

# Effective Single Drug Treatment of Lymphatic Filariasis through Enhanced Transdermal Delivery of Ivermectin Liposomes using Solid and Dissolving Microneedles

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## ABSTRACT

**Objectives:** The present investigation was to study the combination of liposomes (LP) and microneedles (MNs) as a single drug treatment approach for the delivery of an antifilarial drug, ivermectin (IVM) in which the role of MN arrays (commercial solid MNs of different lengths and laboratory fabricated polymeric dissolving MNs of length 0.6mm) in increasing the *in vitro* permeation of IVM-LP across pig ear skin was studied. **Experimental:** IVM-LP was formulated and optimized using solvent injection method and thin layer film hydration method. The optimized IVM-LP formulation F4 were then incorporated into the dissolving MN arrays and tested for the increased permeation of IVM by the assistance of MNs. A transdermal patch with IVM-LP was prepared as passive permeation study. Solid MNs (poke and patch) were tested for assisting the penetration of IVM from IVM-LP patch. *In vitro* skin permeation studies were carried out using Franz diffusion cells for a period of 24 h. **Results and Discussion:** The optimized IVM-LP was < 100 nm in diameter suitable for lymphatic uptake and MNs of IVM-LP had good mechanical strength, insertion capabilities. From the dermatokinetic study it was evident that the delivery of IVM into the excised porcine skin by MNs was significantly higher than that from passive studies, with apparent permeability coefficient of  $0.798 \pm 0.009$  cm/h for 0.6mm dissolving MNs. **Conclusion:** MN assisted transdermal delivery of IVM-LP could be used to target specifically human lymphatic system where single drug treatment for lymphatic filariasis could be made possible.

**Key words:** Lymphatic filariasis, Ivermectin, Liposomes, Microneedles, Transdermal drug delivery systems, Bioavailability.

Submission Date: 05-12-2019;  
Revision Date: 22-06-2020;  
Accepted Date: 07-09-2020

## INTRODUCTION

Human lymphatic filariasis, (LF) commonly known as elephantiasis, is a neglected tropical disease in which infection occurs through mosquitoes.<sup>1,2</sup> Infection that is usually acquired in childhood shows hidden damage to the lymphatic system.<sup>3</sup> The painful and disfiguring lymphoedema, elephantiasis and scrotal swelling occur that can lead to permanent disability. These patients also suffer mental, social and financial losses contributing to stigma and poverty.

Current mass drug administration (MDA) given by WHO for LF, contain

combinations of Ivermectin (IVM, 0.2mg/kg), Diethylcarbamazine (DEC, 6mg/kg) with Albendazole (ALB, 400mg).<sup>1</sup> These drugs kill microfilariae (MF) and late embryonic stages inside the adult female worms. However, they show little effect on adult worms themselves, therefore the aim of MDA is to break transmission.<sup>2</sup> Doxycycline (200mg/day for 4–6 weeks) an antibiotic is also used in combination with MDA (some studies have shown adult worm killing with treatment with doxycycline).<sup>3</sup> Doxycycline kills the adult worms by killing the gram negative bacteria *Wolbachia* which

DOI: 10.5530/ijper.54.3s.148

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exhibits mutualistic symbiosis (it is proven that *Wolbachia* helps the adult female worm in reproduction, without this bacteria the adult worm is unable to reproduce).

Persons with MF in their blood can appear healthy but are infectious. The persons with chronic filarial swellings cannot further spread the infection. A challenge to the MDA programme is to get people to take four medicines simultaneously, especially when they have no symptoms. Lack of proper diagnostic tests is another challenge. IVM, which is a part of MDA has proven adult worm as well as MF reduction and inhibition of female worm from production of MF (similar to Doxycycline), when administered alone. But the existing oral and parenteral IVM formulations have several disadvantages and unable to reach human lymphatic system to show adult worm suppression. When IVM is properly formulated and administered through suitable route, single drug therapy with IVM may be sufficient for LF. The present research better addresses the challenges. The question, "How an infective larva of *Wuchereria bancrofti* finds its way to human lymphatic system?" raised curiosity and served a stimulation that sparked the fire to the present research concept. The infective larvae from the mosquito saliva when deposited over the human skin find their way to the human lymphatic system by themselves through a tiny bore made by the mosquito proboscis. Similar to the journey of the infective larva, IVM is formulated into liposomes to target the human lymphatic system besides blood through the tiny bore made by the microneedles of transdermal delivery system.

Drugs administered via the lymphatic system could be more easily distributed in the lymphatic system and less in blood circulation, which helps strengthen the therapeutic effects and which is particularly useful for lymphatic system associated disease.<sup>4</sup> The following technologies are found successful for lymph targeting, namely liposomes,<sup>5</sup> polymeric nanoparticles,<sup>6</sup> solid lipid nanoparticles,<sup>7</sup> self-emulsifying self-nanosuspension drug delivery systems,<sup>8</sup> nanosuspensions<sup>9</sup> and microneedle array delivery systems.<sup>10</sup>

Ivermectin paralyzes the MFs<sup>11</sup> and stops the production of MF.<sup>12</sup> IVM paralyzes adult female worm that results in the absence of iron, which is a prerequisite for parasite growth and for production of MF. Also, IVM has the ability to kill the mosquito that feeds on blood of IVM treated patient.<sup>13</sup>

Current marketed dosage forms of IVM in India are oral (tablet-3mg, 6mg, 12mg, suspension) and parenteral (Subcutaneous and Intramuscular injection, Tivomac-10mg/10ml, IVM -0.1%/10ml). Being a substrate of permeability-glycoprotein (P-gp) and having water insoluble nature, IVM shows poor bioavailability

through oral route. IVM shows prolonged absorption from the injection site due to drug precipitation.<sup>14</sup> Hence alternate route of administration such as transdermal route is an ideal choice for IVM. The very low dose (approximately 10 mg) and poor bioavailability through oral route make this drug suitable for transdermal delivery. Transdermal drug delivery (TD) represents a novel and alternative approach of IVM delivery to the existing IVM formulations.<sup>14</sup>

The successful route of administration of drugs for specific targeting to the lymphatic system is the intradermal route to deliver drugs into the lymph because of higher lymph flow rates in the skin compared to other interstitial sites.<sup>15</sup> Through the skin, particles of size range 10 -100nm are taken up by the lymphatic capillaries and particles of less than 10 nm are absorbed by blood capillaries.<sup>16</sup> Particles of more than 100 nm are retained at the administrative site.<sup>16</sup> Liposomes are one of the technologies in which the formulated particle has a size range of 10 nm–100 nm. Hence liposomes are the best formulation for lymphatic uptake.

