BJPS

Brazilian Journal of
Pharmaceutical Sciences
vol. 45, n. 1, jan./mar., 2009

Article

Influence of ceramide 2 on *in vitro* skin permeation and retention of 5-ALA and its ester derivatives, for Photodynamic Therapy

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Photodynamic therapy (PDT) based on topical 5-aminolevulinic acid (5-ALA), an endogenous precursor of protoporphyrin, is an interesting approach for the treatment of skin cancer. However, 5-ALA is a hydrophilic molecule and such a characteristic limits its appropriate cutaneous penetration and retention. In this way, more lipophilic molecules, such as esterified 5-ALA derivatives, have been under investigation in order to improve the skin penetration of this molecule. Drug formulation can also alter 5-ALA skin penetration. Therefore, the aim of this work was to study the influence of ceramide 2 - the main lipid of the SC- on the cutaneous delivery of 5-ALA and its ester derivatives in vitro, using Franz diffusion cell. The skin permeation of all studied drugs was decreased in the presence of ceramide, representing a desirable characteristic in order to avoid the risk of systemic side effects. Nevertheless, the SC and [epidermis + dermis] retention after 16 h has also been decreased in the presence of ceramide, as compared to control. In conclusion, ceramide was not a good adjuvant, meaning that research of other vehicles could be useful to improve cutaneous delivery of 5-ALA.

Uniterms: 5-aminolevulinic acid. Ceramide 2. *In vitro*/Skin permeation. *In vitro*/Skin retention. Photodynamic therapy.

A Terapia Fotodinâmica (TFD) tópica com um precursor das porfirinas endógenas, o ácido 5-aminolevulínico (5-ALA), constitui uma nova modalidade para o tratamento do câncer de pele. Entretanto, o 5-ALA é uma molécula hidrofílica, o que limita sua penetração e retenção cutânea apropriadas. Moléculas mais lipofílicas, tais como derivados esterificados do 5-ALA, estão sob intensa investigação para melhorar a penetração cutânea desta molécula. A formulação que contém o fármaco também pode alterar a penetração cutânea do 5-ALA. Desta forma, o objetivo deste trabalho foi estudar a influência da ceramida 2 – o principal lipídeo do EC- sobre a penetração cutânea de 5-ALA e seus derivados esterificados usando células de difusão de Franz. A permeação de todas as drogas estudadas através da pele foi diminuída na presença de ceramida, o que é desejável, evitando riscos de efeitos colaterais sistêmicos. Entretanto, a retenção no EC e [epiderme + derme] também foi diminuída na presença da ceramida, após 16 horas, comparado ao controle. Concluindo, a ceramida não foi um bom adjuvante, sendo necessária a pesquisa de outros veículos para melhorar a liberação cutânea do 5-ALA.

Unitermos: Ácido 5-aminolevulínico. Ceramida 2. Permeação cutânea/*in vitro*. Retenção cutânea/*in vitro*. Terapia fotodinâmica.

INTRODUCTION

Exogenous 5-aminolevulinic acid (5-ALA) is becoming widely useful as an inducer of endogenous synthesis of protoporphyrin IX (PpIX), a photosensitizer used in photodynamic therapy (PDT). Topically ALA-based PDT has been successfully used in the treatment of cutaneous disease of non-melanoma skin cancer type, mainly in basal cell carcinomas and squamous cell carcinomas (Zeitouni *et al.*, 2003; Murmur *et al.*, 2004).

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Despite the promising results obtained with 5-ALA, a significant limitation for its topical application is the poor ability of this molecule to diffuse through biological membranes like the *stratum corneum (SC)*, due to its higher hydrophilicity. Then, a high dose of 5-ALA must be administered, in order to increase PpIX in the tissue at a level that is useful for PDT treatment. Hence, improving the uptake of 5-ALA would be expected, to increase the efficiency of PDT (Lopez *et al.*, 2004).

There are several methods to improve 5-ALA skin penetration: (i) changing the drug formulation by adding a penetration enhancer (Pierre *et al.*, 2006) or by packaging it into a drug delivery system, e.g., liposomes (Pierre *et al.*, 2001); (ii) applying an electrical current (Lopez *et al.*, 2001); (iii) modulating the biosynthetic pathway by the addition of iron chelators (Curnow *et al.*, 1998) and (iv) increasing the lipophilicity of 5-ALA by its own esterification, for example (De Rosa *et al.,* 2003). In topical applications, esters can improve penetration depth as a result of their enhanced lipophilicity and also yield a more homogeneous tissue distribution (Van Den Akker *et al.*, 2000) leading to shorter application times, lower drug doses, lower cost, reduced side-effects (e.g. pain), and improved drug stability (Kloeck *et al.*, 1998; Gaullier *et al.,* 1997).

