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Investigation of different formulations for drug delivery through the nail plate

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ABSTRACT

Topical therapies for nail diseases are limited by keratinized cells in the human nail plate. An optimal permeation enhancer would not only improve drug delivery through the nail plate, but would also open new possibilities for treating neighboring target sites if systemic circulation is reached. The aim of the present work was to identify permeation enhancers and to improve the understanding of physicochemical parameters that influence drug permeation. Caffeine served as the model drug, and formulations were prepared in water and 20% (v/v) ethanol/water solutions. Tested enhancers were urea, dimethyl sulfoxide (DMSO), methanol, N-acetyl-L-cysteine (NAC), docusate sodium salt (DSS), boric acid, and fungal proteins, such as hydrophobins. Permeability studies employed cadaver nails in modified Franz-type diffusion cells. The permeability coefficient of caffeine in ethanol/water was determined to be 1.56E−08 cm/s and was improved to 2.27E−08 cm/s by the addition of NAC. Formulations containing either methanol or DMSO showed the highest permeability coefficients in the range of 5–7.5E−08 cm/s. Enhancers could be classified according to their permeation enhancement: methanol > class II hydrophobins > DMSO > followed by class I hydrophobins and urea. Ethanol at a concentration of 20% (v/v) in water did not influence swelling of nail samples. Hydrophobins are suggested to be efficient in drug delivery through the nail plate.

1. Introduction

The human nail plate is a much more complex structure than it looks at the first sight. It protects the nail bed, the part directly under the nail plate filled with blood vessels, and the nail matrix, the part at the proximal ventral surface of the nail responsible for the cell's proliferation and nail growth (Farren et al., 2004). Although thin, the nail plate has 80–90 layers of dead cells (Achten et al., 1991; Murdan, 2008) in which we distinguish three macroscopic strata: dorsal, intermediate, and ventral (Kobayashi et al., 1999). It mainly consists of keratins, 4/5 is hard hair-type keratin and 1/5 is soft skin-type keratin. In the dorsal and ventral layer, skin-type keratin forms a net and these filaments are oriented parallel and perpendicular to the nail growth axis. In the intermediate layer, hair-type keratin is oriented perpendicular to the growth axis (Garson et al., 2000). The relatively high amount of water in the nail plate, 10–30%, and simultaneously ten times lower lipid content comparing with the stratum corneum of the skin, 0.1–1%, support the theory that the human nail plate behaves like a hydrophilic gel membrane (Walters and Flynn, 1983; Murdan, 2002).

Common nail diseases are onychomycosis and psoriasis. Onychomycosis is a fungal infection which occurs in the elderly rather than in children (Debruyne and Coquerel, 2001). It is responsible for approximately 50% of all nail disorders (Scher, 1994; Ghannoum et al., 2000). The most frequently reported symptoms are discoloration, thickening, and deformity of the toenails (Lubeck, 1998). Treatment options for this persistent disease include oral, topical, mechanical, and chemical therapies or a combination of these modalities. Orally applied antifungal drugs are the most effective agents available to treat onychomycosis, among them terbinafine is the first choice. Griseofulvin, which was the first oral antifungal drug approved by the US Food and Drug Administration or ketoconalzole from the Azole group are currently not used much (Gupta and Tu, 2006; Gauwerky et al., 2009). However, oral systemic antifungal therapy is limited by its toxicity, drug interactions, contraindications, high cost of medication, increased microbe resistance, a long duration of treatment, and relapse is very common (Murdan, 2002; Repka et al., 2004). Topical therapies, on the other hand, have difficulties, too. The active drug from the applied formulation has to permeate and overcome highly restrictive barrier properties of the human nail (Elewski, 1998; Khengar et al., 2007). On the market there are nail lacquers and nail solutions available for topical treatment of onychomycosis. After application, the solvent from the lacquer formulation evaporates leaving an occlusive film on the nail in which the drug concentration is higher than in the original formulation. This increases the diffusion gradient and permeation through dense keratinized nail plate occurs (Marty, 1995). For the use of nail solutions, patients are advised to apply the formulation not only on the nail surface, but on the surrounding skin as well, usually by using the provided brush. Active drug can be

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eventually delivered through an alternative pathway, i.e., the surrounding skin above the nail matrix. To increase drug permeation rate, different techniques are described in the literature, for example iontophoretic drug delivery, where the driving force of ions is an electrical field. Some of the physical approaches are nail abrasion using sandpaper (Di Chiacchio et al., 2004) or controlled nail trephination (Boker, 2007). Influence of chemicals, such as phosphoric acid on human nail samples (Repka et al., 2004) and keratinolytic enzyme, keratinase, on bovine hoof membranes (Mohorčič et al., 2007) as pretreatment, showed an increase in drug permeation rate. Further, Kobayashi et al. (1998) described the effects of N-acetyl-L-cysteine and 2-mercaptoethanol on the nail, substances which facilitate drug permeation by interacting with disulphide bridges in keratin molecules and/or nail swelling and softening.

