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Next-generation topical antifungal therapy: Biocompatible voriconazole nanostructured lipid carriers with enhanced skin penetration for cutaneous candidiasis

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ABSTRACT

Fungal infections caused by *Candida albicans* represent a growing global health concern. The increasing resistance to conventional antifungals and limitations of current topical formulations highlights the urgent need for more effective and targeted therapies. The main objectives of this work were to develop and evaluate voriconazole-loaded nanostructured lipid carriers (VCZ-NLCs) for treating cutaneous candidiasis. In this sense, four formulations (A-D) were prepared by the high shear homogenization and ultrasonication technique, differing mainly in polysorbate 80 concentration, phospholipid type and cholesterol presence. All the obtained VCZ-NLCs were spherical, small (<150 nm), relatively homogeneous (polydispersity indexes: 0.22–0.57), neutral or slightly negative charged and showed good entrapment efficiencies (>70 %). The Peppas-Shalin model exhibited the best fit to the release profiles, suggestive of a predominantly non-Fickian behavior. Formulation A, with the highest concentration of polysorbate 80 and cholesterol, gave rise to the most stable particles with the smallest mean diameter and polydispersity index values, and the highest entrapment efficiency, but exhibited a weak cytotoxicity and lower skin penetration than the other formulations (A: 0.94 % accumulated in the receptor compartment vs. B: 2.28 %, C: 2.25 % and D: 1.37 %). In contrast, formulations C and D, lacking cholesterol and with reduced concentration of polysorbate 80, were more biocompatible and showed deeper skin penetration, with 50 % of VCZ accumulated in 1 g of skin (epidermis and dermis). Antifungal susceptibility tests of *C. albicans* to VCZ resulted in an MIC_{50} value between 15 and 30 ng/mL. No significant differences were observed among formulations in antifungal activity. Overall, our findings suggest that VCZ-NLCs represent a promising strategy for the topical treatment of more invasive cutaneous candidiasis, with formulations C and D showing the greatest potential.

1. Introduction

An estimated 20–25 % of the global population is affected by fungal infections, with tropical regions experiencing a markedly higher burden due to favorable environmental conditions [1]. Candidiasis, predominantly caused by *Candida albicans*, remains one of the most prevalent fungal infections in humans, ranging from superficial mucosal infections to severe systemic disease. Reflecting its global clinical significance, *C. albicans* has recently been classified by the World Health Organization (WHO) as one of the four critical priority fungal pathogens for public health [2]. Despite the availability of a broad spectrum of antifungal agents, the efficacy of conventional treatments has been increasingly compromised by the emergence of drug-resistant strains. This growing resistance highlights the urgent need for alternative therapeutic strategies, including the use of new antifungal drugs and the development of novel drug delivery systems.

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Voriconazole (VCZ), a second-generation triazole antifungal agent, exhibits broad-spectrum activity and improved pharmacokinetic properties compared to earlier azoles [3]. However, its clinical utility in topical applications is limited by poor aqueous solubility and variable bioavailability [4], hampering its penetration into the deeper layers of the skin where fungal infections may persist.

Conventional topical formulations often fail to provide adequate drug delivery to the infection site, suffer from poor retention and tend to accumulate in the stratum corneum, reducing its effectiveness in the topical treatment of more invasive fungal infections [5]. In addition, these formulations may cause irritation or require high doses and frequent reapplication, reducing patient adherence and increasing the risk of both local and systemic toxicity [6].

Nanotechnology-driven drug delivery systems have recently emerged as promising strategies to overcome these limitations and enhance antifungal therapy performance [7–9]. Among these, nanostructured lipid carriers (NLCs) stand out as an innovative and versatile delivery system for the administration of different therapeutic agents, capable of addressing solubility, stability and release challenges of lipophilic drugs [10]. Composed of a blend of solid and liquid lipids, NLCs form an amorphous lipid matrix that accommodates higher drug loading and enables sustained and controlled release. This not only improves drug residence time in the skin but also enhances penetration through the stratum corneum [11,12]. The biocompatibility and biodegradability of NLCs further reduce the risk of local irritation, making them suitable for chronic or long-term use. In addition, NLCs form an occlusive film upon application, which increases stratum corneum hydration and facilitates drug permeation without disrupting the skin barrier. Their large surface area ensures intimate contact with the affected site, enabling more efficient drug delivery. From a technological perspective, NLCs also demonstrate superior physicochemical stability during storage, reducing the risk of drug expulsion and preserving formulation integrity over time, advantages that distinguish them from other nanosystems such as liposomes, nanoemulsions or polymeric nanoparticles [13]. Given their multiple advantages, NLCs have been used for the topical, dermal and transdermal administration of anti-rheumatoid [14], anti-inflammatory [15], anti-aging [16], anti-oxidant [17], anti-bacterial [18] and anti-fungal drugs [19], among others. Specifically, VCZ-loaded NLCs have been designed as targeted carriers for ocular [20], nail [21] and skin [22–24] drug delivery; however, none of these studies evaluated their antifungal activity against *C. albicans*. In addition, other azoles, such as fluconazole and luliconazole, have demonstrated improved transdermal delivery when formulated in NLCs [25,26]. Therefore, NLCs are considered a promising alternative for the formulation of the second-generation azole VCZ and for the treatment of cutaneous candidiasis.

In this sense, the aim of this study was to develop and characterize novel VCZ-loaded NLC formulations optimized for topical application, and to evaluate their physicochemical properties, skin compatibility and antifungal activity against *Candida albicans*. This approach seeks to establish a more effective and targeted therapeutic strategy for managing superficial fungal infections while addressing the challenges associated with conventional antifungal formulations.

2. Materials and methods

2.1. Materials

The following compounds were purchased from Sigma-Aldrich (Madrid, Spain): dimethyl sulfoxide (DMSO), Poloxamer 188, RPMI 1940 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt. Polysorbate 80 was obtained from Guinama (Valencia, Spain) and ethanol from PanReac Applichem (Barcelona, Spain). VCZ was provided by Glentham Life Sciences (Corshman, UK). Sabouraud dextrose agar (SDA), sodium hydroxide and acetonitrile (AcN) were obtained from VWR chemicals (Barcelona, Spain). 4-Morpholinepropanesulfonic acid (MOPS) and cholesterol came from Alfa Aesar (Kandel, Germany) and glucose and oleic acid from Thermo scientific (Madrid, Spain). Phospholipon® 90 G and Phospholipon® 90 H were provided by Lipoid GmbH (Ludwigshafen, Germany) and cetostearyl alcohol from Acofarma (Madrid, Spain). Cell medium, fetal bovine serum (FBS), penicillin, streptomycin, fungizone and all the other reagents for cell studies, unless otherwise specified, were purchased from Gibco (Paisley, UK).

