

Surfactant-based Transdermal System for Fluconazole Skin Delivery

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Abstract

The development of a controlled-release dosage form of antifungals is of crucial importance in view of the side-effects of conventional oral and intravenous treatments of Sporotrichosis. In this study, systems composed of polyoxypropylene (5) polyoxyethylene (20) cetyl alcohol (PPG-5-CETETH-20) as a surfactant, oleic acid as an oil phase, and water were developed as a possible fluconazole transdermal drug delivery system. The systems were characterised by polarised light microscopy (PLM), SAXS, and rheological analysis, followed by cellular and histological analyses, *in vitro* release assays, and *ex vivo* skin permeation and retention studies using porcine ear tissue and a Franz diffusion cell. PLM and SAXS results indicated that the mixtures of surfactant, oil and water formed micellar and lamellar phases. The incorporation of fluconazole in these systems was greater than in water and conventional dosage forms. Micellar systems behave as Newtonian fluids, being more viscous than elastic in rheological analysis, and lamellar phases behave as pseudoplastic fluids with high elastic moduli. *In vitro* and *in vivo* biological assays showed that the formulations did not affect normal cell macrophages and did not promote skin irritation. The release profile indicated that fluconazole could be released in a controlled manner. It was found that the systems enhanced drug permeation and skin retention by changing only the composition of the components in the formulations. Therefore, PPG-5-CETETH-20-based systems have great potential as transdermal systems with different structural and rheological characteristics for Sporotrichosis treatment using antifungal drugs.

Keywords: PPG-5-CETETH-20; Transdermal drug delivery system; Sporotrichosis treatment; Fluconazole; Phase behaviour; Surfactant systems; Liquid crystals

Introduction

Sporotrichosis is the most prevalent subcutaneous mycosis and is caused by a dimorphic fungus, *Sporothrix schenckii*, which has been isolated from soil, decaying vegetation, plants, timber, hay, and moss. The disease usually begins after trauma that inoculates the microorganism into the skin and subcutaneous tissue [1]. Sporotrichosis has a worldwide distribution, although it is more frequent in tropical and subtropical areas with warm and humid climates. In South America, the estimated annual incidence is 48 to 60 cases per 100,000 population [2].

Several modalities have been used to treat cutaneous Sporotrichosis, such as local hyperthermia, cryotherapy, and antifungals (potassium iodide, itraconazol, amphotericin B, terbina and fluconazole), which are mainly administered by intravenous or oral route for a systemic effect [2,3]. However, most of them cause side-effects, including gastric irritation, diarrhoea, nausea, vomiting and stomach pain, hypothyroidism, fever, chills, headache, impairment of renal function, and anaemia [3].

One strategy for avoiding gastrointestinal side effects is transdermal delivery [4]. There are several instances in which the most convenient drug intake method (the oral route) is not feasible, and thus, alternative routes must be investigated [5]. Although the intravenous route may avoid gastrointestinal side-effects, it is invasive, and few patients are compliant. These disadvantages have encouraged research on alternative strategies, and the transdermal route has great potential for optional drug delivery [5]. Transdermal drug delivery not only avoids the digestive system, it also effectively reduces the applied dose [4].

Drug diffusion through the skin may limit the drug action of transdermally delivered drugs. Surfactant systems such as microemulsions and liquid crystals have great potential as topical

vehicles. Surfactants are able to stabilise mixtures of components with different polarities, thus creating additional regions for hydrophilic and lipophilic solubilisation of drugs. The drug can be protected against the dissolution medium due to the interfacial membrane, which is composed of the surfactant layer that the drug must pass through to be released. In this manner, surfactant systems also confer reservoir properties. The phase behaviour of surfactants enable the formation of microemulsions and liquid crystals, which is extremely attractive for nanotechnology because these structures are organised on the nanometric scale. In addition to the advantage of increased stability, their production can be scaled up easily. Varying the composition and proportions of surfactant, oil, and aqueous phase, it is possible to control the viscosity and texture of the systems above, which could lead to the immobilisation or localisation of the formulation at specific sites in the body and routes of administration. Therefore, surfactant systems have great potential as transdermal nanostructured drug delivery systems [6].

Recently, our research group has demonstrated that the surfactant polyoxypropylene (5) polyoxyethylene (20) cetyl alcohol (PPG-5-CETETH-20) is capable of stabilising systems that form nanostructures, including microemulsions and several liquid crystalline mesophases,

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such as lamellar, hexagonal, and cubic phases, depending on the proportion of the components and the oily phases [6]. This capacity has been exploited as a technological platform to develop new drug delivery systems. Zidovudine (AZT)-loaded PPG-5-CETETH-20 systems were shown to enhance the permeation of the drug across the nasal mucosa in *ex vivo* tests employing a diffusion chamber and excised porcine nasal mucosa. The *in vivo* results of the nasal administration of AZT-loaded PPG-5-CETETH-20 systems in rats revealed fast absorption ($C_{\max} = 6.67$ min) compared to commercial oral formulations ($C_{\max} = 0.5\text{--}1.5$ h) [7]. Another study of a Propolis microparticle-loaded PPG-5-CETETH-20-based system designed for periodontal pocket administration showed a prolonged release (more than 7 days) [8].

Therefore, the objective of this study was to develop and characterise PPG-5-CETETH-20-based systems for transdermal administration of antifungals. Fluconazole was used as the drug model because it is a triazole antifungal agent with broad-spectrum activity. It is effective for deep seated mycosis caused by *Candida*, *Cryptococcus* spp., and others and also for superficial infections caused by dermatophytes such as *Microsporum* and *Trichophyton* spp [9].

The systems were characterised by polarised light microscopy (PLM), small-angle X-ray scattering (SAXS), and rheological measurements. The safety was also tested using an *in vitro* cellular assay and *in vivo* histopathological analysis. In addition, *ex vitro* studies were conducted to examine the extent of the binding of fluconazole-loaded PPG-5-CETETH-20-based systems to the skin and their transdermal behaviour by *in vitro* permeation experiments using porcine ears and a Franz diffusion cell.

