Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 298 (2005) 1–12
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes

J.M. López-Pinto, M.L. González-Rodríguez, A.M. Rabasco *

Department of Pharmaceutical Technology, University of Seville, C/ Prof. García González 2, 41012 Seville, Spain

Received 24 March 2004; received in revised form 21 January 2005; accepted 9 February 2005
Available online 17 May 2005

Abstract

The main objective of the present work was to compare the dermal delivery of minoxidil (Mx), a lipophilic drug from ethosomes versus classic liposomes, containing different cholesterol (CHOL) concentrations. All the systems were characterized for shape, lamellarity, particle size and entrapment efficiency percentage (EE), by transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), laser diffraction and ultracentrifugation or dialysis methods, respectively. Multilamellar vesicles (MLVs) were obtained and one to six lamellae were visualized by CLSM. The presence of ethanol in the formulations affects the particle size in terms of reducing this parameter. In addition, it was possible to appreciate the influence of CHOL on the vesicle size, because it was increased, as CHOL concentration was higher. When the EE was determined by two different methods (ultracentrifugation and dialysis methods), a clear losing of entrapped drug by the ultracentrifugation method was observed, because the strong energy transmitted to the samples disrupted vesicles.

Vesicles were non-occlusively applied on rat skin and the permeation pattern of the different systems, depth into the skin and the main permeation pathway were studied by using β-carotene as a fluorescent probe. CLSM studies showed that ethosomal systems were much more efficient at delivering the fluorescent substance into the skin in terms of quantity and depth, than either liposomes or hydroalcoholic solutions.

Keywords: Liposome; Ethosome; Lamellarity; Confocal laser scanning microscopy; Franz cell; Minoxidil

1. Introduction

For the last 2 decades, topical delivery of drugs from liposomal formulations has evoked a considerable interest. Many reports on enhancing percutaneous delivery focus on the use of liposomes, because they can aid the transport of hydrophilic and lipophilic compounds (Coderch et al., 1996). However, it is generally agreed that classic liposomes are of little or no value as carriers for transdermal drug delivery, because they do not deeply penetrate the skin, but rather remain confined to the upper layer of the stratum corneum. Only specially

* Corresponding author. Tel.: +34 954 556724; fax: +34 954 556726.
E-mail address: amra@us.es (A.M. Rabasco).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.02.021

designed vesicles were shown to be able to allow transdermal delivery, such as transfersomes (Cevc et al., 1998, 2002).

Ethanol is known as an efficient permeation enhancer. This solvent is commonly believed to act by affecting the intercellular region of the stratum corneum (Magnusson et al., 1997), although other mechanisms have been reported, such as coadjuvant to diminish the melting point of drugs, such as lidocaine, achieving a higher concentration in the oil phase of an emulsion and prolonging the anaesthetic latency time (Kang et al., 2001). Its inclusion in liposomes to form ethosomes has already been investigated by other authors (Kirjavainen et al., 1999; Dayan and Touitou, 2000; Godin and Touitou, 2004).

In this study, Mx was selected as a model lipophilic drug. It has potential applications in the case of androgenetic alopecia (Olsen et al., 2002). However, the appearance of some complaints in the patients with this treatment (pruritus and scaling of the scalp) as a consequence of irritant contact dermatitis, allergic contact dermatitis or an exacerbation of seborrheic dermatitis, has been reported (Friedman et al., 2002).

In this paper, vesicle systems (liposomes and ethosomes) were prepared. Ethosomal formulations included ethanol in relatively high concentrations, incorporating it in the aqueous phase. Mx was dissolved in the hydroethanolic solution and was included in the aqueous compartment of vesicles.

This paper focuses on the preparation and characterization of ethosomal formulations, for Mx transdermal delivery. So, the effect of ethanol and CHOL on the permeation of two lipophilic substances (Mx and carotene) through rat skin was evaluated.

2. Materials and methods

2.1. Materials

High-purity α-dipalmitoylphosphatidylcholine (α-DPPC) and CHOL were purchased from Sigma (Barcelona, Spain). The lipid purity was higher than 99%, and it was used without further purification. Mx was purchased from Genox Farma (Barcelona, Spain) and was used as a model drug because of its poor water solubility. β-carotene (βC) was used as a fluorescent probe, obtained from Faisa (Seville, Spain).

Chloroform and ethanol were received from Panreac Chemistry (Barcelona, Spain). All other chemicals were at least reagent grade and used as received.

2.2. Mx solubility measurements

The solubility was determined in water at 25 °C. Briefly, an excess of Mx was added to 2 mL of solvent; the suspensions were vigorously shaken for 7 days (*Selecta Rotaterm*) and then left to equilibrate. Samples were ultracentrifuged (*PACISA FP-S10*), at 40,000 rpm for 1 h, the supernatant was filtered (*Millipore* 0.45 μm) and diluted. The Mx concentration was determined by HPLC as further described.

2.3. HPLC assay for Mx quantification

Mx was quantified by an HPLC method proposed by other authors (Touitou et al., 2000). It was used a Hitachi system manager D-7000, equipped with a Rheodyne injector 77251, isocratic pump L-7100 and detector L-7455. Separation was carried out on Merck LiChrospher® 100 RP-18 column with a particle size of 5 μm, 12.5 cm × 4 mm and kept at room temperature. The mobile phase was methanol:water:glacial acetic acid (7:3:0.1) pH 3.0 and 3 g/L of sodium docusate. Mx was detected at 254 nm, and the flux rate was 1 mL/min.

