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Formulation and in vitro assessment of minoxidil niosomes for enhanced skin delivery

Prabagar Balakrishnan[a], Srinivasan Shanmugam[a], Won Seok Lee[a], Won Mo Lee[a], Jong Oh Kim[a], Dong Hoon Oh[a], Dae-Duk Kim[b], Jung Sun Kim[c], Bong Kyu Yoo[a], Han-Gon Choi[a], Jong Soo Woo[a,**], Chul Soon Yong[a,*]

[a] College of Pharmacy, Yeungnam University, 214-1, Dae-Dong, Gyongsan 712-749, South Korea
[b] College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Ku, Seoul 151-742, South Korea
[c] Department of Biotechnology, Dongseo University, Pusan, 617-716, South Korea

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ABSTRACT

Niosomes have been reported as a possible approach to improve the low skin penetration and bioavailability characteristics shown by conventional topical vehicle for minoxidil. Niosomes formed from polyoxyethylene alkyl ethers (Brij™) or sorbitan monoesters (Span™) with cholesterol molar ratios of 0, 1 and 1.5 were prepared with varying drug amount 20–50 mg using thin film-hydration method. The prepared systems were characterized for entrapment efficiency, particle size, zeta potential and stability. Skin permeation studies were performed using static vertical diffusion Franz cells and hairless mouse skin treated with either niosomes, control minoxidil solution (propylene glycol–water–ethanol at 20:30:50, v/v/v) or a leading topical minoxidil commercial formulation (Minoxyl). The results showed that the type of surfactant, cholesterol and incorporated amount of drug altered the entrapment efficiency of niosomes. Higher entrapment efficiency was obtained with the niosomes prepared from Span 60 and cholesterol at 1:1 molar ratio using 25 mg drug. Niosomal formulations have shown a fairly high retention of minoxidil inside the vesicles (80%) at refrigerated temperature up to a period of 3 months. It was observed that both dialyzed and non-dialyzed niosomal formulations (1.03 ± 0.18 to 19.41 ± 4.04%) enhanced the percentage of dose accumulated in the skin compared to commercial and control formulations (0.11 ± 0.03 to 0.48 ± 0.17%) except dialyzed Span 60 niosomes. The greatest skin accumulation was always obtained with non-dialyzed vesicular formulations. Our results suggest that these niosomal formulations could constitute a promising approach for the topical delivery of minoxidil in hair loss treatment.

1. Introduction

Minoxidil, a pyrimidine derivative (2, 4-diamino-6-piperidinopyrimidine-3-oxide, Fig. 1), is the only topical medical treatment with proven efficacy for the treatment of Androgenetic alopecia (AGA). AGA is hereditary and is the progressive, androgen-dependent thinning of scalp hair, which follows a definite pattern. The US Food and Drug Administration approved treatments for AGA are oral finasteride at a dose of 1 mg per day and topical solutions of 2 and 5% minoxidil (Price, 1999). Little is known of the effect of minoxidil on normal human hair growth and studies have been limited mainly to the response of androgenetic alopecia to topical minoxidil. Recently, Han et al., 2004 reported that minoxidil stimulates hair growth in human by prolonging anagen through proliferative (by activating both ERK and Akt) and antiapoptotic (by increasing the ratio of Bcl-2/Bax) effects on dermal papilla cells (DPCs) of human hair follicles. Minoxidil have been reported for its poor skin penetration ability, which limits minoxidil usefulness as a potent drug in the use of hair growth treatment.

Minoxidil is poorly soluble in water and most of the water-immiscible organic solvents such as chloroform. Therefore, it has been formulated for topical use in an ethanol-based solution containing ethanol, propylene glycol and water (Tata et al., 1995). One drawback of ethanol-based formulations is the tendency of the minoxidil to revert to an insoluble crystalline form when applied to the skin, as the ethanol solvent evaporates. Whether due to the tendency of the drug to crystallize or other factors, the minoxidil formulation shows relatively inefficient uptake by the skin. Further, evaporation of ethanol, when the formulation is applied to the skin, leaves a viscous propylene glycol/water residue which may be objectionable to many users. Moreover, typical side effects of the topical treatment with ethanol-based minoxidil formulations

* Corresponding author. Tel.: +82 53 810 2812; fax: +82 53 810 4654.
** Corresponding author. Tel.: +82 53 810 2824; fax: +82 53 810 4654.
E-mail addresses: woojongsoo@yu.ac.kr (J.S. Woo), csyong@yu.ac.kr (C.S. Yong).

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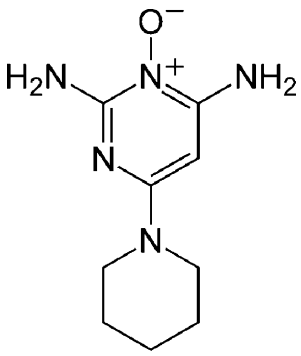

Fig. 1. Chemical structure of minoxidil (3-hydroxy-2-imino-6-(1-piperidyl) pyrimidin-4-amine).

include irritative dermatitis going along with pruritus, erythema, scaling and dryness occur at the onset of therapy (Williams and Barry, 2004). In some cases, allergic contact dermatitis or exacerbation of seborrhoic dermatitis has been reported. While most of the patients with allergic contact dermatitis described in the literature showed a positive sensitization to the vehicle substance propylene glycol evaluated by patch testing, reactions to the active ingredient minoxidil are rare (Hagemann et al., 2005). Since most of the conventional topical minoxidil formulations consist of propylene glycol–water–ethanol solution, to minimize the side effects and to improve the therapeutic efficiency, new dermatological preparations with free of organic solvents and propylene glycol are required.