Methodology

Materials

The surfactant PPG-5-CETETH-20 is available commercially as Procetyl AWS (Croda, Campinas, Brazil). Oleic acid was acquired from Synth (Diadema, Brazil), and fluconazole was acquired from Purifarma, São Paulo, Brazil. High-purity water was prepared with a Millipore Milli-Q plus purification system.

Preparation of the formulations

The formulations were prepared by manual stirring using PPG-5-CETETH-20 as a surfactant, oleic acid as an oily phase, and water as an aqueous phase in a beaker. The proportions of the constituents are indicated in Table 1. The formulations were prepared 24 h before the experiments and left at room temperature to allow the system to reach complete equilibrium. Fluconazole was incorporated by dissolving the drug powder directly into the systems.

Evaluation of the fluconazole solubility in the formulations

The solubility of fluconazole in the formulations was investigated by adding crescent specific amounts of the drug powder (0.01% w/w) to 2 g of each system and to their isolated components. The mixtures

were manually homogenised using a glass rod and maintained at room temperature for 24 h. This procedure was repeated until precipitation was observed. Loaded samples were named AO1F, AO2F, AO3F, and AO4F.

Fluconazole was analysed by HPLC, with the UV detector set at 210 nm (Varian Pro Star 330). A reverse-phase Varian Chromsep C18 column (250×4.6 mm i.d., 5 µm pore size) was used. The mobile phase was a mixture of methanol/water (1:1 v/v), the flow rate was set at 1 mL/min, and the injection volume was 20 µL. A calibration curve was constructed by preparing working solutions of fluconazole in the mobile phase at concentrations ranging from 20 to 400 µg/mL.

Samples were prepared by diluting the formulation into the mobile phase to obtain a drug concentration of 150 µg/mL. The dilution was cleaned up by solid phase extraction (SPE) using 500 mg per 3 mL C18 cartridge. The SPE procedure included conditioning the samples with 1 mL of the mobile phase. The spiked samples (2.5 mL) were then loaded into the conditioned cartridge, and the analytes were collected, filtered through 0.45 µm-pore membranes, and injected into the HPLC system.

Studies of phase behaviour by polarised light microscopy (PLM) and small-angle X-ray scattering (SAXS)

PLM was performed in an optical Jenamed 2, Carl Zeiss (Jena, Germany) microscope. The isotropic or anisotropic behaviour of the samples was observed, and pictures were taken at a 20,000 × magnification. Samples were prepared by placing a drop of formulation between a cover slip and a glass slide. They were then examined under polarised light at room temperature.

SAXS data were collected at the Synchrotron SAXS beam line of the National Laboratory of Synchrotron Light (LNLS, Campinas, Brazil), equipped with an asymmetrically cut and bent Si (1 1 1) monochromator ($\lambda = 1.608$ Å) that yields a horizontally focused beam. A vertical position-sensitive X-ray detector and a multichannel analyser were used to record the SAXS intensity, $I(q)$, as a function of the modulus of the scattering vector q ($q = (4\pi/\lambda)\sin(\epsilon/2)$), with ϵ being the scattering angle. The samples were placed in a cell at 25°C. The parasitic scattering produced by the slits was subtracted from the total scattering intensity.

Rheological studies

The rheological measurements were taken with a controlled stress Rheostress RS1 rheometer (Haake, Karlsruhe, Germany) and analysed with Rheowin 3.5 software. Plate-plate geometry was used with a gap of 200 µm between plates 35 mm in diameter. Samples were carefully applied to the lower plate, ensuring that sample shearing was minimised, and were allowed to equilibrate for at least 3 min prior to analysis.

The flow properties were determined with shear rates within the range of 0.01–100 s⁻¹, which was chosen on the basis of the strength of resistance to the applied stresses. The rheological measurements were

	Composition (w/w)			Fluconazole solubility (mg/g)
	Oleic acid	PPG-5-CETETH-20	Water	
AO1	20	40	40	24.43 ± 0.99
AO2	30	40	30	26.34 ± 1.00
AO3	40	40	20	29.78 ± 1.68
AO4	50	40	10	26.28 ± 2.08
Fluconazole solubility (mg/g)	4	15	36	

Table 1: Composition, phase behaviour, and fluconazole solubility of the studied formulations.

performed on both the up and down curves. The flow curves were fitted to a power law model using the program Origin 7.0. All rheological determinations were carried out at $25 \pm 0.25^\circ\text{C}$.

Oscillatory analysis of each sample was performed after determination of its linear viscoelastic region at 25°C , where stress was directly proportional to strain and the storage modulus remained constant. Frequency sweep analysis was performed over the frequency range of 0.1–10 Hz at a constant stress of 1 Pa. The systematic error in the frequency of the rheometer was approximately 0.01 Hz.

Safety evaluation of the formulations

In vitro unspecific cytotoxicity: Mammal cytotoxicity of the formulations was studied *in vitro* using J-774 mouse macrophages as the cellular model. Cells were seeded at a density of $2.5\text{--}10.0 \times 10^5$ cells/well in 96-well flat bottom microplates (Nunclon) and exposed for 48 h to different doses of the formulations and free-drug (18.6, 10, 5 and 1 μM) for control. After treatment, the compounds were removed, and the cells washed once with PBS. Cell viability was then colorimetrically assessed by measuring the mitochondrial-dependent reduction of MTT to formazan. For this purpose, the cells and MTT (0.4 mg/mL) were incubated in air at 37°C for 3 h. After the incubation period, the supernatant was removed, and formazan crystals were dissolved with DMSO (180 μL). The plates were shaken for 10 min, and the optical densities were measured at 560 nm in a multiwell spectrophotometer. Each concentration was assayed three times, and six additional controls (cells in medium) were used in each test. Data were exhibited in per cent of cellular viability.

