21.166±6.916	28.333±1.820

Data shown as mean ± SD (n =3)

ISSN 2319-1074

Effect of Nature of Surfactants and Concentration

To evaluate the effect of nature and concentration of Surfactant on NPs, 3 surfactants i.e. Cremophor RH 40, PVA and poloxamers were used at the different concentration of 1%, 2% and 3%. The results of the study are summarized in **Table 8**.

Table 8 Effect of Surfactant on Characteristics of NPs

Surfactant	Conc. (%)	Particle Size (nm)	PDI	Zeta Potential	Entrapment Efficiency (%)
Cremophor RH 40	1	632.25±4.62	0.321±0.050	-10.37±0.84	47.07±0.90
	2	604.89±2.12	0.261±0.048	-12.51±0.54	43.39±0.49
	3	555.64±3.21	0.312±0.075	-12.17±0.75	39.89±0.13
PVA	1	468.42±3.64	0.215±0.028	10.54±1.12	35.89±1.56
	2	342.54±2.06	0.204±0.031	14.57±0.85	35.07±0.90
	3	284.25±2.58	0.147±0.024	20.87±0.90	34.05±0.73
Poloxamer	1	520.67±5.13	0.288±0.02	6.50±0.20	39.65±1.41
	2	494.67±6.03	0.282±0.01	8.23±0.97	42.64±0.86
	3	560.67±5.13	0.229±0.02	9.48±0.60	42.10±0.58

Data shown as mean ± SD (n =3).

The particle size with PVA was significantly less in comparison to Cremophor and poloxamers. The entrapment of drug was high with Cremophor in comparison to PVA and poloxamer. There was no significant change in the entrapment between PVA and Poloxamer. The zeta potential of NPs was significantly different with the different surfactant. However, the value was near to zero for poloxamer stating the possibility of less stability of NPs. Considering the high zeta potential which can lead to high stability, lower particle size and good entrapment efficiency, PVA 3% was selected as a surfactant for further studies. The effect of the surfactant on the PDI was negligible and can not be correlated with the type and concentration of the surfactant.

The reason for the fine particle size with PVA may be due to great reduction of surface tension between organic and aqueous phase by PVA in comparison to other 2 surfactants. Due to high surface tension, the agglomeration between the particles can be reduced which contributes to the decrease in particle size of NPs. This effect is increased with increase in the concentration of the surfactant. Further, the increase in breakage of NPs causes the liberation of the drug and faster solvent diffusion. This leads to reduced entrapment of the drug.

There was significant difference in the zeta potential was observed with each of the surfactant. The zeta potential was observed in the negative range for the Cremophor while in the positive range for the PVA and poloxamers. With the increase in the concentration of the surfactant, the zeta potential moved far from the zero value. This may be due to adsorption of the surfactant on the surface of NPs which attracts more opposite charged ion. This forms a tightly bound layer which is responsible for the generation of zeta potential. A more concentration of the surfactants causes more adsorption on NPs and more tight formation of the surface bound layer.

ISSN 2319-1074

Effect of Drug Loading

To evaluate the effect of drug loading, the concentration of the Rosuvastatin was varied from 5-15% in internal phase. The results of drug loading study are summarized in **Table 9**.

Table 9 Effect of Drug Loading on Characteristics of NPs

Drug Loading	Particle Size (nm)	PDI	Zeta Potential	Entrapment Efficiency (%)
5 %	286.33±2.245	0.137±8.791	19.266±4.794	34.048±0.8123*
10 %	283.33±5.738	0.168±6.628	15.93±4.083	31.095±0.733**
15 %	322.00±5.084	0.208±7.259	15.13±12.699	29.857±0.5150***

Data shown as mean ± SD (n =3)

The entrapment of Rosuvastatin was highest with 5 % drug loading which then decreased with increase in drug loading with no linear correlation. The particle size was increased with increase in the drug loading. There was no significant change in zeta potential and PDI with varied drug loading. Considering the very high amount of the drug entrapment with 15% drug loading even though the % entrapment was less, 15% drug loading was selected for further experimentation.

There are several explanations for the loss of drugs which were not entrapped within NPs. Differences in the entrapment efficiencies was observed with different drug loadings may be because the differences in osmotic pressures between internal and external aqueous phases. The osmotic pressure of the internal aqueous phase was higher in case of high drug loading, leading to rupture of the lipophilic droplets, and an exchange between internal and external aqueous phases with consequent loss of drugs. Due to high volume of the polymer solution, it is also possible that the polymer layer was not precipitated quickly and a loss of drug occurs with solvent. .