Vesicular system, both liposomes and niosomes are uni- or multilamellar spheroidal structures composed of amphiphilic molecules assembled into bilayers. They are considered primitive cell models, cell-like bioreactors and matrices for bioencapsulation. In the recent years, nonionic surfactant vesicles known as niosomes received great attention as an alternative potential drug delivery system to conventional liposomes. Moreover, compared to phospholipid vesicles, niosomes offer higher chemical and physical stability (Vora et al., 1998) with lower cost and greater availability of surfactant classes (Manconi et al., 2006). Niosomes have been reported to enhance the residence time of drugs in the stratum corneum and epidermis, while reducing the systemic absorption of the drug and improve penetration of the trapped substances across the skin. In addition, these systems have been reported to decrease side effects and to give a considerable drug release (Schreier and Bouwstra, 1994). They are thought to improve the horny layer properties both by reducing transepidermal water loss and by increasing smoothness via replenishing lost skin lipids (Junginger et al., 1991). Moreover, it has been reported in several studies that compared to conventional dosage forms, vesicular formulations exhibited an enhanced cutaneous drug bioavailability (Manconi et al., 2006; Mura et al., 2007).

In this work, the effects of minoxidil entrapped niosomes on the drug penetration in hairless mouse skin were investigated by in vitro permeation experiments, and compared with those of control minoxidil solution 5 mg/mL (propylene glycol–water–ethanol at 20:30:50, v/v/v) and a leading commercial topical formulation "Minoxyl™" containing 5% minoxidil and 1% dexpentanol (Hyundai Pharma Ltd. South Korea). Niosomal formulations were prepared by thin film-hydration (TFH) method using cholesterol and nonionic surfactants polyoxyethylene 2 cetyl ether (Brij 52), polyethylene glycol octadecyl ether (Brij 76), and sorbitan monoesters (Span 20, Span 40, Span 60 and Span 80). This paper focuses on the properties of thin film-hydrated niosomes as potential new minoxidil carriers for effective skin delivery.

Table 1
Composition of vesicles as minoxidil carriers (mg/batch).

Components	HLB	*Tc* (°C)	1:1 (surf:chol) (mg)	1:1.5 (surf:chol) (mg)
Cholesterol Surfactants			38.6	57.9
Brij 52	5.3	32.5	33	33
Brij 76	12.4	34	71.1	71.1
Span 20	8.6	16	34.6	34.6
Span 40	5.7	42	40.2	40.2
Span 60	4.7	53	43.6	43.6

*In all the formulations, DCP 8.2 mg used.
*Minoxidil used (20, 25, 30, 40 and 50 mg).
*Gel–liquid transition temperature (*Tc*).

2. Materials and methods

2.1. Chemicals

Cholesterol, dicetyl phosphate (DCP), polyoxyethylene 2 cetyl ether (Brij 52) and polyethylene glycol octadecyl ether (Brij 76) were purchased from Sigma Aldrich (St. Louis, USA). Minoxidil (MW 209.25, 99% purity) was kindly provided by Hunmi pharmaceutical company (Seoul, South Korea). Methanol and ethanol were supplied by Burdick and Jackson (Ulsan, South Korea). Span 20 and Span 80 were purchased from Junsei chemicals Co. Ltd. (Tokyo, Japan). Span 40 and Span 60 were obtained from Yakuri pure chemicals Co. Ltd. (Kyoto, Japan). Propylene glycol and chloroform were supplied by D. C. chemical Co. Ltd. (Seoul, South Korea). A 5% Minoxyl® topical solution sold by Hyundai pharm. Co. Ltd. (Seoul, South Korea) was purchased from a local market. All other materials and solvents used in this study were of analytical grade.

2.2. Vesicle preparation

Niosome preparation: Minoxidil niosomes were prepared using thin film-hydration method. Accurately weighed quantities of the surfactant (Brij™ or Span™)) and cholesterol in different molar ratios, viz. 1:0, 1:1 and 1:1.5, were dissolved in 8 mL of chloroform:methanol mixture (2:1, v/v) in a round-bottom flask (Agarwal et al., 2001). Afterwards, minoxidil and DCP dissolved in 5 mL of chloroform:methanol mixture (2:1, v/v) was added to the lipid solution (Table 1). The organic solvents were removed under vacuum in a rotary evaporator at 40 °C for 20 min to form a thin film on the wall of the flask, and kept in a desiccator under vacuum for 2 h to ensure total removal of trace solvents. After removal of the last trace of organic solvents, hydration of the surfactant film was carried out using 10 mL of distilled water at 55 °C, which is above the gel–liquid transition temperature (*Tc*) of sorbitan monoesters and polyoxyethylene alkyl ether surfactants (Abbas et al., 2007; Azeem et al., 2008). The resulting niosomal suspension was mechanically shaken for 1 h using a horizontal mechanical shaking water bath at 55 °C. Then, the vesicle suspension was sonicated (Branson 5510R-DTH, USA) in 3 cycles of 1 min "on" and 1 min "off" leading to the formation of multilamellar niosomes. The niosomal suspension was left to mature overnight at 4 °C and stored at refrigerator temperature for further studies.

