Revista Brasileira de Ciências Farmacêuticas
Brazilian Journal of Pharmaceutical Sciences
vol. 38, n. 1, jan./mar., 2002

RBCF

Glycerol monooleate/solvents systems for progesterone transdermal delivery: i*n vitro* permeation and microscopic studies

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Transdermal delivery of most drugs is precluded by the barrier characteristics of the stratum corneum (SC). Chemical penetration enhancers are capable of interacting with SC constituents, inducing a temporary reversible increase in the skin permeability. The aim of this work was to assess the influence of glycerol monooleate (GMO)/solvents systems on percutaneous absorption across hairless mouse SC of a lipophilic drug, progesterone (PG), as well as its effect on the SC structural characteristics, by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). The morphological changes observed in the hairless mouse SC suggest a GMO effect on the skin barrier. In addition, the increase in the in vitro PG flux and in vivo penetration of a fluorescent label point towards GMO as a potential absorption enhancer. The results obtained showed that GMO/solvents systems provoked changes in the SC that could be causing increased permeation of PG across hairless mouse skin, optimising in this way the transdermal delivery of this drug.

Uniterms:
- Glycerol monooleate
- Penetration enhancer
- Scanning Electron Microscopy
- Confocal Laser Scanning Microscopy
- *In vitro* permeation study

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INTRODUCTION

The skin has attracted much attention as an alternative route for administering systemically active drugs. The potential advantages associated with transdermal drug delivery are well documented (Guy *et al.*, 1987). However, very few drugs can be administered transdermally due to the low permeability of the skin, predominantly attributed to its outermost layer, the stratum corneum (SC), a multilayered wall-like structure in which keratin-rich corneocytes are embedded in an intercellular lipid-rich matrix. It has been assumed that the transport of substances across the SC occurs via both the intra and intercellular routes, the intercellular lipids being the most important for the percutaneous absorption of most permeants (Abraham *et al.*, 1995).

Chemical and physical approaches to increase transdermal transport have been explored in efforts to enhance skin permeability and expand the range of drugs, which can be delivered transdermally (Chang *et al.*, 2000; Obata *et al.*, 2000; Sung *et al.*, 2000). In theory, the rate of skin permeation of drugs can be increased either by varying vehicle composition in order to increase the solubility of drugs in the skin, or by altering skin permeability to the drug. In this case, chemical penetration enhancers have been extensively used. Ideally, an enhancer should be chemically and pharmacologically inert, non-toxic, non-irritant and non-allergenic. It might have a rapid and

reversible onset of action, be potent at low concentrations and compatible with the formulation ingredients. Various vehicles have been identified as penetration enhancers. Many of these substances however, have been associated with untoward reactions such as acute and chronic inflammation of subcutaneous tissue. To overcome this problem, compounds that cause relatively less skin irritation have been studied as new candidates for percutaneous absorption enhancers, including natural components of skin lipids which have long-chain saturated or unsaturated fatty acids (Williams, Barry, 1992).

Glycerol monooleate (GMO) is a fusogenic and polar lipid of interest in a number of areas ranging from controlled uptake to release of cosmetic, food and pharmaceutical formulations (Qiu, Caffrey, 2000). It is capable of interacting with phospholipid bilayers and, like other lipids, has been proposed as a penetration enhancer (Maggio, Lucy, 1976; Ogiso *et al.*, 1995).

In the present work, the influence of GMO on the percutaneous absorption of progesterone (PG) through hairless mouse skin was studied by evaluating *in vitro* permeation parameters. Morphological methods, such as scanning electron microscopy (SEM), which provide visualization of the structure of SC, were useful to determine the influence of penetration enhancers on SC structure (Bentley *et al.*, 1997). Confocal laser scanning microscopy (CLSM) was used to provide information about the *in vivo* penetration of a fluorescent label (fluorescein) across hairless mouse skin under the influence of GMO.

MATERIALS AND METHODS

Chemicals

Glycerol monooleate (GMO), progesterone (PG), fluorescein and type III trypsin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals used were of analytical grade; solvents used in HPLC were of HPLC grade.

