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Permeation studies of novel terbinafine formulations containing hydrophobins through human nails *in vitro*

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ABSTRACT

Existing treatments of onychomycosis are not satisfactory. Oral therapies have many side effects and topical formulations are not able to penetrate into the human nail plate and deliver therapeutical concentrations of active agent *in situ*. The purpose of the present study was to determine the amount of terbinafine, which permeates through the human nail plate, from liquid formulations containing enhancers, namely hydrophobins A–C in the concentration of 0.1% (w/v). The used reference solution contained 10% (w/v) of terbinafine in 60% (v/v) ethanol/water without enhancer. Permeability studies have been performed on cadaver nails using Franz diffusion cells modified to mount nail plates and filled with 60% (v/v) ethanol/water in the acceptor chamber. Terbinafine was quantitatively determined by HPLC. The amount of terbinafine remaining in the nail was extracted by 96% ethanol from pulverized nail material after permeation experiment and presented as percentage of the dry nail weight before the milling test. Permeability coefficient (PC) of terbinafine from reference solution was determined to be 1.52E−10 cm/s. Addition of hydrophobins improved PC in the range of 3E−10 to 2E−9 cm/s. Remaining terbinafine reservoir in the nail from reference solution was 0.83% ($n = 2$). An increase of remaining terbinafine reservoir in the nail was observed in two out of three tested formulations containing hydrophobins compared to the reference. In all cases, known minimum inhibitory concentration of terbinafine for dermatophytes (0.003 μg/ml) has been exceeded in the acceptor chamber of the diffusion cells. All tested proteins (hydrophobins) facilitated terbinafine permeation after 10 days of permeation experiment, however one of them achieved an outstanding enhancement factor of 13.05 compared to the reference. Therefore, hydrophobins can be included in the list of potential enhancers for treatment of onychomycosis.

1. Introduction

Nail and hoof are two different modifications of claws occurred in mammalian evolution and were produced by differential growth (Spearman, 1985), thus these two biological materials show some structural similarities. The structure and properties of the human nail plate are elucidated with the development of new technologies and analysis techniques. Evidence that the nail plate is produced by the underlying matrix is obtained by the researchers Zaias and Alvarez in 1968 (Zaias and Alvarez, 1968). Commonly used values of growth rate are 3 mm per month for fingernails and 1 mm per month for toenails, which means that the complete renewal of fingernails is around two times faster than the complete renewal of toenails (Murdan, 2008). Desmosomes as a type of junction between the membranes of the cells forming the nail plate and between adjacent corneocytes all along the tortuous intracellular boundaries are described in the works of Caputo et al. (1982) and McCarthy (2004). Cells on the dorsal side of the nail are thinner than those on the ventral side and intracellular spaces are frequently observed (Achten et al., 1991). Nail plate mainly consists of keratin filaments and Garson et al. determined their orientation (Garson et al., 2000). The energy to cut nails transversely (3 kJ/m^2) is only half that is needed to cut them longitudinally (6 kJ/m^2), which is similar with values found for horse hoof (Farren et al., 2004). Mean carbon content in the fingernails of healthy adults is 45% and increases with aging. Females have more sulfur and less nitrogen, but the sulfur content does not change with aging (Dittmar et al., 2008). Understanding the structure and properties of the nail plate is fundamental for the development of potent drugs, which can be used in the treatment of nail diseases. Further, the impact of toxins and drugs on nails can be better understood. Thus, although dead tissue, the human nail plate can provide useful information for retrospective analysis. For example poisons such as thallium and arsenic are stored in nails and a large number of trace elements can be detected in nails giving a picture about pollution or level of exposure to toxins at the working place. Nail clippings as

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easy available and non-invasive samples can be utilized for measuring long-term control of blood glucose levels in diabetic patients or detection of hepatitis B virus DNA in positive patients (Daniel et al., 2004). Cancer chemotherapeutic agents can induce changes in the nail. Paclitaxel, methotrexate, or taxanes, which includes paclitaxel induce mostly but not only changes where cell proliferation occurs in the nail matrix (Uyttendaele et al., 2004). The affinity of some substances to accumulate in nails and/or alter the nail structure, beside the retrospective analysis and information about condition of human organism in general, can serve as a starting point for drug development in the treatment of nail diseases, such as onychomycosis. Onychomycosis is a common nail disease and the number of patients rises among the elderly population. This fungal nail infection needs a prolonged treatment period and has a low cure rate. Drake et al. conclude in their study with 258 subjects with a median age of 51.5 years that the mean duration of the nail disease is 9.5 years (Drake et al., 1998). Potent and the latest widely accepted antifungal agent terbinafine with its lipophilic and keratinophilic nature fulfills the requirements for an effective drug. Terbinafine was developed from naftifine, which was discovered serendipitously in 1974 during the synthesis of compounds active in the central nervous system at Sandoz research institute Wander (Berney and Schuh, 1978; Stütz, 1988). The mode of action for this synthetic allylamine involves inhibition of enzyme squalene epoxidase in fungal ergosterol biosynthesis, which induces accumulation of intracellular squalene and cell's death (Ryder, 1992). It was suggested that orally administered terbinafine reaches the nail plate by diffusion from the nail matrix and the nail bed (De Doncker, 1999). The molecular mechanism by which terbinafine inhibits the enzyme reminds unclear because structural information of squalene epoxidases is currently unavailable (Fuglseth et al., 2009). Terbinafine can be applied systemically or topically. Oral tablets are usually prescribed for the treatment of onychomycosis, while topical formulations are indicated for skin infections. Due to adverse effects topical therapy in onychomycosis treatment would be preferable. Although oral terbinafine is since 19 years present on the world market (Newland and Abdel-Rahman, 2009), by our knowledge there is no efficient and approved topical terbinafine formulation for the treatment of onychomycosis on the market yet, mainly because of the restrictive barrier properties of the nail plate. Amorolfine and ciclopirox have been approved for onychomycosis treatment in Europe and ciclopirox has been approved by the FDA (Elkeeb et al., 2010). Usually, the treatment includes oral and topical application in combination according to a time schedule. Currently, investigations of drugs to be applied topically are divided in several directions: (I) iontophoretic delivery, which was demonstrated in the work of Nair et al. (2009), (II) addition of chemical enhancers as it was shown by Brown et al. (2009), (III) investigation of novel pharmaceutical formulations/carriers, such as transfersomes licensed by Celtic Pharma or lacquer formulations (Lehman et al., 2005; Jan et al., 2008), (IV) physical removal of certain parts of the nail, for example, by forming microconduits in the nail plate (Boker and Burks, 2007), and (V) discovery of new drugs, such as AN2690 by Hui et al. (2007).

