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Research paper

Development of microemulsions to topically deliver 5-aminolevulinic acid in photodynamic therapy

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ABSTRACT

The aim of this study was to obtain and to characterize microemulsions containing 5-aminolevulinic acid (5-ALA) and to investigate the influence of these systems in drug skin permeation for further topical photodynamic therapy (PDT). 5-ALA was incorporated in water-in-oil (W/O), bicontinuous (Bc), and oil-in-water (O/W) microemulsions obtained by the titration of ethyl oleate and PEG-8 caprylic/capric glycerides:polyglyceryl-6 dioleate (3:1) mixtures with water. Selected systems were characterized by conductivity, viscosity, size of the droplets, and drug release. The stability of the drug in the microemulsions was also assessed. Moreover, the *in vitro* and *in vivo* skin permeation of 5-ALA was investigated using diffusion cells and confocal scanning laser microscopy (CSLM), respectively. Despite the fact that the O/W microemulsion decreased the 5-ALA diffusion coefficient and retarded the drug release, it also significantly increased the *in vitro* drug skin permeation when compared to other 5-ALA carriers. It was observed by CSLM that the red fluorescence of the skin increased homogeneously in the deeper skin layers when the 5-ALA microemulsion was applied *in vivo,* probably due to the formation of the photoactive protoporphyrin IX. The microemulsion developed carried 5-ALA to the deeper skin layers, increasing the red fluorescence of the skin and indicating the potentiality of the system for topical 5-ALA-PDT.

1. Introduction

Topical photodynamic therapy is used for a variety of malignant and pre-malignant skin disorders, including Bowen's disease and superficial basal cell carcinoma. A haem precursor, typically 5-aminolevulinic acid (5-ALA), acting as a prodrug, is absorbed and converted by the haem biosynthetic pathway to the photoactive protoporphyrin IX (PpIX), which accumulates preferentially in the cells that multiply fast, such as tumour cells. Cell destruction occurs when PpIX is activated by an intense light source at the appropriate wavelength [1]. However, despite the fact that 5-ALA can permeate the skin's barrier, the local bioavailability of the drug is normally insufficient for a complete therapeutic effect [2]. In the last two decades, a considerable number of studies have been conducted on the development of carriers and on the synthesis or modification of valuable photosensitisers [3]. To date, many carriers have been used to deliver 5-ALA topically, including emulsions, liposomes, lipid sponge forms, and nanocolloid lotions [4–6]. Therefore, in this work, it was intended to assess the potential of microemulsions for the enhancement of 5-ALA skin permeation.

During recent decades, various colloidal systems have been investigated as suitable pharmaceutical vehicles for the dermal and transdermal delivery of active substances [7]. Microemulsion systems, due to their thermodynamic stability, ease of preparation, transparency, low viscosity, and considerable potential for solubilising a variety of drugs, have often been the object of investigations as drug delivery systems [8,9]. In a typical system, microemulsion components form non-spherical aggregates, which may be more or less continuous in the phase with the highest volume fraction [10–12]. For the majority of microemulsion systems, these aggregates fluently change into bicontinuous structures by titration with the phase of the lowest volume fraction and, through these structures, fluently invert to 'reversed' aggregates [9]. These characteristics depend on the physico-chemical properties of the microemulsion components and on the system internal structure, which is very important for the diffusivity of the phases and thereby also for the diffusion of a drug in the respective phases. Microemulsions can also change skin membrane properties and, consequently, drug diffusivity into this tissue [13,14]. Therefore, the results of numerous studies have suggested that microemulsions have a significant potential to increase the penetration of hydrophilic, lipophilic, and amphiphilic substances into and

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through the skin compared to conventional vehicles [7,13]. In this way, the aim of this work was to obtain and to characterize microemulsions containing 5-ALA and to investigate the influence of these systems for *in vitro* and *in vivo* skin permeation of this drug for further topical PDT.

2. Materials and methods

2.1. Chemicals

5-ALA hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA), PEG-8 caprylic/capric glycerides (Labrasol®) and polyglyceryl-6 dioleate (Plurol Oleique®) were from Gattefossé (Lyon, France), and ethyl oleate was from Acros Organics (Geel, Belgium). Tissue Tek® (O.C.T. Compound) was purchased from Sakura (Torrance, CA, USA). Distilled water was filtered through a Mili-Q® filter (Millipore, Bedford, MA, USA) prior to use. Solvents were of HPLC grade, and all other chemicals were of analytical grade.

2.2. Skin

Pig ears were collected immediately after the slaughter of the animals (Frigorífico Pontal Ltda, Brazil). Ears were then kept at 4 °C during transportation to the laboratory to be dermatomed. This whole process took no more than 2 h. Afterwards, the dermatomed skin (700 μm) was kept at −20 °C for a maximum of 7 days before use.