2.4. Preparation of liposomes

Multilamellar liposomes were prepared by the hydration method. In a typical procedure, 60 mg (81.7 μmol) DPPC, 0–40 mg (0–103.44 μmol) CHOL and 40 mg (191.16 μmol) Mx or fluorescent probe (5 mg) were dissolved in a small amount of methanol:chloroform (1:3) mixture. The solution was placed in a rotary evaporator (*Rotavapor R 200/205, Buchi*) at 55 °C until a thin lipid film on the wall of a round-bottomed flask, was obtained. The resulting lipid film was kept under vacuum in order to eliminate the traces of organic solvent. This lipid film was then hydrated with 4 mL of the appropriate aqueous solution, by mixing in a vortexing device, consisting in 1 min of vortex treatment and 5 min in a thermostatized bath at 55 °C, value that is above the phase-transition temperature (T_c) of DPPC (41 °C) according to Lasic et al. (1998). All the samples were sonicated for 10 min by using an ultrasound bath (*Transonic 460 H, Singen*),

Table 1
Composition of all formulations

Batches	Composition			
	DPPC (mM)	CHOL (mM)	EtOH (%, v/v)	Mx (mg)
1	20.43	–	–	40
2	20.43	6.47	–	40
3	20.43	12.93	–	40
4	20.43	25.87	–	40
5	20.43	–	40	40
6	20.43	6.47	40	40
7	20.43	12.93	40	40
8	20.43	25.87	40	40
Control 1	–	–	–	40
Control 2	–	–	40	40

When β-carotene was included in the formulations, a concentration of 0.05% (w/v) was used. Batches 1–4 were liposome formulations, and batches 5–8 were referred to ethosome formulations. An aqueous Mx suspension was fixed as *control 1*, and a Mx water–ethanol mix solution was named as *control 2*.

then, quickly sealed and stored in darkness at a temperature of 4 °C. In Table 1, the batches composition corresponding to the all formulations, have been reported.

2.5. Preparation of ethosomes

Ethosomes were prepared following the classic mechanical-dispersion method; 60 mg (81.7 μmol) DPPC and 0–40 mg (0–103.44 μmol) CHOL were dissolved in a small amount of methanol:chloroform (1:3). This solution was completely dried in the rotary evaporator for 60 min, to form a lipid film on the wall of a round-bottomed flask. This lipid film was then hydrated with a water–ethanol mixed solution (40% (p/v) ethanol) containing Mx or probe previously dissolved. The preparation was mixed by using a vortexing device. All the samples were sonicated for 10 min, then, immediately sealed and stored in darkness at a temperature of 4 °C.

2.6. Vesicle characterization

2.6.1. Visualization by transmission electron microscopy (TEM)

Liposomal and ethosomal preparations were examined by TEM. A drop of the sample solution was placed on a copper grid and the material excess was removed with a filter paper. A 2% uranyl acetate solution was dropped onto the grid. The excess of staining solution was removed with a filter paper in 60 s. Finally, the grid was examined under a transmission electron microscope (*Philips, CM-10*) at 80 kV.

2.6.2. Vesicle size distribution

The vesicle size distribution was determined using a laser diffraction technique on a Mastersizer X, with a MSX17 sample preparation unit (*Malvern Instruments*) at 25 °C. For size measurements, the preparation was mixed with the appropriate medium (40% (w/w) ethanol–water solution for ethosomes and purified water for liposomes). The measurements were performed five times, using a 45 mm focus objective, a beam length of 2.4 mm and obscuration levels up to 10% at a stable count rate.

2.6.3. Determination of trapping efficiency

The drug-entrapment percentage was determined by two methods: ultracentrifugation and by using dialysis bags. In the first one, vesicle preparations were kept overnight at 4 °C and ultracentrifuged (*PACISA FP-S10*) for 2 h at 40,000 rpm. Free Mx was assayed by HPLC in the supernatant. The Mx entrapment percentage was calculated from the relationship:

$$\mathrm{EE} = \frac{Q_t - Q_s}{Q_t} \times 100$$

where EE is the entrapment efficiency, Q_t the theoretical amount of Mx that was added, and Q_s is the amount of Mx detected only in the supernatant.

The drug EE determination was also carried out by using dialysis bags. Cellulose acetate membranes

(*Spectra/Por MWCO 12-14.000*) were kept into saline solution for 1 h before dialysis to ensure the whole wetting of the membrane; 2 mL of the drug-loaded vesicles or free drug in aqueous solution was placed into the dialysis bag, which was then transferred into 500 mL of phosphate buffer solution (PBS) pH 7.0. The receiver medium was stirred with a magnetic stirrer. Samples of 800 μL were withdrawn at fixed time ranges from the receiver medium and replaced with equal volumes of PBS solution. Samples were analyzed by HPLC.

In this case, the EE was calculated from the same equation that previously described.

2.6.4. Lamellarity

This parameter was quantified by confocal laser scanning microscopy (CLSM). The equipment used was a Leica TCS SP II confocal unit (*Leica, Heidelberg, Germany*) equipped with a Krypton–Argon–Helium/Neon laser and mounted on a Leica DM IRE 2 inverted microscope (*Leica, Heidelberg*) using HC PL Fluotar Leica lens with magnifications of 10, 20× (dry) and HCX PLAN APO Leica lens 40× (0.85 multi immersion objective) on its oil position. For excitation of the label the 488 nm laser line was used and the fluorescence emission was detected above 520 nm.

Following parameters were used for the confocal microscope starts: magnification 10, 20 and 40×, laser power 5%, scan modus slow, gain values 400–600, *x–y–z* mode, 488 nm emission line at 31% maximum power, transmission light channel activated and bidirectional scanning pattern.

Samples containing Mx were analyzed by using the transmitted light, which will be compared with the samples containing the probe, whose images were obtained by fluorescence emission.

2.6.5. Stability studies

Formulation stability was studied in terms of morphology, by TEM and in terms of size, by laser diffraction at several times (1, 2 and 4 weeks) after preparation.

