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Enhanced Oral Bioavailability of Atorvastatin via Oil-in-Water Nanoemulsion using Aqueous Titration Method

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Abstract
Atorvastatin, a highly lipophilic anti-hyperlipidemic drug, has poor oral bioavailability (14%) due to hepatic first pass effect. The present study aimed at developing an optimal oral nanoemulsion formulation containing an atorvastatin using different proportions of oil and surfactant systems for enhancing its oral bioavailability. Pseudoternary phase diagrams were constructed by aqueous titration technique and various nanoemulsion formulations were prepared. Formulations selected from the o/w nanoemulsion region were subjected to various thermodynamic stability and dispersibility tests. Optimized formulations were evaluated for various physicochemical characterization tests. The *in vitro* dissolution studies revealed that release of atorvastatin from nanoemulsion was faster than the conventional tablet (Ozovas ™) and pure drug suspension. The formulation used for assessment of bioavailability contained 15% of oil (Oleic acid), 18% mixture of surfactants (Tween 80 and Brij 35), 6% of co-surfactant (ethanol) and 61% of double distilled water. The absorption of atorvastatin from nanoemulsion resulted in 2.87- and 2.38-fold increase in bioavailability as compared to conventional tablet and pure drug suspension respectively. Thus, the study confirmed that the nanoemulsion formulation can be used as a possible alternative to traditional oral formulation of atorvastatin to improve its bioavailability.
Keywords: Bioavailability, First-pass metabolism, Lipids, Nanoemulsion, Solubility.

INTRODUCTION

With the aid of computer aided drug design and high throughput screening several highly potent new molecular entities (NMEs) have been designed. Unfortunately, the translation of these molecules into finished products has been hampered by their inherent physicochemical properties like solubility and/or permeability. This is supported by the fact that number of NMEs approved by FDA (Food and Drug administration) has been gradually decreasing over the last decade. It is a challenge for the formulation scientists to develop novel strategies for the effective delivery of such molecules as billions of dollars are invested to develop NMEs. In recent years, lipid based formulation approach, with a particular emphasis on nanoemulsion delivery systems have been considered as an ideal alternative for improving the oral bioavailability of BCS (Biopharmaceutical drug classification system) Class II and IV drugs [1,2].

Atorvastatin is a cholesterol-lowering agent widely used to treat hyperlipidaemias. It is a highly lipophilic molecule having logP (Octanol/water) of 6.36 with absolute bioavailability of only14% [3,4].The low systemic availability is attributed to very low solubility of the drug in water and its presystemic clearance in gastrointestinal mucosa or hepatic first-pass metabolism [5].The attraction of formulating oil-in-water (o/w) nanoemulsion systems lies in their ability to incorporate hydrophobic drugs into the oil phase and is delivered by the lymphatic route, thereby restraining hepatic first-pass metabolism and thus reducing the dose related side effects of the drug like myopathy, elevation of cretinine kinase (CK) and rhabdomyolysis [6]. Under the aforementioned circumstances, the current work endeavours to design and characterize an optimal oil-in-water nanoemulsion (o/w) system of Atorvastatin using minimum surfactant concentration, with an aim to increase the solubility and bioavailability.

MATERIALS AND METHODS

Materials

Atorvastatin calcium was kindly provided by Ind Swift Pvt. Ltd. (Chandigarh, India) and Capmul MCM (glyceryl mono/dicaprylate) from Indchem International (Mumbai,India), were received as gift samples. Oleic acid, Soyabean oil and sunflower oil were purchased from Kamani Oil Industries Ltd. (Mumbai, India). Isopropyl myristate (IPM), Olive oil, Polyoxyethylene (20) sorbitan mono oleic acid (Tween80®), Acetonitrile (HPLC grade) and Methanol (HPLC grade) were purchased from Merck (Schuchardh, Hokenbrunn,Germany).Brij 35 (Polyoxyethylene lauryl ether) was purchased from Sigma Aldrich (U.S.A.). Ethanol was purchased from S.D. Fine Chemicals (Mumbai, India). Dialysis bags (Molecular weight cut-off (MWCO), 12 000g/mole) were procured from HiMedia, India. Deionized water for HPLC analysis was prepared by a Milli-Q-purification system. All other chemicals were of analytical grade. Double distilled water was prepared freshly whenever required.

Solubility Studies

The most important aspect for the selection of oils for nanoemulsion is the solubility of poorly soluble drug in oils. An excess amount of drug was added in 2 ml of each oil separately in 5 ml capacity stoppered vials, and mixed using a vortex mixer. These vials were then kept at 25±1.0°C in an isothermal shaker (IKA® KS 400i, Germany) for 72 hours to reach equilibrium. The equilibrated vials were removed from shaker and centrifuged at 10000 rpm for 30 min using centrifuge

(Remi, India). The supernatant was taken and filtered through a 0.45 µm membrane filter. The concentration of atorvastatin was determined in different oils by using HPLC at detection wavelength of 246nm. The HPLC system consisted of a mobile phase delivery pump (LC-20 AD; Shimadzu, Japan), a photodiode array detector (SPD-M20 A; Shimadzu, Japan) and a 20µL loop (Rheodyne). A C_{18} reverse-phase column (Phenomenex Gemini C_{18}, 250 x 4.6 mm i.d., 5µ) was utilized for drug separation, using Acetonitrile-25mM Potassium Dihydrogen Orthophosphate (50:50,v/v), adjusted to pH 6.5 as mobile phase. The mobile phase was pumped at a flow rate of 1.0 ml min^{-1} at an ambient temperature of 25±2 ^oC with retention time of 5.9min. Solubility studies were carried out in triplicate.