2.3. Microemulsion preparation

The three pseudo-ternary phase diagram of microemulsion regions was constructed by titration of a given blend of Labrasol®/Plurol Oleique® (1:1, 1:2 and 1:3) and oil (ethyl oleate) with water. The titrated mixture was continually stirred at room temperature. Microemulsion regions were identified as transparent, single phase, low viscous and isotropic mixtures. To assess isotropy, an Axioplan 2 Imaging Polarized light microscope (Carl Zeiss, Germany) was employed.

2.4. Electrical conductivity

Electrical conductivity measurements were performed at 27 °C (±2 °C) on samples marked as points along the surfactant/oil 8:2 dilution line of the diagram obtained with Labrasol®/Plurol Oleique® 3:1 using a conductivity meter (MCA 150, Tecnopon, Brazil). The electrode was dipped in the microemulsion samples until equilibrium was reached.

From these measurements, three microemulsions, marked in the pseudo-ternary phase diagram as W/O, Bc, and O/W (Fig. 1C), were selected for further studies.

2.5. Viscosity

Viscosity was measured by using a Brookfield LVDVIII Rheometer operated with Rheocalc software (Brookfield, Middleboro, MA, USA) at 27 °C (±2 °C). The cone and plate geometry was used.

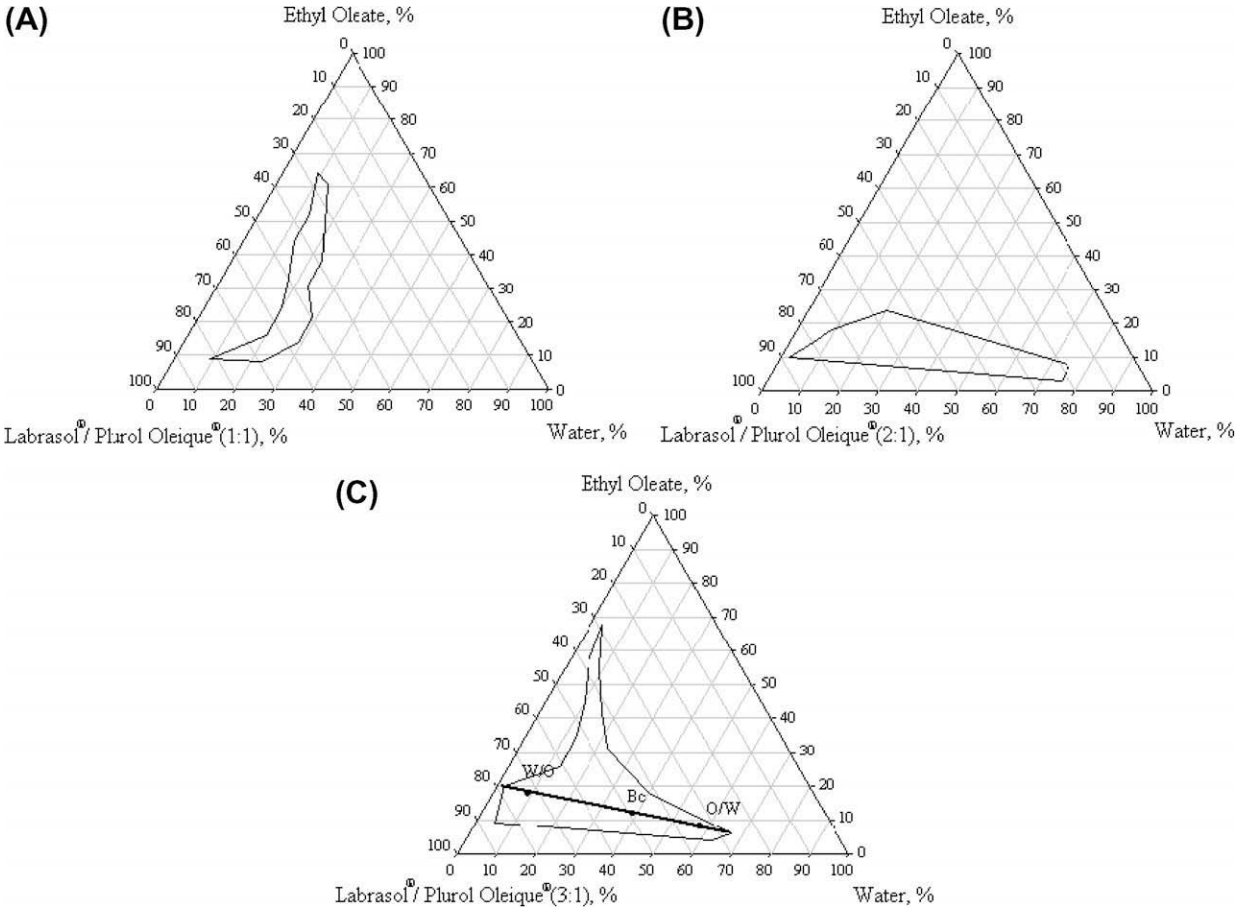

Fig. 1. Pseudo-ternary phase diagrams of the region of existence of the microemulsion systems (within the connected lines) obtained with three different Labrasol/Plurol Oleique blends: (A) 1:1, (B) 2:1, and (C) 3:1. Samples at a constant surfactant-to-oil ratio of 8:2 (line in the shaded area of diagram C) indicated as W/O, Bc, and O/W were chosen for further investigations.