The problem that arises when a compound (IVM has a log P of 5.83 and highly lipophilic, poorly water soluble) has a log p value above 2 is that the drug is retained in the Stratum corneum(SC), which will create problems with achieving steady plasma concentrations within a reasonable time span. Lipophilic IVM easily penetrates skin and is delayed in SC, also it is unable to pass through aqueous epidermis. Hence IVM may not permeate through the skin at a sufficient amount to reach dermis. Novel transdermal permeation enhancement methods such as microneedles (MN) application may address this limitation. MN application relies on the creation of transient disruption in the SC. As a result, the skin (both SC and epidermis) barrier properties are compromised and drug permeation is facilitated. MN offer several advantages over other enhancement methods and can be seen as a hybrid between a traditional transdermal delivery and subcutaneous injections.

Microneedles are less invasive and painless method by avoiding the barrier properties of skin for increased delivery of drugs and they can be used for self-administration.<sup>17,18</sup> MNs are more advantageous when compared to hypodermic injection<sup>19</sup> and are known to improve the permeation of drug molecules, including macromolecules like insulin, growth hormone.<sup>20,21</sup> Moreover, this technique makes use of the powerful delivery capabilities of the needle systems while improving patient compliance and safety by avoiding pain, fear and the need for expert training to administer,<sup>22</sup> thus may be expected to deliver drugs at rates similar to that achieved using conventional injection methods. In

MN devices, a small area is covered by hundreds of MNs that pierce only the SC, thus allowing the drug to bypass this important barrier. MNs of variable diameters and shapes of solid and hollow are used to poke the skin samples.<sup>23</sup>

Liposomal uptake by reticulo-endothelial system is significantly less in microneedle assisted transdermal delivery (MNTD) than intravenous route.<sup>5</sup>

Hence in the present research work, IVM was formulated into liposomes and delivered through MNTD with an aim of single drug therapy for LF. Overall, MNTD may very well bridge the need for patient friendly single drug treatment for LF and clinically efficient liposomes of IVM which may improve the mental, economic, social status of LF patients to lead normal lives. No reports were published so far on the MNTD of IVM liposomes. The present research forms the basis in the future for bridging the MNTD of anticancer drugs, vaccines, nucleic acid constructs for gene therapy with liposomes for lymphatic targeting to avoid permeability-glycoprotein (p-gp) efflux mechanism and first pass metabolism in oral route.

## MATERIALS AND METHODS

### Materials

Ivermectin of analytical grade (purity, > 98%) was a gift sample from Parkinson Pharma, Mohali, India. Lecithin was prepared from egg yolk in house. Cholesterol (LOBA CHEMI Laboratories-Mumbai), n-Butanol (LOBA CHEMI laboratories, Mumbai) and methanol (Merck Specialities Pvt. Ltd, Mumbai, India) were used. Distilled de-ionized water was used. All the materials used were of pharmacopoeial and analytical grades. Adminpatch MN arrays were bought from Admin Med, Suunnyvale, USA. Pluronic F127 (PF127) was gifted. Hematoxylin and eosin stains, poly (vinyl pyrrolidone) (PVP) (MW 58 kDa, 360 kDa), poly (vinyl alcohol) (PVA) (31-50 kDa) were bought from Sigma Aldrich, India. Pig ear skin was obtained locally.

### Methods

#### Analytical method

A UV-VIS ultraviolet-visible spectrophotometric method was used in the present research work in which the absorbance was measured at 261nm in methanol stock solution for the estimation of IVM in *in vitro* and *ex vivo* studies.

#### Stability of IVM in phosphate buffer pH 7.4

The stability studies of IVM were performed in phosphate buffer pH 7.4. The samples (20 µg/ml) were

placed at 37°C in an orbital shaker for a period of 48 hr and were withdrawn at different time points that were analyzed by UV-VIS spectrophotometric method.

#### Preparation of lecithin

Fresh egg yolks were separated from egg and taken into a beaker. After breaking, fine stirring, acetone was added to the egg yolk, filtered. Mixture of chloroform and ethanol (2:1) was added to the residue obtained after filtration and this mixture was kept aside for 3 hrs for extraction of lecithin. After 3 hrs, the mixture was filtered, filtrate is collected. The filtrate was allowed to evaporate chloroform and ethanol from it to form a layer of lecithin.

#### Preparation of IVM Liposomes

Some of the parameters that affect the final properties of liposomes are cholesterol, lecithin amounts and the method of preparation of liposomes. Three variables at two levels of formulations were investigated in full factorial design (2<sup>3</sup>) and finally, eight different formulations were prepared by two different methods (Solvent injection method and thin layer film hydration technique) and also by varying the ratios of cholesterol and lecithin (1:1, 1:2, 1:0.5, 0.5:1). (Table 1)

#### Preparation of IVM liposomal formulation by solvent injection method

Liposomes were prepared by solvent injection method. In a beaker, accurately weighed amounts of 100mg of freshly prepared egg lecithin and 100mg of cholesterol were taken and dissolved in 10ml of n-butanol (Lipid phase). In another beaker, 10mg of drug was taken and 0.2%w/v Pluronic (PF127) was added as a stabilizer and the entire mixture was dissolved in 5ml of methanol and to this 10ml of pH 7.4 phosphate buffer was added (Aqueous phase). The beaker with aqueous phase and the beaker with lipid phase were kept stirring at 200 rpm on thermostatically controlled magnetic stirrer (Remi Magnetic Stirrer) at a temperature of 45°C. To the aqueous phase at 45°C, lipid phase (which is also at 45°C) was added by injection at one jet. The mixture was continued for stirring for 1 hr to obtain uniform vesicular dispersion. After that the suspension was subjected to cyclomixer (CM101 REMI) for 30 m and then sonicated for 1 hr. After adding 5ml of cryoprotectant solution (2.5%w/v of PVP) into the suspension lyophilization was performed. Dry powder particles were obtained after the suspension was lyophilized by bench top freeze drier system (SP Scientific Warminster) for 26 h (before which the mixture was pre-frozen at -80°C in an ultra-low temperature freezer for 2 hr). Finally, the liposome

freeze dried powder was stored in airtight container at 2-8°C.

