Research paper

Voriconazole-loaded nanostructured lipid carriers (NLC) for drug delivery in deeper regions of the nail plate

Kamilla Amaral David Rocha, Anna Paula Krawczyk-Santos, Lígia Marquez Andrade, Luana Clara de Souza, Ricardo Neves Marreto, Tais Gratieri, Stephânia Fleury Taveira

Universidade Federal de Goiás (UFG), Rua 240, Setor Leste Universitário, 74. 605-170, Goiânia, GO, Brazil

Laboratory of Nanosystems and Drug Delivery Devices (NanoSYS), School of Pharmacy, Universidade Federal de Goiás (UFG), Rua 240, Setor Leste Universitário, 74. 605-170, Goiânia, GO, Brazil

Laboratory of Food, Drugs and Cosmetics (LTMAC), University of Brasília (UnB), Campus Universitário Darcy Ribeiro, Asa Norte, 70.910-900, Brasília, DF, Brazil

ABSTRACT

Voriconazole-loaded nanostructured lipid carriers (VOR-NLC) were developed and drug penetration evaluated in porcine hooves in vitro. Synergistic effect of urea (Ur), selected among other known chemical enhancers according to hoof hydration potential, was also evaluated. VOR-NLC presented a high encapsulation efficiency (74.52 ± 2.13%), approximate mean diameter of 230 nm and were positively charged (+27.32 ± 2.74 mV). Stability studies indicated they were stable under refrigeration (4 ± 2 °C) for up to 150 days. SEM images revealed hooves treated with VOR-NLC and VOR-NLC-Ur suffered a disturbance on the surface depicting high roughness and porosity. Permeation data showed a substantial VOR amount retained in superficial hoof sections independent of the formulation used (2.42 ± 0.26; 2.52 ± 0.36 and 2.41 ± 0.60 μg/cm² for unloaded VOR, VOR-NLC and VOR-NLC-Ur, respectively, p > 0.05). Still, successive extractions, revealed the amount of VOR retained in deeper regions was significantly higher when VOR-NLC or VOR-NLC-Ur was used (0.17 ± 0.04, 0.47 ± 0.14 and 0.36 ± 0.07 μg/cm² for unloaded VOR, VOR-NLC and VOR-NLC-Ur, respectively, p < 0.05). Such results indicate NLC are promising formulations for the management of onychomycosis. Further studies in diseased nail plates are necessary.

1. Introduction

Nail infections can affect about 20% of the population (Elkeeb et al., 2010). Usually caused by dermatophytes such as Trichophyton rubrum, which use the nail keratin as a nutrient source, they are very difficult to treat (Grumbt et al., 2013). Treatment options are limited; oral administration of antifungal agents relies usually on long-duration therapies, associated with severe side effects, drug interactions and high recurrence rates (Nogueiras-Nieto et al., 2011; Van Hoogdalem et al., 1997).

Topical treatments would obviously be more convenient, however, their effectiveness is restrained by the rigid and cohesive characteristic of the nail plate, which do not present any alternative route for drug penetration as appendage structures of the skin (Nair et al., 2009). Hence, nail drug delivery to deeper layers (intermediate and ventral nail plate) is extremely limited (Naumann et al., 2014).

Many alternatives have been proposed to enhance topical nail drug delivery, such as chemical (Brown et al., 2009; Nogueiras-Nieto et al., 2011; Vejnovic et al., 2010) and physical methods (Dutet and Delgado-Chorro, 2009, 2010; Hao et al., 2009).

In addition, colloidal carriers combined or not with other techniques, have been proposed to increase drug penetration to the nail plate. Examples include the use of liposomes and ethosomes (Tanriverdi and Ozer, 2013), nano/microemulsions (Naumann et al., 2014), cyclodextrins (Nogueiras-Nieto et al., 2013), polymeric nanoparticles associated with microneedles (Chiu et al., 2015) and nanovesicles associated to permeation enhancers (nPEV) (Bseiso et al., 2016). To our knowledge, until the present date no research has evaluated the use of lipid nanoparticles for nail drug delivery despite indications they could be very promising systems to increase drug nail penetration.

