ROSUVASTATIN CALCIUM LOADED NANOSTRUCTURED LIPID CARRIER FOR THE MANAGEMENT OF DIABETIC RETINOPATHY

Manodhini Elakkiya[#], Kousalya Selvaraj[#], Gowthamarajan Kuppusamy[#], Veera Venkata Satyanarayana Reddy Karri[a].

Department of Pharmaceutics, JSS College of Pharmacy, Ootacamund, JSS University, MysoreIndia-643001.

Corresponding Authors: Gowthamarajan kuppusamy and Manodhini Elakkiya,Department of Pharmaceutics, JSS College of Pharmacy, Ootacamund, JSS University, MysoreIndia-643001.

E-mail:gowthamsang@gmail.com and manodhini.elakkiya@gmail.com, Tel: +91-9751242662.

Abstract

Purpose: Repurposing of Rosuvastatin Calcium as antiangiogenic molecule for the better management of diabetic retinopathy and to formulate surface modified Rosuvastatin Calcium loaded NLC to improve the permeability. To study the toxicity of the formulation and *In vitro* antiangiogenic activity of Rosuvastatin calcium and formulation.

Methods: Rosuvastatin calcium was formulated my micro emulsion technique. Permeability of drug solution and formulation was studied using goat cornea and antiangiogenic property of drug and formulation was studied by *Ex vivo*.

Results: Rosuvastatin calcium was more soluble in Tripalmitin, oleic acid and tween 80 (2%), Eutragit RS PO was used as surface modifier. particle size of without surface modified and with surface modified formulation was found to be 140.5nm and 151.8nm. Zeta potential was changed from -24 to +22.8 after surface modification. Entrapment efficiency of optimized F3 was found to be 88%. SEM analysis showed particle size of without surface modified and with surface modified formulation was found to be 133nm and 168nm. Formulation showed sustained release and followed peppas model in release kinetics. Permeability study was done with drug solution (8.28), uncoated formulation (25.88%) and coated formulation (34.7%). Formulation was found to be sterile FTM, NB and SCDM. IC50 value of drug and formulation was found to be 100µg/ml and 4000µg/ml. Formulation does not show toxicity at 15µg/ml. From antiangiogenic study Rosuvastatin Calcium loaded NLC showed a reduction in blood vessel growth.

Conclusions: Rosuvastatin calcium showed antiangiogenic activity and % permeability is increased by surface modification of the formulation. So Rosuvastatin Calcium can be used for the better management of DR.

Keywords: Diabetic retinopathy, antiangiogenic activity, Repurposing of drug, Ocular drug delivery.

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INTRODUCTION

Globally, as of 2014, 387 million people had diabetes its prevalence will increase to 592 million individuals by 2035 as per IDF.[1]So, diabetes can affect sight by causing cataracts, glaucoma, and most importantly, damage to blood vessels inside the eye, a condition known as "Diabetic Retinopathy (DR)”.[2] DR is a complication of diabetes that is caused by changes in the blood vessels of the retina. When blood vessels in the retina are damaged, they may leak blood and grow fragile, brush-like branches and scar tissue. This can blur or distort the vision images that the retina sends to the brain. [3-16]

METERIALS AND METHODS

Materials

Rosuvastatin calcium was purchased from IPCA Mumbai India, Tripalmitin was purchased from KenbhasolMumbai India, Tween 80 was purchased from sigma chemicals Mumbai India, Oleic acid was purchased from SD fine chemicals Mumbai India, Ethanol form SD chemicals India, Viru cell was purchased from NSS Mumbai India, was received and performed the following methods.

Preparation of Rosuvastatin Calcium loaded NLC

Drug loaded nanostructure lipid carrier were prepared by micro emulsion method using tripalmitin and Oleic acid as lipids, Rosuvastatin Calcium as drug, Tween 80 surfactant, Eudragit RS PO was used as positive charge inducer.[21-22] Different ratios of liquid and solid lipids are taken and drug is added, alcohol is added to dissolve and maintained at 60ºC in magnetic stirrer with constant speed. 2% solution of tween 80 was prepared which was maintained at 60º in magnetic stirrer with constant speed. Organic phase is added to aqueous phase by injecting drop wise, then after 5mins this was added to ice cooled water of 250 ml by injecting drop wise and it was kept for 2 hrs and 2000 rpm of speed.