In vivo evaluation:

Animals: During the experiment, the animals were maintained in accordance with the guidelines established by Olfert et al. [10] and the Statement of Principles adopted by the Federation of American Societies for Experimental Biology (FASEB) Board. The animals had free access to food and water. Twenty-four hours before the start of treatment, areas of 4 cm x 5 cm were outlined on the back of each animal. The raw materials and formulation AO3F were tested. Each experimental group consisted of five rats. The animals were given the following treatments: oleic acid (OA), PPG-5-CETETH-20 (PROC), formulation AO3F, benzalkonium chloride (BC) solution 15% (positive control, C+), and propanol (negative control, C-). All areas, except the control area, were subjected to a daily treatment with the indicated formulations for 21 days, always at the same time, accompanied by frictional circular massage.

Histological procedures: The animals were anesthetised with xylazine hydrochloride / ketamine hydrochloride and sacrificed in a CO_2 chamber, after which the areas of treated skin were removed, fixed in Bouin's solution for 48 hours, rinsed, and treated routinely for embedding in paraffin (Histosec, Merck). Five non-serial histological sections (5 μm) were cut for each slide, with three slides per treatment area. The sections were stained in hematoxylin and eosin (HE) for histomorphometric and histopathological analyses.

Histomorphometric analysis was performed by capturing images of random cuts through an optical microscope Leica DMZ 2000 connected to a computer with the software Motic Images Advanced. Thirty areas were randomly chosen, and the thickness of the epidermis and dermis for each treatment area was measured. From these measurements, a correction for the micrometre scale was calculated for an objective of 20x, which yielded the mean thickness and standard deviation for each experimental group.

Histopathological analysis consisted of counting the number of leukocytes and fibroblasts using a Leica DMZ 2000 optical microscope connected to a computer with the software Motic Images Advanced 3.2. For each experimental group, three slides with 5 nonserial sections per slide were obtained. Six areas of $250 \mu\text{m}^2$ of skin per experimental group, always in the region of the papillary dermis, were measured. Therefore, each treatment comprised a total area of $30,000 \mu\text{m}^2$ per animal. From these results, the mean and standard deviation for each cell type was determined for each treatment.

Statistical analysis was performed using the Kruskal-Wallis statistical test, followed by the Wilcoxon-Mann-Whitney test with Bioestat version 5.0. The level of significance adopted was 5% ($p < 0.05$).

Proof of concept of the formulations by drug release, drug skin permeation, and drug skin retention studies

These studies comprise drug release, skin permeation, and skin retention assays [11]. The loaded-formulations and loaded-oleic acid containing fluconazole were tested at 10 mg/mL.

In the drug release study, a 45 μm -pore size cellulose acetate membrane was used to separate the formulations from the release medium. For drug permeation and retention studies, porcine ear skin obtained from healthy 6-month-old Brazilian pigs of a local slaughterhouse and prepared for the test as described elsewhere [12] were used. The ears were cleaned with water ($25 \pm 0.5^\circ\text{C}$), and the ears with injury were discarded. The undamaged skins were removed from the cartilage with a scalpel, and a 500 μm thick layer stratum corneum and epidermis were separated from the adipose tissue with a dermatome (Nouvag TCM 300, Goldach, USA). The prepared skins were frozen at -20°C and stored no longer than 4 weeks. On the day of the experiment, the skin was thawed in physiological saline solution containing 0.9% (w/v) NaCl (Merck, Germany) at $25 \pm 0.5^\circ\text{C}$ for 30 min; then, its hair was cut with a scissor.

The cellulose acetate membranes or skin pieces were placed between the donor and receptor chambers of a Franz diffusion cell (Microette Plus, Hanson Research, Chatsworth, EUA) equipped with diffusion cells with an exposition area of 1.77 cm^2 for formulation deposition. The receptor chambers of the cells (7 mL) were filled with 10 mM monobasic buffer (pH 7.4) stirred at 300 rpm. The samples (400 mg) were dropped on the donor chambers in contact with the receptor chambers. The total test time was 24 h, and 2 mL of the receptor fluid was collected and replaced by an equal volume of fresh receptor solution. The assays were carried out on six replicates for each sample.

Fluconazole was quantified by HPLC using the same conditions described for the fluconazole solubility study. A calibration curve was constructed by preparing working solutions of fluconazole in 10 mM monobasic phosphate buffer (pH 7.4) at concentrations ranging from 1 to 250 $\mu\text{g}/\text{mL}$. Samples of collected receptor fluid were filtered and injected into the HPLC system.

The results are represented by a graph of the cumulative mass of the drug that passes through a unit area of the membrane or skin versus time, calculated according to equation 1:

$$Q = C_i \times V_r + \sum V_c \times C_c \quad (1)$$

where Q ($\mu\text{g}/\text{cm}^2$) is the total amount of drug in the receptor fluid up to time t , C_i ($\mu\text{g}/\text{mL}$ cm^2) is the concentration measured at time t , V_r (mL) is the volume of receptor solution (7 mL), C_c ($\mu\text{g}/\text{mL} \cdot \text{cm}^2$) is the concentration at the previous sampling, and V^c (mL) is the volume sampled.

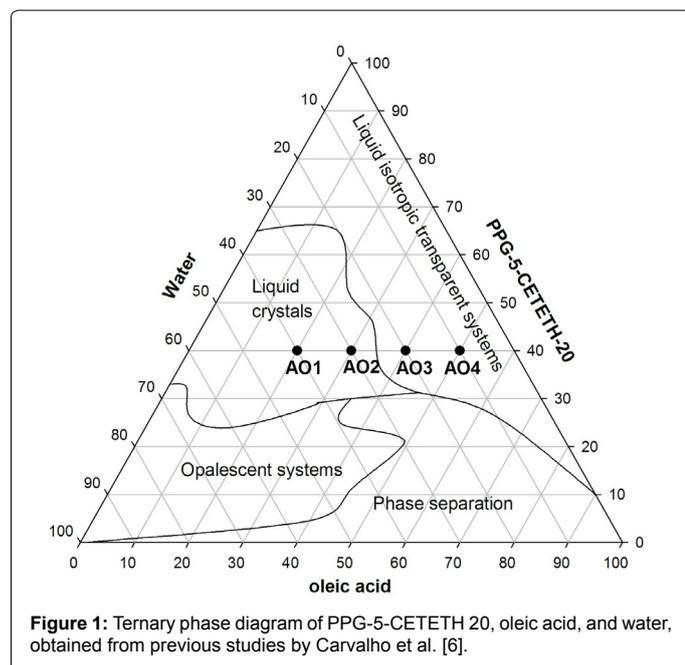


Figure 1: Ternary phase diagram of PPG-5-CETETH 20, oleic acid, and water, obtained from previous studies by Carvalho et al. [6].