Preparation of hairless mouse SC samples for the pre-treatment and *in vitro* permeation studies

Abdominal full-thickness skin was excised from male, one month old, HRS/J-Jackson Laboratories, Bar Harbor, ME hairless mice; subcutaneous fat and connective tissue were removed using forceps. The SC was prepared by floating abdominal full-thickness skin for 14 h on a water solution containing 0.1% (w/v) trypsin and 0.5% (w/v) sodium bicarbonate at room temperature. The mushy epidermis was removed by rubbing with moistened cotton tipped applicator. The transparent SC sheets obtained, were briefly rinsed with distilled water, blotted dry and kept in a desiccator until ready for use. SC sheets were examined in optical microscope in order to verify the presence of holes. Samples presenting holes were not used in the experiments.

Pre-treatment procedure

Hairless mouse SC sheets were hydrated by floating on phosphate buffered saline (pH 7.2) with stirring, for 3 h at 37 °C. During this period, a formulation consisting of 20% (w/v) GMO in mineral oil was placed over the SC. Only mineral oil was also studied. After 3 h, the SC sheets were rinsed with ethanol (50% v/v) and used for *in vitro* experiments. SC sheets without pre-treatment were used as controls. For each formulation, 10 hairless mouse SC sheets were used.

In vitro permeation studies

The *in vitro* permeation study was carried out at 37 °C, using ten modified Franz-type diffusion cells assembled with a hairless mouse SC sheet mounted between the donor and acceptor chambers. The receptor solution was 10% (v/v) ethanol in distilled water, changed at each sampling time to maintain sink conditions. The donor solutions [1 mL of saturated PG (infinite dose) in mineral oil containing 20% (w/v) GMO] were applied on upper surface of SC non-occlusively. Controls without GMO was also tested. Samples from the receptor phase were withdrawn at predetermined times and analysed by HPLC. When pre-treated hairless mouse SC sheets were used, the donor solutions were 1 mL of saturated solutions of PG in mineral oil.

HPLC assay

Analyses of all samples of the *in vitro* permeation studies were performed according to the method proposed by Pereira *et al.* (2000). A Shimadzu Instruments HPLC System, UV detector at 254 nm, C_{18} reversed-phase column 125 mm x 4 mm (5 mm), C_{18} pre-column 4 mm x 4 mm (5 mm) was used. A methanol:water (70:30) mixture was used as the mobile phase, at a flow rate of 1 mL/min and an injection volume of 20 μL. The extraction was carried using chloroform. Medroxyprogesterone was the internal standard. The retention times were 8.0 min and 10.0 min for the internal standard and progesterone, respectively. The detection sensitivity of this HPLC method for progesterone was 300 ng/mL, with less than 1% intra-day variation, and less than 3% inter-day variation.

Scanning electron microscopy (SEM)

Freshly excised abdominal skin samples (~1 cm², n=6) from hairless mice were incubated for 2 h at room temperature in 50% (v/v) ethanol aqueous solution containing 20% (w/v) GMO. After fixation with 3% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 h and post-fixation with 1% (w/v) osmium tetroxide for 2 h, the samples were dried using increasing concentration of ethanol, coated with gold and viewed under a Jeol JSM Scanning Electron microscope. Approximately 10 skin samples were studied for each formulation. Control skins treated only with 50% (v/v) ethanol water solution were also tested.

Confocal laser scanning microscopy (CLSM)

Formulations containing 100 μg/mL of the fluorescent label (fluorescein) and 20% (w/v) GMO in a 50% (v/v) ethanol water solution were applied on the dorsal region of the mice and left for 3 h. The controls were untreated skin samples or skin treated with formulations without GMO. After the treatment, the animals (n=6) were sacrificed by cervical dislocation and treated skin areas removed. A mechanical cross-section (perpendicular series) was made from the skin samples. The samples were embedded in a matrix, frozen at –17 °C and sectioned at 40 μm thickness. To avoid interference by fluorescence from damaged cells, the mechanical cross-section of the skin was examined by CLSM, 10 μm below the cutting surface (De Rosa *et al.*, 2000). A krypton-argon laser line at 488 nm was used for excitation; emission was detected at 530 nm. Confocal Microscope LEICA-DMIRBE, software LEICA TSCNT 1.5.451, equipped with Kripton-Argon laser and a 16X immersion objective was used. To investigate the autofluorescence properties of the skin, samples were first observed in the absence of fluorescein. The autofluorescence of hairless mouse skin was found to be very low for the confocal settings used in this study. Because fluorescein is not chemically similar with PG, it was used only as fluorescent probe for visualization of GMO effect in the skin.