### Preparation of IVM liposomal formulation by thin layer film hydration method

Liposomes were prepared by using thin layer film hydration technique. Drug; Egg Lecithin; Cholesterol were dissolved in a few ml n-butanol and the mixture was rotated in rotary evaporator at 150rpm for 45 m. Very thin film of dry lipids was formed on the inner surface of the round bottomed flask after the slow evaporation of organic solvent. The dry film was slowly hydrated with 7.4 Phosphate buffer and 0.2%w/v Pluronic (PF127) was added as stabilizer. The Liposomal suspension was left overnight at 4°C, to ensure full lipid hydration. After that the suspension was subjected to cyclomixer (CM101 REMI) for 30 m and then sonicated for 1 hr. After adding 5ml of cryoprotectant solution (2.5%w/v of PVP) into the suspension, lyophilization was performed. Dry powder particles were obtained after the suspension was lyophilized by bench top freeze drier system (SP Scientific Warminster) for 26 h (before which the mixture was pre-frozen at -80°C in an ultra-low temperature freezer for 2 hr). Finally, the liposome freeze dried powder was stored in airtight container at 2-8°C.

### Evaluation of liposomes

The particle size of the liposomes was determined by Phase contrast microscopy (Olympus) where the size of the liposomal vesicles along with its shape and its distribution can be measured. The morphology of the vesicles of the liposomes was also analyzed by the Binocular I 20 light microscope.

### Drug entrapment efficiency

The entrapment efficiency of liposomal suspension was determined by ultra-centrifuge at 15000 rpm at 25°C for 15m. A clear solution of supernatant and pellets of liposomes were formed. The pellets containing liposomes were suspended in absolute alcohol for 10min. Accurately 100 µl of liposomal suspension was added to 100 µl of absolute alcohol. The lipid vesicles were broken to release drug which were then estimated for the drug content. After rupture of the IVM loaded liposomes, the entrapment efficiency was determined through the calculation of the drug concentration by the measurement of IVM absorbance at 261 nm in triplicate using spectrophotometer with reference to the blank solution prepared.

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

### In-vitro drug release studies

Vertical type Franz diffusion cells (area 1.44 cm<sup>2</sup>) with a dialysis membrane were used for *in vitro* drug release studies to determine the release rate of IVM from different lyophilized liposomal formulations (equivalent to 10 mg of each drug) and IVM pure drug. Hydration of the Dialysis (molecular weight G12000) membrane was performed in distilled water at 25°C for 24 hr. The membrane was clamped between the donor and receptor compartments of the cell. The receptor chamber contained 14 ml of methanol-phosphate buffer pH 7.4 (2:1) and was continually stirred using a magnet stirrer (300 rpm) at 37°C. Two ml of the sample was withdrawn from each batch at definite time intervals (0.5, 1, 2, 3, 4, 5, 6, 24 h) and replaced with the same amount of buffer phosphate to maintain sink conditions. Single beam UV/Visible Spectrophotometer (Elico SL-150) at 261 nm was used to determine the release concentrations of IVM. The results were plotted as cumulative release drug percent versus time. Various kinetic models such as zero order, first order<sup>24</sup> were employed to explain drug release from liposomal formulations. The formulation with higher *r*<sup>2</sup> was selected. *In vitro* drug release studies were conducted in triplicate.

### FTIR-Fourier transform infrared spectroscopy studies

ATR-FTIR Spectrometer (Burker Germany) analysis the samples over the wave number range of 4000-500 cm<sup>-1</sup> at a resolution of 1.0 cm<sup>-1</sup>. The powder sample is placed onto the ATR crystal and the sample spectrum is collected. ATR analysis is easier than using KBR pellets. It is a fast process and a very small amount of the sample is needed.

### Preparation of Needle free patch and two step casting of dissolving MNs by injection molding

Many formulations were investigated in order to optimize the MNs for delivery of IVM in liposomes. Various aqueous gel formulations were prepared using selected polymers of various concentrations consisting of 20%w/w or 30%w/w of F4 lyophilized liposomal formulation of IVM and 50% w/w of PVP, 15%w/w of PVA or combination of 15% w/w of PVA and 5% w/w of PVP. Initially, the lyophilized liposomes were added to the aqueous formulations of selected polymers and mixed until homogenous. Following this, 100 mg of the aqueous blend was then poured into the MN molds, manufactured using micro-injection molding, (Wittmann-Battenfeld Micro-Power 15 micro-injection molding machine, microneedle array components consisted of 25 conical needles, each 0.6mm in length,

with a base diameter of 0.3 mm in a 5 x 5 array over a 0.5 x 0.5 cm<sup>2</sup> area, supported by a circular substrate of diameter 17.5 mm and thickness 0.5 mm). A combination of 15% w/w PVP (MW 360 kDa) and 1.5% w/w glycerol was placed behind the needles to prepare a precast dry baseplate. The cavity of the molds was filled by placing the formulations in a positive pressure chamber with a pressure of 3-4 bar that was applied for 15 m. Lastly the MNs were dried at room temperature for 24 hr and were removed from the molds. For the preparation of transdermal patch, similar formulations were prepared. The aqueous blend (100 mg) was poured onto the top of a flat silicon sheet and a pre-cast dry baseplate was attached behind the formulations which were allowed to dry 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 IVM liposomal incorporated microneedles

#### Mechanical characterization studies

The MNs were visually examined using an Olympus vertical scanning laser confocal microscope LEXT OLS 4000 to accurately measure the tip radius and height of MN arrays, a Hitachi TM-3000 table-top scanning Electron Microscope (Tokyo, Japan) to analyse MN insert and needle geometry.