Effect of Homogenization Speed

The NPs were prepared using 3 different homogenization speed i.e. 8000 rpm, 10000 rpm and 12000 rpm. The results of homogenization speed study are summarized in **Table 10**.

Table 10 Effect of Homogenization Speed on Characteristics of NPs

Homogenisation Speed	Particle Size (nm)	PDI	Entrapment Efficiency (%)
8000	373.667±5.716	0.238±8.030	30.333±0.459
10000	322.00±5.084	0.208±7.259	29.857±0.5150
12000	293.333±3.031	0.1667±7.233	24.190±0.929

Data shown as mean ± SD (n =3)

The entrapment of the Rosuvastatin as well as the particle size was decreased with increase in homogenisation speed. There was no significant effect on the PDI.

ISSN 2319-1074

The decrease in entrapment of drug with increase in homogenization speed may be due to increase of surface area of the particles. More the surface of the particles exposed to the medium, more the drug will be released from it.

Effect of Dilution Volume

The NPs were prepared by varying the external phase volume i.e. 10 ml, 20 ml and 30 ml. The results of dilution volume study are summarized in **Table 11**.

Table 11 Effect of Dilution Volume on Characteristics of NPs

External phase Volume	Particle Size (nm)	PDI	Entrapment Efficiency (%)
10 ml	401.333±3.491	0.246±15.024	27.619±1.163
20 ml	384.000±4.864	0.247±9.043	29.095±0.786
30 ml	322.000±5.084	0.208±7.259	29.857±0.515

Data shown as mean ± SD (n =3)

The particle size of NPs was reduced with increase in the external phase volume. Further, there was no significant effect on the particle size as well as entrapment efficiency.

Since the manufacturing process involves the diffusion of the organic solvent in external phase, the volume of external phase is critical since it will affect concentration gradient of the organic solvent during diffusion process. The high amount of external phase will lead to high concentration gradient and hence more amount of the diffusion of organic solvent. This will reduce the amount of organic solvent in nanoparticles and hence much lower particle size.

Effect of Final Mixing Time

The NPs were prepared by varying the final mixing time of NPs suspension i.e. 4h, 8h and 12h. The results of study are summarized in **Table 12**.

Table 12 Effect of Mixing Time on Characteristics of NPs

Mixing Time	Particle Size (nm)	PDI	Entrapment Efficiency (%)
4 hrs	375±12.533	0.205±5.404	29.714±1.755
8 hrs	322±5.084	0.208±7.259	29.857±0.515
12 hrs	**289.667±8.323**	**0.186±5.456**	**28.476±0.861**

Data shown as mean ± SD (n =3)

The particle size of the NPs was reduced with increase in the final mixing time. Further, there was no significant effect on the particle size as well as entrapment efficiency.

The increase in the final mixing time will allow more time for the diffusion of organic solvent from NPs. This will lead to reduction in particle size.

ISSN 2319-1074

Freeze Drying Study

To improve the stability of NPs, the freeze drying was performed. The literature reports suggest that the various sugar molecules will act as cryoprotectants and lyoprotectants in freeze drying process. The four different sugars i.e. Dextrose, Sucrose, Trehalose and Mannitol were selected based on the literature data and screened at the concentration of 6 %w/v. The results of screening of different lyoprotectants are provided in **Table 13** and **Figure 2.**

Table 13 Effect of Different Sugars (6% w/v) on Characteristics of NPs during Freeze Drying Study

Name of Lyoprotectant	Reconstitution Time	Particle Size (nm)	PDI	Entrapment Efficiency (%)*
Initial	-	317.00±1.446	0.195±10.010	30.000±0.756
Trehalose	2 min	334.00±8.43	0.185±4.58	28.095±1.072
Mannitol	4 min	419.67±7.43	0.229±7.19	23.857±0.756
Sucrose	2 min 30 sec	387.33±5.94	0.299±16.97	24.333±1.551
Dextrose	2 min	366.33±4.57	0.236±6.35	26.667±0.577

Data shown as mean ± SD (n =3)