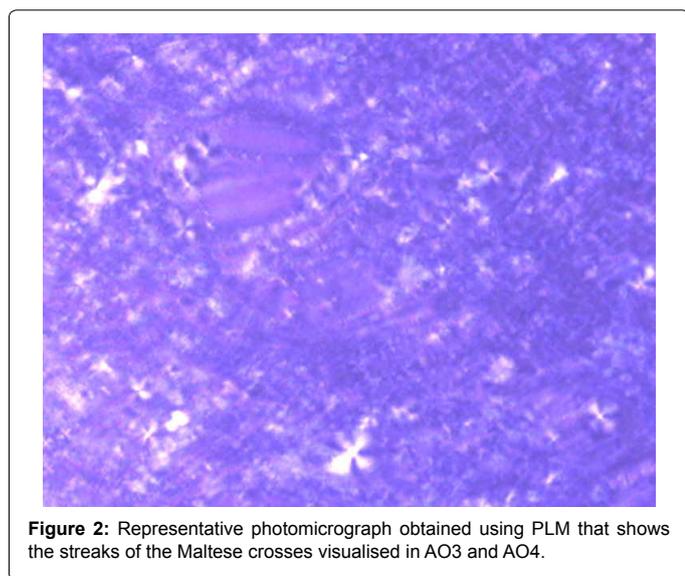


Figure 2: Representative photomicrograph obtained using PLM that shows the streaks of the Maltese crosses visualised in AO3 and AO4.

For retention studies, the tape stripping method [13-15] was performed. The skins derived after 6 h and 12 h of permeation assay were cleaned using soft paper. The stratum corneum was removed from sixteen tapes (Scotch 750, 3M), and retained fluconazole was evaluated by solvent extraction. The tapes were placed in an assay tube containing 5 mL of methanol and vortexed for 2 min. Then, the sample was immersed in an ultrasonic bath for 30 min. The solvent was filtered through a 0.45 μm membrane and injected into the HPLC system. The remaining skin was cut into small pieces, placed in a tube containing 5 mL of methanol, and tested according to the same procedure.

Results and Discussion

Preparation of the formulations

The composition of the formulations was chosen based on previous studies in which the ternary phase diagram of oleic acid, PPG-5-

CETETH-20 and water mixtures was obtained (Figure 1). For this study, thermodynamically stable systems, characterised by their spontaneous formation, transparency and high stability, were selected. Samples AO1 and AO2 correspond to semisolid transparent systems, and AO3 and AO4 correspond to liquid and transparent systems. Their compositions are given in Table 1. For AO1-4, the concentration of the surfactant was fixed at 40% (w/w), whereas the other component concentrations were varied. These compositions were chosen to avoid the translucent dispersion and phase separation areas.

Evaluation of fluconazole solubility in the formulations

The incorporation of fluconazole into the individual oil, surfactant, water, and formulations was measured, and the results are displayed in Table 1. The formulations incorporated more drug than water and surfactant but less drug than oleic acid by itself. These results confirm the lipophilic character of fluconazole. It is worth highlighting that the formulations incorporated more fluconazole than conventional topical pharmaceutical formulations (10 mg/g) due to the mixture of components with different polarities, namely, oil and water, in the presence of a surfactant that forms aggregates and allows additional regions of drug solubilisation.

Phase behaviour study by PLM and SAXS

The semisolid samples (AO1 and AO2) were anisotropic under polarised light, which is characteristic of liquid crystals that are birefringent as a result of their molecular ordering. The images obtained by PLM for AO1 and AO2 are shown in Figure 2, in which Maltese crosses characteristic of lamellar phases can be seen.

The transparent liquid systems investigated (AO3 and AO4) showed a dark background under PLM, and thus, they are isotropic systems that are characteristic of micellar systems, microemulsions, or cubic phases. Cubic phases are well known by their high stiffness, and AO3-AO4 flowed easily. Therefore, they may not be organised structurally as a mesophase.

The phase behaviour of the systems was confirmed by the SAXS measurements. The data that were collected from the scattering profiles were plotted as intensity, I , versus the scattering vector module, q (\AA^{-1}). The resulting curves are shown in Figure 3.

Surfactants can form different aggregate types, which can be identified by the peak positions on the scattering vector axis. From the scattering profile of loaded and unloaded AO1-AO2 formulations, two peaks were observed, possibly because of a degree of structural organisation. The ratio between the first and second peak is 2:1, which is characteristic of lamellar liquid crystals [16]. This result correlates with the PLM experiments because Maltese crosses were visualised for the AO1 and AO2 systems. For the AO3 and AO4 formulations, a maximum intensity value of $q \neq 0$ followed by a long tail was observed, which is associated with short-range 3D spatial correlation and may be characteristic of micellar structures [17].

Rheological studies

The flow properties of these systems are displayed graphically in Figure 4, in which the shear stress is plotted versus the shear rate. In the continuous shear rheometry assay, both unloaded and loaded AO3-AO4 systems exhibited Newtonian behaviour. This result was expected because the samples consisted of isotropic liquid systems. The curves of AO1-AO2 showed a nonlinear relationship between shear stress and shear rate, thus exhibiting a non-Newtonian flow characteristic. These results are in accord with literature data showing that liquid crystalline phases are found to exhibit shear-thinning behaviour.