Pseudo-ternary phase diagram study

The pseudo ternary phase diagrams consisting of oil, smix (surfactant-co-surfactant mixture) and double distilled water were developed using the aqueous titration method [7]. Surfactant and cosurfactant were mixed in different volume ratios (1:0, 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, and 4:1) selected on the basis of increasing concentration of cosurfactant with respect to surfactant and vice versa. For each phase diagram, oil and a specific smix ratio was mixed properly in different volume ratios from 1:7 to 7:1 in separate glass vials. Thirteen different combinations of oil and smix, (1:1, 1:2, 1:3,1:4, 1:5, 1:6, 1:7, 2:1, 3:1, 4:1, 5:1,6:1,7:1) were slowly titrated with aqueous phase and visually inspected for transparency and flowability. The physical state of the nanoemulsion was marked on the phase diagrams with three axis representing an aqueous phase, oil and smix. For each phase diagram, anoemulsion area was plotted and the wider region indicated the better self nanoemulsifying efficiency. From each phase diagram, constructed, different formulations were selected from nanoemulsion region varying the proportion of oil (10-30%v/v) at minimum concentration of smix. Selected formulations were subjected to stability and dispersibility tests.

Stability tests

Centrifugation and freeze thaw cycling were used to assess the physical stability of the prepared nanoemulsion [8]. The formulations were centrifuged at 3500 rpm for 30min. Those formulations that did not show phase separation were subjected to freeze thaw studies. Three freeze thaw cycles between – 20^oC and +25^oC with storage at each temperature for not less than 24 h was done for the formulations. Those formulations, which passed these thermodynamic stress tests, were further taken for the dispersibility tests.

Dispersibility tests

One ml of each formulation was added to 500 ml of 0.1 N HCl in USP Dissolution apparatus Type II at 37 ± 0.5^oC to assess its efficiency of self emulsification [7]. A standard stainless steel dissolution paddle rotating at 75 rpm provided gentle agitation. The formulation was visually assessed using the following grading system:

Grade A: Rapidly forming (within 1 min) nanoemulsion, having a clear appearance.

Grade B: Rapidly forming, slightly less clear emulsion.

Grade C: Fine milky emulsion that formed within 2 min.

Grade D: Dull white emulsion having slightly oily appearance that is slow to emulsify (longer than 2 min).

Grade E: Formulation, exhibiting either poor or minimal emulsification with large oil globules present on the surface. Among the formulations which passed the stability and also dispersibility tests in Grade A and B were selected for preparing drug loaded batches utilizing minimum concentration of smix for each percentage of oil.

Preparation of atorvastatin loaded nanoemulsion

The drug loaded nanoemulsions were prepared by dissolving 10mg (single dose) of atorvastatin in oil (10%, 15%, 20% and 25% v/v). Respective smix ratio was added to the oil, mixed using vortex mixer and followed by addition of aqueous phase to obtain o/w nanoemulsion.

Physicochemical characteristics

Droplet size analysis

The average droplet size and polydispersity index (PDI) of nanoemulsions were determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion of the particles using a zetasizer ZS 90 (Malvern instruments, UK) [9]. Light scattering was monitored at 25^oC at a 90^o angle.

Samples were diluted 100 times with double distilled water and were directly placed into the module. Three replicate analyses were carried out for each formulation, and data presented as mean ± S.D.

Viscosity determination

The rheological property of the formulations was determined as such without dilution using Brookfield DV-II ultra+ viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) using spindle # CPE 40 at 25 ± 0.5ºC. The software used for the calculations was Rheocalc V2.6. Experiments were performed in triplicate for each sample, and results were presented as mean ± S.D.

Electroconductivity studies

Electroconductivity of the resultant system was measured by an electroconductometer (Conductivity meter 305, Systronic). For the conductivity measurements, the tested nanoemulsions were prepared with a 0.01 N aqueous solution of sodium chloride instead of doubled distilled water. The measurements were made in triplicate at 25±1^oC [10].

Refractive index and percent transmittance

The refractive index of the system was measured by an Abbe refractometer (Bausch and Lomb optical company, NY) by placing a one drop of nanoemulsion on the slide in triplicate at 25^oC. The percent transmittance of the system was measured at 650 nm using UV spectrophotometer (Shimadzu, Japan) keeping doubled distilled water as blank [10] .The measurements were made in triplicate.