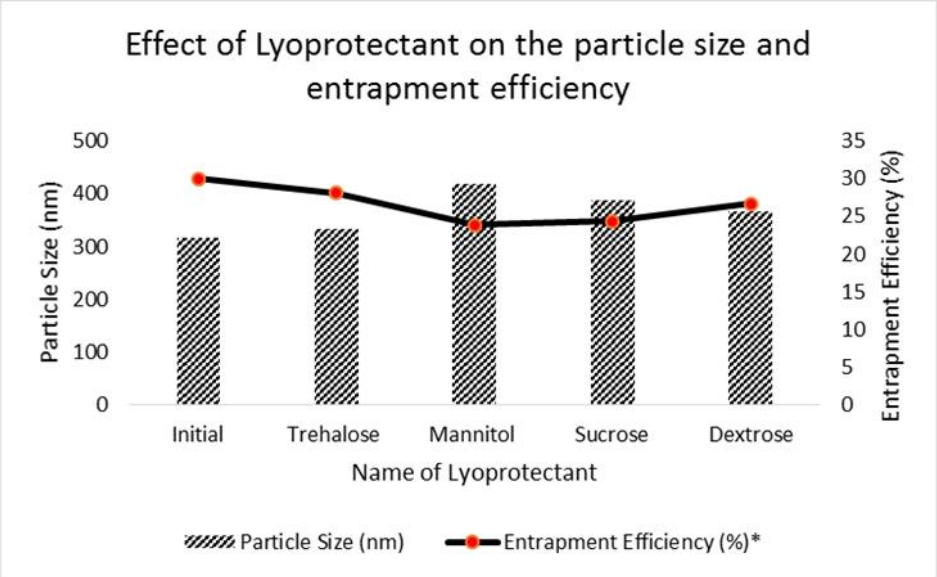

Fig. 2 Effect of Different Sugars (6% w/v) on Characteristics of NPs during Freeze Drying Study

ISSN 2319-1074

The particle size for freeze dried NPs were lowest with trehalose in comparison to other sugars with highest entrapment. The study also suggests the freeze drying process will also cause a slight increase in particle size and decrease in entrapment of the drug. However, considering the improvement of the stability of the product during storage, these changes are negligible. The reconstitution time was also good with all the sugars except mannitol which increased the reconstitution time to 4 minutes which is quite high. Further the entrapment of the drug is also lower with the mannitol which proves unsuitability of the mannitol for Rosuvastatin loaded PLGA NPs. The literature report suggests the conversion of the mannitol in the crystalline form during freeze drying. This might be responsible for breaking of the NPs and formation of the Clumps with broken NPs.

The increase in the particle size and decrease in the entrapment may be due to effect of the freezing on NPs which confirms that the sugars are not 100% effective in preventing the negative effect of freezing on NPs. The NPs collides with each other causing increase in particle size and breakdown of the particles which causing leaking and thus decrease in the entrapment of the drug.

In the second step, the concentration of Trehalose vas varied (4, 6, 8 and 10 %w/v) to check its effect on characteristics of NPs. The results are provided in **Table 14** and **Figure 3.**

Table 14 Effect of Concentration of Trehalose on Characteristics of NPs during Freeze Drying Study

Concentration of Trehalose	Reconstitution Time	Particle Size (nm)	PDI	Entrapment Efficiency (%)
Initial	-	317.00±1.446	0.195±10.010	30.000±0.756
4%	2 min	364.00 ± 2.44	0.186 ± 4.581	29.000 ±1.450
6%	2 min	334.00 ±8.43	0.185 ± 8.571	28.095 ± 1.072
8%	2 min	315.66 ± 2.54	0.194 ± 7.161	28.857 ± 1.143
10%	2 min	310.00 ± 4.34	0.169 ± 7.828	27.476 ± 0.861

Data shown as mean ± SD (n =3)