Lipid nanoparticles such as solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) has been extensively used for topical application (Pardeike et al., 2009; Yoon et al., 2013), since its lipid matrix can interact with the lipid membrane of stratum corneum, facilitating skin drug delivery. Moreover, the occlusive film formed on the skin surface significantly increases tissue hydration and drug skin...
permeation (Fan et al., 2013; Jenning et al., 2000; Obeidat et al., 2010). Thus, denoting these systems could also be capable of increasing nail plate hydration enhancing drug penetration.

In fact, nail hydration is one of the most important factors that influences nail physical properties (Wessel et al., 1999). When hydrated, nail behaves like a hydrogel with a network of pores through which molecules could diffuse (Hao et al., 2008; Nogueiras-Nieto et al., 2013). Indeed, the nail plate is capable of absorbing approximately 0.3 to 0.5 g H2O/g of dried nail (Benzéval et al., 2013), resulting in a softer and more flexible structure. Hydration also has been demonstrated to increase nail pores in number and size (Nogueiras-Nieto et al., 2011).

Here, the potential of NLC for antifungal delivery to deeper regions of the nail plate is evaluated in vitro using porcine hooves. NLC containing voriconazole (VOR), a broad-spectrum triazole agent that has proved action against 541 different fungal isolates, including nail dermatophytes (Carrillo-Munoz et al., 2007; Palacio et al., 2000; Silva et al., 2014) were applied alone or in combination with a known chemical permeation enhancer as an attempt to elucidate the mechanisms involved in drug penetration through the nail structure (Vejnovic et al., 2010).

2. Materials

2.1. Reagents

Polysorbate 80 (Tween® 80), sorbitantrioleate (Span® 85), cetylpyridinium chloride (CPC), glycerin and L-cystein were purchased from Sigma Aldrich (St. Louis, MO). Glyceryl behenate (Compritol®) and chloride (CPC), glycerin and L-cystein were purchased from Gattefossé (Weil am Rhein, Germany). Capric and caprylic triglycerides (Miglyol® 812 N) were purchased from Sta?llegenes SA (Antony, France). Salicylic acid was purchased from Laby nth (Diadema, Brazil). Propylene glycol was purchased from Vetec (São Paulo, Brazil). VOR was purchased from AK Scientific (Union City, EUA). Acetonitrile and HEPES buffer were obtained from VOR (São Paulo, Brazil). Propylene glycol was purchased from Lusiana et al., 2013), thus porcine hooves were used as a model of biological membrane (Myoung and Choi, 2003). Although bovine hooves are more commonly used, studies show there are no relevant structural differences between bovine and porcine hooves (Thatai and Sapra, 2014).

Hooves were obtained at Frigorífico Sol Nascente (CNPJ 73.918.757/0001-31) and Vigorsíco Caçula (CNPJ 01.684.299/0001-53), which are properly regulated by local Health Surveillance. Hooves were removed from the animal immediately after slaughter with a scalpel, washed with distilled water and stored at −20 °C (Naumann et al., 2014). Twenty-four hours before the studies, hooves were maintained at 25 ± 2 °C for equilibration (Chouhan and Saini, 2012).

2.2. Hooves

Human nail is difficult to obtain in great amounts (Lusiana et al., 2013), thus porcine hooves were used as a model of biological membrane (Myoung and Choi, 2003). Although bovine hooves are more commonly used, studies show there are no relevant structural differences between bovine and porcine hooves (Thatai and Sapra, 2014).