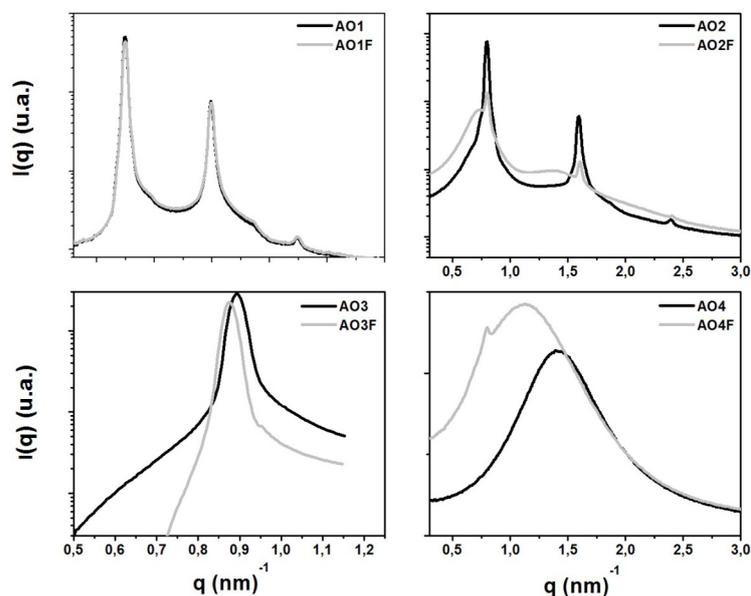


Figure 3: SAXS patterns of samples of fluconazole-loaded (AO1F-AO4F) and unloaded (AO1-AO4) formulations.

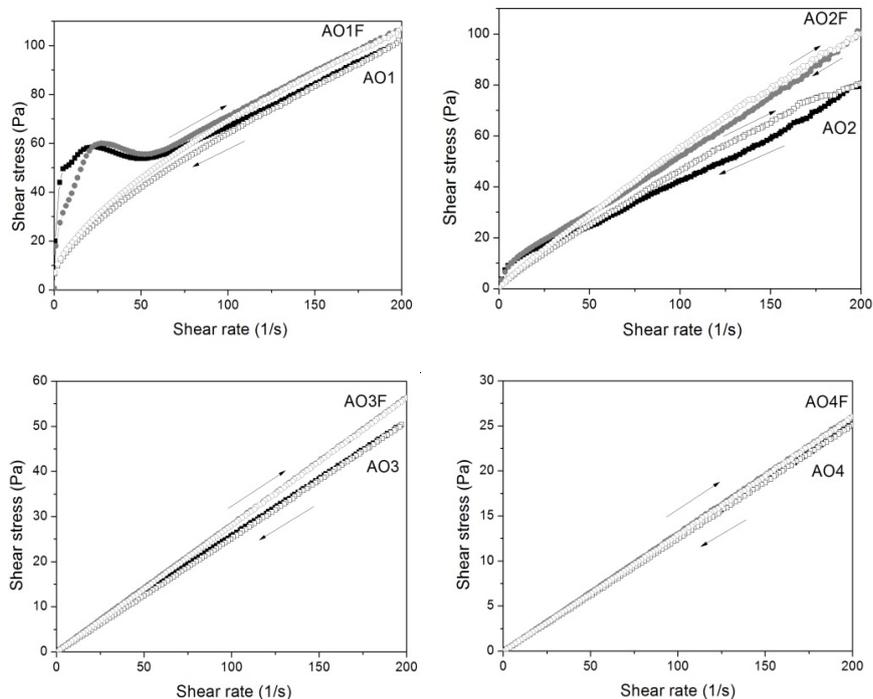


Figure 4: Continuous shear rheometry assay of fluconazole-loaded (grey symbols) and unloaded formulations AO1-AO4 (black symbols). The closed symbols represent upward flow curves, and the open symbols represent downward flow curves.

The oscillatory rheological data are displayed in Figure 5 as the storage modulus (G') and loss modulus (G'') as a function of the oscillatory frequency. The storage modulus is a measure of the energy stored and recovered per deformation cycle and represents the solid-like component of a viscoelastic material. The storage modulus is large when a sample is predominantly elastic or highly structured. The loss

modulus is a measure of the energy dissipated per cycle and represents the liquid-like component [18]. The frequency sweep analyses suggest that AO3-AO4 are more viscous and less elastic at the selected frequency because $G'' > G'$ and because both moduli are frequency dependent. For AO1-AO2, the G' values were high and relatively independent of frequency. The slopes of the lamellar phase formulations (AO1-AO2)