Batch	Rosuvastatin calcium(mg)	Tripalmitin (mg)	Oleic acid (µl)	Tween 80	Time (hrs)	Speed (rpm)
F1	50	90	11.2	2%	2	2000
F2	50	80	22.4	2%	2	2000
F3	50	70	33.7	2%	2	2000
F4	50	60	44.9	2%	2	2000
F5	50	50	56.2	2%	2	2000

Table.1:Evaluation of nanostructure lipid carrier

Particle size and zeta potential

Particle size and zeta potential of the solid lipid nanoparticles were measured by photon correlation spectroscopy using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK), which works on the Mie theory. All size and zeta potential measurements were carried out at 25°C using disposable polystyrene cells and disposable plain folded capillary zeta cells, respectively, after appropriate dilution with original dispersion preparation medium. In order to investigate the effect of Eutragit RS PO on zeta potential two batches were prepared with and without Eutragit RS PO and their zeta potential were measured. [19]

Polydispersity Index

The polydispersity index (PI) is the measure of size distribution of the nanoparticle formulation. PI was measured using Malvern zetasizer. PI values range from 0.000 to 1.000 i.e. monodisperse to very broad particle size

distribution. PI values of all the formulations indicate that particle size distribution was unimodal.[19]

Scanning electron microscopy (SEM)

The surface morphology of NLC was determined using Scanning Electron Microscopy (SEM). Samples are diluted with ultra-purified water to obtain a suitable concentration. Then the samples are spread on a sample holder and dried using vacuum. They are subsequently coated with gold (JFC 1200 fine coater, Japan) and examined by a scanning electron microscopy (SEM) scanned by the refractive index at 30 s run time.

Drug loading and entrapment efficiency

To separate NPs the nanosuspension was ultra centrifuged at 8,000 rpm for 1 h. The supernatant was removed and the pellets of the F3 were collected. The collected NPs were dissolved in 5ml of Phosphate buffer (PB) (7.4 pH) and then 10ml of methanol was added to precipitate the lipids. Then the samples were taken and the amount of drug was estimated by UV-Visible. The drug loading and entrapment efficiency was calculated by the following equation.

$$\text{Drug loading (\% w/w)} = \frac{\text{Mass of drug in nanoparticle}}{\text{Mass of nanoparticle recovered}} \times 100$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{Mass of drug in nanoparticle}}{\text{Mass of drug used in the formulatio}} \times 100$$

***In vitro* release studies**

The release of Rosuvastatin Calcium from the drug suspension and formulations was studied under sink conditions. 70:30 (S/L) showing higher drug content and entrapment efficiency were evaluated for *invitro* release. 10ml of NLCs were placed in dialysis bag. The dialysis bag were placed in 100mL of dissolution medium and stirred under magnetic stirring at 37°C. Aliquots of the dissolution medium were withdrawn at each time interval and the same volume of fresh dissolution medium was added to maintain a constant volume. Samples withdrawn from PB 7.4 pH were analyzed for Rosuvastatin Calcium content spectrophotometrically at 241 nm against solvent.

Permeation Experiment

Goat cornea was purchased from slaughter house in normal saline solution, then fatty layers are removed and then corneal layer was separated. Freshly excised goat cornea was fixed between clamped donor and receptor compartments of an all-glass modified Franz diffusion cell in such a way that its epithelial surface faced the donor compartment. The corneal area available for diffusion was 0.67 cm2. The receptor compartment was filled with 10 ml of freshly prepared normal saline, and all air bubbles were expelled from the compartment. An aliquot (1 ml) of test solution, uncoated and coatedformulation was placed on the separate corneas, and the opening of the donor cell was sealed with a glass cover slip, while receptor fluid was kept at 37°C with constant stirring using a Teflon-coated magnetic stir bead. The permeation study was continued for 120 minutes; samples were withdrawn from the receptor and analyzed for rosuvastatin calcium content by measuring absorbance at 241 nm in a UV spectrophotometer. Results were expressed as amount permeated and percent permeation or in vitro ocular availability. The permeation (%) or in vitro ocular availability was calculated as follows. [17]

% Permeability= KD/Δx

Where:

K= Solubility value

D= Diffusion Coefficient

Δx= Thickness of membrane

Sterility study

The sterility testing was performed according to Indian Pharmacopoeia (IP) 2007 method on four different media namely, FTM, NB andSCDM to investigate the presence or absence of aerobic bacteria, anaerobic bacteria and fungi. FTM media was incubated at 37 ± 0.5°C under aerobic condition, NB at 30 ± 2.5°C under anaerobic condition in a bacteriological incubator while SCDM, was incubated at 25 ± 0.5°C under aerobic condition in a fungal incubator for 7 days. The experiment was performed in triplicate.