2.2. Selection of penetration enhancers by hydration studies

Hydration studies were performed according with Chouhan and Saini (2012) in triplicate. Hooves had their size and thickness adjusted with the aid of a scalpel and a digital caliper (Digimess®, São Paulo, Brazil) to be 0.25 ± 0.05 cm² and of 700 ± 50 µm, respectively. Hooves were dried overnight in a drying oven at 45 ± 0.5 °C and before the study, they were left at 25 ± 2 °C for 2 h, for equilibration. Then, each sample of hoof was weighted and put on a glass vial with 1 mL of penetration enhancer solutions (urea 5% w/v, urea 10% w/v, salicylic acid 1% w/v, L-cystein 1% w/v, L-cystein 1% w/v, L-cystein 1% w/v, glycerin 5% v/v and propylene glycol 5% v/v). Control samples were obtained by immersing the hooves in 1 mL of deionized water. Vials were sealed and incubated at 25 ± 2 °C for 24 h. After this period, hooves were removed and dried with paper towels to remove any solution residue or water from their surfaces. Then, hooves were weighed and the hydration factor for each promoter was calculated according to Eq. (1):

$$\text{HEF24} = \frac{W_p}{W_c}$$

Where HEF24 is hydration enhancement factor calculated in 24 h. Wp is weight gain of hooves exposed to penetration enhancer solution (% w/w). Wc is weight gain of hooves exposed to water (control) (% w/w).

2.3. Production of nanostructured lipid carriers (NLC) containing voriconazole (VOR)

NLC were prepared according to Andrade et al. (2016) with some modifications. The amount of liquid lipid was increased and the ratio between solid and lipid liquid was 5:3 (glyceryl behenate/caprylic caprylic triglycerides). Briefly, glyceryl behenate and caprylic triglycerides (15.6 and 9.4 mg/mL, respectively), polysorbate 80 (8.7 mg/mL), sorbitan trioleate (3.7 mg/mL), and cetylpyridinium chloride (6.2 mg/mL) were heated and 250 mL of distilled water was added to obtain a hot microemulsion which was dispersed into cold water (2–4 °C) under vigorous stirring (13,400 rpm for 10 min; IKA T25® Ultra-Turrax, Staufen, Germany). The final dispersion was sonicated using a 20-KHz low frequency ultrasonic processor (Misonix S-4000; New York, USA). The best penetration enhancer was added to VOR-NLC by simple dissolution in the aqueous dispersion, resulting the formulation VOR-NLC-Ur.

3.3.1. Characterization of nanostructured lipid carriers (NLC)

Dynamic light scattering was used to assess the mean diameter and polydispersity index (PDI) of the NLC dispersions using a Zetasizer Nano S (Malvern Instruments Ltd., Worcestershire, UK). Zeta potential was determined using Zeta Plus (Malvern Instruments, United Kingdom). Drug recovery (DR%) and entrapment efficiency (EE%) were determined as previously described (Andrade et al., 2016). Briefly, EE% was determined indirectly calculating the amount of entrapped drug inside the NLC. Separation of free drug in NLC dispersion from encapsulated VOR was performed by centrifuging 500 µL of freshly prepared NLC dispersions for 5 min at 3000 rpm (centrifuge model 3–18 K SIGMA, Osterode am Harz, Germany) using Vivaspin2 filter (Sartorius, Germany).
3.7. Permeation studies

Stability study was performed for VOR-NLC and VOR-NLC-Ur. Characterization parameters mean diameter, PdI, zeta potential, DR% and EE% (according to section 3.3.1) was assessed immediately after prepared and after 7, 15, 30, 60 and 150 days. The analysis was performed in triplicate. Samples were kept under refrigeration (4 ± 2 °C) throughout the stability study.

3.5. Hooves scanning electron microscopy (SEM)

SEM images were taken of hooves surface before and after treatment with each penetration enhancer. As wet samples cannot be analyzed in SEM, they were dried appropriately after treatment for 4 h in room temperature before analysis. Hooves had their size and thickness adjusted as in hydration studies (see Section 3.4). Analyzed samples were: non-hydrated hoof, water-hydrated hoof, VOR-NLC and VOR-NLC-Ur treated hooves. Images were taken using the same procedure as described for NLC images (see Section 3.3.1).