2.3. Content of drug in vesicles

The prepared minoxidil niosomes were separated from unentrapped minoxidil by exhaustive dialysis (Mura et al., 2007) for 3 h in distilled water using Spectra-Por® membranes (12,000–14,000 cut-off, Spectrum laboratories, Inc., USA). The amount of entrapped minoxidil was determined by lysis of the dialyzed vesicles with absolute ethanol. A 100 μL sample of niosomes was mixed with 5 mL of absolute ethanol and covered well with parafilm to pre-

vent evaporation. The solution was then sonicated for 15 min in a bath type sonicator to obtain a clear solution. The concentration of minoxidil in absolute ethanol was determined by a previously reported high performance liquid chromatography method with slight modification (Chen et al., 2005). The HPLC (Jasco UV-975, Japan) was equipped with an Inertsil ODS-3 C18 column (GL science, 0.5 μm, 15 cm × 0.46 cm i.d.) and UV detector (Model L-7450). The mobile phase was consisted of methanol/water/glacial acetic acid (750/250/10, v/v/v, pH 3.0) and the eluent was monitored at 281 nm with a flow rate of 1 mL/min.

The entrapment efficiency is defined as follows:

$$\text{Minoxidil entrapment efficiency (\%)} = \frac{\text{Amount of minoxidil entrapped}}{\text{Total amount of minoxidil used}} \times 100$$

2.4. Characterization of minoxidil niosomes

2.4.1. Particle size and zeta potential determination

Vesicle properties such as particle size diameter, zeta potential and size distribution were determined by ELS-8000 Electrophoretic light scattering particle size and zeta potential analyzer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at room temperature. The particle size and zeta potential of the samples were determined over a period of three months. The values of mean particle size diameters (MPSD) and zeta potential of formulated niosomes prepared with different surfactants were compared.

2.4.2. Physical stability of minoxidil niosomes

Physical stability studies were carried out to investigate the leaching of drug from niosomes (in a liquid form) during storage. The minoxidil niosomal formulations, composed of Brij 52 or Span™ with 1 cholesterol ratio, and Brij 76 with 1.5 cholesterol ratio, were sealed in 20 mL glass vials and stored at refrigerator temperature (2–8 °C) for a period of 3 months. Samples from each batch were withdrawn at definite time intervals, the residual amount of the drug in the vesicles was determined as described previously in Section 2.3 after separation from unentrapped drug (Shahiwala and Misra, 2002).

2.5. Skin penetration and permeation studies

In vitro skin penetration and permeation studies were performed using static vertical diffusion Franz cells with an effective diffusion area of 1.64 cm^2. The control solution was prepared by dissolving minoxidil (5 mg/mL) in propylene glycol/water/ethanol solution (20/30/50, v/v/v) (Mura et al., 2007). Fresh hairless mouse skin specimens were sandwiched securely between donor and receptor compartments with the epidermis side facing the donor compartment. The receptor compartment was filled with 13 mL saline solution, which was continuously stirred and thermostated at 37 ± 1 °C throughout the experiment. After 12 h equilibrium, 0.2 mL of either minoxidil entrapped vesicle in aqueous suspension (dialyzed or non-dialyzed sorbitan ester and Brij 52 niosomes with 1 cholesterol ratio), control minoxidil solution or Minoxyl commercial formulation were placed onto the skin surface (n = 6 skin specimens per tested formulation). At predetermined time intervals for 24 h (0.25, 0.5, 0.75, 1, 2, 4, 8, 12 and 24 h), 0.2 mL of aliquots were withdrawn and were replaced with an equal volume of fresh saline solution to ensure sink conditions. After 24 h, the surface of skin specimens was washed 10 times with 1 mL distilled water and dried with filter paper. The effective surface area of the skin was separated and minced with a surgical sterile scalpel then finally homogenized in a vial filled with methanol by using ultra turrax homogenizer at an rpm of 16,000 for 5 min (T25 Basic, Germany) on ice bath. The tissue suspension was centrifuged for 15 min at 9000 × g and then the supernatants were filtered. Then, 100 μL of the supernatant tissue suspension was further extracted with 500 μL methanol and filtered. The supernatants from receptor solutions and tissue suspensions as well as washing solutions were assayed for their minoxidil content by HPLC as described previously in Section 2.3.

2.6. Statistical analysis of data

Data analysis was carried out with the software package Microsoft Excel version 2007. Results were expressed as a mean ± standard deviation. Statistically significant difference was determined using the student t-test with $P < 0.05$ as a minimal level of significance.

3. Results and discussion

The low cost, greater stability and resultant ease of storage of nonionic surfactant vesicles (Uchegbu and Vyas, 1998) have lead to the exploitation of these vesicles as alternatives to phospholipid vesicles for the enhancement of dermal and/or transdermal bioavailability of drugs and substances. Minoxidil is a potent antihypertensive drug which acts on the peripheral arteriolar smooth muscle. Thus, it is necessary to focus on providing a means to limit the efficacy of hypertrichosis to local sites with the percutaneous delivery of minoxidil and to avoid excess minoxidil entering the blood circulation, which can produce side effects, including systemic hypotensive effects. In topical drug delivery, vesicular systems were reported for significantly reduced systemic absorption and side effects with increased drug concentration in the various skin layers (Choi and Maibach, 2005).