Drug content

The dose of the drug is well below the saturation point; hence it is presumed that the amount of drug incorporated will be available for the release. Since surfactant and cosurfactant (smix) are added, there are chances of precipitation of the drug. Hence, the drug content was calculated by UV visible spectrophotometer. The formulation was diluted to required concentration using methanol as solvent and the absorbance was measured at 246nm against a solvent blank. Encapsulation efficiency

was expressed as a percentage of atorvastatin found in the system to the theoretical quantity of the drug added. All the measurements were made in triplicate.

Transmission electron microscopy

Transmission electron microscopy (TEM) TOPCON 002B operating at 200 kV was employed to study the morphology and structure of the resulting nanoemulsion. Prior to the analysis, the nanoemulsion samples were diluted 100 times with double distilled water, stained with 2% (w/v) phosphotungstic acid for 30s and placed on carbon-coated grid and observed after drying.

***In vitro* drug release**

The quantitative *in vitro* release test was performed in 500 ml of Phosphate buffer pH 6.8 using USP Dissolution apparatus Type II at 75 rpm and 37±0.5^{o}C using dialysis bag technique. Dialysis membrane (MWCO 12000 g/mole) was soaked in double-distilled water for 12h before use for experiment. Two milliliter of nanoemulsion formulation (containing single dose 10mg of atorvastatin) was placed in treated dialysis bag. Samples (5ml) were withdrawn at regular time intervals (0, 0.5, 1, 1.5, 2, 4, 6, 8 and 12 h) and an aliquot amount of phosphate buffer was replaced. The release of drug from nanoemulsion formulation was compared with the conventional tablet formulation (Ozovas ^{TM} 10, Atorvastatin 10 mg) and the suspension of pure drug. To prepare drug suspension, drug and methylcellulose mucilage (3% w/v) were ground in a mortar to obtain a 5mg/ml drug suspension; this suspension was ultrasonicated for 2 minutes. The samples were analyzed for the drug content using UV-Visible spectrophotometer (Shimadzu, Japan) at 246nm. The *in vitro* drug release data were analyzed by one-way analysis of variance (ANOVA) using Dunnett's test.

Pharmacokinetic Studies

Approval to carry out *in vivo* study was obtained from Institutional Animal Ethics Committee, J.S.S. College of Pharmacy, Ooty, India and their guidelines were followed throughout the studies. The nanoemulsion formulation (NE2), which showed the highest release profile of drug based on *in vitro* studies, was taken for *in vivo* studies. The animals used for *in vivo* experiments were adult male Wistar albino rats (200±20g). Dose for the rats was calculated based on the surface area ratio of a rat to that of human being [11]. Animals were divided into four groups comprising six animals in each group (n=6). The animals of group I served as control. All the animals of group II were given an oral dose of pure drug suspension; group III were given an oral dose of conventional tablet and group IV was given optimized batch of nanoemulsion (NE2) at dose of 9 mg/kg orally using a ball-tipped feeding needle. The animals were kept under standard laboratory conditions, temperature at 25±2^{o}C and relative humidity 55±5%. The animals were kept in polypropylene cages, 6 per cage with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water *ad libitum.* The rats were anesthetized using diethyl ether and blood samples were withdrawn from the tail vein at 0 (pre-dose), 0.5, 1, 2 , 3, 4, 6, 8 and 24 hours in centrifuge tubes containing 0.2 ml of anticoagulant solution (citrate solution), mixed and centrifuged at 5000 rpm for 20 minutes. The plasma was separated and stored at -20^{o} C until drug analysis was carried out using HPLC.

Quantification of plasma concentration

Atorvastatin plasma concentration was determined by HPLC analysis as described above. A 200µl plasma sample was placed into a centrifuge tube and 200 µl of 10% perchloric acid was added and shaken vigorously for 30s at room temperature. After centrifugation at 4000 rpm for 15 min, the supernatant was separated and analyzed. Calibration curves were prepared by linear regression analysis of the plot of the peak area against concentration of atorvastatin. The concentration of plasma samples was determined from the area of chromatographic peak using the calibration curve.

Data analysis

Peak concentration (C_{max}) and time of peak concentration (T_{max}) were obtained directly from the individual plasma concentration-time profiles. The area under the concentration-time curve from time zero to time t ($AUC_{0\rightarrow t}$) and area under mean concentration (AUMC) were calculated using the trapezoidal method. The area under the curve (AUC) determines the bioavailability of the drug for the given the same dose in the formulation. The area under the total plasma concentration–time curve from time zero to infinity was calculated by:

$AUC_{0\rightarrow\infty} = AUC_{0\rightarrow t} + C_t/K_e$

where, C_t is the atorvastatin concentration observed at last time, and K_e is the apparent elimination rate constant obtained from the terminal slope of the individual plasma concentration–time curves after logarithmic transformation of the plasma concentration values and application of linear regression. The relative bioavailability (F_r) at the same dose was calculated as:

$Fr = AUC_{NE,\ 0\rightarrow t} / AUC_{TAB/SUSP,\ 0\rightarrow t}$.

The mean residence time (MRT) was estimated from MRT = $AUMC_{0\rightarrow\infty}/AUC_{0\rightarrow\infty}$.The data obtained from pharmacokinetic parameters were analyzed by one-way analysis of variance (ANOVA) using Tukey test. All the values are expressed as their mean ± S.D.

