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Original Article

A Novel Targeted Nanoliposomal Atorvastatin Transdermal Patch Assisted with Solid Microneedles for Improved Bioavailability

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ABSTRACT

Objectives: The present study investigated the alternate route of delivery to avoid first pass metabolism and to increase bioavailability by nanosized Liposomes (LP) with Microneedles (MNs) Assistance for the Delivery of Atorvastatin (AVT), a HMG CoA reductase inhibitor in which the role of commercial Epyz derma Roller (poke and patch) MN arrays containing 540 Titanium Microneedles (MNs) with dissimilar micro-needle lengths (0.25, 0.5, 1.5, 2.5 mm) in improving the *in vitro* permeation of AVT-LP over pig ear skin was evaluated. **Materials and Methods:** AVT-LP were formulated and optimized using four methods and evaluated for microscopy, % drug entrapment efficiency, drug-excipient compatibility studies, *In vitro* drug release studies, *In vitro* skin permeation studies. Formulation F4 prepared by thin film hydration method with egg lecithin: cholesterol ratio 1:2, found to be having 92.219±0.965 drug entrapment efficiency and 92.53±0.621 drug release within 24 hr with significant physical and chemical stability. The optimized AVT-LP formulation F4 was prepared into a transdermal patch and using Franz diffusion cells, *in vitro* skin permeation studies were evaluated for a period of 24 hr. **Results and Discussion:** The optimized AVT-LP, were <100 nm in diameter and showed an ordered state of good lamellarity. From the dermatokinetic study it was noticeable that the delivery of MN assisted AVT into the excised porcine skin was significantly higher (p<0.05) with cumulative % drug permeation of 90.13±0.231 for 2.5 mm MNs than that from AVT-LP patch without the help of MNs. **Conclusion:** Nanosized MN assisted AVT-LP could avoid oral route first pass metabolism and increase bioavailability.

Keywords: Liposomes, Atorvastatin, Microneedles, Transdermal drug delivery systems, Bioavailability, Anti hyperlipedemic.

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INTRODUCTION

The major cause of death internationally is cardiovascular disease[1] and atherosclerosis is the dominant cause of cardiovascular diseases. Chronic plaque (cellular waste products, fat, cholesterol, calcium, and fibrin) deposition on the arterial walls occurs in atherosclerosis. This plaque ruptures, detaches and clogs the local blood flow leading to myocardial infarction or ischemic stroke.[2] The mechanisms of plaque formation are not clear but linked to several theories[3] that show plasma cholesterol levels as the leading cause for plaque formation.[4-6] HMG-CoA (3-hydroxy-3 -methyl-glutaryl-coenzyme A) reductase is responsible for the biosynthesis of cholesterol with a feedback regulation. HMG-CoA reductase is inhibited by statins. Hence statins are widely used for atherosclerosis and are successful as cholesterol-lowering agents. Besides cholesterol lowering, statins are also used for respiratory, carcinogenic, viral and neurodegenerative protective agents.[7-10] Even though statins are successful, their off-target mechanisms claim a challenge to formulation development. Targeted statin therapy is needed for complete therapeutic efficacy.

Atorvastatin (AVT) is a statin; US-FDA approved HMG-CoA reductase inhibitor. Poor oral bioavailability of AVT is mainly attributed to its low solubility (BCS class II). The absolute bioavailability of the Atorvastatin is only 15%. Atorvastatin shows peak plasma concentrations after 14 hr of oral administration and 99% protein binding.[11] Presently Atorvastatin was approved worldwide under different brand names Lipitor Atoris, Atorlip, Atorva, Atorvastatin Teva, Atorvastatina Parke-Davis, Avas, Cardyl, Liprimar, Litorva, Mactor, Orbeos, Prevencor, Sortis, Stator, Tahor, Torid, Torvacard, Torvast, Totalip, Tulip, Xarator,

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and Zarator. Pfizer also makes its own generic version under the name Zarator.

From the literature review it was understood that all the research works of AVT published are based on film coated tablets, cyclodextrin complexation,[12] solid dispersions,[13] novel polymorph (form II) by crystallization for increasing solubility, dissolution, bioavailability of AVT. However, there were no research works published on the liposomal formulations to improve bioavailability of AVT.

The physicochemical characteristics of AVT such as poor solubility, high lipophilicity, drug efflux pathways, dose dependent metabolism and non-linear pharmacokinetic profile result in very less efficacy of existing marketed formulations. The extensive first-pass metabolism of AVT and degradation within the gastro-intestinal tract is the main cause of its less oral bioavailability (10-15%) which is a serious issue that should be corrected immediately. Hence a change in the administration route and novel formulation development are needed for AVT. Nanoliposomal AVT administered through transdermal route is investigated in the present research work.

Liposomes are microscopic vesicles in which aqueous volume is enclosed by a membrane composed of lipid bilayers.[14,15] The deeper relationship among critical material attributes, critical process parameters, physicochemical properties with critical quality attributes that influence the performance of liposomes through transdermal patch assisted with microneedles are investigated. The critical quality attributes that would affect the final AVT liposomes pharmacokinetic and pharmacodynamics performance such as particle size, particle size distribution, lamellarity, AVT entrapment efficiency, *in vitro* AVT release from AVT liposomes are evaluated after the final formulation development.

There will be a problem with AVT for transdermal delivery (log P of 4.23 and highly lipophilic, poor water solubility). The highly lipophilic AVT will be remained in the lipophilic Stratum Corneum (SC) for a longer time creating a problem in achieving steady plasma concentrations. Also, AVT after easily penetrating the skin will be delayed in SC. As the epidermis is aqueous, lipophilic AVT will be unable to cross it. Hence required quantity of AVT may not permeate through the skin to reach dermis where blood vessels are present. To avoid this problem novel transdermal permeation enhancement methods such as Microneedles (MNs) application will help to solve the limitation of skin barrier properties and AVT LP permeation is easily facilitated in required concentration.[16-22] Microneedles are less invasive and painless, hence can be used for infants and neonatal seizure treatment also. The drug delivery rates attained through the application of MNs are similar to that attained using conventional injection methods. There is a disadvantage of significant uptake by reticulo-endothelial system in intravenous route compared to that in Microneedle Assisted Transdermal Delivery (MNTD).

MATERIALS AND METHODS

Materials

Atorvastatin (AVT) of analytical grade (purity, >98%) was kindly gifted by Mylan laboratories, Hyderabad, India. Lecithin was prepared from egg yolk extemporaneous. Soya lecithin (LOBA CHEMI Laboratories-Mumbai), Cholesterol (LOBA CHEMI Laboratories-Mumbai), n-Butanol (LOBA CHEMI laboratories, Mumbai) and methanol (Merck Specialities Pvt. Ltd., Mumbai, India) Polyethylene glycol-400 (gift sample from S.D Fine chemicals Ltd, Mumbai), Propylene glycol (gift sample from Central drug house, Bombay), and Ethyl alcohol of HPLC grade (gift sample from Changshu Yang chemicals, China), Butylated Hydroxy toluene, (Merck Specialities Pvt. Ltd., Mumbai, India) were used. Pluronic F127 (PF127) was gifted. Distilled de-ionized water was used. All the materials used were of pharmacopoeial and analytical grades. Pig ear skin was obtained locally. Epyz derma Roller with various micro-needle lengths (0.25, 0.5, 1.5, 2.5 mm) containing 540 Titanium microneedles were bought from Amazon.in.

Methods

Analytical Method for Estimation of Atorvastatin (AVT)

The estimation of AVT in *in vitro, ex vivo* studies was carried out by using an ultraviolet-visible (UV-vis) spectrophotometric method in the present research work and the absorbance was measured at 246 nm in methanol stock solution. In 7.4 Phosphate buffer solution, AVT was estimated by subsequently diluting the stock solution to get a series of dilutions 10,20,30,40 and 50 µg/mL of solution and the absorbance was measured at 246 nm (UV-VIS spectrophotometer, SL-150, Elico) against the same dilution as blank.

Stability of AVT in phosphate buffer pH 7.4

The stability of AVT was estimated in phosphate buffer pH 7.4 by placing the samples (20 µg/mL) at 37°C in an orbital shaker for a period of 48 hr. The samples were taken out at different time points to analyze using UV-vis spectrophotometric method.

Extraction of lecithin from fresh egg yolk

Fresh egg yolks were taken into a beaker and were stirred. Acetone was added to the fine stirred egg yolks and filtered. The residue that was obtained after filtration was taken. After filtration the residue was added to a mixture of chloroform and ethanol (2:1). For the complete extraction of lecithin this resultant mixture was left aside for 3 hr after which it was filtered. Here filtrate is collected and the residue is discarded. The filtrate was kept aside to evaporate chloroform and ethanol. After the evaporation of ethanol and chloroform, a layer is formed which contain lecithin.