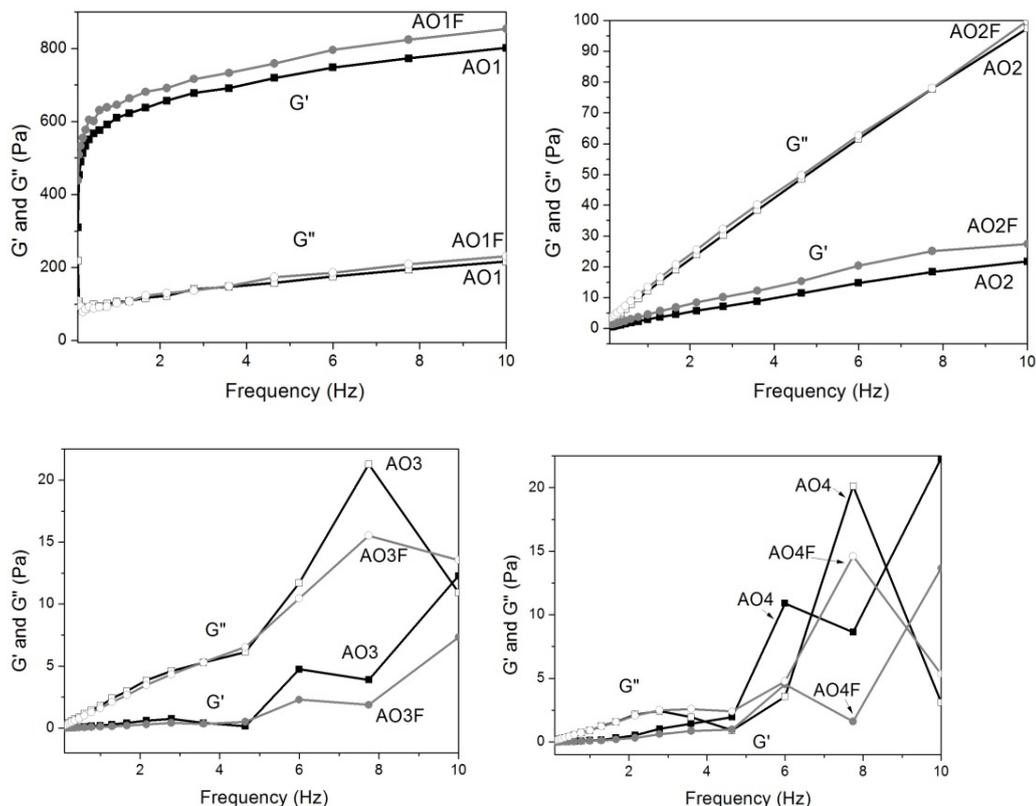


Figure 5: Frequency sweep profile of the storage modulus (G' – closed symbols) and loss modulus (G'' – opened symbols) of fluconazole-loaded (grey symbols) and unloaded formulations AO1-AO4 (black symbols) at 25°C.

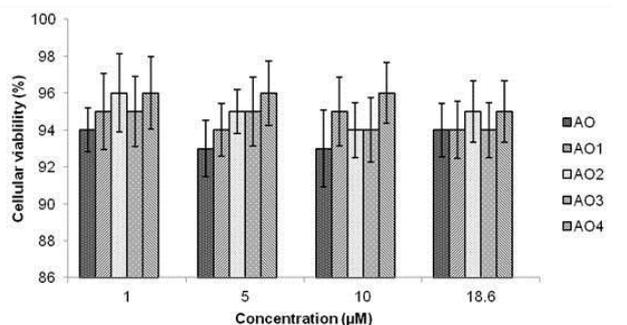


Figure 6: Cellular viability of unloaded formulations (AO1, AO2, AO3, AO4) and oleic acid (AO).

were lower than the slopes of the liquid isotropic systems AO3-AO4, indicating a more structured character for the lamellar phases.

Safety evaluation of the formulations

In vitro unspecific cytotoxicity: *In vitro* unspecific cytotoxicity studies of the formulations were performed using J-774 mouse macrophages as the cellular model. The data (Figures 6 and 7) were exhibited in per cent of cellular viability. The assay results of cellular viability showed that the unloaded samples (AO, AO1, AO2, AO3, AO4) and loaded samples (AOF, AO1F, AO2F, AO3F, AO4F) were unable to kill normal cell macrophages. All samples exhibited cellular viability greater than that 92%, and therefore, it is suggested that the formulations and the drug do not present toxic activity or cytotoxicity.

In vivo evaluation:

Histomorphometric analysis of the epidermis and dermis: The results for the thickness of the epidermis and dermis are presented in Table 2. Analysis of variance (ANOVA) verified a normal distribution of data, and the experiment followed a completely randomised design. The F test was used to detect differences between treatments. The means of groups were compared, two by two, by the Tukey test.

The data suggest that the groups AO, PPG-5-CETETH-20, and formulation AO3F caused significantly higher thickening of the epidermis and dermis than the control (Figure 8). This result suggests an increase of skin hydration that would be revealed by an increase in the interstitial spaces. It is known that some cosmetic formulations, with or without active substances, may promote skin hydration, to the benefit of the skin [19].

According to Libardi [20], what makes the skin stay healthy and soft, with good flexibility and elasticity is the maintenance of skin hydration and the ability of the organism not only to stimulate cell renewal but also to synthesise components of the epidermis. For good functioning of the mechanism of skin hydration, the stratum corneum should be able to retain water so that its rate of evaporation is limited to a normal level. Proksch et al. [21] claim that skin hydration is intrinsically related to the integrity of the stratum corneum.

The properties of occlusion and moisturising of the components were observed for the formulation AO3F, characterised as a micellar system. The occlusive activity conferred by nano-systems has been cited in other works such as Brinon et al. [22] Those authors suggest that this

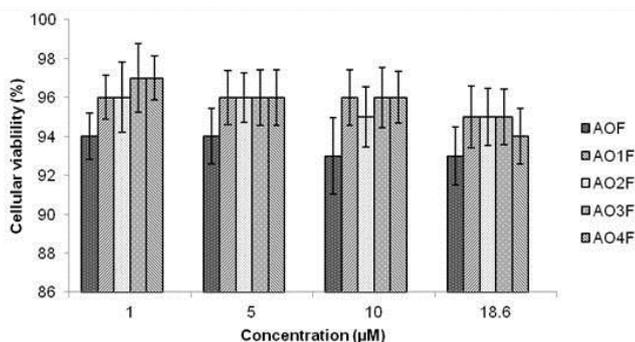


Figure 7: Per cent cellular viability of fluconazole-loaded formulations (AO1F, AO2F, AO3F, AO4F) and loaded oleic acid (AOF).

Group	Epidermis (µm)		Dermis (µm)		Number of fibroblasts		Number of leukocytes	
	Average	SD	Average	SD	Average	SD	Average	SD
S	32.92	1.66	1136.76	37.20	12.47	2.30	2.6	1.65
AO	35.56	5.79	1097.83	47.64	12.27	2.30	1.87	1.53
AO3F	30.24	3.63	1014.82	66.79	12.67	2.59	0.27	0.52
C+	37.58	3.00	1269.11	53.04	7.83	1.68	6.10	3.00
C-	23.95	1.62	672.95	51.48	9.10	1.65	0.67	0.88

Table 2: Thickness of the epidermis and dermis (µm). Number of fibroblasts and leukocytes in 250 µm² of the papillary dermis in each experimental group. Key: (S) PPG-5-CETETH-20, (AO) oleic acid, (AO3F) fluconazole-loaded tested formulation, (C+) positive control, (C-) negative control, (SD) standard deviation.