The purpose of the present work was to investigate the permeability of the antifungal drug terbinafine hydrochloride through the human nail plate from liquid formulations containing various enhancers. Terbinafine (written terbinafine in the text indicates used terbinafine hydrochloride) has been chosen because it is the most potent antifungal drug against dermatophytes (Ghannoum et al., 2000). Due to its lipophilicity, formulations were prepared with the addition of ethanol and therefore studies to evaluate a potential influence of ethanol on the human nail plate were performed. The amphiphilic fungal proteins called hydrophobins showed an enhancement effect in our previous permeability studies using caffeine as model drug (Vejnovic et al., 2010) and therefore it is hypothesized and tested if hydrophobins can be potential and promising universal enhancers to be applied to the human nail plate. Thus, three different hydrophobins, small proteins with astonishing features of self-assembling properties were used in this study and permeability coefficients of terbinafine were determined with their presence using human cadaver nails. The relation between the amounts of terbinafine penetrated into the nail, which forms a reservoir in the nail and enhancement factor was investigated after the permeation experiment and performed milling test. Finally the formulations were discussed in respect to their enhancement factors and known minimum inhibitory concentration of terbinafine for dermatophytes.

2. Materials and methods

2.1. Materials

Terbinafine hydrochloride was purchased from Molekula, Germany and hydrophobins A–C were a gift from CIBA, Switzerland. Hydrophobin A is a class I hydrophobin TT1 from the thermophile fungus *Talaromyces thermophilus* produced in *E. coli* as a fusion protein with glutathione-S-transferase. Hydrophobin B is a chimeric protein consisting of the N-terminal part of the class I hydrophobin SC3 from *Schizophyllum commune* and the C-terminal portion of the class II hydrophobin HFB2 from the fungus *Trichoderma reesei*, also expressed in *E. coli*. Hydrophobin C is a class I hydrophobin POH3 from the fungus *Pleurotus ostreatus* produced in *E. coli* as a fusion protein with glutathione-S-transferase. 96% (v/v) ethanol (Ph.Eur. III) was purchased from Synopharm, Basel, Switzerland. Double distilled and filtrated water was produced in-house. All other reagents were of analytical grade.

2.2. Collection and characterization of nail samples

Human cadaver nail samples were collected from corpuses at the Institute of Anatomy and Cell Biology, Freiburg, Germany. Method of collection was developed in a previous study and is explained in detail elsewhere (Vejnovic et al., 2010). Information about age and sex were recorded for all nail samples and then kept at −20 °C. One day before the permeability studies were performed, nail samples were left over night for equilibration at open air and room temperature. After equilibration nail samples were characterized by the measurement of weight (Analytical balance, type AT261, Mettler Toledo, Switzerland), thickness (Digit cal SI, TESA S.A., Switzerland), transonychial water loss (Tewameter TM210, Courage&Khazaka electronic GmbH, Germany), and visioscan images (Visioscan VC98, Courage&Khazaka electronic GmbH, Germany). Subsequently, they were hydrated in double distilled and filtrated water for 60 min in order to achieve maximal hydration. Thickness and visioscan images of swollen nails were recorded. The weight of cut nail used in the experiment was calculated from difference in weight of the whole nail sample and the nail rest after cutting. Transepidermal water loss indicates the integrity and state of the skin and can be measured with the Tewameter. The same method applied to the nail delivers useful information about nail state and hydration rate. Transonychial water loss (TOWL) of dry nails and wet nails mounted in Franz diffusion cells without and with 60% (v/v) ethanol/water in the acceptor chamber was measured. The distance of 7.78 mm between the sample and the sensors in the measuring sonde, which represents the thickness of a gum ring of the modified Franz diffusion cell set up, was kept constant. Visioscan VC98 is equipped with an UV-A light video camera and it was originally developed to study skin surface characteristics. All characterization measurements were repeated directly after the permeation experiments and once again after 24 h.

2.3. Preparation of formulations and saturation effect

According to series of solubility tests, 10% (w/v) terbinafine in 60% (v/v) ethanol/water was chosen as the best formulation to be tested by permeation studies. Formulations with 10% (w/v) terbinafine in 40% (v/v), 50% (v/v), and 60% (v/v) ethanol/water were also investigated in respect to solubility before the selection was done. All formulations were kept at room temperature and protected from light by covering the glass flasks with aluminum foil and placing them in a shadowy place. Changes were observed in the formulations containing 40% (v/v) and 50% (v/v) of ethanol. In the case of 40% (v/v) ethanol solution, the formulation became slightly milky at the junction of the two solvents during addition of water and after 1 h the formulation remained still milky. Further, it was tested if the use of an ultrasonic bath can improve the formation of one phase and clarify the solution. On the contrary, the formulation became heavily milky and precipitation increased. A possible explanation can be found in the field of sonochemistry (Suslick, 1989). The ultrasound waves can compose cavities, which in one moment can implode and the gas inside the cavity can generate heat. The dynamics of cavity growth and implosion are dependent on local conditions, for example the form of a material. Shock waves can drive small particles to hit each other at speeds of more than 500 km/h and thus, collisions of particles can cause particles to agglomerate. The formulation containing 50% (v/v) ethanol was promising, but after 3 days of storage crystals appeared and therefore the stability of the formulation was questionable during the complete duration time of the permeation experiment. Therefore, the formulation with 10% (w/v) terbinafine in 60% (v/v) ethanol/water was selected as reference. Hydrophobins were tested as enhancers in the concentration of 0.1% (w/v). Results of pH measurements were recorded for the reference and for the formulations with hydrophobins as well as for the acceptor solutions at the beginning and end of each experiment.

Due to the high lipophilicity of terbinafine, the saturation concentration in 60% (v/v) ethanol/water was important to be determined. In a glass vial covered by aluminum foil and closed with a gum lid to prevent evaporation 10 ml of 60% (v/v) ethanol/water was poured. Saturated terbinafine solution was prepared in the vial by stirring an excess of the drug at 400 rpm at room temperature of 25 °C. Samples taken from the suspension were centrifuged at 10,000 min^{-1} for 5 min. Subsequently supernatants were diluted and quantified by high pressure liquid chromatography (HPLC). The samples were taken until a plateau of three measurement points was reached and no replicate measurements were done.

