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Bicellar systems for *in vitro* percutaneous absorption of diclofenac

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ABSTRACT

This work evaluates the effect of different bicellar systems on the percutaneous absorption of diclofenac diethylamine (DDEA) using two different approaches. In the first case, the drug was included in bicellar systems, which were applied on the skin and, in the second case, the skin was treated by applying bicellar systems without drug before to the application of a DDEA aqueous solution. The characterization of bicellar systems showed that the particle size decreased when DDEA was encapsulated. Percutaneous absorption studies demonstrated a lower penetration of DDEA when the drug was included in bicellar systems than when the drug was applied in an aqueous solution. This effect was possibly due to a certain rigidity of the bicellar systems caused by the incorporation of DDEA. The absorption of DDEA on skin pretreated with bicelles increased compared to the absorption of DDEA on intact skin. Bicelles without DDEA could cause certain disorganization of the SC barrier function, thereby facilitating the percutaneous penetration of DDEA subsequently applied. Thus, depending on their physicochemical parameters and on the application conditions, these systems have potential enhancement or retardant effects on percutaneous absorption that result in an interesting strategy, which may be used in future drug delivery applications.

1. Introduction

Over the past few decades, there has been wide interest in exploring new techniques to modulate drug absorption through the skin (Barry, 2001; Williams, 2003; Honeywell-Nguyen and Bouwstra, 2005). The first lipid vesicles studied for skin delivery were liposomes (Mezei and Gulasekharam, 1980). Over the past 15 years, a new class of lipid vesicles has been developed, the highly deformable liposomes, termed transfersomes (El Maghraby et al., 2001, 1999; Qiu et al., 2008; Trotta et al., 2002, 2004). Other lipid carriers recently developed are ethosomes (Ainbinder and Touitou, 2005; Paolino et al., 2005; Touitou et al., 2000), solid lipid nanoparticles (Fang et al., 2008; Puglia et al., 2008) and micelle-based surfactants (Spernath et al., 2008). These studies on skin delivery point to the need to obtain vehicles of appropriate sizes, high stability and biocompatibility. Bicelles are bilayered aggregates with a discoidal shape composed of long- and short-chain phospholipids. Long-chain lipids commonly used are dimyristoyl and dipalmitoyl phosphatidylcholine (DMPC and DPPC) and the short-chain lipid most frequently used is dihexanoyl phosphatidylcholine (DHPC) (Visscher et al., 2006). The long-chain phospholipids of bicelles form a bilayer section that is surrounded by a rim of short-chain phospholipids (DHPC) (Vold and Prosser, 1996). These systems have the propensity to align in magnetic fields; in fact, their use is mainly based on this property (Whiles et al., 2002). Thus, bicelles are used to orient membrane proteins that can be inserted in the bilayer structure and also to study the superficial interactions between proteins and the phospholipids bilayer. Considering the structure, composition and nanodimensions of these systems, their use as delivery systems for topical applications may be interesting. The use of bicellar systems for skin purposes has been explored and the results obtained indicate that depending on their composition, these systems are able to work in two ways: as permeabilizing agents of the skin or as reinforcing agents of the lipid structures present in the intercellular domains of the outermost layers of the skin (Barbosa-Barros et al., 2008a,b).

In vitro percutaneous absorption studies are a good way to identify how far a given drug penetrates into the skin. These *in vitro* studies with animal or human skin membranes are an elegant tool for obtaining data on the passage of test substances through the skin as well as on its distribution over the different cutaneous compartments (Elias and Feingold, 2006; OECD, 2004; Schaefer and Redermeier, 1996). Also, these studies permit the evaluation of the effectiveness of some vehicles and enhancers (Gwak and Chun, 2002).

Diclofenac is a potent non-steroidal anti-inflammatory drug (NSAID) with analgesic effects. Diclofenac may cause side effects, such as gastrointestinal disorders when it is administered by the oral route and cutaneous lesions if it is administered by an intramuscular injection (Galer et al., 2000). Thus, the identification of strategies to reduce toxicity and to increase the pharmacological effect of the NSAID may be highly relevant.