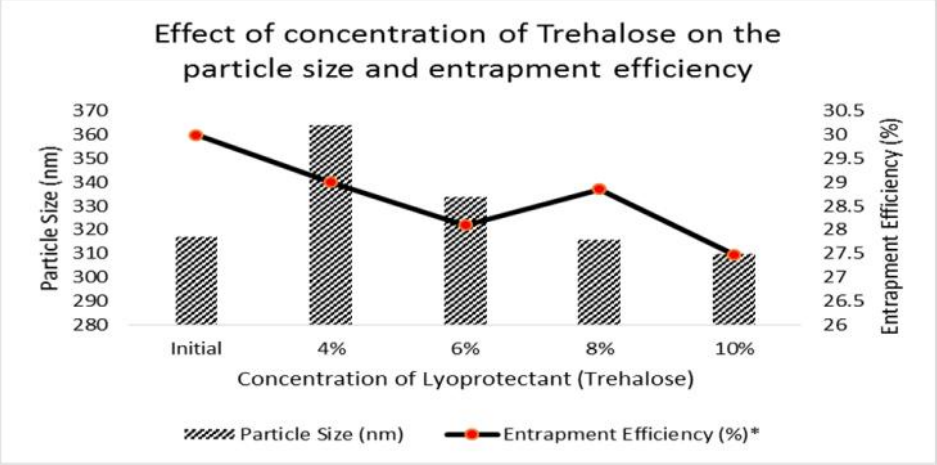

Fig.3 Effect of Concentration of Trehalose on Characteristics of NPs during Freeze Drying Study

ISSN 2319-1074

From this study, it was observed that the 10 %w/v of Trehalose will give NPs with the lowest size without much impact on the entrapment of the drug. The particle size, PDI and entrapment of the final formulation observed were 310.00±4.34 nm, 0.169±7.828 and 27.476 ± 0.861% respectively.

The size ratio was measured with each sugar. Size ratio is the ratio of particles size after free drying and before freeze drying. The lower ratio in comparison to other sugar indicates best sugar for freeze drying. The lower size ratio was observed in case of Trehalose which indicated its potential in FD of this formulation. Reconstitution score is the ease with which the NPs can be resuspended in water (2 ml). The higher reconstitution score indicates the better resuspendability which was observed in case of NPs with all four sugars but not with the NPs without sugars. The physical appearance, reconstitution score and the ratio with different sugars is shown in **Table 15**. The AFM image of freeze dried nanoparticles is provided in **Figure 4.**

Table 15 Characterization of Freeze Dried NPs

Sr. No.	Sugar (6% w/v)	Physical appearance	Reconstitution score	Ratio (Sf/Si)
1	NPs without sugar	Collapsed cake	*	1.00
2	Trehalose	Intact fluffy cake	***	1.05
3	Mannitol	Collapsed cake	***	1.32
4	Sucrose	Collapsed cake	***	1.22
5	Dextrose	Collapsed cake	***	1.16

Data shown as mean ± SD (n =3)

***indicates reconstitution in 2 ml water with simple inversion of vial and redispersed within few seconds while

* indicates reconstitution needed vortexing for 2 minute

Sf/Si – Ratio of particle size after freeze drying to particle size before freeze drying

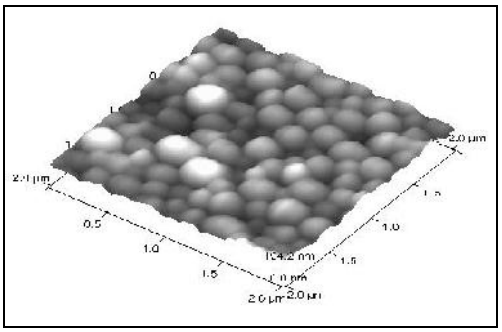

Fig. 4 AFM image of the freeze dried NPs of Rosuvastatin

ISSN 2319-1074

In vitro Drug Release Study

The *in vitro* drug release study was performed at pH 7.4 to simulate the *in vivo* condition of plasma. The drug release profile of Rosuvastatin from NPs is shown in **Figure 5**.

