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Research paper

Liquid crystalline phases of monoolein and water for topical delivery of cyclosporin A: Characterization and study of in vitro and in vivo delivery

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Abstract

Reverse cubic and hexagonal phases of monoolein have been studied as drug delivery systems. The present study was aimed at investigating whether these systems enhance the cutaneous penetration of cyclosporin A (CysA) in vitro (using porcine ear skin) and in vivo (using hairless mice). Different mesophases were obtained depending on CysA concentration. CysA at 4% allowed the formation of reverse cubic and hexagonal phases in a temperature range of 25–40 °C. At 8%, CysA induced the formation of other phases, which might be due to an interaction between the polar groups of the peptide and monoolein. In vitro, the cubic phase increased the penetration of CysA in the stratum corneum (SC) and epidermis plus dermis ([E + D]) at 12 h post-application. The reverse hexagonal phase increased CysA penetration in [E + D] at 6 h and percutaneous delivery at 7.5 h post-application. In vivo, both liquid crystalline phases increased CysA skin penetration. Topical application of these systems, though, induced skin irritation after a 3-day exposure. These results demonstrate that liquid crystalline systems of monoolein are effective in optimizing the delivery of peptides to the skin. The skin irritation observed after topical application of cubic and hexagonal phases should be minimized for their safe use as topical delivery systems.

Keywords: Cubic phase; Reverse hexagonal phase; Monoolein; Skin penetration; Cyclosporin A

1. Introduction

Peptides and proteins have been increasingly investigated as therapeutic agents. Although they are usually systemically administered, their topical and transdermal administration is more advantageous for providing patient commodity, minimization of discomfort, and avoidance of first passage hepatic metabolism [1]. However, the penetration of drugs in the skin and their percutaneous delivery are limited by the barrier function of the highly organized structure of stratum corneum [2]. Several methods to improve cutaneous delivery can be used, including chemical penetration enhancers and more complex physical strategies, such as electroporation and iontophoresis [3–6]. In addition, drug delivery systems such as liposomes and other colloidal systems have been studied in an attempt to promote the cutaneous delivery of macromolecules [7,8].

Monoolein is a biodegradable polar lipid that has no marked toxic effects [9]. By promoting ceramide extraction and enhancement of lipid fluidity in the stratum corneum, monoolein is a penetration enhancer by itself [10]. It also swells in water, forming liquid crystalline phases that can be used as drug delivery systems [11,12]. Depending on

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water content and temperature, several phases can be formed, including reverse cubic and hexagonal phases. The reverse cubic phase of monoolein and water is formed at room temperature, and has been shown to accommodate and sustain the release of drugs with various physical chemical properties, including proteins and peptides [13–17]. The reverse hexagonal phase of monoolein and water is obtained only at high temperatures, unless a third non-polar component (e.g., triglycerides or oleic acid) is added to the system [18]. This phase also presents ability to sustain the delivery of incorporated compounds [11].

Cubic phases of monoolein and water have been shown to improve the topical/transdermal delivery of relatively small molecules such as nicotine, salbutamol, acyclovir, and aminolevulinic acid esters [19–21], but it remains to be determined whether this phase is capable of promoting the topical delivery of larger molecules, such as several peptides and proteins. Reverse hexagonal phases of monoolein have not been studied as a topical delivery system to date. In the present study, reverse cubic and hexagonal phases of monoolein (here referred to as cubic or hexagonal phase, for the sake of simplicity) were evaluated as topical delivery systems for a model peptide. We selected cyclosporin A (CysA) as the model peptide for two reasons. First, CysA is a cyclic and highly lipophilic peptide that presents extremely poor skin penetration, unless a chemical or physical strategy is used [6,22–26]. Second, this peptide is an immune-suppressant agent and has therapeutic potential in the treatment of skin inflammatory disorders [22,25].

2. Materials and methods

2.1. Materials

Commercial grade monoolein (Myverol 18-99, 98.1% monoglycerides) was obtained from Quest (Naarden, The Netherlands). The ability of Myverol 18-99 to form liquid crystalline phases has been shown to be remarkably similar to monoolein of analytical grade [27]. Oleic acid was supplied by Sigma (St. Louis, MO) and CysA by Boechel Co. (Hamburg, Germany). Acetonitrile and methanol were purchased from Ominsolve (Merck, Darmstadt, Germany).

2.2. Preparation of the formulations

To determine the optimum ratio of monoolein, water, and CysA to obtain cubic phase, formulations containing different amounts of these compounds (54–95% monoolein, 0.5–10% CysA, and 4.5–40% water, w/w/w) were prepared. Monoolein was melted (42 °C) and CysA was added under vortex stirring. Immediately thereafter, water pre-warmed to 42 °C was added, and the resulting formulation was allowed to rest in closed vials for 1 week at room temperature to reach equilibrium [14]. To determine the optimum ratio of monoolein, water, oleic acid, and CysA to obtain hexagonal phase, formulations were prepared as described above, except that oleic acid was added to monoolein immediately before CysA. The final concentration of oleic acid in the system was 5% (w/w).

2.3. System characterization and partial phase diagram

The equilibrated formulations of monoolein:CysA:water and monoolein:oleic acid:CysA:water were examined by visual inspection to verify sample homogeneity. They were also examined through a polarized light microscope equipped with a hotstage from 25 to 37 °C (Carl Zeiss, Oberkochen, Germany) to verify whether the liquid crystalline phases formed were anisotropic (characteristic of hexagonal and lamellar phases) or isotropic (characteristic of cubic phase). Under a polarized microscope, the lamellar phase displays a distinct woven structure and/or a mosaic or Maltese cross pattern, whereas the hexagonal phase discloses a fan like and angular texture [28]. On the other hand, the stiff transparent cubic phase is nonbirefringent and only a black background with no well-characterized texture should be displayed [28].

The cubic structure of the formulations composed of monoolein:water at 70:30 (w/w) and monoolein:CysA: water at 67:4:29 (w/w/w) was further investigated using small angle X-ray diffraction (SAXRD). The measurements were performed at the Brazilian Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil, using the D11A-SAXS beamline. The white photon beam was extracted from the ring through a high-vacuum path. After passing through a thin beryllium window, the beam was monochromatized and horizontally focused by a cylindrically bent and asymmetrically cut (1 1 1) silicon single crystal. The selected wavelength was 1.608 Å. The focus was located at the detection plane. The scattered intensities were collected by a one-dimension position sensitive detector located at 736.5 mm from the sample. An ionization detector monitored the intensity of the incident beam. The data were corrected by detector homogeneity, incident beam intensity, and sample absorption.

Formulations containing 4% of CysA were also examined in a temperature range of 25–40 °C. Because the cubic phase composed of monoolein:CysA:water at 67:4:29 (w/w/w) and the hexagonal phase composed of monoolein:oleic acid:CysA:water at 75:5:4:16 (w/w/w/w) presented no phase transition in a wide temperature range (25–40 °C; see Section 3 for details), they were chosen for spectroscopic and skin penetration experiments.