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This work seeks to analyze the influence of different vehicles based on bicellar systems on the percutaneous absorption of diclofenac diethylamine (DDEA). To this end, some physicochemical aspects of the nanostructures were investigated. Also, the effective location of the drug in the skin compartments, as a function of the different bicellar systems used, was evaluated. Our results show that these new systems should be considered as appropriate vehicles for topical applications.

2. Materials and methods

2.1. Chemicals

DDEA was supplied by Novartis (Basel, Switzerland). Dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC) and dihexanoyl phosphatidylcholine (DHPC) were supplied by Avanti Polar Lipids (Alabaster, USA). Methanol (HPLC Grade), sodium dihydrogen phosphate monohydrate and orthophosphoric acid 85% were obtained from Merck (Darmstadt, Germany). Purified water was obtained by an ultra-pure water system, Milli-Q plus 185 (Millipore, Bedford, USA).

2.2. Preparation of bicellar systems

Bicellar systems were formed with DMPC or DPPC as long-chain phospholipids and DHPC as a short-chain phospholipid. In Fig. 1 a general schema of the formation of bicellar systems, with and without DDEA, is shown.

For the preparation of the bicellar systems, an appropriate amount of DMPC or DPPC was weighed and mixed with a DHPC chloroform solution to get DMPC/DHPC or DPPC/DHPC in the molar ratio 2:1. This molar ratio was chosen to ensure the small size of the bicelles, which is more appropriate for skin purposes. The chloroform was eliminated by rotaevaporating the solvent until a lipid film was obtained. Then, this lipid film was hydrated to reach 20% (w/v) of total lipid concentration and sonicated until a transparent solution was obtained. To obtain bicellar systems containing diclofenac, the procedure was the same, but the lipid film was hydrated with an aqueous solution of DDEA 1.16% (w/v) (see Fig. 2). This concentration was chosen as normally this drug is topically administered in the form of a 1.16% gel.

2.3. Characterization of the bicellar systems

2.3.1. Dynamic light scattering technique

The hydrodynamic (HD) diameter of the bicellar systems was measured using the Zetasizer nano ZS90 (Malvern Instruments, UK). This apparatus employs the dynamic light scattering (DLS) technique to determine particle sizes between 1 nm and 3 μm. DLS measures the Brownian motion of the particles and correlates this to particle size (Probstein, 1994).

2.3.2. Cryo-transmission electron microscopy

Bicellar systems of DPPC/DHPC with and without DDEA were visualized by Cryo-transmission electron microscopy (Cryo-TEM). Vitrified specimens were prepared using a Vitrobot (FEI Company, Eindhoven, Netherlands). 5–10 μL of sample was placed onto a glow-discharged holey carbon grid. After, the grid was blotted with filter paper, leaving thin sample films spanning the grid holes. The blotted samples were vitrified by plunging the grid into liquid ethane at its freezing point (−196 °C) and stored under liquid nitrogen (LN_2) prior to examination in the microscope (Honeywell-Nguyen et al., 2002). The vitreous sample films were transferred to a microscope Tecnai F20 (FEI Company, Eindhoven, Netherlands) using a Gatan cryotransfert (Barcelona, Spain) cooled with LN_2 to temperature between −170 and −175 °C. The visualization was taken at 200 kV and using low-dose imaging conditions.

2.4. HPLC analysis of diclofenac

The quantitative determination of DDEA was performed by HPLC using a Hitachi LaChrom Elite equipment (Darmstadt, Germany). The apparatus consists of an L-2130 pump, L-2200 autosampler and an L-2400 UV detector. The system was operated from the software Merck EZChrom Elite v3.1.3. Then 20 μL of injection volume were eluted in a Lichrocart 250-4/Lichrosorb RP-18 (5 μm)