In the present study, correlation between different enhancers and caffeine permeability through the human nail plate was examined. Thus, by comparison of the obtained permeability coefficients, the suitability of the chemical enhancers was evaluated. Further aim was to improve the understanding of physicochemical parameters that influence drug permeation through the nail plate in order to treat not only topical nail diseases, but also to consider the possibility to reach systemic circulation and neighboring target sites. Caffeine was the chosen model drug, because it is water soluble, has relatively low molecular weight of 194.2 g/mol, is easy to detect, and inexpensive. Substances tested for enhancing properties were boric acid (BA), dimethyl sulfoxide (DMSO), docusate sodium salt (DSS), methanol (M), N-acetyl-L-cysteine (NAC), urea (U), and hydrophobins. BA was choosen as potential transungual permeation enhancer due to the following reasons and thoughts: (I) BA stops microbiological growth and can be used as preservative. (II) A new class of antifungal agent, called oxaboroles, was identified by Hui et al. and therefore selected as a clinical development candidate in order to improve transungual permeation (Hui et al., 2007). According to the reasons (I) and (II) it was hypothesized in the present work that the bor atom could have properties which influence the nail structure. Dimethyl sulfoxide is an aprotic solvent and it is known to augment the transdermal drug delivery. It has been shown that DMSO increases permeability by disordering or "fluidizing" the lipid structure of the stratum corneum and interacts with keratin in the corneocytes in a concentration dependent manner (Benson, 2005). Although Walters and Flynn (1983) did not assort DMSO as promising enhancer in nail permeation, we have chosen it for this study. A large molecule, such as DSS was not expected to penetrate easily, but as an anionic detergent it decreases the surface energy and therefore, we tested it. The common laboratory solvent for poorly water soluble substances, methanol, can be absorbed through the skin in toxic amounts. Nevertheless, we selected it for the present study because of its small molecule size and good solvent properties. N-acetyl-L-cysteine belongs to the compounds which contain a sulfhydryl group and thus can act as permeation enhancer. Urea ointment containing 40% of urea is used under occlusion for nonsurgical avulsion of nails. In lower concentrations urea is a humectant, i.e., it improves the skin's ability to retain moisture. It can also act as keratolytic agent by unfolding, thus solubilising and/or denaturing keratin (Murdan, 2008). These properties were utilized for the present study. Hydrophobins are small, amphiphilic fungal proteins, constituted of 100–125 amino acid residues. They have the ability to decrease surface tension and adhere to hydrophilic or hydrophobic surfaces (De Stefano et al., 2007). Hydrophobins are divided into two classes, class I and class II, according to the number and type of amino acid residues, and the way they assemble (Kallio et al., 2007). We tested the influence of hydrophobins from both classes on the permeation rate of caffeine through the nail plate. Finally, all formulations with and without enhancers are compared using the calculated enhancement factors and the most promising enhancers are suggested in this work.

2. Materials and methods

2.1. Materials

Caffeine (Böhringer Ingelheim, Germany) was the chosen model drug. Potential enhancers were boric acid (Merck, Germany), dimethyl sulfoxide (Fluka, Sigma–Aldrich, Switzerland/Germany), docusate sodium salt (Sigma–Aldrich, Germany/USA), methanol (99.9% purity, Merck, Germany), N-acetyl-L-cysteine (Sigma–Aldrich, Germany/USA), urea (Sigfried AG, Zofingen, Switzerland), and hydrophobins (CIBA, Switzerland). Class I type of hydrophobins was obtained from Talaromyces thermophilus and class II type was obtained from Trichoderma reesei. Isotonic phosphate buffer saline (PBS) of pH 7.4 was prepared by dissolving 8 g sodium chloride (Hänseler AG, Herisau, Switzerland), 0.2 g potassium chloride (Siegfried AG, Zofingen, Switzerland), 3.63 g sodium monohydrogenphosphate with twelve molecules of water (Siegfried AG, Zofingen, Switzerland), 0.24 g potassium dihydrogenphosphate (Hänseler AG, Herisau, Switzerland), and 0.1 g sodium azide (Fluka, Sigma–Aldrich, Switzerland/Germany), as preservative, in 1 l double distilled and filtrated water, attained in-house. Ethanol formulations contained 20% (v/v) of 96% ethanol (Ph.Eur. III, Synopharm, Basel, Switzerland).

