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Research Article

A Novel Anionic Atorvastatin Loaded Nanostructured Lipid Carriers for Oral Delivery: Formulation Development, *In Vitro* and *In Vivo* Pharmacodynamic Study

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ABSTRACT

Atorvastatin calcium (ATR) is the choice of an anti-hyperlipidemic agent having only 12% oral bioavailability because of its poor aqueous solubility (log *P* 5.7) and extensive first-pass metabolism. The prime motivation for this work was to develop nanostructured lipid carriers (NLCs) to enhance solubility and avoid first-pass metabolism on oral administration of ATR. Amongst various lipids, stearic (solid) and oleic acid (liquid) were screened to prepare five different batches of ATR loaded lipidic nanoparticles (ATR-LPs) by employing hot homogenization technique for four homogenization cycles and at a pressure of 500 bar. Results concluded that as content of oleic acid increased from 0-30%, there was a decrease in particle size with increase in zeta potential, entrapment efficiency, and drug loading capacity. The optimized ATR-NLCs showed mean particle size of 147.8±7.4, PDI of 0.211±0.050 with a zeta potential of -22.5±3.64 mV. Compatibility was studied by Fourier transform infrared (FTIR) spectroscopy technique. Powder X-ray diffraction and differential scanning calorimetry studies which revealed that the crystalline ATR has converted into an amorphous form in ATR-NLCs. SEM photomicrographs confirmed the non-spherical shape of ATR-NLCs. *In vitro* dissolution study of ATR-NLCs established the biphasic release pattern. Triton-induced hyperlipidemic model was used for examining *in vivo* pharmacodynamic activity of ATR-NLCs, results of which was found to be significant than plain ATR suspension, making NLCs as a robust, promising perspective colloidal vehicles for oral delivery of ATR. Accelerated stability studies proved the robustness of ATR-NLCs on three months storage at 25±2 °C/60±5 % RH.

INTRODUCTION

Nanotechnology is an emerging interdisciplinary technology and widely used as a drug carrier system, which is designed in such way that it can achieve adequate stability, improved absorption, controlled release, quantitative transfer and, therefore, the expected pharmacodynamic activity. Polymeric nanoparticle was the first emerging nanotechnology for the enhancement of solubility and thereby bioavailability thus in our previous research work polymeric nanoparticles were investigated [1].

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Despite their interesting properties, not many products made it to market because of the presence of solvent residues left over from production, the cytotoxicity of the polymers, the lack of low-cost, and unavailability of some good techniques for the production of nanoparticles at large scale [2].

In the present scenario, oral drug delivery is continuously looking into newer avenues and since the last two decade, the oral drug delivery has taken a new dimension with the increasing application of lipid as a carrier for the delivery of poorly water soluble, lipophilic specially BCS class II drugs. The unique properties of lipids reported recently made them very attractive candidature as carriers for oral formulations [3]. The emerging field of lipid-based oral drug

delivery systems is expected as promising carriers because of their potential to increase the solubility and improve oral bioavailability of poorly water soluble, lipophilic drugs and has attracted considerable academic attention [3, 4].

Lipids are typically classified according to their chemical structure, polarity, character and degree of interaction with water. When accessing lipids as vehicles, it is useful to consider them in terms of their digestibility. Non-digestible lipids such as mineral oil (liquid paraffin) and sucrose polyesters essentially remain unabsorbed in the intestinal lumen, and can actually limit drug absorption by retaining a proportion of the co-administered drug. Digestible lipids consist of dietary lipids (glycerides, fatty acids, phospholipids, and cholesterol esters) as well as various synthetic derivatives [5, 6].

The gastrointestinal tract is richly supplied with blood and lymphatic vessels. Since the rate of fluid flow in portal blood is about 500 fold higher than that in intestinal lymph, the majority of the dietary compounds are transported to portal blood [7]. It is now well established that many lipophilic compounds are absorbed, to a certain extent, via the lymphatic route following oral administration [8]. Various mechanisms of targeting drugs to intestinal lymphatics include a paracellular mechanism, transport through M cells of Peyer patches and transcellular mechanism. Among these, the transcellular mechanism is the most relevant for the transport of lipid carriers [7] .

Lipid drug delivery system offers versatility for drug administration as they can be formulated as solutions, suspensions, emulsions, self-emulsifying systems, and microemulsions. Lipid nanoparticles (LPs) such as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are colloidal systems. SLNs were developed in 1991 as a first generation of LPs and have been explored for many other drug delivery applications thus in our previous research work SLNs were investigated [9]. NLCs are the second generation of LPs and overcome the limitations associated with the SLNs. They are considered smarter, a new type of submicron particulate drug delivery system produced from a blend of solid lipid with liquid lipid. The resulting matrix of the lipid particles shows a melting point depression compared to the original solid lipid; however, the matrix remains solid at body temperature [10]. In contrast, SLN being yielded from solid lipid or blends of solid lipids, the incorporation of liquid lipids to solid lipids leads to massive crystal order disturbance and yields NLCs. The resulting matrix shows great imperfections in the crystal lattice and leaves enough space to accommodate drug molecules, leading to improved drug loading capacity, preventing the risk of gelation and avoiding drug expulsion or leakage during storage (long term stability) caused by lipid polymorphism. They have the ability to form highly concentrated dispersions (dispersions with solid contents from 30–80%) is one of the major potential advantages of NLCs over SLNs [2, 10]. Some common advantages are high oral bioavailability, use of physiologically tolerated lipids exhibiting low systemic toxicity, large scale production, protection of drugs from degradation, avoidance of organic solvents, suitable for sterilization, controlled release characteristics and no problems with multiple routes of administration, such as oral, intravenous, pulmonary and transdermal administration [11-13]. NLCs can be employed as an alternative to liposomes, microemulsions, self-emulsifying drug delivery systems, SLNs and polymeric nanoparticles [7]. Due to the lipophilic nature of the matrix produced, NLCs are considered more useful for the oral administration of lipophilic drugs by considering their absorption via intestinal lymphatic transport system [14].