***In vitro* cytotoxicity assay**

i. The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using DMEM medium containing 10% FBS.
ii. To each well of a 96 well microtitre plate, 100µl of the diluted cell suspension (approximately 10,000 cells/well) was added.
iii. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100µl of different sample concentrations prepared in maintenance media were added per well to the partial monolayer in microtitre plates. The plates were then incubated at 37ºC for 24 hrs in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded.
iv. After 24 hours, the sample solutions in the wells were discarded and 20 µl of MTT (2mg/ml) in MEM-PR (MEM without phenol red)/PBS was added to each well.
v. The plates were gently shaken and incubated for 3 h at 37ºC in 5% CO_2 atmosphere.
vi. The supernatant was removed and 100 µl of iso-propanol was added and the plates were gently shaken to solubilize the formed formazan.
vii. The absorbance was measured using a microplate reader at a wavelength of 540 nm.

The percentage growth inhibition was calculated using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line.

$$\% \text{ Growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

***In-vitro* Antiangiogenesis study (HET-CAM Test)**

White Leghorn chicken eggs were obtained from the Poultry Research Centre, Namakkal. White Leghorn chicken eggs were selected for the study because they have no hereditary defects and yields very consistent and reproducible results. Eggs were incubated in the incubator for 9-10 days at 37 ºC. On day 9, eggs were tested with candling light to ensure that all were viable. On day 10, the air cell was marked with a pencil and removed the shell by tapered forceps. The membrane was carefully moistened with 0.9 % NaCl solution at 37 ºC. The eggs were replaced in the incubator until ready for assay. The moistening solution (0.9 % NaCl) was poured off from the opened egg and the membrane was carefully removed without injuring any underlying blood vessels. 0.1 N NaOH and 0.9 % NaCl were used as negative and solvent control respectively. The CAM of each egg was applied directly with 0.3 ml of the solvent/negative control and with different concentration of placebo (without drugloaded) nanoparticles and placebo NLC. Two eggs for controls and three for test substances were used for eachassay. The reactions are the CAM was observed. [22].

RESULTS

Zeta Potential: The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. It has been reported that positively charged nanostructured lipid carriers have a better uptake than neutral or negatively charged particles**.** Hence two batches were prepared with and without Eutragit RS PO. It was found that the batch with Eutragit has a zeta potential was +22.8 mV which confers stability to the dispersion.