2.7. Skin penetration and permeation studies

2.7.1. Skin cryosectioning for CLSM

Abdominal skin of Wistar rats (aged 17–22 weeks, weight 250–300 g) was used for permeation studies. Rats were killed by diethyl ether inhalation. After depilation and washing, abdominal skin was excised, thoroughly washed with the pH 7.4 buffer solution, dried and carefully cleaned from the subcutaneous fat and then preserved at −25 °C.

For skin cross-section CLSM studies, the skin was taken out from the refrigerator and frozen in liquid nitrogen. The treated skin was mechanically cross-sectioned with a cut blade into 200–300 μm slices immediately after taking out the skin section from the liquid nitrogen. The cross-sectioned skin was mounted on a sample holder in such a way that the freshly obtained cutting surface was positioned against the cover glass and examined 5–10 μm below the cutting surface to avoid any interference from fluorescence of damaged cells (Kuijk-Meuwissen et al., 1998).

2.7.2. Depth of β-carotene skin penetration from liposomes and ethosomes, visualized by CLSM

Depth of skin penetration was determined by using β-carotene vesicles. All the formulations were non-occlusively applied for 8 h to the abdominal skin of rats, placed in Franz diffusion cells at 37 °C. At the end of the experiment, the remaining preparation was carefully washed with purified water from the skin surface. Specimen was viewed by CSLM through the *z*-axis, as described above.

The fluorescence intensities of the CLSM images were semi-quantitatively scored and classified as follows: (−) very weak fluorescence; (+) weak fluorescence; (++) medium fluorescence; (+++) bright fluorescence.

The images were corrected for autofluorescence of rat skin, with the black level setting.

2.7.3. Mx skin permeation

The Mx permeation process from vesicles was measured through the rat abdominal skin, in experiments carried out at 37 °C in Franz diffusion cells for 24 h. The skin pieces were rinsed in PBS pH 7.0 and mounted in the diffusion cell with an exposed skin area of 2.545 cm^2 and a volume of 15 mL in the receiver compartment. The upper compartment of the Franz cell was covered with parafilm to avoid any evaporation process. The dermal side of the skin was immersed in PBS and the liposomal formulations were in contact with the skin outer layer. The treatment was carried out at 37 °C, and the chambers were kept protected from light.

Two non-vesicular systems were tested as control solutions: 1% (w/v) Mx suspension in water and 1% (w/v) Mx in hydroethanolic solution (40%, v/v). Samples of 200 μL were withdrawn from the receiver medium for 24 h and quickly analysed; 200 μL of fresh medium (PBS pH 7.0) was added to keep a stable receiver volume. The drug concentration in the receiver medium at different times was determined by HPLC.

2.7.4. Statistical data analysis

The cumulative amount of penetrant, Q (μg/cm^2), which permeated the skin per unit surface area was plotted against time. The linear portion of the plot was taken as being the steady-state flux, (J_s). The permeability coefficient (K_p) was calculated as:

$$K_p = \frac{J_s}{C_v}$$

where C_v is the concentration of penetrant in the donor solution.

Statistically significant differences were determined using the Student's *t*-test and the analysis of variance (ANOVA) with $P < 0.05$ as a minimal level of significance (Šentjurc et al., 1999).

The significance of the differences between groups (different formulations) was tested using the non-parametric Kruskal–Wallis test. Results were expressed as the mean value ± standard error ($n = 5$ independent samples).

3. Results and discussion

3.1. Vesicle characterization

3.1.1. Morphology

For an initial vesicle characterization, liposomes and ethosomes were examined by TEM. In all cases, the presence of spherical-shaped vesicles was predominant. The absence of CHOL into the formulations favours the aggregation process among the structures (Fig. 1A). Batches without CHOL (1 and 5) exhibited some vesicle aggregates in the whole vision field (Fig. 1B). This fact has been corroborated by other authors (Coderch et al., 2000; Brisaert et al., 2001), as a consequence of CHOL effect as stabilizing agent in the lipid bilayer.

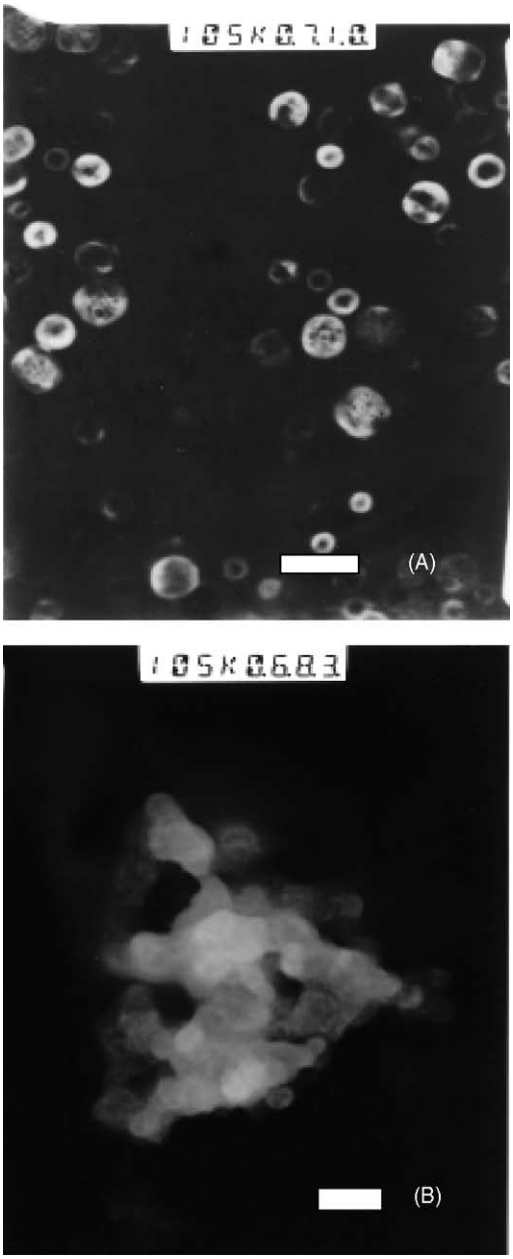

Fig. 1. Microphotographs by TEM (scale bar = 500 nm) corresponding to isolated spherical vesicles from batch 3 (12.93 mM CHOL), aggregates formed in batch 1 (without CHOL).

