In vitro skin permeation and antifungal activity of naftifine microemulsions

Meryem Sedef Erdal, Aslı Gürbüz, Seher Birteksöz Tan, Sevgi Güngör, Yıldız Özsoy

[1]Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 34116, Istanbul, Turkey

[2]Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 34116, Istanbul, Turkey

INTRODUCTION: Microemulsions are fluid, isotropic, colloidal systems that have been widely studied as drug delivery systems. The percutaneous transport of active agents can be enhanced by their microemulsion formulation when compared to conventional formulations. The purpose of this study was to evaluate naftifine loaded microemulsions with an objective to improve the skin permeation of the drug.

METHODS: Microemulsions comprising oleic acid (oil phase), Kolliphor EL or Kolliphor RH40 (surfactant), Transcutol (co-surfactant) and water were prepared and physicochemical characterization was performed. In vitro skin permeation of naftifine from microemulsions was investigated and, compared with that of from its conventional commercial formulation. ATR-FTIR spectroscopy was used to evaluate the interaction between the microemulsions and the stratum corneum lipids. Candida albicans ATCC 10231 and Candida parapsilosis were used to evaluate the antifungal susceptibility of naftifine loaded microemulsions.

RESULTS: The microemulsion formulation containing Kolliphor RH40 as co-surfactant increased naftifine permeation through pig skin significantly when compared with the commercial topical formulation ($p<0.05$). ATR-FTIR spectroscopy study showed that microemulsions increased the fluidity of the stratum corneum lipid bilayers. Drug loaded microemulsions possessed superior antifungal activity against Candida albicans ATCC 10231 and Candida parapsilosis.

DISCUSSION AND CONCLUSION: In conclusion, this study demonstrated that microemulsion could be suggested as an alternative topical carrier featuring a potential for enhanced skin delivery of naftifine.

Keywords: naftifine, microemulsion, colloidal drug carrier system, topical antifungal

Introduction

Naftifine HCl (naftifine) is a synthetic, topical *allylamine* antifungal compound, which is effective in the management of superficial dermatomycoses. Naftifine shows primarily fungicidal activity against *Candida* species including *Candida albicans* (*C. albicans*) and *Candida parapsilosis* (*C. parapsilosis*) and it has been proven to be especially effective in moderate to severe cutaneous candidiasis caused by these two species.[1,2]

The strongly lipophilic nature of naftifine (log P: 5.4) leads to its accumulation of high concentrations within the *stratum corneum*, which is the outermost barrier layer of the skin. However, the therapeutic efficacy of antifungal drugs applied topically depends

on the ability of the formulation to overcome the *stratum corneum* barrier and improve the uptake in deeper skin layers.[3] Therefore, a drug delivery system aiming to increase the topical penetration of naftifine is important to enhance its local antifungal efficacy.

Microemulsions are one of colloidal nano-sized carriers with a dynamic microstructure that form spontaneously by combining appropriate amounts of oil, water, surfactant and a co-surfactant.[4,5] They are thermodynamically stable and isotropic systems, and they have been extensively explored for a variety of pharmaceutical applications including dermal and transdermal drug delivery.[6–9] The high solubilization capacity of microemulsions and their ability to modify the diffusional barrier of the *stratum corneum* can facilitate drug penetration into deeper skin layers compared to conventional formulations.[10,11]

The aim of the present study was to formulate and evaluate the *in vitro* skin permeation and antifungal activity of naftifine loaded microemulsion formulations comprising oleic acid (oil phase), Kolliphor EL or Kolliphor RH40 (surfactant), Transcutol (co-surfactant) and water. Attenuated Total Reflectance Fourier Transformed Infrared (ATR-FTIR) spectroscopy was used to evaluate the interaction between the microemulsions and the stratum corneum lipids. The antifungal susceptibility of naftifine loaded microemulsions was assessed against two Candida species, namely *C*. ATCC 10231 and *C*. *parapsilosis*.

Materials and methods

Materials

Naftifine was kindly provided from Eczacıbaşı Drug Company (Istanbul, Turkey), polyoxyl castor oils (Kolliphor® EL and Kolliphor® RH40) were kind gifts of BASF (Limburgerhof, Germany). Oleic acid was purchased from Sigma (St. Louis, MO, USA). Diethylene glycol monoethyl ether (Transcutol P®) was kindly provided by Gattefossé (Lyon, France). RPMI-1640 medium and 4-morpholinepropanesulfonic acid (MOPS) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in the study were of analytical grade.