3.1. Vesicle-forming ability of surfactants

It has been reported that niosomes prepared without cholesterol formed a gel and only on the addition of cholesterol, homogenous niosome dispersion was obtained (Yoshioka et al., 1994). Thus, in this study cholesterol was included at 0, 1 and 1.5 molar ratio, and the total concentration of surfactant and cholesterol was adjusted to 100 μM. However, niosomes must be stabilized by the addition of a charged molecule to the bilayers such as dicetyl phosphate (Cable, 1989). Dicetyl phosphate was added at a concentration of 15 μM per batch to prevent the aggregation of niosomes.

It appeared that all the surfactants studied were able to form vesicles at all cholesterol ratios studied. The microscopic observations showed the formation of vesicular structures from these surfactants by classic thin film-hydration method. However, crystals of cholesterol were observed in the optimized formulations of Brij 52, Brij 76, Span 60 and Span 40 niosomes, and a very few or no crystals of cholesterol observed in Span 20 niosomes. Moreover, due to aggregations occurred in Span 80 niosomes, further studies on these surfactant vesicles were not carried out.

3.2. Effect of cholesterol

The entrapment efficiency is the most important parameter from pharmaceutical viewpoint in niosomal formulations. A high percentage of entrapment would mean less time and effort involved in removal of unentrapped material. To study the effect of increased cholesterol ratio on the amount of drug entrapment in niosomes, a series of formulations were prepared with increasing cholesterol molar ratio (0, 1 and 1.5) at a fixed amount of minoxidil (50 mg). The effect of cholesterol on minoxidil entrapment was varied according to the nonionic surfactant used. Cholesterol was found to have insignificant effect on the minoxidil entrapment into sorbitan ester and Brij 52 niosomes (Fig. 2). For Brij76, entrapment of minoxidil was significantly increased ($P < 0.05$) from 8.4 ± 1.1%

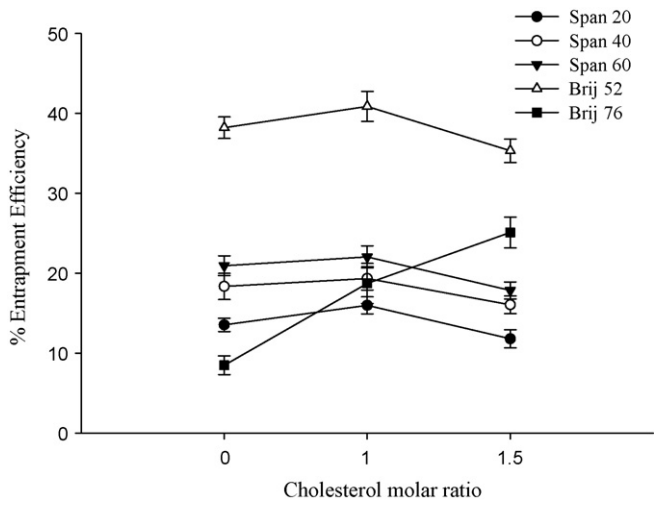

Fig. 2. Effect of cholesterol molar ratio on the entrapment efficiency of minoxidil into niosomes ($n = 3$).

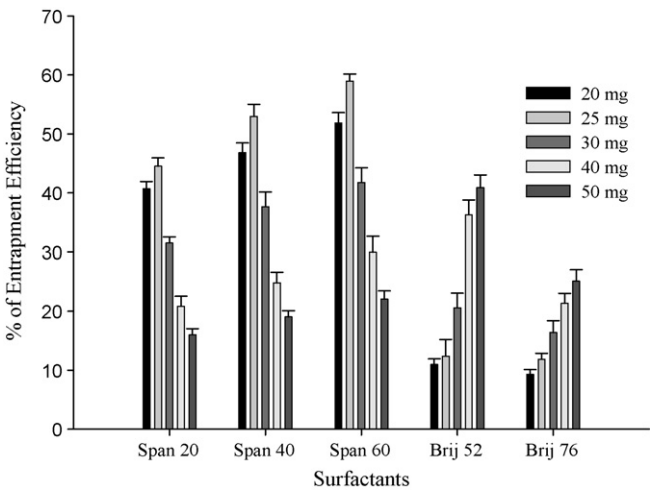

Fig. 3. Effect of the content of minoxidil on the entrapment efficiency of niosomes ($n = 3$).

to 25.11 ± 1.9% with increasing cholesterol ratio from 0 to 1.5. As the HLB of the surfactant increases above 10, the minimum amount of cholesterol necessary to form vesicles increases (Uchegbu and Vyas, 1998). More cholesterol is necessary to compensate for the larger head group. In the present study, Brij 76 ($C_{18}EO_{10}$) has the highest HLB value of 12.6 indicating low hydrocarbon chain volume in comparison with hydrophilic surface area. Thus, increased cholesterol content might have increased the lipophilic behavior of the lipid bilayer of Brij 76 niosomes and crystallinity of the bilayer (Uchegbu and Vyas, 1998). Hence, higher drug entrapment of Brij 76 niosomes was observed in the presence of higher content of cholesterol. However, Brij 52 (HLB 5.3) did not show a significant increase in the entrapment efficiency with respect to higher cholesterol content. Brij 52 niosomes showed an insignificant increase in the entrapment efficiency of minoxidil when cholesterol content increased from 0 to 1 molar ratio (38.6 ± 2.7% to 40.87 ± 2.2%); further increase resulted in decreased entrapment efficiency. Abbas et al. reported that the increasing cholesterol content reduced the volume diameter of Brij 52 ($C16EO_2$) niosomes significantly ($P < 0.05$). This might explain the result obtained for Brij 52 niosomes.