Storage Stability studies

Three batches of the optimized nanoemulsion formulation, NE2, were prepared and stored at a temperature of 40±2^{o}C and 75±5% R.H. for three months. Samples were withdrawn after specified time intervals (0, 30, 60 and 90 days) and examined visually for any physical change in the formulation. Refractive index, viscosity, droplet size and remaining drug content were determined using UV Visible spectrophotometer at 246 nm [12].

Statistical analysis

The results were expressed as mean values ± S.D. The analysis of variance (ANOVA) was applied to examine the significance of differences in nanoemulsions' properties (such as droplet size, polydispersity index, percent transmittance, refractive index, viscosity, conductivity and drug content). In all cases, $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Solubility Studies

Atorvastatin Calcium lipophilicity and vulnerability to enzymatic degradation restrict its oral bioavailability.

Nanoemulsion exhibited potential to improve oral bioavailability of similar lipophilic drug facing metabolic deterrents, such as ramipril and saquinavir [7, 13]. Solubility studies were aimed at identifying a suitable oil phase for the development of atorvastatin nanoemulsion to achieve optimum drug loading [14]. The higher solubility of the drug in the oil phase is important for the nanoemulsion to maintain the drug in the solubilized form.

In the oil phase tested, the solubility of atorvastatin was found to be highest in oleic acid (55.25±2.69 mg/ml) as compared to other oils (Fig. 1). Thus, oleic acid was selected as the oil phase for the development of the formulation

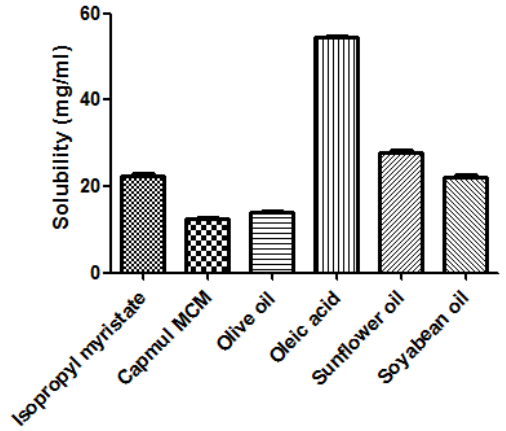

Fig.1 Solubility of Atorvastatin in different oils (*n* =3)

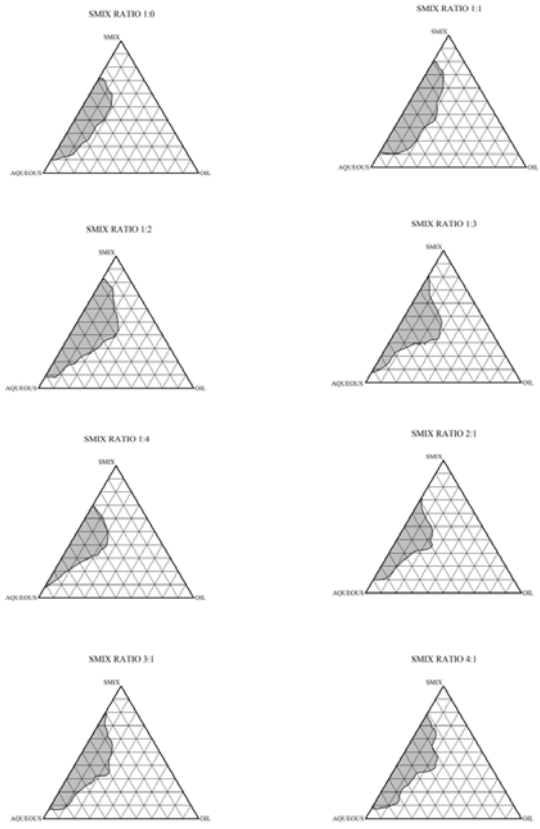

Fig.2 Pseudoternary phase diagrams indicating o/w nanoemulsion region at different smix ratios