It is known that the main skin barrier resides on the stratum corneum (SC) due to the combination of unique properties of its components: corneocytes and lipidic barrier (Scheuplein *et al.,* 1969; Kessner *et al.*, 2008). The lipids, 2% to 10% of the SC, form a hydrophobic layer between cells, avoiding the diffusion of water and hydrophilic substances across SC. Therefore, intercellular lipids have an important role in the maintenance of SC barrier function (Feingold, 2007). Efforts to modulate this barrier, in order to increase the penetration of drugs to or through the skin have been focused.

SC lipids are composed mainly by free fatty acids, cholesterol, ceramides and cholesteryl sulfates, which represent approximately 25 %, 25 %, 40 % and 10 % (w/w) of the SC lipids, respectively (Yardley, Summerly, 1981; Long *et al.*, 1985). In this way, ceramides (CER) constitute the major group of lipids in the mammalian SC.

The CER consist of a long-chain or sphingoid base linked to a fatty acid via an amide bond (Figure 1a). CER are formed as key intermediates in the biosynthesis of all complex sphingolipids, in which the terminal primary hydroxyl group is linked to carbohydrate, phosphate, etc. Unlike the sphingoid precursors, they are not soluble in water.

The CER of the SC can be subdivided in three main subgroups, based on the nature of their head group architectures: sphingosine (S), phytosphingosine (P) or 6-hydroxysphingosine (HS) (Figure 1b). Through an amide bond, long-chain non-hydroxy (N) or α-hydroxy (A) fatty acids with varying acyl chain lengths are chemically linked to the sphingosine bases (Wertz, 1992).

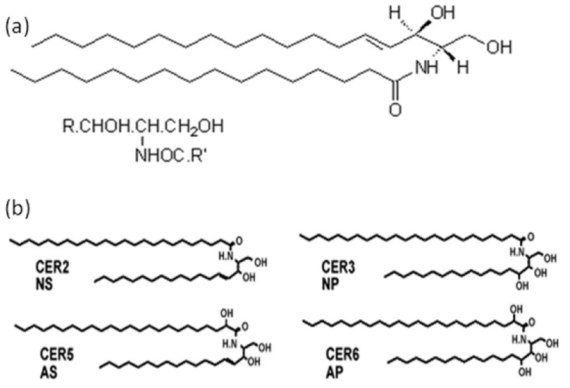

FIGURE 1 - (a) General structure of the ceramides. **(b)** Representative molecular structure of SC ceramides according to WERTZ (1992). **NS**: long-chain non-hydroxy fatty acid sphingosine, **AS**: α-hydroxy fatty acid sphingosine, **NP**: long-chain non-hydroxy fatty acid phytosphingosine and **AP**: α-hydroxy fatty acid sphingosine.

The CER consist of, at least, nine different species with a wide range of long to very long fatty acid chains (Jeong, Oh, 2007). The human skin CERs are classified by Arabic numbers, changing from 1 to 9 according to their position on thin layer chromatography plate (Robson *et al.*, 1994). On the other hand, the Roman numbers indicate the commercially available ceramide types, so that CER III corresponds to CER 2 while CER IV to CER 5 of the human skin (Motta *et al.,* 1994).

The CER 2 represents the most important fraction between all other ceramides that are present in the SC (18% w/w) (Wertz *et al.*, 1986). According to Wertz and Downing (1983), they protect the epidermal barrier from water loss.

The proposal of this work was to study the *in vitro* influence of ceramide 2 on the skin penetration of the hydrophilic 5-ALA and its lipophilic esters derivatives. Since ceramides constitute an abundant component in the SC, their exogenous addition could lead to a "reservoir" effect, increasing the retention of lipophilic drugs and facilitating their penetration into the skin. In this case, they could improve the effect of topical delivery of 5-ALA or its derivatives in 5-ALA based-PDT treatment of skin cancers.