2.2. Analytical method

VCZ was quantified by means of a high-performance liquid chromatography assay (HPLC) with ultraviolet (UV) detection at 255 nm. The HPLC equipment consisted of a quaternary pump SpectraSYSTEM P4000, an autosampler SpectraSYSTEM AS3000 and a spectrophotometric detector SpectraSYSTEM UV 6000LP. Data were processed through “Chromquest Chromatography Workstation Software Version 1.63”. The column was a Waters “Nova-Pack” C_{18} (4 μm, 3.9 mm × 150 mm), and the mobile phase consisted of a mixture of acetonitrile, water and 0.6 % trietylamine, pH 6 (35/65, v/v). The injection volume was 25 μL, and the flow rate was 1 mL/min.

2.3. Method validation

The calibration curves (peak area versus nominal concentration) were constructed using a least square linear regression analysis for the calculation of the slope, intercept, and correlation coefficient. The accuracy (bias) and precision (relative standard deviation; RSD) of the assay were determined from VCZ standards prepared at four concentrations (0.1, 1, 10 and 50 μg/mL).

The limit of detection (LOD) was estimated as the concentration of VCZ giving rise to a peak whose height is 10 times the signal-to-noise ratio. The lower limit of quantification (LLOQ) was determined as the concentration of the lower standard with an accuracy between 80 and 120 % and RSD within 20 %.

2.4. Preparation of NLCs

VCZ-loaded NLCs were prepared melting the solid lipids (cholesterol, Phospholipon® 90G or 90H and cetostearyl alcohol) using a water bath at 80 ± 1 °C. The liquid lipid (oleic acid) was dissolved in ethanol and placed into the melted lipid phase. The lipid phase and a solution of VCZ in ethanol were added to the hot aqueous phase comprised of water containing various amounts of Polysorbate 80 with two different syringes and dispersed using a high shear homogenizer (Ultra-Turrax T25, Janke & Kunkel IKA Labortechnik, Germany) at 8500 rpm for 3 min. Thereafter, an aqueous solution of Poloxamer 188 was added to the mixture with a syringe and homogenized for 2 more minutes, followed by magnetic stirring for 10 min at 200 rpm. Poloxamer 188 was added in this way to be absorbed by the particle surfaces. Formulations were sonicated with 2 cycles of 3 min (3 s on and 1 s off, 60 % and 45 % amplitude) with an ultrasonic disintegrator (CY-500, Optic Ivymen system, Barcelona, Spain) to homogenize the preparation. The mixture was then cooled whilst being stirred for 2 h at 200 rpm at room temperature. Finally, formulations were sterilized by filtration (CA syringe filter; cellulose acetate; 0.22 μm). The final concentration of VCZ in NLCs was 0.5 mg/mL. Empty NLCs were also prepared to assess the effect of VCZ on NLC assembly.

2.5. Characterization of NLCs

Mean diameter (MD) and polydispersity index (PI) were determined by Photon Correlation Spectroscopy using a Zetasizer nano (Malvern Instruments, Worcestershire, UK). The same equipment was also used to measure the zeta potential (ZP) by means of the M3-PALS (Phase Analysis Light Scattering) technique, which measures particle electrophoretic mobility.

Transmission electron microscopy (TEM) confirmed vesicle formation and morphology. The samples were stained with 2 % phosphotungstic acid aqueous solution and examined under a JEM-1010 (Jeol Europe, Paris, France) transmission electron microscope equipped with a digital camera AMT RX80 and the AmtV602 software, version 602.579 at an accelerating voltage of 80 kV.

The amount of VCZ loaded into the NLCs was evaluated by dialyzing an aliquot against water. Previously, an experiment was performed to verify that the dialysis membrane had no retention effect on VCZ and to determine the equilibration time between the inner and outer compartments of the dialysis tube. A 1 mL aqueous solution of VCZ (500 μg/mL) was placed inside the dialysis membrane (Spectra/Por® membranes, 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, the Netherlands). The membrane was immersed in 100 mL of water to ensure sink conditions and maintained under continuous stirring at room temperature (~25 °C) for 24 h. At predetermined time intervals, 0.5 mL samples were withdrawn from the external medium, mixed with 0.5 mL of AcN, and analyzed by HPLC to quantify the VCZ concentration. Equilibrium was considered achieved when the concentration remained constant in consecutive samples. To confirm this, an aliquot from the internal compartment was also analyzed at the end of the experiment. To verify that the dialysis membrane did not retain VCZ, samples were collected from both the inner and outer compartments at the equilibration time. The VCZ concentrations were then determined and the total amount of VCZ was calculated and compared with the initial amount introduced.

To determine the percentage of the free (unentrapped) drug, 1 mL of each formulation was transferred into the dialysis tube and dialyzed against distilled water (100 mL) under continuous stirring at room temperature. After 6 h, that was the time estimated for reaching the dialysis equilibrium, a sample of 0.5 mL of the exterior aqueous medium was taken and added to 0.5 mL of AcN. The mixture was injected into the HPLC to determine the concentration of VCZ in the external aqueous medium, which is assumed to be equal to the free (unencapsulated) concentration of VCZ in the NLCs. Additionally, the total concentration of VCZ inside the dialysis tube was quantified by HPLC after disruption of vesicles with AcN (1/10). The entrapment efficiency (EE) was calculated as the percentage of the VCZ concentration inside the NLCs (defined as the total VCZ concentration inside the dialysis tube minus free concentration of VCZ) versus that initially used, as was described by Usach et al. previously [27].

2.6. Stability of NLCs

The stability of VCZ-loaded NLCs was evaluated during storage at 4 ± 1 °C for a period of 3 months. Samples were collected at predetermined time points (0, 1, and 3 months) and analyzed for MD, PI and ZP.

2.7. In vitro drug release study

The release studies of the NLCs were conducted using the dialysis membrane method. 1 mL of NLCs was loaded into a Spectra/Por® 2 standard regenerated cellulose dialysis tube with an MWCO of 12–14 kDa and clipped by standard closures. The dialysis tube was immersed into 100 mL of distilled water with a magnetic stirrer stirring at 300 rpm. At 0, 1, 2, 3, 4 and 6 h, 0.5 mL of the medium were removed and replaced with an equal volume of distilled water. VCZ released amounts were determined by HPLC.