Pseudo-ternary phase diagram study

To study the relationship between the components of the nanoemulsion and their phase behaviour, phase diagrams were constructed (Fig. 2a to 2 h) using oleic acid as the oil phase, Brij 35 and Tween 80 as surfactant mixture and ethanol as co-surfactant. In the present study, non-ionic surfactants were selected as they are less toxic, have lower critical micellar concentrations compared to ionic surfactants and offer better *in vivo* stability [15] .Also, the surfactants selected for o/w nanoemulsions should have HLB>10 [16]. In the present study, two surfactants viz. Brij 35 and Tween 80 were selected having HLB values 16.2 and 15 respectively. Further, to obtain nanoemulsion at low concentration of surfactant, ethanol was used as a co-surfactant. The darkened areas enclosed by lines roughly indicate the zone of nanoemulsion formation. The rest of the region on the phase diagrams represent the turbid and conventional emulsions. The optimal nanoemulsion formations can be selected from the nanoemulsion region of the phase diagrams. In Fig. 2a, smix ratio 1:0 it can be observed that when surfactants alone were used without co-surfactant, a low nanoemulsion region was obtained. Probably, when the co-surfactant is absent or present at lower concentrations, the surfactant is not able to sufficiently reduce the o/w interfacial tension. The maximum amount of oil that could be solubilized was 19% (v/v) using 50% (v/v) of smix. As concentration of surfactant increased, solubilization of oil decreased. When co-surfactant was incorporated along with the surfactant in equal proportion, i.e., smix ratio 1:1 (Fig. 2b), a higher nanoemulsion region was observed. This may be due to the addition of co-surfactant leading to further decrease in the interfacial tension, which will lead to increase in the fluidity of the interfacial film, thus increasing the entropy of the system [17].The maximum amount of oil that could be solubilized was found to be 23% (v/v) using 42% (v/v) of smix. On further increase in the co-surfactant concentration, i.e. at smix 1:2 (Fig. 2c), the nanoemulsion region increased in the size as compared to the region in smix 1:0 and smix 1:1. The maximum amount of oil that could be solubilized was observed to be 30% (v/v) using 38% (v/v) of smix. There was slight decrease in nanoemulsion region when smix ratio 1:3 (Fig. 2d) was used, indicating that a proper ratio of smix is important to obtain the wide nanoemulsion region, but the maximum amount of oil that could be solubilized still remains the same as that of 1:2. When co-surfactant concentration was further increased to 1:4 (Fig. 2e), nanoemulsion area decreased considerably making just 24% (v/v) oil solubilized with 38% (v/v) of smix. In contrast, when surfactant concentration was increased as compared to co-surfactant, smix ratio 2:1 (Fig. 2f), the concentration of oil that could be solubilized was increased upto 25% (v/v) using smix concentration of 35% (v/v) but the nanoemulsion area decreased as compared to smix ratio 1:1. On further increasing the concentration of surfactant in the smix to 3:1 (Fig. 2g), it was observed that the nanoemulsion region increased in size as compared to region in smix 2:1. There was no change in the maximum amount of oil that could be solubilized using this smix ratio. No appreciable increase in the nanoemulsion region

was observed on further increasing the proportion of surfactant in the smix ratio 4:1 (Fig. 2 h). The maximum amount of oil that could be solubilized still remained the same as that of 2:1 and 3:1 smix ratio, but relatively at higher amount of 40% (v/v) smix. The surfactant and co-surfactant ratio is a key factor in influencing the area of nanoemulsion region [18]. Smix ratio 1:2 showed the maximum area as compared to other ratios. While going through pseudoternary phase diagrams, oil could be solubilized upto the extent of 30% v/v. Therefore, from each phase diagram different concentrations of oil which could solubilize 10mg (single dose) of atorvastatin was selected at 5% intervals (10%, 15%, 20%, 25% and 30%). So that, largest number of formulations could be selected covering the nanoemulsion area of the phase diagram (Table 1). For each percentage of oil selected, only those formulations were taken from the phase diagram which used minimum concentration of smix for the formation of nanoemulsion.

Stability tests

Nanoemulsions can be differentiated from ordinary emulsions due to their thermodynamically stability [19, 20]. In order to avoid phase separation, creaming or cracking, stability tests like centrifugation and freeze thaw cycle were performed. Those formulations, which survived stability tests (Table 1), were taken for dispersibility test in order to estimate the efficiency of dispersibility.

Dispersibility tests

Since the objective of the present research work was to develop an oral nanoemulsion formulation of atorvastatin, dispersibility studies were of paramount importance. The formulations that passed the dispersibility test in 0.1N HCL in grade A and B (as specified in Table 1) were considered to pass the dispersibility test and were selected for further study.

Table 1- Thermodynamic stability and dispersibility tests of different formulations selected from phase diagrams at a difference of 5%v/v of oil

Smix Ratio	*Oil (%v/v)*	*Smix (%v/v)*	*Aqueous(%v/v)*	*Cent.*	*Freez*	*Dispersibility grade*	*Inference*
1:0	10	13	77	√	X	---	FAIL
	15	25	60	√	√	B	PASS
1:1	10	10	80	√	√	A	PASS
	15	12	73	X	---	---	FAIL
	20	30	50	√	√	B	PASS
1:2	10	16	74	√	√	D	FAIL
	15	20	65	√	√	C	FAIL
	20	27	53	√	√	A	PASS
	25	38	37	√	√	B	PASS
	30	40	30	√	√	C	FAIL
1:3	10	23	67	√	√	A	PASS
	15	28	57	√	√	B	PASS
	20	30	50	√	√	A	PASS
	25	32	43	√	√	A	PASS
	30	35	35	√	X	---	FAIL
1:4	10	21	69	√	√	B	PASS
	15	28	57	√	√	B	PASS
	20	32	48	√	√	A	PASS
2:1	10	15	75	√	X	---	FAIL
	15	30	55	√	√	B	PASS
	20	32	48	√	√	A	PASS
	25	36	39	√	X	---	FAIL
3:1	10	8	82	X	---	---	FAIL
	15	24	61	√	√	A	PASS
	20	32	48	√	√	B	PASS
	25	36	39	√	√	B	PASS
4:1	10	9	81	X	---	---	FAIL
	15	25	60	√	√	A	PASS
	20	35	45	√	√	B	PASS
	25	40	35	√	√	A	PASS

