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In Vitro Transungual Permeation of Ciclopirox from a Hydroxypropyl Chitosan-Based, Water-Soluble Nail Lacquer

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ABSTRACT Commercial antimycotic nail lacquers are commonly based on water-insoluble resins. The present study was aimed at evaluating a novel, experimental nail lacquer (P-3051, Polichem SA, Lugano, Switzerland) based on the water-soluble film-forming agent hydroxypropyl chitosan (HPCH). The in vitro permeation of ciclopirox (CPX) from P-3051 and from a commercial, water-insoluble lacquer based on a vinyl resin (Penlac™, Aventis Pharma), was investigated using thin membranes obtained from bovine hooves, an accepted model for human nails. Similar CPX permeation fluxes at steady state through the membranes, but significantly different lag times were observed for P-3051 and Penlac™, when these were tested as dry films. The formulations thus appeared to influence only the time required by CPX to saturate the membrane, and not the final drug concentration gradient in the membrane. Permeation experiments performed on the same membranes and on hairless mouse skin with P-3051 and with a similar, HPCH-free vehicle (ERV), both tested in liquid form, disproved the possibility that HPCH might act as a permeation enhancer for CPX in either substrate. The possible reasons for the greater efficiency of the HPCH vehicle in terms of CPX transfer from the vehicle itself to the keratin membrane are discussed. This effect might be tentatively attributed to a particular affinity of HPCH for the membrane, resulting in intimate contact and strong adhesion of the HPCH lacquer to the keratin substrate.

KEYWORDS Onychomycosis, Ciclopirox, Hydroxypropyl chitosan, Transungual permeation, Transdermal permeation, Nail lacquer

INTRODUCTION

Onychomycosis (tinea unguium) is a fungal infection of the nail bed or nail plate. It accounts for approximately 50% of all nail diseases and is the most common nail disorder in adults, affecting up to 18.5% of the U.S. adult population (Ghannoum et al., 2000). Most of the infections (90–95%) are caused by dermatophytes, the rest being caused by yeasts and moulds (Migdley

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et al., 1994). Infected nails appear unsightly, discolored, thickened, and dystrophic: this condition can have a significant impact on the quality of life, reducing physical and social functioning (Drake et al., 1998). Among the superficial fungal infections, onychomycosis is the most difficult to manage: the disease is frequently chronic, difficult to eradicate, and tends to recur.

In the past, the only available treatment for onychomycosis consisted of surgical avulsion; this procedure, however, is extremely traumatic and painful (Niewerth & Korting, 1999). Onychomycosis is currently treated with systemic or topical antimycotic agents. Systemic therapy consists of oral administration of potent antifungals, such as terbinafine and itraconazole (Baran et al., 1996), which diffuse from the blood vessels into the nail plate via the nail bed. Unfortunately, a large number of patients does not respond; moreover, relapse, severe side effects, and drug interactions have been observed (Hay, 2001; Roberts, 1999). Topical therapy is based on application of nail lacquers containing ciclopirox or amorolfine directly onto the infection site. This approach circumvents the problems associated with oral treatment. However, drug diffusion into the highly keratinized nail plate is poor and the treatment is of long duration, so that topical therapy is currently recommended only for the earlier stages of the disease or as support to systemic treatment (Murdan, 2002). Nevertheless, an active interest in methods aimed at improving the efficacy of topical treatment is testified by recent reports (Kim et al., 2001; Myoung & Choi, 2003; Repka et al., 2002).

Commercial medicated nail lacquers are based on water-insoluble vinyl resins. The present study was aimed at evaluating the effect of a water-soluble, film-forming agent, hydroxypropyl chitosan (HPCH), on in vitro permeation of the broad-spectrum antimycotic ciclopirox across bovine hoof membranes, an accepted model for human nails (Mertin & Lippold, 1997a, 1997b, 1997c). A commercial nail lacquer (Penlac™, Aventis Pharma) was used as reference.

MATERIALS AND METHODS

Chemicals

Ciclopirox (CPX, Erregierre S.p.A, Bergamo, Italy); hydroxypropyl chitosan (HPCH, MW 400–800 KD, Laserson S.A., Etampes Cédex, France); ethyl alcohol 95°; ethyl acetate; cetostearyl alcohol NF (Lanette™ O, Cognis Deutschland GmbH, Dusseldorf, Germany); phosphoric acid; 1.0 N sodium hydroxide; acetonitrile; and water HPLC grade.