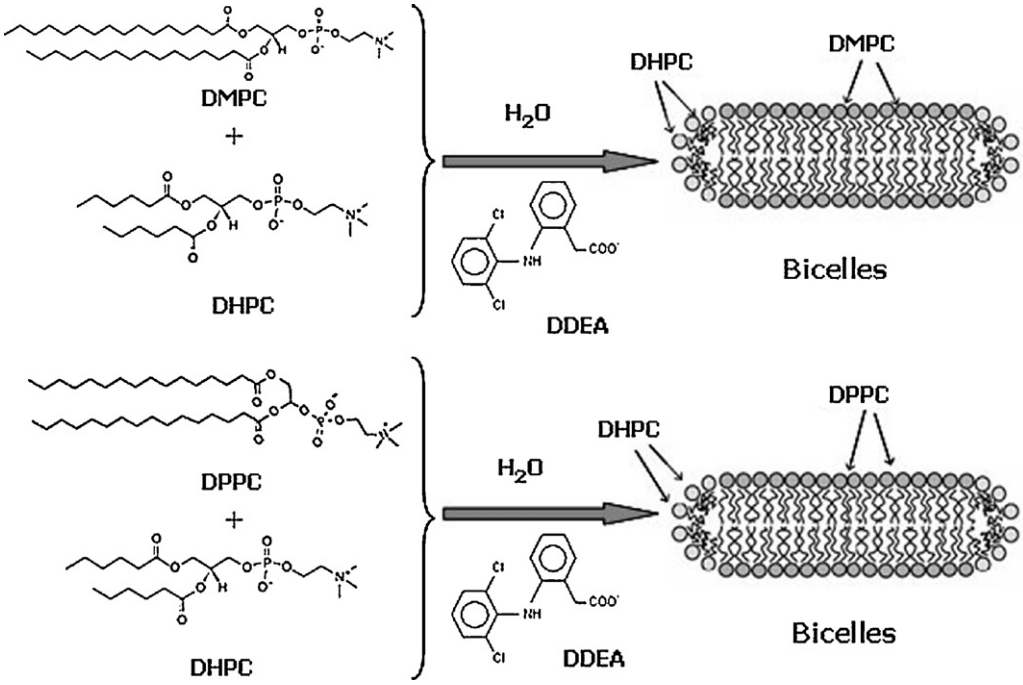

Fig. 1. Schema of the bicellar systems formation.

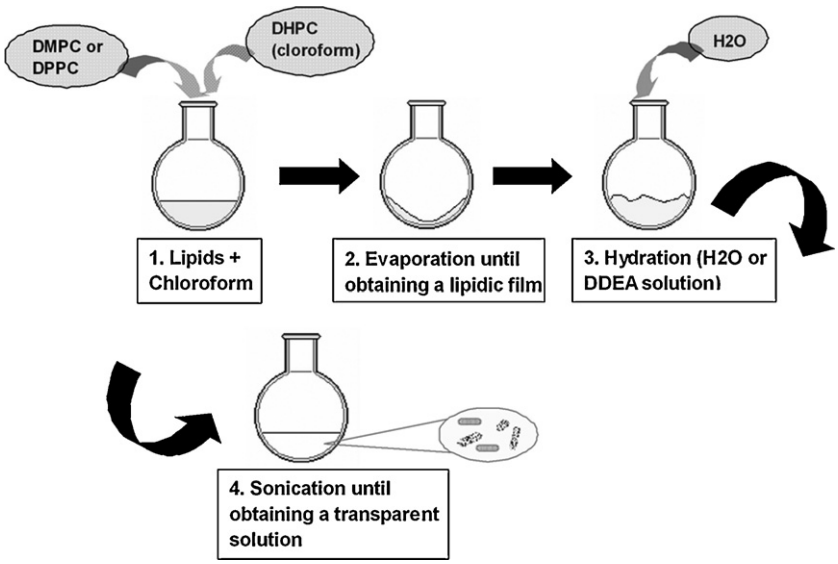

Fig. 2. Preparation of bicellar systems.

column (Merck, Germany) at room temperature. DDEA was monitored by UV detection at 254 nm. A mobile phase consisting of a 66% methanol and 34% phosphate buffer (pH 2.5) was used at a flow rate of 1 mL/min. The area under the peak was used to calculate the concentration of DDEA using external standards that showed linearity over the concentration range of 0.33–83 μg/mL. The intraday and interday variations of the method were less than 2%.