Formulation	Zeta potential
F4	-17.5

Table: 2 Zeta potential values of different formulations

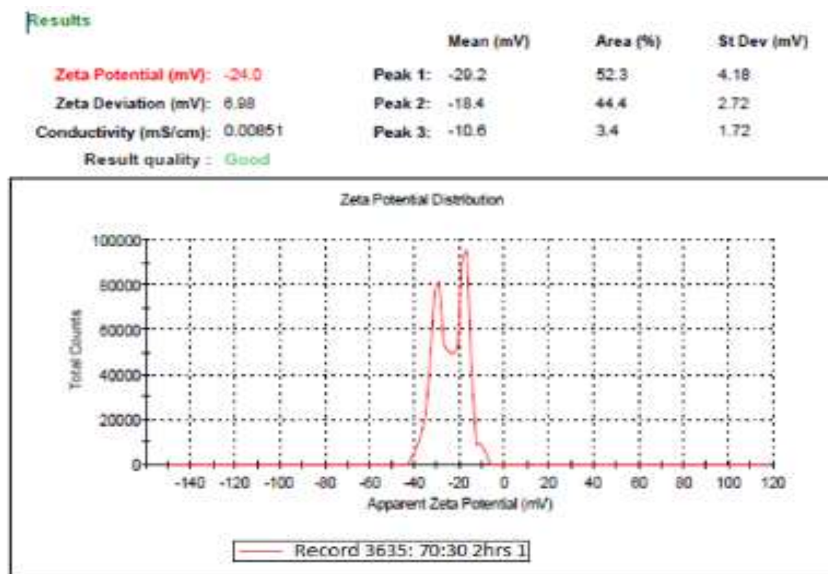

Fig. 1:Zeta potential for uncoated F3

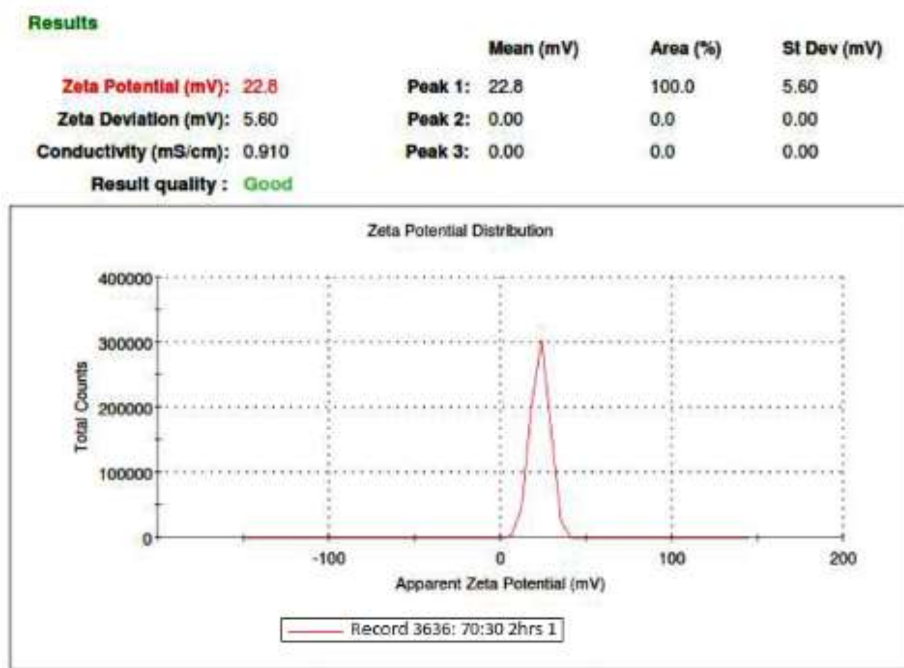

Fig. 2:Zeta potential of coated F3

Particle size and Polydispersity Index (PDI)

Among various batches, F3 have shown good particle size. F3 has shown avg. particle size of 140 nm with PI of 24 indicating narrow particle size distribution and follows unimodal distribution. The reduced particle size may be attributed to higher ratio of liquid lipid when compared to other batches.

Results

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	140.5	Peak 1:	243.2	77.2	148.0
PdI:	0.610	Peak 2:	38.75	16.6	10.47
Intercept:	0.932	Peak 3:	5006	6.2	607.8
Result quality :	Good				

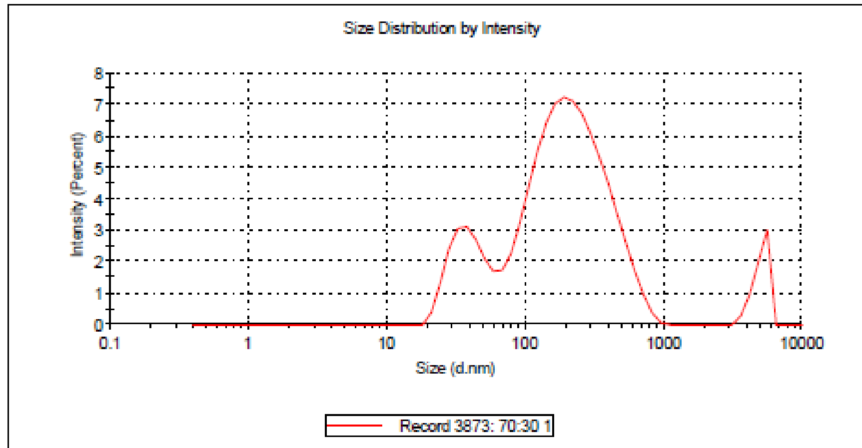

Fig. 3:Particle size and polydispersity index of uncoated F3

Results

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	151.8	Peak 1:	163.4	92.3	64.20
PdI:	0.315	Peak 2:	5012	5.2	601.8
Intercept:	0.919	Peak 3:	30.97	2.5	6.877
Result quality :	Good				