2.2. Collection, evaluation, and characterization of nail samples

2.2.1. Collection of nail samples

Human cadaver nail samples were collected from human corpuses at the Institute of Anatomy and Cell Biology, Freiburg, Germany. One or two years old corpuses, which have been used in anatomy courses, were filled with 3% solution of formaldehyde, thymol, and glycerin by a connected tube under a pressure of 1.5 bar directly into the Arteria femuralis. Hands and feet were kept in cotton sacks sodden with formaldehyde solution. The whole body was sprinkled with 3% formaldehyde solution for disinfection and preservation, and covered with a cotton lid and nylon. Nails were wet, mostly soft, and strongly bound to the surrounding tissue. A collection technique, without the use of a scalpel, was developed in this work, and thus guaranteed to collect the whole nail plate. By removing the skin, nail edges were liberated and then the nail bed was pressed down along the whole nail plate by easily placing forceps between the ventral nail plate and the nail bed, moving in the direction of nail matrix. Using claws, the nail plate was uprooted. This technique insured fast collection of the whole nail plates, without breaking them. For each of the corpuses, information about age, sex, and the cause of death were recorded. Only healthy nail plates were used in this study. Kobayashi et al. (2004) suggested that the permeability through healthy and fungal nail plates is not significantly different. Thus, the fungal nail permeability can be estimated from healthy nail permeability data. They also noted that the flux of drug through very heavily infected fungal nail plate may be higher than through a healthy nail plate. Nail samples were kept at −20 °C.

2.2.2. Evaluation of nail samples

Since nail samples were from one or two years old corpuses, possible interaction of formaldehyde might have occurred. Zhai et al. (2007) investigated the decontamination capacity of three model decontaminant solutions, tap water, isotonic saline, and hypertonic saline in human cadaver skin dosed with radio-labeled [^{14}C]-formaldehyde. Data suggested that isotonic saline provided a slight enhancement in removal of applied dose of formaldehyde. We expanded these findings on human cadaver nails. To confirm or decline the presence of formaldehyde in biological material, the following experiment was done. Two fingernail samples, 1 and 2, both from the same corpus, were left over night in an open Petri dish to dry. On the next day, only sample 2 was immersed

for 60 min in PBS, as we did with all nail samples used in this study. Afterwards, Raman spectroscopy was applied and data were obtained. Measurement was performed from the nail surface to a depth of 100 μm, with a measurement interval of 5 μm. In total, 21 measurements were taken per point. Raman spectra in the high wavenumber region from 2500 to 4000 cm^{-1}, as well as in the fingerprint area from 400 to 1800 cm^{-1} were recorded. Additionally, water and lipid content per nail were noted.

To determinate whether there was a significant difference (*t*-test) in water intake between healthy nail clippings from cadavers and healthy fresh nail clippings from volunteers under the same storage conditions, the following experiment was performed. Six fresh clippings from female, six from male, and six from cadavers were stored for 24 h in an exsiccator with a relative humidity in the range of 0.8–4.3% and temperature of 23.4 °C. After the storage period, weight and thickness of the nails were measured. Subsequently, the nails were placed in an exsiccator with relative humidity in the range of 92–93.3% and temperature of 23.3 °C. Again, after one day, weight and thickness were noted and increase in weight was compared.

2.2.3. Characterization of nail samples

Nail samples were left over night for equilibration at open air and room temperature. On the next day, weight of the whole nail, thickness (Digit cal SI, TESA S.A, Renens, Switzerland), transonychial water loss (TOWL), and visioscan images (Visioscan VC98, Courage&Khazaka electronic GmbH, Germany) were noted. Visioscan VC98 is an additional tool recommended to dermatologists for skin surface evaluation and comparison before and after the treatment. By our knowledge, this is the first time that it has been applied to evaluate variations of nail surfaces. Samples were placed in PBS solution for 60 min, in order to achieve maximal hydration. In the literature, contradictory results were found in respect to this method. Wessel et al. (1999) found that saturation appeared soon after 10 min, which was explained by a defined water holding capacity. While, Hao and Li (2008) demonstrated that the nails approached 90% of complete hydration within half an hour named as fast phase, followed by a slow phase in which equilibrium was observed within one day. Thickness and visioscan images of swollen nails were recorded. Bigger nails were cut by metallic puncher of 16 mm in diameter and afterwards thickness and weight were measured. Nail rests were collected, left over night, and weighed (mwhole nail – mnail rest = mnail in experiment). TOWL (Tewameter TM210, Courage&Khazaka electronic GmbH, Germany) of wet nails mounted in Franz diffusion cells without and with PBS in the acceptor chamber was measured. All measurements were again performed directly after the permeation experiments and once repeated after 24 h.