3.6. In vitro drug release

Drug release was evaluated using Franz-type diffusion cell supplied by Hanson Research (Chatsworth, USA). A cellulose acetate membrane (MWCO 12,000–14,000, Fisher Scientific, Waltham, USA) was used between donor and receptor chambers. Donor chamber was filled with 250 μL of the formulation (VOR-NLC or VOR-NLC-Ur) or unloaded drug (VOR dispersed in acid water, acidified with acetic acid until reach pH 4) containing 90 μg/mL of VOR. The receptor chamber was filled with HEPES buffer, pH 7.4. All experiments were performed under sink conditions (n=6) and stirring rate kept at 600 rpm. At appropriate time intervals (0.5; 1; 2; 3; 4; 6; 7; and 8 h), 1 mL aliquots of the receptor media were withdrawn and immediately replaced with equal volumes of fresh HEPES buffer. VOR released amounts were determined by HPLC.

Drug release kinetics was determined by applying three kinetics models to the data to find the best fitting equations:

\[ F = F_0 + k_0 t \]  
**Zero-Order equation**

\[ \ln F = \ln F_0 - k_1 t/2 \]  
**First-order equation**

\[ F = k_2 t^{1/2} \]  
**Higuchi equation**

Where F represents the fraction of drug released over time t, F₀ is the initial amount of drug in the solution (most times F₀ = 0), and k₀, k₁, and k₂ are the apparent rate constants for zero-order, first-order, and Higuchi release constants, respectively (Dash et al., 2010). The curve with higher linear coefficient was used to calculate flux (J).

3.7. Permeation studies

For permeation experiments standardized hooves were mounted in the diffusion cells with ventral part facing downward into the receiving media (HEPES buffer, pH 7.4) using a nail adapter PermeGear (PermeGear, Inc., USA), with permeation area of 0.19 cm². Donor chamber was filled with 250 μL (containing 90 μg/mL of VOR) of the same formulations as release studies. Experiments were performed under sink conditions (n = 6) and stirring rate kept at 600 rpm. After 48 h, an aliquot of receptor chamber was analyzed by HPLC, following extraction process for drug quantification within the hooves.

### 3.7.1. Extraction of VOR from hooves after permeation

Hooves were removed from the diffusion cells, washed with distilled water and dried with paper towel. Then, hooves were sliced using a scalpel and splinters, transferred to a glass vial and immersed in 0.5 ml of methanol. Samples were kept under magnetic stirring for 2 h at 300 rpm and 25 °C. After that, they were centrifuged at 10,000 rpm for 5 min, to precipitate the hooves fragments and supernatant was collected for drug assay. This first assay was denominated as VOR extraction immediately after permeation experiments. After supernatant collection a further extraction procedure was performed adding fresh extractor solvent to reminiscent hooves fragments and leaving them under stirring for 72 and 144 h, after which sample were further centrifuged and VOR in the supernatant quantified. Samples were sealed and kept at controlled temperature to avoid evaporation. VOR solutions were submitted to the same procedures and no degradation was observed as also demonstrate in the literature (Shaikh and Patil, 2012).

### 3.8. Statistics

Graphpad Prism 5.3 (Graphpad Prism inc., USA) was used for statistical analysis. The statistical significance of each experiment was determined using t-test nonparametric. Tests yielding p values < 0.05 were considered significant.

### 4. Results and discussion

#### 4.1. Selection of penetration enhancer by hydration study

Selection of penetration enhancers was performed according to HEF24 and results are presented in Table 1.

Natural moisture content of the nail may vary according to membrane model and applied technique used to determine this parameter. Human nails have been reported to present 11.78 ± 0.6% of moisture by thermogravimetric technique (Barba et al., 2009) while such value was 23 ± 10% for cadaver nails subjected to water hydration for 1 h following moisture determination by transonychial water loss (TOWL) (Vejnovic et al., 2010). In the present study, porcine hooves absorbed 31.14 ± 2.79% of water after hydration for 24 h, as determined by gravimetric study.