In niosome formulations prepared using sorbitan monoesters, Span 60 showed the maximum entrapment efficiency at 1 cholesterol molar ratio, as it has the longest saturated alkyl chain, followed by Span 40 and Span 20 (Fig. 2). Increasing cholesterol content from 0 to 1 molar ratio lead to an insignificant increase ($P > 0.05$) in the entrapment efficiency of sorbitan ester niosomes, from 13.5 ± .0.8% to 15.9 ± 1.08%, 18.4 ± 1.6% to 19.4 ± 1.46% and 20.5 ± 1.2% to 22.05 ± 1.38% for Span 20, Span 40 and Span 60 niosomes, respectively. The improvements in drug entrapment with increased cholesterol content (0–1) and the major reduction in drug entrapment ($P < 0.05$) when cholesterol content was further increased (1–1.5) may be due to two conflicting factors: (1) with increased cholesterol, the bilayer hydrophobicity and stability increased (Bernsdorff et al., 1997) and permeability decreased (Kirby et al., 1980), which may lead to efficiently trapping the hydrophobic drug into bilayers as vesicles formed. (2) In contrast, higher amounts of cholesterol may compete with the drug for packing space within the bilayer, hence excluding the drug as the amphiphiles assemble into the vesicles. This explains why sorbitan monoester niosomes at 1:1 cholesterol molar ratio showed higher entrapment efficiencies than those showed at high cholesterol molar ratio at 1.5. It was reported that the nature of the hydrophobic alkyl chain affects the encapsulation efficiency of CF (Yoshioka et al., 1994) and DOX (Uchegbu and Florence, 1995) by unsonicated Span surfactant niosomes. Span 60 (C18) and Span 40 (C16) gave the greatest encapsulation efficiency for CF niosomes due to the fact that these Span surfactants had the highest phase transition temperature (Yoshioka et al., 1994). This accounts for the higher entrapment observed for both Span 40 and Span 60 than Span 20 niosomes at 1:1 molar ratio.

3.3. Effect of minoxidil concentration

The effect of increasing minoxidil content on the entrapment efficiency in the range of 20–50 mg in the niosomes prepared from sorbitan monoester surfactants (Span 20, 40 and 60), Brij 52 (1:1), and Brij 76 (1:1.5) is profiled in Fig. 3. The effect of drug content on minoxidil entrapment varied according to the nonionic surfactant used. In polyethylene alkyl ether niosomes, entrapment efficiency of minoxidil were increased from 10.29% to 40.87% and 10.96% to 25.11%, and the amount of drug entrapped increased from 2.54 to 21.49 and 2.51 to 12.56 mg for Brij 52 (1:1) and Brij 76 (1:1.5), respectively, as the drug concentration was increased from 20 to 50 mg. The entrapment efficiency of minoxidil was increased in Span 20, Span 40 and Span 60 niosomes, as the drug concentration was increased from 20 to 25 mg (Fig. 3). The increased entrapment efficiency of minoxidil with higher amount of drug used in the formulation could be due to the saturation of the media with minoxidil that forces the drug to be encapsulated into niosomes (EL-Samaligy et al., 2006). However, further increase in drug concentration from 25 to 50 mg showed a decrease in the entrapment efficiency ($P < 0.05$). This might be due to the fact that the saturation of the bilayers of Span niosomes might be reached at 25 mg of drug incorporation. Drug crystals dispersed in-between the niosomal pellets were observed under the optical microscope, when higher amount of drug was used. This leads to the assumption that niosomal formulations could enhance the solubility of certain poorly soluble drugs but to a maximum limit after which any increase in the drug concentration leads to drug precipitation (Mokhtar et al., 2008).

3.4. Vesicle characterization

Characterization of minoxidil niosomal suspensions is reported in Table 2. All of the vesicles formed were in the mean area-number diameters (dAN) ranging from 0.2 μm to 1.3 μm. It has been widely known that the diameter of the vesicles is dependent on the length of the alkyl chain of the surfactants. Surfactants with longer alkyl chains generally give larger vesicles (Uchegbu and Vyas, 1998). This might be the reason for smaller particle size of span 20 and

Table 2
Characterization of minoxidil niosomes prepared by thin film-hydration method: Zeta potential, Particle diameter (nm) and Polydispersity index (PI).

