Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 4, 2013

Research Article

BIOAVAILABILITY AND HYPOCHOLESTEROLEMIC EFFECT OF PRONIOSOMAL SIMVASTATIN FOR TRANSDERMAL DELIVERY

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Received: 15 July 2013, Revised and Accepted: 12 Aug 2013

ABSTRACT

Objective: Simvastatin (SIM) existing oral formulations suffer from poor bioavailability (less than 5%) as a result of extensive first-pass effect as well as dissolution rate-limited *in vivo* absorption. In the present study, a proniosomal system was designed for SIM transdermal delivery.

Methods: *In vitro* evaluation of proniosomal SIM was performed in different aspects; drug entrapment, vesicle size, zeta potential, vesicular morphology, *in vitro* release, skin permeation and stability. The optimized formula was assessed for transdermal permeation in rats and for hypocholesterolemic effect in hypercholesterolemic rats compared to oral SIM dispersion.

Results: The proniosomal formula consisted of lecithin: Tween 20 in molar ratio of 1:9 exhibited significantly (P<0.05) lower vesicular size, high SIM entrapment, sustained release pattern as well as significantly higher skin permeation. The topical application of optimized proniosomal SIM showed significantly (P<0.05) higher values of AUC_{0-8} and T_{max}, and significantly (P<0.05) lower values of C_{max} compared to SIM oral dispersion. The mean relative bioavailability of proniosomal SIM to oral dispersion was 120.40 ± 11.44%. The investigated proniosomal SIM showed a significantly (P<0.05) higher hypocholesterolemic effect compared to oral SIM dispersion in treatment of hypercholesterolemic rats.

Conclusion: The obtained results were very encouraging and offered an alternative approach to enhance the bioavailability and the hypocholesterolemic effect of SIM.

Keywords: Proniosomes, Simvastatin, Bioavailability, Hypocholesterolemic effect, Transdermal delivery.

INTRODUCTION

Proniosomes are liquid crystalline-compact niosomal hybrid which can be converted into niosomes upon hydration with water. Modification of vesicle composition or surface charge can adjust drug release and/or affinity for the target site. Proniosomes are known to avoid many problems associated with the nature of aqueous niosomal dispersions. Although niosomes show good chemical stability, they also show problems of physical instability (as vesicles' aggregation and fusion, sedimentation upon storage, leakage of entrapped drug molecules or hydrolysis of encapsulated drugs), which leads to reducing their shelf life [1]. Ease of manufacture of niosomal vesicles, with good stability and low cost of materials used during formulation, makes niosomes and in turn proniosomes, more attractive to be scaled up for industrial manufacturing [2]. The additional advantages as ease of transportation, distribution, dosing and storage make proniosomes; 'dry niosomes' a promising industrial product [1].

Recently, there is an increasing interest towards transdermal drug delivery systems. This route of administration has various advantages like avoiding drug metabolism or chemical degradation in the gastrointestinal tract in addition to hepatic first pass effect [3]. But on the other hand, the skin represents a great barrier towards transdermal delivery of drugs, providing resistance to penetration of drug molecules. Thus, several strategies and technologies had been developed to overcome skin barrier properties and facilitate percutaneous penetration of drugs [4]. Proniosomes offer various types of vesicles with different composition representing potential candidates for transdermal delivery of drugs. Previous research investigated the transdermal delivery of drugs using proniosomes composed of non-ionic surfactants as: sorbitan esters (Spans) and polyoxyethylene sorbitan esters (Tweens), in addition to cholesterol or lecithin [5 -8].

Simvastatin (SIM) is a lipid-lowering agent used for treatment of hypercholesterolemia in humans and animals. Simvastatin (lactone compound) undergoes hydrolysis to be converted to the β, δ-dihydroxy acid form, which is a potent competitive inhibitor of 3-hydroxyglutaryl-CoA reductase, the enzyme catalyzing the rate-limiting step of cholesterol biosynthesis. When administered orally, SIM is subjected to extensive first-pass metabolism in the liver leading to its low bioavailability (5%) and short half life (2 hrs) [9]. Thus, the objective of our study was to formulate and optimize a stable transdermal formulation for delivering SIM by using proniosomes in order to avoid the first-pass effect, and consequently enhance SIM bioavailability and therapeutic efficacy leading to reduction in dosing frequency and improvement of patient compliance.

MATERIALS AND METHODS

Materials

SIM was kindly donated by Hikma Pharma S.A.E (6th of October City- Egypt). Lecithin, Sorbitan monostearate (Span 60), and Methanol were purchased from Sigma Aldrich (St. Louis, USA). Polyoxyethylene (20) sorbitan monolaurate (Tween 20) was purchased from Oxford Chemicals (Mumbai, India). All other chemicals and solvents were of analytical grade and obtained from El-Nasr Company for Pharmaceutical Chemicals, Cairo, Egypt.

Preparation of proniosomes

Proniosomal formulae were prepared by a method reported in literature [6]. Briefly, in wide-mouth glass tube, accurately weighed amounts of the surface-active agent were mixed with lecithin to make 1 mmol total lipids. Simvastatin was added to the surfactant/lipid mixture. Absolute ethanol (about 400 mg) was then added to the above mixture, and then the open end of the glass tube was tightly sealed and warmed in water bath at 65 ± 3°C for 5 min with shaking till complete dissolution of lipids. About 1.6 ml hot distilled water (65 ± 3°C) was added to the formed transparent solution, while warming in the water bath for 3-5 min till a translucent solution was obtained. The mixture was allowed to cool down to room temperature till the dispersion was converted to proniosomal gel. The obtained formulae were kept in the same closed glass tubes in dark for further characterization.