2.6. Light scattering studies

For accurate measurement of the droplet size, the refractive index of microemulsions was analysed by a refractometer (Digital ABBE Mark II-REICHERT, Depew, NY, USA). The refractive index and the viscosity were employed during the analysis of the mean droplet size. Microemulsion samples were then analysed in a Zetasizer Nano-ZS apparatus (Malvern Instruments, England) to calculate the mean droplet size and polydispersity of the microemulsions. Measurements were performed at a permanent angle of 173° at 25 °C.

2.7. Drug incorporation

5-ALA was solubilised in the microemulsions at a final concentration of 2% (w/w) by stirring at room temperature until a clear and transparent system was obtained.

The influence of drug incorporation on the microemulsion pH and mean droplet size was analysed. Moreover, an accelerated stability study of 5-ALA in the O/W microemulsion was also performed. The system was stored in sealed amber glass ampoules at 8 °C, 27 °C, 40 °C, and 60 °C and removed at the appropriate intervals. The remaining 5-ALA was assayed according to the method described below by extracting the drug from the microemulsion with 25 mL of ethanol. This extraction procedure was validated by spiking the microemulsion with different known amounts of 5-ALA. The recovery percentages obtained were not less than 97.96%.

2.8. Analytical procedures

The amount of 5-ALA that diffused into or through the skin was determined by a fluorometric high-performance liquid chromatography (HPLC) method [15]. Because 5-ALA is not fluorescent, it was derivatised before analysis as follows: 50 μL of a 5-ALA sample was reacted with 3.5 mL of an acetylacetone reagent (15% vol./vol. acetylacetone, 10% ethanol and 75% distilled water) and 0.45 mL of 10% formaldehyde solution for 15 min at 100 °C. The fluorescent products were quantified in a high-performance liquid chromatograph (LC 10-AD, Shimadzu Instruments, Japan) equipped with an automatic sample injector (SIL10AD, Shimadzu Instruments, Japan). Twenty microlitres of the sample were injected onto a Lichrosphere 100 RP-18 column (125 mm × 4 mm, 5 μm, Merck, Germany,). Elution was performed at 40 °C with a mobile phase consisting of methanol/water (60:40) at a flow rate of 1 mL/min. The fluorescence intensity of the eluate was monitored (Shimadzu fluorescent detector RF-10AXL, Japan) at 370/460 nm (excitation/emission). The calibration curve was linear ($y = 19901x - 156{,}435$, $r = 0.999$) for 5-ALA over the concentration range of 8–600 nmol/mL. The method was validated in accordance with FDA guidelines [16].

2.9. Release studies

5-ALA release rates from the microemulsions and from a phosphate buffer solution (control) were measured through a 23-μm cellulose membrane (MW 12,000–14,000, Fisher Scientific, USA) in a Franz-type diffusion cell with a diffusion area of 1.4 cm^2. The formulation (0.5 mL) was placed on the membrane surface in the donor compartment while the receptor contained 5.5 mL of pH 5.0 phosphate buffer. During the experiments, the receptor solution was stirred at 500 rpm and kept at 37 °C by a circulating water bath (Ecoline 003, E100 from Lauda, Germany). The receptor was perfused continuously at 1 mL/h using a peristaltic pump (Pump Pro MPL580 – Watson-Marlow Bredel Pumps, United Kingdom). Samples were collected automatically every hour (Fraction collector PTFCII-Pharmatest, Germany) up to 12 h. At the end of the experiment, the amount of the drug that permeated across the membrane, i.e., the amount of the drug in the receptor solution, was analysed as described previously.

The diffusion coefficient (D) of the drug in the microemulsions was calculated according to the equation

$$Q = 2C_0\sqrt{\frac{D \cdot t}{\pi}} \quad (1)$$

where Q is the amount of drug released per unit area, C_0 is the initial concentration of the drug in the microemulsion, and t is the time elapsed since the release experiment started [17].

2.10. In vitro skin permeation studies

The skin was mounted between the upper and lower parts of vertical, flow-through modified Franz diffusion cells, with the dermal side facing downwards into the receptor medium: 5.5 mL of phosphate buffer, pH 5.0 [18,19]. To achieve higher reproducibility, the skin samples were allowed to pre-hydrate with receptor fluid for 1 h before the formulation was applied. The donor compartment was then filled with 0.5 mL of the microemulsions (O/W, Bc and W/O) containing 2% of 5-ALA. All experimental conditions were identical to those of the release studies.