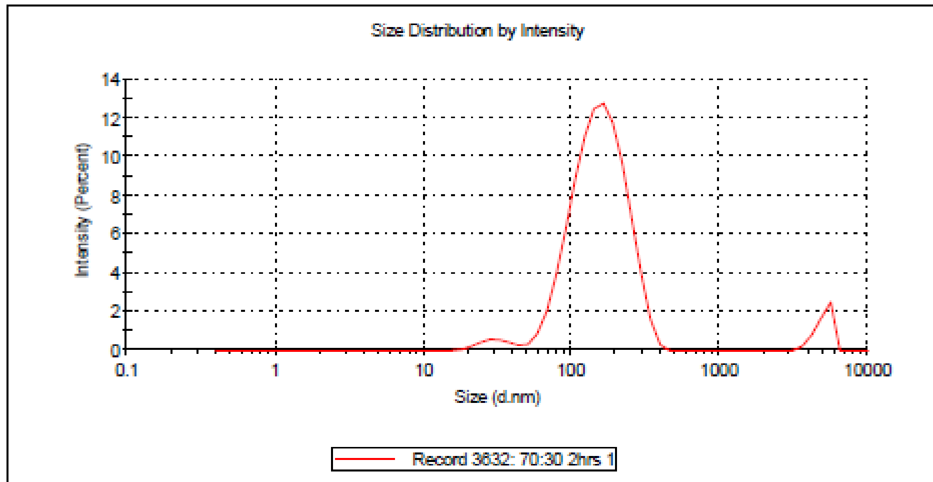

Fig. 4:Particle size and Polydispersity index of coated F3

Entrapment efficiency

The ratio of solid lipid to liquid lipid was found to affect the extent of entrapment efficiency. Among all the above batches, F3 has

shown maximum Entrapment Efficiency of 88%.The entrapment efficiency was higher when total amount of lipid used was 70:30 (S/L). It was also found that the as the quantity of liquid lipid increased the entrapment efficiency increased. The EE value of F1, F2, F3, F4 and F5 was found to be 61.79%, 70.35%, 88%, 81% and 56%.

Batch	Entrapment efficiency (%)
F1 (90:10)	61.79
F2 (80:20)	70.35
F3 (70:30)	88
F4 (60:40)	81
F5 (50:50)	56

Table:3Entrapment efficiency for different drug loaded NLC formulation

SEM analysis

Scanning electron microcroscopy (SEM) was used to verifythe uniformity of particle size and shape. The SEM microphotographs of ROS nanoparticles revealed that the particles were spherical in shape having smooth surface. The formulated NPs size is about 151nm.

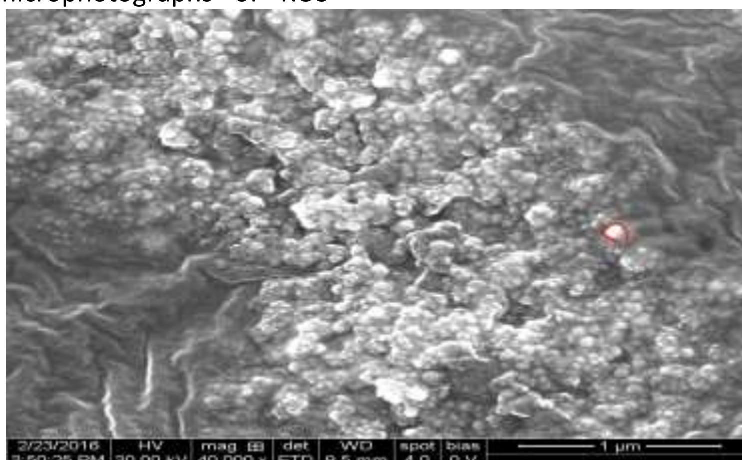

Fig.5: SEM image of uncoated F3

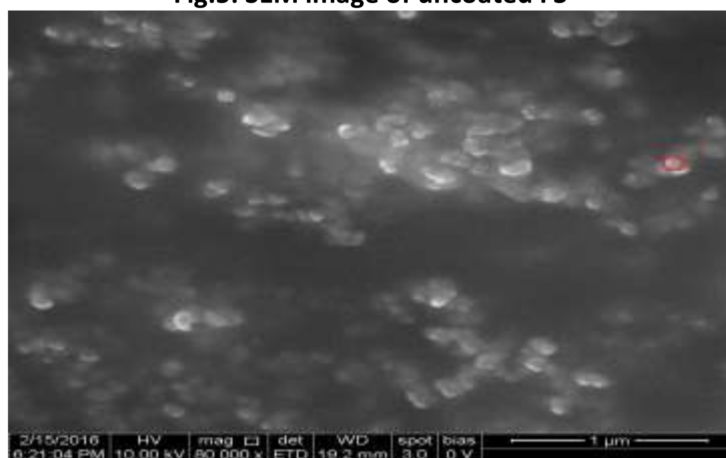

Fig. 6:SEM image of coated F3

S. No	*Coated F3*	*Uncoated F3*
1	*168*	*133*

Table. 4: SEM of formulations

In vitro Release studies

In vitro release studies were carried out in Phosphate buffer pH 7.4. The releaseprofiles indicate that NLC formulations showed a retarded release of the drug from the lipid matrix when compared with plain Rosuvastatin calcium solution (ROS-SOL). The *in vitro* release data and graph of NLC formulation and ROS-SOL is shown below. It was observed that ROS-SOL showed 97.34% release in 48h compared to 92.05% release for NLC at the end of same time. This is due to fact that there is no barrier for diffusion at dialysis membrane interface for Rosuvastatin calcium molecules. Hence, higher release was observed in case of ROS-SUS. The NLC formulation showed sustained release behaviour.