Preparation and characterization of microemulsions

We previously reported the preparation and physicochemical characterization of microemulsions loaded with naftifine.[12] Briefly, the concentration range of components necessary for the formation of microemulsions was determined by construction of pseudoternary phase diagrams based on water titration method at ambient temperature (25±0.5°C). The oil phase to surfactant/cosurfactant mixture ratio varied from 1:9-9:1 (w/w) and the ratio of surfactant/cosurfactant (Km) was fixed as 1:2. The microemulsions were formed spontaneously at room temperature as a clear monophasic liquid (Table 1). Naftifine was solubilized in the oil phase at 1% concentration before preparation of the microemulsions.

In order to verify the isotropic nature of the optimized microemulsions ME1 and ME2, cross-polarized light microscopy (Olympus BX51 U-AN 360, Tokyo, Japan) imaging was performed. The droplet size and the polydispersity index of the microemulsions were determined using dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK) after pre-filtering (0.45 μm, Millex) the microemulsions. The electric conductivity and pH of the microemulsions was measured using a combined device (EuTech PC 700; Eutech Instruments, Landsmeer, the Netherlands) at room temperature. The refractive index of the microemulsions was determined by a digital Abbe Refractometer (Atogo Co.Ltd., Tokyo, Japan). In order to measure the viscosity and rheological behavior of microemulsions a Brookfield Rheometer (Brookfield DV3THACJ0, Middleboro, MA, USA) with a cone-plate measuring device was used. All measurements were conducted in triplicate in a temperature controlled environment at 25°C.

In vitro permeation study

Preparation of skin

Pig skin (obtained from a local slaughterhouse) was carefully cleaned from fat and muscle and then dermatomed to a thickness of 750 μm (Zimmer Electric Dermatome, Warsaw, IN, USA). The integrity of the skin was confirmed by transepidermal water loss (TEWL) measurement with an open chamber device (Tewameter TM 300; Courage – Khazaka Electronic, Cologne, Germany) as is stated in the literature[4].

Permeation experiments

In vitro permeation experiments were performed using Franz type diffusion cells (diffusion area of 1.76 cm^2, Permegear V6A Stirrer, Hellertown, PA, USA). Pig skin was placed on the receiver chambers with the *stratum corneum* facing upwards and then the donor chambers were clamped in place. The receptor chamber was filled with 12 mL of phosphate buffer pH 5.0 containing 30% ethanol to ensure sink conditions. The receptor phase maintained at 37°C under constant stirring (300 rpm) with a magnetic bar. Microemulsion formulation (500 µl) was placed in the donor compartment of diffusion cells up. The donors were sealed by Parafilm M® (Bemis, Oshkosh, WI, USA) immediately after the addition of the formulation in order to prevent evaporation. The permeation of naftifine from microemulsions was followed to 8 h. The commercial topical cream formulation of naftifine (Exoderil) was used as control formulation. Samples of 500 µL were removed at appropriate time intervals for analysis and replaced immediately by fresh receptor medium.

The samples were analyzed for their drug content by HPLC. Permeation profiles of naftifine were constructed by plotting time (hour) against the cumulative amount of the drug (µg/cm^2) as measured in the receptor medium. Steady state flux (J_{ss}, µg/cm^2.h) was calculated from the steady state part of the curve. The effect of microemulsions as carrier on dermal administration (enhancement ratio, ER) is determined by the following equation:
ER= Flux from microemulsion formulation / Flux from commercial formulation
Data were determined from the average of at least six experiments.

HPLC analysis

For the HPLC (Shimadzu Model LC 20AT, Kyoto, Japan) analysis of naftifine we used a reversed phase C18 Column (4.6 mm x 150 mm, 5 µm, Merck, Darmstadt, Germany) preceded by a guard column (4x4mm, 5 µm, Merck, Darmstadt, Germany). The mobile phase consisted of acetonitrile: tetrahydrofuran: tetramethyl-ammonium hydroxide buffer (pH 7.8) (62:10:28) and the flow rate was fixed at 1.2 mL/min. The wavelength of detection was set at 280 nm. The limit of quantification was found to be 0.025 µg/mL. The method was validated for selectivity, linearity, accuracy, and precision. It was found to be linear between the concentration range of

0.025 µg/mL and 100 µg/mL with a high correlation coefficient (r^2 0.999) and was precise (intra- and interday variation 2%) and accurate (mean recovery 99%).

ATR-FTIR Spectroscopy

The effect of microemulsions ME1 and ME2 and marketed formulation (Exoderil) on pig skin was investigated by ATR-FTIR spectroscopy. For this purpose, the surface of 4cm^2 pieces of dermatomed pig skin was treated with 300 µl of ME1, ME2 or commercial cream for 8h. At the end of the treatment period ATR-FTIR Spectra were recorded as the average of 40 scans with a spectral resolution of 4cm^{-1} in the 4000-650 cm^{-1} range using a Perkin Elmer Spectrum 100 FTIR spectrometer (UK) equipped with a ZnSe ATR crystal. Attention was focused on characterizing the occurrence of peaks near 2850 and 2920 cm^{-1} which were due to the symmetric and asymmetric CH_2 stretching vibrations, respectively. In order to minimize inter-sample variation, the same piece of skin before treatment was used for normalization[13].