Statistical analysis

Statistical comparison was made using the non-parametric Kruskal-Wallis test and Dunn's multiple range test with the help of an SAS program. The level of significance was taken as $P< 0.05$.

RESULTS AND DISCUSSION

In this work the potential use of GMO/solvents systems for PG transdermal delivery, a lipophilic model drug, was investigated. The influences of GMO in mineral oil and pre-treatment on the *in vitro* percutaneous absorption profiles of PG across hairless mouse SC are shown in Figure 1. A linear relationship was obtained when the total amount of progesterone in the receptor phase was plotted against time. GMO/mineral oil systems

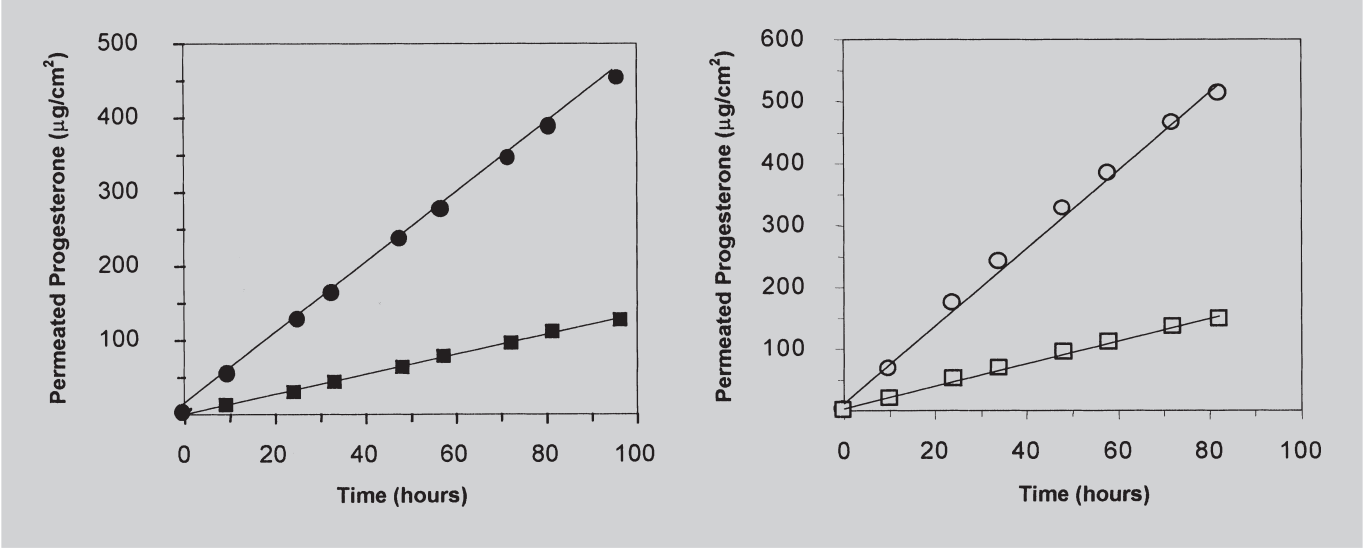

FIGURE 1 - *In vitro* permeation of progesterone across hairless mouse SC: (a) from (●) 20% (w/w) GMO in mineral oil (■) mineral oil (control); (b) following the pre-treatment with (○) 20% (w/w) GMO in mineral oil (□) mineral oil (control). SC: stratum corneum; GMO: glycerol monooleate.

increased the PG flux with or without the pre-treatment with these systems (Table 1). The linear relationship observed in the Figure 1 suggests that the percutaneous transport of PG from GMO in mineral oil across hairless mouse SC followed zero order kinetics. PG fluxes were significantly greater ($P< 0.05$) than the control. The increase of PG permeation can be mainly ascribed to the enhancer effect of GMO/mineral oil system and not to the increase of the drug solubility in the system, since saturated preparations of PG were used (Table I). In addition, the pre-treatment experiments showed that GMO/mineral oil systems might have caused changes on the hairless mouse SC, which provoked an increase on the PG flux (about 4.5 times compared to controls). Since the permeability of lipophilic drugs through human skin has been found to be slightly higher than in hairless skin (Morimoto *et al.*, 1991), a greater permeation rate might be expected in man. The profound hydration effect on skin or SC under *in vitro* experimental conditions must also be considered (Scheuplein *et al.*, 1969). Nevertheless, our results are still very useful, because tissue is hydrated easily, and more under occlusion, than in most drug transdermal administrations (patch) (Guy *et al.*, 1987).