Atorvastatin calcium (ATR) [R-(R*, R*)]-2-(4-fluorophenyl)-ß, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenyl amino)carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate is a selective and competitive inhibitor of hydroxyl methyl glutaryl-coenzyme A reductase (HMG-Co A reductase). HMG-Co A reductase catalyzes the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-Co A) to mevalonate, which is rate-limiting step in hepatic cholesterol biosynthesis. Inhibition of the enzyme decreases *de novo* cholesterol synthesis, increasing expression of low-density lipoprotein receptors (LDL-receptors) on hepatocytes. This increases LDL uptake by the hepatocytes, decreasing the amount of LDL-cholesterol in the blood. It is used in the treatment of hyperlipidemia, including hypercholesterolaemias and combined (mixed) hyperlipidemia (type IIa or IIb hyperlipoproteinaemias), hypertriglyceridemia (type IV), and dysbetalipoproteinemia (type III). ATR can be effective as adjunctive therapy in patients with homozygous familial

hypercholesterolemia who have some LDL-receptor function. It is also used for primary and secondary prophylaxis of cardiovascular events in patients with multiple risk factors, including diabetes mellitus. ATR is rapidly absorbed from the gastrointestinal tract. However, the oral bioavailability of ATR is about 12% due to extensive first-pass metabolism in the liver, and it belongs to the Biopharmaceutical Classification System (BCS) "Class II" drugs (i.e., low solubility and high permeability) having log *P* value and mean plasma elimination half-life of 5.7 and 14 h, respectively [15]. All above parameters make ATR promising drug for formulation into NLCs. An alternative parenteral route of administration could have provided greater bioavailability, however, until the date for ATR, not a single parenteral formulation is reported in the pharmaceutical market. Delivering the existing drug molecules, by using advanced technology will be a more preferred strategy to improve its therapeutic efficiency. Thus in the present study, it was undertaken to formulate ATR-NLCs and conquer its extensive first pass metabolism. Nano-sized lipid based drug delivery system gains direct access to blood circulation via the intestinal lymphatics instead of portal circulation leading to avoidance of first-pass metabolism. Transportation of such lipid based drug delivery system through intestinal lymphatic has been already worked out and reported by Aji Alex et al, [7].

In present investigation ATR-NLCs were prepared by high-pressure homogenization technique because of its prominent advantages such as its simplicity, ability for production at industrial scale up, avoids the use of organic solvents and it produces the narrow and uniform size of nanoparticles [12]. Furthermore, it was also reported by Mehnert and Mader [16] that high-pressure homogenization is a more effective method for the production of submicron sized dispersions of solid lipids compared to high shear mixing or ultrasound and dispersions produced by this method have low microparticle content. Hu et al. successfully used stearic acid (solid lipid) and oleic acid (liquid lipid) to prepare NLCs with high-pressure homogenization [17]. Physicochemical characteristics of ATR-NLCs were investigated in detail. The role played by the oily component of the NLC was also judged by comparing the particle size, zeta potential, entrapment efficiency (EE %) and even with *in vitro* release study. The surface morphology of the ATR-NLCs was assessed by using scanning electron microscopy. The study was further extended to assess the *in vivo* pharmacodynamic activity of ATR-NLCs in male Albino Wistar rats.

EXPERIMENTAL

Materials

ATR was a generous gift from Biochem Pharmaceutical Industries Ltd., Mumbai, India. Stearic acid and poloxamer 188 were procured from HiMedia Lab. Pvt. Ltd., Mumbai, India. Oleic acid was purchased from S.D. Finechem Ltd., Mumbai, India. Soya lecithin (Phospholipon 90® G) was received as a kind gift from PHOSPHOLIPID GmbH Nattermannallee, Germany. Methanol and other reagents used were of analytical grade. Water used in all the studies was double distilled and filtered through 0.22 µm nylon filter paper before use.

Screening of Components

Selection of components for development of ATR-NLCs was based on the solubility of ATR in them. The solubility of ATR was determined in different solid lipids, liquid lipids, and surfactants [10].

Table 1: Screening of different solid lipid, liquid lipid and surfactant for ATR

Solubility of ATR in different liquid lipids, surfactants and solid lipids	
Ingredients	**Solubility (mg/g) [a]**
Liquid lipids	
Oleic acid	29.23 ± 3.4
Castor oil	24.14 ± 2.5
Soya bean oil	19.06 ± 2.7
Surfactants	
Poloxamer 188	284.74 ± 20.11
Tween 80	259.27 ± 11.34
Tween 60	243.45 ± 9.16
Tween 20	231.15 ± 18.96
Amount of solid lipid required to solubilize 50 mg of ATR	
Solid lipids	**Amount (g) [a]**
Stearic acid	1.53 ± 0.05
GMS	2.00 ± 0.50
Precirol ATO 5	2.33 ± 0.28
Bees wax	2.66 ± 0.28

[a] Data represented as mean ± standard deviation, (n = 3).

An excess of ATR was added individually to liquid lipid and surfactant listed in Table 1 (5 mL each) in screw-capped tubes. After 24 h, each sample was centrifuged and 0.5 mL of the clear supernatant layer was diluted suitably with

methanol and analyzed at 241 nm by UV-visible spectrophotometer (UV 1700, Shimadzu, Japan). One of the most important factors that determine the loading capacity of the drug in the lipid is the solubility of the drug in melted lipid. However, equilibrium solubility studies cannot be carried out in this case. Hence, a modified method described by Joshi and Patravale [18] was used to select the solid lipid having better solubilization potential for ATR. For studying the solubility in solid lipids (Table 1), accurately weighed 50 mg of the ATR was taken in a test tube; the solid lipid was added in increments of 0.5 g, and the test tube was heated in a controlled temperature water bath kept at 80 °C till the clear melt was achieved. By this way, the quantity of lipid required to solubilize 50 mg of ATR was estimated.

Preparation of ATR loaded NPs

ATR loaded NLCs were prepared by hot high-pressure homogenization technique according to the method previously reported by Muhlen et al, [19]. All the five formulations were prepared as shown in **Table 2**.