2.3. Preparation and characterization of the formulations

Formulations were prepared in parallel using double distilled and filtrated water (w) and 20% (v/v) ethanol/water (e). References were formulations with 2% (w/v) caffeine in water and 2% (w/v) caffeine in 20% (v/v) ethanol/water solution. Each enhancer was added in the concentration of 5% (w/v) or (v/v), in the case of liquids, to the solvent(s), except for DSS with 1% (w/v) and hydrophobins with 0.1% (w/v). All formulations contained caffeine as model drug in a concentration of 2% (w/v). Khengar et al. (2007) reported that saturated caffeine concentration in PBS is 21.6 mg/ml, prepared by incubating an excess of solid caffeine in PBS solution at room temperature. Hence, caffeine concentration was nearly saturated and thus provided faster achievement of steady state permeation through the nail plate according to the highest possible concentration gradient. The formulations were characterized by pH (pH-meter, Metrohm 827, metrohm Herisau, Switzerland),

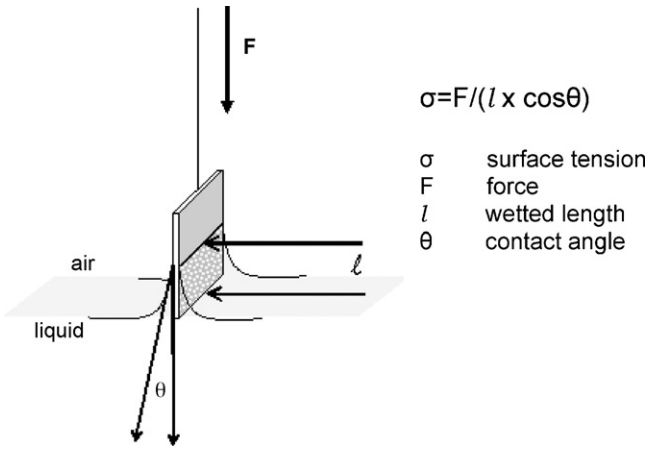

Fig. 1. Wilhelmy plate method.

viscosity (Haake Viscotester 550, Thermo Scientific, Tracomme AG, Adliswil, Switzerland), and contact angle values (Tensiometer K100, Krüss GMBH, Germany). Additionally, stability studies and capillary constant measurements (Tensiometer K100, Krüss GMBH, Germany) were performed. Formulations which contained hydrophobins were not tested thereon.

2.3.1. Surface tension and contact angle measurements

To fulfill contact angle measurements, it was necessary to measure the surface tension of solutions. Formulations were freshly prepared, left for minimum 30 min in an equilibration room where the temperature was constant at 22 °C. Wilhelmy plate method was applied, shortly plate method, to determine the surface tension. A roughened platinum plate of known dimensions was connected with a sensitive balance. The force between the platinum plate and the formulation when they were in contact was noted and the surface tension was calculated according to the equation presented in Fig. 1. If the roughened platinum plate is optimally wetted by hexane, the contact angle is virtually 0° and the cosine therefore 1. The same method was applied for the contact angle measurements. Instead of platinum plate, human cadaver nails were used, since we were interested in the wetting properties of the examined formulations on the nail surface. Chosen nail samples, flat as much as it was possible, were equilibrated over night at room temperature of 22 °C and subsequently mechanically cleaned from the remained skin, cut by scissors, and settled to be immersed in the direction of nail growth. The wetted length of nails was in the range of 8.5–9.16 mm and no statistical difference between wetted lengths was found (spss software, one-way analysis of variance (ANOVA), post-hoc test Tukey, $p > 0.05$). Detection speed was 6 mm/min, detection sensitivity 0.01 g, maximum immersion depth 4 mm, and minimum immersion depth was 1 mm. Regression was calculated from the minimum position of 1.9–4 mm.

2.4. Permeation studies

Specially modified Franz diffusion cells with a diffusion area of 0.785 cm^2 were used. Cut nail plates were mounted between a rubber and a plastic ring over the glass cells. The whole diffusion cell was put in a metal construction, which was tightened by screws in order to fix the nails. The acceptor chamber was filled with 5 ml PBS. On the nail within the rubber ring 400 μl of formulation was applied. The glass cells were temperated by a water jacket at 32 ± 1 °C. Each acceptor solution was mixed with a magnetic stirrer at 400 rpm. The donator chamber and the extensions for taking samples were covered with parafilm. Thus, an occlusive effect was attained. A sample of 400 μl was taken from each cell acceptor twice