2.11. Preliminary in vivo experiments – confocal scanning laser microscopy (CSLM)

The formation of PpIX induced by 5-ALA was investigated *in vivo* by the application of the O/W microemulsion containing 2% of the drug in hairless mice, 6–9 weeks old (strain HRS/J Faculty of Pharmaceutical Sciences, Ribeirão Preto, São Paulo, Brazil). The animals were housed at 24–26 °C, exposed to daily 12:12 h light:-dark cycles (lights on at 6 am) and had free access to standard mouse chow and tap water. The animal protocol was approved by the University of São Paulo Animal Care and Use Committee (Authorization number: 06.1.569.53.1).

After mouse immobilization, a buffer solution or the O/W microemulsion containing 5-ALA was applied on the dorsal region of the skin. The formulations were left there for 4 h to guarantee the 5-ALA skin penetration and its conversion in the fluorescent PpIX. Untreated skin was used as a control. The animals were also treated with O/W microemulsions free of 5-ALA. At the end of the experiment, the mice were sacrificed by carbon dioxide vapour. The formulation-exposed skin areas were removed from the animals for confocal scanning laser microscopy analysis. For that, skin samples had their fluorescence preserved by the application of Tissue Tek® solution and were frozen in liquid nitrogen. Cryosections (60 μm) were made perpendicular to the skin surface using a cryomicrotome (Microm D-6900, Germany). Skin slices were then analysed using a confocal microscope (LEICA TCS-SP2 SE, Manheim, Germany) equipped with an argon laser, a 20×/NA 0.70 immersion objective and a 488/543-nm double dichroic mirror. For excitation, the argon laser line at 488 nm was used; fluorescence emission was detected using the spectral detector set in the 590–800 nm range. Detector settings were kept constant for all images.

2.12. Data analysis

At least 4–6 replicates of each experiment were used. All results were expressed as the mean ± standard deviation. Statistical comparisons were performed using ANOVA followed by the parametric Tukey's test. A 0.05 level of probability ($p < 0.05$) was taken as the level of significance.

3. Results and discussion

3.1. Microemulsion preparation and characterization

Pseudo-ternary phase diagrams at three different surfactant/co-surfactant (S/CoS) ratios are shown in Fig. 1. In Fig. 1A, it is possible to observe that the maximum water solubilisation capacity of the microemulsion systems obtained with a S/CoS ratio of 1:1 was around 30%. On the other hand, the microemulsions presented in Fig. 1B and C, obtained with S/CoS ratios of 2:1 and 3:1, respectively, solubilised almost 80% of the water. Furthermore, the increase in the surfactant/co-surfactant ratio also improved the oil proportions within the isotropic area, as shown in the Labrasol®/Plurol Oleique® 3:1 diagram (Fig. 1C). Large microemulsion regions indicate the flexibility of the surfactant/co-surfactant film, which allows the existence of continuous structural transitions with an increasing water phase volume fraction in the selected oil/surfactant/co-surfactant mixture [8]. In this way, microemulsions formed with Labrasol®/Plurol Oleique® 3:1 were chosen for further studies.

The electro-conductivity behaviour of the microemulsion samples in diagram 1C prepared along the dilution line 8:2 (surfactant mixture/oil) is shown in Fig. 2. It can be seen that while the water volume ratio (ϕ_w) increases from 5 to 20%, the electrical conductivity (σ) of the system increases slightly. Up to this value, a linear and sharp increase in σ is observed until $\phi_w > 60\%$, when the conductivity of the system is not significantly affected by further addition of water. The sudden increase in σ is a phenomenon known as percolation [20,8]. Djordjevic and colleagues [8] obtained a very similar profile for microemulsion systems composed of the same non-ionic surfactant/co-surfactant mixture but with a different oil phase. According to those authors and others [20–22], it is possible to correlate the electro-conductivity behaviour with the structure of the microemulsion system. In the region of low water content, O/W microemulsion is formed. Beyond the percolation threshold, a network of conductive channels exists, which corresponds to the formation of non-spherical W/O-bicontinuous-non-spherical O/W structures. In the region of high water content ($\phi_w > 50\%$), an O/W microemulsion is formed [8,13]. Therefore, three structural regions (W/O, bicontinuous-Bc and O/W) were found in the analysis of the electro-conductivity behaviour of the systems studied, and they are discriminated in Fig. 2.