To determine the release kinetics of VCZ from NLCs, different commonly used mathematical models including zero order, first order, Higuchi, Korsmeyer–Peppas and Peppas–Sahlin were used. The values of the kinetic parameters were obtained by using Sigmaplot 10.0® (Systat Software, Inc., San Jose, CA, USA). The equations that represent each drug release model are the following:

$$\text{Zero order equation}: Q_t = Q_0 + K_0 \cdot t \quad (1)$$

$$\text{First order equation}: Q_t = Q_0 \cdot e^{-K_1 \cdot t} \quad (2)$$

$$\text{Higuchi equation}: Q_t / Q_\infty = K_H \cdot \sqrt{t} \quad (3)$$

$$\text{Korsmeyer} - \text{Peppas}: Q_t/Q_\infty = K_{K-P} \cdot t^n \quad (4)$$

$$\text{Peppas} - \text{Sahlin}: Q_t/Q_\infty = K_{P-S(1)} \cdot t^n + K_{P-S(2)} \cdot t^{2n} \quad (5)$$

where Q_t represents the amount of VCZ released over time t, Q_0 the initial amount of VCZ in the formulation, Q_t/Q_∞ the fraction released of VCZ and K_0, K_1, K_H are the VCZ release rate constants for zero-order, first-order and Higuchi release kinetics, respectively. $K_{K\text{-}P}$ and $K_{P\text{-}S(1)}$ are the diffusion constants, $K_{P\text{-}S(2)}$ is the relaxation constant and n is the exponent that characterizes the diffusion process. Thus for $n < 0.5$, the release is Fickian, between $0.5 < n < 1.0$, indicating that an anomalous process has occurred, while for $n = 1$, the release obeys zero-order kinetics [28].

2.8. In vitro cytotoxicity of formulations

Some 75 cm^2 flasks were used to let immortalized human keratinocytes (HaCaT, ATCC collection, Manassas, VA, USA) grow as monolayers using an incubator set at 37 °C, 100 % humidity and 5 % carbon dioxide and Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX with 1 g/L glucose and pyruvate, supplemented with 10 % of FBS and 1 % of penicillin and streptomycin (10,000 units/mL of penicillin and 10,000 g/mL of streptomycin) and 0.1 % fungizone as the growth medium. Cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well, incubated for 24 h and then treated with the VCZ in aqueous dispersion or loaded in NLCs properly diluted with the cell medium to achieve the desired concentrations (5, 50 and 5000 ng/mL). These concentrations were chosen to simulate the potential dilution that may occur *in vivo*, with 500 ng/mL considered a realistic upper limit for the amount of VCZ capable of effectively penetrating into the deeper layers of the skin [29]. After 48 h of incubation, the medium was replaced with an MTT solution in phosphate buffered saline (PBS, 0.5 mg/mL). Three hours later, the MTT solution was removed, the formed formazan crystals were dissolved with DMSO and the absorbance was measured at 570 nm using Multiskan EX (Thermo Scientific, Waltham, MA, USA). The experiments were repeated six times, each time in triplicate. The results are reported as cell viability (%) in comparison with non-treated cells (100 % viability).

2.9. Skin permeability studies

Skin permeability was assessed by quantifying both the drug retained in the different skin layers and the drug that permeated into the receptor compartment. To this end, VCZ in aqueous dispersion or loaded in NLCs was evaluated using vertical Franz-type diffusion cells with an effective area available for diffusion of 0.785 cm^2 and a receptor compartment capacity of approximately 6 mL (Vidrafoc, Barcelona, Spain). Donor chambers were filled with 500 μL of the formulations containing 0.5 mg/mL of VCZ and the receptor chambers with 0.9 % sodium chloride solution. A 600 μm thickness dermatomed porcine ear skin was placed between the two compartments. Pig ears were provided by the Faculty of Medicine, University of Valencia (Valencia, Spain) following the death of the animal used for other research purposes. The complete cells were placed in a thermostatic bath at 37 ± 1 °C, ensuring a skin temperature of 32 ± 1 °C, and continuously stirred. After 6 h, the VCZ content of both compartments was determined and cross sections of the skin (10, 40, 100 and 450 μm thickness) were prepared using a cryostat (Leica CM1950). Thereafter, the VCZ content was extracted from the skin and quantified by HPLC.

2.10. Microbial strains

The antifungal activity of VCZ both in aqueous dispersion and formulated as NLCs was tested against *Candida albicans* strain of Colección Española de Cultivos Tipo (CECT) 1394 (Valencia, Spain). Cultures were kept for 24 h at 36 ± 1 °C. After 24 h of incubation, the fungal suspensions were diluted with PBS in order to obtain an adequate density expressed as colony forming units per milliliter (CFU/mL).

2.11. Antifungal susceptibility of C. albicans to VCZ

The antifungal activity of VCZ against *C. albicans* was evaluated by performing the broth micro-dilution method, using 96-well microtitration plates.

The yeast suspension was made by an initial dilution with PBS followed by another in RPMI 1940 according to the protocol of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) [30]. The inoculum should be approximately 1-5 x 10^5 CFU/mL 100 μl of *C. albicans* inoculum were seeded in 96-well microtitration plates and treated with 100 μl of VCZ at different concentrations (2000, 1000, 500, 250, 120, 60 and 30 ng/mL). To obtain the desired concentrations, VCZ was first dissolved in DMSO (20 mg/mL) and subsequently diluted with water. In addition, a positive control was prepared in each test, in the absence of VCZ. The negative control consisted of RPMI 1940 without inoculum. The 96-well plates were incubated at 36 ± 1 °C for 24 h and the turbidity in each well was measured with a spectrophotometer (Hitachi U-2900, Tokyo, Japan) at a wavelength of 530 nm and compared with that obtained in positive growth control wells (with inoculum and without the assayed VCZ concentrations) to determine the minimum concentrations of VCZ required to inhibit the growth of microorganisms by 50 (MIC_{50}) and 90 % (MIC_{90}). This value can also be estimated visually thanks to the absence of turbidity in the cases of inhibition.

2.12. Growth curves of C. albicans

The procedure followed to carry out this type of test was described previously by our research group [31]. Briefly, vials with 500 μl of aqueous solutions of VCZ were used: 1000, 400, 200, 100, 10 and 0.1 μg/mL. All solutions were prepared from a stock solution of VCZ in DMSO (100 mg/mL). In addition, a negative control containing 500 μl of water was used in each assay. 500 μl of inoculum containing 1-5 x 10^5 CFU/mL was added to all vials.

These tests were also carried out with the different VCZ-based formulations. For the preparation of the 500 μl of the different formulations, a 1/5 dilution with sterile water of the initial formulations was made, which contained a concentration of 500 μg/mL. Thus, the concentration of VCZ in the diluted formulations was 100 μg/mL and the final concentration in the assay vials was 50 μg/mL. The assays also included a positive control composed of an aqueous dispersion of VCZ (50 μg/mL), in which maximal *C. albicans* inhibition was expected.