In vitro antifungal activity

Yeasts

C. albicans ATCC 10231 and *C. parapsilosis* were used to evaluate the antifungal susceptibility of naftifine loaded microemulsions. The yeasts were cultured and maintained on Sabouraud dextrose agar for 24 hours at 30°C prior to testing.

Assay

The minimum inhibitory concentration (MIC) of microemulsion formulations was determined by the microdilution technique using 96-well microplates. The serial two-fold dilutions ranging from 5000 to 5 µg/Ml were done following the Clinical Laboratory Standards Institute (CLSI) recommendations, rule CLSI M27-A3 for yeasts.[14,15] RPMI-1640 medium (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS (Sigma, St. Louis, MO, USA) was used for the dilutions. The inoculum was prepared with RPMI-1640 medium, using a 24 h cultures adjusted to a turbidity equivalent to a 0.5 McFarland standard, to give a final concentration of $0.5x10^3$ to $2.5x10^3$ cfu/mL in the test tray. The trays were covered and placed in plastic bags to prevent evaporations and incubated 35°C for 46-50 h. The MICs were defined as the lowest concentration of compound giving complete inhibition of visible growth.[16] Naftifine was used as reference antifungal for yeast. The MIC values of the naftifine

were within the accuracy range in CLSI throughout the study[14]. All tests were performed in triplicate.

Statistical analysis

All results are the mean ±SD of at least three experiments. The statistical analysis was performed using the Tukey's Multiple Comparison test, with $p<0.05$ as level of significance (GraphPad Prism 5 Software, La Jolla, CA, USA).

Results and Discussion

Physicochemical characterization of microemulsions

The small droplet size (7.34±0.03 nm - 11.17±0.25 nm) together with its narrow distribution (0.15±0.01 - 0.19±0.01) indicated the homogenous nature of the microemulsions and explained the clarity and isotropicity of the systems. The conductivity values revealed that the microemulsions were in oil in water (o/w) form and naftifine in salt form led to an increased conductivity. The pH values of the microemulsions were reasonable for their cutaneous application (4.26±0.01 - 5.71±0.03). The refractive indices of the microemulsions confirmed the transparency of the formulations. The apparent viscosity values of the microemulsions were between 70.76±0.12 Cp - 83.58±0.46 Cp and, they showed Newtonian flow behavior.[12]

In vitro permeation study

The permeability of naftifine from microemulsions was measured *in vitro* using excised pig skin. The cumulative percutaneous penetrating amounts of naftifine from the microemulsions and from the commercial cream are illustrated in Figure 1 and the permeation parameters are summarized in Table 2.

Flux of naftifine from the microemulsion ME1 (109.99±1.58 **$\mu g.cm^{-2}.h^{-1}$**) was significantly higher than those of ME2 (45.59±2.10 **$\mu g.cm^{-2}.h^{-1}$**) and commercial cream (21.32±1.56 **$\mu g.cm^{-2}.h^{-1}$**) ($p<0.05$). It has been reported that percutaneous permeation is an essential requirement for satisfactory topically applied antifungals in order to achieve therapeutic concentration for deep fungal infections.[17] The ability of a microemulsion to improve the transport of drugs to and across the skin is largely influenced by the composition and concentration of the microemulsion components,

as well as the internal structure and type of the microemulsion used (o/w, w/o or bicontinuous systems).[10,18] Various oils have been used as components of the oil phase in microemulsions. Oleic acid is one of the most frequently selected ones and, it has been demonstrated that oleic acid containing microemulsions increased the penetration of lipophilic drugs through the skin.[19,20] Transcutol P is a well-known penetration enhancer in skin delivery and by its amphiphilic nature it has been known to reduce the interfacial tension resulting in balanced microemulsions systems.[21] Although both of the microemulsions contained oleic acid and Transcutol P, the highest permeation was obtained by ME1 demonstrating a possible synergistic effect of the added surfactant Kolliphor®RH40. The content of surfactant mixture in ME1 influenced the permeation rate of naftifine. Also the lower viscosity of ME1 (75.62±0.24 cP) than ME2 (83.58±0.46 cP) might provide better mobility for the drug molecules, and consequently a faster release with a diffusion controlled mechanism.[12,22]