To confirm theses regions, viscosity measurements were also determined along the 8:2 surfactant/oil dilution line (Fig. 2). The viscosity data can also be explained by reference to the phenomenon of percolation [8,23]. The initial increase in viscosity with increasing ϕ_w represents the water percolation threshold. With the increase in the water phase concentration, the viscosity of the samples decreases, suggesting the transformation of W/O microemulsion, via a bicontinuous system, to an O/W system ($\phi_w > 50\%$). Therefore, three microemulsion compositions (Table 1), one for each region of the electro-conductivity and viscosity diagrams (Fig. 2), were then selected to be incorporated with 5-ALA.

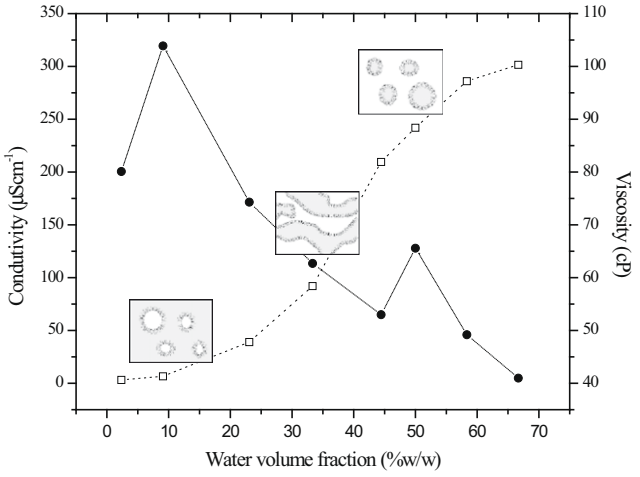

Fig. 2. Electrical conductivity (□) and apparent viscosity (●) of microemulsions at a constant surfactant-to-oil ratio of 8:2 as a function of the water volume fraction in the system obtained with Labrasol/Plurol Oleique 3:1.

3.2. 5-ALA microemulsions

Table 2 summarises the refractive index, viscosity, and particle size of the microemulsions studied containing 2% of 5-ALA. After the addition of the drug, the systems continued to be isotropic and transparent. No phase separation was observed with centrifugation. However, the size of the droplets increased slightly with the addition of the drug. It is worth emphasizing that the light scattering analyses were performed without any dilution of the systems because, according to the conductivity and viscosity studies (Fig. 2), it could change the type of microstructure formed. Therefore, the second droplet population observed for the O/W system (Table 2) could be due to aggregates formed by these structures.

The pH of O/W and Bc microemulsions also changed from around 7.4 to 4 when the 5-ALA was added. The carboxylic group present in the drug structure is probably responsible for this decrease in pH. It is possible that the pH difference before and after 5-ALA addition contributed to a rearrangement of the microstructures, which is reflected in the size of the droplets. However, given that the stability of 5-ALA in aqueous systems at pH 6 and above is notoriously poor [24], no adjustment of this pH was done.

Because of the known 5-ALA instability in aqueous solutions, an accelerated stability study of the drug in the O/W microemulsion was performed. 5-ALA stability depends on the pH, concentration, temperature, and degree of oxygenation of the medium where it is dispersed [24–27]. Novo et al. [24] showed that the 5-ALA molecule condensates and that this condensation reaction depends on the non-protonated form of the amino group of the molecule that permits its reaction with the ketone group of a neighbouring ALA molecule to form the product 2,5-(b-carboxyethyl) dihydropyrazine (CHPY). This product is further oxidised to pyrazine-2,5-

Table 1
Composition and possible microstructure of microemulsions used to incorporate 5-ALA.

Microemulsion type[a]	Composition (%)		
	Water	Ethyl oleate	Labrasol/Plurol Oleique (3:1)
W/O	9.09	18.18	72.73
Bc	39.39	12.12	48.48
O/W	58.33	8.33	33.33

[a] W/O: water-in-oil, Bc: bicontinuous, O/W: oil-in-water.

Table 2
Physical properties of microemulsions containing 2% of 5-ALA.

Microemulsion type[a]	Refractive index	Viscosity (cP)	Particle size (nm)			
			Without 5-ALA		With 5-ALA	
W/O	1.45	103.9	209		354	
Bc	1.33	69.3	94.4		106	
O/W	1.38	49.2	33.6	245	32.4	252

[a] W/O: water-in-oil, Bc: bicontinuous, O/W: oil-in-water.

dipropionic acid (PY), considered to be the major degradation product in aerated solutions [26,27]. Both CHPY and PY could not be quantified by the analytical method proposed in this work because the condensation of the drug blocked the fluorescent derivatisation reaction used to quantify 5-ALA. In this way, this method only permitted the quantification of 5-ALA and not its degradation products. Therefore, the drug stability was assessed by the measurement of the remaining amount of 5-ALA in the microemulsion.