Stearic acid (F1) or mixture of stearic with oleic acid (F2 to F5) were prepared by adding oleic acid to stearic acid (**Table 2**) and then heated together with soya lecithin in water bath at 80 °C to form a uniform and clear oil phase, ATR (0.05 % w/v) was incorporated in it. Simultaneously, the aqueous phase was also separately prepared by dispersing poloxamer 188 in double distilled water and heated to the above same temperature [17]. Then the hot oil phase was added dropwise to the aqueous phase maintained at 80 °C under magnetic stirring (Remi Instruments Ltd., Mumbai, India) at 600 rpm to form pre-emulsion. Then the final ATR-NLC dispersion was produced by passing this hot pre-emulsion through a high-pressure homogenizer (Panda 2K, Niro Soavi, Italy).

Determination of particle size, PDI and zeta potential of the ATR-LPs

Mean particle size of ATR-LPs and Polydispersity index (PDI) as a measure of the width of particle size distribution was determined by photon correlation spectroscopy (PCS) using a Zetasizer (Nano ZS 90, Malvern Instruments, UK). ATR-NLC formulations were diluted with double distilled water to get optimum 50–200 kilo counts per second (kcps) for measurements. Based on the Smoluchowski equation, the surface charge of the LPs was determined by measuring the zeta potential using the same equipment. Zeta potential measurements were run at 25 °C with an electric field strength of 23 V/m [1, 9, 19-21].

Determination of percent entrapment efficiency (EE %) and drug loading capacity (DL %) of ATR-LPs

Percent EE of ATR-NLCs was calculated by measuring the concentration of the unentrapped or free drug in suspension [22, 23]. About 2 mL of the dispersion was placed in the Eppendorf tubes and centrifuged at 15,000 rpm for 45 min at 4 °C (Remi Instruments Ltd., Mumbai, India). The amount of ATR in the aqueous phase was estimated spectrophotometrically at λ_{max} 241 nm. The EE % and DL % of all formulated batches were calculated using the following Eqs. (1) and (2), respectively;

$$EE\ (\%) = [(W_{Total} - W_{Free})/W_{Total}] \times 100 \quad \text{..... Eq. (1)}$$

$$DL\ (\%) = [(W_{Total} - W_{Free})/W_{Lipid}] \times 100 \quad \text{..... Eq. (2)}$$

Where, W_{Total}, W_{Free}, and W_{Lipid} are the weight of total drug in NLC, the weight of free drug in the aqueous phase and the weight of lipid used in the system, respectively.

Solid state characterization of ATR-NLCs

Fourier transform infrared (FTIR) spectroscopy studies

Fourier transform infrared (FTIR) spectra of pure ATR, stearic acid, a physical mixture of ATR and stearic acid and optimized ATR-NLC formulation were obtained on FTIR spectrophotometer (8400S, Shimadzu, Japan) using the KBr disk method (2 mg of sample in 200 mg of KBr). The scanning range was 400–4000 cm^{-1}.

Differential scanning calorimetry (DSC) studies

DSC analysis was performed of pure ATR, bulk stearic acid, a physical mixture of ATR and stearic acid in 1:1 ratio and freeze dried ATR-NLCs of the optimized batch using differential scanning calorimeter (DSC 1 STAR^e System, Mettler-Toledo, Greifensee, Switzerland). Accurately weighed 2 mg of each sample was placed in aluminum pans and sealed with a lid. In the scanning process, a heating rate of 10 °C/min was applied in the temperature range from 35 to 300 °C (except for stearic acid, because of its low melting point, temperature range was kept from 35 to 100 °C) with a nitrogen flow of 5 mL/min.

Table 2: Composition and physicochemical characterization of ATR-LPs

Formulation code	Composition of different ATR loaded LPs formulations (%, w/v)			
	Total lipid (g)	Ratio of liquid lipid to solid lipid	Surfactant concentration	Co-surfactant concentration
F1 [b]	2	0:100	1	1
F2 [b]	2	5:95	1	1
F3 [b]	2	15:85	1	1
F4 [b]	**2**	**30:70**	**1**	**1**
F5 [b]	2	45:55	1	1
	Physicochemical Characterization of different ATR loaded LPs			
	Mean particle size (nm) [a]	**PDI** [a]	**Zeta potential (mV)** [a]	**EE %** [a]	**DL %** [a]
F1 [b]	372.8 ± 12.5	0.278 ± 0.034	-7.48 ± 1.37	46.67 ± 1.59	11.5 ± 0.40
F2 [b]	289.0 ± 8.3	0.221 ± 0.064	-12.5 ± 2.32	61.22 ± 1.53	15.3 ± 0.35
F3 [b]	221.6 ± 10.1	0.218 ± 0.062	-17.6 ± 3.72	67.53 ± 2.31	16.88 ± 0.14
F4 [b]	**147.8 ± 7.4**	**0.211 ± 0.050**	**-22.5 ± 3.64**	**76.34 ± 0.58**	**19.08 ± 0.22**
F5 [b]	152.21 ± 8.2	0.357 ± 0.058	-40.1 ± 4.25	65.47 ± 1.73	17.12 ± 0.30

[a] Data represented as mean ± standard deviation, (n = 3); [b] In each formulation ATR 50 mg (i.e. 0.05 % w/v) was used.

Table 3: Pharmacodynamic evaluation (*in vivo)* of optimized batch (F4) of ATR loaded-NLCs

Treatment group	Triglyceride (mg/dL) [a]	Cholesterol(mg/dL) [a]	HDL(mg/dL) [a]	LDL(mg/dL) [a]
Phase I (24 h)				
Control	310 ± 10.51	449.34 ± 5.64	32.17 ± 1.26	335.17 ± 4.63
Test	223.8 ± 3.05*	314.9 ± 9.19*	67.54 ± 1.79*	202.62 ± 9.59*
Standard	264.9 ± 7.28	368.5 ± 5.48	48.68 ± 2.60	266.84 ± 4.89
Normal	90.17 ± 8.14	151.9 ± 8.37	62.33 ± 1.03	71.54 ± 8.03
Phase II (48 h)				
Control	254.9 ± 4.42	329.2 ± 6.45	44.59 ± 1.29	183.63 ± 5.62
Test	123.53 ± 7.14*	212.1 ± 8.60*	73.39 ± 3.97*	105.8 ± 8.63*
Standard	230.79 ± 4.28	316.3 ± 7.19	51.82 ± 8.16	165.32 ± 8.39
Normal	95 ± 4.14	143.5 ± 6.78	68.43 ± 2.36	78.27 ± 7.32

[a] Data represented as mean ± standard deviation, (n = 6).* indicates significant. Statistically significant difference between test group and control group.