Table 1: Formulation table of Atorvastatin (AVT) Liposomes by Thin layer film hydration method and Solvent injection method.

Formulation	Ingredients							
	AVT (mg)	Lecithin (mg)	Cholesterol (mg)	Butanol (mL)	Buffer (mL)	Lecithin:Cholesterol	Pluronic (PF127) (mg)	Method of preparation
F1	10	50	50	5	10	1:1(Soy lecithin)	0.25	TFH
F2	10	50	100	5	10	1:2(Soy lecithin)	0.25	TFH
F3	10	50	50	5	10	1:1(egg lecithin)	0.25	TFH
F4	10	50	100	5	10	1:2(egg lecithin)	0.25	TFH
F5	10	50	50	5	10	1:1(Soy lecithin)	0.25	SIM
F6	10	50	100	5	10	1:2(Soy lecithin)	0.25	SIM
F7	10	50	50	5	10	1:1(egg lecithin)	0.25	SIM
F8	10	50	100	5	10	1:2(egg lecithin)	0.25	SIM

TFH: Thin layer film hydration method, SIM: Solvent injection method.

Preparation of AVT Liposomes

Liposomal composition, type of lecithin (soya lecithin/freshly prepared egg lecithin) and method of preparation of liposomes are the discriminating factors that affect their final properties. Full factorial design is used to investigate two variables at four levels of formulations (4^2) and finally, sixteen different formulations were formulated by four novel customized methods (Solvent injection method, thin layer film hydration method, Freeze-thaw method, Reverse Phase evaporation technique) and also by investigating the ratios (1:1,1:2) of lecithin and cholesterol (Table 1).

Preparation of AVT liposomal formulation by solvent injection method

In 10 mL of n-butanol, 77.5 micromoles of egg lecithin/soya lecithin and 129 micromoles of cholesterol were dissolved (Lipid phase). In another beaker, 10 mg of drug and 0.2%w/v Pluronic (PF127) were dissolved in 5 mL of methanol and to this resultant mixture 10 mL of pH 7.4 phosphate buffer was added (Aqueous phase). Both the above beakers were kept stirring at 200 rpm on thermostatically controlled magnetic stirrer (Remi Magnetic Stirrer) at a temperature of 45°C. To obtain uniform vesicular dispersion, lipid phase (which is at 45°C) was added to the aqueous phase at 45°C by injection at one jet with stirring for 1 hr. For 30 m the suspension was subjected to cyclomixer (CM101 REMI) after which sonicated for 1 hr. Lyophilization was carried out by adding 5 mL of cryoprotectant solution (2.5%w/v of PVP) into the suspension. The mixture was pre-frozen at -80°C in an ultra-low temperature freezer for 2 hr. Dry powder particles were resulted by lyophilization process carried in bench top freeze drier system (SP Scientific Warminster) for 26 hr. The liposome freezes dried powder obtained was stored in airtight container at 2-8°C.

Preparation of AVT liposomal formulation by thin layer film hydration method

Egg/soya Lecithin, Cholesterol and AVT were dissolved in a few mL of n-butanol and the mixture was rotated in rotary evaporator at 150 rpm for 45 m. The slow evaporation of organic solvent results in the formation of very thin film of dry lipids on the inner surface of the round bottomed flask. To hydrate the dry film, 7.4 Phosphate buffer was added slowly and to stabilize the resultant liposomal suspension, 0.2%w/v Pluronic (PF127) was added.

To allow for complete lipid hydration the liposomal suspension was kept aside overnight at 4°C. The suspension was cyclomixed (CM101 REMI) for 30 m and sonicated for 1 hr. Lyophilization process was carried out by the addition of 5 mL of cryoprotectant solution (2.5%w/v of PVP) into the suspension. The liposome dry powder particles which were formed were stored in airtight container at 2-8°C.

Preparation of Atorvastatin liposomal formulation by Freeze-thaw method

In a beaker 50 mg of cholesterol and 50 mg of soya lecithin, 10 mg of pure drug of AVT were taken, to this 10 mL of n-Butanol (organic phase) was added (Table 2). This beaker containing the mixture was sonicated for 5 min. Then this mixture was transfered into 250 mL round bottom flask, which was connected to vaccume pump used for solvent evaporation for a period of 30 min. To the buffer solution, 250 µL of PEG400 and 250 µL of PG were added. After the evaporation of solvent, to the remaining residue of cholesterol and lecithin present in the round bottom flask, 10 mL of buffer (containing 250 µL of PEG400 and 250 µL of PG) was added (aqueous phase). This flask was kept in freezer(Freeze Drier) at temperature of -20°C for 5 min. Then this flask was thawed at 50°C for 20 min by using double boiling process on hot plate. This cycle of freezing and thawing was carried for 3 cycles.

Table 2: Formulation table of Atorvastatin (AVT) Liposomes by Freeze thaw method and Reverse phase evaporation method.

Formulation	Ingredients							
	AVT (mg)	Soy/egg Lecithin (mg)	Cholesterol (mg)	Butanol (mL)	Buffer (mL)	Lecithin:Cholesterol	Pluronic (PF127) (mg)	Method of preparation
F1	10	50	50	5	10	1:1(Soy lecithin)	0.25	FTM
F2	10	50	100	5	10	1:2(Soy lecithin)	0.25	FTM
F3	10	50	50	5	10	1:1(egg lecithin)	0.25	FTM
F4	10	50	100	5	10	1:2(egg lecithin)	0.25	FTM
F5	10	50	50	5	10	1:1(Soy lecithin)	0.25	RPM
F6	10	50	100	5	10	1:2(Soy lecithin)	0.25	RPM
F7	10	50	50	5	10	1:1(egg lecithin)	0.25	RPM
F8	10	50	100	5	10	1:2(egg lecithin)	0.25	RPM

FTM: Freeze thaw method, RPM: Reverse phase evaporation method.

After 3 cycles the formed suspension was collected and stored in centrifuge tubes at opitimal temperature.

Preparation of Atorvastatin liposomal formulation by Reverse phase evaporation

In a beaker 50 mg of cholesterol and 50 mg of soya lecithin, 10 mg of pure drug of AVT were taken, to this 10 mL of n-Butanol (organic phase) was added (Table). This beaker containing the mixture was sonicated for 5 min. Then this mixture was transfered into 250 mL round bottom flask, which was connected to rotary evaporator (Rota Evapourator) at 100-150 rpm for solvent evaporation. After the evaporation of solvent, to the remaining residue of cholesterol and lecithin present in the round bottom flask, organic phase, diethyl ether was added. To this, 10 mL of buffer (containing 250 µL of PEG400 and 250 µL of PG) was added (aqueous phase). This results in the formation of W/O emulsion. This emulsion was again subjected to rotary evaporation for solvent evaporation accompanied by freeze thaw. To this, buffer was added which results in the formation of liposomal suspension, collected and stored at temperature between 2-8°C.

Evaluation of Liposomes

The prepared AVT liposomes were evaluated for microscopy, drug entrapment efficiency, *in vitro* drug release studies, *ex vivo* drug release studies, drug-excipient compatibility studies, *ex vivo* skin permeation studies.

Characterization of liposomes

Optical Microscopy

The prepared Atorvastatin liposomes were viewed for observing the vesicle formation and discreteness of dispersed vesicles. A slide was prepared by placing a drop of liposomal dispersion on a glass slide and cover slip was placed over it which was viewed under optical microscope at 10X/40X magnification. Photographs were taken to prepared slides using digital camera**.** Particle size of liposomal formulation was determined as mean diameter which includes two steps:

Calibration of eye piece micrometer by using stage micrometer

The eyepiece micrometer should be calibrated with stage micrometer for every optical measurement. The eyepiece micrometer has a size ranging from 0-100 µm. The exact value at the smallest division of eye piece micrometer has to be determined.

Procedure

Eyepiece micrometer was placed in eyepiece of the microscope. The stage micrometer was placed under microscope and focused through 10x and then through 40x. The stage micrometer and eyepiece micrometer are adjusted either by rotating the eyepiece micrometer or by adjusting the stage micrometer so that both of them are parallel and superimposed on each other. The two lines which are perfectly caught into coincidence are identified as x and y. Each division of stage micrometer is equal to 10 micrometer or 0.01 mm. The number of eyepiece divisions and stage divisions are counted between x and y. One eyepiece division is equal to number of stage micrometer divisions (x) divided by number of eye piece divisions (y) and multiplied by 10.

Measurement of globule size

A droplet of liposomal formulation was mounted on glass slide and placed on mechanical stage of microscope, then the globule diameter was measured by martin's diameter which is the most frequently used for particle sizing and recorded for 100 globules.