All vials were incubated at 36 °C ± 1, taking samples from each vial at 0, 6, 24 and 48 h. To collect them, 50 μL of each were diluted in 5 mL of PBS and seeded in Petri dishes with SDA. The plates were kept in an oven for 48 h and the colonies observed were counted.

2.13. Statistical analysis

Data are presented as mean ± standard deviation (SD). The Student's *t*-test was used for comparisons of two groups. One-way analysis of variance (ANOVA) was used for comparisons of more than two groups; when statistically significant differences were found, Tukey's test was applied to determine which groups were statistically different. P values of <0.05 were considered statistically significant. All calculations were performed with IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL).

3. Results

3.1. Assay validation

A linear relationship was found between the VCZ peak area and their concentrations in standards in the range of 0.1–100 μg/mL (Peak area = 145,072 · Conc (μg/mL) + 60,946, r > 0.999. LOD was approximately 0.02 μg/mL, and LLOQ was established at 0.1 μg/mL. Bias and RSD values of the method were lower than 7 % and 14 %, respectively.

3.2. Preparation of NLCs

A pre-formulation study was carried out to find the most adequate and promising formulation. NLCs were prepared by the high shear homogenization and ultra-sonication technique using different experimental conditions, types and concentrations of solid and lipid liquids, surfactants or co-solvents. Some of the problems found were the obtention of unstable formulations that precipitated in short time and high particle sizes, among others. Finally, four mixtures were selected as they seemed to be the most promising (Table 1), with the main difference being the type and concentration of phospholipid used (Phospholipon® 90G or 90H), the presence or absence of cholesterol and the concentration of polysorbate 80.

3.3. Characterization of NLCs

NLCs loaded with VCZ were visualized by TEM and it was observed that they were small in size, spherical shape and a monodisperse size distribution, indicating the reproducibility of the process (Fig. 1A and B). Empty formulations were also prepared in order to assess the effect of VCZ on NLCs assembly (Fig. 1C and D).

In the dialysis assay, the external concentration remained stable from 6 h until the end of the experiment (24 h); therefore, it was estimated that equilibrium was reached at 6 h. At this time point, the concentration inside the dialysis tube (5.0 μg/mL) was very close to that in the external medium (4.9 μg/mL), confirming the establishment of equilibrium. The total amount of VCZ recovered from both compartments was 99 % of the initial amount introduced in the dialysis tube, indicating minimal or negligible retention by the dialysis membrane.

The physicochemical properties of NLCs were evaluated measuring the mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) (Table 2). Formulation A containing the highest concentration of polysorbate 80 (17.5 mg/mL) gave rise to the particles with the smallest MD (110 nm) and PI (0.22) values. No significant differences in size were obtained by the incorporation of VCZ in NLCs. Moreover, formulations with cholesterol (A and B) showed higher amount of VCZ effectively incorporated (87.3 % and 77.7 %, respectively) than the formulations without this compound. All formulated NLCs exhibited a neutral surface charge, ranging between −10 and + 10 mV [32].

Table 1
Composition of VCZ loaded NLCs.

	Formulation			
Component (%)	A	B	C	D
Oleic acid	0.2	0.2	0.2	0.2
Cetostearyl alcohol	0.5	0.5	0.5	0.5
Phospholipon 90H	0.2	–	0.2	–
Phospholipon 90G	–	0.4	–	0.4
Cholesterol	0.02	0.02	–	–
Polysorbate 80	1.6	0.8	0.8	0.8
Poloxamer 188	0.5	0.5	0.5	0.5
Voriconazole	0.05	0.05	0.05	0.05
Ethanol	11.5	11.5	11.5	11.5
Water	88.5	88.5	88.5	88.5

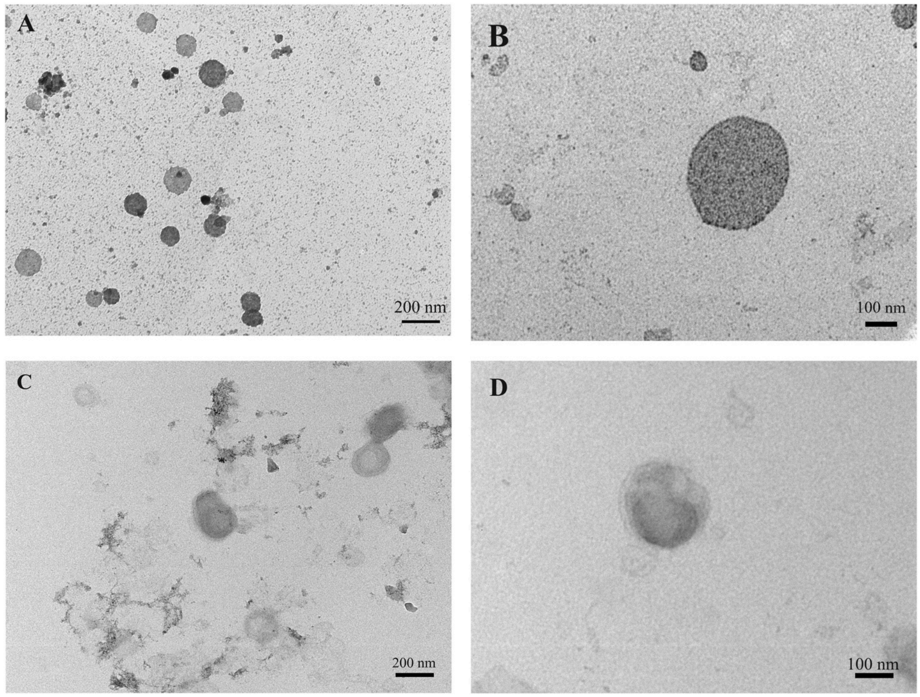

Fig. 1. Transmission electron microscopy (TEM) images of formulation A (A and B) and empty formulation A (C and D).

Table 2
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of empty and VCZ loaded NLCs. Each value represents the mean value ± standard deviation (SD) of three replicates (n = 3).