Formulations	*z*-zeta potential (mV)		*z*-diameter (nm)		(PI)	
	Fresh	3 month old	Fresh	3 month old	Fresh	3 month old
Brij 52						
Non-Dialyzed	−30.48 ± 1.1	−32.55 ± 1.1	1160 ± 41	922 ± 61	1 ± 0	1 ± 0
Dialyzed	−27.96 ± 1.6	−31.05 ± 1.4	1292 ± 64	1012 ± 21	0.905 ± 0.03	0.754 ± 0.05
No DCP	–	–	976 ± 35	–	0.815 ± 0.02	–
Brij 76						
Non-Dialyzed	−41.31 ± 0.8	−44.71 ± 1.3	777 ± 89	961 ± 43	0.927 ± 0.02	0.811 ± 0.12
Dialyzed	−36.23 ± 0.7	−37.12 ± 0.9	905 ± 73	1312 ± 34	0.739 ± 0.01	0.651 ± 0.06
No DCP	–	–	1082 ± 28	–	0.860 ± 0.08	–
Span 20						
Non-Dialyzed	−37.54 ± 1.6	−38.84 ± 1.8	214 ± 32	210 ± 28	0.334 ± 0.02	0.321 ± 0.03
Dialyzed	−32.16 ± 1.2	−33.48 ± 1.6	289 ± 29	262 ± 49	0.282 ± 0.03	0.244 ± 0.02
No DCP	–	–	441 ± 22	–	0.623 ± 0.03	–
Span 40						
Non-Dialyzed	−33.22 ± 1.7	−34.81 ± 1.9	252 ± 26	247 ± 56	0.422 ± 0.02	0.418 ± 0.03
Dialyzed	−29.07 ± 1.2	−31.01 ± 1.7	498 ± 11	468 ± 31	0.363 ± 0.05	0.358 ± 0.03
No DCP	–	–	575 ± 28	–	0.763 ± 0.2	–
Span 60						
Non-Dialyzed	-28.11 ± 2.1	−31.61 ± 1.8	1240 ± 47	1182 ± 39	1 ± 0	1 ± 0
Dialyzed	-26.73 ± 1.1	−29.74 ± 1.2	1368 ± 39	1291 ± 56	0.968 ± 0.04	0.943 ± 0.03
No DCP	–	–	843 ± 93	–	0.728 ± 0.03	–

Span 40 vesicles and larger particle size of Span 60 niosomes. The size distribution could be observed from the polydispersity index shown in Table 2, which indicates that all the formulations were multi dispersed niosomes. Among the formulations, Span 20 showed comparatively a lesser degree of polydispersity (0.334 and 0.282 for non-dialyzed and dialyzed niosomes respectively). Zeta potential value of Span niosomal formulations increased with the hydrophilicity of the surfactants increased (Table 2). This could be due to the fact that the surface free energy of the Span surfactants increases with increased HLB value (Uchegbu and Florence, 1995). Inclusion of the negatively charged lipid DCP in the formulation decreased the vesicle size compared to DCP-free niosomes in Span 20, Span 40 and Brij 76 niosomes. This might be due to a disproportionate distribution of DCP in the bilayers, which may increase the curvature of the bilayer through the effect on electrostatic repulsion between the ionized head group, thus increasing the hydrophilic surface area. This effect will result in smaller vesicles (Cable, 1989). This is similar to *Z*-average diameter decrease of polyoxyethylene alkyl ether and the sugar ester vesicles, following the insertion of DCP (Van Hal, 1994). However, in Span 60 and Brij 52 inclusion of DCP increased the polydispersity index, and vesicle size. The negatively charged lipid DCP might have increased the hydrophilicity of the bilayers of these surfactant vesicles, thereby increased the water intake of the bilayers of these vesicles, which resulted in increased particle size and polydispersity index (Carafa et al., 1998). It must be considered that the size of vesicles depends on the properties of the molecules in the bilayer as well as interactions between bilayers, with the ultimate size of the vesicle determined by the distance between the bilayers and the number of bilayers present. The inclusion of charged molecules in the composition may increase the separation between bilayers of vesicles. This effect was observed in formulation of vesicles of alkyl glycerol ethers prepared by hand shaking method (Cable, 1989). The dialysis procedure increased the mean particle size and decreased the polydispersity index of the formulations because of the osmotic stress suffered by the vesicles during the purification procedure (Mura et al., 2007).

3.5. Stability studies

Stability studies were performed on Span 20 Span 40 and Span 60 (1:1) prepared with 25 mg of drug as well as Brij 52 (1:1) and Brij 76 (1:1.5) with 50 mg drug for a period of 14 weeks by subjecting them to ageing at 4 °C (Fig. 4). A direct relationship between the percentage leaching of the drug out of the vesicles and aging was observed, i.e., as the storage period increased, the degree of leaching also increased. It was observed that Span 40 (79.8 ± 3.5% of entrapment) and Span 20 (76.56 ± 2.1% of entrapment) niosomes showed higher stability over other formulations. In our study, it was observed that the niosome formulations Span 60 (69.526 ± 2.9%), Brij 52 (61.4 ± 3.9%) showed low stability; this could be due to low electrostatic repulsion due to low zeta potential of these systems (Uchegbu and Florence, 1995). Moreover, their larger particle size and polydispersity index also may have contributed to the lower stability of these systems (Uchegbu and Florence, 1995). The lowest stability of 44.5 ± 8.4% in terms of drug retention was observed for Brij 76 niosomes at the end of 14 weeks compared to initial drug entrapment. Such a low stability for Brij 76 niosomes was also observed by Abbas et al. (2007). Hence, further study on Brij 76 niosomes was not performed.

3.6. In vitro skin permeation studies

Niosomes are composed of nonionic surfactants, which are biocompatible and relatively nontoxic and themselves serve as

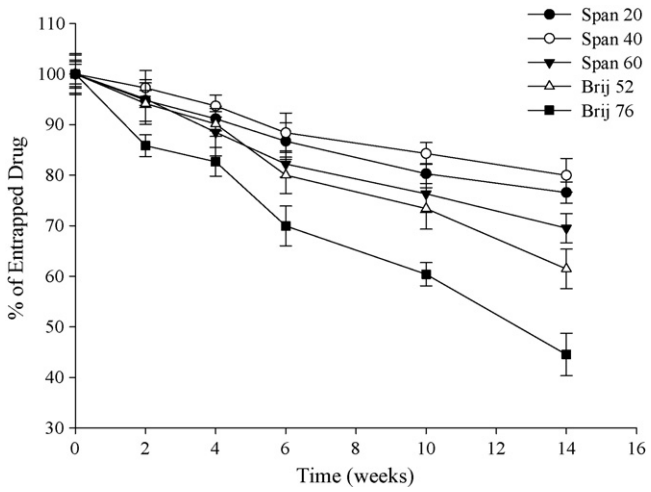

Fig. 4. Stability of Span niosomes (1:1) prepared with 25 mg minoxidil as well as Brij 52 (1:1) and Brij 76 (1:1.5) niosomes with 50 mg minoxidil stored at 4 °C for 14 weeks. Each value represents the mean ± S.D. ($n = 3$).