The permeability coefficient (Kp) of naftifine in ME1 was fivefold higher than the value obtained for commercial formulation. The microemulsion formulation ME1 had highest ER_{Flux} (5.16) with the ~ 5 fold enhancement rate compared to commercial formulation. It was also found that the lag times shortened by using microemulsions (Table 2). This result was consistent with previous studies reporting that microemulsions remarkably shortened the lag time of formulations.[23]

ATR-FTIR Spectroscopy

In Figure 2, spectra of untreated pig skin (control), and pig skin treated with either microemulsion formulations (ME1 and ME2) or commercial cream (Exoderil) are shown. The most characteristic bands are the asymmetric and symmetric CH_2 stretching absorbances at 2920 cm^{-1} and 2850 cm^{-1} arise from endogenous skin lipids. We observed that both the CH_2 stretching vibration bands shifted towards higher wave number after microemulsion application, which could be attributed to a decrease in the conformational order of the stratum corneum lipids. Thus, disorder of barrier might further have enhanced permeation of naftifine across the stratum corneum.[17,24]

In vitro antifungal activity of naftifine microemulsions

Naftifine is a topical antifungal agent having shown a specific inhibitory effect in sterol biosynthesis of *C. albicans* and *C. parapsilosis*.[25] The *in vitro* antifungal activity of naftifine loaded microemulsions ME1 and ME2 against *C. albicans* ATTC 10231 and *C. parapsilosis* (port blood) was higher than the commercial topical cream (Exoderil) as can be seen in Figure 3. These results proved that microemulsions had superior fungicidal efficacies due to considerable penetration of the drug into fungal cell membranes.[26] Jadhav et al. (2011) observed greater activity of fluconazole loaded microemulsions than that of conventional gel formulations and this fact was attributed to the better diffusion of the drug provided by the presence of surfactants and oil phase.[27] Similarly, El-Hadidy et al. (2012) observed considerably higher antifungal activity with voriconazole microemulsions against *C. albicans* when compared to the supersaturated solution of the same drug.[28]

Microemulsion formulation M1 which contains Kolliphor EL as surfactant, showed higher antifungal activity as compared to that of the microemulsion M2 and Exoderil. Kolliphor EL is a non-ionic surfactant and it is a well-known formulation vehicle of various hydrophobic drugs, including anesthetics, photosensitizers, sedatives, immunosuppressive agents and anticancer drugs.[29] Kolliphor EL containing microemulsions have been considered as effective topical carriers to deliver the antifungal agents oxiconazole and terconazole.[30,31] The composition of microemulsion M1 in our study might alter the permeation of fungal cells more than the formulation M2 and commercial cream which consequently resulted in higher penetration of microemulsion globules containing naftifine through fungal cell walls.

Conclusion

Our results showed significant naftifine permeation enhancement by microemulsion formulation comprising oleic acid, Kolliphor RH40, Transcutol and water when compared with its conventional topical formulation. ATR-FTIR spectra revealed that microemulsions increased the fluidity of the stratum corneum lipid bilayers. Drug loaded microemulsions showed superior antifungal activity against two *Candida* species. In conclusion, this study demonstrated that microemulsion formulation of naftifine could be a potential alternative to conventional formulation of drug.

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Figure Legends

Figure 1. Comparison of skin permeation rates of naftifine from microemulsions and from the topical commercial formulation. At least 6 experiments (n≥6) were performed for each formulation.

Figure 2. Comparison of ATR-FTIR spectra of untreated and formulation (ME1, ME2 and commercial cream) treated pig skin samples

Figure 3. Values of MIC determined either for naftifine containing microemulsions (ME1 and ME2) or for the commercial topical formulation (Exoderil).

Table 1. The composition of naftifine loaded microemulsion formulations

Formulation Components	**ME1 (% w/w)**	**ME2 (% w/w)**
Oleic Acid	8.0	8.7
Kolliphor®RH40	19.0	-
Kolliphor®EL	-	17.2
Transcutol P®	38.0	34.8
Naftifine HCl	1.0	1.0
Water	34.0	38.1

Table 2. Effect of microemulsions (ME1 and ME2) on percutaneous permeation of naftifine

Formulation	**Flux (J_{ss}) ($\mu g.cm^{-2}.h^{-1}$)**	**Permeability coefficient (K_p) ($cm.h^{-1}$)x10^{-3}**	**Lag time (h)**	**ER_{Flux}**
ME1	109.99±1.58	10.10±0.23	1.22±0.87	5.16
ME2	45.59±2.10	4.56±0.41	1.55±0.56	2.14
EXODERIL	21.32±1.56	2.13±0.85	3.50±0.49	1.00

Each data point is the mean ±SD of six determinations