2.5. In vitro percutaneous absorption studies

For this study pig skin from the unboiled back of Landrace large white pigs weighing between 30 and 40 kg was used. The pig skin was provided by the Clínic Hospital of Barcelona, Spain. The bristles were removed carefully with an animal clipper and then the skin was washed with tap water. The excised skin was dermatomed to 500 ± 50 μm thickness (Dermatome GA630, Aesculap, Tuttlingen, Germany). Discs of the dermatomed skin were obtained with an iron punch (2.5 cm inner diameter) and fitted into Franz type diffusion cells. The skin discs were stored at −20 °C until use. One hour prior to the diffusion experiments, the skin was thawed at room temperature.

Franz cells (Lara-Spiral, Courtenon, France) consisted of an upper donor compartment and a receptor chamber (3 mL of volume). These two parts were separated by the skin biopsy, leaving an exposed surface area of 1.86 cm^2. A magnetic stirring bar was introduced into the receptor chamber. The skin disc was mounted with the SC side up in the Franz cell. The receptor chamber was filled with a receptor fluid (RF) which was PBS (pH 7.4) in distilled water, containing 1% of bovine serum albumin and 0.04% gentamicin sulphate, this was stirred continuously.

Franz cells were kept at 37 ± 1 °C by means of a circulating water bath (Julabo Labortechnik GmbH, Germany) to ensure that the surface skin was maintained at 32 ± 1 °C. The integrity of each skin sample was checked by determining the transepidermal water loss (TEWL) using a Tewameter TM210 (Courage-Khazaka, Köln, Germany). The diffusion experiment was initiated by applying to the entire surface, delimited by the upper cell, 10 μL of each of the following solutions: aqueous solution of DDEA, DMPC/DHPC bicelles with DDEA or DPPC/DHPC bicelles with DDEA. A control cell was also used (only with the application of 10 μL of water). After the exposure time (24 h), the test formulation remaining on the skin surface was removed with a specific wash: first with 0.5 mL of sodium lauryl ether sulphate solution (at 0.5%, w/v) and then twice (2×0.5 mL) with distilled water. After that, the skin surface was dried with a cotton swab. Water aliquots, all tips of the micropipette, all cotton swabs as well as the top of the cell were pooled, constituting the fraction of the active compound remaining in the surface. Then, the receptor fluid was removed from the receptor compartment and brought up to 5 mL in a volumetric flask. The SC of the treated skin area was removed by 8 successive tape-strippings using adhesive tape (D-Squame®, CuDerm Inc., Dallas, USA). After that, the viable epidermis was separated from the dermis after heating the skin at 80 °C for a few seconds.

The amount of DDEA in the different layers and in the washing solution was extracted with a solution of methanol: water (50:50) for 20 h. Then, samples were shacked for 30 min at room temperature and sonicated for 15 min. Before the analytical determination by HPLC, the samples were filtered through a 0.45 μm Acrodisc filter (Pall Gelman Sciences, Northampton, UK).

2.6. Pretreatment of the skin with DMPC/DHPC and DPPC/DHPC bicelles

To evaluate the effect of the pretreatment of skin with bicelles in the subsequent percutaneous absorption of DDEA, 10 μL of bicelles (without diclofenac) were topically applied for 1 h and after that a careful aqueous washing of the skin surface was carried out. This process was repeated four times. Then, an *in vitro* percutaneous absorption test of an aqueous solution of DDEA (1.16%, w/v) was performed as described in Section 2.5.

2.7. Statistical analysis

Each value is expressed as the mean ± S.D. for six determinations. For group comparisons, analysis of variance (ANOVA) with a one-way layout was applied. The software used was the STATGRAPHICS plus 5. Significant differences in the mean values were evaluated by the Student's unpaired *t*-test. A p value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Characterization of bicellar systems

The characterization of the bicellar systems have been carried out by two different techniques: DLS and Cryo-TEM.

Table 1
Particle size of the bicellar systems investigated, measured with Zetasizer nano ZS90 at 37 °C.