3.1.2. Vesicle size distribution

The vesicle mean diameters for all formulations are shown in Table 2. Laser diffraction studies showed some differences in size between liposomes and

Table 2
Mean particle size for the different formulations, measured by laser diffraction techniques

Batches	Mean size (nm)	EE (%)	
		Method 1	Method 2
1	412 ± 45	30.5 ± 2.46	27.0 ± 2.86
2	470 ± 37	38.1 ± 3.24	36.2 ± 1.52
3	504 ± 59	44.5 ± 2.96	43.7 ± 2.34
4	520 ± 24	66.1 ± 1.45	65.6 ± 1.23
5	136 ± 42	42.7 ± 2.21	37.4 ± 3.21
6	158 ± 35	55.8 ± 2.14	54.1 ± 2.56
7	195 ± 15	60.2 ± 3.95	57.9 ± 1.95
8	230 ± 27	84.4 ± 4.52	84.5 ± 1. 88

Method 1, dialysis bags for determining the EE. Method 2, ultracentrifugation method for determining the drug-entrapment. Mean ± S.D. ($n \geq 3$).

ethosomes. In liposomes, diameters ranged from 412 to 520 nm, while for ethosomal systems, diameters ranged from 136 to 230 nm. In accordance with other authors, this generalized decrease in the mean diameter is due to the presence of ethanol in vesicle composition (Touitou et al., 2000). Probably, ethanol causes a modification of the net charge of the system and confers it some degree of steric stabilization that may finally lead to a decrease in the mean particle size (Lasic et al., 1998).

On the other hand, it was seen that the increase in CHOL concentration resulted in an increase in the mean particle size. As it was illustrated in Fig. 2, in the case of liposomal systems, this rise was 26.21% (between batch 1, without CHOL and batch 4, with 25.87 mM CHOL), whereas for ethosomal systems it was 69.11% (between batch 5, without CHOL and batch 8, with 25.87 mM CHOL). Several hypotheses

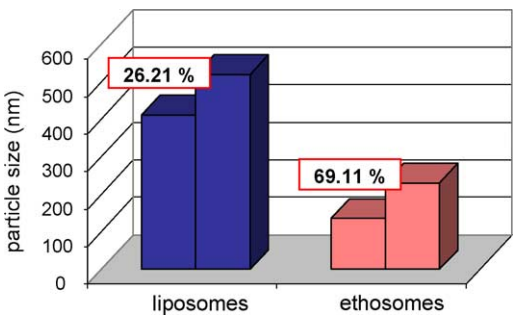

Fig. 2. Variation range (%) between minimum and maximum values of the mean particle size obtained for liposomes and ethosomes.

were taken into account to explain this result (Socaciu et al., 2002). Recent studies carried out by DSC (Socaciu et al., 2000) showed that the addition of steroids to liposomes tends to eliminate the phase-transition temperature (T_c) peak of DPPC and thus the range of the gel state of vesicles increased, remaining in a solid state and preventing the partial dilution of the bilayers.

The differences in size among all the batches studied were statistically significant ($P < 0.05$; Kruskal–Wallis test); so, it could be concluded that both ethanol and CHOL have a remarkable effect on the vesicle mean size of the studied formulations.

3.1.3. Entrapment efficiency

Two different methods were used to quantify the Mx amount entrapped into the systems: ultracentrifugation and dialysis methods, in order to compare both data series. Results obtained were reported in Table 2. It was observed that liposomes were more stable at higher ethanol concentrations than in its absence. Maximum entrapment percentage was reached at 40% (v/v) ethanol.

When the ultracentrifugation method was used, EE data were slightly lower than that obtained by the dialysis method. This fact suggests a losing of entrapped drug during the ultracentrifugation process, probably due to the deformation phenomenon of lipid membranes because of the high speed reached with this method. This conclusion was corroborated taking into account that formulations without CHOL (both liposomes and ethosomes), showed statistically significant differences ($P < 0.05$) in EE values. Other authors (Barry and Cullis, 1995) have demonstrated that, although a decrease in the membrane thickness of vesicles containing high ethanol concentrations has been observed, corresponding to the formation of a phase with interpenetrating hydrocarbon chains, however, CHOL contributes to vesicle stability, since it provides a greater rigidity to the lipid layers and confers to the system a higher resistance value, when the high rotational energy was transferred (Maurer et al., 2001).

3.1.4. Lamellarity

In this work, we have demonstrated that it is possible to determine both, the outer morphology and the lamellarity of vesicle systems by means of CLSM. Transmitted light CLSM pictures show a main distribution of