Penetration enhancers evaluated were selected based on literature information about their mechanism in increasing skin and nail permeation of drugs. For instance, propylene glycol has been described to solubilize α keratin in stratum corneum (Paliyil et al., 2013). Glycerin

<table>
<thead>
<tr>
<th>Penetration enhancers</th>
<th>% (w/v)</th>
<th>HEF24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerin</td>
<td>5</td>
<td>0.952 ± 0.068</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>5</td>
<td>0.952 ± 0.008</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>1</td>
<td>1.421 ± 0.176</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>1</td>
<td>1.074 ± 0.167</td>
</tr>
<tr>
<td>Urea</td>
<td>5</td>
<td>1.166 ± 0.083</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.738 ± 0.011</td>
</tr>
</tbody>
</table>

* L-cysteine and salicylic acid presented HEF24 values significantly different from glycerin, propylene glycol, salicylic acid (p < 0.05). *Urea 10% presented HEF24 values significantly different from the other penetration enhancers (p < 0.05).
and urea are used in commercial skin care lotions because of their hygroscopic properties, acting as humectants (Björklund et al., 2013). Urea and salicylic acid have been used in nail studies as a penetration enhancer suggesting nail softening and superficial alterations (Akhbar et al., 2016; Joshi and Patrawale, 2008; Murdan, 2002). L-cysteine has been reported to promote disulfide linkage reduction in the nail keratin matrix destabilizing integrity of the barrier (Shivakumar et al., 2014; Walters et al., 2012).

Table 1 demonstrates neither glycerin nor propylene glycol favored nail hydration. In fact, the HEF24 values were below 1.0, demonstrating nail dehydation. Salicylic acid did not improve HEF24 value, while urea promoted a higher hydration of the hooves, followed by propylene glycol and glycerin. This result differs from the literature (Chouhan and Saini, 2012), in which glycerin was the best substance to increase hydration. Nevertheless, this difference can be attributed to the use of human nails in cited study instead of porcine hooves used here.

Urea 5% HEF24 was approximately 1.16, statistically different from glycerin HEF24 values, but similar to all other penetration enhancers. Still, urea 10% provided the highest HEF24 value (p < 0.05). Thus, it was chosen as the one to be combined with NLC formulation (VOR-NLC-Ur) in penetration studies.

NLC were also evaluated for HEF24 hydration. Nanoparticles presented a HEF24 of 1.19 ± 0.09, slightly higher than salicylic acid and lower than urea. Surprisingly, when 10% of urea was added to NLC dispersions, no changes in HEF24 value were observed (1.18 ± 0.04). Probably, NLC formed a film on hoof surface hindering urea access and consequently impairing its moisturizing effect, once urea is soluble in the formulation and not entrapped.

4.2. NLC characterization

VOR-NLC were prepared using the microemulsion technique. Characterization data is presented in Table 2. No significant variation was observed for mean diameter, PdI, zeta potential, EE and DR after urea addition (p > 0.05). DR values of about 78% are probably different from glycerin HEF24 values, but similar to all other penetration enhancers. Still, urea 10% provided the highest HEF24 value (p < 0.05). Thus, it was chosen as the one to be combined with NLC formulation (VOR-NLC-Ur) in penetration studies.

Table 2

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Mean Diameter (nm)</th>
<th>PdI</th>
<th>Zeta Potential (mV)</th>
<th>EE (% w/w)</th>
<th>DR (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOR-NLC</td>
<td>259.90 ± 25.41</td>
<td>0.235 ± 0.010</td>
<td>27.32 ± 2.74</td>
<td>74.52 ± 2.13</td>
<td>86.58 ± 3.57</td>
</tr>
<tr>
<td>VOR-NLC-Ur</td>
<td>210.74 ± 15.86</td>
<td>0.263 ± 0.041</td>
<td>27.18 ± 1.95</td>
<td>70.19 ± 5.08</td>
<td>78.78 ± 5.06</td>
</tr>
</tbody>
</table>

4.3. Stability studies

VOR-NLC were characterized at 0, 7, 15, 30, 60 and 150 days and the results are shown in Table 3. The average size and PdI were around 245 nm and 0.285, respectively. The surface charge of the particle remained positive. Furthermore, EE and DR of the formulations did not show significant changes (p > 0.05), demonstrating system stability under the analyzed conditions.