### Ex vivo evaluation for the relative efficiency of microneedles in transdermal permeation enhancement of IVM liposomes using porcine ear skin as membrane model

#### 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 m. After thawing, the skin surface was carefully washed with saline and the skin was equilibrated in phosphate buffered saline,

pH 7.4, for 30 m. (Figure 5-8) The Admin patch with different micro-needle lengths (0.6, 0.9, 1.2, 1.5 mm) and laboratory fabricated polymer MN arrays PM (0.6 mm) were pressed over the skin surface under thumb pressure. (Figure 9) In the case of PM, single insertion- PM-1 and triple insertion-PM-3 at different places within a 1.77 cm<sup>2</sup> skin area were made in order to maintain the MN density closer to ADM 0.6 mm. For checking of any damage acquired on the needles, a stereo microscope was used periodically in between the experiments.

### Histological examination and calculation of penetration depth

The PZRM-700 microscope (Quasmo, Haryana, India) fitted with 10x objective was used to observe the histological sections of the skin samples prepared with and without MN treatment. For visualization of skin layers and to display a clear indentation of MN penetration they are stained with hematoxylin and eosin. The depth of penetration was also calculated with the help of Toup View 3.2 Software from AmScope FMA050 microscopic attachment (AmScope, Irvine, USA). Skin samples without MN treatment were also prepared as a control.

### In vitro skin permeation studies

Vertical type Franz diffusion cells equipped with a water circulation system, a water heater and an eight-stage magnetic stirrer (Orchid Scientifics, Nasik, India) with a diffusion area of 1.77 cm<sup>2</sup> and a receptor volume of 14ml was used to conduct *in vitro* transdermal permeation studies. The skin samples were taken from the freezer and thawed at room temperature for about 30m. After thawing, the skin surface was carefully wiped with cotton wool balls wetted with fresh distilled water.

### For solid stainless-steel ADM MN arrays

The pig ear skin which was pressed with ADM MNs was taken. The skin sample was clamped in between the donor and receptor compartments with SC surface facing towards the donor cell. By using a magnetic stirrer the receptor medium was stirred for uniform drug distribution at a speed of 600 rpm. The surface of the skin was maintained at 32°C using a circulating water bath. After equilibrium for 30 m, needle free patch preparation containing F4 liposomal suspension was applied on to the skin which was pressed/poked with commercial solid ADM MNs. For passive studies, needle free patch containing F4 liposomal suspension was applied on to the skin that was not pressed with MNs. The receptor compartment is filled with fresh phosphate buffer, pH 7.4 solution. The temperature

of the receptor compartment is maintained at  $37 \pm 1^\circ\text{C}$  with stirring at 600 rpm. Samples (500  $\mu\text{l}$ ) will be withdrawn from the receptor fluid at regular intervals (0.5, 1, 2, 3, 4, 5, 6, 24 hr). Fresh phosphate buffer, pH 7.4 solution will be replaced accurately to maintain the constant volume. The samples obtained will be analyzed by Single beam UV/Visible Spectrophotometer (Elico SL-150) at 261 nm.

### For laboratory fabricated dissolving PM MN arrays

The skin sample not pressed was taken for both dissolving MN arrays study and passive studies. The skin was clamped in between the donor and receptor compartments with SC surface facing towards the donor cell. By using a magnetic stirrer, the receptor medium was stirred for uniform drug distribution at a speed of 600 rpm. The surface of the skin was maintained at  $32^\circ\text{C}$  using a circulating water bath. After equilibrium for 30 m, the laboratory fabricated PM dissolving MNs arrays containing F4 IVM liposomal formulation were inserted into the skin using manual force for 30 s and a circular stainless-steel weight of 5.0 g placed on top to hold the MNs in place. Needle free patch preparation containing F4 liposomal suspension was applied on to the skin for comparative studies. The receptor compartment is filled with fresh phosphate buffer, pH 7.4 solution. The temperature of the receptor compartment is maintained at  $37 \pm 1^\circ\text{C}$  with stirring at 600 rpm. Samples (500  $\mu\text{l}$ ) will be withdrawn from the receptor fluid at regular intervals (0.5, 1, 2, 3, 4, 5, 6, 24 hr). Fresh phosphate buffer, pH 7.4 solution will be replaced accurately to maintain the constant volume. The samples obtained will be analyzed by Single beam UV/Visible Spectrophotometer (Elico SL-150) at 261 nm.

Graphs will be plotted for cumulative amount of drug permeated vs. time for passive (needle free-patch onto the skin which was not pressed), solid MN poked skin samples, and dissolving PM inserted skin samples. The slope of the linear portion of the graph gives flux and permeability will be obtained from the flux and concentration of drug in donor solution. Enhancement ratio or Enhancement fold will be calculated by dividing the active to passive fluxes or permeability coefficients i.e. flux obtained after microneedle pre-treatment (solid ADM MN/dissolving lab fabricated PM MN treatment) to the flux obtained by passive permeation (without solid ADM MN/dissolving lab fabricated PM MN treatment).

### IVM content in the skin

Drug concentration in the skin was measured at the end of the experiment. The exposed skin tissue (1. after

removing transdermal patch on pressed/poked skin-solid commercial ADM MNs study, 2. After removing dissolving MN insert from the skin-laboratory fabricated dissolving MNs PM study, 3. After removing transdermal patch on skin that was not poked-passive comparative study) was cut with a scalpel, rinsed with water in order to remove the adhered drug to the surface. The skin was minced and placed in a pre-weighed vial. IVM was extracted from the skin by placing in 5 ml of acetonitrile and shaken (100 rpm) for 24 hr at room temperature in an orbital shaker. Samples were analyzed by Single beam UV/Visible Spectrophotometer (Elico SL-150) at 261 nm.

### Statistical analysis of the data

One-way ANOVA (analysis of variance) for statistical difference using SYSTAT 13 software (Systat software, Inc. San Jose, USA) was used for the interpretation of results. Results with  $p$  value less than 0.05 were considered to be statistically significant variance.

## RESULTS AND DISCUSSION

### Stability of IVM in phosphate buffer pH 7.4

The samples were investigated for a period of 48 hr in order to assess the stability of IVM and analyzed using UV-VIS spectrophotometric method. No significant degradation of IVM was observed in phosphate buffer and therefore phosphate buffer was selected as the receptor fluid.