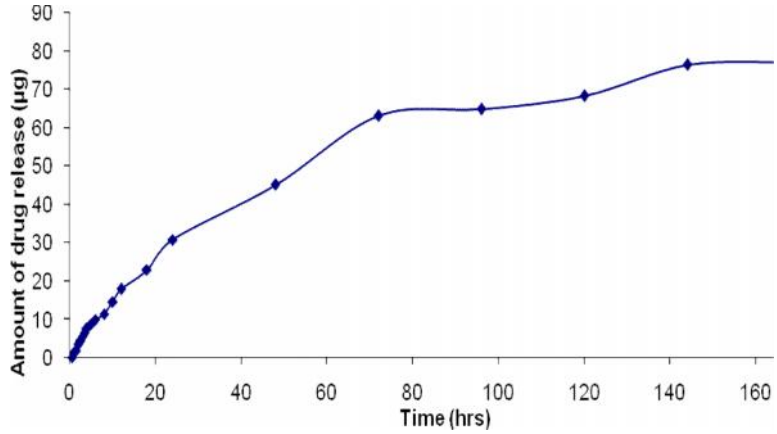

Fig. 5 Cumulative Rosuvastatin released from NPs at pH 7.4

The release of Rosuvastatin from the NPs can be divided in 3 phases from the drug release curve. The Phase 1 has shown rapid release of the drug which continued for 0-24 hours. The Phase 2 has shown intermediate drug release of drug which continued for 24 -72 hours. The Phase 3 has shown very less drug release of drug which continued for 24 -72 hours. The release of the drug from nanoparticles will continue for about 7 days. The sustained drug release can also reduce the dose of the drug as well as can reduce the side effects of the drug.

Accelerated Stability Study

To confirm the shelf life of freeze dried NPs, the accelerated stability study was performed. The freeze dried NPs were kept for 6 months at the ICH recommended accelerated condition (25°C and 60%RH) for the products to be stored in refrigerator in the stability chamber. The results are summarized in **Table 16.**

Table 16 Accelerated Stability Study Results

Time (Months)	Particle Size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)
0	315.33±6.22	0.194±0.125	11.45±3.13	28.61±1.2
1	321.66±4.62	0.256±0.066	13.21±2.16	28.14±3.18
2	308.22±9.56	0.230±0.114	11.36±1.54	27.55±3.12
3	328.25±4.56	0.224±0.082	12.25±1.65	29.12±1.12
6	402.55±6.85	0.222±0.056	13.21±1.11	27.96±2.10

Data shown as mean ± SD (n =3)

ISSN 2319-1074

The stability results show no significant change in particle size as well as entrapment of the drug after 3 months of stability studies. However, a slight increase in particle size with no change in entrapment was observed as the interval of 6 months. Further, the PDI and zeta potential were not affected at the accelerated stability study. The results suggest that the NPs are stable upto period of 6 months at the accelerated condition. Based on the results, atleast 12 months of shelf life can be awarded to NPs.

pH Dependent Stability of Nanoparticles

To evaluate the effect of different pH conditions which NPs will be exposed after oral administration, the pH dependent stability of NPs was evaluated in media simulation the pH of GIT. The results of pH dependent stability are summarized in **Table** 17.

Table 17 Effect of pH on Characteristics of NPs

Media	Particle Size (nm)	PDI	Entrapment Efficiency (%)
Initial	312.18±7.65	0.152±0.113	27.56±2.52
0.1 N HCL (pH 1.2)	338.52±2.52	0.138±0.021	27.12±1.23
pH 4.5 acetate buffer	326.25±3.65	0.185±0.065	28.18±2.1
pH 6.8 phosphate buffer	322.11±2.24	0.218±0.056	27.15±2.81
pH 7.4 phosphate buffer	318.16±3.58	0.134±0.111	26.11±2.1

Data shown as mean ± SD (n =3)

No significant impact of pH of GIT on any physic-chemical characteristics of NPs was observed. There was no significant impact on the particle size, PDI as well as the entrapment of the drug. There was a slight increase in the particle size with the 0.1N HCl. The impact was much lesser at the pH 7.4 phosphate buffer.

In vivo study

The study showed that the biochemical parameters PTC, PTG and LDL-C levels were elevated, whereas HDL-C levels decreased significantly during 4 weeks of HFD treatment to the rats. Further the level of the glucose was also elevated while the level of the SGOT and LDH was remained unaffected during the treatment with HFD.

The treatment of Rats using Rosuvastatin formulations caused significant decrease in the parameters related with Hyperlipidemia i.e. PTC, PTG and LDL-C level while the level of HDL-C was significantly increased. This shows that Rosuvastatin is causing significant effect on these lipid profiles and alleviate Hyperlipidemia.

The Crestor tablets (reference product of Rosuvastatin) causes decrease in PTC, PTG and LDL-C level during 4-6 weeks of the treatment period. However, after withdrawing the treatment from 6^{th} week, the PTC, PTG and LDL-C level again starts increases in plasma. This confirms that the

ISSN 2319-1074

effects are only observed during the treatment phase and requires continuous administration of the drug. The reverse trend was observed for HDL which increases during the treatment phase and again starts decreasing after stopping the treatment **(Figure 6 and 7).**