Vehicles

The following vehicles, all containing 8% w/w CPX were tested. 1) Experimental nail lacquer (P-3051, Polichem SA, Lugano, Switzerland) having the following w/w composition: 8.0% CPX; 1.0% HPCH; 1.0% cetostearyl alcohol; 73.0% ethyl alcohol (95°); 4.0% ethyl acetate; 13.0% purified water. 2) Experimental reference vehicle (ERV), with the same composition as P-3051 but without HPCH. 3) Commercial nail lacquer (Penlac™, Aventis Pharma), chosen as reference, having the following composition: 8.0% CPX; ethyl acetate; isopropyl alcohol; butyl monoester of poly(methylvinyl ether/maleic acid) in isopropyl alcohol.

In Vitro Transungual and Transdermal Permeation Experiments

Hooves from freshly slaughtered cattle, free of adhering connective and cartilaginous tissue, were soaked in water for 24 hours. Membranes of about 150 μm thickness were then cut from the distal part of the ball horn with a criotome (2800 Frigocut E, Reichert-Jung). In vitro permeation experiments were carried out using vertical permeation cells (Gummer et al., 1987), consisting of a donor and a receiving chamber (respective volumes 1.0 mL and 5.0 mL) fastened together by a clamp; the hoof membranes were placed between the two compartments and the surface area available for permeation was 1.23 cm^2. The magnetically stirred (600 rpm) receiving phase was isotonic, 66.7 mM, pH=7.4 phosphate buffer containing 0.003% w/v sodium azide to prevent bacterial growth. The experimental temperature was maintained at 32°C by circulation of thermostated water inside the cell jacket.

Prior to the experiments, the hoof membranes were hydrated in distilled water for 15 hours to reduce rigidity and to allow them to assume a planar shape. The effective thickness of the hydrated membranes was determined after this preliminary soaking step.

The membranes, after being placed inside the permeation apparatus with empty receiving compartment, were dried in a warm air stream for a few minutes. Then, 75.0 µl of the test vehicle were evenly applied on the exposed membrane surface; a subsequent treatment with warm air for at least 15 minutes ensured removal of the volatile components and formation of a homogeneous film on the membrane. The receiving phase was then introduced into the lower compartment.

Each experiment lasted 30 hours: at predetermined time intervals, 5.0 mL of receiving solution was collected for analysis and simultaneously substituted with an equal volume of fresh buffer, using an automatic apparatus controlling inflow and outflow of the solution from the receiving chamber. Each experiment was replicated at least six times.

Chitosan derivatives have been reported to enhance drug permeation through different tissues (Kato et al., 2003; Sandri et al., 2004). To evaluate possible effects of HPCH on transungual and transdermal permeation of CPX, in vitro permeation tests were also performed by applying the P-3051 and ERV vehicles to excised hairless mouse skin (Monti et al., 1995) and hoof membranes. The experiments were carried out by the procedure previously described; the air-drying step, however, was omitted and the donor chamber was kept occluded to avoid possible separation of CPX in the case of the ERV vehicle, due to the absence of HPCH and evaporation of the volatile components. Also, due to the high skin permeability to CPX, the transdermal permeation experiments had a duration of 5 h instead of 30 h.

Determination of CPX Content in the Hoof Membranes at End of Experiments

Treated membrane samples (10–20 mg) were weighed, cut into small fragments, and transferred to 5.0 mL test tubes. The samples were treated with 5.0 mL of 1.0 N NaOH and left at room temperature for 48 hours to produce complete degradation of the keratin structure. The resulting solution was vortexed and centrifuged (12000 rev/min, 5 min); 100 µL of the supernatant were diluted to 5.0 mL with the high-performance liquid chromatography (HPLC) mobile phase and directly injected into the HPLC apparatus. A standard curve was obtained from CPX solutions containing digested keratin from untreated membranes: this procedure excluded any interference deriving from alkali-induced keratin degradation products (Dykes et al., 1990).