MATERIALS AND METHODS

Chemicals

5-ALA hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other 5-ALA esters

(Table 1) were synthesized as previously described (Kloeck *et al.*, 1996), with purity over 95%. Ceramide 2 (CER III) that corresponds to synthetic non-hydroxylated fatty acid N-acyl sphingosine, prepared from the action of phospholipase C, a bovine brain sphingomielin, was purchased from Sigma Aldrich Chemie GmbH (Schnelldorf, Germany). All other chemicals were of analytical grade.

TABLE I - 5-ALA and alkyl esters: general structure = $HCl{\cdot}NH_2{-}CH_2{-}CO{-}CH_2{-}CH_2CO{-}OR^1$

Drugs	Abbreviation	R^1
5-ALA	5-ALA	H
Hexyl-ester	H-ALA	$(CH_2)_5CH_3$
Octyl-ester	O-ALA	$(CH_2)_7CH_3$

Skin

Permeation experiments were performed in full thickness pig ear skins. Tissue was obtained less than 2 h after animal slaughter (Frigorífico Pontal Ltda, Brazil) and used at once or stored frozen for a maximum of 30 days before use.

Fluorimetric assay of 5-ALA and its esters

The amounts of 5-ALA were determined after conversion of 5-ALA, H-ALA and O-ALA onto its fluorescent derivatives, by reaction with acetylacetone and formaldehyde (OISHI *et al.*, 1996); the derivative was estimated spectrofluorometrically using a spectrofluorometer (FLUOROLOG 3-Spex, Ivon Jobin), with excitation at 378 nm and emission at 464 nm (bandwidth 1.5/2.5 nm), by reference to standard curves.

Determination of partition coefficients for 5-ALA and its ester derivatives

Partition coefficient octanol/water

The measurements of the 1-octanol/water partition coefficients were carried out using the shake-flask method according to the OECD guideline 107 (OECD, 1995). The 5-ALA and its ester derivatives were dissolved in water previously saturated with octanol at a concentration of 60 µg/mL, and mixed with the same volume of octanol previously saturated with water. All samples were shaken for 30 minutes, centrifuged to separate the two phases, and then the amount of 5-ALA and its esters in the aqueous phase was quantified. The amount of drugs in octanol phase was calculated by subtraction of the measured concentration (C) from the initial concentration. The partition coefficients ($K_{o/w}$) were calculated using the following equation:

$$K_{o/w} = \frac{C_{octanol}}{C_{water}}$$

Skin/water partition coefficient

This methodology was based on the method proposed previously by Scheuplein *et al.* (1969). Full-thickness skin was excised from the dorsal surface of 4-6 weeks old hairless mice HRS/J strain (Jackson Laboratories, Bar Harbour, ME). Skin samples (≈ 150 mg) were cut and transferred to extraction tubes containing 3.0 mL of 5-ALA aqueous solution or its esterified esters, at a concentration of 60 µg/mL. The skin and drug aqueous solutions were stirred for 6 minutes, filtered by filter paper and then by nitrate cellulose membrane of 0.45 µm porosity. The $K_{S/W}$ was determined in the aqueous phase by spectrofluorimetric assay of 5-ALA and its derivatives, according to the following equation: $K_{S/W=} C_{skin}/C_{water,}$ where C_{skin} was calculated by the difference between the measured concentration and the initial concentration in the water.

In vitro skin permeation studies

Mixtures containing CER III at 2.0% (w/w) and 5-ALA, H-ALA or O-ALA at 1.0 % (w/w) were obtained after their dissolution in chloroform: methanol (2:1) solvent mixture, followed by drying under nitrogen flow, forming a thin film. Next, this film was dispersed in propylene glycol (PG). Control solutions (without CER) were represented by 5-ALA or its derivatives at a 1% concentration, dispersed in PG.

Full thickness porcine ear dorsal skins were excised and mounted in a modified Franz diffusion cell (Microette-Hanson Research, Chatsworth, CA, USA) with the dermal side facing downwards into receptor medium: 7 mL of isotonic phosphate buffer, pH 5.0 for 5-ALA and pH 7.2 for its ester derivatives (De Rosa *et al.*, 2000). The donor compartment was filled with 120 µL of the mixtures containing 5-ALA, H-ALA or O-ALA at 1% concentration (w/w), and CER III (2% w/w) in PG. The total available diffusion area of the cell was 1.7 cm^2. The system was maintained at 37°C, giving a temperature of 32 ºC measured at the SC, and the receptor medium was stirred at 300 rpm. At regular intervals for up to 16 hours, 1 mL of the receptor phase was removed for determination of the total drug content permeated through the skin by fluorescence derivatization, followed by spectrofluorimetric assay and replaced by an equal volume of fresh receptor solution.