Time (Hrs)	Cummulative release of ROS-SOL (%)	Cummulative release of NLC (F3) (%)
0	0	0
1	28.67	35.8
2	52.79	37.03
4	80.65	56.87
8	95.34	58.05
12		61.88
24		68.59
48		92.36

Table. 4:Comparative *in vitro* release data for NLC formulation compared to pure drug in PB pH 7.4.

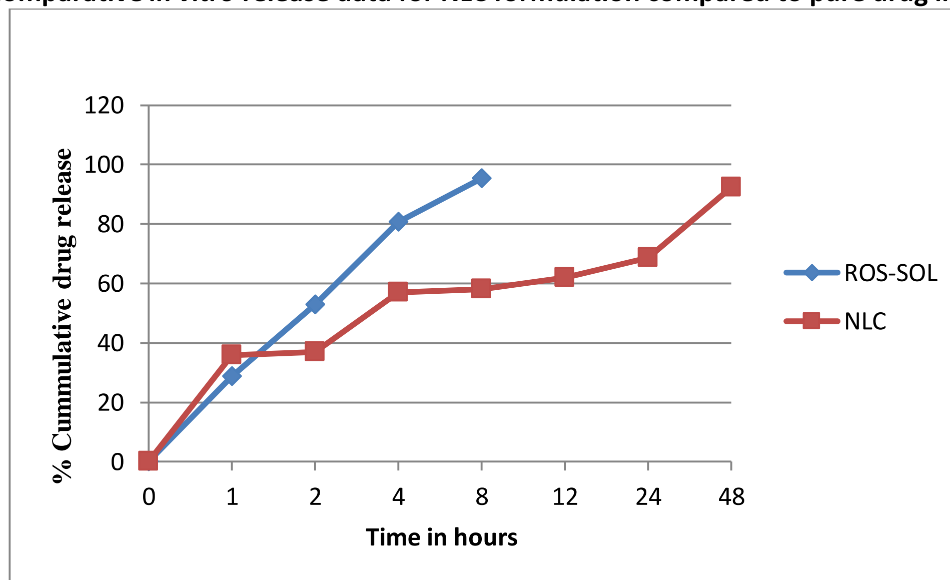

Fig.7:Cumulative release of Rosuvastatin Calcium drug solution and F3 formulation

Release kinetics

In order to elucidate mode and mechanism of drug release, the *invitro* data was transformed and interpreted at graphical interface constructed using various kinetic models. The *in vitro* release data obtained for NLC formulations, in phosphate buffer pH 7.4, was fitted into various kinetic models. The results are shown in Table

In order to elucidate mode and mechanism of drug release, the *invitro* data was transformed and interpreted at graphical interface constructed using various kinetic models. The *in vitro* release data obtained for F3, in phosphate buffer pH 6.8, was fitted into various kinetic models. The results are shown in Table.

	Release Kinetics				
	Zero	**Higuchi**	**Peppas**	**First**	**Hixson Crowell**
	1	**2**	**3**	**4**	**5**
	R(CvT)	**R(CvRoot(T))**	**Log T vs Log C**	**TIME vs LOG % Remaining**	**TIME Vs (Q1/3-Qt1/3)**
Slope	1.350	11.255	0.237	-0.011	0.044
Correlation	0.8086	0.9260	0.9650	-0.9587	0.9288
R 2	0.6539	0.8574	0.9313	0.9191	0.8626

Table. 5:Regression value for various kinetic models

The best linearity was obtained in Peppas for NLC formulation. Peppas model indicates the mechanism of drug release is by diffusion, erosion, swelling and may by the combination of diffusion and swelling.
The release study for F3 is shown below.