	Diameter (nm)[a]	%Vol
DMPC/DHPC	14.0 ± 0.2	99.8
DPPC/DHPC	15.2 ± 0.3	99.9
DMPC/DHPC/DDEA	2.4 ± 0.1	99.8
DPPC/DHPC/DDEA	2.8 ± 0.1	100

[a] Mean ± S.D.

The results obtained on the particle size of the bicellar systems investigated by DLS are indicated in Table 1. It is appreciated that similar size values were detected for both bicellar systems not including DDEA, with diameters in the range of 14–15 nm. Almost 100% of particles analyzed by volume were in this size range. The incorporation of DDEA in the bicellar systems led to a drastic decrease in the particle size for both systems (2.4 and 2.8 nm). These particle sizes are in the range of the formation of bicellar systems. It is known that the minimal size of vesicles is 20 nm. (Cornell et al., 1982). The size decrease by effect of DDEA can be understood considering that surface-active drugs, as non-steroidal anti-inflammatory compounds, are reported to self-associate and bind membranes causing partial disruption and solubilization. Several authors have described this behavior for diclofenac and other drugs (Lopes et al., 2004; Kriwet and Müller-Goymann, 1994; Lopes et al., 2006; Rades and Müller-Goymann, 1997; Schreier et al., 2000; Schutze and Muller-Goymann, 1998).

DDEA could have a similar effect as DHPC in the discoidal structure of the bicellar systems, showing a tendency to locate at the edges of the lamellar structure. This fact would induce a decrease in the molar ratio between molecules in the bilayer and in the edges, and, hence, the size would decrease, as indeed occurred in our experiments. Everything seems to indicate that the discoidal morphology of the bicelles is reduced by the effect of DDEA taking on a spherical structure similar to that described for mixed micelles (Schutze and Muller-Goymann, 1998).

The mechanism of DDEA incorporation in the bicelles is probably similar to that published by Lopes et al (Lopes et al., 2004) when this active principle was encapsulated in soya phosphatidylcholine (PC) liposomes. The amphiphilic nature of diclofenac would permit its incorporation into the lipid bilayer (Lopes et al., 2004). This incorporation would be possible given the hydrophobicity of the diclophenil ring of the drug, which would be oriented toward the hydrophobic core of the bilayers.

Fig. 3 shows Cryo-TEM micrographs of DPPC/DHPC bicellar systems with (Fig. 3a) and without (Fig. 3b) DDEA. In Fig. 3a little structures with sizes smaller than 5 nm are observed in agreement with data reported by DLS experiments (Table 1). In the case of the bicellar systems of DPPC/DHPC without DDEA (Fig. 3b), the micrograph shows discoidal bicelles in edge-on (white arrow) and face-on dispositions (black arrow). Micrographs of DMPC/DHPC bicellar systems showed very similar structures (data not shown)