The amount of drug permeated was calculated according to the equation:

$Q_{real,\,t} = (C_{measured,\,t} \times V_r + (V_a \times \sum^{n-1} C_a)$,

where Q= accumulated permeated amount; Q_{real}= real value at the time t; $C_{measured,\,t}$= concentration measured from the sample at time t; V_r= volume of the diffusion cell; V_a = volume of the removed sample and C_a= concentration of the removed sample.

Finally, the amount of drugs permeated through the skin was divided by the skin area, and these values were plotted as a function of the time (μg/cm^2).

In vitro skin retention studies

At the end of the above described experiment (after 16 h), the amount of drug present into the skin was also evaluated. For that, the skin was removed from the diffusion cell and pinned to a piece of Parafilm™ with the SC face up. That part of the skin, which had been exposed to the formulation (1.7cm^2), was then tape-stripped 10 times using Scotch Book Tape n° 845 (3M, St Paul, MN). The tape-strips were subsequently immersed in 10 mL of methanol in a vial, and shaken for 1 min to extract the permeant, before an aliquot of the resulting solution was subjected to derivatization and fluorimetric analyses, to evaluate the compound in the SC. The remaining skin [epidermis without SC + dermis], was cut into small pieces and homogenized with 5 mL of methanol for 1 min, sonicated for 30 min and filtered. An aliquot of the filtered homogenate was then analyzed by fluorimetric assay, to determine the compound quantity in the skin without the SC ("viable epidermis").

RESULTS AND DISCUSSION

Drugs that are highly hydrophilic or lipophilic cannot lead to an optimum passive transport through the skin and others biological barriers. Then, the 5-ALA esterification aims to increase their lipophilic properties, in order to verify if this modification in the 5-ALA molecule could really improve skin penetration of the drug. Then, partition coefficient studies were realized to evaluate (i) the lipophilicity grade, represented by $K_{O/W}$ and (ii) the affinity to the full thickness skin ($K_{S/W}$) of 5-ALA and its more lipophilic derivatives. Table II shows partition coefficients studies of 5-ALA, H-ALA and O-ALA.

As can be seen at Table II, the Ko/w of 5-ALA is low, indicating that this molecule is a highly hydrophilic compound. Also, its Ks/w was low, meaning 5-ALA limited affinity to the skin. On the contrary, the esterification of the drug with hexyl and octyl alcohol increased significantly its lipophilicity. Table II shows that this lipophilicity

TABLE II - Octanol/water ($K_{O/W}$) and skin/water ($K_{S/W}$) partition coefficients (K) of 5-ALA and its n-alkyl esters

Partition coefficients	5-ALA	H- ALA	O-ALA
$\mathbf{K}_{o/w}$ [a]	0.055 (± 0.007)	0.1720 (± 0.035)	0.3205 (± 0.010)
$\mathbf{K}_{s/w}$ [b]	0.032 (± 0.007)	0.2740 (± 0.135)	0.8265 (± 0.020)
$\mathbf{K}_{sc/w}$ [c]	0.042	8.169	10.522

[a, b] Statistic test ONE WAY ANOVA (Tukey's multiple comparisons). [c] De Rosa *et al.*, 2003

increases with the alkyl chain length of the alcohol used in the esterification. In this way, H-ALA is about 3 times more lipophilic than 5- ALA, and O-ALA is about 6 times more lipophilic than the mother molecule. The esterification improves as well the affinity of the drug for the skin, as showed by $K_{S/W}$ results.

Recently, stratum corneum/water partition coefficients of 5-ALA and several alkyl esters were determined by De Rosa *et al.* (2003) and also showed this effect, that is, an increase in the K with the alkyl chain length of the ALA ester.

However, the $K_{SC/W}$ of ALA esters (De Rosa *et al.*, 2003) is much higher than the $K_{S/W}$ (Table II). These results indicated that the affinity of ALA esters for the SC is significantly higher than their affinity for the viable epidermis, pointing out retention of these esters in the most external barrier of the skin, i.e, the SC. They also indicate that the esters can penetrate the skin thanks to improvement of drug skin affinity. It is well known (Aulton, 2005) that to have an efficient topical effect, a drug has to release itself from the formulation and enter, by partition and diffusion, into the skin. Therefore, the more drug enters the skin, the more drug can diffuse to deeper layers of the tissue. In this way, the affinity of ALA esters for the SC can lead to an improved amount of the drug into skin. It can improve the 5-ALA bioavailability in the tissue and, possibly, might even lead to a more homogeneous distribution of the resulting photosensitizer.