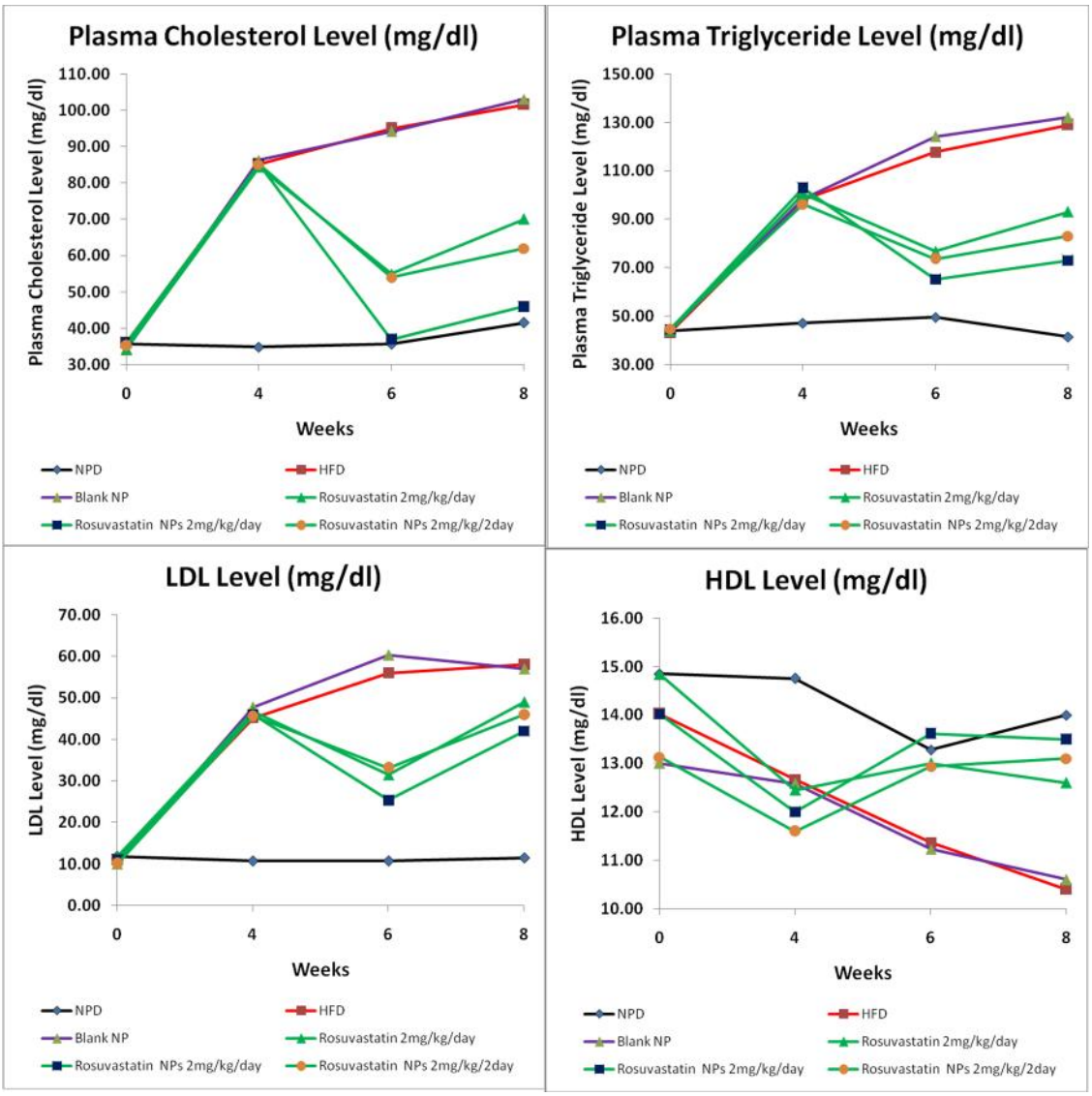

Fig. 6 a) Plasma Cholesterol Level b) Plasma Triglyceride level c) Plasma LDL Level d) Plasma HDL Level