2.4. Influence of ethanol content in the formulations on cadaver nails

Two sets of experiments were performed to evaluate the influence of ethanol concentration in the formulations on the nail swelling behavior and drying process. The nail weight was chosen as the parameter to study nail swelling and drying. It has to be mentioned that nails chosen for the first set of experiment were additionally pretreated. They were immersed in phosphate buffer saline for 1 h, a day before they were left to dry over night. In both cases, the nail samples were left to dry over night at room temperature and open air. The day after, weights of dry nail samples were recorded. In the first set of experiment nail samples were immersed for 1 h in water, 20%, 40%, and 60% (v/v) ethanol, respectively. Hereafter, they were taken out, softly dried with a paper to remove solution adhered to the surface, and immediately weight was measured on an analytical balance. Due to the fast drying of the samples and time dependent differences in weight, the first value after the stabilization of the balance was noted. Thereafter, the nails were placed in an open Petri dish in a way that the up-taken solution could evaporate and hence the nails dried. Their weights after 5, 10, 30, 60, 120, 360, and 1440 min were recorded. The immersion of nail samples and the measurements of weights followed a special plan to avoid overlapping. In the second set of experiment, the same procedure of nail's immersion into water and hydro-ethanolic solutions was repeated, but after each measurement nails were returned into the corresponding solution. The schedule of 1 h, 24 h, 48 h . . . 240 h, 20 days, and 34 days was fulfilled. During that time the glass vials with solutions were kept at room temperature of 23 °C and protected from light.

2.5. HPLC analysis

The amount of terbinafine was quantified by high pressure liquid chromatography (HPLC) system (Agilent 1100 Series) using an EC 250/4.6 Nucleodur 100-5 C18 ec HPLC column (Macherey-Nagel AG, Switzerland) with the following specifications: length of 250 mm, an internal diameter of 4.6 mm, a particle size of 5 μm, and a pore size of 110 Å. Mobile phase was a mixture of 75% acetonitrile, 5% tetrahydrofurane, and 20% 10 mM phosphate buffer (pH 4.5). Injection volume was 40 μl, flow rate 0.7 ml/min, and wavelength was set to 224 nm. All samples were measured at a temperature of 20 °C. The stock solution was 10% (w/v) terbinafine in dimethyl sulfoxide and dilutions were prepared in 60% (v/v) ethanol/water. The method was validated in terms of linearity, precision, and accuracy. The range of the calibration curve was from 0.01 to 20 μg/ml and correlation coefficient was >0.999. Stability of the standard solutions was confirmed within 28 days. Precision and accuracy were evaluated from three different formulations and five measurements within 24 h. Results for coefficient of variation were in the range of 3.99–7.48% and for accuracy between 88.71 and 102.06%. One of the tested formulations contained 0.1% (w/v) hydrophobin C and no outlier value was observed. It was concluded that hydrophobins used as enhancers in the formulations did not show any influence on the quantitative measurements of terbinafine.

2.6. Permeation studies

Permeation studies were preformed using Franz diffusion cells with a diffusion area of 0.785 cm^2. The acceptor chamber was filled with 6 ml of 60% (v/v) ethanol/water solution and constantly stirred with a magnetic stirrer at 400 rpm. The water jacket retained a temperature of 32 ± 1 °C. The formulation (400 μl) was applied on the nail surface. The same volume of sample was taken from the acceptor once per day during 10 days of the experiment and acceptor chamber was refilled each time with 400 μl of 60% (v/v) ethanol/water solution, which was kept in a dark place at room temperature. An occlusive effect was attained throughout the experiment and the whole set up was protected from daily light by cardboard. The amount of terbinafine in the collected samples was determined by HPLC. The flux is defined as the amount of drug permeated through the nail per time and unit area and here it was calculated within the last 6 days of the experiment to assure steady state flux. The permeability coefficient, PC (cm/s) was derived from terbinafine flux and measured terbinafine concentration of the applied formulation after 10 days of storage in a dark place and room temperature in order to fulfill stricter requirements. Approximation that 1 cm^3 is equal to 1 ml was used for PC calculation. The enhancement factor represents the permeability coefficient of the formulation with enhancer divided by the permeability coefficient of the reference containing no enhancer. In order to statistically evaluate the data in comparison to the refer-

Table 1
TOWL of dry nails before and after permeation experiments.

TOWL (g/h m^2) before experiment		TOWL (g/h m^2) after experiment	
Reference	0.4	Reference	0.5
	0.4		0.5
	0.9		1.0
Hydrophobin A	0.5	Hydrophobin A	0.5
	0.9		0.5
	0.6		0.5
Hydrophobin B	0.1	Hydrophobin B	0.9
	1.1		0.1
	0.8		0.8
Hydrophobin C	1.0	Hydrophobin C	1.0
	1.3		0.0
	1.3		0.1

ence, a preliminary test for the equality of variances was performed prior *t*-test.

2.7. Milling test

The milling test was performed using the Freezer/Mill 6750 (SpexCertiPrep, Matuchen, USA), which was settled in the fridge room to avoid environmental temperature changes. The sample was pre-cooled for 10 min in liquid nitrogen and then milled for 2 min in one cycle. After an equilibration time at room temperature, the pulverized nail was suspended in 25 ml of 96% ethanol. Ultrasonic bath was avoided for an extraction process of terbinafine and replaced with shaking by hand for 5 min. Immediately after centrifugation for 5 min at 10,000 min^{-1}, supernatants were diluted and measured by HPLC. Taking into account a dilution factor of 500, the amount of terbinafine remained in the nail plate was presented as a percentage of the dry nail weight before the milling test.