Hydration step and formation of niosomes

Proniosomes-derived niosomes were prepared by hydration of the gels prepared as previously described above. About 7 ml phosphate buffer (pH 7.4) was added into each glass tube followed by heating for 10 min at a temperature above 65°C in a water bath. The niosomal suspension was sonicated using probe sonicator

(Hielscher UP50H ultrasonic processor, Germany) to ensure uniform dispersion. The final volume was adjusted to 10 ml using phosphate buffer (pH 7.4). The resulting niosome dispersion was used for the determination of the entrapment efficiency, particle size analysis, and morphological studies.

Entrapment efficiency %

Free SIM was separated from SIM-loaded niosomes by centrifugation [8]. One ml sample of the prepared niosomal dispersion (equivalent to 0.5 mg SIM) is centrifuged at 14000 rpm for 40 min at 4°C by cooling centrifuge (Centurion Scientific Ltd). The supernatant was collected and the niosomal pellets were re-dispersed in phosphate buffer (pH 7.4) and then centrifuged again. This washing procedure was repeated two times to ensure that the free drug was no longer present in the voids between the niosomes. The collected supernatant fractions were diluted with phosphate buffer (pH 7.4) and analyzed for SIM concentration spectrophotometrically at λ = 238 nm (Perkin Elmer UV/Vis spectrometer, Lambda EZ 201). The amount of entrapped SIM was calculated by the following equation:

Eq. 1

$$EE(\%) = \left[\frac{C_t - C_f}{C_t}\right] \cdot 100$$

Where C_t is the concentration of total drug incorporated in 1 ml niosomal dispersion and C_f is the concentration of free drug.

Particle size and zeta potential determination

Vesicles particle size diameter, zeta potential and size distribution were determined by Zetasizer Nano-ZS (Nano series, Malvern Instruments Ltd. Malvern, UK). The vesicle size measurements were performed at temperature 25°C, using a 45 mm focus lens and a beam length of 2.4 mm. Three replicates were taken for each sample.

Transmission electron microscopy

The morphology of the niosomes derived from proniosomal formulae was determined by transmission electron microscopy (Tecnai G20, FEI, Hillsboro, Oregon, USA- Super twin, double tilt, LaB6 Gun, with applied voltage of 200 kV and magnification range up to 1,000,000 X). A drop of niosomal dispersion was applied to a carbon film-covered copper grid and was stained with 2% phosphotungstic acid.

***In vitro* drug release studies**

Prior to testing, a piece of cellulose membrane (Molecular weight cut off 12,000–14,000 Da, Spectra Por®, Spectrum Medical Inc., Los Angeles, CA, USA) of suitable dimensions was soaked in sufficient amount of distilled water for about 24 h. The membrane was fixed in position to cover one end of a top-cut plastic syringe used to represent a dialyzing tube of 1.9 cm internal diameter to provide an effective release area of approximately 2.84 cm^2. The membrane was made water tight by rubber band and 1 ml of the washed niosomal pellets suspended in phosphate buffer (pH 7.4) was placed in the designed release assembly. The tube enclosing the test sample was then attached to the shaft of a dissolution apparatus I (Hanson Research, California, USA) instead of its basket. The dialyzing tube was carefully lowered and adjusted so that the membrane just touched the surface of the release medium. A volume of 200 ml phosphate buffer (pH 7.4) maintained at 37°C and stirred at a speed of 100 rpm was used as the release medium [10]. Aliquots (2 ml) of the medium were withdrawn at 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h, and replaced with equal volume of the fresh release medium. The samples were filtered through 0.2 µm Millipore membrane filters and analyzed for SIM content spectrophotometrically.

***Ex vivo* skin permeation**

Skin was obtained from the abdominal region of male mice weighing 25 ± 2 g. The full-thickness skin, free of bites and scratches was excised after removing hair with a depilatory cream. Subcutaneous fat was carefully removed without damaging the epidermis; the skin washed with physiological saline followed by phosphate buffer (pH 7.4) and then visually inspected for its integrity. When not in use, the skin was stored at −21°C and used within 1 week of skin harvest. Prior to testing, the skin was left to thaw till room temperature then equilibrated in phosphate buffer saline (pH 7.4) for 1 h before the experiment. This membrane was then fixed in position to top-cut plastic syringe representing a dialyzing tube of 1.9 cm internal diameter. The skin was placed with its stratum corneum facing upward (donor compartment) and dermal side facing downward (receptor compartment). The position-fixed skin was made leak-proof by a rubber band. One gram of the tested proniosomal gel (equivalent to 1 mg simvastatin) was accurately weighed in the plastic tube (representing donor compartment), which was then attached through its other end to the shaft of the dissolution tester. The whole assembly was adjusted in the same manner as previously described under the *in vitro* release study. The receptor compartment was filled with 50 ml phosphate buffer saline (pH 7.4). The temperature was maintained at 37 ± 0.5°C to simulate human body temperature; the medium was constantly stirred at 100 rpm. Two ml samples were withdrawn from the receptor compartment at various time intervals up to 12 h, and replaced with an equal volume of fresh buffer. The samples were then assayed for their SIM content by HPLC.