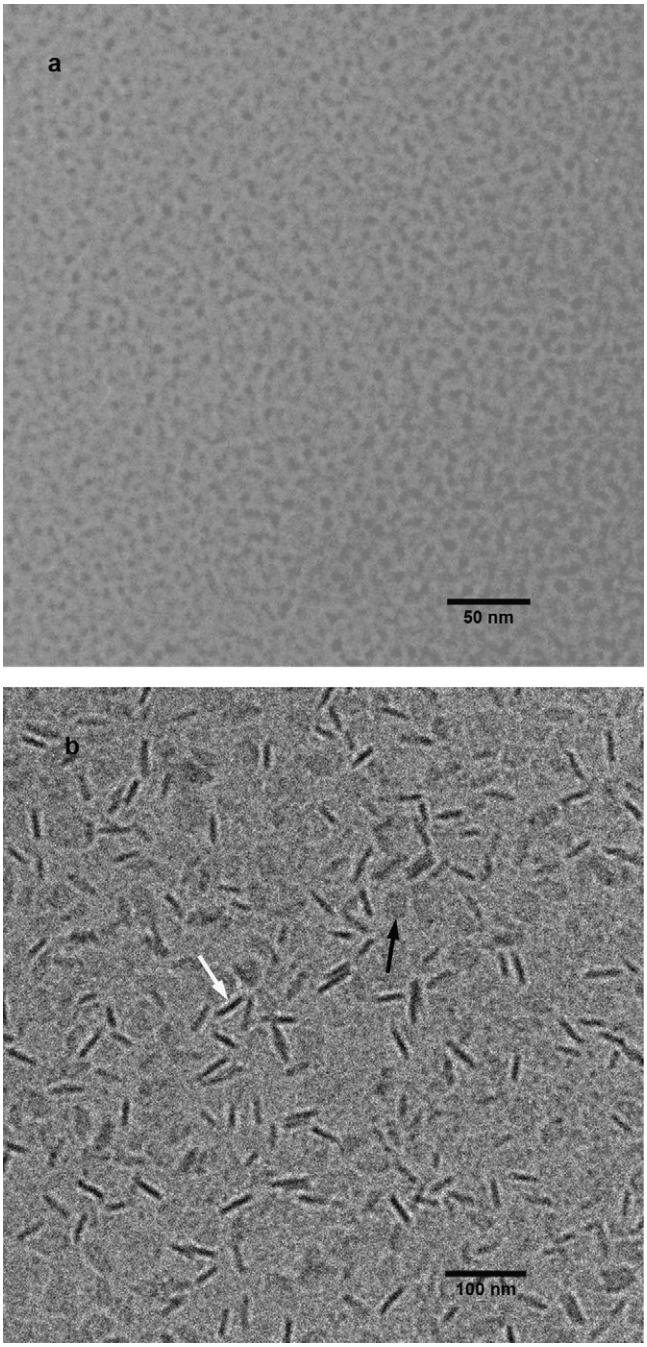

Fig. 3. Cryo-TEM micrographs of DPPC/DHPC bicellar systems with (a) and without (b) DDEA.

3.2. Bicelles as a drug delivery system

Systems formed by DMPC/DHPC or DPPC/DHPC were able to incorporate 1.16% DDEA in a similar way to other colloidal drug carrier systems such as liposome, microemulsions, mixed micelles, etc. (Boinpally et al., 2003; Kriwet and Müller-Goymann, 1996; Kweon et al., 2004; Lopes et al., 2004; Parsaee et al., 2002; Kriwet and Müller-Goymann, 1994; Lopes et al., 2006; Rades and Müller-Goymann, 1997; Schreier et al., 2000; Schutze and Muller-Goymann, 1998). The bicellar systems with DDEA showed a transparent appearance without phase separation and/or precipitates. These systems remained stable for at least 1 week and exhibited a gel aspect, which facilitated its application on the skin compared to the application of an aqueous solution of DDEA (much more fluid).

The percutaneous absorption profiles of diclofenac vehiculized in the mentioned bicelles compared with DDEA in an aqueous solution (1.16%, w/v) is shown in Fig. 4. The results are expressed as a percentage of the applied dose on the skin. As it can be shown, most DDEA remained in the skin surface. However, it is interesting to note that among all the skin layers, a higher percentage of DDEA was detected in the SC. This behavior is noted especially when the drug was applied in an aqueous solution. The inclusion of DDEA, in both types of bicelles, decreases the percutaneous absorption of the drug compared to that of an aqueous solution of diclofenac.

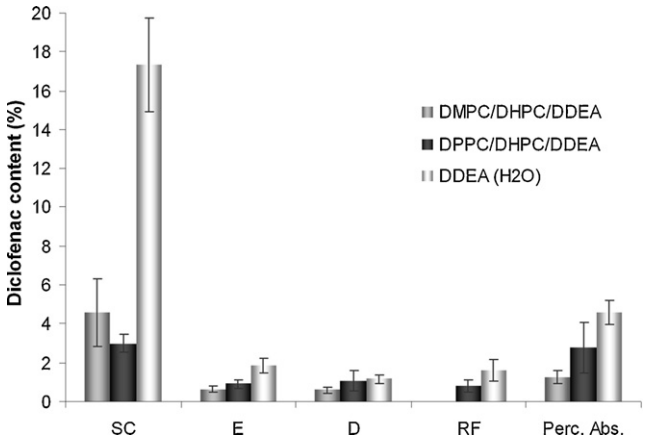

Fig. 4. Percutaneous absorption profiles of all the diclofenac formulations studied (mean values ± SD, $n = 6$). Distribution in the different layers of the skin: stratum corneum (SC), epidermis (E), dermis (D), receptor fluid (RF) and total percutaneous absorption (Perc. Abs.).