Phase contrast microscopy

The particle size determination of the liposomes was carried by OLYMPUS Phase contrast microscopy where the size of the liposomal vesicles along with shape and distribution are measured. Sample was prepared by spreading a drop of the liposomal formulation on the glass slide. After a cover slip was placed over it, then the slide was viewed. The structure of the vesicles of the liposomes was analyzed by the Binocular I 20 light microscopes.

Drug entrapment efficiency

To determine the entrapment efficiency ultra-centrifuge was used at 15000 rpm for 15 min. A transparent supernatant and pellets of liposomes were separately formed. The pellets were separated that contain purely liposome particles. These pellets were suspended in 10 mL of absolute alcohol for 10 min. To estimate the drug content, the vesicles were broken to release the drug. The prepared atorvastatin loaded liposomes were ruptured to determine the entrapment efficiency.

%Entrapment efficiency=Entrapped drug/Total drug added×100

In vitro drug release studies

The *in vitro* drug release studies for AVT liposomal formulation were carried out on a magnetic stirrer in a 250 mL beaker containing 100 mL Phosphate buffer (pH 7.4). 5 mL of liposomal suspension was taken in a test tube of opening diameter of 20 mm covered with a semi-permeable dialysis membrane (Himedia Laboratories Pvt. Ltd.,) and tied with a thread. And the test tube was inverted and placed on the surface of the phosphate buffer (pH 7.4) (100 mL) contained in a 250 mL beaker. The test tube was clamped securely with a stand. The buffer in the beaker was stirred with the temperature maintained at 37°C with an agitation speed of 150 rpm. The drug released entered into the receptor chamber medium. The Samples of 2 mL were collected, Filtered, diluted and analyzed at 246 nm using UV-Visible Elico SL150 spectrophotometer against the fresh blank medium (phosphate buffer). The receptor compartment was replaced with the fresh buffer medium (2 mL) to maintain sink conditions. Dissolution experiments were conducted three times.

Drug release Kinetics and Mechanisms

There are number of kinetic models, to describe the overall release of drug from the dosage form. As both the qualitative and quantitative changes in a formulation may influence drug release and *in vivo* performance, developing a drug product performance assessment tool is needed. The rate of release of AVT from prepared dosage form was estimated by fitting drug release data into first order release kinetics equation:

$$\text{Log } C = \log C_0 - k\, t/2.303.$$

C_o is the initial concentration of the drug; k is the first order constant. Log cumulative of % drug unreleased were plotted against time to get a line from which slope was calculated. The k value was estimated from the slope.

Drug-Excipient Compatibility Studies

Samples were analyzed using an ATR-FTIR Spectrometer (Burker Germany). ATR Spectra were measured over the wave number range of 4000-500 cm^{-1} at a resolution of 1.0 cm^{-1}. The powder or film sample was simply placed onto the ATR crystal and the sample spectrum was collected. The sample was then cleaned from the crystal surface and the accessory was ready to collect additional spectra. ATR analysis is less complicated than using KBR pellets. It is a fast process and a very small amount of the sample is sufficient.

Preparation of Transdermal Patch of Atorvastatin

The F4 formulation with all the best material, process and quality attributes was selected as optimized formulation and the preparation of transdermal patch was initiated. At first various aqueous gel formulations were prepared using 50% w/w of PVP, 15%w/w of PVA or combination of 15% w/w of PVA and 5% w/w of PVP. The selected F4 lyophilized liposomal formulation of AVT in concentration of 20%w/w or 30%w/w was added to the aqueous gel formulations of selected polymers and mixed until homogenous. A flat silicon sheet was taken and onto it 100 mg of the above resulted aqueous blend was poured. A combination of 15% w/w PVP (MW 360 kDa) and 1.5% w/w glycerol was placed behind the formulation to prepare a precast dry baseplate. The final combination was dried at room temperature for 24 hr.

Collection and storage of pig ear Skin

As per the protocol approved by the Institutional Animal Ethics Committee (IAEC) the pig ears of samples 12 in number were collected from the local abattoirs (pigs aged about 6-7 months) immediately after animals were killed by electric current and processed accordingly. With the help of an electrical hair clipper the hair was removed from the external part of pig ear from which the full-thickness skin was separated from the underlying cartilage using a scalpel and excess fat under the skin was removed to a thickness of 1.2 mm for all the skin samples. Dermis side was wiped with isopropyl alcohol cotton balls to remove residual adhering fat. The processed pieces of skin obtained were individually wrapped in plastic bags without air entrapment and stored in a deep freezer at -20°C till further use.

Evaluation of AVT Liposomal Transdermal Patch Assisted with Solid Microneedles

Ex vivo evaluation of AVT liposomal transdermal patch assisted with solid microneedles was carried out for determining comparative efficiency of microneedles in increasing the

transdermal permeation of liposomal AVT. The membrane model used was porcine ear skin.

Skin Perforation by Micro-Needle Arrays

Prior to the skin permeation experiments, the skin samples were taken from the freezer and brought to room temperature for about 30 min. After thawing, the skin surface was carefully washed with saline and the skin was equilibrated in phosphate buffered saline, pH 7.4, for 30 min. Epyz derma Roller was disinfected before every use. Epyz derma Roller with various micro-needle lengths (0.25, 0.5, 1.5, 2.5 mm) containing 540 Titanium microneedles were rolled over the skin surface vertically, horizontally, and diagonally on the coverage areas about 4-5 times each before applying ATV patch within a 1.44 cm^2 skin area in order to maintain the Franz diffusion cells area. Periodically a stereo microscope was used for checking of any needle damage in between the experiments.

In vitro skin permeation studies

To investigate *in vitro* transdermal permeation studies vertical model Franz diffusion cells of diffusion area 1.44 cm^2 containing a water circulation system, a water heater and an eight-stage magnetic stirrer (Orchid Scientifics, Nasik, India) with a 14 mL receptor volume was used. The freezed skin samples were thawed at room temperature for about 30min and fresh distilled water wetted cotton balls were used to wipe them.

Epyz derma Roller was rolled on the pig ear skin and was investigated by clamping in between the donor and receptor compartments with donar cell faced Stratum Corneum (SC) surface. The receptor medium was stirred with magnetic stirrer at a speed of 600 rpm to ensure uniform drug distribution. A circulating water bath is used to maintain the skin surface at 32°C. Then transdermal patch with F4 liposomal formulation was applied on the above treated skin surface after attaining equilibrium for 30 min.

For passive studies, the skin not rolled with derma roller MNs was investigated by the application of transdermal patch with F4 liposomal formulation. Fresh phosphate buffer, pH 7.4 solution was taken in the receptor compartment and maintained at 37 ±1°C with stirring at 600 rpm. From the receptor medium 500 µL samples were taken for analysis at time intervals of 0.5, 1, 2, 3, 4, 5, 6, 24 hr and again to maintain constant volume, accurately replaced with fresh phosphate buffer pH 7.4 solution.

Atorvastatin content in the skin

At the end of the experiment, drug concentration in the skin (both rolled and untreated) was estimated. Scalpel was used to cut the exposed skin tissue and washed with water to remove any drug adhered to its surface. After mincing, the skin was taken in a pre-weighed vial. The skin was dipped in 5 mL of acetonitrile with shaking (100 rpm) for 24 hr at room temperature in an orbital shaker to extract AVT and then analyzed.

Statistical Analysis of the data

The estimations were calculated as mean±SD. One-way ANOVA analysis (using Fisher's Least-Significant-Difference post hoc test) using SYSTAT 13 software (Systat Software Inc., CA, USA) was used for interpreting the results and analyzing the data statistically. Results with p value less than 0.05 were taken as statistically significant variance.

Stability Studies on AVT Liposomal Transdermal Patch

As per ICH guidelines, the stability of AVT liposomal transdermal patch formulated in the present investigation was studied. The patches packed in amber colored bottles were stored at 40°C, 75% RH for 12 months. During the period of storage at 3rd, 6th and 12th months, the products were studied for *in vitro* drug release studies, drug entrapment efficiency, *in vitro* skin permeation studies.

RESULTS

Analytical Method for Estimation of Atorvastatin (AVT)

The present analytical method obeyed beers law in the concentration range of 10-50µg/mL and it is suitable for the estimation of AVT in pH 7.4 phosphate buffer. The value of r^2 for the linear regression was found to be 0.9950. The linear regression equation for the calibration curve is Y=0.027x+0.036.

Stability of AVT in Phosphate Buffer pH 7.4

To assess the stability of AVT the samples were investigated for a period of 48 hr for any decomposition/degradation and estimated using UV-VIS spectrophotometric method. Phosphate buffer was selected as receptor fluid because significant degradation of AVT was not observed in it.