Analytical Methods

The quantitative determination of CPX in the samples was carried out by HPLC [Merck ChromBook, 2004; http://www.chromatography.co.uk/apps/hplc/hplc0009.htm (accessed June 2004)]. The apparatus consisted of a Shimadzu LC-6A system with an UV SPD-10A detector and a C-R4A analyzer. The injection valve was a Rheodyne with a capacity of 20 µL. A LichroCART® 5 µm 125 × 4.0 mm Purosphere® RP-18 endcapped column was employed. The mobile phase consisted of a (60:40) mixture of acetonitrile and 20 mM H_3PO_4; the flux and the detection wavelength were 1.0 mL/min and 302 nm, respectively. The retention time was 3.10 min. The concentration of permeant in each sample was determined from standard curves, obtained by plotting the concentration of known solutions vs. the corresponding peak areas of HPLC chromatograms.

Data Analysis

Linear regression analysis of pseudo steady-state diffusion plots allowed calculation of the following parameters: steady-state flux (J), given by Q/At, where Q is the amount of permeant diffusing across the area A in time t; lag time (time required by the drug to establish a uniform concentration gradient within the membrane) calculated from the X-axis intercept values

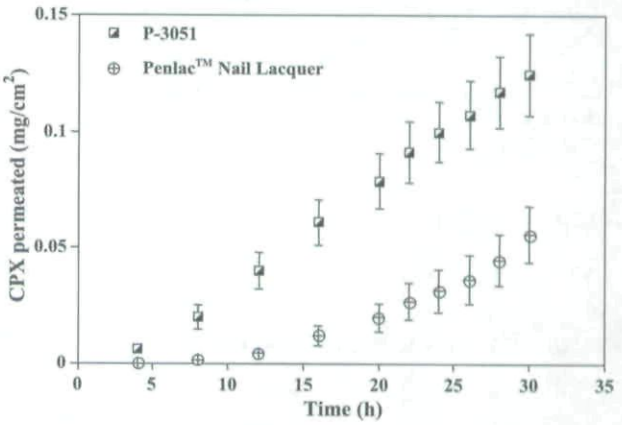

FIGURE 1 Permeation Plots of CPX Through Hoof Membranes from Nail Lacquers P-3051 and Penlac™.

TABLE 1 Permeation Data of CPX Through Hoof Membranes from Dry Nail Lacquers P-3051 and Penlac™

Nail lacquer	J ($\mu g/cm^2h \pm SE$)	Lag time (h±SE)	$Q\%_{30\ h}$ (±SE)	$CPX_{memb.}$ (μg/mg membrane±SE)	$\%CPX_{memb.}$ (±SE)
Penlac™	3.05±0.63	12.48±1.31	1.06±0.25	9.68±0.77	11.57±0.66
P-3051	4.70±0.60	3.36±0.46[a]	2.58±0.36[a]	9.08±0.94	11.02±0.77

[a]Statistically significant differences (Fisher PLSD test, $p<0.05$).

of the regression lines; percent drug permeated at end of the experiments ($Q\%_{5h}$, $Q\%_{30h}$); promotion factor (PF), given by the ratio between CPX fluxes in the presence and in the absence of HPCH. Moreover, the keratin degradation procedure allowed calculation of the CPX content ($CPX_{memb.}$, μg/mg) and percent drug retained in the membranes ($\%CPX_{memb.}$) at end of permeation studies.

All data are the average of six determinations± standard error (SE). Statistical differences between permeation parameters were assessed by one-way analysis of variance (ANOVA) (StatView software, Abacus Concepts Inc., Berkley, CA), followed by multiple comparisons using the Fisher PLSD (protected least significant difference) test. In the relevant tables, significant difference is $P<0.05$.

RESULTS

The results of CPX permeation experiments through bovine hoof membranes, performed with P-3051 and Penlac™, are illustrated in Fig. 1; the relevant permeation parameters (J, lag time, and $Q\%_{30h}$) and CPX retained in the membranes at end of experiments ($CPX_{memb.}$, $\%CPX_{memb.}$) are summarized in Table 1.

The CPX flux values at steady state observed for the two formulations were in the same range (4.70±0.60 $\mu g/cm^2h$ for P-3051 and 3.05±0.63 $\mu g/cm^2h$ for Penlac™). The lag times, however, were significantly different: 3.36±0.46 h for P-3051 vs. 12.48±1.31 for Penlac™. The percent CPX permeated ($Q\%_{30h}$) was also significantly different for the two formulations: 2.58% for P-3051 vs. 1.06% for Penlac™. The percent drug retained in the membranes at end of the permeation studies ($\%\ CPX_{memb.}$) was similar for the two vehicles (11.02±0.77 for P-3051 and 11.57±0.66 for Penlac™).