	MD (nm)	PI	ZP (mV)	EE (%)
Formulation A	110 ± 1^{a}	0.22	− 8.06 ± 1.28	87.3 ± 2.8^{a}
Empty Formulation A	108 ± 1^{a}	0.24	− 2.51 ± 0.05	/
Formulation B	137 ± 2^{b}	0.34	− 3.41 ± 0.68	77.7 ± 1.5^{a}
Empty Formulation B	145 ± 3^{b}	0.33	− 4.50 ± 0.21	/
Formulation C	148 ± 3^{b}	0.37	− 0.30 ± 0.02	70.2 ± 7.4^{b}
Empty Formulation C	140 ± 2^{b}	0.45	− 5.92 ± 0.28	/
Formulation D	135 ± 3^{b}	0.56	− 4.95 ± 0.22	74.7 ± 1.4^{b}
Empty Formulation D	141 ± 2^{b}	0.57	− 3.18 ± 0.51	/

The same superscript letter indicates values that are not statistically different ($p > 0.05$).

3.4. NLCs stability

The stability of NLCs over time was evaluated storing them at 4 °C for 3 months and measuring their MD, PI and ZP at scheduled intervals (Fig. 2). The MD of formulations A and D remained unchanged during the first month. However, it increased significantly in the third month, reaching 127 and 161 nm, respectively. In contrast, the MD diameter of formulations B and C was significantly larger after one month of preparation. The PI of the stored formulations ranged from 0.23 to 0.55, with the highest heterogeneity observed in formulations C and D (Fig. 2A). ZP did not undergo major changes during the entire storage period, remaining in the neutral range (Fig. 2B).

3.5. Release studies

The percentages of VCZ released as a function of time from the formulations assayed are shown in Fig. 3. About 100 % of VCZ was released from NLCs in 6 h. VCZ release from formulation A was the slowest, with 56 % released after 2 h, with this percentage being around 70–75 % in the case of formulations B, C and D. To describe the release kinetics, zero-order, first-order, Higuchi, Korsmeyer–Peppas and Peppas–Shalin models were tested and compared (Table 3). Among the applied models, the Peppas-Shalin model exhibited the best fit with the experimental data, giving rise to the highest correlation coefficient values (0.9989–0.9997). As can be observed in Table 3, the *n* values obtained were equal or higher than 0.5 in all cases. Therefore, the non-Fick or anomalous diffusion ($0.5 < n < 1$) process was dominant [28]. Moreover, as can be seen from the results, the relaxation rate constants ($K_{P\text{-}S(2)}$) have much lower values than the diffusion rate constants ($K_{P\text{-}S(1)}$), suggesting that the matrix nature has a relative importance compared to the Fick diffusion.

3.6. Biocompatibility of formulations

The viability of the human keratinocytes (Fig. 4) incubated for 48 h with VCZ in dispersion or loaded in NLCs was, in general, higher than 85 % (value calculated versus untreated cells, with 100 % of viability), confirming high formulation biocompatibilities [33]. However, this percentage was reduced to 73 % in the case of the lowest dilution of formulation A indicating a weak cytotoxicity, with statistical differences with the dispersion at all the dilutions tested. The VCZ in dispersion was used as a reference with the aim of understanding the effect of both the drug and vehicles on cell viability.

3.7. Skin permeability of formulations

Percentages of VCZ present in the remaining formulation in the donor compartment, in the skin and accumulated in the receptor compartment at the end of the transdermal absorption study are summarized in Table 4. No statistically significant differences between formulations and dispersion were obtained when the percentage of VCZ load on the skin was compared, probably because the skin was saturated with VCZ. However, the penetration of formulation A through the skin to the receptor compartment was significantly lower than dispersion. The study of the drug distribution across skin layers revealed differences between the dispersion and formulations C and D. In these formulations, VCZ was distributed more homogeneously, reaching the deepest layers of the dermis. This highlights a greater drug load in both the epidermis and dermis, which may represent a more effective reservoir for the treatment of more invasive fungal skin diseases (Fig. 5).

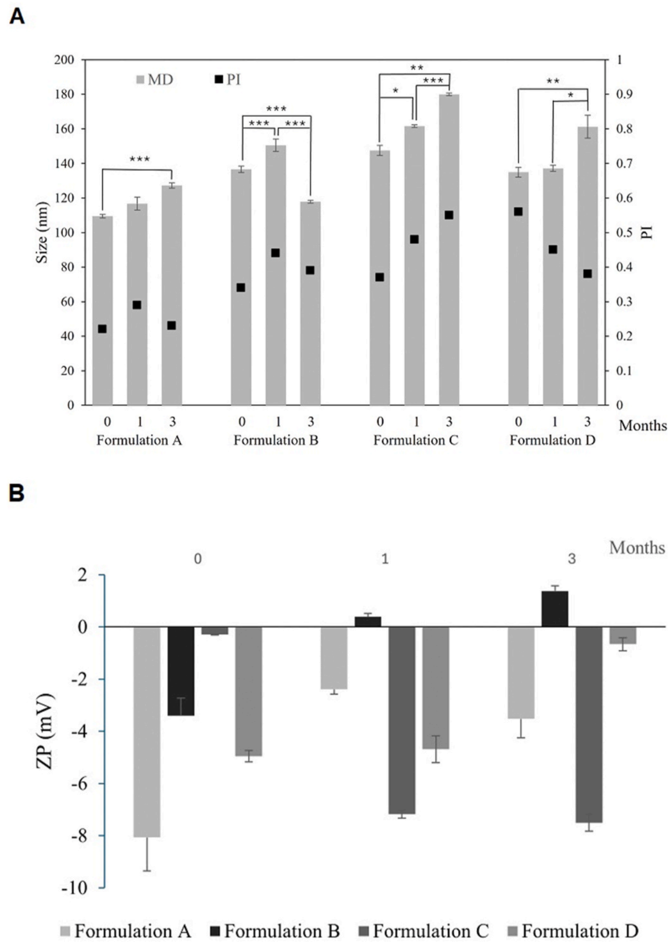

Fig. 2. Mean diameter (MD), polydispersity index (PI) (A) and zeta potential (ZP) (B) of VCZ loaded NLCs stored for 3 months at 4 °C. Data are reported as mean values ± standard deviations (SD) (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

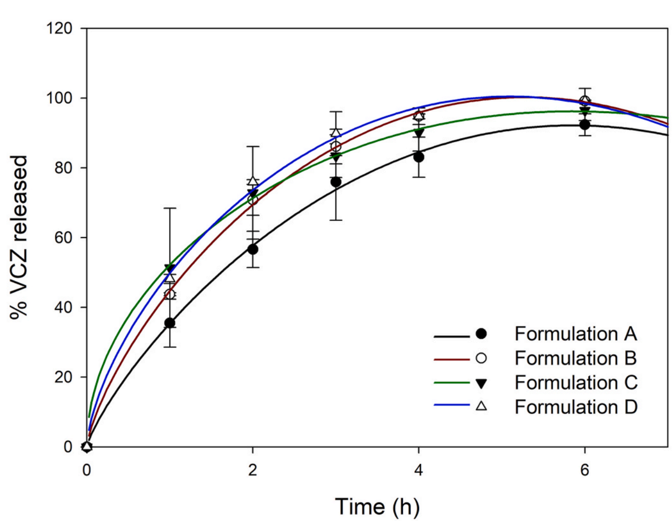

Fig. 3. Release profiles of VCZ obtained with the different formulations assayed (mean ± S.D., n = 3). The continuous curves show the predicted values using the Peppas-Sahlin equation.