ISSN 2319-1074

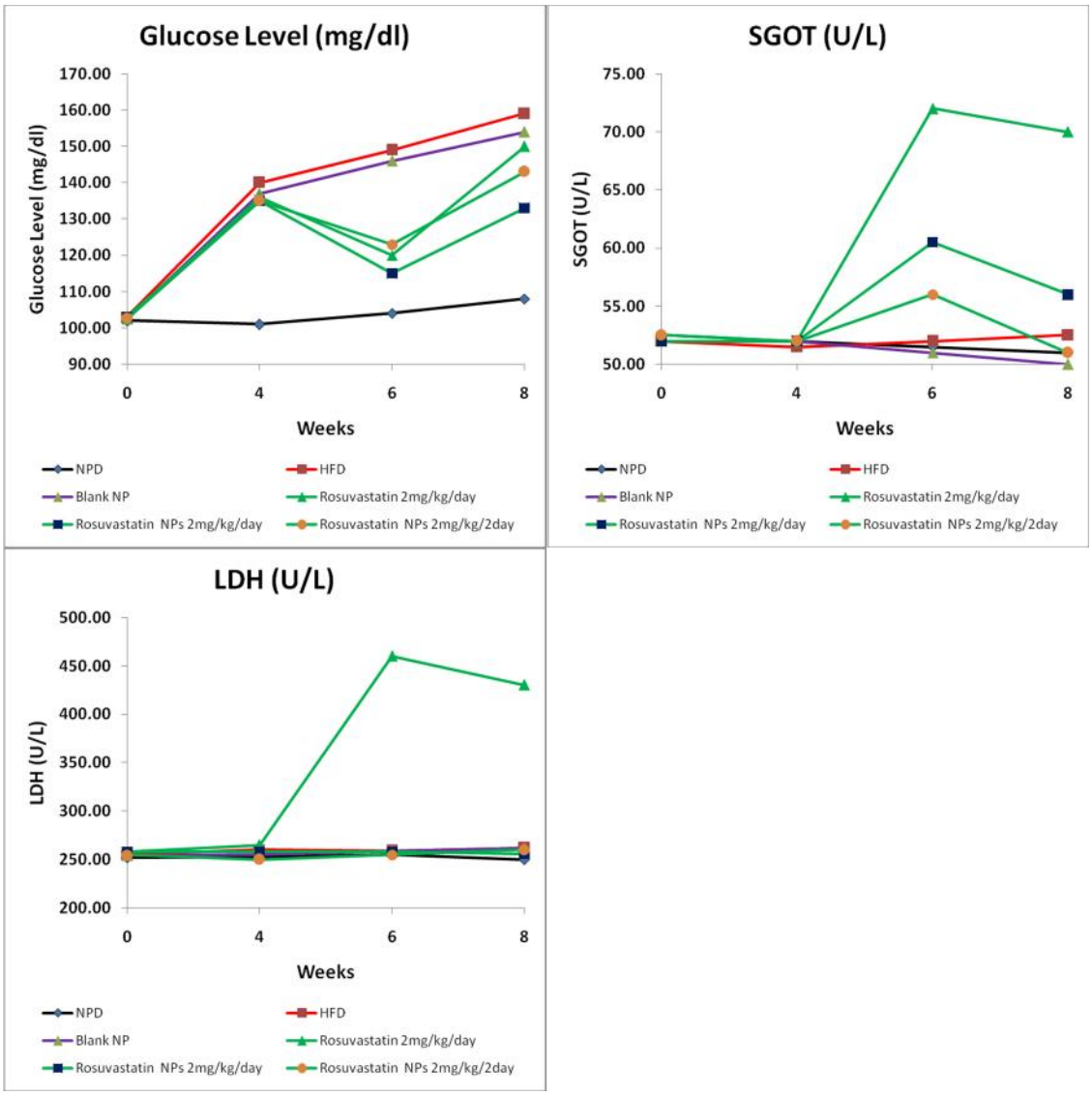

Fig. 7 a) Plasma Glucose level b) Plasma SGOT Level c) Plasma LDH Level

The effect of Rosuvastatin loaded NPs with the same dose as reference product was very prominent. The PTC, PTG and LDL-C level decreased while HDL level increased more sharply during the treatment phase. Further, the drug effect was also observed after stopping the treatment. The level of these parameters was well maintained during the 6-8 weeks without treatment of rats. This indicates that the NPs remains circulating in the plasma, releasing the drug for longer duration of time. Further, half reduction in Rosuvastatin dose of NPs does not compromised the efficacy much and the effect were found comparable to the full dose of reference product. This confirms that approximately half dose reduction of Rosuvastatin can be done by encapsulating it in NPs.