Table 3
Determination of minoxidil accumulation in hairless mouse skin after permeation study ($n = 6$).

Formulations	Minoxidil in formulations (mg/mL)	Minoxidil in 1 cm^2 Skin (μg) (Total Skin)	Dose Minoxidil absorbed (%) in skin (Total Skin)
Control solution	5.05 ± 0.02	3.81 ± 1.38	0.48 ± 0.17
Minoxyl	48.76 ± 2.3	10.29 ± 3.14	0.11 ± 0.03
Brij 52			
Non-Dialyzed	4.85 ± 0.83	32.06 ± 9.43*	5.42 ± 1.59
Dialyzed	2.03 ± 0.05	5.23 ± 2.03*	1.78 ± 0.42**
Span 20			
Non-Dialyzed	2.51 ± 0.06	59.41 ± 12.37	19.41 ± 4.04
Dialyzed	1.11 ± 0.13	3.91 ± 0.39*	2.43 ± 0.24**
Span 40			
Non-Dialyzed	2.48 ± 0.1	48.92 ± 13.24	16.37 ± 4.43
Dialyzed	1.32 ± 0.11	4.71 ± 0.41*	2.62 ± 0.22**
Span 60			
Non-Dialyzed	2.44 ± 0.05	3.07 ± 0.54	1.03 ± 0.18
Dialyzed	1.47 ± 0.08	NA	NA

** $P < 0.01$ compared to non-dialyzed, control solution and Minoxyl commercial formulation.
* $P < 0.01$ compared to Non-Dialyzed and Minoxyl formulation. NA = Not available.

excellent penetration enhancers (Van Hal, 1994). In this study, in order to assess the influence of the drug carriers on the accumulation into and diffusion of drug through the skin, in vitro permeation studies using hairless mouse skin and static vertical Franz diffusion cells were carried out. The in vitro permeation experiments were carried out in non-occlusive conditions to better simulate the usual topical application of minoxidil lotion. In this study, permeation of minoxidil through the hairless mouse skin into the receptor compartment was never detected, taking account the limit of quantification of the HPLC system. Thus, at the end of the experiments, minoxidil accumulation in the skin was assessed after 24 h treatment with either vesicular systems (i.e., Span 20, Span 40 and Span 60 with 25 mg of drug, and Brij 52 with 50 mg drug at 1:1 molar ratio, both dialyzed and non-dialyzed niosomes), control minoxidil solution or commercial formulation as donors. Minoxidil content in skin layers is reported in Table 3. Skin permeability values from all the systems were expressed as (a) the drug amount per 1 cm^2 of hairless mouse skin (b) the percentage of absorbed drug compared to drug applied onto the total surface area (1.64 cm^2) of the skin.

The Franz cell diffusion experiments with hairless mouse skin showed that the dialysis of vesicle systems decreased minoxidil accumulation into the skin layers. Our results suggest that the purification of drug carriers by dialysis, which led to an increase in vesicle size, greatly reduced the skin penetration of minoxidil. Superior minoxidil skin penetration was obtained with the non-dialyzed smaller niosomes, Span 20 and Span 40 (214–252 nm) than with the larger Brij 52 and Span 60 niosomes (1160–1240 nm). This could be due to better release of drugs from Span 20 and Span 40 niosomes due to low phase transition temperature of these surfactants and smaller size of these niosomes (Yoshioka et al., 1994). As shown in Table 3, 2.43–2.62% of the total applied minoxidil was detected in the skin with Span 20 and Span 40 dialyzed niosomes. When larger dialyzed niosome (Brij 52) suspensions containing minoxidil were applied to the mouse skin, only 1.78% of the drug was identified in the skin. It was interesting that the non-dialyzed larger Brij 52 niosomes gave higher skin content than dialyzed smaller Span 20 and 40 niosomes. This could be explained by two factors (1) higher drug concentration, (2) about 30% Brij 52 niosomes were in the mean particle size of 300 nm. Surprisingly, very low accumulation of 1.03% and no accumulation of minoxidil was observed for non-dialyzed and dialyzed Span 60 niosomes, respectively. It was suggested that the niosomes prepared from low phase transition surfactant mixtures (more fluid membranes) showed higher penetration than the one having higher phase transition temperature (Uchegbu and Vyas, 1998). Moreover, it was reported that sorbitan ester niosomes exhibit an alkyl chain length-dependent drug release; the higher the chain length, the lower the release rate (Devaraj et al., 2002; Guinedi et al., 2005). The low skin accumulation of minoxidil from Span 60 niosomes could be due to (1) high cholesterol content, (2) high phase transition temperature, (3) low drug release, and (4) larger particle size. In this study, the pH of the Span 20, Span 40 optimized niosomes were 5.97 ± 0.03 and 6.14 ± 0.02, respectively at 25 °C (pK_a of minoxidil ~ 6.30) the partial ionization of minoxidil (positive charge in ionized state as it is a weak base) may have contributed to the higher skin accumulation profiles of these niosomal formulations, since the skin acts as a negative barrier (Hadgraft and Valenta, 2000). However, The degree of ionization of minoxidil in Span 60 and Brij 52 niosomes could not be significant, as the pH of these formulations were 6.38 ± 0.03 and 6.27 ± 0.02, which is very near to the pK_a value of minoxidil.