In order to evaluate a possible enhancing effect of HPCH on both transungual and transdermal permeation of CPX, experiments with vehicles P-3051 (1.0% HPCH) and ERV (no HPCH) were performed. The relevant permeation parameters are reported in Table 2.

The transungual and transdermal CPX fluxes at steady state from the ERV vehicle were 5.95±1.12 and 121.65±31.93 $\mu g/cm^2h$, respectively; the CPX fluxes through the two substrates from formulation P-3051 were similar to those observed for ERV (6.47±2.03 and 127.45±46.29 $\mu g/cm^2h$) with promotion factors (PF) close to the unity. The permeability of mouse skin to CPX was about 20 times higher than that of keratin hoof membranes: a behavior to be ascribed to the different structural and biological nature of the two experimental barriers. The transungual fluxes obtained in these experiments,

TABLE 2 Permeation Data of CPX Through Hoof Membranes and Hairless Mouse Skin from Liquid Vehicles P-3051 (HPCH) and ERV (No HPCH)

	Permeation parameters	ERV vehicle	P-3051 lacquer
Transdermal permeation experiments	J ($\mu g/cm^2h \pm SE$)	121.65±31.93	127.45±46.29
	Lag time (h±SE)	1.23±0.22	1.51±0.20
	$Q\%_{5\ h}$ (±SE)	11.11±2.52	10.82±3.83
	PF (±SE)	–	1.04±0.38
Transungual permeation experiments	J ($\mu g/cm^2h \pm SE$)	5.95±1.12	6.47±2.03
	Lag time (h±SE)	7.24±1.49	10.44±1.96
	$Q\%_{30\ h}$ (±SE)	3.55±0.75	2.81±0.52
	PF (±SE)	–	1.09±0.34

where CPX *solutions* were tested, are of the same order of those observed with *dry* P-3051 lacquer (4.70 ± 0.60 μg/cm^{2}h, Cf. Table 1). Conversely, a substantially longer transungual lag time was observed in the case of the solution (10.44 hours, vs. 3.36 hours for the dry lacquer). This difference might be attributed to the affinity of the permeant for the alcoholic vehicle, resulting in a slower partitioning process with the barrier. On the basis of the ERV/P-3051 comparison, a promoting effect of HPCH on transungual or transdermal permeation of CPX could be excluded.

DISCUSSION

Bovine hoof membranes, although more permeable than human nails and less selective toward large permeants, are a commonly accepted model for human nails and in vitro testing of medicated nail lacquers (Mertin & Lippold, 1997a, 1997b, 1997c). When compared to human nails, they have a less dense keratin network that, when incubated in water, swells to a larger extent: a behavior possibly due to a significantly lower content of half-cystine and disulfide linkages with respect to human nails (Baden & Kubilus, 1983; Baden et al., 1973). This factor may result in a different drug motility into the two different three-dimensional structures, hence in a different permeability, a kinetic process influenced by pathway accessibility. On the other hand, the affinity (a chemical similarity not influenced by the physical structure) of a penetrant for human nails and bovine hooves, both mainly consisting of keratin, should be similar.

The experimental nail lacquer P-3051 is characterized by the presence of hydroxypropyl chitosan (HPCH), a water-soluble derivative of chitosan. Chitosans, polysaccharides similar in structure to chitin, are natural components of the exoskeleton of crustaceans widely employed in medicine for their wound healing, bacteriostatic, skin moisturizing, and protecting properties. In particular, HPCH was chosen on account of its favorable properties, such as high solubility in water, high plasticity, affinity to keratin, wound-healing activity, high compatibility with human tissues, etc. (Goosen, 1997; Muzzarelli, 2002).

According to available, still unpublished studies, the P-3051 nail lacquer, due to its particular composition, does not produce nail and skin irritation, is easily removed by water washing without need of organic solvents, and can be applied to the perungual skin where the proliferation of fungi is favored by the presence of hyphae. It forms an invisible film, nonirritating and well accepted by patients (Baudet, 2002). Penlac™ Nail Lacquer, on the contrary, contains a water-insoluble vinyl resin [butyl monoester of poly (methylvinyl ether/maleic acid)], which forms on nails a hard, water-resistant film requiring weekly removal, either mechanically (nail filing) or with organic solvents. These procedures may render the newly growing nail and the adjacent perungual skin less resistant to diffusion of dermatophytes causing onychomycosis.