### Preparation of liposomes and screening of stabilizers

Major variables that influence the liposome properties include cholesterol, lecithin amounts and method of preparation. IVM resulted into successful F4 formulation of liposomes with small particle size ( $< 100\text{nm}$ ) and negative charges with Pluronic (PF127) as a stabilizer. The stabilization of liposomes by Pluronic (PF127) may be because of its hydrophobic poly propylene oxide that promote the polymer to adsorb on to IVM surface<sup>25,26</sup> and hydrophilic poly ethylene oxide chains that extend into the aqueous phase thereby giving steric stabilization by stoppage of the aggregation.<sup>25,27</sup>

### Evaluation of liposomes

The size of liposomal vesicles was found to be in the range of  $0.06\mu\text{m}$ - $10.89\mu\text{m}$  ( $60\text{nm}$ - $10890\text{nm}$ ) which clearly supports the fact that the sizes between  $10\text{nm}$  and  $100\text{nm}$  are taken up by the lymphatic capillaries<sup>16</sup> (Figure 1). The liposomes were discrete, smooth with spherical shape, Uni-lamellar in Solvent Injection

method and multi-lamellar in Thin layer hydration technique.

### Drug entrapment efficiency

Formulation F4 showed highest % entrapment efficiency  $86.76 \pm 1.001$  among all the other formulations. Formulation F4 was prepared by solvent injection method and consists of equal ratios of the cholesterol and Lecithin. The presence of optimum amount of cholesterol in F4 is the major reason for highest entrapment efficiency which may be because of increased stability of the liposomal membrane that increased the rigidity of the bilayer.

### In-vitro drug release studies

*In vitro* release studies were performed to assess the dissolution parameters like drug percent released and first order release kinetic data for the prepared IVM liposomal formulations. The release profiles of the IVM liposomes are evident to show that the liposomal formulations were able to increase the release. In contrast, only  $38.14 \pm 0.432$  of IVM was released in case of pure IVM. The IVM release profiles from IVM liposomes were found to be significantly higher than that of pure IVM. The rapid diffusion rate of IVM from liposomes is a result of the increase in surface area after reduction of particle size to nano-size formulations. Therefore, lymphatic uptake of IVM liposomes should be achieved before the dissolution of the liposomes in the skin layers. In order to investigate the kinetic modelling and release mechanism of liposomes, the release profiles were fitted to several kinetic models. The release profiles exhibited best fit to first order model which showed that the drug release from the formulation matrix depends on the drug concentration within the matrix. The F4 formulation showed cumulative percent drug release of  $92.53 \pm 0.612$  at the end of 24 hr which may be due to the presence of optimum ratio of lecithin: cholesterol

(1:1) and solvent injection method as the method of preparation (Figure 2). Hence the F4 formulation was selected as optimized formulation. All the drug release studies were carried out in triplicate and in each case mean values and standard deviation values were calculated.

Liposomes with highest cholesterol content showed the lowest percent drug release and highest entrapment efficiency, lowest leakage of the drug. Even though F2 and F5 have highest cholesterol content, F4 showed highest entrapment efficiency because of optimum ratio of lecithin: cholesterol (1:1). The *in vitro* release study of IVM showed no burst effect which indicates that the drug transport out of the liposomes was mainly diffusion controlled mechanism. Release of IVM from liposomes is prolonged which could be because of Pluronic (PF127), its hydrophobic poly propylene oxide that promote the polymer to adsorb on to IVM surface and hydrophilic poly ethylene oxide chains that extend into the aqueous phase thereby giving steric stabilization by stoppage of the aggregation.

The F1-F8 liposomes showed characteristic drug release profiles with an initial fast drug loss followed by slower rates of drug loss. The IVM release from liposomal surface contributed to initial fast rate of release while the later slow release resulted from sustained drug release from the inner lamellae. In the first 4h, the release rate was fast and approximately reached 50% of the total dose and this phase is regarded as rapid release phase which could be because of the release of IVM that was adsorbed on the surface of the liposomes by a week binding force. The drug release became relatively slow after 4h and that phase is regarded as slow release phase. After 12 h, the amount of drug released gradually decreased with time.

Formulations F7 and F8 were prepared and subjected to drug release studies in order to evaluate the effect

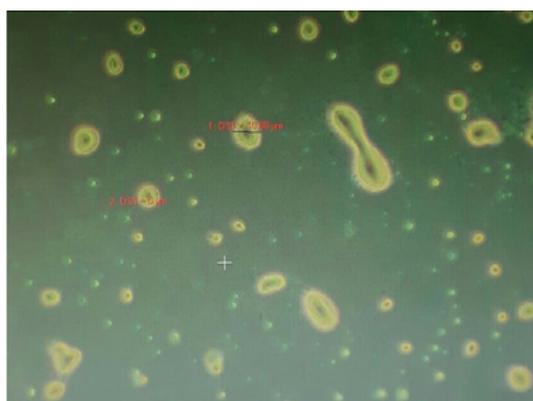


Figure 1: Phase contrast microscopy of IVM Liposomal Formulation.

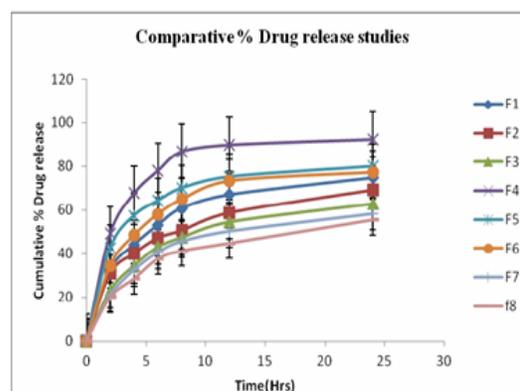


Figure 2: Comparative *in-vitro* % Drug release studies.

of drug : phospholipid (lecithin) ratio which is 1: 2.5 in F7 and F8 where as in F2, F5, in all other formulations drug: phospholipid (lecithin) ratio is 1: 5. The ratio of 1:5 IVM: lecithin ratio is considered optimum in terms of percent drug release and drug entrapment efficiency. Formulations F1, F2, F3 and F7 were prepared by thin layer film hydration method where as Formulations F4, F5, F6, F8 were prepared by solvent (ethanol) injection method. Ethanol injection method, one of the solvent dispersion method facilitated cholesterol to stabilize the lipid bilayer and decrease the leakage of entrapped IVM where as in thin layer film hydration method, one of the mechanical dispersion method cholesterol did not prevent leakage of entrapped IVM.