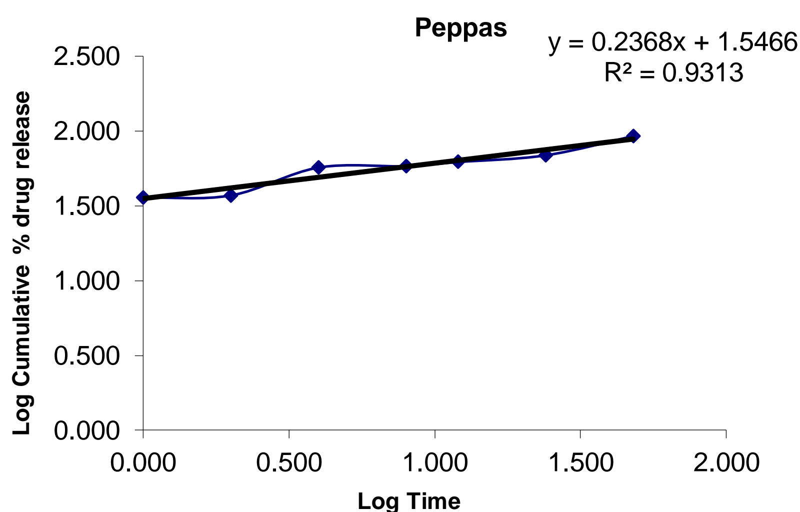

Fig. 8: Peppas release

Permeation experiment

Freshly excised goat cornea was fixed between clamped donor and receptor

compartments of an all-glass modified Franz diffusion cell in such a way that its epithelial surface faced the donor compartment. The corneal area available for diffusion was 0.7 cm^2. The receptor compartment was filled with 10 ml of freshly prepared normal saline, and all air bubbles were expelled from the compartment.An aliquot (1 ml) of test solution was placed on the cornea, and the opening of the donor cell was sealed with a glass cover slip, while receptor fluid was kept at 37°C with constant stirring using a Teflon-coated magnetic stir bead. The permeation study was continued for 120 minutes; samples were withdrawn from the receptor and analyzed for Rosuvastatin calcium content by measuring absorbance at 241 nm in a spectrophotometer. Results were expressed as amount permeated and percent permeation or in vitro ocular availability. The permeation (%) or in vitro ocular availability was calculated. The permeation characteristics of Rosuvastatin calcium from 1% solution of crude drug and optimized formulation were also evaluated through freshly excised paired goat, sheep, and buffalo corneas.

$$\%P = KD/\Delta x$$

Where

P=Premeability

K= Solubility Value, D= Diffusion Coefficient

Δx = Thickness of the membrane

Fig. 9: Permeability study

S. No	Solutions	% Permeability
1	1% solution of Rosuvastatin calcium	8.28%
2	Uncoated NLC of Rosuvastatin calcium	25.88%
3	Eutragit RS PO coated NLC of Rosuvastatin Calcium	34.7%

Table. 6:Permeability values

Sterility studies

Sterility is one of the most vital requirements for an ophthalmic preparation to determine the absence of viable microorganisms that may harm the eye of the patient. FTM, NB and SCDM to investigate the presence or absence of aerobic bacteria, anaerobic bacteria and fungi. FTM media was incubated at 37 ± 0.5°C under aerobic condition. No growth was observed in all media

on the second day compared with positive control and Gram positive (-*S.aureus, B.pumilis* and Gram negative-*E. coli, P. aeroginosa*) and two fungal strains (*C. albicans, A. niger*). It was concluded that moist heat sterilization and dry sterilization by filtration for drug solution and nanoparticles could be done to achieve the sterility.

In vitro cytotoxicity assay (MTT)

The percentage growth inhibition was calculated using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line.

$$\% \text{ Growth inhibition} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

The IC 50 value of the formulation is about greater than 4000µg/ml and IC 50 value of the drug is about 1000 µg/ml.