The mechanism of topical delivery involves mainly the direct transfer of drugs to the lipid phase of the SC (Golden *et al.*, 1987). The SC lipids are arranged in multiple bilayers providing alternate hydrophobic and hydrophilic barriers (Abraham *et al.*, 1995). In general, routes of skin penetration are classified into two pathways, polar and non-polar in the intercellular domain. In a study of the action of enhancers on transdermal delivery, Ogiso *et al.* (1995) observed that GMO and oleic acid increased the flux across skin of a lipophilic drug (indomethacin) and of a hydrophilic drug (urea), and also the fluidity of SC lipids.

Fatty acids are the most abundant lipids in biological membranes, where they exist in free form but also as components of more complex lipids such as ceramides, triglycerides and phospholipids. Administration of exogenous free fatty acids, mainly of the *cis*-unsaturated variety, has been reported to increase membrane permeability (Potts *et al.*, 1991; Tanojo *et al.*, 1997; Gao, Singh, 1998). GMO has a similar structure to oleic acid, with a *cis*-unsaturated double bound in the molecule. It was initially proposed that the presence of *cis* double bounds introduces an accentuated flexion of the hydrocarbon chain, which prevents the formation of well-ordered compact crystals (Golden *et al.*, 1986). Results obtained by attenuated total reflectance infrared spectroscopy ATIR suggest that the action of oleic acid could be due to two mechanisms, lipid fluidity and lipid phase separation (Tanojo *et al.*, 1997). It has been proposed that lipids like oleic acid and GMO which have a polar head and a carbon chain presenting a low melting point increase membrane permeability by promoting intercellular lipid disorder (Ogiso *et al.*, 1995) and interactions between a hydroxyl group of GMO and the anionic oxygen in the polar head of phospholipids (Maggio, Lucy, 1976).

Morphological methods for visualization of the structures of SC such as SEM are useful for the determination of the structural changes caused by different types of penetration enhancer and the impact of different types of vehicle on SC membrane structure (Bentley *et al.*, 1997; Pflucker *et al.*, 1999). In fact, the present SEM studies showed some changes occurred in the intercellular space among the corneocytes for all skin samples treated with GMO formulation (Figure 2).

The corneocytes appear in their characteristic polygonal shape and seem to be intact, and judging from the intercellular spaces, to lie very close to each other (Figure 2a and 2b). Treatment with GMO/ethanol aqueous solution caused a change in the intercellular regions; junctions between the cells were loosened, leading to increased cell separation (Figure 2c and 2d). Considering the resolution characteristics of SEM technique, it can be

TABLE I - Influence of GMO/mineral oil on *in vitro* permeation of progesterone across hairless mice stratum corneum*

Formulation	Progesterone concentration** (mg/mL)	Flux ($\mu g/cm^2.h^{-1}$)	Flux after pre-treatment*** ($\mu g/cm^2.h^{-1}$)
20%(w/w) GMO in mineral oil	3.5	4.7 (± 0.1)	6.3 (± 0.32)
Mineral oil (Control)	1.2	1.4 (± 0.07)	1.8 (± 0.08)

* Data refer to means ± SEM of 10 experiments. Non parametric Kruskal-Wallis statistical analysis: $P< 0.05$ significant; ** Saturated PG solutions; *** Pre-treatment period: 3 h; formulation applied after pre-treatment: saturated PG solutions. GMO: glycerol monooleate.

suggested the effect of GMO on the SC junction, which is mainly formed by lipids. Because of the atoxicity of GMO, even for food use, and the constant turn over of the skin *in vivo* these changes caused by GMO should not be considered a problem for human use.