Table 3 shows the remaining percentage of 5-ALA in the O/W microemulsion as a function of time and temperature. It is possible to observe that the remaining concentration of 5-ALA in the formulations did decrease as a function of the period of storage. Nonetheless, it might be also assumed that the temperature of storage is not critical for 5-ALA microemulsion stability. The half-life for decomposition of 2% of 5-ALA in the O/W microemulsion at pH 4 was around 30 days.

The stability of 5-ALA in macroemulsions currently used to topically administer the drug was not found in the literature. It is common to incorporate 5-ALA in O/W emulsions immediately before application. Elfsson and collaborators [25] determined that the decomposition of 5-ALA in an aqueous solution (1%, pH 4.8) followed a second-order kinetics, presenting a half-life of around 10 days. They also showed the effect of temperature on 5-ALA stability. In their study, the 5-ALA concentration decreased significantly with increasing temperature. Considering that the concentration of the drug in this solution was twice as low as the one found in the microemulsion developed in the present work, and that the drug degradation is accentuated when its concentration is higher [25], it could be assumed that the microemulsion protected the drug against condensation reactions. However, the pH of the solution was higher than the pH of the microemulsion developed, which could benefit the stability of the drug in the microemulsion. In any event, the microemulsion does protect the drug against the effects of temperature.

3.3. Release studies

To obtain more information about the microstructures of the systems developed, a release study of 5-ALA from the microemulsions was verified. Table 4 shows the calculated diffusion coefficient (D) value of 5-ALA from the control (phosphate buffer solution at pH 5) and from the microemulsions. It can be seen in this Table that microemulsions decreased the 5-ALA D value compared to the D value of the control solution, retarding drug release. The W/O microemulsion decreased the 5-ALA diffusion by a factor of four, while the O/W system retarded drug diffusion by only a factor of two. Therefore, it seems that 5-ALA is really in the internal phase of the microemulsion called W/O and in the dispersant phase of the O/W one. The Bc system showed a D value between the ones presented by the O/W and W/O microemulsions.

Table 3
Remaining percentage of 5-ALA in the O/W microemulsion as a function of time and temperature.

Time (days)	Remaining 5-ALA (%)			
	8 °C	27 °C	40 °C	60 °C
7	98.77 (±7.06)	93.01 (±0.29)	90.70 (±2.7)	85.48 (±1.68)
15	78.02 (±6.31)	79.80 (±1.55)	75.88 (±5.13)	58.10 (±28.78)
30	57.26 (±7.07)	52.60 (±3.46)	50.95 (±13.16)	46.15 (±33.56)

Table 4
Diffusion coefficient (D) of 5-ALA in the formulations.

Formulation	$D \times 10^{-6}$ (cm^2/s)
Phosphate buffer solution	2.32 (±0.24)
W/O microemulsion	0.58 (±0.06)
Bc microemulsion	0.83 (±0.05)
O/W microemulsion	1.30 (±0.15)

3.4. In vitro skin permeation experiments

In an attempt to evaluate the influence of the internal structure (W/O, O/W and Bc) of the microemulsions on the 5-ALA skin penetration, the three different formulations were applied over the pig skin used as a membrane for Franz diffusion cells. The drug skin permeation of the phosphate buffer solution pH 5 was used as a control. Table 5 shows that the flux of 5-ALA was significantly improved when the O/W microemulsion was applied. In contrast, the amount of 5-ALA that permeated the skin when this drug was dispersed in the W/O microemulsion was very small, below the quantification limit (8 nmol/mL) of the analytical method. Therefore, the 5-ALA flux calculated from this experiment is not reliable but allows us to affirm that, despite the fact that the W/O microemulsion is the one that has the highest surfactant/co-surfactant proportion (73%), it did not improve 5-ALA skin permeation when compared to the control (5-ALA in phosphate buffer solution). In addition, the stratum corneum remained intact after all the experiments (no visible damage was observed) when the skin in contact with the microemulsions studied was compared to the control. Therefore, it seems that the high percentage of surfactant in the microemulsion does not enhance the penetration. Moreover, these results indicate that the internal structure of the microemulsion (O/W, bicontinuous or W/O) may influence the 5-ALA skin permeation. Differences in the internal structure of the microemulsion can modify the thermodynamic activity of the drug, which could favour its partitioning to the stratum corneum [28].

It is important to bear in mind that the different internal structures of the microemulsions studied in this work are due to the different ratios among their components (oil, water, and surfactants). Delgado-Charro et al. [13] also verified the transdermal delivery of a hydrophilic compound, sucrose, from microemulsions composed of components very similar to those employed in this work. They observed that, in general, microemulsions containing a higher percentage of the aqueous phase delivered sucrose better. Our results are in agreement with this conclusion. Nevertheless, the 5-ALA flux from the O/W microemulsion was much higher than from Bc microemulsion, which had only 1.5 times less water than the former. It seems that the percentage volume of water/amphiphilic significantly influences the drug skin permeation as well as the microstructure formed. The O/W system with a water/surfactant mixture ratio of 2 increased 5-ALA skin permeation by a factor of 17.5 when compared to the Bc formulation with a water/surfactant ratio of 0.8. Because the D value of 5-ALA from the Bc microemulsion was only 1.5 times smaller than the one from the O/W system (Table 4), it is possible that the O/W microemulsion penetrates into the skin in a different manner than the Bc microemulsion, changing the drug partitioning across the membrane. It is clear, however, that further investigation is necessary to define the O/W skin penetration mechanism.