The SC lipidic lamellae consists mainly of ceramides, cholesterol, free fatty acids and cholesteryl sulphate, however it is devoid of phospholipids, which are bilayer forming components in all other cellular and intracellular membranes (Wertz *et al*, 1986). It was suggested that lipids such as ceramides and cholesterol participate of the bilayer formation in the SC, providing selectivity in the transcutaneous permeation of lipophilic and hydrophilic substances (Gray, Yardley, 1975). In this way, the similarity

of composition of the drug delivery system with the main SC lipid, the CER III, could lead to a higher interaction between formulation and skin, and consequently, a higher drug accumulation/retention in the tissue. Thus, in order to verify the influence of CER III on cutaneous delivery of 5-ALA and its esters, the *in vitro* skin permeation and retention studies were carried out.

According to Robert (1997), the skin permeability to polar solutes can be favored by hydrocarbon based vehicles, as CER. In this way, we have expected an improved permeation for 5-ALA dispersed in CER formulation. However, it was not observed in the experiment *of in vitro* skin permeation of our work. Figure 2 shows the cumulative amount of 5-ALA, H-ALA and O-ALA present in the receptor solution after permeation through full-thickness porcine ear skin, after 16 h. It is possible to notice that the presence of CER did not increase the permeation of 5-ALA and its esters. In the case of 5-ALA, it has even decreased significantly the amount of drug in the receptor solution.

A reasonable explanation for the results obtained is that the propylene glycol, also present in the formulations, had changed solubility pattern of the skin (William, Barry, 2004), increasing the partition coefficient of the drug (together with CER) to this layer and, therefore, decreasing its permeation through the skin. These results, however, can be advantageous, once systemic risk effects could be avoided by this low permeation and for a topical treatment, the drug should be present into the skin and not in the circulation (represented by the receptor solution in the *in vitro* experiments).

To verify the influence of CER in the skin retention of 5-ALA and its esters, their presence in the SC and "viable epidermis" was also evaluated. As can be seen in Figure 3, drugs skin retention was not improved by CER presence in the formulation. Moreover, for H-ALA and O-ALA CER presence has even decreased drug amount in the SC (a) and viable epidermis (b), respectively. Therefore, CER III was not efficient to improve neither 5-ALA/esters derivatives permeation nor retention, when dispersed in a propylene glycol solution.

It has been postulated (Coderch *et al.,* 2003; Bouwstra, Ponec, 2006) that formulations containing lipids identical to those in skin, in particular, some ceramide supplementation could have two divergent actions: (i) improve intercellular lamellae disorder or (ii) increase the skin lamellar phase, which could difficult the penetration of some drugs. These at first sight contradictory effects are dependent of the formulation that contains the ceramide. It seems that dispersion of CER in propylene glycol leads to increase of the skin lamellar phase (Jager *et al.*, 2003).

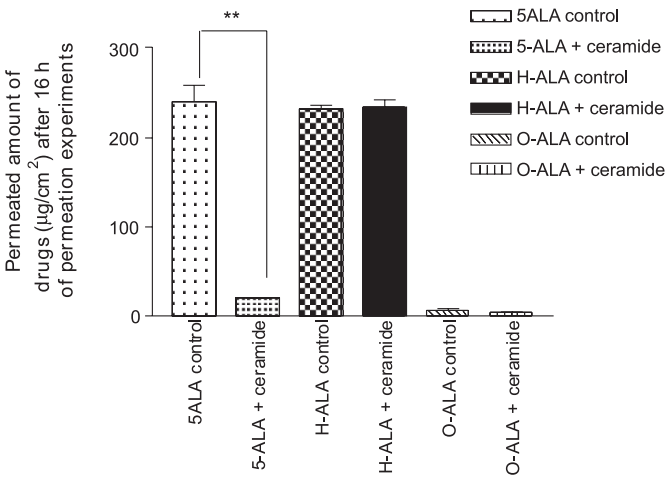

FIGURE 2 - *In vitro* permeation of 5-ALA, H- ALA and O-ALA (µg/cm^2) through porcine skin, from PG solution and mixture with ceramide (2.0% w/w) in PG, after 16 h of permeation experiments. Percentage of 5-ALA and its ester derivatives in the formulation: 1.0% (w/w). Values are expressed as S. M. E (n=4) for each group. Values are significantly different only between 5-ALA control and ceramide + 5-ALA ($P < 0.001$**). Statistical analysis: One- way Anova (Tukey´s multiple comparison test).