Powder X-ray diffraction (PXRD) studies

Powder X-ray diffraction (PXRD) was performed to analyze crystalline or amorphous nature of ATR loaded NLCs. They were performed by powder X-ray diffractometer (Bruker AXS, D8 Advance, Germany) using the Cu-Kα line as a source of radiation. The samples were scanned over a 2θ scale at a scan rate of 3°/min. Samples used for the study were pure ATR, stearic acid, a physical mixture of ATR and stearic acid and lyophilized ATR-NLCs.

Scanning electron microscopy (SEM) studies

The surface morphology of NLCs was visualized by scanning electron microscopy (LEO 440i, Leo Electron Microscopy Ltd., Cambridge, UK). The pure ATR and optimized freeze dried ATR-NLC formulation was kept onto a metal plate and dried under vacuum to form a dry film which was then observed under the scanning electron microscope.

Determination of *in vitro* drug release from ATR LPs

The *in vitro* drug release from all ATR-LPs (i.e. ATR-SLNs F1 whereas ATR-NLCs F2 –F5) were carried out in phosphate buffer saline (PBS) solution (pH 6.8) by using the dialysis bag diffusion technique. As reported earlier by Liu et al, [24], dialysis bag (molecular weight cut off 12–14 kDa) was equilibrated in PBS medium for 12 h prior to study. Dialysis bag was sealed from one end; 2 mL of NLCs dispersion was poured into it and tightly sealed from another end. Then it was

placed in a beaker containing 200 mL PBS media at 37 ± 2 °C and magnetically stirred at 50 rpm. At predetermined time intervals up to 48 h, 5 mL of samples were withdrawn by filtration through 0.22 µm filter (Millipore, USA) and sink condition was maintained by replacing with fresh PBS solution of the same temperature. The filtrate was suitably diluted if necessary and analyzed by using UV spectrophotometer at λmax 241 nm.

In vivo pharmacodynamic studies (Triton-induced hyperlipidemic model) of ATR-NLCs

The animal experiments were carried out in full compliance with the protocol approved by institutional animal ethical committee (Registration no. IAEC/RCPIPER/2015-16/09 under the (CPCSEA) Committee for the Purpose of Control and Supervision of Experiments on Animals, India). The *in vivo* study was carried out according to a previously reported method by Patel and Vavia [25] and Maurya et al,[26]. Male Wistar rats, weighing 150-200 g, were divided into four groups such as control, test, standard and normal group, each containing six rats. The rats fasted overnight and then (except normal group) intraperitoneally injected with 250 mg/kg of Triton WR 1339 (isooctyl-polyoxyethylene phenol) (Tyloxapol; Sigma Chemical Co, St Louis, MO, US) dissolved in 0.9% saline. Control group of rats was given with vehicle (saline solution), test groups of rats were treated with optimized ATR-NLCs formulation (equivalent to 25 mg/kg ATR), standard groups of rats were treated with plain ATR (25 mg/kg bodyweight), and normal groups of rat were given with normal diet. Without anesthesia and by restraining rats by hand, the oral dosing was performed by intubation using an 18 – gauge feeding needle (the volume to be fed was 1.0 mL in all cases). Blood samples were collected in Eppendorf tubes at 0, 24 and 48 h post oral dose. Serum was separated by centrifugation (Remi Instruments Ltd., Mumbai, India) at 16770 g/min and used for biochemical analysis. Serum cholesterol, triglycerides, and high-density lipoprotein (HDL) were estimated in all above groups as per methods reported by Patel and Vavia [25] and Maurya et al, [26]. Statistical analysis of the collected data was performed using one-way analysis of variance (Kruskal-Wallis test followed by Dunnett's-test) to evaluate the individual differences between the treatment groups.

Accelerated stability studies of ATR-NLCs

The purpose of stability study is to provide evidence on the quality of a drug substance or drug product that varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light. The final optimized ATR-NLC batch (F4) was lyophilized and utilized for carrying out accelerated stability studies according to International Conference on Harmonization (ICH) Q1A (R2) guidelines and previously reported methods [1, 7, 9, 27]. Accelerated stability study was performed with the prime aim to assess the stability of ATR-NLCs at 25 ± 2 °C/60 ± 5% RH (relative humidity) with respect to particle size, zeta potential, and EE %. Freshly prepared a freeze-dried powder of ATR-NLCs was filled in three different amber colored glass vials, sealed and placed in stability chamber (CHM-10S, Remi Instruments Ltd., Mumbai, India) maintained at 25 ± 2 °C/60 ± 5% RH for a period of total three months. The dried powder samples subjected to stability test were re-dispersed in double distilled water and analyzed with a sampling interval of one month for particle size, zeta potential, and EE % of the ATR-NLCs over three months period.

RESULTS AND DISCUSSION

Screening of components

Among the liquid lipids that were screened (**Table 1**), maximum solubility of ATR was found in oleic acid followed by castor oil and soya bean oil. Among the surfactants (**Table 1**), poloxamer 188 proved to be the best followed by Tween 80, Tween 60 and Tween 20. Thus the saturation solubility studies helped to streamline the choice of liquid lipid and surfactant. Of the solid lipids screened, ATR showed maximum solubility in stearic acid as compared to glyceryl mono stearate (GMS), precirol ATO 5, and beeswax (**Table 1**). Thus stearic acid, oleic acid, and poloxamer 188 as the solid lipid, liquid lipid, and surfactant respectively were screened for this study.