3. Results and discussion

3.1. Characterization of the nail samples

In order to ensure hydration and higher flexibility of nails used in the permeation studies, nails were immersed in double distilled and filtrated water prior to the experiment. After the immersion time of 1 h, detected weight increase was found to be 19.5% with a standard deviation of ±4.8%. The obtained value was in agreement with the findings of the previous study, in particular 23 ± 10% is recorded after maximal hydration in phosphate buffer saline solution and 22 ± 8% is found after the experiment performed in an exsiccator under controlled relative humidity of 92–93.3% and temperature of 23.3 °C (Vejnovic et al., 2010). The weight of dry nails before and 24 h after the experiments was compared. Slight increase of weight was detected in dry samples measured after the permeation studies, with an average of 1.25%. In our previous research, a decrease of weight after the experiments in 95.6% of all nail samples is noted, as well as an increase of TOWL in 69.8% of all cases. The results of TOWL are summarized in Table 1. Four nail samples showed increase, five showed decrease, and three no change, which gave 33.3% for increase, 41.7% for decrease, and 25% of all 12 nails for no change. In contrast to the previous work where different chemical enhancers with different modes of action are used, in the present study three types of hydrophobins with a similar mode of action, but different characteristics were tested. Some previously rendered enhancers, such as methanol or dimethyl sulfoxide interact with keratin from the nail plate and cause irreversible structural changes. On the other hand, hydrophobins did not show such aggressive structural changes in nail samples, which was here demonstrated by a slight increase of weight after the permeation studies. Although the formulations were prepared in 60% (v/v) ethanol/water solutions, it seems that ethanol addition was not significantly contributing to a recordable damage of the nail plates. This slight increase of weight and decrease or no change of TOWL values in more than half of the samples could be due to the adsorption of hydrophobins on the nail surface. If this hypothesis can be confirmed then hydrophobins could act as enhancers and as protectors at the same time. Visioscan images of nail surfaces could be distinguished according to the type of applied formulation. In our previous work Visioscan VC98 was first applied to study the human nail surface and it is found to be a useful method to provide data about nail surface conditions. Fig. 1 shows dry surfaces of nail plates before and 24 h after the experiment. Formulations with hydrophobins A and B in some cases left a thin, transparent, and mostly well and equally distributed film, while after the application of reference solution no film was observed. Formulation containing hydrophobin C left like clusters on the nail plate surface.

3.2. Characterization of the formulations and saturation effect

Terbinafine was soluble and stable in the prepared formulations and no changes were observed by the naked eye during the period of 2 months. Neither of the hydrophobins was dissolved completely, which assured saturated effect of proteins and therefore their sufficient contribution in terbinafine permeation through the nail plate by interactions with the drug or the nail plate. Hydrophobin A showed minimum solubility in 60% (v/v) ethanol/water solution, which can be one of the reasons for its low enhancement effect. Hydrophobin C showed improved solubility, but it needed a longer time to achieve it and hydrophobin B showed the best solubility. Development of an analytical method to determine the saturation concentration of proteins, hydrophobins, was beyond the scope of the present work. Ethanol/water solution used for the replacement in the acceptor chamber had an average pH of 6.71 measured at temperatures in the range of 24.1–25.3 °C. The pH values for reference and other three formulations were between 3.44 and 3.93. The acceptor solutions on the last day of permeation experiments showed decreased pH values in the range of 4.95–5.41 due to the pH of the applied formulations. Fig. 2 presents changes in the formulations after 6 months. In the reference solution long and transparent crystals could be observed. Mat, cubic crystals and protein particles were found in the formulation with 0.1% (w/v) of hydrophobin A. Since the formulations were not checked in the period between 2 and 6 months, the critical stability point cannot be exactly concluded. As no crystallization effect was noted in the other two formulations, it was hypothesized that hydrophobins contributed to the physical stability of terbinafine. It is likely that a higher solubility of hydrophobins leads to higher terbinafine stability in the formulation.

Saturation concentration of terbinafine in 60% (v/v) ethanol/water solution was determined to be 191.63 mg/ml at room temperature during the measurement period of 48 h. This is four times less than the result found in literature for terbinafine (not indicated hydrochloride) saturation concentration in analytical grade absolute ethanol of 772 mg/ml (Alberti et al., 2001). Predicted water solubility of terbinafine (not indicated hydrochloride) is 7.38E−4 mg/ml (DrugBank, 2009). In the monograph of terbinafine hydrochloride is indicated that the drug is slightly soluble in water and acetone, and easily soluble in water free ethanol and methanol (Ph.Eur., 2008). From the study conducted by Alberti et al. (2001), the highest solubility of terbinafine is in neat isopropyl myristate, followed by a 50:50 (v/v) mixture of neat isopropyl myristate and analytical grade absolute ethanol, and finally in analytical grade absolute ethanol. Even though terbinafine had the same degree of saturation in all

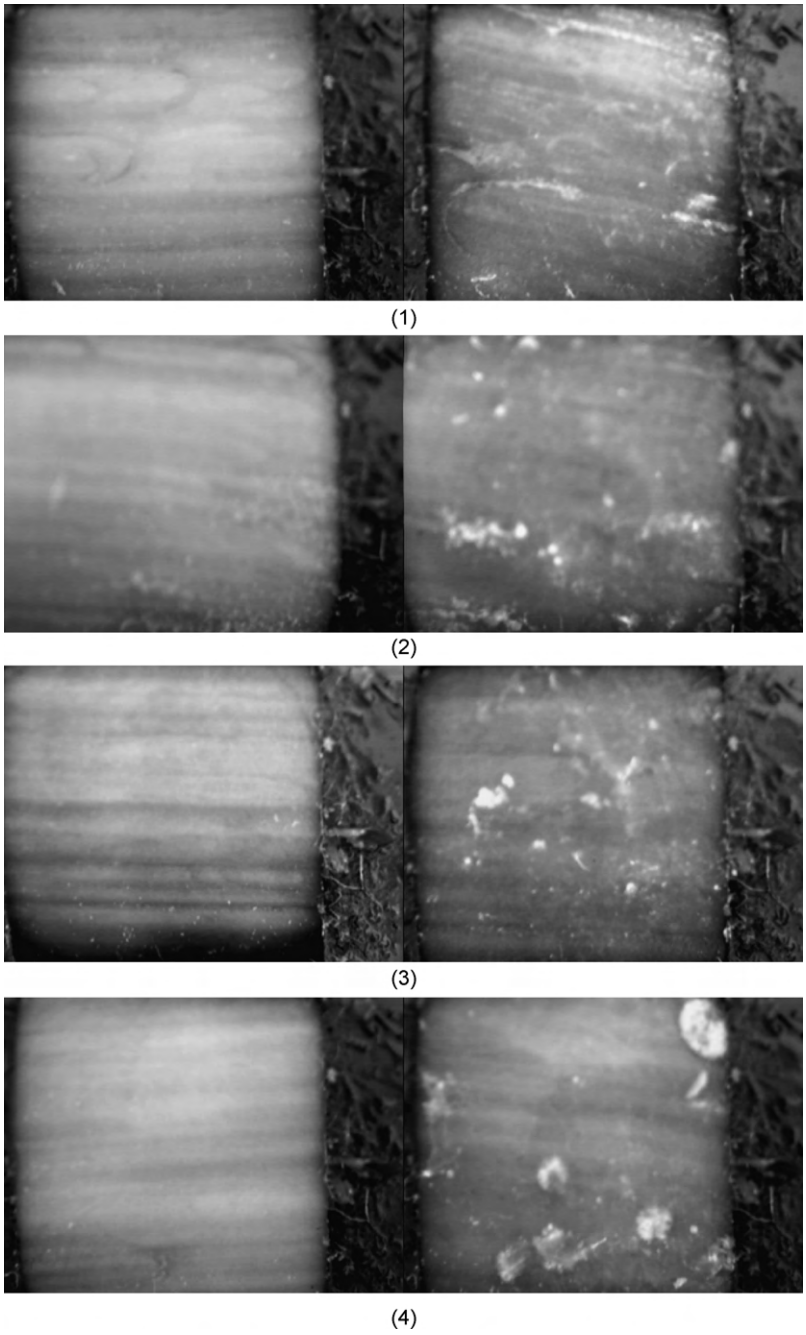

Fig. 1. Visioscan images of dry nail surfaces before (left) and 24 h after (right) the experiment (1) with reference, (2) with hydrophobin A, (3) with hydrophobin B (4), with hydrophobin C.