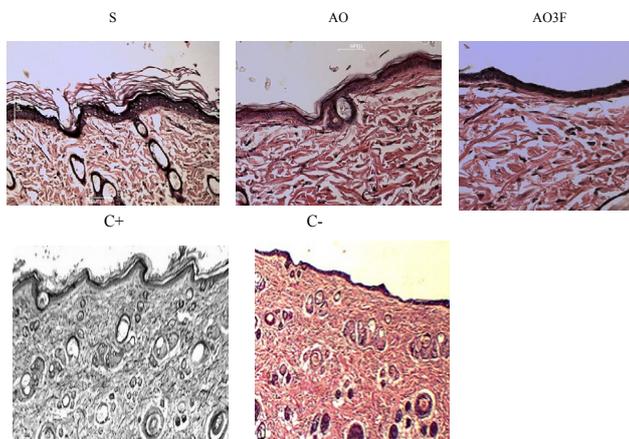


Figure 8: Photomicrographs of sections of the epidermis and dermis of rats in the following treatment groups (10x). S: PPG-5-CETETH-20 surfactant; AO: oleic acid; AO3F: sample; C+: positive control; C-: negative control.

action is related to the characteristic colloidal structure of the emulsified system, in which the organisation of the surfactant interfacial layer is similar to that of the lipid bilayers that join keratinocytes, increasing cohesion and, consequently, the ability to form a continuous film [23].

Histopathological analysis of the dermis: The presence of fibroblasts and leukocytes in larger amounts during skin irritation can be used to predict irritation. The number of fibroblasts and leukocytes counted in the papillary dermis of the various experimental groups is given in Table 2. By ANOVA, the distribution of the data for the number of fibroblasts and leukocytes per area was found to be non-normal, so the Kruskal-Wallis test ($p < 0.05$) was used to analyse the results.

The most common adverse reactions of topical formulations is skin irritation, which can be seen as local intolerance and may elicit minor discomfort, as well as acute reactions that vary in intensity from

burning to itching. This irritation may lead to corrosion and tissue destruction. All of these reactions are restricted to the area in direct contact with the product. Generally, the degree of irritation is related to the concentration of an ingredient in the final product and the cosmetic formulation [24].

It can be said that irritation causes a skin condition similar to that produced by a lesion. Lesions in skin tissue are characterised as any events that impair the structure or function of the tissue. They produce changes in the ability of cells to perform their normal homeostatic functions, and they trigger various histochemical and biological processes that lead to vasomotor action to mobilise all the cells responsible for fighting infection and/or the offending agent. These processes are intended to replace losses of substances as quickly as possible and to restore the physiological balance and contribute to the survival of the organism [25].

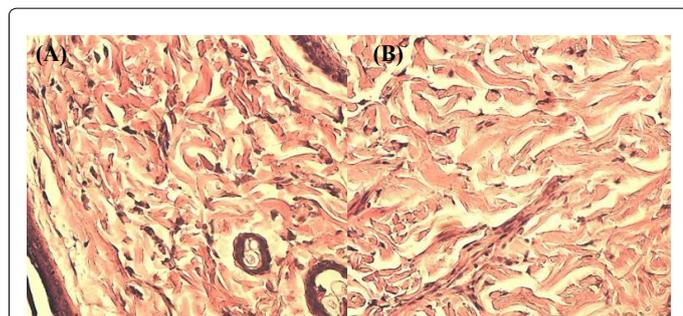


Figure 9: Photomicrographs showing the papillary dermis in areas: (A) C-; (B) picture representative of the treatments with S, AO, AO3F and C+, where a greater number of fibroblasts are seen in the dermis (1000x).

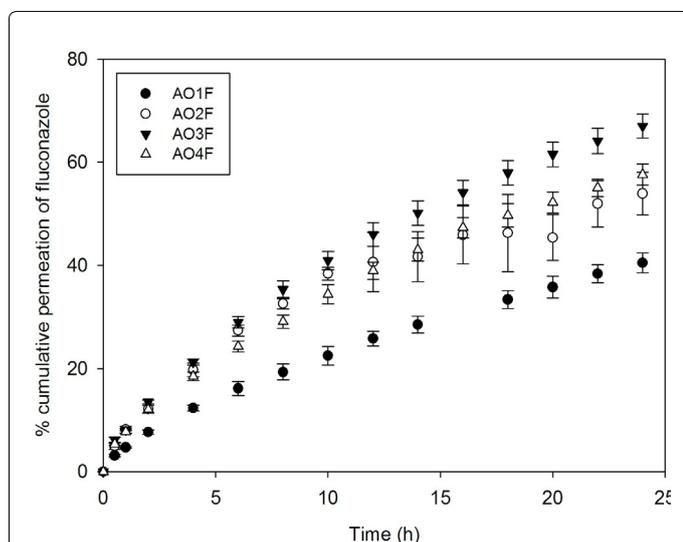


Figure 10: Drug release profile of fluconazole-loaded AO1F-AO4F formulations, 10 mg/mL (n=6).

At an early stage, an irritation process releases inflammatory mediators and mobilises a sequence of cells, especially neutrophils and lymphocytes, to combat the offending agent or to start the immune processes that facilitate healing [26]. Inflammatory mediators act as chemotactic signals and growth factors for the inflammatory cells, which are represented by polymorphonuclear leukocytes (PMNs), macrophages, and lymphocytes. These cells also release growth factors for endothelial cells, fibroblasts and keratinocytes. The release of all of these factors can lead to the formation of granulation tissue [27].

Although the results of the leukocyte count of PPG-5-CETETH-20 and oleic acid were higher than those obtained for the control, these counts still remain low. Importantly, these results may have occurred through the use of pure raw materials in the animal's skin.

The results obtained for the formulation AO3F were not significantly different from those obtained for the control group, suggesting no occurrence of inflammatory processes when administering a formulation containing 40% oil, 40% surfactant and 20% water.

Other promising data are related to the count of fibroblasts. It was observed that the number of fibroblasts was significantly higher for the various experimental groups than for the control (Figure 9). This result indicates that the formulations may also improve properties related to

skin support and cell regeneration because these cells will originate collagen and elastin fibres.