DISCUSSION

Preparation of Liposomes

The present study is mainly focused on Critical Quality Attributions (CQA) of prepared liposomes that influence majorly their pharmacokinetic and pharmacodynamics performance such as particle size, lamellar structure, surface properties, drug encapsulation efficiency, drug release rate. The CQA depend on the variables such as cholesterol: lecithin ratio; lecithin: AVT ratio; type of lecithin (egg lecithin/soy lecithin/hydrogenated egg lecithin) used in the formulation; and method of preparation to a greater extent.

Passive AVT-loading approach was executed in the present preparation method which involves encapsulating AVT during the process of preparation of liposomes. The lipophilic AVT drug was embedded in the lipid bilayer by steric interactions between

AVT and lecithin. The free AVT was removed which was not encapsulated in the liposomes.

Among soy lecithin and egg lecithin, egg lecithin containing formulations were preferred because egg lecithin comprises 78% of Phosphatidylcholine (PC) whereas soy lecithin comprises only 33% of PC. Lipid bilayer is formed by PC and is stabilized by cholesterol which modulates PC membrane fluidity, elasticity and permeability. The unsaturated fatty acid structure of PC is easily oxidized and results in less stable liposomes with less phase transition temperature making the PC bilayer disordered and leaky *in vivo*. Hence in the present study, the prepared egg lecithin was subjected to hydrogenation to obtain white solid with less unpleasant odour. The Hydrogenated Egg Lecithin (HPC) used has a higher phase transition temperature than unsaturated egg lecithin resulted in the formation of stable liposomes. Also, the HPC resulted into solid dispersions with the lipophilic AVT increasing the dissolution rate, solubility and bioavailability.

In phosphate buffer pH, polar heads of HPC impart neutral charges to the liposomes. Hence Pluronic (PF127) a stabilizer was used to get negatively charged stable AVT liposomes that avoid aggregation. The stabilization of liposomes by Pluronic (PF127) was resulted through hydrophobic poly propylene oxide of PF127 that adsorbed on the surface of AVT. The hydrophilic poly ethylene oxide chains of Pluronic (PF127) extended into the aqueous phase and resulted in steric stabilization to avoid the aggregation.

Cholesterol promotes the PC bilayer formation and act as bilayer membrane fluidity/rigidity modulator effecting drug release and kinetics of exocytosis and resulted in the formation of stable liposomes. The effect of cholesterol on the PC bilayer membrane properties is dependant majorly on its concentration. The ordering effect of cholesterol at high concentrations led to the reduction of fluidity in the PC bilayer membrane hence avoiding it from being leaky.

The F4 formulation prepared by Thin-film hydration method and consisting of 1:2 weight ratios of HPC and cholesterol formed stable liposomes. Molecular weight of lecithin (643.9) being approximately double that of cholesterol (386.6), 1:2 weight ratio of HPC and cholesterol formed 155 micromoles of lecithin with 100 mg and 517 micromoles of cholesterol with 200 mg. Cholesterol formed 258 micromoles only with 100 mg (1:1 weight ratio). The presence of optimum amount cholesterol improved rigidity of the HPC bilayer and led to stable AVT liposomes.

Method of liposome preparation influences majorly the properties of them. Thin-film hydration method was beneficial for the loading of lipophilic drugs such as AVT. A thin film was created by evaporating the lipid-solvent solution during flask rotation under vaccum as shown in the Figures 1-3. The aqueous buffer solution addition resulted in the hydration of the thin lipid film. As a result of thermodynamic response of lipid bilayer in order to reduce the entire liposome surface area-to volume ratio, different lamella formation occurred due to water egress caused by external osmotic change. Small unilamellar vesicles of liposomes were resulted after homogenization for size reduction. The free drug removal is minimized by effective loading process and excellent encapsulation efficiency.

The solvent injection method resulted in unilamellar liposomes spontaneously formed through passive loading of AVT resulting from interfacial turbulence. The ethanol is commonly used as solvent for injection. The flow-rate, stirring-rate, temperature of

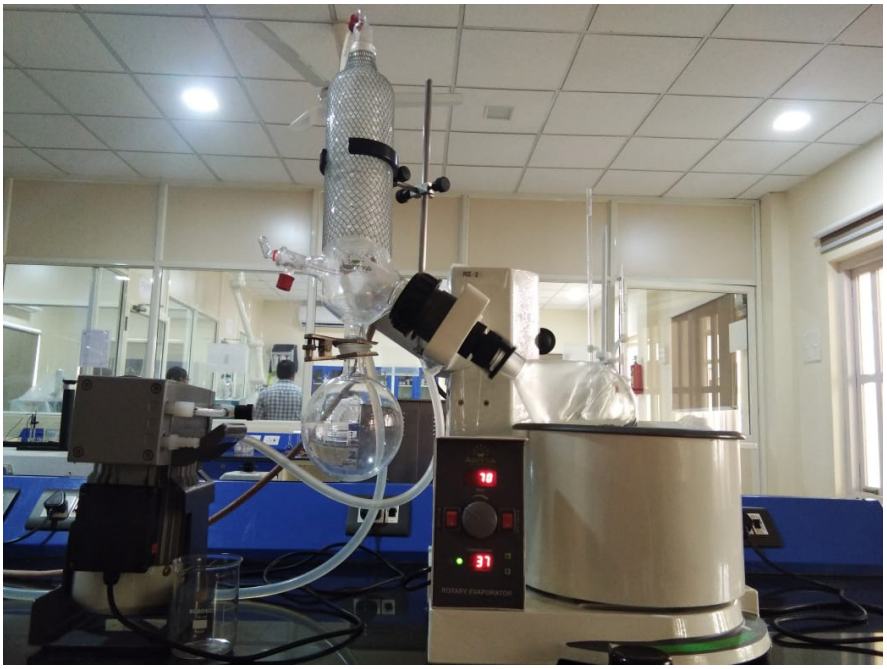

Figure 1: Rotary Evaporator.

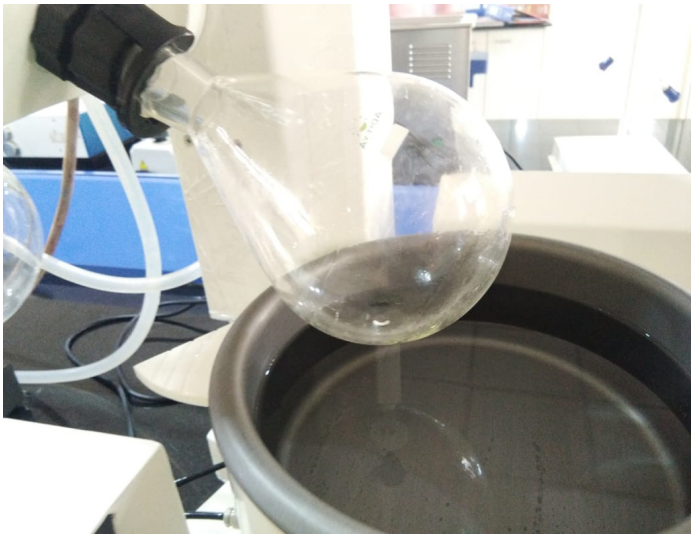

Figure 2: Formation of Thin Film Layer After Solvent Evapouration.

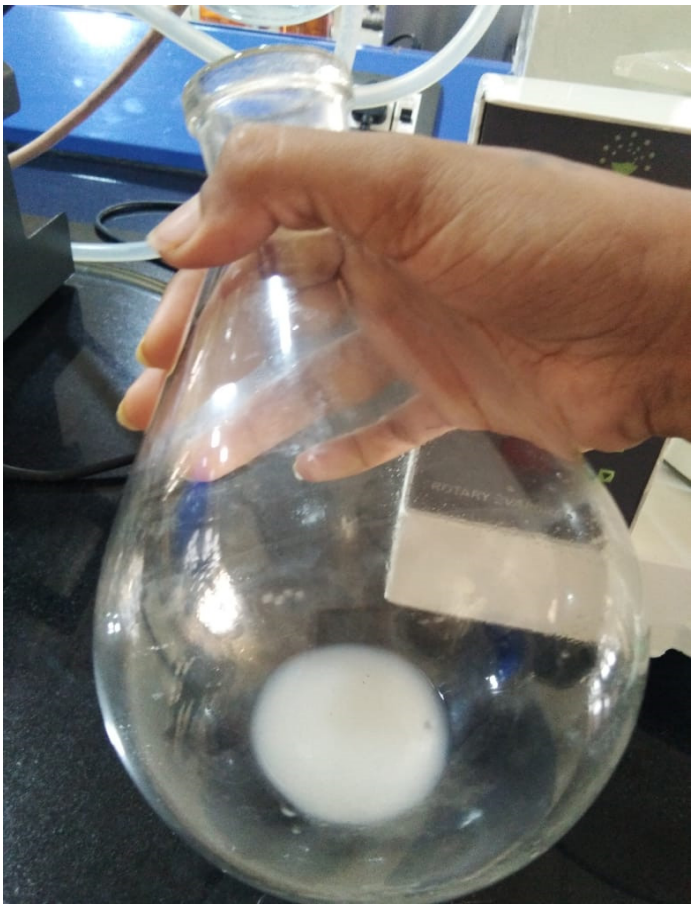

Figure 3: Formation of Milky White Suspension After Reconstituted With 7.4 pH Phosphate Buffer.

both organic solvent and aqueous solution while injection, lipid concentration largely influenced the liposome properties. The water extrusion due to external osmotic change resulted in the reduction of surface area-to volume ratio of liposomal particles as a thermodynamic response in several lamellae formation by lipid bilayer of liposomes. The resulted AVT Liposomes were coated with PEG chains to prevent aggregation and prolong the circulation time *in vivo*.