Cent=Centrifugation, Freez=Freeze-thaw cycle

Table 2-Optimized formulations selected from phase diagram at a difference of 5% w/w of oil having least smix concentration

Batch Code	*Smix ratio*	*Oil (%)*	*Smix (%)*	*Aqueous (%)*	*Oil: Smix ratio*	*Dispersibility grade*
NE1	1:3	10	23	67	1:2.3	A
NE2	3:1	15	24	61	1:1.6	B
NE3	1:2	20	27	53	1:1.35	A
NE4	1:3	25	32	43	1:1.28	A

Oil used: Oleic acid; Surfactant used: Tween 80 and Brij 35; Co-surfactant used: Ethanol;
Aqueous phase: Double distilled water

Table 3- Mean (± S,D., *n*=3) droplet size, polydispersity index, percent transmittance, refractive index, viscosity, conductivity and drug content

Batch Code	*Droplet Size (nm)*	*Polydispersity Index*	*Percent Transmittance*	*Refractive Index*	*Viscosity (cP)*	*Conductivity (µS/cm)*	*Drug Content (%)*
NE1	66.4±0.6	0.288±0.022	99.43±0.03	1.352±0.017	18.08±1.14	412.3±3.47	98.62±0.56
NE2	62.2±0.4	0.250±0.032	99.62±0.03	1.354±0.014	20.12±0.95	387.2±2.43	99.41±0.14
NE3	75.9±1.2	0.271±0.025	99.51±0.04	1.366±0.021	23.25±0.97	366.5±1.21	98.44±0.23
NE4	90.1±0.9	0.264±0.036	99.48±0.05	1.412±0.013	28.55±1.16	342.2±4.22	98.84±0.63

The main purpose of the dispersibility test is to evaluate the stability of nanoemulsion upon dispersion in GI fluids. Due to dilution, there may be phase separation eventually leading to precipitation of drug [17]. The optimized formulations were evaluated further for *in vitro* characterization (Table 2).

Droplet size analysis

Droplet size has been found to affect the degree of drug absorption, the smaller the droplet size, the larger the interfacial area for drug absorption [21]. Droplet size of the prepared nanoemulsion was determined and results are shown in Table 3 along with polydispersity indices. From the table, it can be seen that formulation NE2 has smallest particle size (62nm) which was significant ($p < 0.05$) in comparison to other formulations. The minimum droplet size observed in NE2 may be attributed to the composition of smix. In case of NE2, the % of surfactant in smix is higher as compared to other NE formulations. The higher droplet size was observed in case of NE1 compared to NE2 which may be due to the expansion of the interfacial film by the cosurfactant leading to increased droplet size [22].Droplet size analyses of NE3 and NE4 showed that the size increased with the increase in concentration of oil. This may be due to the fact that smix is unable to disperse the increased amount of oil. NE2 exhibited smallest PDI of 0.250±0.032. The PDI of all the formulations was less than one, indicating narrow size distribution and was statistically insignificant.

Viscosity

The viscosity of the optimized formulations was determined and results are given in Table 3. As the oil content was increased from 10%v/v to 25%v/v, an increase in the viscosity of the formulations was observed. Overall, the viscosity of the optimized formulations was low as expected for o/w nanoemulsion. NE1 had the minimum viscosity (18.08±1.14 cP), perhaps because of higher aqueous content and results were significant ($p < 0.05$) as compared to formulations NE3 and NE4.

Electroconductivity

Electroconductivity of the optimized formulations was determined to assert the nature of formulation. The measurements were performed with sample containing sodium chloride in the water phase. Formulation NE1 had a significant ($p < 0.05$) difference in electroconductivity compared to other formulations (Table 3). The higher conductivity of NE1 may be due to higher percent of conducting ions in the aqueous media.

Refractive Index (RI)

The RI of the selected formulations was determined using an Abbe type refractometer. It indicates the isotropic nature of the formulation and was found to be in the range of 1.352-1.412. The results (Table 3) indicate that RI values increased with increase in concentration of oil and corresponding decrease in aqueous content. NE 4 exhibited highest RI of 1.412 which was significant in comparison to other formulations ($p < 0.05$). The RI of the developed system was found to be similar to that of the water (1.334).

Transmittance

The transmittance of the developed formulations was found greater that 99% (Table 3). Amongst the selected formulations, formulation NE2 had highest percentage of transmittance which was significant ($p < 0.05$) in comparison to other formulations. The observed transparency of the system is due to the fact that the maximum size of the droplets of dispersed phase is not larger than $1/4^{th}$ of the wavelength of visible light [23]. Thus, nanoemulsions scatter little light and therefore transparent or translucent.