2.4. Spectroscopic experiments

To verify whether CysA interacts with the lipid component of the system, the formulations were analyzed by Fourier Transformed Infrared Spectroscopy using a Nicolet Protégé apparatus (mod.460; Nicolet Instrument Corporation, Madison, WI, USA, resolution of 2 cm^{-1}). The formulations studied were: cubic phase containing CysA (monoolein:CysA:water, 67:4:29, w/w/w) or not (monoolein:water, 70:30, w/w); hexagonal phase containing CysA

(monoolein:oleic acid:CysA:water, 75:5:4:16, w/w/w/w) or not (monoolein:oleic acid:water, 77:5:18, w/w/w); mixtures of monoolein:CysA (96:4 w/w) and monoolein:oleic acid:-CysA (91:5:4 w/w/w) prepared by simply mixing the components; and CysA. Each spectrum, except that of pure CysA, was obtained from a film of each formulation in a ZnSe cell. The spectrum of pure CysA was obtained from a KBr disk.

2.5. In vitro skin penetration and percutaneous delivery

The penetration of CysA in the skin and its percutaneous delivery were assessed in an in vitro model of porcine ear skin, as previously described [6]. Briefly, the skin from the outer surface of a freshly excised porcine ear was carefully dissected (making sure that the subcutaneous fat was maximally removed), stored at −20 °C, and used within a month. On the day of the experiment, the skin was thawed and mounted in a Franz diffusion cell (diffusion area of 1.77 cm^2; Hanson Instruments, Chatsworth, CA), with the stratum corneum facing the donor compartment (where the formulation was applied) and the dermis facing the receptor compartment. The latter compartment was filled with 100 mM phosphate buffer (pH 7.2) containing ethanol (10%). Ethanol increases drug solubility, thus allowing quantification of CysA in this solution. Previous studies have used receptor phases containing up to 33% of ethanol [24,26]. The receptor phase was under constant stirring and maintained at 37 ± 0.5 °C [6]. One hundred milligrams of CysA-bearing cubic (monoolein:CysA:water at 67:4:29, w/w/w) or hexagonal (monoolein:oleic acid:CysA:water at 75:5:4:16, w/w/w/w) phases was applied to the surface of the stratum corneum. Based on previous studies [22,23], a solution of CysA (4%, w/w) in olive oil was used as the control formulation.

At 3, 6, or 12 h post-application, skin surfaces were thoroughly washed with distilled water and wiped with a cotton swab to remove excess formulation. To separate the stratum corneum (SC) from the remaining epidermis (E) and dermis (D), skin sections were subjected to tape stripping. The skin was stripped with 15 pieces of adhesive tape, the first one was discarded, and the other tapes containing the SC were immersed in 5 mL methanol, vortex stirred for 2 min, and bath sonicated for 30 min. The methanolic phase was filtered using a 0.45 μm membrane, and the resulting filtrate assayed for CysA. The remaining [E + D] was cut in small pieces, vortex mixed for 2 min in 2 mL of methanol and bath sonicated for 30 min. The resulting mixture was then filtrated using 0.45 μm membranes, and CysA was assayed in the filtrate. The integrity of the liquid crystalline structure of the formulations in the donor compartment was confirmed after the experiments.

At 3, 6, 7.5, 9, or 12 h post-application, samples (500 μL) of the receptor phase were withdrawn. CysA was extracted from each sample using 3 mL chloroform. After the chloroform phase was evaporated, the residue was suspended in 50 μL of an acetonitrile:water solution (67:33, v/v), and CysA was assayed. The amounts of drug detected in SC and in [E + D] are indicatives of drug penetration in the skin, whereas the amount of drug in the receptor phase is indicative of its percutaneous delivery.

2.6. In vivo skin penetration

Hairless mice (males, 6 to 8 weeks old) were obtained from the colony of the Pharmacy School of Ribeirão Preto (University of Sao Paulo, Ribeirão Preto, SP, Brazil). They were housed separately at 24–26 °C, exposed to a daily 12:12-h light:dark cycle (lights on at 6 AM), and had free access to standard mice chow and tap water. To reduce the stress associated with the experimental procedure, the mice were handled daily for 1 week before experimentation. The protocols were in accordance with the guidelines of the University of Sao Paulo Animal Care and Use Committee.

At the day of the experiment, 100 mg of CysA-bearing cubic phase (monoolein:CysA:water at 67:4:29, w/w/w), hexagonal phase (monoolein:oleic acid:CysA:water at 75:5:4:16, w/w/w/w), or control formulation (CysA in olive oil at 4%, w/w) was applied on a limited area (∼2 cm^2) of the skin on the back of each mouse. At 6 h post-application, the animals were killed with an overdose of carbon dioxide, and the skin area where the formulations were applied was dissected. The skin was subjected to tape stripping (as described for the in vitro penetration experiment), and the amount of CysA in the SC and [E + D] was determined.

2.7. Evaluation of skin irritation

Histological changes in hairless mice skin were examined after treatment with cubic and hexagonal phases for 2 days to evaluate the potential of the formulations to cause skin irritation. One hundred milligrams of either formulation (cubic or hexagonal phase gels without CysA) was applied topically and non-occlusively on a limited area (∼2 cm^2) [29] of the skin on the back of each mouse once a day for 2 days. On the third day, the animals were killed with an overdose of carbon dioxide and the skin area where the formulations were applied was dissected, fixed by immersion in Bouin liquid at room temperature for 24 h, processed for inclusion in paraffin, sectioned with 6 μm of thickness, and stained with Masson tricromic. This regimen was chosen to simulate a short-term treatment. Skin sections were examined under conventional light microscopy (Carl Zeiss, Oberkochen, Germany) for epidermis thickening, edema, and infiltration of inflammatory cells in the dermis [29]. Epidermis thickness was measured using the AxiVision software (Carl Zeiss, Oberkochen, Germany). Skins of untreated animals and animals treated with saline were used as the controls.

2.8. Analytical methodology for CysA

CysA was assayed by HPLC using a Shimadzu equipment, which consisted of a Model LC10 AD solvent pump,

a Rheodyne injector, a 20 μL loop, a Model SPD-10A variable wavelength UV detector, a Model CTO-10A column oven, and a Model SCL-10A controller system. The separation was performed by a Lichrospher 100 RP-18 column (5 μm, Merck, Darmstadt, Germany), which was equipped with a RP-8 precolumn (Merck, Darmstadt, Germany) and equilibrated at 60 °C. A mobile phase of 67% acetonitrile and 33% water (flow rate of 1 mL/min) was used, and CysA was detected at 210 nm. Under these conditions, the retention time of CysA was 9.1 min. When methanolic solutions of CysA were injected, linearity was achieved over the concentration range between 0.15 and 500.00 μg/mL, presenting a correlation coefficient (r) of 0.999. Calibration curves of extracted CysA from aqueous solutions were used to assay CysA in the receptor phase of the diffusion cell. Phosphate buffered solutions were spiked with known amounts of CysA, and the drug was extracted from these solutions with chloroform (3 mL). The chloroform was evaporated, the residue was suspended in the mobile phase (50 μL), and the latter solution was assayed. In this assay, linearity was achieved for concentrations in the range of 20 ng/mL–2 μg/mL. In both assays, the error and the intra-day and inter-day variations were less than 10%. These values are considered adequate for an analytical method [30]. Using this HLPC procedure, unidentified peaks were not detected.