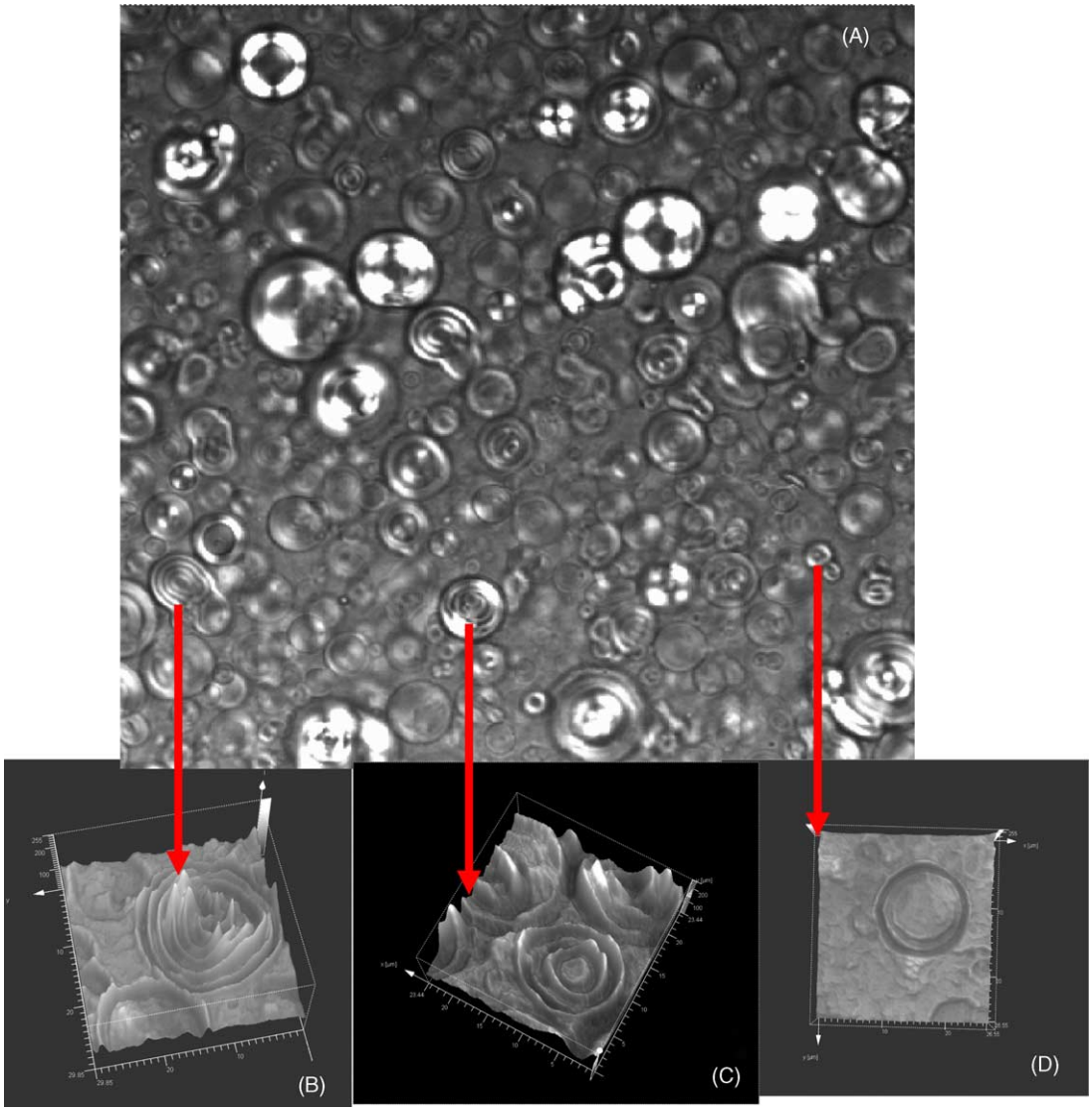

Fig. 3. Microphotographs corresponding to DPPC multilamellar liposomes (batch 3) by CLSM using transmitted channel: (A) the whole field; (B) six lamellae; (C) three lamellae.

MLVs in all the systems studied (Fig. 3A), with a number of concentric bilayers ranged between 6 (Fig. 3B) and 3 (Fig. 3C). Sonicated vesicles showed a clear tendency to decrease their lamellarity, finding a representative number of unilamellar vesicles in the formulations, as it is shown in Fig. 3D.

3.1.5. Stability of formulations

The stability of vesicular systems was investigated by TEM and laser diffraction for a period of 4 weeks, in order to follow the vesicle evolution in terms of mean size.

These results were shown in Table 3. The mean diameter of vesicles tends to increase with time, only for liposomal systems the difference in size was statistically significant ($P < 0.05$), while ethosomes showed a higher stability and the mean particle size remained constant during 4 weeks.

Ethanol may exert a stabilizing effect in the formulation. It confers a surface negative net charge,

Table 3
Mean particle size for the different formulations after 4 weeks of storing

Composition	Mean diameter (nm)			
	$t = 0$ week	$t = 1$ week	$t = 2$ weeks	$t = 4$ weeks
1	412 ± 45	445 ± 32	496 ± 24	588 ± 57
2	470 ± 37	489 ± 25	504 ± 15	505 ± 13
3	504 ± 59	517 ± 41	548 ± 29	552 ± 22
4	520 ± 24	535 ± 32	542 ± 43	557 ± 34
5	136 ± 42	138 ± 37	132 ± 35	139 ± 23
6	158 ± 35	162 ± 28	165 ± 38	159 ± 44
7	195 ± 15	193 ± 21	197 ± 19	194 ± 26
8	230 ± 27	234 ± 42	245 ± 33	246 ± 41

These measurements were carried out by laser diffraction techniques. Mean ± S.D. ($n \geq 3$).

avoiding or, at least, delaying the formation of vesicle aggregates, due to the electrostatic repulsions. This was found to constitute the main cause of growing in vesicle mean size for those formulations without ethanol.

With respect to the effect of CHOL concentration, it was observed that high concentrations of this steroid with respect to the whole lipid amount, provide a lower tendency to rise in diameter at the end of 4 weeks: 7.11, 9.52 and 7.24% for 25.87, 12.93 and 6.47 mM CHOL, respectively, than the batches without CHOL, 42.72% for liposomes. CHOL, a rigid steroid molecule, is able to stabilize the bilayers when vesicles remain in gel state, making these structures more rigid, and therefore, making it more difficult for vesicle fusion.