### FTIR studies

FTIR studies were conducted for the pure IVM and the physical mixtures of the drug and other excipients within the formulation. From the overlay it was observed that there was no interaction between the IVM and other excipients. The presence of all major functional groups of IVM confirmed that there were no chemical interactions between the pure IVM and any of the excipients used.

### Evaluation of IVM liposomes incorporated microneedles

Fabrication of the dissolving MN formulation to load high concentrations of lyophilized IVM liposomes was done by using different biocompatible polymers. Water-soluble, biocompatible polymers, such as PVA,<sup>28</sup> PVP<sup>29</sup> have been used in the preparation of dissolving MNs. It is evident from the present investigation that MNs prepared showed homogenous polymer blends and final prepared MN having sharp needle tips. A dry baseplate prepared from 15% w/w PVP and 1.5 % w/w glycerol was used for supporting all the formulations.

### Mechanical characterization studies

The ability of a MN array to get inserted properly is crucial to its use because the stratum corneum must be penetrated for the MN array to have its effect. Incorporation of IVM liposomes into the polymeric solution in the preparation of an MN array can show either a weak or strong effect on the MNs.<sup>30</sup> Mechanical tests are carried out as a part of initial formulation studies for MN arrays. The results obtained revealed that F4-E containing the combination of PVP and PVA with 30% w/w F4 IVM liposomes exhibited optimum mechanical strength and F4- F resulted in lack of mechanical strength due to increase of drug loading to 40% w/w. The baseplate had sufficient mechanical

strength. The same force was applied to commercially available solid MN devices AdminPatch arrays (ADM) (0.6, 0.9, 1.2 and 1.5 mm length) and lab fabricated polymeric dissolving MN arrays (PMs) (0.6 mm length). The dimensions of the PM were found to be consistent and repeatable with good tip shape, confirming the complete filling of the PVP/PVA into the MN insert cavity under the maintained processing conditions and the technique used is reliable for the bulk manufacture of PMs.

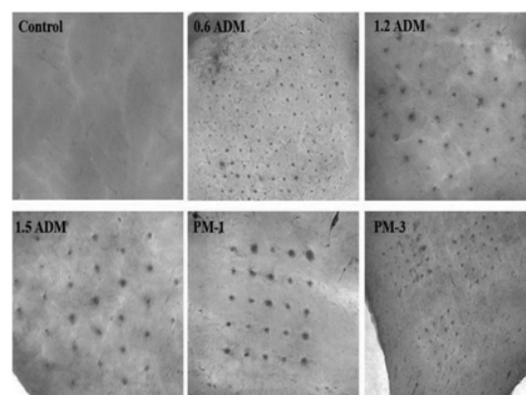
### Ex vivo evaluation studies

#### Evaluation of microneedles for their relative efficiency in transdermal permeation enhancement of IVM using porcine ear skin as membrane model

As IVM is lipophilic, it did not dissolve in the aqueous environment of the MN polymers and did not result in homogeneous distribution of the drug in the arrays and hence MN lacked mechanical strength.

#### Histological examination and calculation of penetration depth

Quasmo PZRM-700 microscope fitted with 10x objective was used to observe histological sections prepared using the hematoxylin and eosin stains. The evidence of breakage of SC barrier after MN treatment was detectable in the histological sections and MN arrays penetration through the corneocytes without merely indenting them (Figure 3) Skin layer disruption and the formation of microconduits were clearly evident from histological section images. The penetration depth was 25-35% of the original needle length for ADM and 55-60% for the PM MN (Figure 3 and 4). Even though the length of the MN differs in ADM, the percentage of MN penetration is almost same, which is an indication of uniformity in thumb pressure under which MNs



**Figure 3: Surface images of stained skin without and with MN treatment.**

were applied at different times. With the ADM devices, as the length of the MNs increased the penetration depth also increased. However, the microconduits were found to be wider and deeper with PM when compared to ADM of similar lengths i.e 0.6 mm. These differences in the efficiency of creating microconduits in skin layers between the two types of MN devices (ADM and PM) may be attributed to the differences in the geometry parameters such as shape, design and type of fabricating material. Regarding the shape/design, both ADM and the PMs were conical (3D) in shape and the microconduits formed by PMs were wider.

### In vitro skin permeation studies

Significant increment in IVM permeation was observed after the application of MNs into the skin ( $p < 0.05$ ) when compared to passive permeation studies (needle free patch onto the skin which was not pressed). With the PM MNs 3.15-fold increase in the cumulative amount of IVM permeated was observed when compared to passive amounts. The IVM flux values were also found to be more with formulation code E. Even though, the skin penetration by PM device (0.6mm array) was

significantly greater when compared to 0.6 and 0.9 mm ADM, the overall permeation enhancement in terms of flux, cumulative amount permeated etc achieved were in the order of 1.5mm ADM > PM Formulation code E > 1.2 mm ADM > 0.9 mm ADM > 0.6 mm ADM > PM Formulation code F > passive (Table 2). Significant higher amounts of IVM were found to be distributed in skin layers at the end of 24 h with MN treated studies and is an indication of potential IVM skin deposition.