2.9. Statistical analyses

The results are reported as means ± SD. As in previous skin penetration studies [6], data were statistically analyzed using nonparametric tests. The Kruskal–Wallis test (followed by Dunns post hoc test) was used to compare more than two experimental groups. Values were considered significantly different when $p < 0.05$.

3. Results

3.1. System characterization and partial phase diagram

We first evaluated the effect of CysA on the phase behavior of monoolein and water systems. At room temperature, formulations of monoolein and water formed lamellar phase when water content was less than 14%, cubic phase when water content was between 15% and 40%, and cubic phase equilibrated with excess water (cubic phase + water) when water content was more than 40%. The presence of CysA in the formulations at concentrations up to 1% (w/w) did not lead to phase transformation (Fig. 1). However, when CysA concentration increased (2–6%), cubic phase was observed at a narrower range of water content, whereas lamellar and [cubic phase + water] were observed at a wider range of water content (Fig. 1). When CysA concentration in the system was 6%, lamellar phase, cubic phase, and [cubic phase + water] were observed when water content was <19%, 21–25%, and >28%, respectively. When drug concentration reached 8%, the cubic phase was no longer observed.

Because a CysA concentration of 4% allowed the formation of cubic phase, and because others have used similar concentrations for topical administration [31,32], formulations containing 4% of CysA were further studied. Based on the fact that topical formulations are usually subjected to temperature variations, we verified the effect of temperature (25–40 °C) on the phase behavior of monoolein:CysA:water systems. When water content was 18–35%, there was no phase transition at the temperature range studied (Fig. 2A).

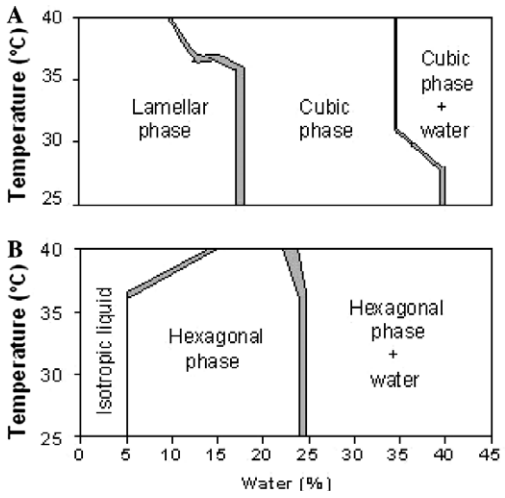

Fig. 2. Liquid crystalline structure of formulations containing CysA in the temperature range of 25–40 °C. Formulations composed of monoolein, water, and CysA (4%) are depicted in (A), formulations composed of monoolein, water, oleic acid (5%), and CysA (4%) are depicted in (B). Areas of uncertainty and/or phase transition are indicated in gray.

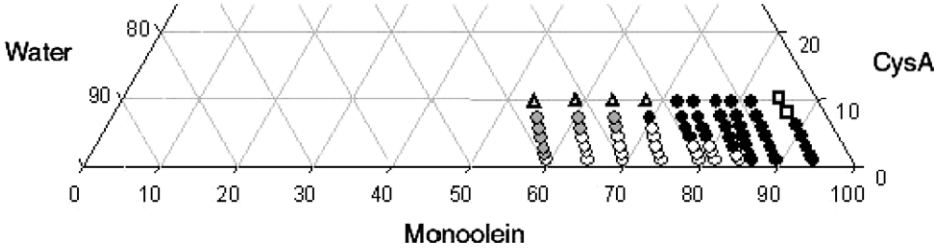

Fig. 1. Partial phase diagram of monoolein/water/CysA at 25 °C. Key: ●, lamellar phase; △, lamellar phase + water; ○, cubic phase; ◍, cubic phase + water; and □, isotropic liquid formulation.

The cubic structure of the formulation composed of monoolein:CysA:water at 67:4:29 (w/w/w) was further investigated by small angle X-ray diffraction and compared to the unloaded system (monoolein:water at 70:30, w/w/w), since polarized light microscopy is not sufficient to unambiguously differentiate between cubic phase and other isotropic systems. The X-ray diffractograms of the unloaded formulation and the formulation containing CysA at 4% displayed 7–10 diffraction lines, which were indexed, and the results demonstrated the cubic structure of the systems (Table 1). It is important to notice that the incorporation of CysA affects the structure of the system, since the G-type phase disappeared, but preserves the D-type cubic arrangement. This tendency of elimination of the G-type cubic phase was also observed in a system containing a small amount of CysA (0.6%), but to a lesser extent. Hence, a cubic phase system composed of monoolein:CysA:water at 67:4:29 (w/w/w) was selected for the spectroscopic and skin penetration experiments.

We also verified whether hexagonal phases of monoolein:oleic acid:water could be formed in the presence of CysA (4%) and oleic acid (5%). At 25 °C, reversed hexagonal phase was formed when the concentrations of CysA, oleic acid, monoolein, and water were 4%, 5%, 68–86%, and 5–23%, respectively (Fig. 2B). Temperature changes from 25 (ambient) to 40 °C (even above body temperature) did not result in alterations in the liquid crystalline structure of the formulations containing water from 15% to 22%. Thus, a hexagonal phase system composed of monoolein:oleic acid:CysA:water at 75:5:4:16 (w/w/w/w) was selected for the spectroscopic and skin penetration experiments.

3.2. Spectroscopic experiment

To evaluate a possible interaction between CysA and monoolein, we performed infrared spectroscopic analysis of cubic and hexagonal phases containing CysA or not.

The spectra obtained were compared to those of monoolein or CysA alone. The spectrum of CysA presented characteristic bands at 1636 cm^{-1} (C=O stretching vibration), 3325 cm^{-1} (NH stretching vibration), 3421 cm^{-1} (OH stretching vibration), and 2959 and 2872 cm^{-1} (CH stretching vibration bands). The spectrum of monoolein had characteristic bands at 1739 cm^{-1} (C=O stretching vibration from the ester group), 1178 cm^{-1} (C–O stretching vibration), 3409 cm^{-1} (OH stretching vibration broad band), and 2925 and 2854 cm^{-1} (CH stretching vibration bands). The spectrum of oleic acid showed a broad band at 3400–2400 cm^{-1} (OH absorption) and at 1711 cm^{-1} (carbonyl stretching band).