The results obtained from the characterization of VOR-NLC-Ur (with addition of 10% urea) are presented in Table 4. Analyzed parameters did not suffer any significant change, including DR (p > 0.05).

4.4. Hooves scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was conducted to analyze the effect of water and formulations VOR-NLC and VOR-NLC-Ur on hooves surface. SEM images are presented on Fig. 2.

Image 2A shows untreated hoof surface. Hoof cells are organized in a smooth and dense surface, resembling the human nail (Nogueiras-Nieto et al., 2011).

As hooves absorb water keratin expands, and surface becomes irregular and swollen with a greater amount of pores, but the overall structure is still compact (Joshi et al., 2015), as it can be seen in Fig. 2B. Van der Waals forces, hydrogen bonds and ionic interactions on nail keratin are highly affected by water, thus nail plate hydration is able to destabilize these bonds causing changes in proteins geometry (Wessel et al., 1999). Alterations on geometry are probably the reason for the new pores.

When hooves were treated with VOR-NLC and VOR-NLC-Ur, Fig. 2C and D, a disturbance on the surface became more evident, as depicted by higher roughness and increased porosity. Probably the positively charged surfactant cetylpyridinium chloride destabilized keratin ionic interactions affecting the appearance of external surface structure and promoting new pores formation (Som et al., 2012).

4.5. In vitro drug release

VOR release profiles from unloaded VOR and loaded on VOR-NLC and VOR-NLC-Ur are exhibited in Fig. 3. At the first 3 h of study, unloaded VOR release is statistically higher than loaded-NLC drug. VOR-NCL and VOR-NLC-Ur presented similar release levels of VOR for 8 h. After which there was no difference in the amount of VOR released among formulations, about 80.55 ± 4.78%.

Besides previous reports on the capability of NLC in controlling drug release (Nagaich and Gulati, 2016; Souto et al., 2004), even hydrophilic drugs (Souza et al., 2011), a near 80% release in 8 h may suggest VOR could be located within the surfactant polar interface rather distributed in the lipid matrix. For instance, genistein-loaded NLC (log P 3.04) (Rothwell et al., 2005) presented a rapid drug release within the first 9 h (drug associated to the surfactant core) followed by slow drug
release within the next hours (drug dispersed in lipid matrix), resulting 60% of drug release over 50 h (Andrade et al., 2014). Still, as topical formulations should be reapplied at least in 12 h, VOR release proportioned by NLC could be sufficient for the desired application.

Kinetic models were applied and the best linear correlation coefficient ($r^2$) for both VOR-NLC and VOR-NLC-Ur was determined by the

Table 3
Stability study data for VOR-NLC after 150 days of storage at 4 °C.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Mean Diameter (nm)</th>
<th>Pdl</th>
<th>Zeta Potential (mV)</th>
<th>EE (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>242.14 ± 36.95</td>
<td>0.280 ± 0.04</td>
<td>28.90 ± 0.21</td>
<td>75.24 ± 4.52</td>
</tr>
<tr>
<td>7</td>
<td>245.62 ± 37.56</td>
<td>0.285 ± 0.02</td>
<td>25.69 ± 2.35</td>
<td>77.52 ± 0.68</td>
</tr>
<tr>
<td>15</td>
<td>252.17 ± 41.84</td>
<td>0.275 ± 0.04</td>
<td>24.05 ± 0.78</td>
<td>80.20 ± 0.38</td>
</tr>
<tr>
<td>30</td>
<td>239.33 ± 29.90</td>
<td>0.303 ± 0.07</td>
<td>27.62 ± 2.33</td>
<td>77.40 ± 1.91</td>
</tr>
<tr>
<td>60</td>
<td>252.28 ± 42.81</td>
<td>0.284 ± 0.04</td>
<td>27.23 ± 1.54</td>
<td>77.33 ± 1.98</td>
</tr>
<tr>
<td>150</td>
<td>239.43 ± 30.81</td>
<td>0.296 ± 0.05</td>
<td>24.84 ± 3.58</td>
<td>75.24 ± 4.52</td>
</tr>
</tbody>
</table>