Drug-Excipient Compatibility Studies

FTIR studies were investigated for the pure AVT and the physical mixtures of the AVT with other excipients used in the formulation. From the overlay obtained it was evident that AVT did not interact with other excipients. All the functional groups of AVT (Aromatic 2°amine (N-H stretch) 3510.77, O-H stretch 3580.2, C=O Amide 1676.8, N-H bending 1580.38, C=C stretch 1560.48, Carboxylic acid (C=O; 1655, C-H bending; 841, O-H stretch) 3580.2) were within the range (Figure 4). Hence liposomal formulation of the present investigation confirmed that AVT and other excipients did not involve in any chemical interactions.

Evaluation of Liposomes

The prepared AVT liposomes were evaluated for microscopy, drug entrapment efficiency, *in vitro* drug release studies, drug-excipient compatibility studies, *ex vivo* studies. Lamellar structure of liposomes influenced the rate of drug release and particle size could influence the circulation time of liposomes *in vivo*. Morphology and surface properties majorly influence the targeting of drugs. In the present research, studies were carried out on basic characterization of liposomes such as liposome size and size distribution, lipid bilayer analysis of liposomes and encapsulated drug analysis that include encapsulation efficiency and *in vitro, ex vivo* drug release profile.

Characterization of Liposomes

Particle size

The particle size and lamellarity are the important critical quality attributions of liposomal dosage form that affect their pharmacokinetic and pharmacodynamics performance. The morphology of liposomes such as spheres, disks, strips is linked to the surface-to-volume ratio. During storage, the particle size and lamellarity played key role in controlling the wobbling, axial, translational motion, flip-flop motion and thickness fluctuations, shape fluctuations in the HPC bilayer, lateral AVT diffusion and liposomal vesicle fusion.

Optical Microscopy

The size of liposomal vesicles was measured at different locations by placing a drop of liposomal suspension on the glass slide and found that all the vesicles were found to be in the size less than 10 µm (Figure 5) and were discrete with smooth nature and spherical in shape, uni-lamellar in solvent Injection method, freeze thaw method and multi-lamellar in reverse phase evaporation technique, thin layer hydration technique. The small size liposomes have the ability to passively target the targeted site with long circulation time *in vivo*.

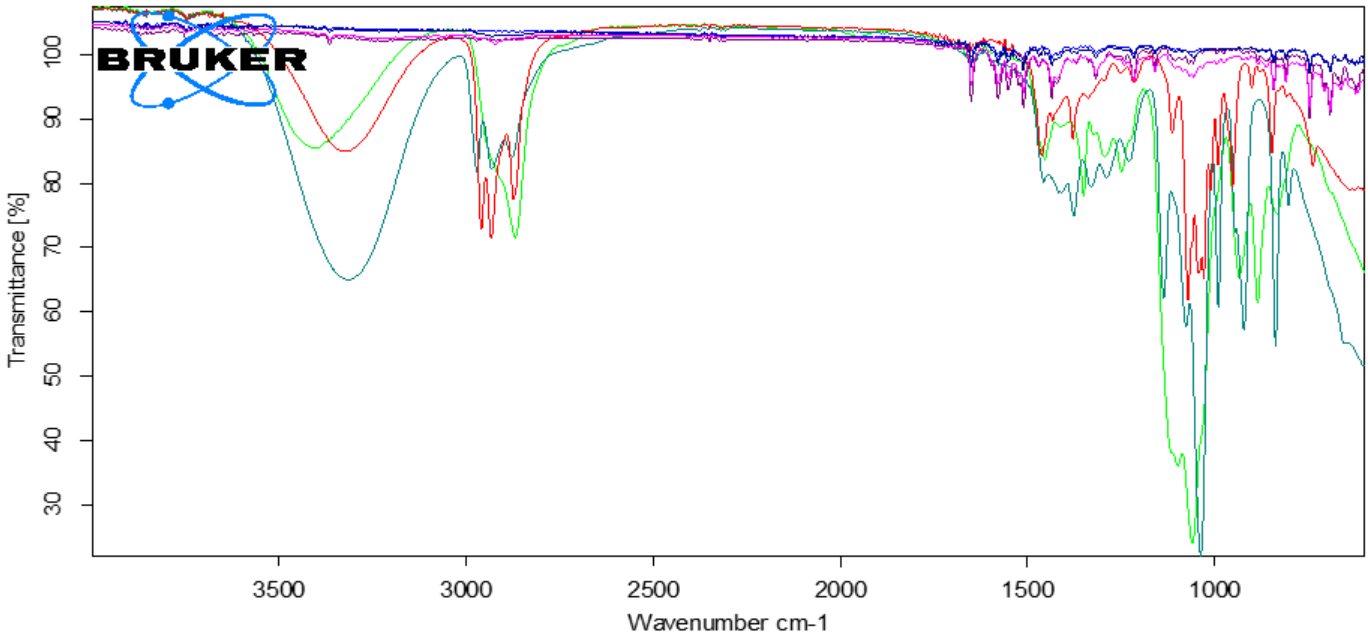

Figure 4: Overlay FTIR Spectra for pure drug (AVT) along with other Excipients.

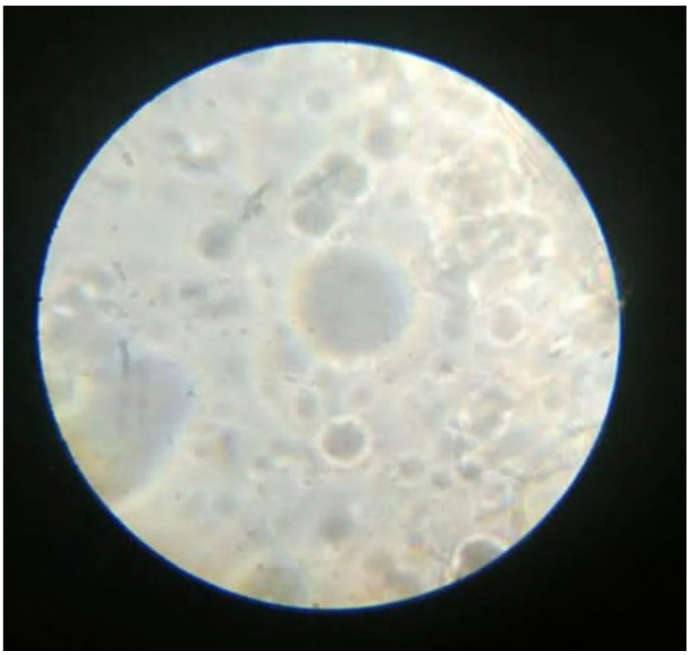

Figure 5: Optical microscopy of F4 liposomal formulation.

The liposomal lipid bilayer was not seen clearly through optical microscopy due to weak non-covalent interactions. Hence phase contrast microscopy was used to evaluate whether the formulation contained lipid bilayer or not and to assess that the formulation is not an emulsion but a liposomal formulation.

Phase Contrast Microscopy

The size of liposomes was between of 0.05 µm to 10.55 µm (50 nm-10550 nm) which clearly supports the size of nanoliposomes between 10 nm and 100 nm (Figure 6). Sufficient care was taken to avoid thick specimens as they cause distorted vision. The liposomes were separate with spherical shape and the lipid bilayer was clearly visualized suggesting that the formulation contained liposomal suspension but not an emulsion.