applied vehicles onto the skin of volunteers at a concentration equal to 1/4 saturation, i.e. the drug was administered at constant thermodynamic activity, authors concluded that the drug uptake into the stratum corneum was vehicle dependent, e.g. terbinafine penetration into the stratum corneum was greater from ethanol and ethanol/isopropyl mixture. They suggested that another factor (or factors) may be playing a role, e.g. that the entry of ethanol into the stratum corneum permits higher entry level of the drug, or that the loss of ethanol causes a supersaturated system. All formulations contained terbinafine in the concentration of 10% (w/v), i.e. 100 mg/ml, which was below the saturation concentration of the used solvent mixture ethanol/water. Thus a maximal concentration gradient was not achieved, but it was sufficient for a positive permeation and a stable solution was a prerequisite for the experiments. In this respect also toxic effects have to be considered when applying high concentrations of terbinafine, since the aim is to minimize side effects, which appear after oral application of terbinafine, by offering topical administration of the drug (Section 3.4).

3.3. Influence of ethanol content in the formulations on cadaver nails

The amount of ethanol present in the formulations showed an influence on the nail swelling behavior and drying process. The greatest swelling behavior of the nails was achieved in water and it decreased with the increase of ethanol addition. Fig. 3 shows results from both sets of experiments. Results were not uniform and certainly did not correspond to the results obtained from Section 3.1. This could be due to the number of samples investigated

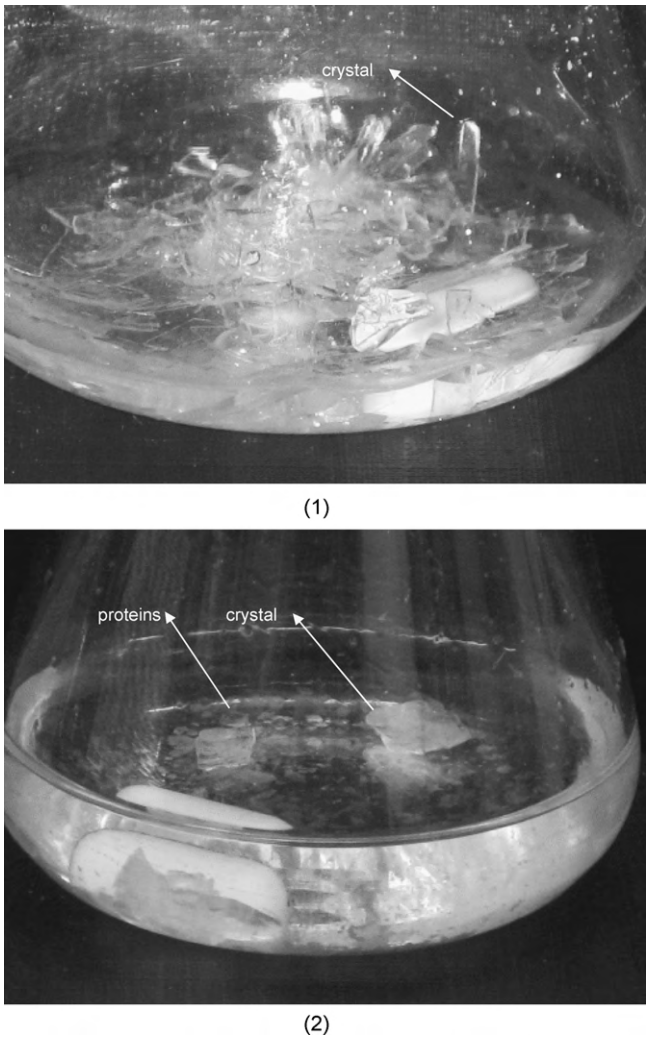

Fig. 2. Formulations after 6 months of storage at room temperature in a dark place (1) reference, (2) with hydrophobin A.

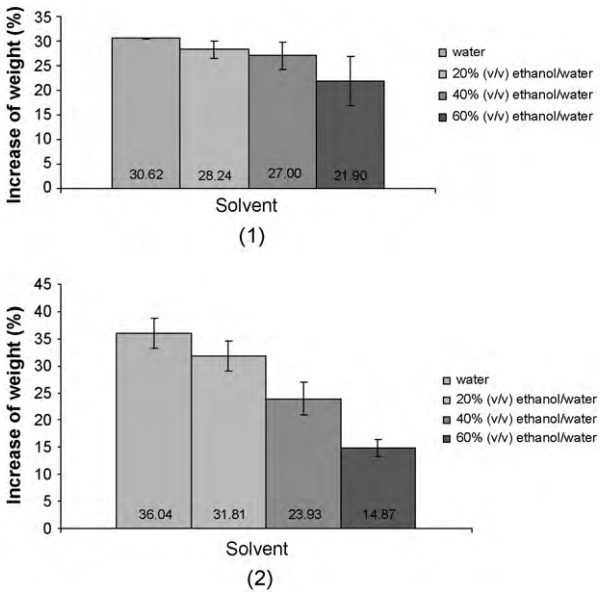

Fig. 3. Average ($n = 3$) of the nail weight increase (%) with standard deviations after immersion in various solvents for 1 h (1) in the first set of exp. and (2) in the second set of exp.