Table 4
Stability study data for VOR-NLC-Ur after 150 days of storage at 4 °C.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Mean Diameter (nm)</th>
<th>Pdl</th>
<th>Zeta Potential (mV)</th>
<th>EE (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>218.58 ± 55.32</td>
<td>0.260 ± 0.03</td>
<td>27.91 ± 1.85</td>
<td>68.61 ± 3.78</td>
</tr>
<tr>
<td>7</td>
<td>211.67 ± 54.82</td>
<td>0.266 ± 0.01</td>
<td>26.58 ± 2.40</td>
<td>69.91 ± 4.51</td>
</tr>
<tr>
<td>15</td>
<td>205.18 ± 41.84</td>
<td>0.284 ± 0.04</td>
<td>28.17 ± 1.56</td>
<td>70.80 ± 1.57</td>
</tr>
<tr>
<td>30</td>
<td>219.02 ± 66.56</td>
<td>0.289 ± 0.07</td>
<td>31.64 ± 1.60</td>
<td>69.01 ± 1.96</td>
</tr>
<tr>
<td>60</td>
<td>209.97 ± 45.19</td>
<td>0.275 ± 0.02</td>
<td>28.57 ± 1.22</td>
<td>69.55 ± 2.68</td>
</tr>
<tr>
<td>150</td>
<td>219.80 ± 21.52</td>
<td>0.328 ± 0.05</td>
<td>27.37 ± 0.71</td>
<td>68.61 ± 3.78</td>
</tr>
</tbody>
</table>

Fig. 1. (a) SEM of VOR-NLC without VOR, and (b) VOR-NLC-Ur, both with a 10.000 magnification.

Fig. 2. SEM images of the surface of porcine hoof – A: untreated (magnification 5000); B: treated with water (magnification 5000); C: treated with VOR-NLC (magnification 5000); D: treated with VOR-NLC-Ur (magnification 5000). Bars length is 5 μm.

Fig. 3. VOR release in different formulations: water pH 4 (unloaded VOR) and from VOR-loaded NLC (VOR-NLC) and VOR-loaded NLC with urea (VOR-NLC-Ur) over 8 h of experiments in Franz-type diffusion cells (n = 6). Receptor media was comprised of HEPES buffer pH 7.4. *Indicate difference between unloaded and encapsulated VOR (p < 0.05).
relation between VOR released concentration (μg/mL) and time1/2, fitting Higuchi model. Kinetic parameters are shown on Table 5.

### 4.6. Permeation study

Different formulations were topically applied on porcine hooves (unloaded VOR, VOR-NLC and VOR-NLC-Ur) and, after 48 h, no VOR was detected in the receiver solution in any of the analyzed cells. Nonetheless, to better treat onychomycosis, drug accumulation within the nail plate is more relevant than permeation trough the tissue (Tanriverdi and Ozer, 2013). Hence, to extract/deplete as much VOR as possible from the hooves, successive extractions were performed, immediately after the experiment, after 72 and 144 h of contact with the extractor solvent.