Preparation and characterization of ATR loaded LPs

Five different batches of ATR-LPs were successfully developed by hot high-pressure homogenization (HPH) technique. HPH is a reliable and suitable technique for the preparation of lipid nanoparticles was one of the reasons behind its choice. Generally, scaling up of a process encounters several problems. Nevertheless, usage of the larger scale machines

during HPH leads to an even better quality of the product with regard to a smaller particle size and its homogeneity. Additionally, HPH technique is widely used and well-established technique in pharmaceutical and food industry. The homogenizer pressure was optimized to 500 bar for four homogenization cycles based on trial and error process.

The mean particle size, PDI, zeta potential, EE %, and DL % of the resulted ATR-LPs are tabulated in Table 2. Particle size plays a crucial role in the gastrointestinal uptake and their clearance by the reticuloendothelial system. Hence, the precise determination of the particle size is very important. PCS is one of the relatively accurate, sensitive, most powerful and widely used technique for the measurement of particle size of LPs. The formulated ATR loaded LPs showed a decrease in mean particle size with increase in EE % and DL % with an increase in oleic acid content from 0 up to 30% while beyond this level there was a decrease in EE %, and DL % while the increase in mean particle size (F5). These results were inconsistent with the previous studies reported by Agrawal et al, [11] . This might be due to the incorporation of liquid lipids into solid lipids, which leads to massive crystal order disturbance, and the resulting matrix of lipid particles indicates great imperfections in the crystal lattice and leaves enough space to accommodate drug molecules, thus, leading to improved DL % and EE %. A similar kind of result was reported by Hu et al, Jenning et al, and Souto et al, [17, 28-29]. The EE % mainly depends on the nature of the drug and used lipids. As ATR is a highly lipophilic drug (log *P* 5.7) and has a greater solubility in the chosen lipids (conclusion drawn from screening study), the EE obviously was found to be high. Thus, it might ultimately decrease the amount of ATR-LPs in the formulation and attain higher plasma concentration through the lymphatic transport system by avoiding its first pass metabolism. Lecithin was added to the system as co-surfactants so as to enhance the ability to emulsify the lipid and stability of the system. The lecithin was mainly distributed at the interface of the oil and the aqueous phase. ATR-SLNs (F1) showed poor EE and DL % as compared to ATR-NLCs i.e. specially with F4 batch where the concentration of liquid to solid lipid was 30:70. This could be because of the oil (liquid lipid) incorporation in the solid matrix of lipid nanoparticles avoids the crystallization process, producing imperfections in the lattice to load a higher amount of active ingredient in comparison to the "old" SLNs. Thus, proving the NLC system better than SLN.

From the **Table 2**, F4 was selected as the optimized batch. It has least mean particle size. Literature survey revealed that if the mean particle size of lipidic nanoparticles (of both SLNs/NLCs) were below 200 nm, it would be transported via lymphatic transport system instead of portal vein thus avoiding the first pass metabolism. Moreover, small particles ranging between 120 – 200 nm rarely undergo blood clearance by the reticuloendothelial system i.e. liver and spleen filtrations are avoided [30]. Thus, altogether, avoids first pass metabolism that will in turn decrease the dose of ATR-LPs in the formulation and attain higher plasma concentration through the lymphatic transport system.

Lipidic nanoparticles such as SLNs or NLCs are usually polydisperse in nature. The measurement of PDI is important to know the size distribution of LPs. It was also measured by using PCS. The lower the PDI value, the more monodispersed is the nanoparticulate dispersion. The PDI of F4 batch was found to be 0.211 ± 0.050. The PDI value is below 0.3 is always considered as narrow and optimum [13, 30]. PDI of F1 to F4 batches was optimum but for F5, it was found more than 0.3.

Stability of nanodispersion during storage can be predicted from the zeta potential values. It indicates the overall a charge a particle acquires in a specific medium. Zeta potential indicates the degree of repulsion between close and similarly charged particles in the dispersion. Zeta potential of all the lipidic nanoparticles was reported in **Table 2**.

To stabilize these systems, both non-ionic and ionic (cationic, anionic and zwitterionic) surfactants are often used in combinations to increase the extent of the microemulsion region. When surfactants are dispersed in water or oil, they self-associated into different equilibrium phases, depending on both inter- and intra-molecular forces and entropic factors. On incorporation of surfactants into immiscible mixtures of oil and water, the location of surfactants at the oil/water interface is thermodynamically favourite [4]. The non-toxic, non-ionic surfactant, Poloxamer 188 (polyethylene–polypropylene glycol) was used in combination with zwitterionic lecithin as cosurfactant. The zeta potential of optimized

ATR-NLCs (F4) was found to be - 22.5 ± 3.64 mV indicating good stability. The surface charge of the different samples was consistently negative. Thus, this negative (anionic nanoparticle) could be because of used lipids [17]. Zeta potential value in the range of – 30 to + 30 mV is good for stabilization of nanodispersion [30]. It was also measured by using PCS. Thus, F4, optimized batch of ATR-NLCs was only carry forwarded for further study. It was subjected for lyophilization and freeze dried ATR-NLCs were utilized for further study except *in vitro* drug release study.
If we compare all the developed formulations on the basis of composition (Table 2) F1 was SLNs while F2 to F5 were of NLCs. Results reported in Table 2 were once again proved that NLCs are best than SLNs.

Indeed, it has been reported by Mehnert and Mader and Santos-Magalhaes et al, [16, 31] that lipid nanoparticles stabilized with surfactant mixtures such as Poloxamer 188 and soya phospholipid have a lower particle size and higher storage stability compared to formulations prepared with an alone surfactant due to the surface-active property of the surfactants. In the preliminary study, the use of alone was proven insufficient as the layer of dispersed lipid melt stuck to the container's wall and was unable to be completely homogenized. When a combination of surfactant and a co-surfactant was used, a complete homogenization could be achieved. The mixture of a lipophilic (soya lecithin) and hydrophilic (poloxamer 188) surfactants in a molar ratio (1:1) was helpful in promoting the stabilization of the system was previously reported by Jenning and Gohla [28]. Results clearly suggested that 1% of poloxamer was sufficient to cover the surface of nanoparticles and thereby preventing agglomeration during the homogenization process.