As it was explained above, the stability of the different systems was evaluated along 4 weeks also in terms of morphology. Both ethosomes and liposomes formulations with more than 6.47 mM CHOL exhibited vesicles individually distributed, while formulations without this steroid (batches 1 and 5) showed vesicle aggregates in the whole system.

3.2. Skin penetration and drug permeation studies

3.2.1. Depth of β-carotene skin penetration

Different formulations can be compared aimed to study the penetration depth of the fluorescent probe and the relative intensity of fluorescence in the skin layers. The results of the scoring for each formulation are summarized in Table 4, where the relative intensity level of the probe substance was qualitatively described. During the first 3 h, a similar penetration depth of the applied probe was visualized in all formulations, remaining the fluorescence in the treated stratum corneum with a low intensity level. However, remarkable differences can be noted after 6 h following the application.

Fig. 4 shows the confocal microphotographs corresponding to various formulations applied onto the rat skin in the same experimental conditions. For every formulation applied, different intensities of fluorescence can be seen. Both, relative intensity of fluorescence and penetration depth of the fluorescent label were higher, when CHOL and ethanol were included in the formulation. The maximum values were reached in ethosomes batches with 12.93 and 25.87 mM CHOL. These later formulations showed high fluorescence intensity in the stratum corneum as well as in viable

Table 4
Flux (J), permeation coefficient (K_p), fraction of total drug quantified in the receiver compartment (P (%)), and the relative intensity of fluorescence of the lipophilic βC probe into the stratum corneum after 3 and 6 h of application

Batches	J (μg/h cm^2)	K_p (cm/h)	P (%)	Relative intensity (3 h)	Relative intensity (6 h)
1	1413.69 ± 13.05	70.68 ± 2.81	23.85 ± 0.91	–	+
2	1750.43 ± 16.19	87.52 ± 3.52	28.77 ± 1.33	–	+
3	2396.60 ± 15.01	119.83 ± 3.84	32.80 ± 2.53	+	++
4	2420.12 ± 24.37	121.01 ± 1.96	33.31 ± 1.56	+	++
5	1732.27 ± 21.28	86.61 ± 2.15	35.48 ± 3.39	+	++
6	2805.48 ± 23.51	140.27 ± 3.37	37.22 ± 2.86	+	+++
7	3021.46 ± 35.01	151.07 ± 2.80	41.68 ± 1.37	+	+++
8	3054.53 ± 43.73	152.72 ± 3.42	42.79 ± 1.57	+	+++
Control 1	461.12 ± 24.12	23.05 ± 1.97	12.29 ± 0.94	–	–
Control 2	4529.43 ± 32.78	226.47 ± 12.45	24.41 ± 1.24	+	++

Mean values ± S.D. ($n \geq 3$).

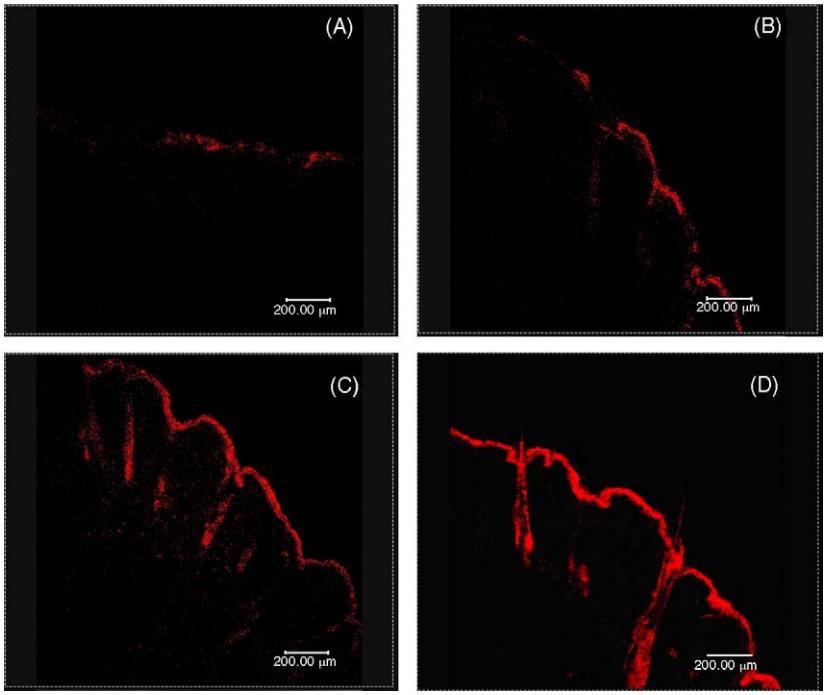

Fig. 4. Optical cross-sections perpendicular to the rat skin surface incubated with fluorescent-labelled formulations: (A) scored (−) corresponding to *control 1*; (B) scored (+) corresponding to the batch 1; (C) scored (++) corresponding to the batch 4; (D) scored (+++) corresponding to the batch 8. All micrographs were made after 6 h after application and recorded in the red channel. The stratum corneum of the skin faces to the upper side of the image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

epidermis. Also, it can be visualized in Fig. 4D that high amounts of CHOL and ethanol in liposome composition make it possible for the drug to reach deeper skin structures, such as the pilosebaceous follicle.

In accordance with other authors, we proposed two hypotheses to explain these results: (A) the recognized effect of ethanol as a skin permeation enhancer (Komatsu and Okada, 1996) and (B) ethosomal systems should be more flexible, and therefore, more effective in enhancing transport (Cevc et al., 2002) although CHOL molecules were included in the formulations.