Table 5
In vitro 5-ALA fluxes through pig skin from phosphate buffer solution (PBS) and from different microemulsions.

Formulations	Flux J (nmol/cm^2/h)
PBS	3.96 (±0.03)
W/O microemulsion	0.12 (±0.04)[a]
Bc microemulsion	4.11 (±1.15)
O/W microemulsion	69.94 (±24.82)

[a] The amount of drug permeated in this case was under the quantification limit of the analytical method.

Table 6
In vitro transdermal 5-ALA delivery from different vehicles across skin models found in the literature.

Vehicle	Initial 5-ALA concentration (% p/p)	Skin model	$K_p \times 10^{-6}$ (cm/h)	References
O/W emulsion + 3% EDTA + 20% DMSO	1.5	Hairless mouse	87	[18]
O/W Emulsion	2	Hairless mouse	217	[19]
20% monoolein in propylene glycol	5	Hairless mouse	9524	[31]
Liposome	30	Wistar rat	1	[32]
Thermogel	10	Human SC	337	[33]
Excipial® Fettcreme	10	Human SC	2	[34]
Excipial® Creme	10	Human SC	4	[34]
Dolgit® Mikrogel	10	Human SC	15/53	[34,33]
Basicreme DAC	10	Human SC	5/46	[34,33]
3.8% l-methyacetato + 40% ethanol	10	Yucatan micropig	85	[35]
10% oleic acid in propylene glycol	1	Pig	63	[36]
Sponge phase (monoolein/propylene glycol/water)	0.25	Pig	133	[6]
Microemulsion O/W[a]	2	Pig	586	

[a] O/W microemulsion developed in this work.

Many approaches have been taken to increase 5-ALA skin permeation because its limited penetration depth is one of the major limitations of topical 5-ALA-PDT [29,30]. Table 6 shows *in vitro* transdermal 5-ALA delivery from different vehicles across skin models found in the literature [6,18,19,31–36]. The transdermal delivery is described in Table 6 in terms of the permeability coefficient (K_p) to allow some comparisons among experiments performed with different initial drug concentrations. The initial ALA concentration in the formulations was also demonstrated in Table 6. However, it is important to bear in mind that the 5-ALA skin passive flux may not increase in proportion to the donor concentration.

It is well established that hairless mouse skin is more permeable than pig skin and human skin for the majority of drugs [37–40]. Table 6 shows that 5-ALA also diffuses easily through hairless mouse membrane when the drug K_p from propylene glycol solutions containing oleic acid [36] and monoolein [31] are compared. According to the literature, monoolein (glyceryl monooleate) is capable of interacting with phospholipid bilayers and has a penetration-enhancing activity similar to oleic acid [31,41–43]. However, *in vitro* studies showed that the permeability coefficient of 5-ALA through hairless mouse skin, in the presence of monoolein, was around 150 times greater than that through pig skin when oleic acid was present (Table 6). On the other hand, the amount of PpIX induced by 5-ALA delivered from monoolein and oleic acid solutions *in vivo* in hairless mouse skin was only two times greater than the more concentrated monoolein formulation [31,36]. Therefore, among the formulations containing 5-ALA applied passively