Fourier transform infrared (FTIR) spectroscopy

Results of FTIR study showed characteristic peaks of ATR between 3700 and 3000 cm^{-1}, specifically at 3670, 3363, 3055, 1728 cm^{-1} in Fig. 1(A). The peak at 3670 cm^{-1} indicated free O-H stretching, other peaks at 3363 cm^{-1} (N-H stretching), 3055 cm^{-1} (symmetrical O-H stretching) were seen in the ATR spectra. All the above characteristic peaks of ATR were not appeared in the spectra of the physical mixture and ATR-NLCs indicating to the molecular dispersion of crystalline ATR in stearic acid (lipids).

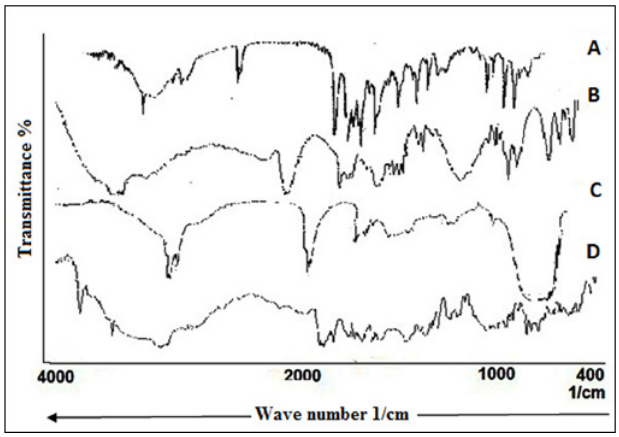

Figure 1: FTIR spectra of A) pure ATR, B) stearic acid, C) physical mixture of ATR and stearic acid, D) lyophilized ATR loaded NLCs.

The intensity of characteristic peaks of ATR was found to be reduced in the physical mixture and ATR-NLCs as seen in Fig. 1(C) and (D) which may be due to traces of ATR in physical mixture and NLCs. When we compare Fig. 1(C) and (D), the poor intensity characteristic peaks of ATR were rare in Fig. 1 (D) as while more in Fig. 1 (C) clearly indicated that more traces were in physical mixture than ATR-NLCs.

Differential scanning calorimetry

The DSC thermograms of bulk ATR, a physical mixture of ATR and stearic acid, bulk stearic acid, and lyophilized ATR-NLCs are depicted in Fig. 2. ATR showed a characteristic sharp endothermic peak at 152.15 °C and the DSC thermogram of the stearic acid showed an endothermic peak at 53.36 °C. The lyophilized ATR-NLCs showed a very small and not sharp endothermic peak around 163.83 °C. This small and not sharp endothermic peak indicates that few traces of ATR may present in ATR-NLCs and most ATR molecules converted from crystalline form of the drug to the amorphous form i.e., maximum entrapment of ATR in the lipid matrix. It was also concluded that ATR is molecularly dispersed in the lipid matrix, indicating its reduction in crystallinity as the peak intensity of the drug was found to be reduced as shown in Fig. 2.

Powder X- ray diffraction (PXRD) studies

X-ray diffractograms of pure ATR, stearic acid, a physical mixture of ATR and stearic acid and freeze dried ATR-NLCs are presented in Fig. 3. The X- ray diffractogram of ATR has sharp peaks at diffraction angles (2θ) 6.24°, 9.26°, 10.36°, 11.92°, 15.38°, 17.10°, 19.54°, 21.70°, 22.75° and

23.38° showing a typical crystalline pattern (Fig. 3A). Fig. 3 B showed the peaks for stearic acid only. In the physical mixture, the few peaks for ATR and stearic acid was evident in Fig. 3(C) but the sharpness of ATR peaks was poor as compared to Fig. 3A. Whereas the ATR-NLC formulation showed the absence of all above peaks for ATR, indicating its conversion from crystalline to amorphous state i.e. molecular dispersion state as shown in Fig. 3(D). DSC studies already supported the same hypothesis, which was further confirmed by X- ray diffractometry.

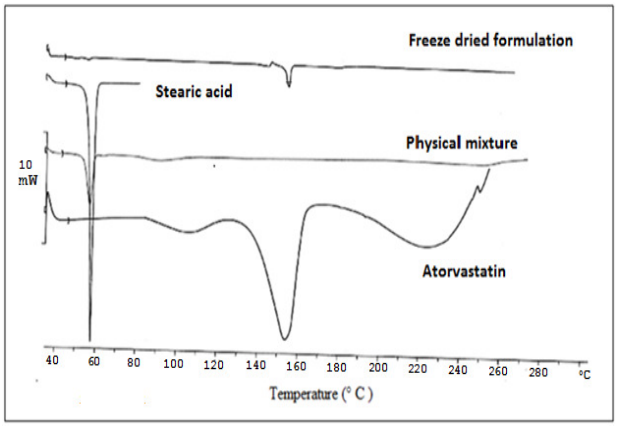

Figure 2: DSC thermogram of Pure ATR, physical mixture of stearic acid and ATR, stearic acid, freeze-dried ATR-NLCs

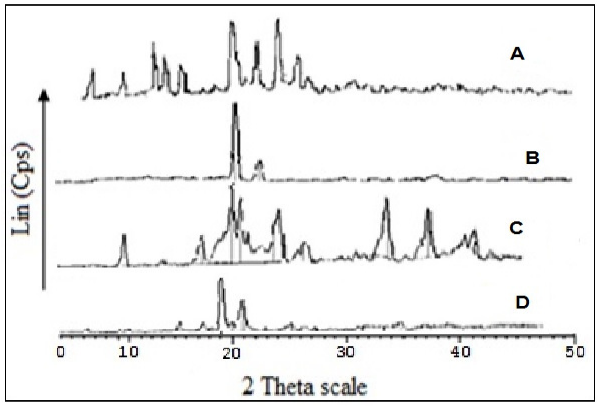

Figure 3: PXRD diffractogram of A) pure ATR, B) stearic acid, C) physical mixture of ATR and stearic acid, D) freeze-dried ATR-NLCs.