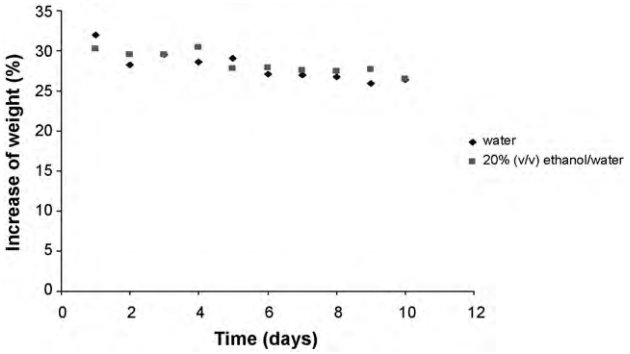

Fig. 4. Average ($n = 3$) of the nail weight increase (%) in water and 20% (v/v) ethanol/water during 10 days.

or differences in the nail plate structure among the samples, as well as due to the pre-step implemented in the first set of experiment. Therefore, the upper and lower limits regarding the swelling behavior of the nails should be observed in a wider range. No significant difference was found between swelling behavior of the nails immersed for 1 h in water or in 20% (v/v) ethanol/water (t-test, $p > 0.05$). In our previous study was found that there is no statistical difference between particular formulations with different solvents and it is concluded that 20% of ethanol in the formulation does not influence negatively the hydration of the nail during 6 days of experiment and therefore permeability coefficient. Indeed, the same conclusion can be made if we look at Fig. 4. Hydration profiles of nails in water and 20% (v/v) ethanol/water during 10 days of incubation were similar. Fig. 5 illustrates faster weight decrease of nails immersed in hydro-ethanolic solutions with higher portion of ethanol within 1 h. The drying process was fast and 50% of all absorbed solutions evaporated between 10 and 30 min (marked in Fig. 5). Interesting was that after 24 h nails in water and 20% (v/v) ethanol/water reached initial dry weight, but nails from solutions with higher ethanol portion did not, which would be expected considering the fact that ethanol has lower boiling point under the same atmospheric pressure. As it can be seen, solutions with higher ethanol portion evaporated faster during the first 60 min, but then the process slowed down. This finding illuminated the data from Section 3.1 by providing one of the reasons for weight increase after the permeation studies. In the second set of experiment nails were returned in the corresponding solution. Full hydration was achieved after 1 h in the case of water and 20% (v/v) ethanol/water solution. Therein, the weight was dropping with an increase of time. The opposite behavior was observed in 40% and 60% (v/v) ethanol/water solutions. The correlation between the increase of weight and time was positive (Fig. 6). Ethanol/water solution with 60% of ethanol at the determined time point of 816 h had a higher absorption rate compared with 40% (v/v) ethanol/water solution. It was suggested that nails exposed to ethanol/water mixture over a longer period of time would swell more till some extend and

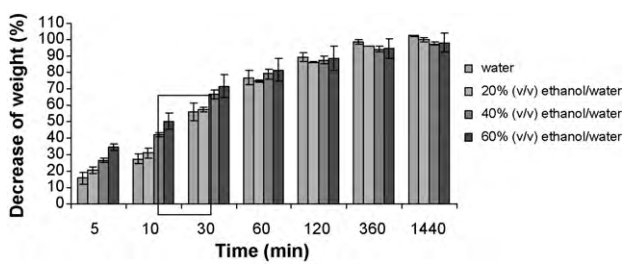

Fig. 5. Average ($n = 3$) of the nail weight decrease (%) with standard deviations during the drying period.

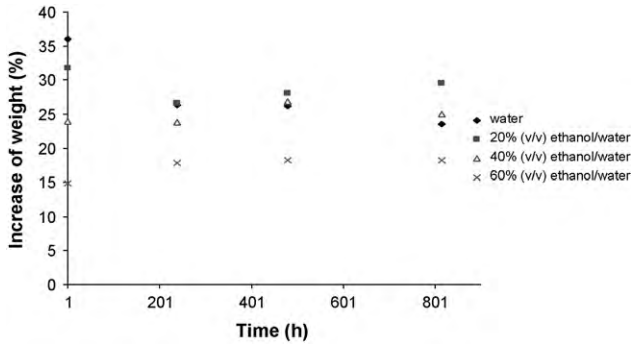

Fig. 6. Average ($n = 3$) of the nail weight increase (%) in various solvents at four time points: 1, 240, 480, and 816 h.

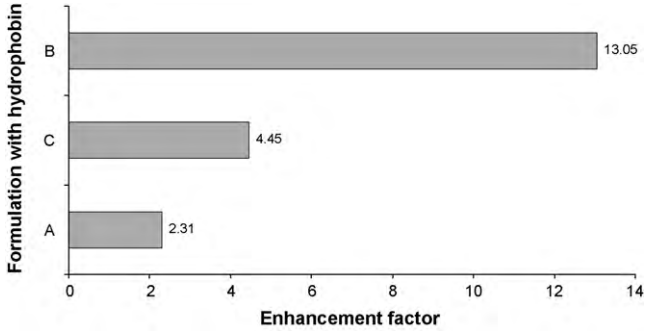

Fig. 8. Enhancement factors calculated to the reference, where the formulation with hydrophobin B shows the highest enhancement factor of 13.05 and formulation with hydrophobin A the lowest enhancement factor of 2.31.

probably this would contribute to increase the permeability of human nails. Khengar and coworkers report an increase in weight of human nail clippings ($n = 10$) after 20 h of incubation in water of $26.9 \pm 4.8\%$ and in 70% (v/v) ethanol of $22.2 \pm 2.5\%$ (Khengar et al., 2007). Increase in weight ($n = 3$) after 24 h of incubation in water was found to be $32.01 \pm 1.88\%$ and in 60% (v/v) ethanol/water $19.86 \pm 1.19\%$, which again indicated that swelling behavior of the nails should be observed in a wider range. Nevertheless, it seems that increase of ethanol in the formulation prolongs the achievement of nail's full hydration status in a defined solvent and nail's drying process in the final stage.

3.4. Permeation studies

Improvement in permeability has been seen in all three formulations. Hydrophobin B was superior to the other tested proteins with a permeability coefficient of 1.99E−9 cm/s. PC of reference was found to be 1.52E−10 cm/s and by the addition of hydrophobin A, it was improved to 3.52E−10 cm/s. For hydrophobin C used in the formulation PC was noted to be 6.78E−10 cm/s. Fig. 7 presents permeation profiles of terbinafine formulations. According to the preliminary *F*-tests which indicated rejection of hypothesis ($p < 0.05$), a two-sample *t*-test was performed that does not assume equal variances. No significant difference in PC values was found between the reference and any of the applied formulations, although in the case of the formulation with hydrophobin B *p*-value one-tail had a border value of 0.05041. Hydrophobin C showed an increase in enhancement effect of 4.45-fold, which was between the values found for hydrophobin A of 2.31-fold and hydrophobin B of 13.05-fold. Fig. 8 illustrates enhancement factors of terbinafine formulations. Differences in enhancement factor between formulations were caused mainly due to different types of used hydrophobins. In general, the whole group of fungi proteins called hydrophobins serves as a coating/protective agent,