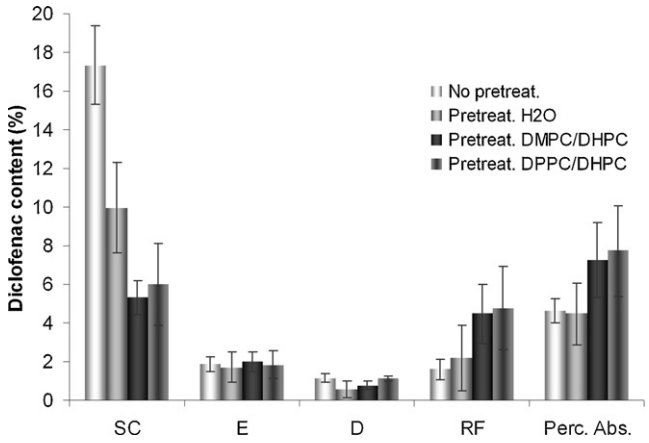

Fig. 5. Percutaneous absorption profiles of diclofenac with skin pretreated with bicellar systems or water (blank) (mean values ± SD, $n = 6$). Distribution in the different layers of the skin: stratum corneum (SC), epidermis (E), dermis (D), receptor fluid (RF) and total percutaneous absorption (Perc. Abs.).

This finding suggests a retarder effect of the percutaneous absorption when the drug is included in bicellar systems. This effect would be interesting to be applied in drugs which have a too fast percutaneous absorption, as it is the case of fentanyl, to prevent an overdose of the drug (Frölich et al., 2001).

It is obvious that the use of both kinds of bicellar systems prevents the passage of the drug to the deeper layers of the skin. This inhibitory effect on skin penetration was more marked for DMPC/DHPC bicelles. For these bicelles, Fig. 4 shows a higher value of DDEA in SC and an absence of drug in the receptor fluid. The results as global percentage of percutaneous absorption (considering the amount of DDEA detected in epidermis (E), dermis (D) plus receptor fluid (RF)) show the following ranking: aqueous solution DDEA ($4.61 \pm 0.62\%$) > DPPC/DHPC bicelles with DDEA ($2.78 \pm 1.62\%$) > DMPC/DHPC bicelles with DDEA ($1.25 \pm 0.33\%$). The significant difference ($p < 0.05$) of skin penetration detected between DPPC and DMPC bicelles could be due to the different transition temperature (T_m) of these two phospholipids. The DPPC at the experimental temperature is in a gel phase, like lipids of the SC (T_m about 60 °C) (Golden et al., 1987); this fact could facilitate the mix between lipids from the SC and from the bicelles promoting skin penetration, with respect to the DMPC bicelles. On the other hand, DMPC at the experimental temperature exhibits a liquid crystalline phase (T_m of DMPC 23 °C) (Lewis et al., 1987). Therefore, the DMPC has a different phase than the lipids from the SC and, as a consequence, the skin penetration could be more difficult.

Due to the small particle size of the lipid systems formed after the encapsulation of DDEA, one might expect that these systems could penetrate more easily through the skin. But there are different factors that are involved in the enhancer effect of a vehicle. Some of these factors are the possible disruption of the organization of the intercellular lipids of the SC, the affinity of the drug to the vehicle and the rigidity of the lipid structure of the vehicle (Gwak and Chun, 2002; Thong et al., 2007). The incorporation of DDEA in a bicellar system may cause a certain rigidity in the bicelles. In fact, the drug is not simply dissolved in the lipophilic region of the phospholipids, but is incorporated in the bilayer lined up with the phospholipids and the diclofenac's carboxyl groups increase the rigidity of the head groups of the phospholipids (Ferreira et al., 2005; Seddon et al., 2009). This possible rigidity would hinder the penetration of DDEA through the skin (Kriwet and Müller-Goymann, 1996; Kriwet and Müller-Goymann, 1994). Also, another possible reason for this low penetration could be related to the difficulty of the DDEA to diffuse out of the bicellar systems.