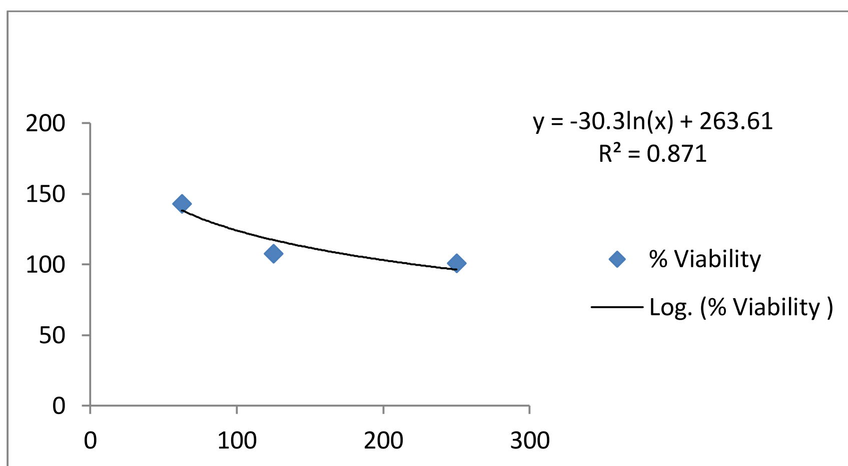

Fig. 10.%Viability of cell in drug

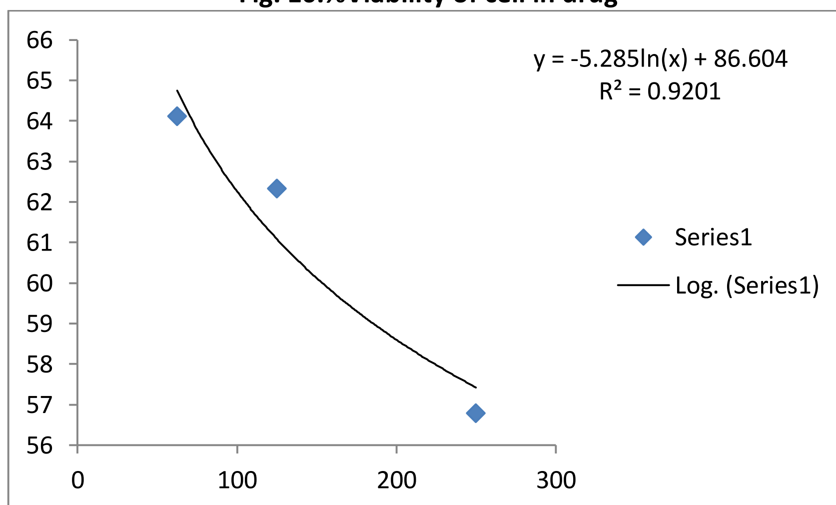

Fig.10:%Viability of cell in F3

HET- CAM assay

The *ex-vivo*antiangiogenesis studies were carried out using HET-CAM method. The drug loaded NLC were prepared without drug by modified coacervation method and subjected for antiangiogenesis study. If drug loaded NLC were subjected for angiogenesis studies then drug will release which inhibit the

growth of blood vessels. The drug was encapsulated into the polymer's core and polymer comes under the direct contact of the eye. So here we antiangiogenesis studies for formulation and were found to be non toxic upto 15 µg/ml. The negative controls 0.1N NaOH exhibited various end points (haemorrhage, lysis, coagulation) at different seconds. In case of solvent control treated with 0.9% w/v of NaCl persistent of blood vessels taken place where as negative control. The F3 shows toxicity at 15015µg/ml , when 10 fold dilution is done F3 does not show toxicity at 15µg/ml.

Fig. 11: Angiogensis activity

DISCUSSION

The solubility of Rosuvastatin calcium was found to be more in Oleic acid and tween 80 was found to be more, so we have taken those lipid and surfactant for the formulation. Tripalmitin was used in the formulation because it was solid lipid maximum used for treating posterior eye disease. FTIR study showed that it is incompatible but still we can use in the formulation because these are the lipids that are already available in the body and showed non toxic to the body. Formulation was optimized in solid lipid and liquid lipid ratio as 70:30 because of higher entrapment efficacy (88%) and less particle size of about 140.5nm. Surface modification was done to increase the permeability from 8.28% to 34.7% which is of particle size 151.9nm and PDI of about 0.315. SEM analysis showed that there is no much particle size difference of uncoated (133nm) and coated (168) formulation. Formulation showed it is a sustained release and followed peppas model in kinetic release (fickian diffusion). Formulation was found to be sterile in NB, FTM and SCDM medium, so that this formulation can be used in ophthalmic delivery. IC 50 value showed that formulation (4000µg/ml) is less toxic when compared to drug (1000µg/ml). Formulation showed antiangiogenic activity by inhibition of new blood vessel growth in egg shell which was showed by HET CAM assay. From the above results, it was concluded that Rosuvastatin calcium has a new indication (drug repositioning) that it is having anti-angiogenetic activity. Hence it may be used for the better management of DR.

CONCLUSION

In order to overcome the limitations of current therapy in the treatment of DR, a surface modified Rosuvastatin Calcium loaded NLC was prepared. Rosuvastatin Calcium was successfully incorporated into the lipids. The surface modified nanostructure lipid carriers showed a significant increase in permeability when compared to drug suspension and also without surface modified formulation. Rosuvastatin calcium loaded NLCs shown sustained release behaviour of the drug with anti-angiogenetic activity. From the above results, it was concluded that Rosuvastatin calcium has a new indication (drug

repositioning) that it is having anti-angiogenetic activity. Hence it may be used for the better management of DR.

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