Scanning electron microscopy (SEM) studies

Scanning electron photomicrographs showed long platy crystals in Fig. 4 (A) for pure drug ATR, whereas the ATR-NLCs showed a smooth surface in which drug and lipid get completely fused to form a uniform molecular component indicating conversion of crystalline to amorphous form Fig. 4 (B) after freeze drying.

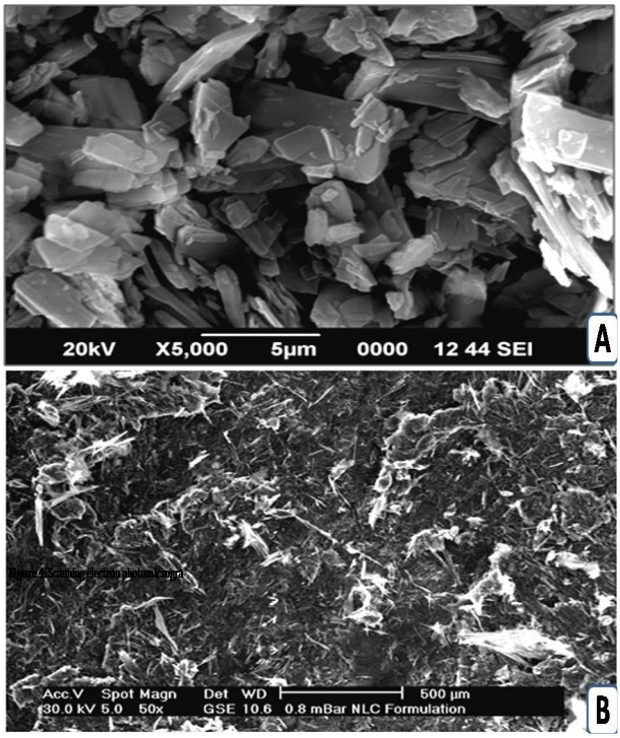

Figure 4: Scanning electron photomicrographs of A) pure ATR, B) optimized lyophilized batch (F4) of ATR-NLCs

The non-spherical shape of ATR-NLCs is clearly visible in photomicrographs. There was no drug crystal or aggregation of particles visible in the photomicrograph Fig. 4 (B).

The lyophilized powder could be re-dispersed in water easily. This would be helpful for the reconstitution of the dry powder and stability during storage. The diameters of the particles observed in the micrographs are in good agreement with the data obtained from Malvern particle size analyzer (Table 2). These ATR-NLCs can preferably be absorbed by Peyer's patches and transported to intestinal lymphatic, bypassing the liver (mean particle size less than 200 nm), thereby, enhancing the oral bioavailability of the drug ATR [30].

In vitro drug release study of ATR-LPs

In vitro drug release study was performed by using the dialysis bag method (diffusion technique). The levels of ATR *in vitro* release from LPs of varying compositions are shown in Fig. 5. The amount of ATR released from each system was plotted as a function of time. The *in vitro* drug release of all the five batches of ATR-LPs showed an interesting biphasic release with an initial burst effect followed by sustained release. However, during the production of lipid nanoparticles, surfactants were also incorporated into the system, which could affect

over the drug release, such as initial burst release of ATR. Similar results were reported previously by Muhlen et al, [19]. If NLCs are prepared with hot high-pressure homogenization (i.e. at high pressure, temperature and high concentration of surfactant), it is believed that these NLCs will produce burst release. This could be the other possible explanation for the burst release, reported by Hu et al, [17]. Further, this type of release could be explained by the large surface area due to the nanosize of the ATR-NLCs with the drug-enriched outer layer NLCs provides a shorter diffusion path of the drug and causes the burst release [32]. Afterward, the drug release followed a steady pattern of sustained release up to 48 h. The low solubility of the ATR in aqueous phase along with the higher half life of drug could be the reasons for this slow and extended release of the drug from the lipid matrices after initial burst release. It was observed from results obtained, as the content of oleic acid in the formulations increased from 0 to 30% (i.e. from ATR-SLNs to ATR-NLCs) there was increase in % of drug release. These results are in accordance with the previously reported study by Hu et al, [17]. This means the oleic acid content is a main factor affecting the drug release rate for ATR-LPs. At the same time, when the oleic acid content increased up to 30 %, the particle size significantly decreased (Table 2), consequently, the specific surface area was increased. Therefore, the fastest release rate in initial stage, emerged in the ATR-NLCs of 30% oleic acid content (F4), was resulted by both of smaller size and higher oleic acid content. However, after that it is interesting to note that the release profiles of five recipes were almost parallel and their slopes tended to be similar with each other. This result revealed that the oleic acid almost did not affect the drug release rate of oleic acid incorporated nanoparticles after initial stage. The oleic acid incorporated nanoparticles possessed a soft and considerable higher solubility for lipophilic drugs like ATR, in which the drug was easily loaded to higher amount and could be easily released as well by the drug diffusion or the matrix erosion manners. Therefore, the oleic acid incorporated nanoparticles showed the burst release at the initial stage and sustained release subsequently.

Among all the ATR-LPs, F4 gave higher initial burst release of 16.45 ± 1.22 % at the end of 2 h (almost double of F1) followed by the prolonged release of ATR of 53.12 ± 1.37%. ATR-SLNs (F1) gave initial burst release of 8.07 ± 1.12 % at the end of 2 h followed by the prolonged release of ATR of 30.07 ± 1.03 %. These results altogether with physicochemical characterization showed that NLCs are always superior to SLNs. NLC showed higher entrapment efficiency due to their liquid parts. In agreement to these results NLC (F4) also showed faster release profile in comparison to SLN (F1).