SIM steady state flux (J) was calculated from slope of the line obtained on plotting mean cumulative amount permeated per area versus time, according to the following equation: [11]

Eq. 2

$$J = \frac{dQ}{dt} \cdot \frac{1}{A}$$

Where A is the diffusional area of the diffusion cell, dQ/dt is the slope in the steady-state region of the amount of permeant (Q) in receiver chamber versus time (t) plot. In other words, dQ/dt represent the permeation rate obtained as the slope of the line obtained on plotting cumulative amount of drug permeated versus time. The total permeability coefficient (P_T) for the permeant (SIM) was then calculated according to the following equation: [11]

Eq. 3

$$P_T = P_L = \frac{1}{AC_D} \cdot \frac{dQ}{dt}$$

Where C_D is the concentration in the donor chamber, P_T can be further divided into parallel lipoidal and pore pathway components P_L and P_P in the stratum corneum, respectively. but the probe permeant SIM transport through skin mainly through the lipoidal pathway, therefore it most conveniently, allows equation (3) to be well-approximated as;

$$P_T \approx P_L$$

HPLC assay of SIM

Sample preparation

To each 0.5 ml sample, 0.5 ml of 2% zinc sulphate solution, a protein precipitant, was added and the mixture, vortexed for 1 min and then centrifuged at 13,000 rpm (Centurion Scientific Ltd) for 10 min. The supernatant was directly injected into HPLC system.

HPLC system

A reported HPLC method [12] for determination of SIM was adopted with slight modifications. The HPLC system consisted of a Shimadzu (Tokyo, Japan) chromatographic system equipped with Shimadzu LC-10 AD VP pump, Shimadzu SPD-10A VP UV/Visible detector, DGU-12A degasser and SCL-10A VP system controller. Samples were injected using Spectra System Auto sampler AS3000 at injection volume of 20 µl and 1 ml/ min flow rate. The used column was a Waters C18 column (10 µm particle diameter 125A, µ Bondpak, 4.6×250 mm). A mobile phase consisting acetonitrile was used in an analysis that was conducted in an isocratic elution mode. Prior to use, the mobile phase was sonicated and filtered through 0.2 µm

membrane filter. Data acquisition and integration were carried out using Shimadzu Class-VP software (version 6.14 SP1). The detection wavelength was 238 nm. All operations were carried out at ambient temperature.

Stability study

The optimized proniosomal formula was stored in sealed glass vials at room temperature (25±0.5°C) and refrigeration temperature (2-8°C) for 3 months. After 3 month-storage period, hydration step was carried out, and the entrapment efficiency as well as the mean particle size of each sample were determined and compared to the freshly prepared proniosomes.

***In vivo* absorption study**

The *in vivo* absorption study was carried out to evaluate bioavailability of SIM-loaded proniosomes formulation applied onto skin compared to oral SIM dispersion.

Study design

The protocol of the study was approved by the Animal Ethics Committee of Faculty of Pharmacy, Helwan University. The study was conducted in accordance with EC Directive 86/609/EEC for animal experiments. Twelve male Wistar rats weighing 250 ± 20 gm were maintained in a light and temperature controlled room. The rats were divided into 2 groups, 6 rats each. The dorsal side of rats in the first group was shaved from hair using electric shaver. All rats were fasted overnight (12 h) with free access to water before the experiments. On day of experiment, the rats dorsal skin of the first group were treated with application of a single dose of SIM loaded proniosomes (equivalent to 20 mg/kg) [13] hydrated in 1% carbopol gel. The applied gel was gently rubbed onto the shaved skin until totally disappeared. The rats of the second group received the same equivalent oral dose of SIM suspension using an animal feeding needle.

Blood sampling

Blood samples (1ml) were collected directly through retro-orbital puncture from each rat under mild anesthesia using diethyl ether, into heparinized tubes at 1 h pre-dose and at 1, 2, 3, 4, 6 and 8 h post dose. The blood samples were centrifuged at 5000 rpm for 10 min and the plasma was transferred to separate glass tubes to be kept frozen until being analyzed using LC-MS/MS.

Analysis of SIM Plasma Levels

SIM plasma concentration was quantified by a reported liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [14] with slight modifications. Prior to analysis of plasma samples, aliquots of plasma (200 µl) spiked with 20 µg/ml Rosuvastatin (internal standard), were vortexed for 1 min with 400 µl of diethyl ether : dichloromethane 7:3 v/v) and centrifuged at 2,000 rpm for 10 min at room temperature. The supernatant was filtered and injected into the LC-MS/MS system.

LC-MS/MS System

The LC system was interfaced to an Agilent 6410 triple quadruple liquid chromatography/mass spectrometry (LC/MS) system (Agilent Technologies Inc., Santa Clara, California, USA). The analytical column used was Inertsil ODS-3 (50 mm x 4.6 mm, 5µm). An isocratic mobile phase was used consisting of 10 m M ammonium formate: Methanol 10:90 (% v/v). The flow rate was 0.7 ml/min under ambient temperature. The temperature of the auto sampler (Model G1367B, Agilent 1200 series) was maintained at 4°C and the injection volume was 20 µL. The run time was 3 min. All analyses and internal standard were detected on a triple quadruple mass spectrometer equipped with an electro spray ion source (Model G1948B, Agilent 1200 series) and operating in the positive ion mode. Data acquisition was performed using the Agilent Mass Hunter workstation software (B.02.01 SP1).