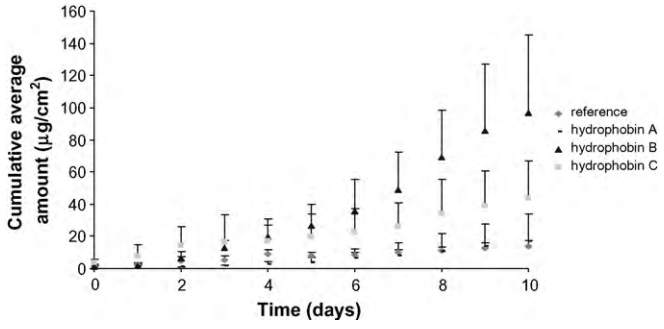

Fig. 7. Permeation profiles of terbinafine formulations ($n = 3$) with standard deviations.

in adhesion, surface modification, or some other types of functions that require surfactants (Linder, 2009). They are divided into two classes, class I and class II. This classification is based on the occurrence of hydrophilic and hydrophobic amino acid residues in primary protein structure and on some biophysical characteristics in different solutions. As the interest for hydrophobins rises, new features and fields of their application are and probably will be found. Since research on hydrophobins is ongoing, few modes of action for their behavior as enhancers on nails can be suggested. Having eight cysteine residues, which form four disulfide linkages (Cox et al., 2007), hydrophobins are not typical surfactants with one hydrophilic and one hydrophobic part, but more stable (Linder, 2009). De Vocht and coworkers conclude that these eight cysteine residues are essential for the function of hydrophobins at hydrophobic–hydrophilic interfaces during fungal development and that they can stabilize the monomeric form of hydrophobins and prevent premature self-assembly while retaining the functionality even after reduction and blocking of them (De Vocht et al., 2000). It is also found that SC3 class I hydrophobin has an ability to shift between α-helical and β-sheet form (De Vocht et al., 1998). Keratin in the nail is mainly highly folded in order to interact with its surroundings to a minimal degree (Gniadecka et al., 1998). In the presence of water keratin changes its geometry (Wessel et al., 1999). It was suggested that in the presence of water and hydrophobins from the formulations, protein interactions became more intensive not only among keratin fibers, but also with fungi proteins through secondary bonds, such as ionic interactions, hydrogen bonds, or van der Waals' forces. Further, hydrophobins adhered to the nail surface, which was demonstrated in Fig. 1. There are differences in binding characteristics among the classes. Hydrophobins from class I adhere strongly, while from class II dissociate more easily (Linder, 2009). Formation of monomers, dimers, and oligomers in solutions, or mixtures at air-water interface rearrange and assemble into films with ordered structure, which again depends on the type of hydrophobin (Szilvay et al., 2007). The other possible interaction could occur between hydrophobins and terbinafine within the formulation. Akanbi and coworkers, demonstrate on animal model that hydrophobin SC3 can be used to prepare suspensions of water-insoluble drugs and show that *in vivo* bioavailability is improved in a reduced but longer lasting peak concentration of cyclosporine A. They also propose that hydrophobin coating of the resulting nanoparticles may function as a stabilizer, preventing agglomeration (Akanbi et al., 2010). Most likely, hydrophobins with a property of self-assembling were able to "coat" terbinafine and in such way to improve terbinafine solubility and physical stability in the formulation. A new "coated" terbinafine molecule could show higher affinity to the hydrophilic gel membrane of the human nail plate compared to the reference. Kobayashi and coworkers suggest that the permeability of a drug

is mainly influenced by its molecular weight, which was later confirmed by Brown et al. (2009), who consider the molecular weight of a compound as more important than its lipophilicity in determining the extent of its permeation potential (Kobayashi et al., 2004; Brown et al., 2009). However, specific packing and imposition of hydrophobins in solutions and suggested reactions with keratin from the nail plate could contribute to the formation of unfixed, but flexible drug-hydrophobin systems, i.e. the mechanism of action might have some similarities with transfersome permeation by adapting the shape to the ambient (Paul et al., 1998). Also, the particle size in the study of Akanbi and coworkers, determined by light microscopy, shows a decrease with increasing concentration of SC3, which has to be taken into account when drug-hydrophobin system is observed (Akanbi et al., 2010). On the other hand, a positive impact on permeability rate shows terbinafine itself, since its ability to concentrate in the lipophilic stratum corneum, hair, nail plate, and adipose tissue is well known (Krishnan-Natesan, 2009). A great contribution to a more effective permeation had the reduction of surface tension of the applied formulations caused by hydrophobins. Hydrophobins are known as the most surface active proteins nowadays (Linder, 2009). There are reported data for surface tension measurements of aqueous solutions of hydrophobins and values are below 45 mN/m, depending on the type and concentration of hydrophobin in the solution. Thus, the wetting property of the formulations on the nail surface was significantly improved. Cox and coworkers propose a surface saturation concentration corresponding to the critical micelle concentration (CMC) of conventional surfactans, since they believe that small aggregates of class II hydrophobins are present in a solution before the surface is saturated with molecules of hydrophobins (Cox et al., 2007). Their observation during Wilhelmy plate method measurements is that an elastic "skin" of hydrophobins forms at the air/water surface and it is previously described by Szilvay and coworkers as a formation of HFB I film, which indicates hydrophobins' specificity as tensides (Szilvay et al., 2007). Possible explanation for such high enhancement factor of 13.05 for hydrophobin B could lie in the primary structure of the protein. As chimeric protein, a combination of features from class I and class II might be present, which could contribute to its better enhancement characteristic.

Terbinafine concentration in the acceptor chamber on the 10th day of experiment performed with the reference formulation was found to be in the range of 0.75–1.69 μg/ml. The determined minimum inhibitory concentration of terbinafine for dermatophytes (0.003–0.006 μg/ml) was exceeded in all experiments (Clayton, 1994). After an oral administration of 250 mg terbinafine bioavailability is 70–80% and serum concentration ranges from 0.8 to 1.5 mg/l, which corresponds to 0.8–1.5 μg/ml with detected concentration in the nail plates of approximately 250–550 ng/mg (Krishnan-Natesan, 2009). This means that the resulting serum concentration *in vivo* after oral application and acceptor concentration *in vitro* after topical application to the nail plate are approximately in the same range. This immediately raises the question of safety in terms of the applied formulations containing more than 10% (w/v) terbinafine. Although the results of the present study are very promising for a topical application on the human nail plate, the formulations should be tested *in vivo* before the final conclusion can be drawn.