Small uni-lamellar vesicular liposomes were visualized from all the methods of preparation. The lipid bilayer embedded with lipophilic AVT was stabilized with cholesterol. The HPC that contained high levels of saturated fatty acids particularly stearic acid with no polyunsaturated fatty acids formed stable liposomes. The length, symmetry, inter- and intra-molecular interactions, branching and unsaturation degree of hydrocarbon chains greatly influenced the thickness and fluidity of the lipid bilayer, phase transition temperature and drug release. The cholesterol preferentially interacted more with saturated HPC than with unsaturated PC. Hence unsaturated PC resulted into the formation of looser lipid bilayer membrane.

Lamellarity

FTIR studies were conducted for the formulations F4, F8, F12 and F16 which were prepared with 1:2 HPC-to-cholesterol ratio, but different preparation methods. From the overlay it was observed that there was a change in the functional group of AVT C-H bending in Carboxylic acid. The F4 formulation showed AVT C-H bending of Carboxylic acid at 841 cm^{-1} which was an ordered state (trans-conformation) preventing leakage of the entrapped drug. But the other 3 formulations F8, F12 and F16 showed AVT C-H bending of Carboxylic acid at 961 cm^{-1} (gauche transformation) which was a disordered state even-though there was almost no change in dipole moment as the bond stretches. Formulations F3, F7, F11, F15 prepared with 1:1 HPC-to-cholesterol ratio could not form stable liposomes because of lack of HPC layer rigidity (gauche transformation) and thereby resulting in AVT leakage.

Hence F4 liposomal formulation confirmed that there was good level of lamellarity which was thermodynamically beneficial for the AVT release *in vivo*.

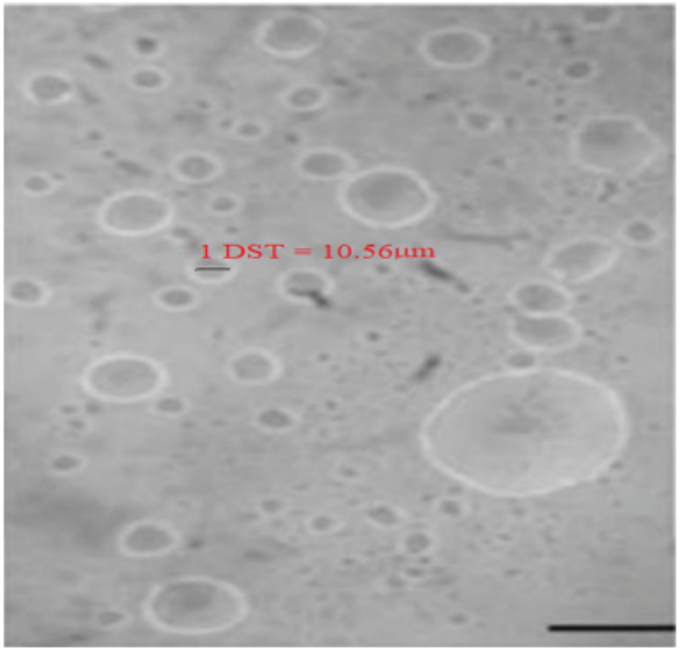

Figure 6: Phase contrast microscopy of F4 Liposomal Formulation.

Drug Entrapment Efficiency

Determination of the Encapsulation Efficiency (EE) began with the removal of the free unencapsulated AVT from the liposomal suspension. The EE was found to be higher with the optimized preparation parameter, AVT-to-HPC ratio 1:5 (drug-to-lecithin) during passive loading of drug. The EE was increased in F4 when compared to the other formulations because F4 formulation was prepared by thin film hydration method that is most successful and beneficial for the encapsulation of lipophilic AVT in the lipid bilayer. Also, F4 contained of 1:2 ratio of HPC-to-cholesterol. Molecular weight of lecithin (643.9) being approximately double that of cholesterol (386.6), 1:2 weight ratio of lecithin and cholesterol forms 155 micromoles of lecithin with 100 mg and 517 micromoles of cholesterol with 200 mg. Cholesterol forms 258 micromoles only with 100 mg (1:1 weight ratio).

The effect of cholesterol on the HPC bilayer properties is concentration dependant. The presence of optimum amount cholesterol improved drug entrapment efficiency because of increased stability of the liposomal membrane by increasing the rigidity of the bilayer. Also, cholesterol increased ordered state of the HPC bilayer preventing it from being leaky by improving the trans conformation over gauchi disordered state that is very beneficial for the stability of liposomes during storage and AVT release *in vivo*. At the same time, cholesterol played an important role in allowing the AVT release easily out of the HPC bilayer by increasing the permeability of lipid bilayer. Formulation

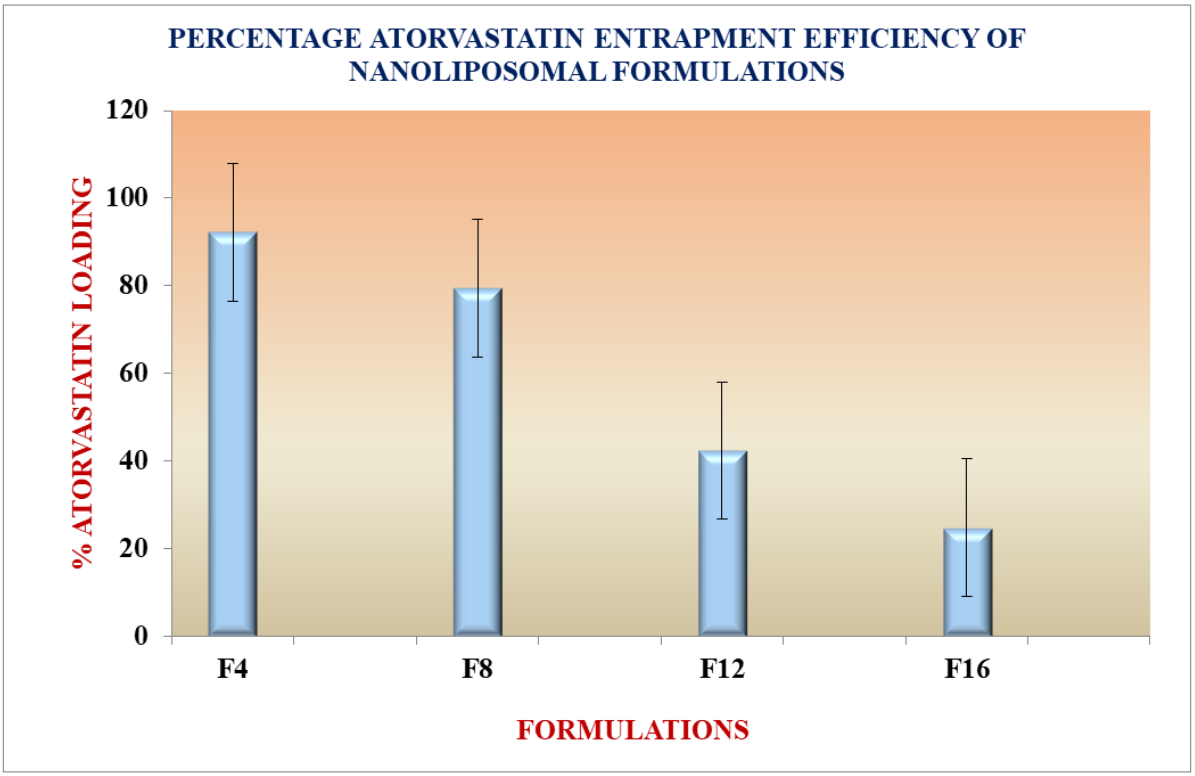

Figure 7: Percentage Atorvastatin Entrapment Efficiency of F4, F8, F12, F16 liposomal formulations.