The results revealed that the incorporation of IVM in liposomes into dissolving MN arrays significantly enhanced the delivery of IVM into the skin and led to the retention of IVM in the dermis layer, a site replete with lymphatic capillaries<sup>31</sup> and hence could potentially target the adult parasitic worms and infective larvae (larvae from mosquito which found their way to lymph nodes from the skin after a mosquito bite) in the lymph nodes. In addition, the part of IVM that was not retained in the dermis layer might be taken up by the blood capillaries which could potentially kill microfilariae in the bloodstream. Many published studies have demonstrated that nanoparticles of size < 100 nm accumulate in lymph nodes.<sup>32-34</sup> Furthermore, work will be carried out

**Table 1: Formulation table of Ivermectin (IVM) Liposomes.**

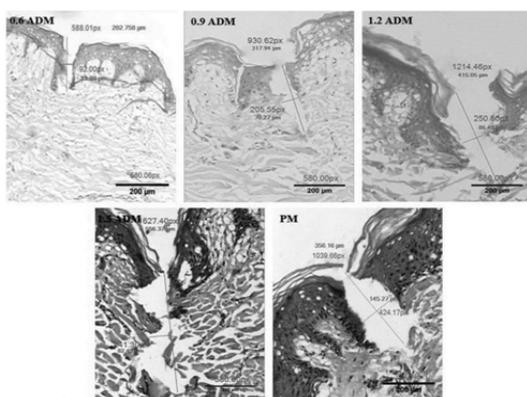
Formulation	Ingredients							Method of preparation
	IVM (mg)	Lecithin (mg)	Cholesterol (mg)	Butanol (ml)	Buffer (ml)	Lecithin :Cholesterol	Pluronic (PF127) (mg)	
F1	10	50	50	5	10	1:1	0.25	FHM
F2	10	50	100	5	10	1:2	0.25	FHM
F3	10	50	25	5	10	1:0.5	0.25	FHM
F4	10	50	50	5	10	1:1	0.25	SIM
F5	10	50	100	5	10	1:2	0.25	SIM
F6	10	50	25	5	10	1:0.5	0.25	SIM
F7	10	25	50	5	10	0.5:1	0.25	FHM
F8	10	25	50	5	10	0.5:1	0.25	SIM

FHM - Thin layer film hydration method

SIM - Solvent injection method

**Table 2: Permeation parameters for IVM Liposomes.**

Permeation parameter/variable	Skin treatment					
	Passive (needle free patch)	1.5 mm solid microneedles	1.2 mm solid microneedles	0.9 mm solid microneedles	0.6 mm solid microneedles	0.6 mm dissolving microneedles
Apparent Permeability Coefficient (cm/h)	0.025±0.012	0.815±0.006	0.725±0.021	0.601±0.010	0.317±0.014	0.798±0.009
Diffusion Coefficient (10 <sup>-6</sup> ) (cm <sup>2</sup> /s)	1.72±0.12	28.12±1.58	22.74±1.35	16.32±1.51	12.72±1.12	25.62±0.71
IVM content in skin(µmol/g)	0.36±0.12	5.72±0.66	4.12±0.39	3.78±0.55	2.19±0.13	4.96±0.81



**Figure 4: Penetration depth calculation of ADM and PM in skin.**

in the near future to demonstrate the incorporation of rhodamine B-encapsulated IVM liposomes of <100 nm into dissolving MNs to deliver the dye to lymph nodes of mice.<sup>35</sup> From the work done by Ryan F. Donnelly it was clearly evident that nanoparticles were not only taken up by the lymphatic circulatory system but also travelled through the lymphatics and entered the systemic circulation.<sup>35</sup> With respect to the concentration of IVM required to kill filarial nematodes, it was reported that 170 µg/ml was able to kill *Brugia malayi* *in vitro* after 4 days<sup>36</sup> which was lower than the  $C_{max}$  obtained in the present dermatokinetic study.

However, further studies are now required to determine the concentration of IVM which reach the infection site *in vivo*. Skin sections will be sliced carefully to separate epidermis from dermis and amount of drug deposited into each slice will be calculated in the future. Accordingly, we propose that this combination of liposomes and MNs could be used to target IVM to the anatomic sites of filarial nematodes for the single drug therapy of Human lymphatic filariasis

Microneedle assisted transdermal drug delivery may very well bridge the need for patient friendly single drug treatment for LF and clinically efficient IVM liposomes which may improve the current mass drug administration.

### IVM content in the skin

Compared to passive permeation (needle free patch) IVM content in the skin was increased due to skin pre-treatment with microneedles.

### CONCLUSION

The present study investigated the potential of solid, dissolving MNs, used in combination with liposomes, to assist in the lymphatic targeted delivery of IVM to the dermis layer. Several optimization steps were

carried out to develop the liposomal formulation with small particle size (< 100nm) and negative charges. Microneedles (solid and dissolving) enhanced the release of IVM from IVM liposomes *in vitro* through transdermal delivery which could have positive implications in the single drug clinical treatment of LF. However, comprehensive studies including lymphatic uptake and pharmacodynamic studies in an appropriate model system are required to support the present novel microneedle assisted IVM liposomal formulations before they achieve patient benefit.

### ACKNOWLEDGEMENT

The authors are thankful to Siddhartha Academy of General and Technical Education, Vijayawada, for providing necessary facilities to carry out the present research work.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ABBREVIATIONS

**LP:** Liposomes; **IVM:** Ivermectin MNs-Microneedles; **TD:** Transdermal delivery; **LF:** Lymphatic filariasis; **ADM:** Admin patch solid microneedles-commercial; **PM:** Laboratory fabricated dissolving polymer microneedles.

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**SUPPLEMENTARY FIGURES**

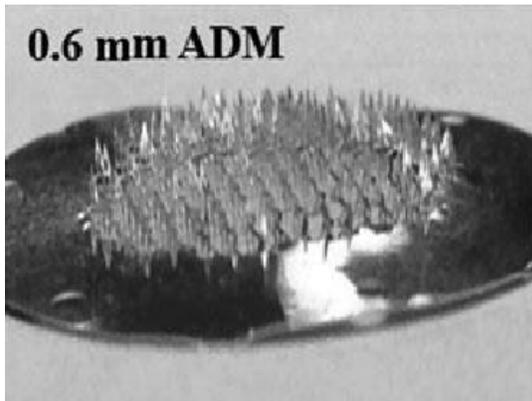


Figure 5: Adminpatch commercial solid microneedles of length 0.6 mm.

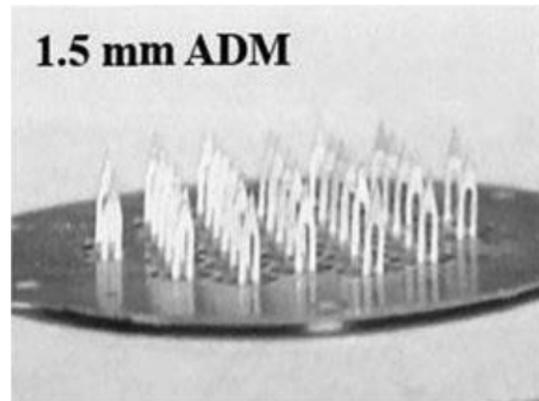


Figure 8: Adminpatch commercial solid microneedles of length 1.5 mm.