Drug content

Atorvastatin content in the nanoemulsion formulations was analyzed spectrophotometrically at 246 nm, against solvent blank. Drug content of the optimized formulations was found in range of 98.84-99.41%. The drug content varied for upto 0.57% between formulations NE1 to NE4 (Table 3). However, there was no significant ($p > 0.05$) difference in drug content among various formulations.

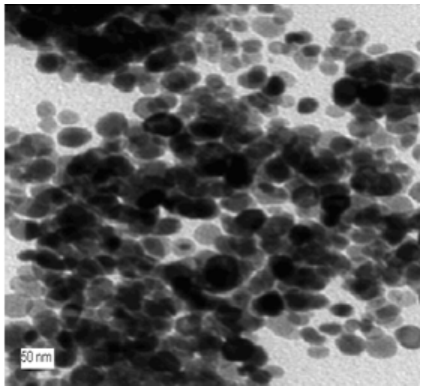

Fig.3 Transmission electron micrograph of atorvastatin nanoemulsion NE2 showing size of oil droplets

Transmission electron microscopy

In order to observe the physical properties of the oil droplets in the nanoemulsion, TEM analysis was carried out with negatively stained samples as shown in Fig. 3, phosphotungstic acid-stained oil droplets were clearly visible and the droplet size correlated well with the results obtained from droplet size analysis using zetasizer. In addition, the morphology of the droplet was spherical and there was no evidence of atorvastatin precipitation in either the oil phase or aqueous phase. This means that atorvastatin was encapsulated in the oil droplet and preferred to remain in the oil phase on addition of water.

***In vitro* release studies**

The release of the drug from the nanoemulsion formulations was extremely significant ($p < 0.001$) in comparison to conventional tablet and pure drug suspension, having same quantity of atorvastatin (Fig. 4). It was observed that NE2 showed 55.84% drug release in 1h compared to tablet and suspension which released less than 18% of the drug at the end of same time. The rate of drug release from formulations NE3 and NE4 was slow in comparison to NE2. This could be attributed to the fact that formulation NE3 and NE4 had higher droplet size, higher viscosity and higher oil content which may restrict the release of highly lipophilic atorvastatin into the medium. Cumulative percent release from NE2 was extremely significant compared to other nanoemulsion formulations. In contrast drug suspension and tablet formulation showed cumulative percent release of 46.28% and 44.1% at the end of 12 hours due to lower aqueous solubility. Therefore, the optimized formulation NE2 having higher drug release, optimal droplet size and minimum polydispersity was selected for the *in vivo* study.

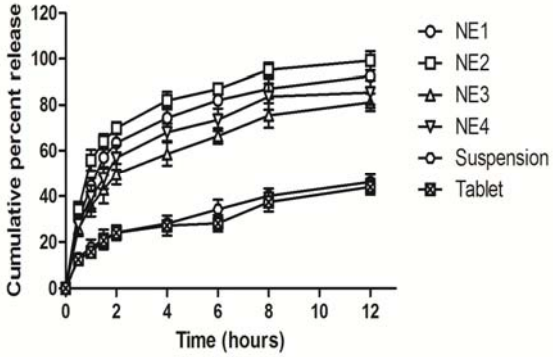

Fig. 4 *In vitro* release profile of atorvastatin from different optimized nanoemulsion formulations (NE1 to NE4), Tablet suspension and Pure drug suspension in Phosphate buffer pH 6.8 ($n = 3$)

Pharmacokinetics Studies

Pharmacokinetic parameters of atorvastatin after oral administration are shown in Table 4. C_{max} and T_{max} of NE2 were11.11 ± 0.99 µg/ml and 2h, respectively, as compared to those of tablet which were 2.501 ± 0.17 µg/ml and 4h and drug suspension 2.725 ± 0.23 µg/mL and 3.33 ± 0.57h, respectively. The difference in C_{max} of NE2 formulation was extremely significant ($p<0.001$) when compared with tablet formulation and drug suspension. Statistically the difference in T_{max} of NE2 was extremely significant ($p<0.001$) when compared to T_{max} of tablet and highly significant ($p<0.01$) when compared to drug suspension. It was also observed that $AUC_{0\rightarrow t}$ and $AUMC_{0\rightarrow\infty}$ of NE2 formulation were 43.96 ± 0.78µg/ml and 233.8 ± 16.58 µg h/ml, respectively. Both the values were extremely significant ($p<0.001$) as compared to tablet and drug suspension. The difference in the values of MRT is not significantly different ($p>0.05$) when the nanoemulsion, tablet or suspension was compared as there is no change in the intrinsic properties of the drug when it is formulated into different formulations.