3.2.2. Mx skin permeation from vesicles

In order to clarify the enhancer effect of ethosomes and the CHOL contribution to the permeation process, Mx permeability studies across the rat skin were carried out. Franz type cells were used for diffusion studies. Vesicle systems and non-vesicle systems were used as donors. Skin permeability values from all the systems were summarized in Table 4. It was observed that the drug flux to the receiver compartment was favoured in the case of ethosomal formulations, indicating the enhancing effect of ethanol for drug penetration across the skin layers. Moreover, taking into account the drug permeation values when the drug was suspended in water or dissolved in a mixed water–ethanol solution, it is possible to appreciate the enhancer effect of ethanol (Fig. 5). The presence of ethanol in the formulation increased the *P*-value (2-folds) and the flux (9.8-folds) and the obstructive capacity of lipid vesicles (Fig. 6). This fact causes a decrease in the Mx flux rate. However, when the percentage of drug permeated was calculated, it was possible to observe that, although the drug flux is higher in the ethanolic solutions of the drug, the total drug amount is higher in the vesicle formulations.

The plots followed a common pattern for all the formulations studied, conventional liposomes, ethosomes and control systems. In general, the profiles were

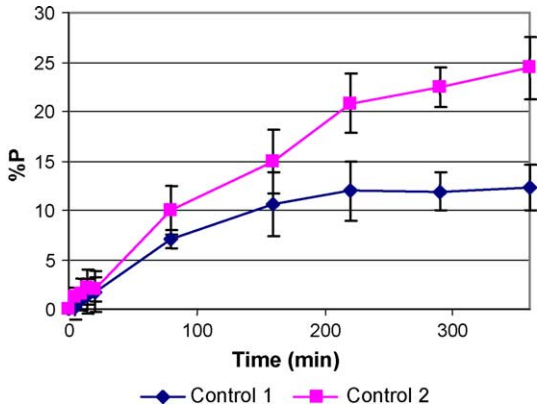

Fig. 5. Mx skin penetration profiles from the drug aqueous solution (*Control 1*) and hydroalcoholic solution (*Control 2*).

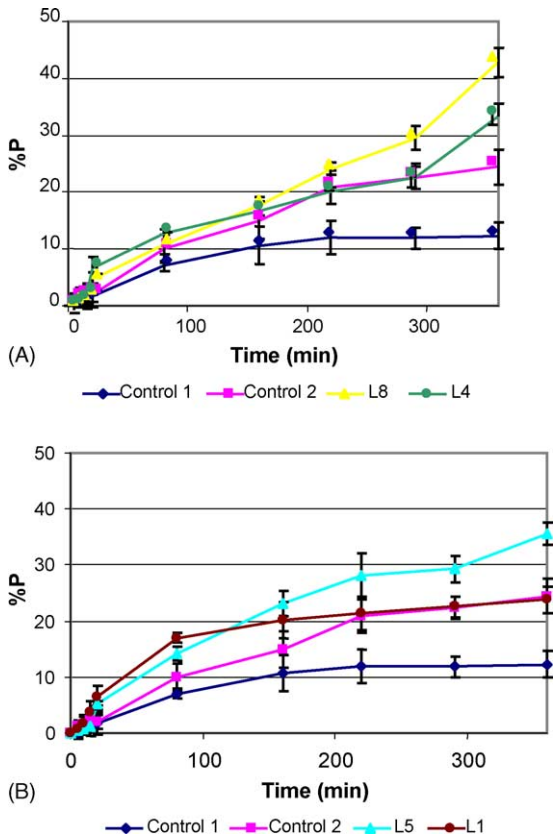

Fig. 6. Effect of ethanol on Mx skin permeation from liposomes without CHOL, batches 1 (L1) and 5 (L5), vs. control solutions; ethosomes containing 25.87 mM CHOL, batches 4 (L4) and 8 (L8), vs. control solutions.

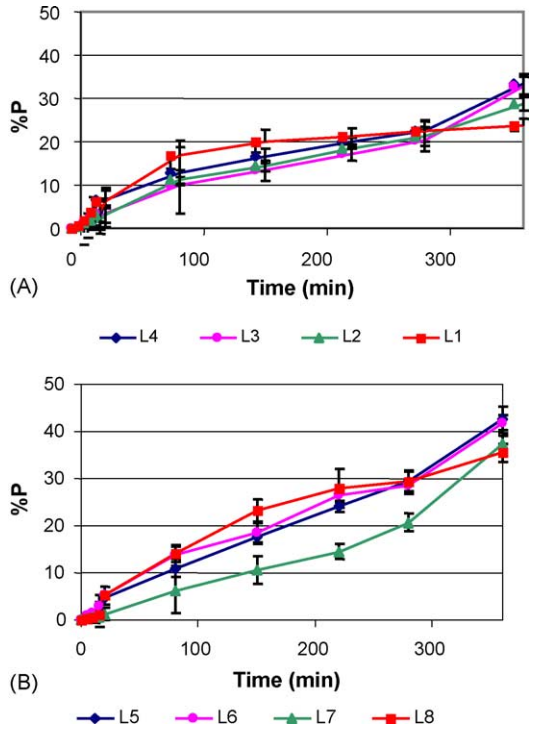

Fig. 7. Effect of CHOL concentration on Mx permeation profiles through rat skin from: (A) liposomes and (B) ethosomes.

adjusted to a main kinetic mechanism of passive diffusion.

However, depending on CHOL concentration, the pattern is different (Fig. 7), and no statistical differences were found among the formulations with and without CHOL. These findings suggest that CHOL regulates membrane fluidity and produces a condensing effect on membranes, decreasing their permeability and making them more rigid. Therefore, due to this rigidity, MLVs are not capable to pass across the stratum corneum, making it more difficult for the drug to permeate across the skin.