It is worth noting that niosomal formulations increased the percentage of minoxidil absorbed in the skin layers more than the propylene glycol/water/ethanol solution as well as the commercial formulation. It was observed that a large net difference in skin deposition of drug was found between the niosomal suspensions and the control or commercial formulation. The commercial formulation showed very low skin penetration ability; in fact minoxidil crystals were observed on the surface of the skin after the permeation study (Fig. 5), which explains the low penetration ability of the commercial formulation. Most of the commercial formulations are ethanol and propylene glycol based formulations. Therefore, evaporation of the volatile solvents could have reduced the solubility of minoxidil in the residual phase of the applied control and commercial solution, hence, the drug thermodynamic activity and drug permeation (Mura et al., 2007). On the other hand, the solvent evaporation also leads to an increased viscosity of the residual applied solution with a consequent reduced minoxidil diffusion coefficient. The much lower dose absorbed (%) in skin by commercial formulation compared to control solution (Table 3) could be due to low solvent volume compared to drug content in the formulation, which might have lead to form a thin film over the skin as the solvent evaporate. In conclusion, results obtained during these in vitro permeation studies have shown that niosomes are better carriers than the control and commercial formulations.

There are several mechanisms which could explain the ability of niosomes to modulate transfer across skin. One of the mechanism by which niosomes may contribute to transdermal drug delivery may be ascribed to the fusion of vesicles on the surface of the skin which might lead to the establishment of large concentration gradients of the intercalated drug across the skin and hence enhanced skin permeation (Touitou et al., 1994; Uchegbu and Vyas, 1998). Moreover, it has been proven that niosomes enhances penetration and retention of topically applied drugs (Van

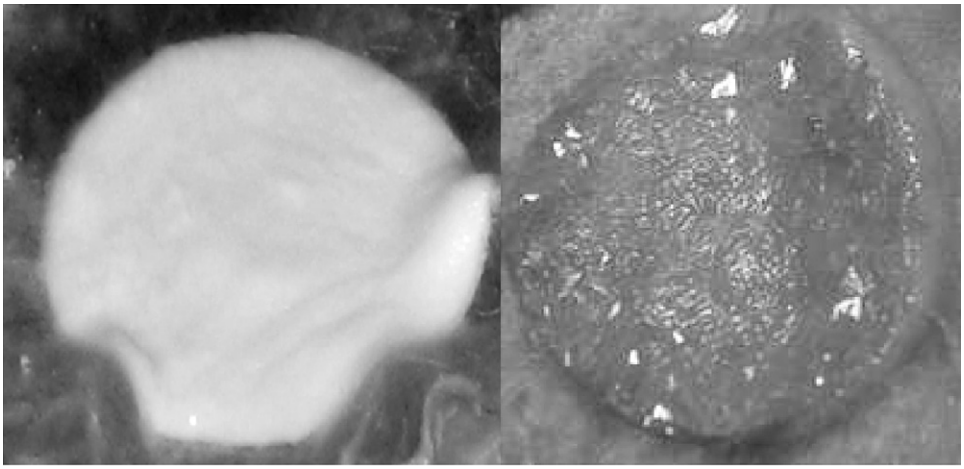

Fig. 5. Photographs of skin specimens after washing with water following permeation study. A: Optimized Span 20 niosomal formulation, B: Minoxyl commercial formulation.

Hal et al., 1996; Azeem et al., 2008). Previous researchers reported that the transdermal delivery of minoxidil-loaded nanoparticles depended on the size of the colloidal carriers and minoxidil-loaded liposomes, although the particle size dependence on the permeation behavior of minoxidil was not observed in hairless guinea pig skin (Shim et al., 2004). In the present study, no permeation of minoxidil through the hairless mouse skin into the receptor compartment was detected. This probably depends on the influence of the components on the skin barrier properties on the different species, types of skin and on different experimental procedures. However, the higher skin content of minoxidil was provided by the smaller niosomes (Verma et al., 2003; Shim et al., 2004). The enhancing penetration effect and smaller particle size of niosomes could be attributed to the enhanced bioavailability of minoxidil within the skin. Thus, the higher skin bioavailability obtained by both dialyzed and non-dialyzed niosomes shows the superiority of the formulation over the control and commercial formulations. The results obtained from the permeation study followed by minoxidil skin content study clearly implicit that niosomes were able to greatly improve cutaneous retention of minoxidil.

4. Conclusion

In the present study, the findings revealed that the process variables critically affect the formulation of niosomes with regards to drug loading and need to be carefully controlled.

The investigations further revealed the potential of niosomes in enhancing transdermal bioavailability of minoxidil in hairless mouse skin even at very low drug concentration unlike control formulations. Permeation experiments of the niosomes showed that the surfactant nature and vesicular size played important role in enhancing minoxidil bioavailability in hairless mouse skin. In conclusion, our study suggests that these niosomal formulations have greater potential for drug cutaneous targeting and could be used as a feasible cargo carrier for the topical delivery of minoxidil in skin diseases such as hair loss.

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