The fibroblast is the main cell involved in the formation of granulation tissue, synthesising hyaluronic acid, fibronectin, collagens type I and III, elastin and proteases, such as collagenase [25]. The formulation AO3F increased the production of fibroblasts. This, in turn, increased the production of collagen, which is responsible for the strength and resistance of greater thickness and dermal trauma, promoting satisfactory skin restructuring [28,29].

Proof of concept of the formulations

The drug release assay enables *in vitro* analysis of drug movement across a membrane using a two-compartment model. The donor compartment contains the formulation, and a non-rate-limiting membrane separates the compartments and supports the dose. The amount of fluconazole released was expressed as a percentage, which was plotted against time in Figure 10. In addition, the pseudo-steady-state flux (J , $\mu\text{g}/\text{min cm}$) derived from Fick's law was calculated as the gradient of the linear portion of the drug release curve [30]. The results are listed in Table 3.

This study revealed that AO3F-AO4F released more fluconazole than AO1F-AO2F. This phenomenon most likely occurs because the latter formulations present a liquid crystalline network structure with higher elasticity than that of AO3F-AO4F. The less ordered AO3F-AO4F systems with reduced viscosity allowed a higher degree of mobility of drug molecules in their matrices.

The OA sample showed the highest value of flux (J), followed by AO3F. The other formulations did not present a significant difference. Because the AO sample presents no structural organisation, such as the liquid crystalline samples or the micellar system, the drug was released faster. The flux of AO3F could have been higher due to its position in the phase diagram of Figure 1. This sample was placed in a phase transition area, and therefore, its structure may not control drug release as much as the AO1F, AO2F or AO4F systems. AO1F was shown to prolong drug release to the greatest extent. This result may be due to the highest elastic modulus in AO1F, which increases the stiffness of the liquid crystalline matrix. In addition to the differences between the drug release rates, all formulations released fluconazole in a controlled manner because an exponential pattern governed drug diffusion, as seen in the curves of Figure 10. Therefore, this study showed that structural modifications of surfactant systems can modify the rate and profile of drug delivery.

Permeation studies were performed in Franz diffusion cells using the skin of porcine ear. The cumulative permeation of fluconazole incorporated into formulations AO1F-AO4F was calculated for 6 h and 12 h of experiment, as was the drug retention in the stratum corneum, dermis, and epidermis. The values obtained are listed in Table 3.

The sample AOF promoted the highest drug permeation, followed by AO4F, which contains the largest quantity of oleic acid among the other formulations. This result indicates the enhancing effect of oleic acid on the *in vitro* permeability of fluconazole. Oleic acid may reduce the lipid packing order in epithelial cell membranes. This effect could increase the fluidity of the lipid layer and decrease the diffusion resistance, thus assisting the permeation of compounds through the skin [31]. Therefore, AO4F may be explored for a systemic effect because it promoted drug transportation across the skin. This suggests that the drug may reach the blood circulation.

	Drug release		Drug permeation ($\mu\text{g}\cdot\text{cm}^2$)		Skin retention ($\mu\text{g}\cdot\text{cm}^2$)			
	J ($\mu\text{g}/\text{cm}^2$)	r^2	6 h	12 h	Stratum corneum		Epidermis+dermis	
					6 h	12 h	6 h	12 h
AOF	132.12	0.98	319.2 ± 37.2	673.6 ± 10.6	2.87 ± 0.31	9.79 ± 2.33	19.87 ± 8.39	13.43 ± 1.58
AO1F	52.39	0.97	0	0	0.61 ± 0.32	11.38 ± 2.56	18.58 ± 3.55	7.19 ± 1.52
AO2F	59.89	0.99	0	0	2.35 ± 0.12	2.15 ± 0.43	5.37 ± 0.41	6.96 ± 2.08
AO3F	71.09	0.98	0.4 ± 0.1	4.3 ± 1.4	2.44 ± 0.54	0.10 ± 0.4	5.42 ± 1.09	2.96 ± 1.14
AO4F	53.38	0.99	85.9 ± 28.4	130.3 ± 5.8	1.02 ± 0.37	9.60 ± 3.03	17.59 ± 1.23	7.59 ± 1.19

Table 3: Flux (J) and correlation coefficient (r^2) derived from drug release curves, drug permeated through the skin at 6 h and 12 h, and drug retained in the stratum corneum and in the epidermis + dermis at 6 h and 12 h. Data were collected for fluconazole-loaded formulations (AO1F, AO2F, AO3F, AO4F) and loaded oleic acid (AOF), all containing 10 mg/mL of drug. The values represent the average ± standard deviation of six replicates.

In contrast, even though AO1-AO3 had retained more drug in the epidermis plus dermis than in the stratum corneum barrier, there was no drug permeation for these systems, which leads us to conclude that these samples promoted skin retention. In comparing AO1F-AO3F, an increase in the drug concentration in the stratum corneum with time was observed. AO1F and AO2F are lamellar phases, but AO1F exhibited a higher elastic modulus, which is characteristic of a more structurally organised system. Therefore, it can be concluded that the higher organisation of the liquid crystalline structure of AO1F contributed to controlling drug release.

It is reported that the concentration of antifungal drug attained in the skin is an important factor in the treatment of dermatomycosis and that the presence of the therapeutically active form in the skin is closely related to the efficacy of the drug. However, this evidence was found for oral treatments [9]. Considering the topical application of the formulations studied here, it is possible to promote drug permeation or skin retention by changing only the composition of the components in the formulations. These results open the way to considering PPG-5-CETETH-20-based transdermal systems for antifungal skin delivery for Sporotrichosis treatment.

Conclusions

The findings of this work noted the great potential of PPG-5-CETETH-20-based transdermal systems for either systemic or local action because it was found that both formulations enhanced drug permeation and skin retention. *In vitro* and *in vivo* biological assays showed that the formulations did not affect normal cell macrophages and did not promote skin irritation. Therefore, the results presented here provide new possibilities for transdermal systems with different structural and rheological characteristics for the treatment of Sporotrichosis using antifungal drugs.

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