Table 3
Parameters obtained from those adjusted using the equations from release kinetics for the models used.

		Formulation			
Model	Parameter	A	B	C	D
Zero order	r	0.9274	0.8913	0.8659	0.8656
	K_0 (%·h^{-1})	14.87 ± 3.00	15.66 ± 3.98	14.37 ± 4.15	15.25 ± 4.41
First order	r	0.9975	0.9950	0.9978	0.9989
	K_1 (h^{-1})	0.42 ± 0.01	0.80 ± 0.04	0.55 ± 0.02	0.79 ± 0.02
Higuchi	r	0.9911	0.9828	0.9786	0.9746
	K_H (%·h$^{-1/2}$)	39.98 ± 2.68	43.45 ± 4.08	40.88 ± 4.30	46.19 ± 4.96
Korsmeyer-Peppas	r	0.9913	0.9880	0.9953	0.9872
	n	0.49 ± 0.06	0.40 ± 0.07	0.33 ± 0.04	0.35 ± 0.07
	$K_{K\text{-}P}$ (%·h^{-n})	40.80 ± 3.60	51.53 ± 4.81	56.0 ± 2.94	56.40 ± 5.11
Peppas-Sahlin	r	0.9993	0.9997	0.9997	0.9989
	n	0.80 ± 0.06	0.73 ± 0.03	0.50 ± 0.03	0.65 ± 0.06
	$K_{P\text{-}S(1)}$ (%·h^{-n})	37.18 ± 1.18	46.80 ± 0.86	53.10 ± 0.95	51.68 ± 1.82
	$K_{P\text{-}S(2)}$ (%·h^{-2n})	~0	~0	~0	~0

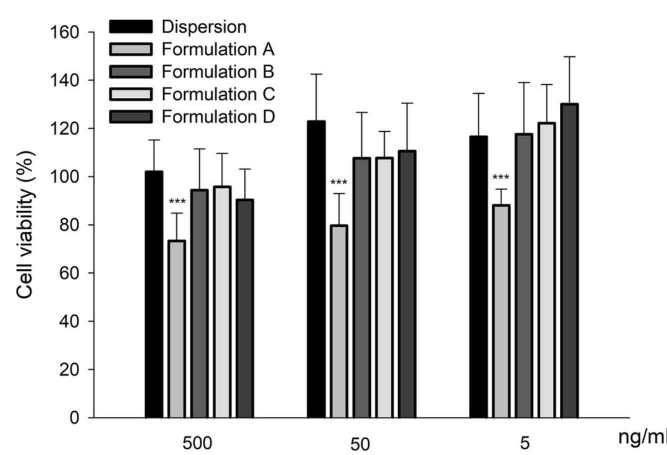

Fig. 4. Cell viability of HaCaT cells treated for 48 h with VCZ in dispersion or loaded in NLCs diluted to reach 500, 50 or 5 ng/mL of VCZ. Data are reported as mean values (n = 17) ± SD of cell viability expressed as the percentage of untreated cells (100 % of viability). Symbols indicate that the viability of cells treated with formulations is statistically different from that of cells treated with the dispersion of VCZ. ***p < 0.001.

3.8. *Antifungal susceptibility of C. albicans to VCZ*

To evaluate the antifungal activity of VCZ against *C. albicans*, the MIC_{50} and MIC_{90} values, as well as the growth curves of *C. albicans* in the presence of different concentrations of VCZ, were determined.

The MIC_{50} and MIC_{90} values of VCZ solutions were estimated using the microdilution method according to the EUCAST protocol, resulting in an MIC_{90} range between 0.5 and 1 μg/mL (Table 5). Furthermore, Fig. 6 shows the growth curves of *C. albicans* in the absence of VCZ (negative control) and in the presence of various concentrations of the drug, with final concentrations in the incubation medium of 500, 200,

Table 4
Percentage of VCZ present in the remaining formulation in the donor compartment (% Donor), in the skin (% Skin) and accumulated in the receptor compartment at the end of the transdermal absorption study (% Receptor). Each value represents the mean value ± SD of four replicates (n = 4). Symbols indicate that the percentage of VCZ remaining is statistically different from that obtained with the dispersion. *p < 0.05, **p < 0.01.

		Formulation			
	Dispersion	A	B	C	D
% Donor	97.2 ± 0.8	98.9 ± 0.1*	97.4 ± 0.5	97.4 ± 1.0	98.4 ± 0.02
% Skin	0.35 ± 0.09	0.20 ± 0.09	0.28 ± 0.06	0.18 ± 0.07	0.21 ± 0.06
% Receptor	2.10 ± 0.23	0.94 ± 0.16**	2.28 ± 0.40	2.25 ± 0.69	1.37 ± 0.03

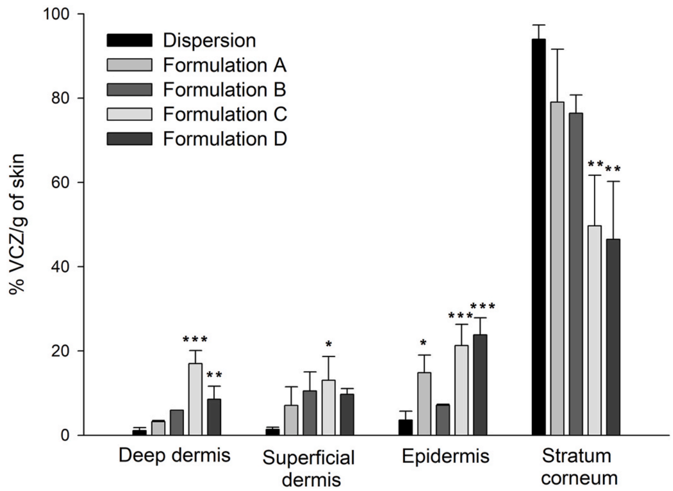

Fig. 5. Percentage of VCZ per gram of tissue accumulated into skin layers after the administration of formulations and VCZ dispersion. Data are reported as mean values (n = 4) ± SD. Symbols indicate that the % VCZ/g of skin treated with formulations is statistically different from that of skin treated with the dispersion. *p < 0.05, **p < 0.01, ***p < 0.001.