3.3. Bicelles as enhancers of skin penetration

Although the bicelles with DDEA inhibit the drug penetration, *in vivo* studies of Barbosa-Barros et al. (2008c) showed an increase of TEWL after a consecutive application of phospholipids bicelles. The effect of bicelles on the barrier function and their possible enhancer effect on the *in vitro* percutaneous absorption of DDEA were investigated. To this end, we performed a pretreatment of the skin discs with bicellar systems of DMPC/DHPC and DPPC/DHPC followed by a topical application of a DDEA aqueous solution. Also, to discard the possible influence of the water contained in the bicellar systems, some skin discs were pretreated with water (blank).

Fig. 5 shows the percutaneous absorption of DDEA using skin pretreated with bicelles, expressed in percentage of applied dose and using the experimental conditions described earlier.

In general terms, the amount of DDEA detected in the SC was higher in skin not pretreated with bicelles than in pretreated samples. In addition, the global results obtained on skin penetration show that a pretreatment of the skin with bicelles promotes the percutaneous absorption of diclofenac. There were no significant differences ($p < 0.05$) in the percentage of percutaneous absorption between the treatment with DMPC/DHPC or DPPC/DHPC bicelles. These results have been obtained despite the fact that microscopy studies have shown that each of these systems change, in different ways, the microstructure of the SC. DMPC/DHPC bicelles did not affect SC lipid microstructure (Barbosa-Barros et al., 2008a) and DPPC/DHPC systems seem to penetrate inside the skin SC and grow forming vesicles (Barbosa-Barros et al., 2008c). In future works it would be interesting to study the eventual histological changes detected not only in the SC but also in whole structure of skin treated with bicelles.

The enhancer effect of the bicellar systems on the percutaneous absorption of diclofenac could be due to an initial interaction of bicelles with the SC. This interaction could cause some disorganization of intercellular lipids, responsible of the SC barrier function. This fact would help the absorption of diclofenac through the skin. The interaction of the specific phospholipids of bicelles with the lipids of the SC would be the mechanism responsible for the event detected. This mechanism is different for both systems because the DMPC/DHPC bicellar systems, apparently, did not produce modifications in the microstructure of the SC, whereas the DPPC/DHPC system did produce changes in the lipid lamellae regions (Barbosa-Barros et al., 2008a,c). Thus, our results seems to indicate that other factors, in addition to the microstructural changes of the SC, must be involved in the penetration of DDEA, since the two types of bicelles

produce a similar effect. In this case, the DDEA is applied in an aqueous solution. For this reason, the limiting rate of drug transport through the SC may not be due to drug release from the vehicle, but could probably be related more to inherent SC resistance.

4. Conclusions

This work demonstrates that bicellar systems are able to incorporate DDEA. This incorporation decreases the particle size with respect to the original bicelles. These bicellar systems, including DDEA, can act as retarders in the percutaneous absorption of the drug; probably, the limiting of the rate of drug transport is dependent on rate of the drug release from the vehicle. The results of our work may be useful to develop bicellar systems for drugs which have a too fast percutaneous absorption as it is the case of fentanyl (Frölich et al., 2001). On the contrary, the previous *in vitro* application of bicelles on skin discs seems to promote the passage of diclofenac through the lipidic interstices of the SC and, as a result, to improve the percutaneous absorption. In this case, there are no problems of drug release since DDEA is in an aqueous solution. As a consequence, a previous application of bicellar systems on the skin seems to be useful to modulate the percutaneous absorption of topically applied DDEA. Further investigations on the use of bicelles as delivery systems, using other drugs with different physicochemical properties and applications, should be considered in future research.

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