The spectra of cubic and hexagonal phases without CysA were very similar to the spectrum of the mixture of the constituents of the systems. Addition of CysA to these systems resulted in a shift in the absorption band of the carbonyl group of CysA (Fig. 3). For the cubic phase, the shift was from 1636 to 1628 cm^{-1}; for the hexagonal phase, it was from 1636 to 1630 cm^{-1}. A similar shift was also observed when CysA was mixed with monoolein. These data suggest the occurrence of an interaction between drug and monoolein involving the carbonyl group of CysA.

3.3. In vitro skin penetration and percutaneous delivery

Compared to the control formulation (olive oil), formulations of liquid crystalline phases significantly enhanced

Table 1
Small angle X-ray diffraction data for the formulations indexed as cubic phase

Sample	2θ (°)	d (Å)	Ratio (hkl)	Structure	Phase
Monoolein:water (70:30)	1.539	57.40	$1/\sqrt{2}$ (110)	Cubic–Pn3m	D type
	1.705	51.81	$1/\sqrt{3}$ (110)	Cubic–Ia3d	G type
	1.903	46.42	$1/\sqrt{3}$ (111)	Cubic–Pn3m	D type
	1.983	44.55	$1/\sqrt{4}$ (200)	Cubic–Ia3d	G type
	2.187	40.39	$1/\sqrt{4}$ (200)	Cubic–Pn3m	D type
	2.646	33.39	$1/\sqrt{7}$	Cubic–Ia3d	G type
	2.730	31.73	$1/\sqrt{6}$ (211)	Cubic–Pn3m	D type
	3.048	28.99	$1/\sqrt{8}$ (220)	Cubic–Ia3d	G type
	3.111	28.38	$1/\sqrt{8}$ (220)	Cubic–Pn3m	D type
	3.234	27.32	$1/\sqrt{10}$ (310)	Cubic–Ia3d	G type
Monoolein:CysA:water (67:4:29)	1.511	58.46	$1/\sqrt{2}$ (110)	Cubic–Pn3m	D type
	1.859	47.52	$1/\sqrt{3}$ (111)	Cubic–Pn3m	D type
	2.131	41.46	$1/\sqrt{4}$ (200)	Cubic–Pn3m	D type
	2.580	34.24*	$1/\sqrt{6}$ (211)	Cubic–Pn3m	D type
	2.965	29.80	$1/\sqrt{8}$ (220)	Cubic–Pn3m	D type
	3.152	28.03*	$1/\sqrt{9}$ (300, 221)	Cubic–Pn3m	D type
	3.330	26.53*	$1/\sqrt{10}$ (310)	Cubic–Pn3m	D type

Samples: unloaded cubic phase (monoolein:water at 70:30, w/w) and cubic phase containing CysA (monoolein:CysA:water at 67:4:29, w/w/w). *Lattice parameter values calculated with this peak present a discrepancy of 0.5 Å, considering the (110) peak as reference. Key: θ is the diffraction angle (in degree Celsius), d is the observing Bragg spacing, hkl denotes Miller indices, Pn3m and Ia3d denote the space group, D and G type phases denote Diamond and Giroid cubic phases, respectively.

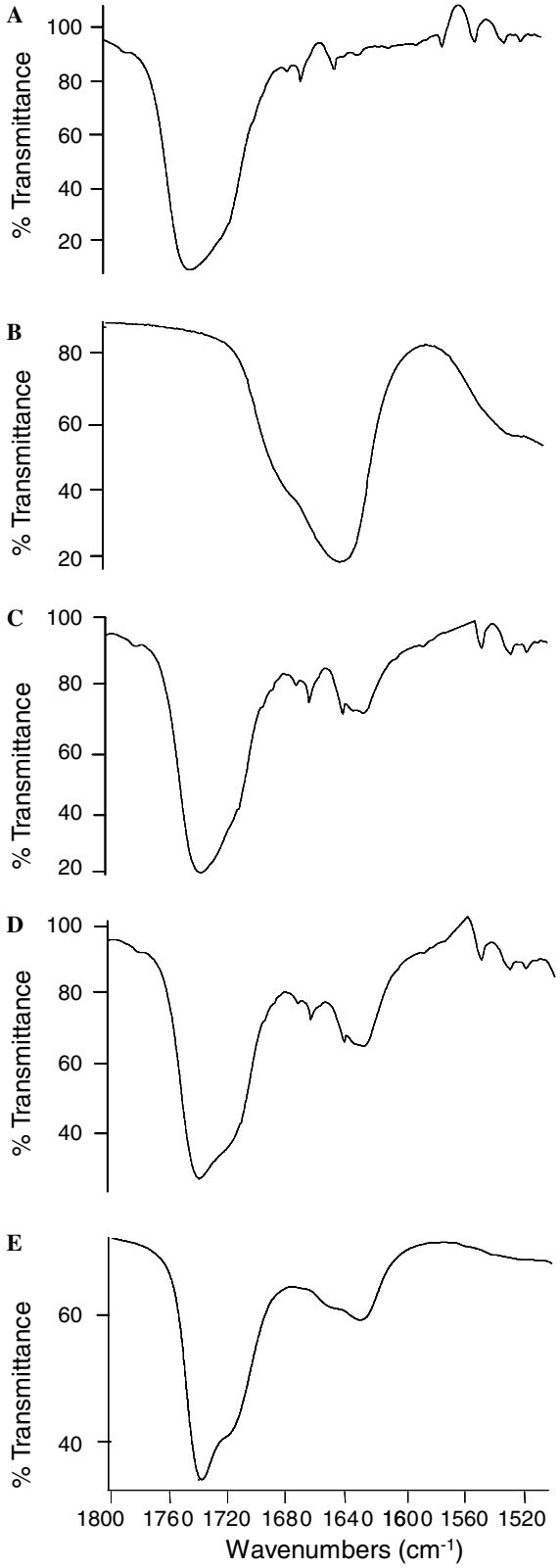

Fig. 3. Expanded infrared spectra of monoolein, CysA, and liquid crystalline phases containing the drug. Monoolein spectra are depicted in (A), CysA spectra are depicted in (B), mixture of CysA and monoolein is depicted in (C), cubic phase containing CysA (monoolein:CysA:water, 67:4:29, w/w/w) is depicted in (D), and hexagonal phase containing CysA (monoolein:oleic acid:CysA:water, 75:5:4:16, w/w/w/w) is depicted in (E). The number of replicates is two per system.