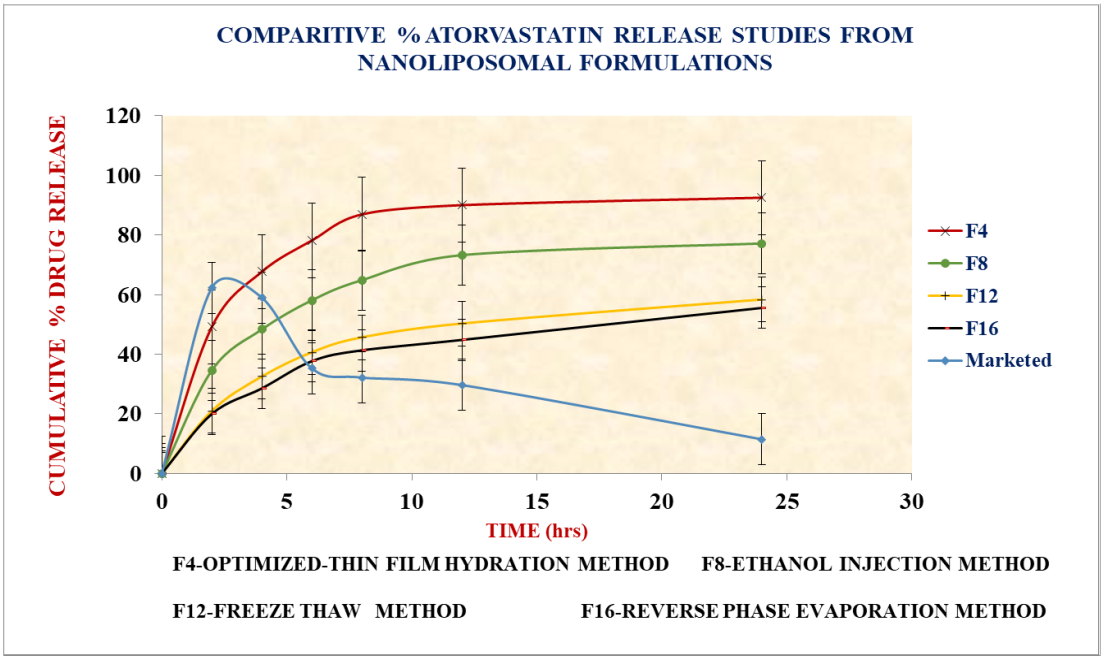

Figure 8: Cumulative Percentage Atorvastatin Release Studies of F4, F8, F12, F16 liposomal formulations.

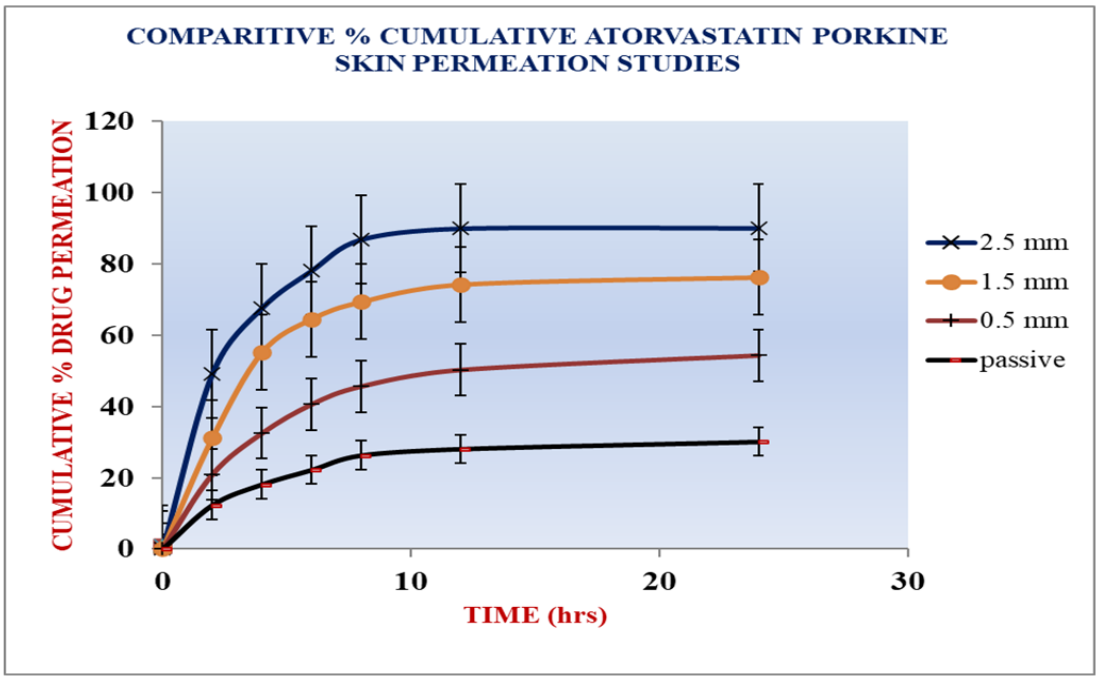

Figure 9: Comparitive Cumulative Percentage Atorvastatin Porkine Skin Permeation Studies of F4 liposomal Transdermal Patch using Microneedles of length 2.5 mm, 1.5 mm, 0.5 mm, Passive Studies.

F4 showed highest entrapment efficiency 92.219±0.965 when compared to other formulations (Figure 7).

In vitro Drug Release Studies

In vitro drug release studies were investigated for the 16 AVT liposomal formulations F1- F16 to study the dissolution parameters. Compared to marketed formulation (Atorva 10 mg tablets of pack size 15 manufactured by Noel Pharma India Pvt. Ltd.,) the release profiles of the developed AVT liposomes were significantly higher and were able to increase the release for a period of 24 hr. The increase in surface area after particle size reduction to nano size resulted in the faster diffusion rate of AVT from liposomes.

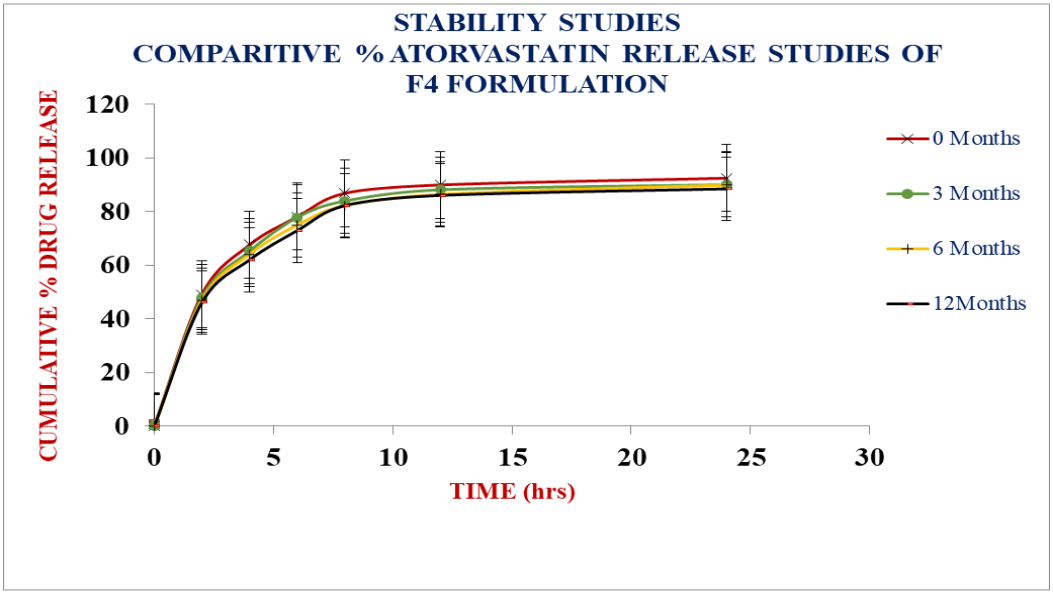

Figure 10: Stability Studies-Comparitive Percentage Atorvastatin Release Studies of F4 liposomal formulation at 0, 3, 6, 12 months.

In vitro drug release studies were carried out in Franz Diffusion cell apparatus by filling the receptor compartment with methanol: 7.4 phosphate buffer (2:1). The diffusion cell was mounted and clamped in a water bath 37±0.50°C. The receptor chamber was stirred continuously with the help of a magnetic bead at 100 rpm.

To study the kinetic modelling and drug release from AVT liposomes, several kinetic models were used to fit the dissolution profiles that showed their best fit to first order model from which it was clear that the AVT release from the HPC bilayer was dependant on the concentration of AVT in the HPC bilayer. Also, the HPC resulted into AVT solid dispersions with the lipophilic AVT increasing the dissolution rate, solubility and thereby bioavailability.

The F4 formulation from thin-film hydration method consisting of 1:2 ratio of HPC and cholesterol showed percent cumulative drug release of 92.53±0.621 at 24 hr which was due to the formation of HPC shells in phosphate buffer with the acyl chain hydrophobic interactions which is very advantageous thermodynamically. Hence this lamellarity is critical for drug release *in vivo* also and F4 formulation was selected as optimized formulation. Triplicate drug release studies were carried out with estimated mean and standard deviation values.

Soy lecithin containing formulations such as F1, F2, F5, F6, F9, F10, F13, and F14 showed very less cumulative percent AVT release of 60% because 33% PC only was present in the soy lecithin compared to 78% PC presence in the egg lecithin. As PC is the major component required for the lipid bilayer formation soy lecithin formulations were not formed with good lamellarity and hence AVT leakage led to less stable liposomes.

Formulations F3, F7, F11, and F15 prepared with 1:1 lecithin-to-cholesterol ratio could not able to form stable liposomes because of lack of HPC layer rigidity and AVT leakage. Hence their cumulative drug release was less than 60%.