CLSM has been used to localize the transport pathways of the macromolecules and fluorescent labels in the skin after electroporation, iontophoresis and application of liposomes and absorption enhancers, to assess the effect of these methods on increasing (trans) dermal and transmucosal transport (Marttin *et al.*, 1997; Van Kuijk-Meuwissen *et al.*, 1998; Kirjawainen *et al.*, 1999; Lombry *et al.*, 2000). The CLSM images parallel to the surface of the skin provide information about the distribution pattern of the fluorescent marker in the SC. In this way, the penetration profiles of the label into the skin can be compared after application of different formulations. In order to obtain information about the penetration of a fluorescent label into deeper layers of the skin, cross-sections perpendicular to the skin were required and subsequently collecting of CLSM images parallel to the plane of these sections.

In the present study fluorescein was used only as a fluorescent probe and no longer can be related to PG penetration. The information that CLSM gives is only about the effect of GMO on the skin, altering its barrier

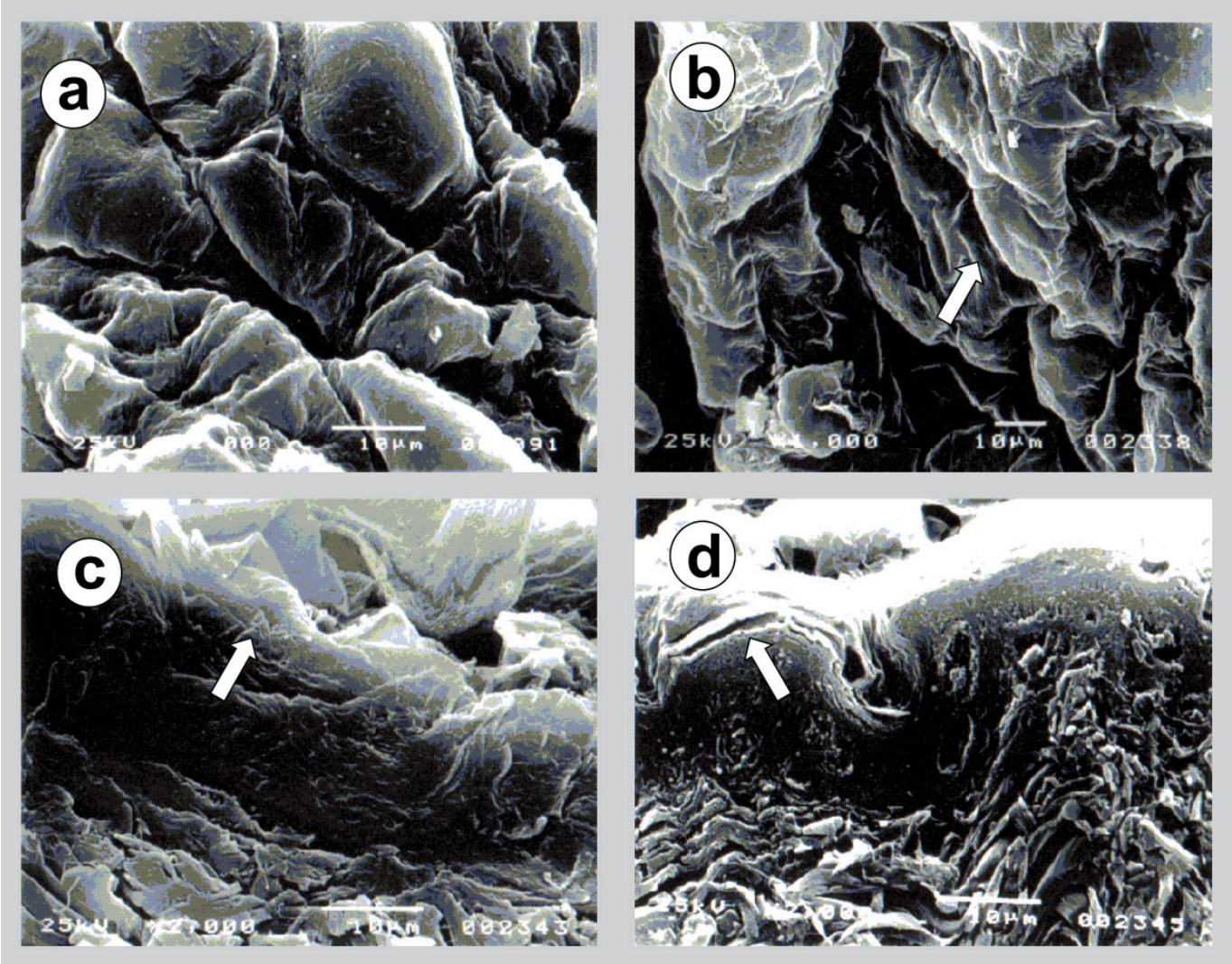

FIGURE 2 - Scanning electron micrographs of hairless mouse SC: (a) surface of SC control (2,000X); (b) surface of SC treated with 20% (w/v) GMO in 50% (v/v) ethanol water solution (2,000X); (c) transverse section of SC control (2,000X); (d) transverse section of SC treated with 20% (w/v) GMO in 50% (v/v) ethanol water solution (4,000X). Bar indications represent 10 mm. SC: stratum corneum; GMO: glycerol monooleate.

effect. As shown in Figure 3, the extent of penetration of fluorescein into the skin after 3 h was visualized by CLSM. This method resulted in images in which the SC, viable epidermis and dermis are visualized in the same focal plane, without a decrease in intensity due to intervening layers of tissue.

Only a slight fluorescent band was observed on the surface of the skin after treatment with fluorescein in the absence of GMO (Figure 3b). When GMO was presented in the formulation, fluorescence spread deeply reached the viable epidermis and dermis (Figure 3c). Confocal microscopy studies showed, therefore, that skin penetration of fluorescein increased remarkably after treatment with GMO.

CONCLUSIONS

Lipids have been studied as potential absorption enhancers for transdermal drug delivery. Considering the non-toxicity of GMO even for internal use and *in vitro* permeation enhancement found in this and other work (Ogiso *et al.*, 1995), it can be proposed as a promising skin absorption enhancer. Additionally, our results of SEM and CLSM in animal model provided understandings about the effects of GMO on the SC and *in vivo* fluorescent probe penetration into the skin, respectively. The present data, while needing further validation by experiments on human skin indicate the possibility of using GMO as an absorption enhancer. Further studies, now in progress, will