Water-soluble polymers as film formers for medicated nail lacquers are in principle superior to water-insoluble ones since they do not require periodical removal. A possible disadvantage of these polymers may consist in loss of medication due to nails' exposure to water. However, the shorter lag time observed for P-3051 with respect to Penlac™ corresponds to a faster penetration/diffusion into the hoof membranes: measurable levels were indeed detected in the receiving solution after 3 hours, i.e., at the first post-application time point, instead of 12 hours for the reference formulation. Based on these results, it was assumed that a standard 6 hr sleep period after application of P-3051, with a reasonable expectancy to avoid exposure of nails to water, could allow CPX penetration into the nails in a sufficient amount before possible removal by washing. This assumption is corroborated by a still unpublished investigation on healthy volunteers (Marzo, 2000), in which 18–35% of the applied CPX dose (1.22 ± 0.82 μg drug/mg nail) was detected in human fingernails washed with water and soap 6 hours after application of P-3051. In the present study, the percent CPX recovered in the hoof membranes treated with 75 μL of lacquer (corresponding to 80 μg of drug/mg membrane) was 11% (8.8 μg drug/mg membrane).

Drug permeation from a lacquer across a hoof membrane can be influenced by three factors: 1) drug release from the dry film resulting after evaporation of volatile components, 2) drug partitioning within the membrane, and 3) drug diffusion through the membrane (Murdan, 2002). In this study, similar CPX permeation fluxes at steady state through the keratin

membranes, but significantly different lag times were observed for P-3051 and Penlac™. Therefore, according to Fick's first law of diffusion (Martin et al., 1993) the two formulations appeared to influence only the time required to establish a uniform concentration gradient within the membrane (lag time), and not the final drug concentration gradient in the membrane. The similar permeation rates observed at steady state also indicate that neither vehicle produced changes in the rate of CPX diffusion, as a result of altered membrane morphology.

The different lag time can be attributed to one or both of the previously mentioned factors: i.e., CPX rate of release from the formulation and CPX partitioning between formulation and membrane. While a detailed physical-chemical analysis of the release/partitioning effects of the two formulations under evaluation is outside the scope of the present investigation, a tentative explanation for the greater efficiency of the P-3051 vehicle with respect to Penlac™, as regards CPX transfer from the vehicle itself into the keratin membrane, might be advanced. Chitosan and its derivatives are known to possess adhesive properties towards different biological tissues: their strong positive charge may result in binding to negatively charged surfaces such as hair and skin, a factor that renders them useful ingredients in wound dressing and cosmetic products (Goosen, 1997; Muzzarelli, 2002; Peh et al., 2001; Woodley, 2001). Moreover, the free hydroxypropyl groups of HPCH may interact with keratin by hydrogen bonding and other weak interactions. A strong adhesion of the medicated HPCH film to the keratin matrix would result in intimate contact with the matrix, hence in improved CPX transport/release into it. Contributions of the other components of the P-3051 vehicle (cetostearyl alcohol NF, ethyl alcohol, and ethyl acetate) to the observed effect, although possible in principle, are difficult to assess on the basis of the present data. Ethyl alcohol and ethyl acetate, due to their quick removal from the nail surface by evaporation, should not affect CPX release or partitioning. The function of cetostearyl alcohol NF (a mixture of stearyl and cetyl alcohol) is to improve the pliability and reduce the brittleness of the final HPCH film. Whether its presence might improve the adhesion of the HPCH film to the keratin matrix and/or contribute to CPX partitioning into the membrane is still an unsolved question, possibly to be further investigated.

Although the nail matrix (hence, the nail plate and bed) is the target of medicated lacquers, untoward effects on soft tissues surrounding the nail, due to CPX absorption facilitated by HCPH, could not be discounted a priori. Chitosan derivatives have indeed been indicated as promoters of drug absorption (Kato et al., 2003; Sandri et al., 2004). However, the permeation experiments performed with P-3051 and ERV on hairless mouse skin and bovine hoof membranes disproved the possibility that HPCH might act as a permeation enhancer for CPX in either substrate.

In conclusion the present data, although obtained on a model (bovine hoof membranes) not fully representative of the human nails, when considered in combination with preliminary studies on humans (Baudet, 2002; Marzo, 2000) point to the validity of HPCH, a water-soluble chitosan derivative, as the main ingredient of nail lacquers for topical treatment of onychomycosis. Further studies are now under way to assess the scope and limitations of chitosan-based water-soluble nail lacquers containing antimycotic agents.

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