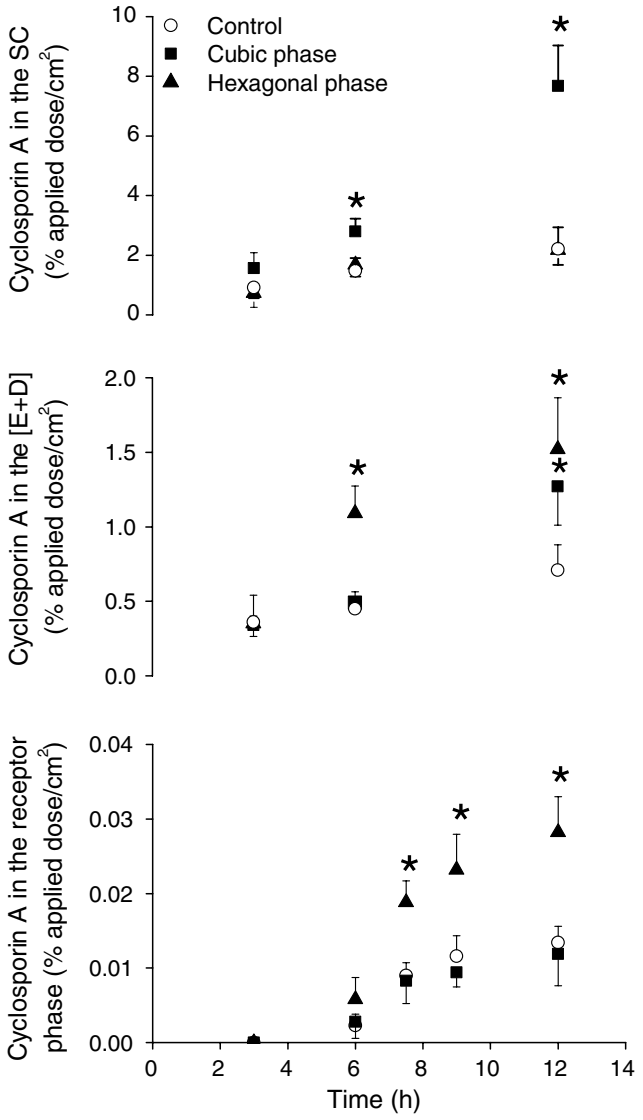

Fig. 4. Time-course of the in vitro skin penetration and percutaneous delivery of CysA incorporated in different formulations. The number of replicates is 6–8 per experimental group. *$p < 0.05$ compared to control formulation.

the skin penetration of CysA (Fig. 4). When CysA was incorporated in the cubic phase, its concentration in the SC was significantly enhanced at 6 ($p < 0.05$) and 12 h ($p < 0.01$) post-application, but its concentration in the [E + D] was enhanced only at 12 h ($p < 0.05$). The maximal concentrations of CysA in the SC and [E + D] were ~3- and 1.8-fold higher than those obtained when CysA was incorporated in the control formulation. When CysA was incorporated in the hexagonal phase, its concentration in the SC was not enhanced at any of the time points studied, but its concentration in the [E + D] was significantly enhanced at 6 ($p < 0.05$) and 12 h ($p < 0.01$) post-application. The maximal concentration of CysA in the [E + D] was ~2.5-fold higher than that obtained when CysA was incorporated in the control formulation.

The use of the hexagonal phase, but not the cubic phase, also enhanced the percutaneous delivery of CysA (Fig. 4).

CysA was barely detected in the receptor phase before 6 h post-application. From 6 to 12 h, increasing levels of CysA were measured in the receptor phase. When CysA was incorporated in the control formulation, $\sim 0.009 \pm 0.002\%$ and $0.013 \pm 0.002\%$ of the applied dose/cm^2 were detected in the receptor phase at 7.5 and 12 h post-application, respectively. Similar concentrations were obtained when CysA was incorporated in the cubic phase. However, when CysA was incorporated in the hexagonal phase, two-times higher concentrations were achieved ($p < 0.05$). Thus, whereas the cubic phase formulation favored retention of CysA in the skin in vitro, the hexagonal phase favored its penetration into deeper skin layers and its percutaneous delivery.

3.4. In vivo skin penetration

The penetration of CysA in the SC and [E + D] was evaluated using an in vivo mouse model. The incorporation of CysA in the liquid crystalline phases led to an increased skin penetration of the peptide in vivo at 6 h post-application (Fig. 5). When CysA was incorporated in the control formulation, we found $1.1 \pm 0.13\%$ of the applied dose/cm^2 in the SC and $0.61 \pm 0.09\%$ of the applied dose/cm^2 in the [E + D]. When CysA was incorporated in the cubic phase, its concentration in the SC was increased $\sim$2.5 times ($p < 0.01$), whereas its concentration in the [E + D] was increased $\sim$2 times ($p < 0.05$). Incorporation of CysA in the hexagonal phase resulted in a $\sim$1.8-fold increase ($p < 0.05$) in peptide concentration in the SC and a $\sim$3-fold increase ($p < 0.01$) in the concentration in the [E + D].

3.5. Evaluation of skin irritation

Photomicrographs illustrating skin tissues of untreated animals and of animals subjected to topical application of the liquid crystalline phases or saline are shown in Figs. 6A–D. Light microscopy indicated no histopathological alteration in the skin of animals treated with saline, as compared to the untreated animals. Topical application of the liquid crystalline systems induced some histopathological alterations. First, we observed an augment in the number of cells in the dermis, probably due to infiltration of inflammatory cells. Second, treatment with liquid crystalline phases significantly increased the thickness of the epidermis approximately 3.5 times ($p < 0.05$); there was no significant difference between the effect of the cubic and hexagonal phases. Fig. 6E shows the thickness of epidermis of animals subjected to topical treatment with saline, cubic, and hexagonal phases. These alterations suggest the occurrence of skin irritation due to the application of cubic or hexagonal phases. No indicator of a more severe reaction (i.e., edema or necrosis) was observed in the skin sections of animals treated with cubic or hexagonal phases.

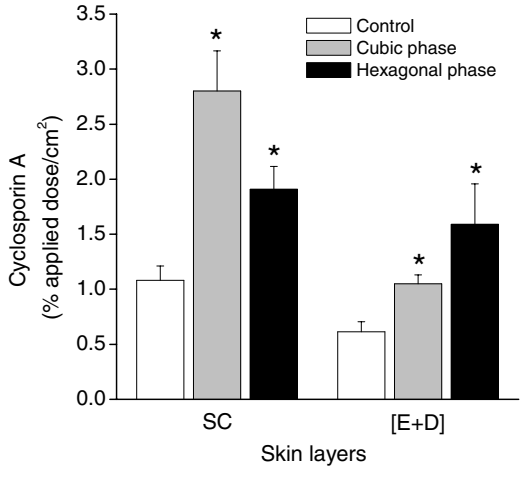

Fig. 5. In vivo skin penetration of CysA incorporated in different formulations. SC: stratum corneum, [E + D]: epidermis without stratum corneum + dermis. The number of replicates is 4–6 per experimental group. *$p < 0.05$ compared to control formulation.