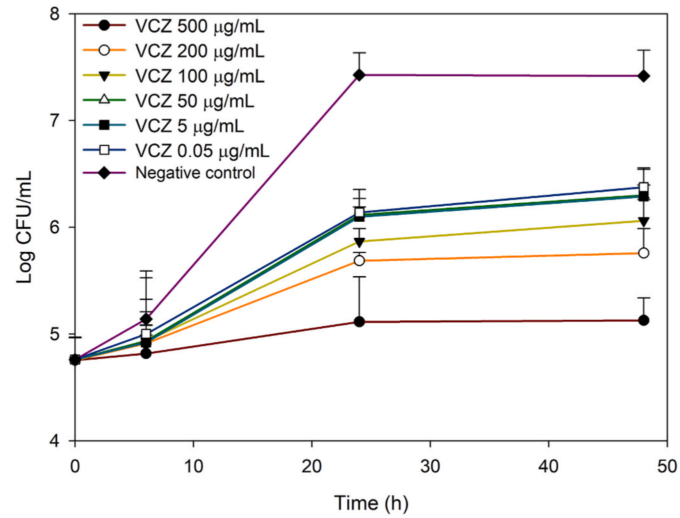

Fig. 6. Growth curves of *C. albicans* in the absence (negative control) and presence of different VCZ concentrations. Mean +SD, n = 6.

Table 5
Growth inhibition of *C. albicans* (n = 4). The last rows show the MIC_{90} and MIC_{50} values obtained.

Concentration (ng/mL)	Growth inhibition (%)
1000	94.3 ± 2.0
500	88.8 ± 7.5
250	81.1 ± 12.7
125	76.1 ± 7.4
60	72.2 ± 6.7
30	62.0 ± 5.5
15	40.9 ± 8.8
MIC_{90}	500-1000 ng/mL
MIC_{50}	15–30 ng/mL

100, 50, 5, and 0.05 μg/mL. As observed, growth inhibition increased with higher VCZ concentrations, while no significant differences were detected between the lowest concentrations tested (0.05–50 μg/mL), where the inhibition was approximately 1.3 log after 24 h of incubation.

3.9. Antifungal activity of NLCs on *C. albicans* growth

The growth curves of *C. albicans* in the presence of different formulations of VCZ are shown in Fig. 7. The final concentration of all formulations in the incubation medium was 50 μg/mL. In this assay, a positive control composed by an aqueous dispersion of VCZ was also tested and it was checked that it provoked the maximum inhibition of the growth. By contrast, negative control marked the absence of inhibition. As can be observed, the growth inhibition achieved by the formulations and the dispersion was similar, with a reduction of approximately 1–1.2 log after 24 h of incubation.

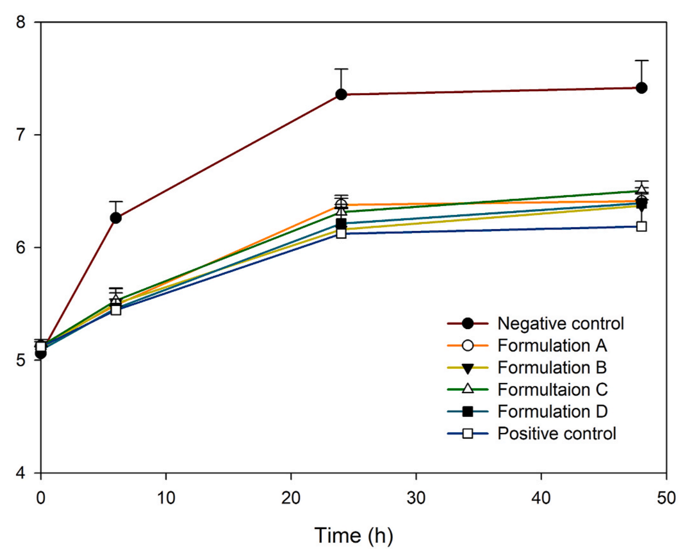

Fig. 7. Growth curves of *C. albicans* obtained in the presence of different VCZ formulations. The curves corresponding to the positive (VCZ 50 μg/mL) and negative (without VCZ) controls are also shown for comparative purposes. Except in the negative control, the VCZ concentration in the incubation medium was 50 μg/mL. Mean +SD, n = 6.

4. Discussion

NLCs represent a promising strategy for the topical delivery of antifungal agents, providing controlled drug release, reduced skin irritation, improved hydration, adhesiveness, occlusion and skin emollience. They also enable targeted delivery to infection sites and enhanced skin penetration, exhibiting deeper action within the dermis [34]. However, specific studies on NLC loaded with VCZ for topical application are still limited. Although previous studies, such as those by Waghule et al. [22], Santos et al. [23] and Song et al. [24] developed and characterized these formulations, they did not evaluate their antifungal activity against *C. albicans*.

To formulate NLCs for the topical administration of a poorly water-soluble drug like VCZ, the selection of suitable solid lipids, liquid lipids and surfactants is critical. The qualitative and quantitative composition of the formulations was therefore established on the basis of the physicochemical properties of the excipients and their expected influence on physical characteristics, stability and skin delivery of VCZ. In this sense, oleic acid was the liquid lipid chosen due to it is a penetration enhancer for delivery of drugs to the skin either topically or transdermally [35]. Moreover, it is widely used in the formulation of NLCs, affecting to the entrapment and release rate of drugs as aceclofenac [14]. A combination of cetostearyl alcohol, cholesterol, phospholipon® 90H and 90G were the solid lipids used. The selection and proportion of each one conditions the encapsulation efficiency of NLCs, due to the formation of structural imperfections in the lipid matrix which results in increased spaces to accommodate the drug [1]. Cetostearyl alcohol is a fatty acid that consists of a mixture of stearyl alcohol and cetyl alcohol and small amount of myristyl alcohol that is used as a stiffening agent and/or emulsion stabilizer in creams, ointments, and other topical preparations [36]. Cholesterol has been used in delivery systems such as nanoparticles, micelles and liposomal formulations improving their long-term stability [37]. Moreover, the presence of cholesterol has been related to a decrease in permeability by increasing membrane rigidity [38]. Phospholipon® 90G is an unsaturated phospholipid that can be converted into a saturated one by hydrogenation giving rise to Phospholipon® 90H [39]. The high phosphatidylcholine levels in both compounds promote the formation of deformable NLCs, which integrate into the lipid matrix of the stratum corneum's enhancing drug permeation [40]. Different concentrations of both components were used in the pre-formulation studies to obtain NLCs with comparable size and stability. The optimal concentration of Phospholipon® 90H appears to be lower than that of Phospholipon® 90G, since hydrogenated phosphatidylcholine (90H) tends to form more compact and less flexible bilayers, thus requiring a lower amount to obtain particles of similar size. Accordingly, concentrations of 0.2 % and 0.4 % were selected for Phospholipon® 90H and 90G, respectively. In addition, some studies have shown that the inhibitory action towards some fungal species of unsaturated fatty acids (like 90G) is considerably higher than that of saturated ones (90H) [41]. Polysorbate 80 and Poloxamer 188 are two non-ionic surfactants commonly used in NLCs for topical application of antifungal drugs that improve the stability of NLCs by steric stabilization [42]. Among the benefits associated with Polysorbate 80 are its low toxicity, low cost and absence of cytotoxicity. Also, it is considered a biodegradable product and a stabilizer of NLCs due to its HLB value (approximately 15) [1]. In line with the procedure followed for phospholipids, two concentrations of Polysorbate 80 were assessed to ensure that both size and stability were maintained. Poloxamer 188 offers an additional steric stabilization effect by covering surfaces of NLCs, preventing aggregation during storage [43,44].