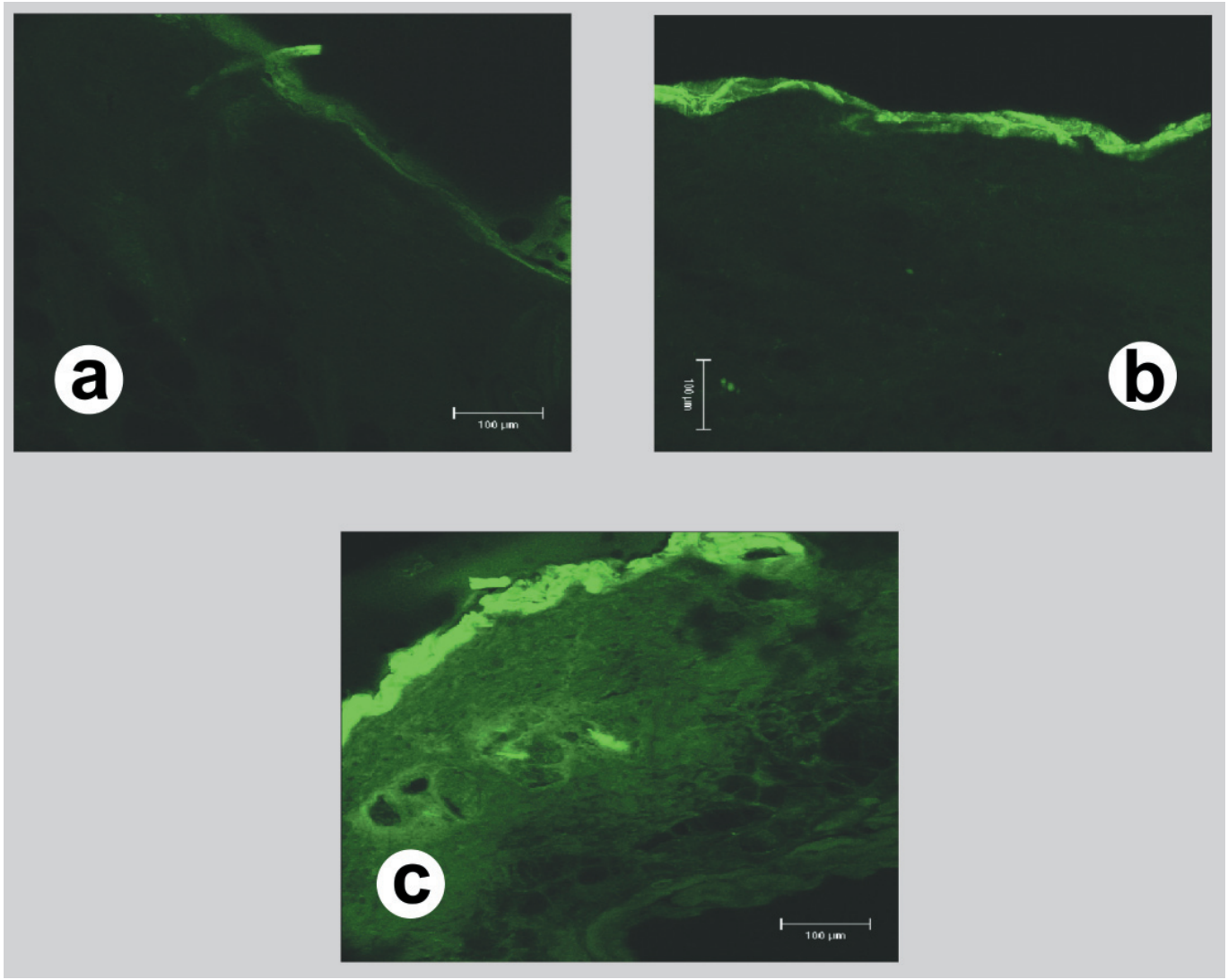

FIGURE 3 - Confocal images of mechanical cross-sections (perpendicular series) of hairless mouse skin, optically sectioned 10 mm below the cutting surface: (a) control (untreated skin); skin treated with: (b) 50% (v/v) ethanol water solution (control): (c) 20% (w/w) GMO in 50% (v/v) ethanol water solution. Both formulations contained 100 mg fluorescein/mL. Bar indications represent 100 mm. SC: stratum corneum; GMO: glycerol monooleate.

be addressed to the evaluation of absorption enhancer effect of GMO for drugs with a range of lipophilicity as well as to elucidate the GMO concentration and time of application influences in this effect by *in vitro* permeation and *in vivo* confocal microscopy studies.

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Maria Dolores Seabra Ferreira and Mrs. Márcia Sirlene Zardin Graeff (Department of Morphology, Faculty of Medicine of Ribeirão Preto, University of São Paulo) for assistance with SEM and CLSM studies, respectively. The authors are also thankful to FAEPA. This research was supported by FAPESP, Brazil.

RESUMO

Sistemas monoleína/solventes para a liberação transdérmica da progesterona: estudos de permeação cutânea e microscópicos