4. Discussion

In the present study, we describe a potential application of cubic and hexagonal phases of monoolein and water as topical delivery systems. These systems have several advantages over other topical/transdermal systems. In addition to their ability to sustain the delivery of drugs, reverse cubic and hexagonal phases of monoolein present several characteristics that are attractive from the pharmaceutical point of view for a topical/transdermal system, such as gel-like texture, bioadhesive properties, and the nontoxic permeation enhancer monoolein as the structure-forming lipid [9,12,20]. Because parameters such as pH, temperature, and the presence of other compounds in the system can influence the packing parameter of the lipid and consequently the liquid crystalline phase formed [18,33], we first studied whether CysA affects the structure of the systems composed of monoolein/water. The addition of CysA from 2% to 6% induced phase transformation from cubic phase to lamellar phase when the water content was low and to [cubic phase + water] when the water content was high. The higher the CysA concentration in the system was, the narrower was the water range in which the cubic phase was obtained. At 8%, CysA precluded the formation of cubic phase.

The phase transformation from cubic to lamellar phase was unexpected. Due to its lipophilic nature, CysA was expected to incorporate within the hydrophobic core of the bilayer. Such incorporation would result in an enhancement of the apparent hydrophobic volume of the lipid and consequent transition to reverse hexagonal phase [33,34]. On the other hand, the observed transition to lamellar phase would be expected if an increase in the polar headgroup area occurred. It is therefore reasonable to suggest that other, presumably polar, types of interactions between the lipid and CysA may occur. The existence of polar interactions is also consistent with the fact that CysA induces the formation of [cubic phase + water] at lower water content.

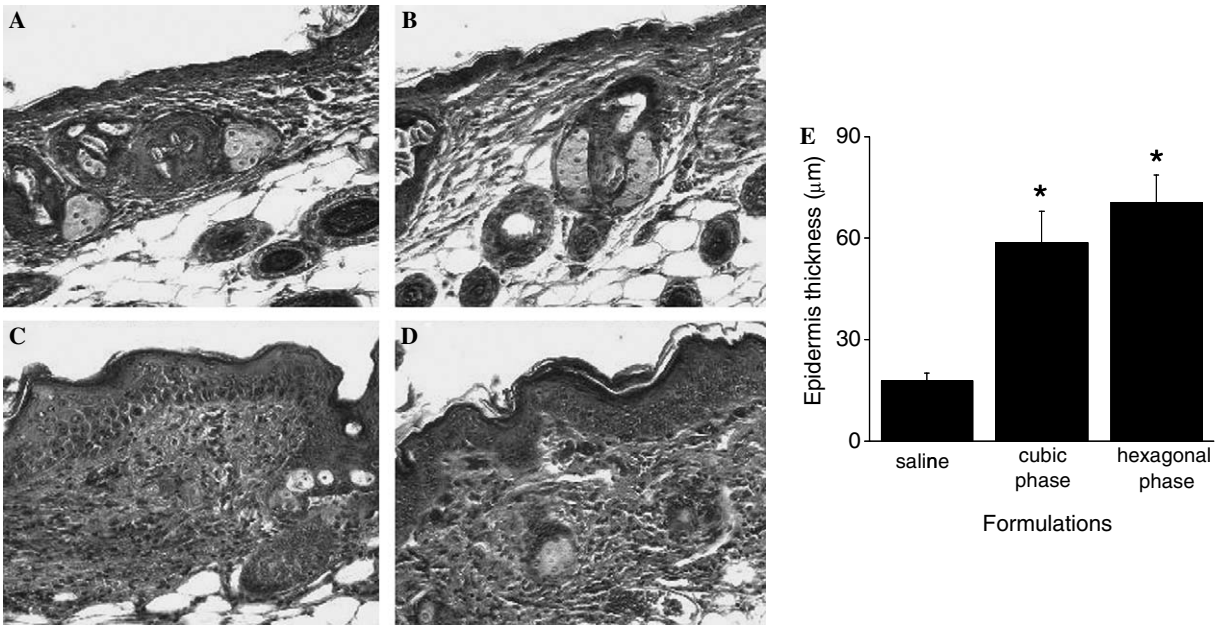

Fig. 6. Photomicrographs of skin sections of untreated animals (A), animals treated with saline (B), hexagonal phase (C), and cubic phase (D). Sections were visualized by conventional light microscopy through a 20× objective. The epidermal thickness of skin sections of animals treated with saline, cubic, and hexagonal phases is depicted in (E). $*p < 0.05$ compared to control formulation.

To assess interactions between monoolein and CysA, we performed infrared analysis. We observed a shift of the carbonyl absorption band of CysA to a lower frequency region of the infrared spectrum when CysA was mixed with monoolein or incorporated in the cubic and hexagonal phases. It is well known that the carbonyl group absorbs strongly in the range of 1850–1650 cm^{-1}, and that the absorbance range can be influenced by electron-withdrawing effects, resonance effects, and hydrogen bonding with other groups [35]. In secondary amides, as those present in the cyclosporin molecule, a resonance structure can be formed due to conjugation of the nitrogen-unpaired electrons with the carbonyl group. The interaction of this resonance structure with the polar headgroup of monoolein (probably the OH group) can explain the observed shift of the carbonyl band in the infrared spectrum. Such an interaction would result in the formation of strong hydrogen bonds between the groups involved and in a shift of the absorption band of CysA to a region of lower frequency.

The interaction of CysA with liquid crystalline phases of MO was also observed by Bonacucina et al. [36]. The authors observed that drug loading resulted in marked changes in rheological and dielectric responses of a MO-based system containing 10% (w/w) of water (lamellar phase). A less marked effect was observed for the system containing 22% of water and, in particular, for the systems containing 30% of water (cubic phase). Alterations in the liquid crystalline structure and system properties by drug loading might have implications on drug release, system stability, and interaction of the system with the site of action (12,33,36). Studying the influence of drug loading on system structure and properties is important to assure the obtainment of a suitable delivery system.

To evaluate whether liquid crystalline phases of monoolein can act as topical/transdermal delivery systems, we studied the skin penetration and percutaneous delivery of CysA (as a model peptide) incorporated in the cubic and hexagonal phases. We observed that incorporation of CysA in both formulations enhanced the in vitro penetration of the peptide in the skin, probably due to the action of monoolein (in combination with oleic acid in the case of hexagonal phase) as penetration enhancer [19]. Indeed, monoolein has been demonstrated to be released from liquid crystalline phases and to influence the buccal and skin permeation of the incorporated compounds [14,19]. In the present study, the cubic phase gel increased the penetration of CysA in the SC and [E + D], but did not influence the percutaneous delivery of the peptide compared with the control formulation. The hexagonal phase increased the penetration of CysA in the [E + D] as well as its percutaneous delivery. No enhancement of the peptide concentration in the SC was observed when the hexagonal phase was used, probably because this formulation increased the penetration of CysA into deeper skin layers and through the skin.