VOR amount extracted immediately after permeation studies was similar for all formulation (2.42 ± 0.26; 2.52 ± 0.36 and 2.41 ± 0.60 μg/cm² for unloaded VOR, VOR-NLC and VOR-NLC-Ur, respectively, p > 0.05). Almost the same amount of VOR was recovered after 72 h of extraction (2.86 ± 0.14; 1.71 ± 0.44 and 2.28 ± 0.13 μg/cm² for unloaded VOR, VOR-NLC and VOR-NLC-Ur, respectively, p > 0.05). The last VOR extraction/depletion (Fig. 4), however, revealed significantly higher amount of VOR following NLC treatment (p < 0.05), suggesting nanoparticles probably increased the permeation of the drug to the deeper regions of the hooves (Fig. 4).

Accordingly, the same physical and chemical bonds responsible for keratin stability, which hinder drug permeation through the nail plate, also difficult their extraction (Murdan, 2002). Therefore, it cannot be said that the drug reached the deeper layers of the nail plate, such as intermediate and ventral layers. This is because, when cutting the membrane in small pieces, all regions were exposed to the solvent extractor. However, the most time-consuming extraction indicates that the drug has penetrated deeper into the nail structure, which may be in the dorsal, intermediate or ventral layer.

VOR values achieved in extraction procedures are expressive taken into account reported MIC values for dermatophytes (0.037–0.107 μg/mL) (Carrillo-Munoz et al., 2007). Obviously, minimum inhibitory concentration (MIC) values may vary depending on the species and isolate. Still, several reports performed to delineate epidemiology of onychomycosis in different regions have encountered Fusarium to be the most resistant species and, in their majority, VOR is pointed out as one of the most potent antifungal drug, together with Amphotericin B (Bueno et al., 2010; Gupta et al., 2016). Despite these promising results, verified even with the unloaded drug, this drug is not yet a routine choice in the clinics. Clearly, clinical studies or administration in diseased nail plate models could provide more information about VOR effectiveness in actual infected tissue. Until the present moment, investigations of diseased nail support the notion that nail pores are opened up by the infection facilitating the passage of hydrophilic molecules whilst keratin binding may keep the penetration of hydrophobic molecules unchanged (McAuley et al., 2016). VOR is a small (349 Da) relatively hydrophobic molecule with a Log P of 1.65, hence, under diseased circumstances, in the worst scenario, its penetration would be unchanged. In fact, urea incorporation in the formulation, which is a keratolytic agent that could in some degree induce such disturbances within the nail plate (Quintanar-Guerrero et al., 1998), had no further penetration enhancing effect. As shown in SEM images, VOR-NLC and VOR-NLC-Ur were capable of disturbing hoof keratin organization, possibly destabilizing ionic interactions, what led pore formation. These alterations had a noteworthy impact on the penetration depth of non-encapsulated drug. Therefore, one assumption is that maybe NLC prevents drug interaction with keratin chains allowing a deeper penetration into the nail plate, as observed with the significant VOR penetration deeper into the hoof membrane from both VOR-NLC and VOR-NLC-Ur in contrast to unloaded-VOR.

### 5. Conclusion

To our knowledge this was the first study proposing the unusual application of NLC, which showed their potential as topical nail delivery systems capable of promoting drug penetration into deeper regions of the hooves in comparison to the unloaded drug. Such results could have a remarkable impact on onychomycosis management. Further studies are necessary to evaluate NLC potential in enhancing drug penetration into diseased nail plates.

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### References


### Table 5

Kinetic parameters of VOR release curves (μg/cm²) in different formulations depending on time1/2.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>J (μg/cm²/h)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unloaded VOR</td>
<td>24.63 ± 3.04</td>
<td>0.87181</td>
</tr>
<tr>
<td>VOR-NLC</td>
<td>19.51 ± 2.67</td>
<td>0.9954</td>
</tr>
<tr>
<td>VOR-NLC-Ur</td>
<td>15.23 ± 1.67</td>
<td>0.9847</td>
</tr>
</tbody>
</table>

* Unloaded VOR flux is statistically different from VOR-NLC and VOR-NLC-Ur (p < 0.05).