Probably, dispersion changes skin pattern, making difficult in this case, the drugs diffusion.

According to Figure 3 (a, b), the H-ALA retention is higher than 5-ALA and O-ALA. The same result in SC was observed by De Rosa *et al.* (2003) for these drugs dispersed in an O/W emulsion, that is, the H-ALA retention in SC was also higher than 5-ALA and O-ALA.

These authors verified also a small amount of O-ALA through the skin, and it is probably related to the small release of this drug from the formulation (O/A emulsion). They have postulated that due to the fact that O-ALA is highly lipophilic, its release from this vehicle is also difficult, because its interaction with the oil phase of the emulsion is also high. The same behavior can be observed in Figure 2 (indicating a low skin permeation of O-ALA) and Figure 3, where the skin retention of O-ALA (SC as well as in epidermis plus dermis) is low, probably due to its high affinity to the vehicle, in this case, CER III in propylene glycol.

The 5-ALA low retention is much probably related to its hydrophilicity, which becomes difficult the drug skin penetration. In conclusion, CER III dispersed in a propylene glycol vehicle did not improve the skin retention and permeation of 5-ALA and its ester derivatives . The composition of the formulation where CER III is dispersed influences directly its performance. The development of appropriate vehicles for 5-ALA and its ester derivatives

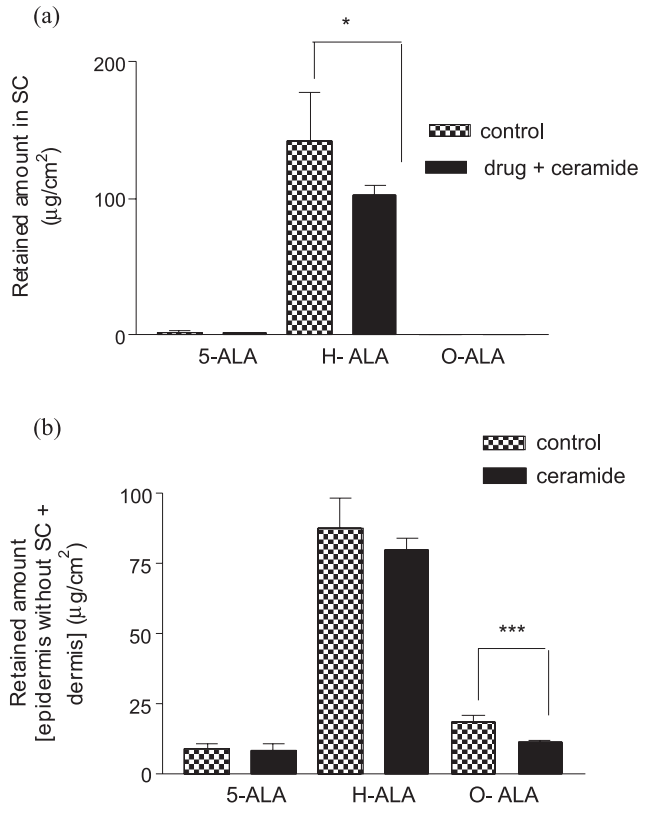

FIGURE 3 - In vitro retained amount of 5-ALA and its esters into: (**a**) SC and (**b**) [epidermis without SC + dermis] in porcine ear skin, after 16 h of permeation study from PG solution and mixture with ceramide (2.0% w/w) in PG. Percentage of drugs in the formulation: 1.0% (w/w). Values are expressed as S. M. E (n=4) for each group. Values signifcantly different between formulation and control for H-ALA retained in SC ($P < 0.01$*), and between formulation and control for O-ALA retained in [epidermis without SC + dermis] ($P < 0.05$). Statistical analysis: Statistical analysis: One- way Anova (Tukey´s multiple comparison test).

is required to improve their skin penetration/retention equilibrium.

ACKNOWLEDGEMENTS

Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Brazil, supplied financial support.

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Recebido para publicação em 29 de janeiro de 2008.
Aceito para publicação em 10 de novembro de 2008.