Bioavailability assessment

Bioavailability parameters of SIM (C_{max} , T_{max} , AUC_{0-t}) after a single dose (80mg/kg) of applied SIM-loaded proniosomal gel and oral SIM dispersion were determined. Peak plasma concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained directly from the individual plasma concentration versus time curve. The area under the plasma concentration-time curve from zero to the last measurable plasma concentration at time t (AUC_{0-t}) was calculated using linear trapezoidal rule. The relative bioavailability (%) of proniosomal SIM applied onto skin to free oral SIM was calculated using the following equation:

Eq. 4

$$\text{Relative bioavailability (\%)} = \left[\frac{AUC_{0-t}\ \text{proniosomal SIM}}{AUC_{0-t}\ \text{SIM oral dispersion}} \right] \cdot 100$$

The elimination rate constant (k_{el}) was estimated by least square regression of plasma concentration–time data points in the terminal log linear region of the curves. Half life ($t_{1/2}$) was calculated as 0.693 divided by k_{el}.

Assessment of hypocholesterolemic effect

This *in vivo* study was carried out in order to evaluate the hypocholesterolemic effect of topically applied SIM proniosomes formulation in hypercholesterolemic rats compared to oral SIM suspension. Approval to carry this study was obtained from the Animal Ethics Committee of Faculty of Pharmacy, Helwan University. Guidelines of the ethics committee were followed for the study. Eighteen male Wistar rats weighing 130 ± 20 g were divided into three groups each containing six rats. All rats were maintained in a light and temperature controlled room. Prior to induction of hypercholesterolemia, blood samples from all rats were withdrawn from the retro-orbital sinus using heparinized glass capillary tubes to a glass slide, then blood was transferred to the cholesterol test strips via 15 µl blood capillary collectors (CardioChek®) to ensure constant blood volume and analyzed for the total cholesterol (TC) using CardioChek® Analyzer (Polymer Technology Systems, Inc., Indianapolis, IN, USA). The rats were fed with atherogenic diet for 30 days to induce hypercholesterolemia. The atherogenic cholesterol diet was composed of 5% hydrogenated fat, 5% butter, 1% cholesterol powder and 0.5% cholic acid from coconut oil, all mixed with powdered chow [15]**.** After one month, rats' TC was recorded. The hypercholesterolemic rats were divided into three groups, each containing 6 rats. One group received no medication (negative control group). The rats dorsal skin of the 2nd group was shaved and SIM-loaded proniosomes formulation was applied onto skin in amount equivalent to 20 mg/kg. While rats of the 3rd group received the same equivalent dose of SIM oral suspension by feeding tube. Both treatments were continued for 7 successive days. On the 8th day, TC level of each rat in the three groups was measured.

Statistical Analysis

In order to compare the results Student's t test, paired t test and ANOVA test (Graph Pad Prism; version 6.0) were used. Data reported as means ± standard deviation (SD). A statistically significant difference was considered at P value <0.05.

RESULTS AND DISCUSSION

Entrapment efficiency % (EE %)

The composition of prepared proniosomes was listed in Table 1. EE % of the prepared proniosomal formulations are presented in Table 2. The results revealed that increasing lecithin ratio in proniosomal formulations significantly increased the EE %. This might be attributed to the higher lecithin concentration that provides an additional space in the bilayer to entrap more drug molecules and reduces the vesicular permeability [16, 17]**.** The proniosomes prepared with span 60 showed higher EE% than their corresponding formulae prepared using tween 20 as surfactant. This could be attributed to the higher gel to liquid phase transition temperature of Span 60 (C18 surfactant) than Tween 20 (C12 surfactant) [18]. Surfactants with higher phase transition temperature are more likely to be in the ordered gel form forming less leaky bilayers than surfactants of lower phase transition temperature which are more likely to be in a less ordered liquid form [2, 19]. Another reason for the higher EE% of Span 60

niosomes is their low HLB value owing to their hydrophobic alkyl chain, which enables efficient trapping of the hydrophobic drug simvastatin (log P = 4.68) [18]. Similar observations have been previously reported in literature [7].

Table 1: Composition of SIM proniosomal formulations

Formulation code*	Molar ratio		
	Lecithin	Span 60	Tween 20
F1	0.1	0.9	_
F2	0.3	0.7	_
F3	0.6	0.4	_
F4	0.1	_	0.9
F5	0.3	_	0.7
F6	0.6	_	0.4

** All formulae contain 50 mg simvastatin*

Table 2: Entrapment efficiency % (EE %), vesicle size, polydispersity index (PDI) and zeta potential (Z) of SIM proniosomal formulations