The cubic and hexagonal phases used in this study differ not only in the internal structure, but also in the composition, since oleic acid was added at 5% to the hexagonal phase but not to the cubic phase. Thus, the differences observed in the in vitro skin penetration of CysA might rely on the difference of constituents of both phases. High monoolein concentrations (up to 70% w/w) have been demonstrated to increase the skin concentration of CysA but had no influence on its percutaneous delivery [6]. The presence of oleic acid in the hexagonal phase might influence the skin permeability, and thus,

result in a higher CysA penetration in deeper skin layers and through this tissue. Oleic acid is a known penetration enhancer, and it has already been shown to increase the skin absorption of several drugs, including peptides [3,37]. A previous study showed that oleic acid was released from a system of cubic phase containing PEG 200 (5% or 10%) and increased the buccal permeation of the incorporated peptide [15].

In addition, parameters related to the structure of the delivery system can also influence the skin penetration of CysA. The release of incorporated drugs has been described as the rate-limiting step in transdermal delivery of at least small, non-peptidic compounds [38,39]. The release is known to be largely influenced by the structure of the delivery system, drug/system interactions, and system physical–chemical characteristics [38–41]. The same types of interactions between CysA and monoolein were observed in both cubic and hexagonal phases, thus, they should not contribute to the differences observed in the skin penetration of CysA. On the other hand, cubic and hexagonal phases present differences regarding the internal structure, viscosity, and drug release properties (including for CysA) [9,39,41], and these differences might influence CysA delivery to the skin. Last, skin penetration and percutaneous delivery of CysA might also be influenced by interactions of the different liquid crystalline systems with the stratum corneum [42].

The in vivo experiment revealed that incorporation of CysA in the cubic and hexagonal phases also results in an increase in the skin penetration of the peptide at 6 h post-application. There were a couple of differences in the results of the in vivo and in vitro experiments. In vivo but not in vitro, the hexagonal phase enhanced the penetration of CysA in the SC and the cubic phase enhanced the penetration of CysA in the [E + D] after 6 h. This might be related to the differences of skin models used. Mouse skin used in the in vivo experiment is more permeable than the porcine skin used in the in vitro experiment; thus, the skin penetration might be faster in the mouse [2]. Additionally, the lack of blood flow in the dermis of the porcine skin in vitro may artificially hinder the skin absorption of lipophilic compounds in this water-rich environment [2,43]. Even though the skin models used are different, the results obtained in vivo confirmed our in vitro observation that monoolein-based delivery systems enhance skin penetration of CysA.

Penetration enhancing effects are very often caused by structural alteration of the stratum corneum [44]. However, this effect can induce changes in the deeper skin layers depending on the penetration enhancer used and its concentration. Histopathological examination is an effective tool for the evaluation of penetration enhancers and their optimal concentration to be used to obtain the enhancing effect associated with minimal damage to the deeper skin layers [29]. Several effective permeation enhancers have been shown to induce some inflammatory effects on the skin in a concentration-dependent manner. Oleic acid and linolenic acid are examples of widely used permeation enhancers that induce some degree of skin irritation [29,44]. We observed some alterations, though not severe, in the skin sections of animals treated with cubic and hexagonal phases, which suggest the occurrence of mild skin irritation in hairless mice after a 3-day exposure. It is important to note that no edema or necrosis was observed in the skin. We also have to consider that skin of hairless mice is thinner and more permeable than human skin [2]. Thus, we can speculate that a more pronounced irritation might occur in these animals compared to humans. Other studies from our laboratory showed that the toxicity caused by subcutaneously injected monoolein formulations are reversible.

In conclusion, the present study shows that cubic and hexagonal phases are formed in the presence of CysA, and the skin penetration of this model peptide was significantly increased when it was incorporated in such systems. Cubic and hexagonal phases induced mild skin irritation, but no severe damage was observed after a 3-day exposure. Hence, efforts should be made to minimize the skin irritation caused by these phases, and in this way provide adequate formulations for clinical application. Strategies to reduce irritation may include altering the proportions of the components of the formulation, diluting and dispersing the liquid crystalline phase in excess water (to decrease the monoolein content), or replacing some components of the formulation.

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References

[1] M.R. Prausnitz, S. Mitragotri, R. Langer, Current status and future potential of transdermal drug delivery, Nat. Rev. 3 (2004) 115–124.
[2] K. Moser, K. Kriwet, A. Naik, Y.N. Kalia, R.H. Guy, Passive skin penetration enhancement and its quantification in vitro, Eur. J. Pharm. Bipharm. 82 (2001) 103–112.
[3] H. Smyth, G. Becket, S. Mehta, Effect of permeation enhancer pretreatment on the iontophoresis of luteinizing hormone releasing hormone (LHRH) through human epidermal membrane (HEM), J. Pharm. Sci. 91 (2002) 1296–1307.
[4] Y.N. Kalia, A. Naik, J. Garrison, R.H. Guy, Iontophoretic drug delivery, Adv. Drug Deliv. Rev. 56 (2004) 619–658.
[5] P. Karande, A. Jain, S. Mitragotri, Discovery of transdermal penetration enhancers by high-throughput screening, Nat. Biotech. 22 (2004) 192–197.