The formulations F4, F8, F12, and F16 prepared with 1:2 egg lecithin-to-cholesterol ratio formed stable AVT liposomes with highest encapsulation efficiency, lowest leakage of the drug and controlled drug release over a prolonged time period of 24 hr. The absence of burst effect in the *in vitro* release study of AVT showed that the AVT transport out of the liposomes was mainly diffusion-controlled mechanism. The steric stabilization and prevention of aggregation of AVT liposomes was attained by the presence of Pluronic (PF127) and PEG.

The formulations F4, F8, F12, and F16 showed an initial fast drug loss followed by slower rates of drug loss. The initial fast drug loss in the first 30 min was the indicative of free AVT release adsorbed by weak forces on the liposomal surface. The free AVT adsorbed on the liposomal surface contributed to less than 10%. The slower rate of AVT release was the indicative of the actual drug release from the liposomal lipid bilayer that contributed to more than 80%. This slower rate of AVT release extended over a time period of 30 min to 24 hr in which the initial 4 hr period contributed to more than 60% which was indicative of longer circulation time of AVT loaded stable liposomes *in vivo.* The drug release slowly decreased with time after 12 hr indicating that the drug loaded stable liposomes were less in number.

Among F4, F8, F12, and F16 formulations which differ only in the method of preparation, F4 prepared by Thin-film hydration method and consisting of 1:2 ratio of egg HPC and cholesterol showed highest percent cumulative drug release of 92.53±0.621 at 24 hr which was due to the formation of stable HPC lipid bilayer

with optimum rigidity (Figure 8). Hence the F4 formulation was selected as optimized formulation. As AVT is lipophilic, Thin-film hydration method, a mechanical dispersion technique is beneficial over other methods. The formulation F8 was prepared by solvent injection method which is a solvent dispersion method and suitable for hydrophilic drugs. The lamellarity was not rigid due to insufficient interfacial turbulence created while injection of the solvent and resulted in leakage of AVT. The free drug was more that created an error in the cumulative percent AVT release.

Freeze thaw method was used to prepare F12 which is also a mechanical dispersion method but the lamellarity formed in this method was not suitable because of heating and cooling cycles. Hence the thermodynamic balance of the HPC bilayer was disturbed and leakage of drug resulted due to lack of optimum rigidity. The cumulative drug percent released was not because of encapsulated AVT in the liposomes but due to the free AVT release.

The formulation F16 was prepared by Reverse phase evaporation method which is a solvent dispersion method and suitable for hydrophilic drugs. The lamellarity was not rigid due to vesicle fusion and wobbling, flip-flop motion of the acyl chains during evaporation. Hence AVT was not encapsulated properly. This lamellarity is critical for drug release *in vivo* also and F4 formulation was selected as optimized formulation. Triplicate drug release studies were carried out along with their calculated mean and standard deviation values.

Drug Release Kinetics and Mechanisms

The dissolution data were analyzed as per zero order and first order kinetics in each case. The r^2 values were higher in the first order than zero order indicating that the release of AVT from the liposomal formulations follows first order kinetics. The first order rate constant 'k' (min^{-1}) values for the formulation were calculated from the dissolution data by fitting the data into first order equation. First order values were significantly higher for F4 formulation compared to others. Overall, F4 formulation gave higher release rate constant values when compared to others.

In vitro Skin Permeation Studies

After the application of Epyz derma Roller containing 540 Titanium Microneedles (MNs) with various micro-needle lengths (0.25, 0.5, 1.5, 2.5 mm) into the skin, significant increase in AVT permeation (p <0.05) was observed from AVT liposomal patch. There was a 5-fold increment in the cumulative quantity of AVT permeated from MN assisted AVT liposomal patch when compared to AVT liposomal patch not assisted with MNs. The AVT flux was found to be more when compared to passive studies. The overall permeation improvement (Figure 9) was in the order of 2.5 mm>1.5 mm>0.5 mm>0.25 mm> passive. Significant higher amount of AVT was found in the skin at 24 hr with MN rolled on it and is an indicative of prolonged AVT skin deposition. During passive studies, lipophilic AVT easily penetrates Stratum Corneum (SC) of the skin and was unable to permeate through aqueous epidermis. Hence AVT was delayed in the SC and sufficient amount could not reach dermis where vasculature starts. Microneedles from dermaroller application were successful in the production of temporary disruption in the SC due to which, the skin barrier properties are compromised and AVT permeation was facilitated. Also, liposomal form of AVT can better penetrate the epidermal barrier compared to non-liposomal formulation due to its epidermis-like lipid composition. These MN assisted liposomal AVT delivery was a novel blend of both traditional transdermal delivery and subcutaneous injections and thus delivering AVT at similar rates compared to injections. Hence the combination of liposomes and MNs could be a novel AVT delivery approach to overcome its limitations such as poor solubility, first pass metabolism and poor bioavailability of 14% through oral route.

Atorvastatin Content in the Skin

The skin pre-treatment with MNs increased AVT amount in the skin than without the usage of MNs.

Stability Studies on AVT Liposomal Transdermal Patch

The formulations were stable up to 6 months (Figure 10) without showing any degradation and retained their integrity in terms of drug entrapment efficiency, *in vitro* skin permeation studies, *in vitro* drug release studies.

CONCLUSION

The present study investigated the challenges of the present marketed AVT dosage forms such as low bioavailability, low solubility, first pass metabolism through oral route and was successful in overcoming them by improving the solubility, bioavailability through nanosized liposomal AVT, alternate MN assisted transdermal route to avoid first pass metabolism. Also, the PEG coated AVT liposomes may show prolonged circulation time *in vivo* avoiding stability challenges. This unique function coupled with biocompatibility, non-toxic and biodegradability makes AVT liposomes attractive. Significant optimization steps were carried out to develop AVT liposomes with nanosize and negative charges coated with PEG and assisted by MNs that showed enhanced AVT release *in vitro* though transdermal delivery. However comprehensive studies are required *in vivo* to assist the present novel MN assisted AVT liposomal transdermal formulations in human beings to achieve patient benefit.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LP: Liposomes; **MNs:** Microneedles; **AVT:** Atorvastatin; **TD:** Transdermal Delivery.

SUMMARY

Atorvastatin (AVT) is a US FDA approved drug, a HMG CoA reductase inhibitor used to prevent cardiovascular disease in those at high risk and to treat abnormal lipid levels. Atorvastatin (AVT) belongs to BCS class II. All the marketed AVT tablets show poor oral bioavailability of AVT (14%) which is mainly attributed to its low aqueous solubility, first pass metabolism, dose dependent and non-linear pharmacokinetics.

Hence alternate route of transdermal was investigated in the present study to avoid first pass metabolism with Nanoliposomes (LP) formulation development for AVT to increase solubility and bioavailability. The sub cellular size of AVT nanoliposomes allows higher intracellular uptake than other particulate systems, thus improving AVT *in vivo* drug bioavailability. Also liposomes are non-toxic, non-haemolytic, non-immunogenic, biocompatible, biodegradable, targeting and enhances the efficacy of the encapsulated drug. The optimized formulation of AVT nanoliposomes of present study was dependent on the type of lipid (egg lecithin being superior over soya lecithin) used, cholesterol to lipid ratio (2:1 ratio being superior than 1:1) and the technique of preparation (thin film hydration method being superior over ethanol injection method, freeze-thaw method, reverse phase evaporation method).

There is a chance of forming AVT reserve issue in the stratum corneum with AVT transdermal delivery (log P of 4.23 and highly lipophilic,) leading to low and insufficient constant plasma concentrations within a reasonable time span. Also, AVT is unable to pass through aqueous epidermis. Hence AVT may not permeate through the skin at a sufficient amount to reach dermis where vasculature starts. Even transferosomes formulation is not suitable for transdermal delivery of AVT because sufficient concentration may not permeate through the skin. Microneedles (MNs) application is the best suitable way to avoid this issue. Liposomal uptake by reticulo-endothelial system is significantly less in Microneedle Assisted Transdermal Delivery (MNTD) than intravenous route.

Hence the present study investigated the Microneedle Assisted Transdermal Delivery (MNTD) of AVT liposomes as a novel approach for the delivery of AVT in which the capability of MN arrays (commercial solid dermaroller MNs 0.5 mm, 1.5 mm, 2.5 mm lengths) in improving the *in vitro* and *ex vivo* permeation of AVT-LP through pig ear skin was studied. The formulation with Poly (Vinyl Pyrrolidine) (PVP), Poly (Vinyl Alcohol) (PVA), 30% w/w F4 AVT liposomes witnessed superior AVT release, AVT flux estimation and fulfilled the regulatory requirements.

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