a day at predetermined time intervals and replaced with the same volume of PBS warmed to 32 °C. The duration of the experiment was six days. Collected samples were stored in the refrigerator at 8 °C till the caffeine detection at maximum wavelength of 273 nm by UV-spectrophotometer (UV-spectrophotometer DU720, Beckman Coulter). The caffeine flux was calculated with the values after a lag time of 48 hours, as till this time steady state permeation was obtained. The permeability coefficient, P (cm/s) was calculated from the caffeine flux in the steady state and initially measured caffeine concentration of the applied formulation. The enhancement factor represents the permeability coefficient of a formulation with enhancer divided by the permeability coefficient of a corresponding formulation without enhancer, i.e., reference. Thus, the possible enhancing effect of ethanol was eliminated. Additionally, possible interference of ethanol on UV measurement was investigated and concluded that enhancement factor did not notably change. The data were evaluated using two calibration curves (with 20% ethanol and without) and no difference was found.

2.5. Milling test

After the permeation experiment nails were milled in order to detect the amount of caffeine remained in the nail (Freezer/Mill 6750, SpexCertiPrep, Matuchen, USA). The remaining formulation on the nail surface was gently removed with paper tissue before the milling step. The mill tube containing the nail sample was placed in a freezer at −70 °C for about half an hour before it was exposed to −196 °C, the temperature produced by liquid nitrogen in the Freezer/Mill apparatus. This step was essential to avoid the damage of mill tube by an abrupt temperature change. In the Freezer/Mill apparatus the sample was pre-cooled for 10 min and then milled for 2 min. Afterwards, the mill's content was suspended in 25 ml of PBS. The caffeine inside the pulverized nail plate was extracted using an ultrasonic bath for 5 min. Subsequently, samples were centrifuged for 5 min at 10 000 min^{-1} in order to measure caffeine concentrations in the supernatants. The amount of caffeine was presented as a percentage of the dry nail weight before the milling test.

3. Results and discussion

3.1. Evaluation of nail samples

In both regions, high wavenumber and fingerprint, compared with a Raman spectrum of 4% formaldehyde solution added to a fitting library, no peak at 1041 cm^{-1} or 1492 cm^{-1} has occurred (see Figs. 2 and 3). Formaldehyde content was not detected in either of the nail samples. This can be owing to the storage conditions and humidity in the plastic bags, in which nails were stored in the freezer at −20 °C. The only difference seen between two samples

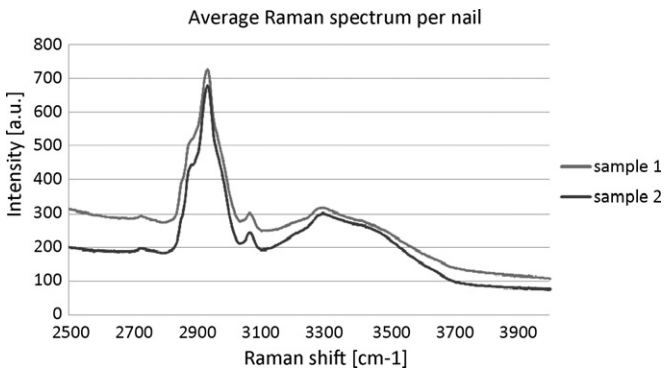

Fig. 2. Average spectrum of 105 measurements taken in the high wavenumber region.

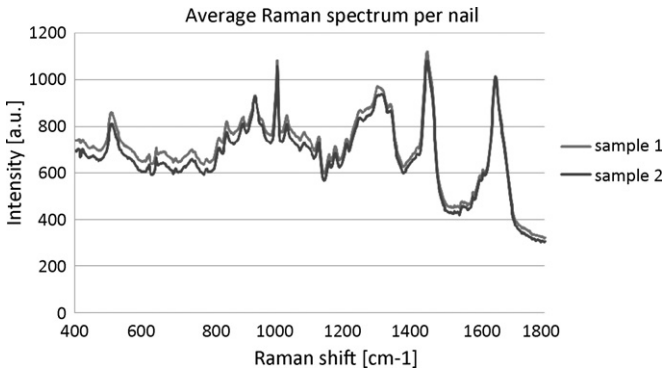

Fig. 3. Average spectrum of 105 measurements taken in the fingerprint region.

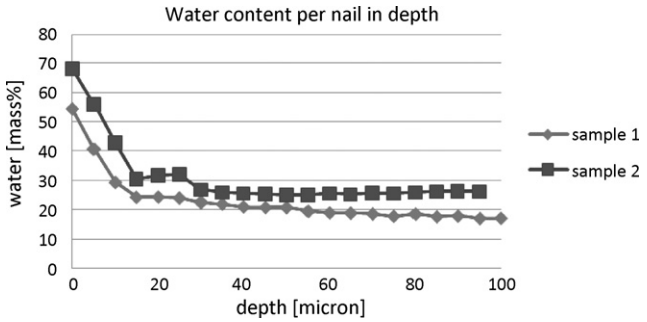

Fig. 4. Average over five measurements of water content per sample per depth.

was in the intensity of the Raman signal. It was lower for the sample 2. Formaldehyde might cause tissue changes, but not in such extent that cadaver nails could not be used as models for permeability studies any more.