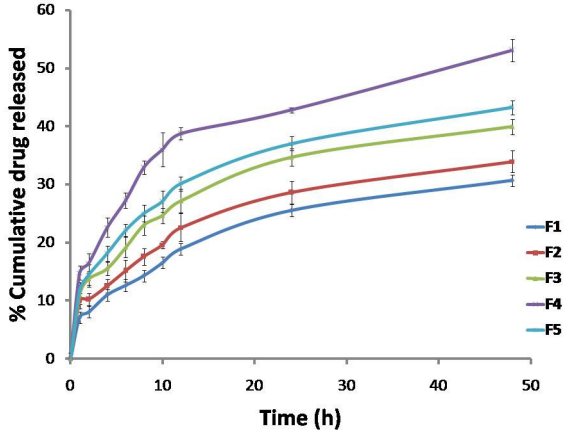

Figure 5: *In vitro* drug release of different batches of ATR-LPs.

In vivo pharmacodynamic studies (Triton-induced hyperlipidemic model) of ATR-NLCs

The study was performed to evaluate the pharmacodynamic potential of developed optimized formulations (F4) against plain ATR using a Triton-induced hyperlipidemia model. Triton is a nonionic surfactant that induces hyperlipidemia by inhibiting peripheral lipoprotein lipase enzymes responsible for removal of lipid particles from the body. The administration of Triton leads to transient elevation of lipid levels, which reach a peak at 18 to 24 hours after administration (phase I) and start to lower again the following day (phase II). It was observed that ATR and its formulation were found to affect the serum lipid level in both phases I and Phase II.

Table 3 gives the effect of treatments on serum lipid levels in phase I (24 h). Plain ATR (in standard group) produced a fall in serum triglyceride, cholesterol, LDL, and rise in protective HDL levels as compared to control group. The ATR loaded NLCs (in test group), as expected, performed better than plain ATR and found significant reduction ($p < 0.05$) in serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to the standard group. It has been reported by Patel and Vavia [25] that there is a natural tapering in

cholesterol and triglyceride values in phase II of the Triton test. Maurya et al, [26] reported similar results previously**.** However, this normal clearance of serum lipid in phase II of the Triton test can also be triggered by the presence of ATR in the circulation, as ATR has long biological half-life of 14 h thus a longer duration of action is guaranteed provided that there is an optimal initial plasma drug level, which is generally determined by the bioavailability of the drug. In phase II of the Triton test, as seen from Table 3, plain ATR (in standard group) lowered serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to control group while ATR loaded-NLCs (in test group), performed better than plain ATR and found significant reduction ($p < 0.05$) in serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to standard group. Thus, the greater lipid-lowering activity of the ATR loaded NLCs in the Triton test might be the probable reason for the faster dissolution of ATR and observed differences in pharmacodynamic activity of ATR-NLCs could suggest enhancement of solubility and thereby oral bioavailability. Earlier low bioavailability of ATR was attributed because of its poor aqueous solubility. As ATR was loaded in NLCs, probably they were transported via intestinal lymphatics (based on lipophilicity and nano size) thus avoiding the hepatic first pass metabolism.

Accelerated stability studies of ATR-NLCs

Accelerated stability studies were conducted on optimized ATR-NLCs (F4) using the particle size, zeta potential, and EE as the prime parameters. There was a slight increase in the particle size during the three months storage from 147.8 ± 7.4 nm to 157.98 ± 3.8 nm with not much change in the zeta potential (i.e. initially which was -22.5 ± 3.64 mV and that after 3 months it was −23.29 ± 2.54 mV). The EE of the optimized batch initially was found to be 76.34 ± 0.58 % while that after 3 months was found to be 74.96 ± 1.14 % indicating that the drug can remain within the nanoparticles for the sufficient period of time. On the storage of ATR-NLCs (F4), there was no significant alteration in the size, zeta potential, and EE % of them. Hence, they were found to be stable at 25±2ºC/60 ± 5% RH for a total period of 3 months.

CONCLUSIONS

In the present study, ATR-SLNs (F1) and ATR-NLCs (F2-F5) for oral administration were successfully prepared to acquire an unmet need of ATR by hot high-pressure homogenization method and could be targeted via intestinal lymphatic transport system. Transportation of such lipid based drug delivery system through intestinal lymphatic's already works out and reported by Aji Alex et al. Recently Chalikwar et al. also reported the intestinal lymphatic transport of nimodipine. The prepared ATR-NLCs found to be narrow and homogeneous in particle size with high EE%. *In vitro* drug release study of designed formulations proved biphasic release pattern of initial burst release followed by sustained release profile of ATR for desired time. *In vivo* pharmacodynamic studies were carried out to compare therapeutic efficacy of prepared ATR-NLCs against pure ATR. Results of analysis of variance demonstrated the significance of suggested model. *In vivo* pharmacodynamic study showed significant ($p < 0.05$) reduction in serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to the standard group in Albino Wistar rats after oral administration. The optimized formulation (F4) showed higher pharmacodynamic potential as compared with plain ATR and found to be stable. The findings of this investigation were statistically significant and conclusively demonstrate the promising role of ATR-NLCs in controlling lipid levels, fostering better patient compliance. These lipid nanoparticles could improve the gastrointestinal absorption of ATR and which could be the probable reason for the remarkable increase in solubility, dissolution rate and thereby may enhance oral bioavailability of ATR. These results altogether with physicochemical characterization showed that NLCs are always superior to SLNs. NLC showed higher entrapment efficiency due to their liquid parts. In agreement to these results NLC (F4) also showed faster release profile in comparison to SLN (F1).

To prove the therapeutic efficacy of ATR-NLCs, a clinical study has to be performed along with the pharmacokinetic study. Then the explored nanostructured lipid carriers can offer a potential and promising approach in improving oral delivery of poorly water soluble drug like ATR. Thus the ATR-NLCs formulation can be a solution not only to avoid first pass metabolism but also to avoid biotoxicity problems as it uses physiological lipids which are generally recognized as safe (GRAS) based on *in vivo* pharmacodynamic activity. Furthermore, clinical studies need to be establishing in patients with hyperlipidemia.

DECLARATION OF INTEREST

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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