Proniosomal SIM formulation	EE (%± SD, n=3)	Vesicle size (nm ±SD, n=3)	PDI (±SD, n=3)	Z (mV ± SD, n=3)
F1 (lecithin/Span 1:9)	81.80 ± 0.33	161.34 ± 11.23	0.11 ± 0.02	-19.90 ± 1.84
F2 (lecithin/Span 3:7)	83.92 ± 0.27	307.55 ± 43.32	0.20 ± 0.06	-18.43 ± 0.97
F3 (lecithin/Span 6:4)	89.61 ± 0.88	494.79* ± 33.10	0.16 ± 0.02	-17.60 ± 1.11
F4 (lecithin/Tween 1:9)	72.90 ± 0.65	68.20** ± 13.11	0.19 ± 0.03	-12.06 ± 0.22
F5 (lecithin/Tween 3:7)	75.86 ± 0.73	164.32 ± 22.20	0.195 ± 0.03	-11.96 ± 0.34
F6 (lecithin/Tween 6:4)	79.32 ± 0.41	251.15 ± 24.66	0.22 ± 0.05	-11.77 ± 0.51

**F3 containing high lecithin content exhibited the largest vesicle size*

*** F4 containing high Tween 20 content exhibited the smallest vesicle size*

Transmission electron microscopy (TEM)

The transmission electron microscopy images of the niosomes prepared from proniosomal formulae F3 and F4 were shown in Figure 1: a, b, respectively. Electron micrographs of formula F3 exhibiting the largest vesicle size revealed spherical nano vesicles with well-identified outline and core, while formula F4 demonstrating the smallest vesicle size had a slightly different outline. This could be attributed to the difference in HLB between the two surfactants used in formulations (Span 60 and Tween 20).

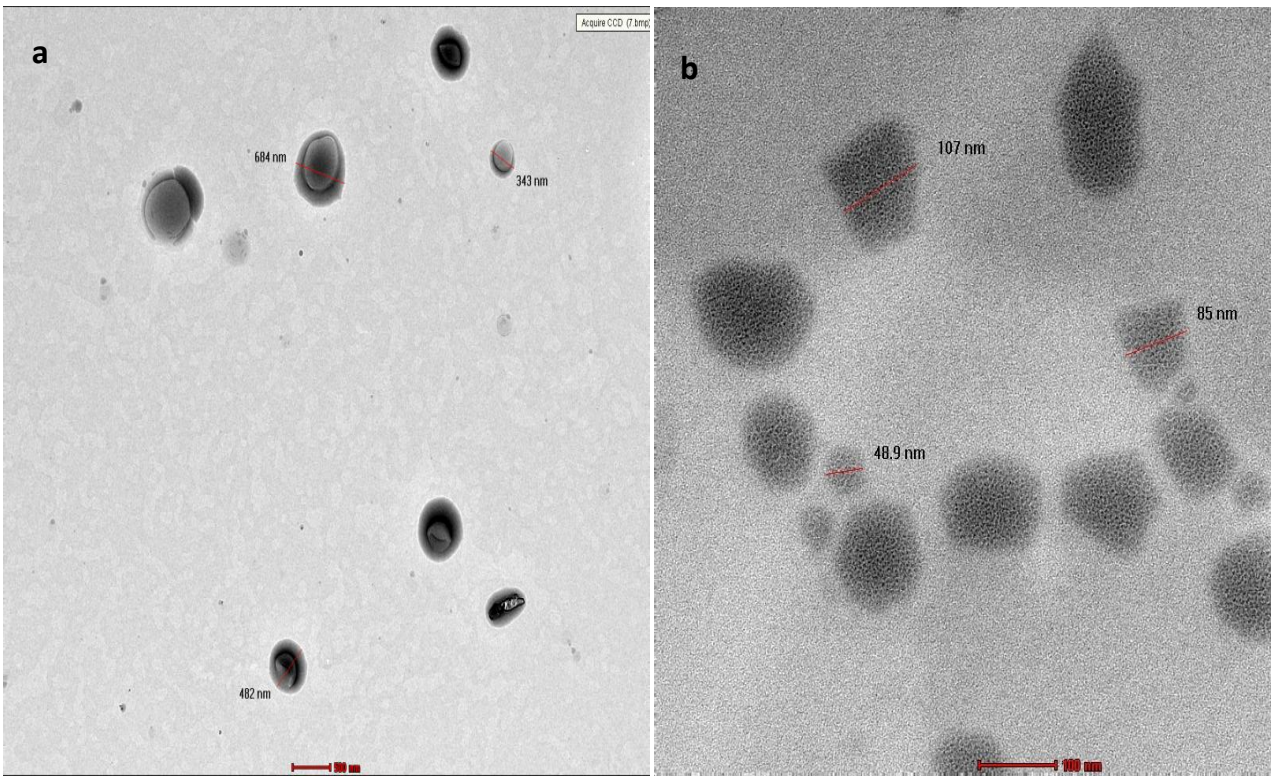

Fig. 1: Transmission electron microphotographs of SIM proniosome-derived niosomes stained with 2% phosphotungstic acid. a; (F3 lecithin/Span 6:4, scale bar = 500nm) b; (F4 lecithin/Tween 1:9, scale bar = 100nm).

Zeta potential analysis

The results of zeta potential were displayed in Table 2. All formulae of proniosomes-derived niosomes carried a negative charge. The zeta potential values ranged from -19.90 to - 11.77 mV which indicated stability of the prepared niosomes with very little or no agglomeration [20].