Results for water content as well as for lipid content measurements are shown in Figs. 4 and 5. Water content decreased up to a depth of 10 μm. In the deeper nail layers the amount of water became constant and was 20% of the total nail weight, which corresponds to literature values, such as 10–30% (Murdan, 2002). Higher values of water content in layers closer to the nail surface can be explained by the preparation procedure of the nail sample for measurement. A drop of water was put between the measurement window and nail sample to achieve an improved contact. The nail absorbed water, which resulted in higher water content values in the upper nail layers. A similar behavior was observed with the lipid content in the nails. Constant values were obtained in a depth of about 30 μm and deeper (see Fig. 5). Measurement was performed till the depth of 100 μm. If we consider that the nail thickness is around 0.25–0.6 mm (Murdan, 2002), then these 100 μm present 40–16.67%, i.e., roughly 2/5–1/5 of whole nail thickness. Knowing that thickness ratio of each stratum of human nail plate (dor-

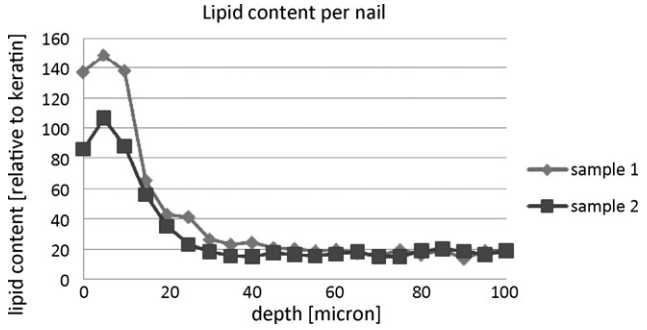

Fig. 5. Average over five measurements of lipid content per sample per depth.

sal:intermediate:ventral) was assessed to be 3:5:2 (Kobayashi et al., 1999), and that lipids are concentrated in dorsal and ventral nail strata (Garson et al., 2000), then observed lipid content in the nails was not surprising.

Increase in weight for female samples was determined to be $21 \pm 1\%$, for male $17 \pm 1\%$, and for cadaver's $22 \pm 8\%$. There was no statistical difference ($p > 0.05$) in water intake between nail clippings from cadavers and fresh nail clippings. Thus, nails from cadavers were suggested as suitable models.

3.2. Characterization of nail samples

Weight, TOWL, and visioscan images were representative parameters to illustrate and detect variations in nail samples. Thickness, although measured three times per each nail edge before cutting, was less reliable. The proximal part of the nail plate, closer to the nail matrix, is thinner comparing with the distal part. The results of nail thickness measurements depended on the applied force, which is especially true for wet nails, and the length of area implied by micrometer. Therefore, results of thickness measurements were rejected. The difference in weight between wet, i.e., for 60 min immersed nails in PBS, and dry nails was expressed as percentage of weight increase. The average weight increase of 36 nail samples was determined to be $23 \pm 10\%$. This correlates very well with $22 \pm 8\%$, the result presented in section 3.1. Nail proteins were able to equally accept water molecules in different aggregate stages, gaseous in an exsiccator and liquid in PBS. In both cases, swelling of the nails was limited by saturation effect. The high standard deviation resulted from the quick drying process. Data of dry nails before and 24 h after experiments were compared. Weight decrease after the experiments was detected in 43 nail samples out of 45. TOWL after the experiment showed increase in 30 cases of 43 in total (Table 1). In two cases no change in TOWL was observed. The parameters, weight decrease and TOWL increase, indicated structural changes in the nails. If an increase in TOWL reflects reduction in the barrier properties of the nail plates (Murdan et al., 2008), then obtained data were not surprising. Some of the chosen enhancers directly influenced the proteins in the nail plate due to keratolytic effect, and/or extraction, and/or washing out of nail constituents. However, no correlation between TOWL and weight or thickness was found. Murdan et al. (2008) reported a strong correlation between nail plate thickness and TOWL, but the objects of the study were individuals, since the measurements were done *in vivo*. Detected structural changes have been confirmed by visioscan images. As Tewameter, Visioscan VC 98 was developed for the evaluation of the skin surface conditions, especially after cosmetic treatment it was applied for the first time in the present work to characterize nail surfaces before and after the permeation experiment. First results confirmed the use also to evaluate nail surfaces and Visionscan is considered and suggested as an appropriate diagnostic tool. The method is based on a graphic depiction of the living skin under special illumination and electronic processing and evaluation of the image according to four clinical parameters. Smoothness, roughness, scaliness, and wrinkles correspond quantitatively and qualitatively to the physiological condition of the skin or nail surface. For example, water content is the most important parameter resorting roughness measurement. A lower degree of water content is expressed by a higher value in roughness detection. According to the obtained images nail surface suffered structural changes comparing them before and after the permeation experiments with the applied formulations. Fig. 6 illustrates the surface change induced by the formulation with methanol. Formulations containing DMSO and urea showed also influence on the nail surface and a clear difference comparing with the reference formulation in water. Exceptions were those with NAC and BA. Boric acid crystallized in most of the Franz diffusion cells after the permeation experiment, which might have hindered it from acting as an enhancer. According to Fick's first law, only dissolved molecules permeate the nail plate. There was no evidence of surface changes with NAC solution. N-acetyl-L-cysteine behaved as an enhancer shortly before it was destroyed, generating H_2S and/or SO_2 gas and sulfhydryl group could not act as keratolytic agent, which led to a lack of the enhancement effect. This was confirmed in the stability tests where NAC formulations had strong smell of rotten eggs. Results were less obvious for the formulations in 20% ethanol/water. Methanol, DMSO, and urea had the greatest influence on the nail surface, but the reference formulation did not show the least surface change, indicating that ethanol itself influenced the surface structure, as well. Solutions with hydrophobins as enhancers could not be compared with other formulations, because in some cases they left a thin, light film in the contact area with the nail plate. Fig. 7 shows the described difference.