4. Conclusions

In this paper, liposome and ethosome formulations have been characterized, containing Mx as lipophilic drug. When drug-entrapment percentage was determined, it was observed that the presence of ethanol in the aqueous compartment of the vesicles, favoured

the Mx-encapsulation, because it was included in the aqueous phase.

In addition, the use of high relative CHOL amount in the lipid fraction of liposomes increased the drug-entrapment percentage, due to the stabilizing effect of this steroid into the lipid bilayers.

Ethanol causes the liposome surface to have a negative net charge. This results in the size of the vesicle to decrease.

CLSM studies showed that the fluorescent lipophilic probe, enclosed in the different formulations, reached deeper skin structures when ethanol was added to the liposomal formulation. In addition, a clear affinity of the fluorescent probe to the pilosebaceous follicle has been shown.

The in vitro Mx permeation studies from liposomes and ethosomes containing different CHOL concentrations were evaluated. The results showed that when ethosomes containing Mx were applied to rat skin, lower percentage of the drug reached the receiver medium if we compared it to the hydroalcoholic Mx solution. This suggests the enhancer effect of the ethanol as well as the occlusive effect of liposomes.

Therefore, the inclusion of ethanol in liposomes plays an important role in the enhancement of Mx permeation. However, the inclusion of CHOL in the formulations must be optimized in future works, because it has been demonstrated that this steroid plays an important role in the stabilization of the systems.

Acknowledgements

We are grateful to the Electron Microscopy Service from University of Seville for providing the CLSM and TEM equipment. We also would like to thank to Mrs. Asunción Fernández for her valuable contribution to this work.

References

Barry, A.L., Cullis, P.R., 1995. Direct NMR evidence for ethanol binding to the lipid–water interface of phospholipid bilayers. Biochemistry 33, 8082–8088.

Brisaert, M., Gabriëls, M., Matthijs, V., Plaizer-Vercammen, J., 2001. Liposomes with tretinoin: a physical and chemical evaluation. J. Pharm. Biomed. Anal. 26, 909–917.

Cevc, G., Gevauer, D., Stieber, J., Schätzlein, A., Blume, G., 1998. Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. Biochim. Biophys. Acta 1368, 201–215.

Cevc, G., Schätzlein, A., Richardsen, H., 2002. Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented: evidence from double label CLSM experiments and direct size measurements. Biochim. Biophys. Acta 1564, 21–30.

Coderch, L., Oliva, M., Pons, M., de la Maza, A., Manich, A.M., Parra, J.L., 1996. Percutaneous penetration of liposomes using the tape stripping technique. Int. J. Pharm. 139, 197–203.

Coderch, L., Fonollosa, J., De Pera, M., Estelrich, J., de La Maza, A., Parra, J.L., 2000. Influence of cholesterol on liposome fluidity by EPR: relationship with percutaneous absorption. J. Control. Rel. 68, 85–95.

Dayan, N., Touitou, E., 2000. Carriers for skin delivery of trihexyphenidyl HCl: ethosomes vs. liposomes. Biomaterials 21, 1879–1885.

Friedman, E.S., Friedman, P.M., Cohen, D.E., Washenik, K., 2002. Allergic contact dermatitis to topical minoxidil solution: ethiology and treatment. J. Am. Acad. Dermatol. 46, 309–312.

Godin, B., Touitou, E., 2004. Mechanism of bacitracin permeation enhancement through the skin and cellular membranes from an ethosomal carrier. J. Contr. Rel. 94, 365–379.

Kang, L., Jun, H.W., Mani, N., 2001. Preparation and characterization of two-phase melt systems of lidocaine. Int. J. Pharm. 222, 35–44.

Kirjavainen, M., Urtti, A., Valjakka-Koskela, R., Kiesvaara, J., Mönkkönen, J., 1999. Liposome-skin interactions and their effect on the skin permeation of drugs. Eur. J. Pharm. Sci. 7, 279–286.

Komatsu, H., Okada, S., 1996. Ethanol enhanced permeation of phosphatidylcholine/phosphatidylethanolamine mixed liposomal membranes due to ethanol induced lateral phase separation. Biochim. Biophys. Acta 1283, 73–79.

Kuijk-Meuwissen, M.E., Mougin, L., Junginger, H.E., Bouwstra, J.A., 1998. Application of vesicles to rat skin in vivo: a confocal laser scanning microscopy study. J. Control. Rel. 56, 189–196.

Lasic, D., Weiner, N., Riaz, M., Martin, F., 1998. Liposomes. In: Lieberman, A., Rieger, M., Banker, G. (Eds.), Pharmaceutical Dosage Forms: Disperse Systems, vol. 3. Marcel Dekker, NY, pp. 43–86.

Magnusson, B.M., Runn, P., Karlsson, K., Koskinen, L., 1997. Terpenes and ethanol enhance the transdermal permeation of the tripeptide thyrotropin releasing hormone in human epidermis. Int. J. Pharm. 157, 113–121.

Maurer, N., Wong, K.F., Stark, H., Louie, L., McIntosh, D., Wong, T., Scherrer, P., Semple, S.C., Cullis, P.R., 2001. Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-desestabilized cationic liposomes. Biophys. J. 80, 2310–2326.

Olsen, E.A., Dunlap, F.E., Funicella, T., Koperski, J.A., Swinehart, J.M., Tschen, E.H., Tranik, R.J., 2002. A randomized clinical trial of 5% topical minoxidil versus 2% topical minoxidil and

placebo in the treatment of androgenetic alopecia in men. J. Am. Acad. Dermatol. 47, 377–385.

Šentjurc, M., Vrhovnik, K., Kristl, J., 1999. Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method. J. Control. Rel. 59, 87–97.

Socaciu, C., Jessel, R., Diehl, H.A., 2000. Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy. Chem. Phys. Lip. 106, 79–88.

Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M., 2000. Ethosomes-novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J. Control. Rel. 65, 403–418.