Vesicle size and polydispersity index (PDI)

Vesicle size and PDI of proniosomes derived niosomes dispersions were listed in Table 2. All formulae showed vesicle size ranged from 68.20 ± 13.11 to 494.79 ± 33.10 nm with a mean vesicle size 321.23 ± 167.15 and 161.22 ± 91.51 nm for proniosomes prepared using Span 60 and Tween 20, respectively. The obtained vesicle size distribution for all samples showed a unimodal pattern which favored transdermal delivery [21]. The results clearly revealed that the lecithin concentration and the type of surfactant have significant impact on the average vesicle size of the prepared niosomes. Niosomes of F3 and F6 containing higher lecithin content showed larger vesicle size (494.79 ± 33.10 nm and 251.15 ± 24.66 nm) compared to their corresponding formulae F1 and F4 (161.34 ± 11.23 and 68.20 ± 13.11, respectively) containing lower lecithin content. The increase in niosomal size could be attributed to the fact that, the presence of high lecithin content in the vesicular bilayer membrane leads to disturbance in the vesicular membrane, thus, increasing vesicle radius in a way to establish a more thermodynamic stable form [22]. The niosomal formulations (F1-F3) prepared using span 60 exhibited an overall increase in the mean particle size in comparison to their corresponding niosomal formulae (F4 -F6, respectively) prepared using Tween 20.

The previous results revealed that F4, F1 and F5 showed the smallest vesicle sizes of 68.2, 161.34 and 164.32 nm, respectively with significantly lower ($P < 0.05$) PDI values in comparison to the other proniosomal formulations. Accordingly F4, F1 and F5 were selected for further evaluation for *in vitro* drug release and *ex vivo* permeation studies.

***In vitro* release studies**

Proniosomal formulations F1, F4 and F5 were chosen for *in vitro* release study, based on their vesicle size, as they showed the smallest vesicle diameter among other formulations (161.34 ± 11.23 nm, 68.20 ± 13.11 nm and 164.32 ± 22.20 nm respectively). *In vitro* release profiles of SIM from the selected proniosome-derived niosomal formulae were illustrated in Figure 2. The release profiles of F1, F4 and F5 were apparently biphasic, with initial fast drug release phase of 39.09 ± 2.72, 20.07 ± 1.35 and 18.90 ± 1.74 % of encapsulated drug in the first hour respectively followed by a slower release phase over the following 12 hours. The initial rapid drug release could be attributed to desorption of SIM from the surface of niosomes, while the slower drug release was regulated by diffusion across the niosomal bilayers [23]. The biphasic release pattern would be beneficial regarding the importance of saturation of skin epidermis with drug, where at the initial fast release phase; this will help to achieve high concentration gradient of drug across skin, required for successful transdermal drug delivery to the blood [24]. These results were in accordance with what had been previously reported in literature [25, 26, 27].

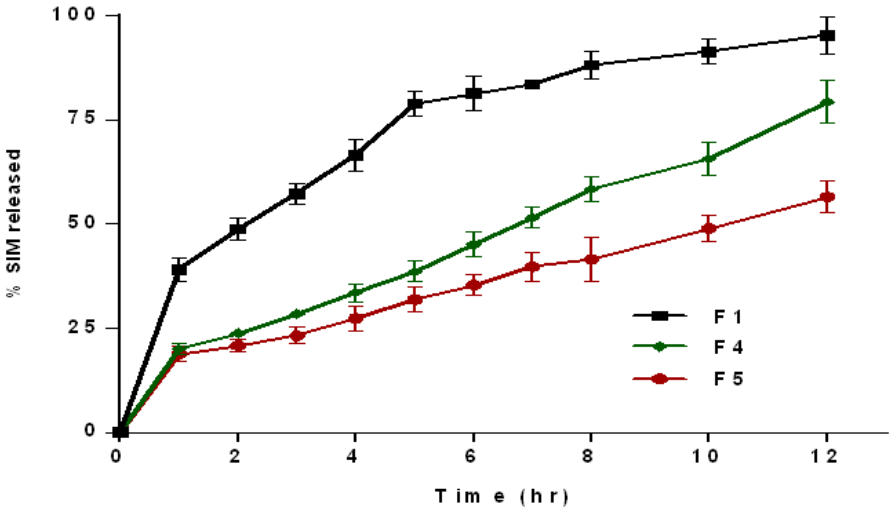

Fig. 2: In vitro release profiles of SIM from proniosomal formulae F1, F4 and F5.

F1 composed of lecithin: Span 60 in molar ratio 1: 9 showed significantly higher initial drug release after 1 hr, and the highest percentage of SIM released after 12 hours in comparison to F4 and F5. The higher SIM release from F1 could be attributed to its lower lecithin content that results in more permeable bilayer [7, 28]. Moreover, F1 has significantly higher zeta potential than that of F4 and F5. The higher negativity of F1 proniosomes bilayer might be unfavorable to keep the negatively charged SIM molecules (due to the presence of (-COO-) group) inside the vesicles and resulted in fast repulsion of SIM molecules outside the vesicles.

F4 (lecithin:Tween 1: 9) exhibited higher percentage of SIM released relative to F5 that composed of lecithin : Tween 20 (3:7). The higher lecithin content in F5 results in forming more rigid bilayers with low permeability of encapsulated molecules within vesicles [29]. Proniosomal formulation with reduced lecithin content gives a faster drug release rate; this could be attributed to the disrupted structure of vesicles with low ratio of lecithin [7, 28].