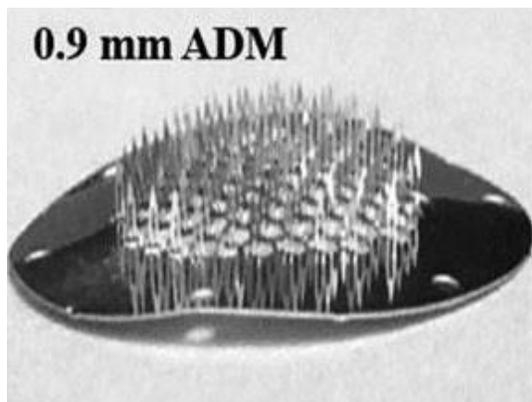


Figure 6: Adminpatch commercial solid microneedles of length 0.9 mm.

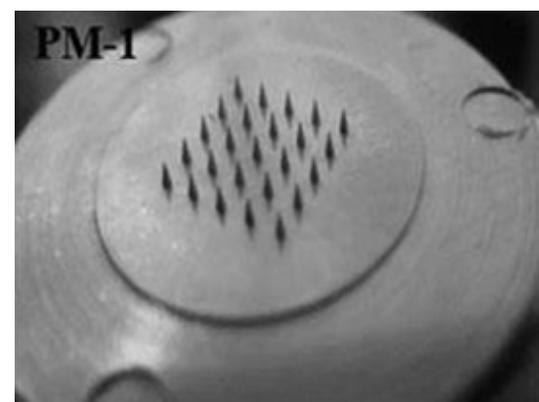


Figure 9: Laboratory prepared dissolving microneedles of length 0.6 mm.

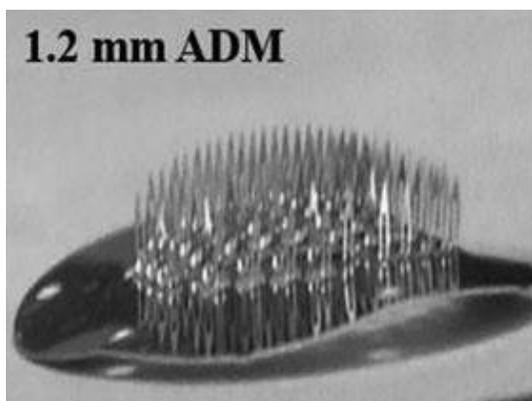


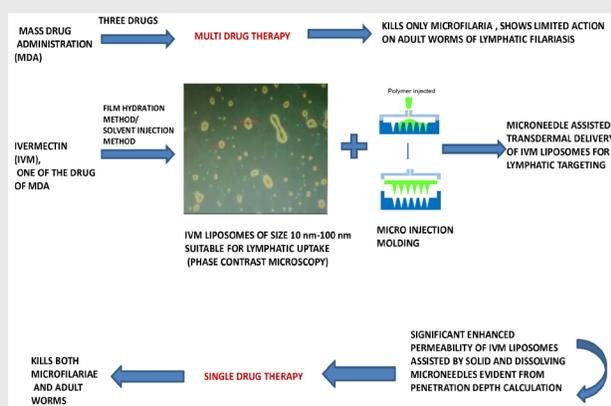
Figure 7: Adminpatch commercial solid microneedles of length 1.2 mm.

## SUMMARY

Conventional oral administration (mass drug administration-MDA) of antifilarial drugs for lymphatic filariasis (LF) results in non-specific targeting of the drugs which control only the transmission of the disease. Ivermectin (IVM), which is a part of MDA for LF has proven adult worm as well as microfilariae suppression when administered alone. But the existing oral and parenteral IVM formulations have several disadvantages and unable to target human lymphatic system to show adult worm suppression. When IVM is properly formulated and administered through suitable route, single drug therapy and specific targeting with IVM may be sufficient for LF. Hence alternate route of administration such as transdermal route is an ideal choice for IVM to specifically target the lymphatic system, due to higher lymph flow rates in the skin compared to other interstitial sites.

Liposomes are one of the technologies in which the formulated particle has a size range of 10 nm–100 nm which are taken up by the lymphatic capillaries. Hence liposomes are the best formulation for lymphatic uptake. The problem that arises with IVM (log P of 5.83 and highly lipophilic,) is that the drug is retained in the Stratum corneum, that create problems with achieving steady plasma concentrations within a reasonable time span and also IVM is unable to pass through aqueous epidermis. Hence IVM may not permeate through the skin at a sufficient amount to reach dermis where lymphatic capillaries are present. Novel transdermal permeation enhancement methods such as microneedles (MN) application may address this limitation. Liposomal uptake by reticulo-endothelial system is significantly less in microneedle assisted transdermal delivery (MNTD) than intravenous route. Hence the present study investigated the combination of liposomes (LP) and microneedles (MNs) as a single drug treatment approach for the delivery of an antifilarial drug, Ivermectin (IVM) in which the role of MN arrays (commercial solid MNs 1.5mm, 1.2mm, 0.9mm, 0.6mm lengths and laboratory fabricated dissolving MNs 0.6mm length) in increasing the *in vitro* permeation of IVM-LP across pig ear skin was studied. Formulation F4-E containing the combination of poly (vinyl pyrrolidone) (PVP) and poly (vinyl alcohol) (PVA) with 30% w/w F4 IVM liposomes exhibited superior IVM release, IVM flux values with optimum mechanical strength and fulfilled the regulatory requirements.

## PICTORIAL ABSTRACT



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**Cite this article:** Devineni J, Pravallika D, Rani BS, Nalluri BN. Effective Single Drug Treatment of Lymphatic Filariasis through Enhanced Transdermal Delivery of Ivermectin Liposomes using Solid and Dissolving Microneedles. Indian J of Pharmaceutical Education and Research. 2020;54(3s):s492-s504.