3.5. Milling test

Table 2 shows average amount of terbinafine accumulated in the nails during the experiments for reference and applied formulations containing hydrophobins. Exception was found for the formulation with hydrophobin B, marked bold in the table, due to the inhomogeneous distribution of terbinafine in all the three nail samples and the fact that not all nail powder could be washed out from the mill tube. Increase in the drug load in nails was detected in two out of three formulations with hydrophobins compared to the reference. Found terbinafine concentration in the nail samples was in the range of 2.28–23.42 μg/mg. The amounts of remaining terbinafine in the nail samples after applied reference and other three formulations were higher than in the case of caffeine, which is hydrophilic drug and has lower molecular weight. Caffeine reservoir after the application of the reference in 20% (v/v) ethanol/water is 0.36% and for the reference in water is 0.33% (Vejnovic et al., 2010). Furthermore, remaining terbinafine after topically administered reference solution surpassed all noted remaining caffeine values, even those recorded for the formulations with enhancers, i.e. the maximum value of 0.61% for methanol as possible enhancer. One of the reasons for such a phenomenon is the different duration of experiments. Permeability experiments using terbinafine formulaltions were conducted 4 days longer compared to the experiments applying caffeine formulations. Also, these results supported the theory of terbinafine affinity towards keratin in the nail plate, which was the other important factor of influence on drug accumulation in the nail plate. In order to investigate a relation between enhancement factor and remaining terbinafine in the nail samples, Fig. 9 was created. Linear dependence was rejected regarding a low value of 0.3 for correlation coefficient. Neither exponential nor logarithmic correlation was found (data not shown).

Table 2
Remaining terbinafine in the nail after permeation experiment with standard deviation (S.D.).

Formulation	Remaining terbinafine (%)	S.D.
Reference	0.83 ($n=2$)	0.02
Hydrophobin A	0.48 ($n=2$)	0.19
Hydrophobin B	1.01 ($n=3$)	**1.16**
Hydrophobin C	1.04 ($n=3$)	0.36

3.6. Toxicity issues regarding hydrophobins

The possibility that hydrophobins may promote further development of fungus in the treatment of onychomycosis was not expected, because they were not solely applied but in combination with terbinafine. Hydrophobins are very new excipients and nearly no data are published yet about their use in pharmaceutical formulations. No literature is found on the use in trans-ungual formulations. Therefore, regarding the toxicity issue not much is known. However, hydrophobins are used as stabilizers of foams in the food industry and therefore low level of toxicity is expected. Studies with hydrophobins done on the mouse cell culture showed that mitochondrial activity is hardly affected when cells are grown on hydrophobins in the β-sheet state and therefore was concluded that hydrophobins are promising candidates to modify the surface characteristics of medical implants in a highly controlled fashion

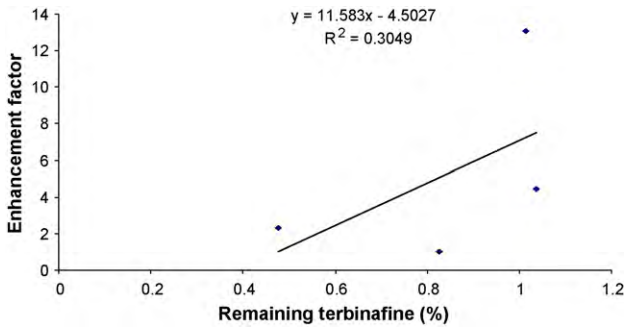

Fig. 9. Correlation between enhancement factor and remaining terbinafine in the nail, where 1 is attributed to the enhancement factor of reference solution.

(Janssen et al., 2004). Another study tested hydrophobins as stabilizers for air-filled emulsions in order to create a new generation of low fat foods in the battle against obesity. It is concluded that the air cells in the air-filled emulsion have a surface elasticity, given by the hydrophobin film, which helps prevent disproportionation and ripening over 45 days (Tchuenbou-Magaia et al., 2009). If the research is ongoing for evaluating safety and efficacy issues of orally administrated hydrophobins or incorporated in the human body, the concern for their topical use should be present in a moderate manner.

3.7. Current status of products in clinical development

As discussed in the introduction section there is no efficient and approved topical formulation containing terbinafine as an active drug on the market yet for the treatment of onychomycosis. However, a need for the topical delivery of terbinafine exists due to severe adverse effects of the oral therapy. Further, more research is needed to have the possibility to understand the nail plate as a membrane in a deeper way. These insights will be helpful to develop therapies for other diseases through this alternative pathway as well, such as for inflammatory and infectious diseases. According to the website www.clinicaltrials.gov there are 19 studies currently performed with terbinafine. Seven of these studies are registered for topical terbinafine application in the treatment of onychomycosis and two include iontophoresis. Based on passive diffusion as it was studied in the present work there are currently three different pharmaceutical formulations which are tested: (I) 10% nail lacquer sponsored by NexMed, Inc. in clinical phase I, (II) 2 and 6% terbinafine loaded organogels sponsored by MediQuest Therapeutics in clinical phase II, (III) 10% solution sponsored by Novartis in clinical phase III. Research is ongoing in various directions and applications, such as Raynaud's Disease, Nail Psoriasis, and Actinic Keratosis using the nail or the skin as application side (MediQuest Therapeutics). Combining all knowledge, expertise, and technical modalities the vision is that the human nail plate becomes one of the conventional routes for drug delivery.

4. Conclusions

An effort to deliver topically applied terbinafine and treat onychomycosis is still a challenge. Although it is on the market since 19 years, the monograph of terbinafine hydrochloride appeared for the first time in Supplement 5.3 of the European Pharmacopeia implemented on 1 January 2006 (Supplement 5.3). Unknown drug properties and side effects and the nail plate as a membrane consisting of death cells do not simplify the task. Even so, there are different approaches, which can increase the permeability coefficient of terbinafine through the nail plate. As demonstrated, a higher terbinafine concentration and therefore higher concentration gradient had a positive influence on passive diffusion. The type of the pharmaceutical formulation and dosage form can contribute to therapeutic efficacy. In respect to terbinafine physicochemical properties, increased ethanol portion was used in the formulations. The swelling behavior of the nails is suggested to be less affected by the formulations with an increased ethanol quantity during exposure over 24 h. Tested enhancers, especially hydrophobin B facilitated terbinafine's permeation and penetration. Considered as stabilizers of foams in the food industry, hydrophobins automatically can be classified among the substances with a low level of toxicity. They are water-soluble and have an amphiphilic nature, which broadens the spectrum of their use, not only as enhancers in onychomycosis therapy, but as universal stabilizers, protectors, and surface modifiers.

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