***Ex vivo* skin permeation**

Figure 3 showed the skin permeation profiles of SIM form formulae F1, F4 and F5 across rat skin and the permeation parameters were illustrated in Table 3. Obviously, no lag phase was detected for all the tested formulae, and simvastatin was detected in the receptor compartment after the first hour indicating that all the processes (water permeation from the receptor compartment to skin, simvastatin release from the formed niosomes and its permeation across the skin) occurred very rapidly. Similar results were previously reported in literature [5, 6, 28].

F4 exhibited the highest amount of SIM permeated after 12 hours (Fig.3 ~ 66.36 µg /cm^2) with a flux value across skin of 4.74 ± 0.62 µg /cm^2 h^{-1} (Table 3). It is well known that Tween 20 acts as an enhancer for drug permeation [6, 30]. Tween 20 contains a lauryl (12 carbons) hydrocarbon chain which previously reported to be the most effective chain length to loosen densely packed lipids in

the stratum corneum by disrupting ceramide-cholesterol or cholesterol-cholesterol interactions, as this chain length corresponds to the chain length present in steroid nucleus of cholesterol molecules [23, 31].

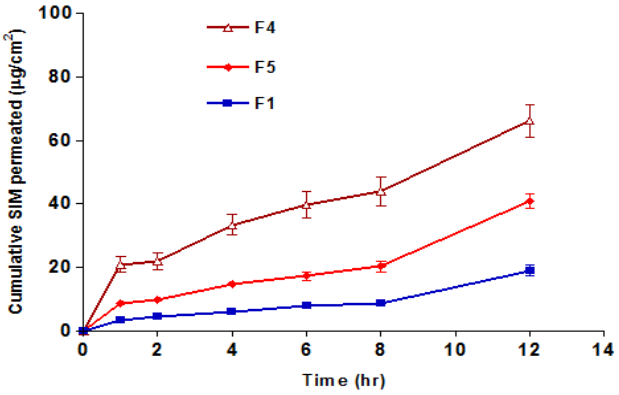

Fig. 3: *Ex vivo* skin permeation profiles of SIM proniosomal formulae F1, F4 and F5 through excised rat skin.

Table 3: *Ex vivo* skin permeation parameters for selected SIM proniosomal formulae

Formulation Code	Flux value *J** (µg/cm² h⁻¹)	Permeability coefficient** (cm / h)
F1	1.37 ± 0.25	0.069 ± 0.012
F4	4.74 ± 0.62	0.237 ± 0.03
F5	2.92 ± 0.39	0.146 ± 0.02

*Flux value (J) was calculated as the slope of the line obtained on plotting mean cumulative amount permeated per unit area versus time.**Permeability coefficient was calculated by the following equation:Permeability coefficient = J / CD , where CD is the concentration of simvastatin in the donor compartment in (µg/ml).

The enhanced drug permeation of F4 relative to F5 could be attributed to higher Tween 20 ratio incorporated in vesicles; which encouraged permeation of the unionized portion of drug by adsorption and fusion of niosomal vesicles onto skin surface, resulting in increased thermodynamic activity of drug at skin interface [5].

The lowest flux value exhibited by F1 composed of lecithin: Span 60 (1: 9), can be explained on the basis of its higher zeta potential value (-19.9 mV). Knowing that skin is generally considered as a negatively charged membrane [32,33] and adsorption of niosomes onto skin surface occurs due to physical or electrostatic forces [34], therefore vesicles with more negative charge will exhibit low skin permeation due to electrostatic repulsion. Similar results were reported in literature [35, 36]. Combining our previous data, F4 was selected as the optimized formula and exposed to stability as well as bioavailability studies.

Stability study

After 3 months of storage at refrigeration temperature and room temperature, the mean vesicle size and EE% of the optimized formula (F4) were determined. Results revealed the mean vesicle size insignificantly (P > 0.05) increased from 68.20 ± 13.11 nm to 81.60 ± 19.02 nm and 102.80 ± 22.31 nm at refrigeration temperature and room temperature, respectively. This slight increase in vesicle size might be due to aggregation and fusion of vesicles upon storage [37]. However, a non-significant decrease in EE% from 72.90 ± 1.65 to 71.70 ± 1.76 and 70.30 ± 2.42 % was observed at refrigeration temperature and room temperature, respectively. The slight reduction in EE% could be due to leakage of drug by desorption from niosomal surface [23]. The results indicated the stability of the prepared proniosomal formulation at refrigerator and room temperature over the tested priod.

Bioavailability study

The mean plasma SIM concentration-time profile following application of single dose of proniosomal SIM onto skin (20mg/kg) and the same equivalent oral dose of SIM dispersion was demonstrated in Figure 4. Bioavailability parameters (C_{max}, T_{max} and AUC_{0-8}) were calculated individually on the basis of concentration-time data. From individual bioavailability parameters, the mean values ± S.D were obtained and presented in Table 4 for both SIM treatments. The bioavailability parameters of SIM proniosomal gel exhibited mean C_{max}, T_{max} and AUC_{0-8} values of 6.01 ± 0.48 ng/ml, 3.33 ± 1.03 h and 31.27 ± 2.67 ng h/ml, respectively. While the values of these parameters were 7.17±0.81 ng/ml, 1.83 ± 0.75 h and 26.17 ± 3.31 ng h/ml, respectively for the oral SIM dispersion.