The physical characteristics of NLCs are influenced by their composition. Studies have shown that particle size decreases as the proportion of polysorbate 80 increases [45]. Accordingly, Formulation A, which contained the highest concentration of polysorbate 80 (1.6 %), produced significantly smaller particles compared to formulations containing only 0.8 % of surfactant (Table 2). Furthermore, consistent with the formulations developed in this study, it has been reported that surfactants enhance drug EE by stabilizing the lipid-drug interface and reduce PI values by preventing particle aggregation through steric stabilization [1,46]. Another component that increases EE is cholesterol [38]. In this sense, formulations containing cholesterol (A and B) showed the highest drug EE. Despite the differences observed in size and EE between formulations, no relevant practical impact is expected, since all obtained NLCs had mean diameters lower than 150 nm, aligning with the optimal size range for enhanced deposition in the epidermis and dermis (reported to be below 300 nm [47,48] or around 100–150 nm [49–51], depending on the source), and high EE values (above 70 %). Additionally, the slight size variations observed after at least 3 months of storage at 4 °C did not compromise the particle size suitability for topical skin administration (MD $<$ 180 nm), considering the above reported optimal size.

In vitro release studies showed a VCZ-release pattern from NLCs consisting of an initial rapid release of 30–50 % of the drug content during the first hour followed by a more sustained release up to 6 h (Fig. 3). However, these initial percentages correspond to the sum of the non-encapsulated VCZ (10–30 %) and the drug released, with the latter averaging around 20 % across all formulations. Therefore, all NLC formulations exhibited similar release rates, which were best described by the Peppas–Sahlin model, which suggested a predominantly non-Fickian behavior. This release mechanism arises from complex interactions between the drug, lipid matrix, and formulation components. In particular, the structural complexity of the matrix and the presence of cholesterol and waxy lipids in the composition of NLCs disrupts simple Fickian diffusion, leading to anomalous transport patterns focused on matrix swelling and/or disintegration. In this sense, the presence of cholesterol and/or Phospholipon® 90G in formulations A, B and D increased the n values to 0.80, 0.73 and 0.65, respectively, compared to formulation C ($n = 0.50$), indicating an anomalous diffusion likely associated with a swelling process due to the high concentration of surfactants (Phospholipon®, polysorbate 80, and poloxamer 188). However, the removal of cholesterol and Phospholipon® 90G in formulation C reduced this value to 0.50, suggesting that swelling may not have played a significant role in the diffusion-controlled mechanism.

As expected, the results from the study of drug distribution across skin layers demonstrated increased VCZ permeation through the stratum corneum and deposition in the deeper layers with the NLC formulations compared to the dispersion [52]. In our study, only 6 % of VCZ was retained in 1 g of skin (epidermis and dermis) from the aqueous dispersion, whereas this value increased significantly to 50 % with formulations C and D (Fig. 5), highlighting these formulations as promising candidates for the treatment of more invasive cutaneous infections. This distribution pattern could be attributed to the formation of microreservoirs of VCZ within the dermis layers, resulting from the interaction between the NLCs and the natural components of the skin. Other properties of the formulations developed such as the sustained release and the nanoscale size of NLCs could contribute to facilitate skin penetration and enhance lipophilicity of the dermal layer, facilitating the effective retention of hydrophobic drugs as VCZ [15]. The presence of cholesterol in formulations A and B may explain the lower distribution of VCZ in the deeper layers of the skin, as cholesterol increases the membrane rigidity of NLCs, making them less deformable and thereby hindering their penetration.

Antifungal susceptibility tests of *C. albicans* to VCZ resulted in an MIC_{50} value between 15 and 30 ng/mL, consistent with the results obtained by Astvad et al. [53] who reported a value $\leq$ 30 ng/mL. Concentrations of VCZ tested in the growth curves assays were greater than 2 x MIC_{50} (Fig. 6), with the highest concentration (0.5 mg/mL) being limited by the low aqueous solubility of VCZ (0.71 mg/mL) [4]. According to this fact, the concentration of VCZ chosen to formulate NLCs was 0.5 mg/mL, coinciding with the concentration previously used when liposomes loaded with clotrimazole, another antifungal drug, were developed by our research group [31]. When the growth inhibition of *C. albicans* by the different VCZ-loaded NLCs was compared to that observed with the VCZ dispersion, similar antifungal activities were obtained, showing an inhibition of approximately 1.2 Log compared to the negative control (without VCZ).

5. Conclusions

This study presents the successful development of four VCZ-loaded NLC formulations, exhibiting small and uniformly distributed particle sizes suitable for enhanced deposition in both the epidermis and dermis and comparable antifungal activity against *C. albicans*. Results underlined that formulations C and D demonstrated a unique and effective combination of excipients that proved their biocompatibility with human keratinocytes and significantly enhanced the penetration and distribution of VCZ into deeper skin layers. These properties may potentially translate into superior antifungal activity in the treatment of more invasive forms of cutaneous candidiasis. These findings not only confirm the therapeutic potential of VCZ-loaded NLCs but also introduce a novel, targeted strategy for improving topical antifungal therapies through NLCs.

CRediT authorship contribution statement

Amparo Nácher: Writing – original draft, Investigation, Data curation, Conceptualization. **José-Esteban Peris:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Octavio Díez-Sales:** Writing – original draft, Investigation, Data curation. **Raquel Taléns-Visconti:** Writing – original draft, Investigation. **Maria Letizia Manca:** Writing – original draft, Data curation. **Maria Manconi:** Writing – original draft, Data curation. **Iris Usach:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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