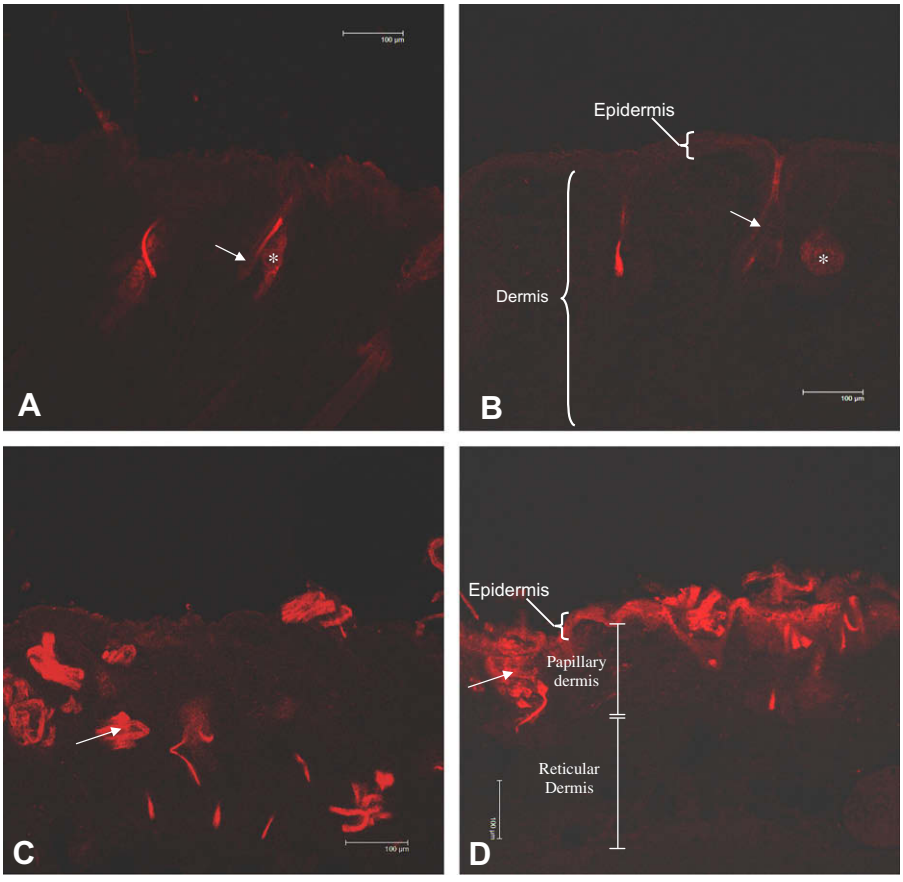

Fig. 3. CSLM micrographs of mechanical cross sections of hairless mouse skin (perpendicular series) after *in vivo* application of formulations for 4 h: (A) control (untreated skin), (B) skin treated with 5-ALA free O/W microemulsion, (C) skin treated with buffer solution containing 2% of 5-ALA, and (D) skin treated with O/W microemulsion containing 2% of 5-ALA. The arrows indicate the hair follicles and the asterisks correspond to the sebaceous glands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in vitro to pig skin (Table 6), the developed O/W microemulsion was the one that showed the best results for skin permeation of this drug.

Some other studies also showed an increase in the permeation of 5-ALA through the skin [44–46]; however, they are not included in Table 6 and are not compared to our results because we do not have enough data to calculate the 5-ALA K_p from these formulations.

The application of a low electrical current, iontophoresis, to improve 5-ALA skin penetration has also been studied by several research groups including ours. The K_p of iontophoretically delivered 5-ALA was around 10 times greater than the one observed when the microemulsion was used to administer the drug [6,15,17,47]. However, iontophoresis is a physical method, and thus its application is more complex than the passive one. Moreover, attention must be paid to the pain and high tumour permeation, which can lead to a systemic toxicity when iontophoresis is applied.

3.5. In vivo experiments – CSLM

The CSLM experiments were performed to visualise the PpIX induced by 5-ALA that penetrates the skin once it has been shown that tissues that accumulate PpIX emit a characteristic red fluorescence when exposed to light of the appropriate wavelength [18,48].

Fig. 3 shows confocal images obtained from mechanical sections from control (untreated skin) and treated skin samples from a hairless mouse. It is important to mention that the images show an intact epidermis, and it is possible to visualise the boundary between the epithelial epidermis and the conjunctiva dermis. The disruption of the basement membrane that underlies the epithelium, characteristic of a non-intact epidermis, would probably lead to an invasion of the typical fluorescence of the epidermis to the dermis, which would make it difficult to distinguish between these two layers. In Fig. 3D there are a few strongly fluorescent points that can be seen over the stratum corneum. These points are artefacts that pollute the image to some extent; however, it is still possible to observe the epidermis boundary, indicating that the skin is intact.

The application of the drug-free O/W microemulsion (Fig. 3B) seems to not change the auto-fluorescence of the skin (Fig. 3A). Fig. 3C shows that the fluorescence in the skin after the application of a 5-ALA buffer solution was heterogeneously distributed. It is possible to observe that it is intense in the skin appendages, such as hair follicles and sebaceous glands, but it is slight in the skin layers. On the other hand, the application of the 5-ALA O/W microemulsion (Fig. 3D) increased the red fluorescence of the skin intensively and homogeneously, especially in the epidermis. These results indicate that 5-ALA skin penetration from the microemulsion induced the formation of the photoactive PpIX in the skin.

In summary, the O/W microemulsion improved the 5-ALA stability. Furthermore, the *in vitro* skin permeation of 5-ALA was significantly increased by its incorporation in the O/W microemulsion developed when compared to other 5-ALA vehicles. Finally, the red fluorescence of the skin was strongly intensified after the application of the 5-ALA microemulsion. Investigations of the cytotoxicity and more conclusive *in vivo* skin permeation experiments with the 5-ALA microemulsion are currently in progress in our laboratory.

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