A liberação transdérmica de muitos fármacos é dificultada pelas características de barreira do estrato córneo. Promotores químicos de absorção cutânea são capazes de interagir com os constituintes do estrato córneo, induzindo aumento temporário e reversível na permeabilidade da pele. O objetivo deste trabalho foi avaliar a influência de sistemas monoleína (monoleato de glicerol)/solventes na absorção percutânea de um fármaco lipofílico (a progesterona), através do estrato córneo de camundongos sem pelo, bem como o efeito da monoleína nas características estruturais do estrato córneo, por meio de microscopia eletrônica de varredura (SEM) e microscopia de varredura confocal a laser (CLSM). As alterações morfológicas observadas no estrato córneo de camundongos sem pelo sugerem efeito da monoleína na barreira da pele. E, ainda, o aumento no fluxo in vitro *da progesterona, bem como na penetração* in vivo *do marcador fluorescente (fluoresceína), apontam a monoleína como potencial promotor de absorção cutânea. Os resultados obtidos mostraram que os sistemas monoleína/solventes provocaram alterações na estrutura do estrato córneo, que poderiam causar o aumento da permeação da progesterona através da pele de camundongos sem pelo, otimizando, deste modo, a liberação transdérmica deste fármaco.*

UNITERMOS: Monoleína. Promotor de absorção cutânea. Microscopia eletrônica de varredura. Microscopia de varredura confocal a laser. Permeação in vitro.

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Recebido para publicação em 12 de setembro de 2001.