Table 1
TOWL before and after experiments.

TOWL (g/hm^2) before experiment		TOWL (g/hm^2) after experiment	
reference	0.1	Reference	0.8
	0.6		1.1
	0.0		0.2
	0.5		1.7
	0.7		0.5
	0.3		1.0
BA	2.1	BA	0.5
	2.8		0.4
	0.1		1.1
	1.5		0.8
	1.5		0.5
DSS	0.6	DSS	0.6
	1.0		0.7
	0.3		0.3
	0.6		0.3
	0.6		0.1
	0.8		0.6
M	0.4	M	4.8
	0.6		2.1
	0.3		1.8
	0.6		2.0
	0.9		2.2
	0.4		1.6
NAC	0.6	NAC	3.7
	0.9		2.3
	0.6		1.6
	0.8		0.7
	0.9		1.1
	0.8		1.0
DMSO	0.0	DMSO	0.8
	0.0		1.6
	0.3		1.2
	0.1		1.0
	0.0		1.3
Class II	0.3	Class II	1.8
	0.6		1.5
	0.4		1.6
	0.4		1.2
	0.4		1.3
	0.5		1.0
Class I	0.3	Class I	0.1
	0.3		0.5
	0.1		0.5
Total	43 nails	Increase	30 cases

3.3. Characterization of the formulations

All formulations had pH values in the range of 5–7.5, except the formulations containing NAC and BA. They were acidic with pH