[6] L.B. Lopes, J.H. Collet, M.V.L.B. Bentley, Topical delivery of cyclosporin A: an in vitro study using monoolein as a penetration enhancer, Eur. J. Pharm. Biopharm. 60 (2005) 25–30.
[7] B.W. Barry, Breaching the skin's barrier to drugs, Nat. Biotech. 22 (2004) 165–167.
[8] B. Godin, E. Touitou, Mechanism of bacitracin permeation enhancement through the skin and cellular membranes from an ethosomal carrier, J. Control Rel. 94 (2004) 365–379.
[9] Ganem-Quintanar, D. Quintanar-Guerrero, P. Buri, Monoolein: a review of the pharmaceutical applications, Drug Dev. Ind. Pharm. 26 (2000) 809–820.
[10] T. Ogiso, I. Masahiro, T. Paku, Effect of various enhancers on transdermal penetration of indomethacin and urea and relationship between penetration parameters and enhancement factors, J. Pharm. Sci. 84 (1995) 482–488.
[11] T. Norling, L.P. Engström, K. Larsson, N. Krog, S.S. Nissen, Formulation of a drug delivery system based on a mixture of monoglycerides and triglycerides for use in the treatment of periodontal disease, J. Clin. Periodontol. 19 (1992) 687–692.
[12] J.C. Shah, Y. Sadhale, D.M. Chilukuri, Cubic phase as drug delivery systems, Adv. Drug Deliv. Rev. 47 (2001) 229–250.
[13] Y. Sadhale, J.C. Shah, Stabilization of insulin against agitation-induced aggregation, Int. J. Pharm. 191 (1999) 51–64.
[14] J. Lee, I.W. Kellaway, Buccal permeation of [D-Ala, D-Leu] enkephalin from liquid crystalline phases of glyceryl monooleate, Int. J. Pharm. 195 (2000) 35–38.
[15] J. Lee, I.W. Kellaway, Combined effect of oleic acid and polyethylene glycol 200 on buccal permeation of [D-Ala^2, D-Leu^5] enkephalin from a cubic phase of glyceryl monooleate, Int. J. Pharm. 204 (2000) 137–144.
[16] M.G. Lara, M.V.L.B. Bentley, J.H. Collett, In vitro drug release mechanism and drug loading studies of cubic phase gels, Int. J. Pharm. 293 (2005) 241–250.
[17] R.F. Turchiello, F.C.B. Vena, Ph. Maillard, C.S. Souza, M.V.L.B. Bentley, A.C. Tedesco, Cubic phase gel as a drug delivery system for topical application of 5-ALA, its ester derivatives and m-THPC in photodynamic therapy (PDT), J. Photochem. Photobiol. B 70 (2003) 1–6.
[18] J. Borné, T. Nylander, A. Khan, Phase behavior and aggregate formation for the aqueous monoolein system mixed with sodium oleate and oleic acid, Langmuir 17 (2001) 7742–7751.
[19] M.G. Carr, J. Corish, O.I. Corrigan, Drug delivery from a liquid crystalline base across Visking and human stratum corneum, Int. J. Pharm. 157 (1997) 35–42.
[20] L.S. Helledi, L. Schubert, Release kinetics of acyclovir from a suspension of acyclovir incorporated in a cubic phase delivery system, Drug Dev. Ind. Pharm. 27 (2001) 1073–1081.
[21] J. Bender, M.B. Ericson, N. Merclin, V. Iani, A. Rosen, S. Engstrom, J. Moan, Lipid cubic phases for improved topical drug delivery in photodynamic therapy, J. Control. Rel. 106 (2005) 350–360.
[22] J.I. Duncan, S.N. Payne, A.J. Winfield, A.D. Ormerod, A.W. Thomson, Enhanced percutaneous absorption of a novel topical cyclosporin A formulation and assessment of its immunosuppressive activity, Br. J. Dermatol. 123 (1990) 631–640.
[23] D.P. Wang, C.Y. Lin, D.L. Chu, L.C. Chang, Effect of various physical chemical properties on the transdermal delivery of cyclosporin through topical application, Drug Dev. Ind. Pharm. 23 (1997) 99–106.
[24] J. Guo, Q. Ping, G. Sun, C. Jiao, Lecithin vesicular carriers for transdermal delivery of cyclosporin A, Int. J. Pharm. 194 (2000) 201–207.
[25] J.B. Rothbard, S. Garlington, Q. Lin, T. Kirschberg, E. Kreider, P.L. Mcgrane, P.A. Wender, P.A. Khavari, Conjugation of arginineoligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation, Nat. Med. 6 (2000) 1253–1257.
[26] H.K. Choi, G.L. Flynn, G.L. Amidon, Percutaneous absorption and dermal delivery of cyclosporin A, J. Pharm. Sci. 84 (1995) 581–583.
[27] J. Clogston, J. Rathman, D. Tomasko, H. Walker, M. Caffrey, Phase behavior of monoacylglycerol (Myverol 18-99 K)/water system, Chem. Phys. Lipids 107 (2000) 191–230.
[28] F.B. Rosevear, The microscopy of the liquid crystalline neat and middle phases of soaps and synthetic detergents, J. Am. Oil. Chem. Soc. 31 (1954) 628–639.
[29] A. Sintov, A. Zeévi, R. Uzan, A. Nska, Influence of pharmaceutical gel vehicles containing oleic acid/sodium oleate combinations on hairless mouse skin, a histological evaluation, Eur. J. Pharm. Biopharm. 47 (1999) 229–303.
[30] R. Causon, Validation of chromatographic methods in biomedical analysis viewpoint and discussion, J. Chromatogr. B 689 (1997) 175–180.
[31] A. Gilhar, G. Winterstein, D.T. Golan, Topical cyclosporin in psoriasis, J. Am. Acad. Dermatol. 18 (1988) 378–379.
[32] M. Mizoguchi, K. Kawaguchi, Y. Ohshuga, Y. Ikari, A. Yanagawa, Y. Mizushima, Cyclosporin ointment for psoriasis and atopic dermatitis, Lancet 339 (1992) 1120.
[33] F. Caboi, G.S. Amico, P. Pitzalis, M. Monduzzi, T. Nylander, K. Larsson, Addition of hydrophilic and lipophilic compound of biological relevance to the monoolein/water system, I – Phase behavior, Chem. Phys. Lipids 109 (2001) 47–62.
[34] S. Engström, Drug delivery from cubic and other lipid–water phases, Lipid Technol. 2 (1990) 42–45.
[35] D.L. Pavia, G.M. Lampman, G.S. Kriz, Introduction to Spectroscopy: A Guide for Students of Organic Chemistry, Harcourt Brace College Publishers, Philadelphia, 1996, 511p.
[36] G. Bonacucina, G.F. Palmieri, D.Q.M. Craig, Rheological and dielectric characterization of monoolein/water mesophases in the presence of a peptide drug, J. Pharm. Sci. 94 (2005) 2452–2462.
[37] A.C. Williams, B.W. Barry, Penetration enhancers, Adv. Drug Deliv. Rev. 56 (2004) 603–618.
[38] N.H. Gabboun, N.M. Najib, H.G. Ibrahim, S. Assaf, Release of salicylic acid, diclofenac acid and diclofenac acid salts from isotropic and anisotropic nonionic surfactant systems across rat skin, Int. J. Pharm. 212 (2001) 73–80.
[39] K. Kriwet, C.C. Müller-Goymann, Diclofenac release from phospholipid drug systems and permeation through excised human stratum corneum, Int. J. Pharm. 125 (1995) 231–242.
[40] E. Farkas, R. Zelkó, Zs. Németh, J. Pálinkás, S. Marton, I. Rácz, The effect of liquid crystalline structure on chlorhexidine diacetate release, Int. J. Pharm. 193 (2000) 239–245.
[41] L.B. Lopes, M.V.L.B. Bentley, Influence of the liquid crystalline structure on cyclosporin A release, Br. J. Pharm. Sci. 39 (2003) 305.
[42] I. Stoye, K. Schröder, C.C. Müller-Goymann, Transformation of a liposomal dispersion containing ibuprofen lysinate and phospholipids into mixed micelles – physico-chemical characterization and influence on drug permeation through excised human stratum corneum, Eur. J. Pharm. Biopharm. 46 (1998) 197–200.
[43] W.G. Reinfarah, G.S. Hawkins, M.S. Kurtz, Percutaneous penetration and skin retention of topically applied compounds: an in vitro–in vivo study, J. Pharm. Sci. 80 (1991) 526–531.
[44] J.Y. Fang, T.L. Hwang, C.L. Fang, H.C. Chiu, In vitro and in vivo evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug, Int. J. Pharm. 255 (2003) 153–166.