ISSN 2319-1074

The level of the glucose was also evaluated during the treatment. The results indicate that Rosuvastatin causes reduction in the glucose level. The literature data suggest that Statins are causing sensitization of insulin receptor which causes reduction in the glucose level. The results show that the NPs were causing more reduction in plasma glucose level when administered at the same dose as reference product. This confirms better control on glucose level in patients suffering from Hyperlipidemia. However, reducing the dose of NPs to the half will give the comparable glucose reduction as reference product.

To evaluate the impact of NPs on safety profile of the drug molecule, the level of SGOT and LDH in plasma were also evaluated. The results indicate that the level of both of these enzymes were same in the Rats with NPS and control rats with HFD. However, exposure of Rosuvastatin to the Rats had increased their level that shows toxicity of these drugs. However, the increase in the level of both of these enzymes was more prominent with the reference product. The administration of the NPs had reduced the level of SGOT to the significant amount. The level of LDH was same for NPs as well as the control rats which indicate no toxicity with the use of NPs. The results are very promising showing the reduction in the toxicity of Rosuvastatin to the significant value. Since the effect of Rosuvastatin was also observed after the treatment phase, the safety parameters were also evaluated during the post-treatment phase. The results confirm the level of the SGOT also decrease after completion of the treatment to the level in the control rats.

The results confirm that the encapsulating the drug in NPs will improve the efficacy and safety in comparison to the reference product. This can increase the marketability of the drug product.

CONCLUSION

The Nanoparticles are very effective in reducing the toxicity and improvement of safety profile of Rosuvastatin. Entrapping Rosuvastatin in nanoparticles will cause 2 times reduction in the dose. This will improve the patient compliance as well as marketability of ROSUVASTATIN.

ACKNOWLEDGEMENT

I am thankful to Cadila Pharmaceuticals Limited for providing Rosuvastatin. I am also thankful to K.B. Institute of Pharmacy to provide me the facility for research work.

REFERENCES

1. Calabresi, L., Donati, D., Pazzucconi, F., Sirtori, C. R., Franceschini, G., 2000. Omacor in familial combined hyperlipidemia: effects on lipids and low density lipoprotein subclasses. Atherosclerosis. 148(2), 387-396. [Journal Article]

2. Wilbert, S., 2006. Management of hyperlipidemia with statins in the older patient. Clin Interv Aging. 1(4), 433–438. [Journal Article]

3. https://www.medicines.org.uk/emc/medicine/11976 [website]

4. Wooltorton, E., 2004. Rosuvastatin (Crestor) and rhabdomyolysis. Health And Drug Alerts. CMAJ. 2004, 171(2), 129. [Journal Article]

5. García-Rodríguez, L. A., Massó-González, E. L., Wallander. M. A., Johansson. S., 2008. The safety of rosuvastatin in comparison with other statins in over 100,000 statin users in UK primary care. Pharmacoepidemiol Drug Saf. 17(10), 943-952. [Journal Article]

ISSN 2319-1074

6. Famularo, G., Miele, L., Minisola, G., Grieco, A., 2007. Liver toxicity of rosuvastatin therapy. World J Gastroenterol. 13(8), 1286-1288. [Journal Article]

7. http://www.fda.gov/Safety/MedWatch/SafetyInformation/ucm200635.htm [website]

8. Calza L., 2009. Long-term use of rosuvastatin: a critical risk benefit appraisal and comparison with other antihyperlipidemics. Drug Healthc Patient Saf. 1, 25–33. [Journal Article]

9. Abdelwahed, W., Degobert, G., Stainmesse, S., Fessi, H., 2006. Freeze-drying of nanoparticles: Formulation, process and storage considerations. Adv Drug Deliver Rev. 58, 1688–1713. [Journal Article]

10. Modi, S., Anderson, B. D., 2013. Determination of drug release kinetics from nanoparticles: overcoming pitfalls of the dynamic dialysis method. Mol Pharm. 10(8), 3076-3089 [Journal Article]

11. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf [website]

12. Kalaria, D. R., Sharma, G., Beniwal, V., Ravi Kumar, M. N. V., 2008. Design of biodegradable nanoparticles for oral delivery of doxorubicin: in vivo pharmacokinetics and toxicity studies in rats. Pharm Research. 26(3), 492-501. [Journal Article]

13. Bhandari, U., Kumar, V., Khanna, N., Panda, B. P., 2011. The effect of high-fat diet-induced obesity on cardiovascular toxicity in Wistar albino rats. Hum Exp Toxicol. 30(9), 1313-1321. [Journal Article]