Statistical analysis of the bioavailability parameters AUC_{0-8}, C_{max} and T_{max} data obtained for transdermal SIM proniosomes showed significantly ($P<0.05$) higher values of AUC_{0-8} and T_{max} and significantly ($P<0.05$) lower values of C_{max} compared to SIM oral dispersion. The individual AUC_{0-8} values for transdermal proniosomal SIM were compared to those for oral SIM dispersion to determine the relative bioavailability. The mean relative bioavailability was 120.40 ± 11.44 %. This result indicated that 20.40 % increase in the bioavailability of SIM was achieved by application of proniosomes onto skin. This enhancement of bioavailability could be due to avoidance of first-pass hepatic metabolism by application on skin.

Although, skin-applied SIM proniosomes improved the bioavailability of SIM in terms of extent of absorption from the skin, it decreased the rate of drug absorption in terms of T_{max} and C_{max} values in comparison to the oral SIM dispersion.

The oral SIM dispersion showed a rapid elimination from the plasma as indicated by the significantly lower values of the $t_{1/2}$ compared to the transdermal proniosomes (Table 4). The higher $t_{1/2}$ of the proniosomal gel indicated extended drug existence in plasma as a result of the absorption of lipophilic drugs such as SIM [log P = 4.68] [38] into the stratum corneum, which in turn serves as the drug reservoir for extended release into the viable epidermis over hours [39].

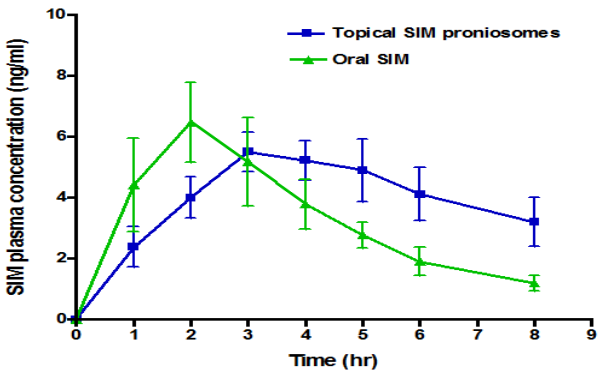

Fig. 4: Mean (± SD) plasma concentration–time curves of SIM in rats (*n=6*) after application of a single dose (20 mg/kg) of proniosomal SIM gel onto dorsal skin and same dose of SIM oral dispersion

Table 4: Bioavailability parameters of SIM in rats (*n=6*) after application of a single dose (20 mg/kg) of proniosomal SIM onto skin or SIM oral dispersion.

Parameter	Transdermal proniosomal SIM	SIM oral dispersion
C_{max} (ng/ml)	6.01 ± 0.48	7.17 ± 0.81
T_{max} (hr)	3.33 ± 1.03	1.83 ± 0.75
AUC_{0-8} (ng.hr/ml)	31.27 ± 2.67	26.17 ± 3.31
k_{el}, (hr^{-1})	0.14 ± 0.07	0.276 ± 0.05
$t_{1/2}$ (hr)	5.66 ± 1.88	2.60 ± 0.58
Relative Bioavailability (%)	120.40 ± 11.44	-

Assessment of hypocholesterolemic effect

After 30 days on standard atherogenic diet, the mean plasma total cholesterol of rats (TC) was elevated by 0.74 fold than normal rats suggesting successful induction of hypercholesterolemia in rats among the different groups. The mean plasma total cholesterol (mg/dl ± SD, *n = 6*) before (baseline) and after 7 successive days of treatment with proniosomal SIM gel or oral SIM dispersion in hypercholesterolemic rats, was presented in Table 5. The mean % reduction of plasma TC of hypercholesterolemic rats was 32.57% and 14.20 % after treatment with proniosomes and oral SIM dispersion, respectively. Treatment of hypercholesterolemic rats for 7 days with SIM topical proniosomes gel significantly (P<0.05, paired t test) decreased the mean plasma TC level relative to not only the mean baseline plasma TC after induction but also to the mean % reduction in the plasma TC achieved after treatment with oral SIM (P<0.05, ANOVA).

Table 5: Mean plasma TC (mg/dl ± SD, *n = 6*) before and after 7 days of treatment with proniosomal SIM onto skin or oral SIM dispersion in hypercholesterolemic rats

Test groups of hypercholesterolemic rats	Mean plasma total cholesterol (mg/dl ± SD, *n = 6*)		
	Before treatment	After treatment	*P value
I Control	172 ± 2.76	174 ± 3.89	0.22
II proniosomal SIM	175 ± 3.16	118 ± 4.59	< 0.05
III Oral SIM	176 ± 4.36	151 ± 4.47	< 0.05
†P value	0.33	< 0.05	

*P values are for before and after treatment of the same group, by paired t test †P values are for among-group comparisons, by analysis of variance (ANOVA)

CONCLUSION

A proniosomal transdermal delivery system of a poorly water soluble drug, SIM, was developed and subjected to *in vitro* and *in vivo* characterization. The obtained results suggested that the application of SIM-loaded proniosomes (composed of lecithin: Tween, 1: 9) onto skin, significantly improved not only the bioavailability of the drug but also its hypocholesterolemic effect in treatment of hypercholesterolemic rats. In conclusion, SIM proniosomes could be considered as very promising candidates for delivering SIM transdermally.

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