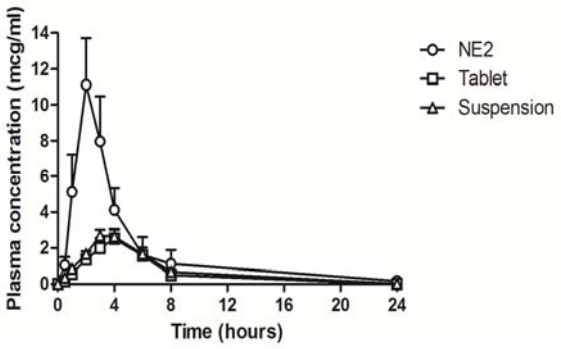

Fig. 5 Plasma concentration-time profile of optimized nanoemulsion formulation (NE2), tablet suspension (Tablet) and pure drug suspension (Suspension) in adult male Wistar rats (n=6)

The relative bioavailability of NE2 to that of conventional tablet and suspension was 2.87- and 2.38-fold higher respectively. It is clear from above results that formulation NE2 was successful in enhancing atorvastatin oral bioavailability and was able to reach maximum concentration in minimum possible time. The plasma concentration-time profile of all the three formulations is given in Fig. 5, which clearly shows the enhanced bioavailability of atorvastatin nanoemulsion over that of marketed tablet and drug suspension.

Table 4- Relative bioavailability and pharmacokinetic parameters of atorvastatin nanoemulsion (NE2), tablet suspension and pure drug suspension (n=6, mean ± S.D.)

Parameter	***Nanoemulsion (NE2)***	***Tablet***	***Suspension***
C_{max} (µg/mL)	11.11 ± 0.99***,ΨΨΨ	2.501 ± 0.17	2.725 ± 0.23
t_{max} (h)	2***,**	4	3.33 ± 0.57
$t_{1/2}$ (h)	3.93 ± 0.2	2.31 ± 0.69	2.66 ± 0.33
$AUC_{0\rightarrow t}$ (µg h/mL)	43.96 ± 0.78***,ΨΨΨ	15.31 ± 1.94	18.47 ± 2.75
$AUC_{0\rightarrow\infty}$ (µg h/mL)	44.90 ± 0.81	15.35 ± 1.98	18.53 ± 2.78
K_{eli} (1/h)	0.176 ± 0.009	0.32 ± 0.11	0.263 ± 0.03
$AUMC_{0\rightarrow\infty}$ (µg h/mL)	233.8 ± 16.58***,ΨΨΨ	72.36 ± 10.88	91.61 ± 11.91
MRT (h)	5.206 ± 0.33	4.717 ± 0.35	4.952 ± 0.16
F_r	2.87	1	---
F_r	2.38	---	1

Statistical analysis was carried out by ANOVA using Tukey's test.
Statistical significance are:
Nanoemulsion vs. Tablet: *** p <0.001
Nanoemulsion vs. Suspension: ΨΨΨ p <0.001 and ** p <0.01

The enhanced bioavailability by the nanoemulsion formulation might be attributed to avoidance of first-pass hepatic metabolism by intestinal lymphatic transport, which circumvents the liver; increased permeability by surfactants, and inhibition of P-glycoprotein efflux mechanism.

A number of studies have reported an improvement in oral absorption of poorly soluble drugs by co-administration of various P-glycoprotein inhibitors [24]. Tween 80 is an inhibitor the P-glycoprotein efflux system, leading to improved oral absorption of atorvastatin [25]. The drug present in the solubilized form as nanolipid globules provides large interfacial area for drug absorption. Furthermore, the presence of surfactants Tween 80 and Brij 35, in nanoemulsion system in the GI tract might have caused changes in membrane permeability which could lead to enhancement of the oral absorption of drug. The absorption enhancing effects of lipids on pharmaceutical actives is well known. Extensive studies on the effect of lipids of absorption have been performed by various research groups. After simultaneous administration of lipid and drug, the lipids are degraded by the enzymes in gut forming surface active mono and diacylglycerols which can solubilize a poorly soluble drug [26] .The use of nanoemulsion opens up new perspectives for the formulation of poorly soluble drugs.

Stability studies

The stability studies revealed that the samples stored at 40±2^{o}C and 75±5% R.H. had stable droplet size for 3 months. There was no significant ($p > 0.05$) change in refractive index, viscosity and drug content (Table 5).

Table 5-Mean (± S.D., $n = 3$) refractive index, viscosity, droplet size and drug content in nanoemulsion NE2 stored at 40±2 ◦C and 75±5% RH.

Time (in days)	*Refractive index*	*Viscosity (cP)*	*Droplet size (nm)*	*Drug content (%)*
0	1.351±0.014	20.12±0.95	62.2±0.4	99.41±0.14
30	1.354±0.008	20.14±0.46	62.4±0.6	99.34±0.06
60	1.356±0.006	20.18±0.34	63.2±0.5	99.24±0.08
90	1.358±0.008	20.21±0.54	63.6±0.8	98.73±0.21

CONCLUSION

In this investigation, lipid nanoemulsion containing atorvastatin was successfully optimized based on physicochemical parameters, *in vitro* and *in vivo* performance. The absorption of atorvastatin from nanoemulsion resulted in 2.87- and 2.38-fold increase in relative bioavailability as compared to conventional tablet and pure drug suspension respectively. The dose of atorvastatin nanoemulsion needs to be corrected in accordance with increased bioavailability; to minimize its dose related adverse effects. Results from stability studies indicate stability of optimized formulation, as there was no significant change in observed physical parameters. Our studies demonstrate that the lipid nanoemulsion formulation is the promising strategy for the formulation of lipophilic compounds with low oral bioavailability.

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