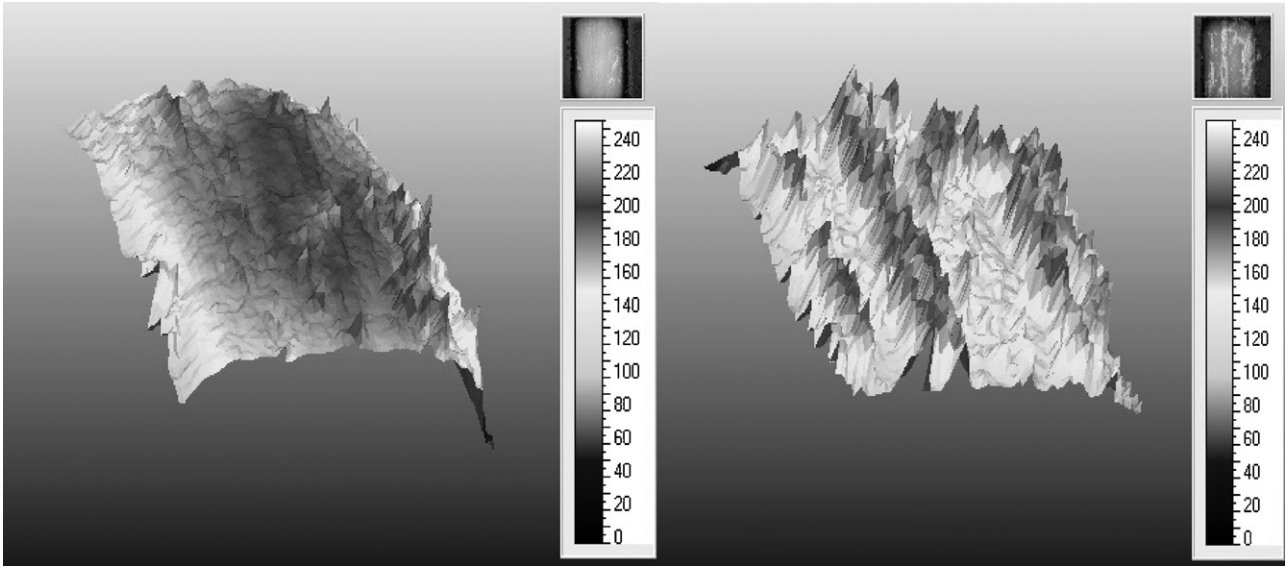

Fig. 6. Changes in the nail surface structure illustrated on the 3D graph. On the left image is dry nail before and on the right after the experiment with methanol in 20% (v/v) ethanol/water formulation.

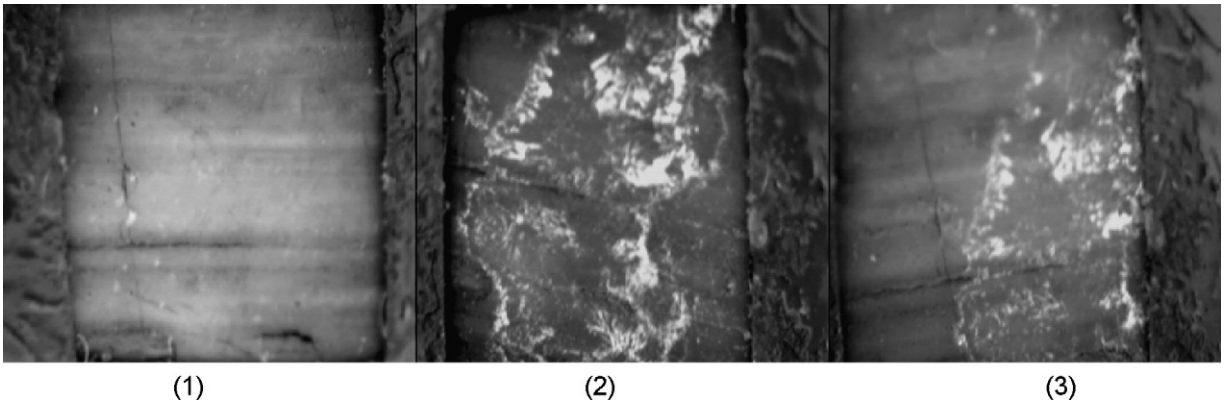

Fig. 7. (1) Dry nail surface before experiment with class I hydrophobin. (2) Nail surface immediately after the experiment with class I hydrophobin. (3) Dry nail surface 24 h after the experiment with class I hydrophobin.

in the range of 1.8–3.7. Isoelectric point of keratin is thought to be around pH 5 (Murthy et al., 2007), which means that the proteins in the nail plate are negatively charged, attracting positively charged molecules. Solubility of caffeine in water is temperature and pH dependent (Keck et al., 2005). Dissociation constant (pKa) for caffeine is 14 at 25 °C (Clarke, 1986). The amount of caffeine in the presented formulations was completely soluble and experimental conditions were kept constant, thus it was suggested that differences in pH range of 5–7.5 between the formulations did not have an influence on the permeability coefficient through the nail plate.

Viscosity measurements are summarized in Table 2. "Blank" is the solution with pure water or 20% (v/v) ethanol/water. All formulations were liquids and their measured viscosity was generally low. Formulations containing 20% ethanol/water as solvent were more viscous than the analogous formulations containing pure water as solvent, which was expected. With the increase of temperature, the viscosity decreased. All formulations showed Newtonian flow behavior. Since the viscosity of all formulations was under 2 mPa s, neither of the formulations could be emphasized nor discussed in respect to their influence on the permeability coefficient.

The formulation was considered as stable if the caffeine concentration after 90 days at room temperature/in the refrigerator, or after six days kept at 32 °C, did not differ more than ± 1.2 mg/ml from the concentration at time zero. Instability was found in three formulations containing BA in 20% ethanol/water, methanol in water, and urea in water for the storage conditions which simulated the conditions during the permeation experiment. Interesting was, that formulations with the same enhancer but another solvent did not show instability, at least not in the defined limits. Thus, it was assumed that these findings did not influence the results obtained from the permeation experiments. All formulations after storage of 90 days at 24 °C and light protected were

Table 2
Viscosity (mPa s) of the formulations measured at 25 °C and 32 °C ($n = 3$, mean ± standard deviation).

Sample	25 °C w	32 °C w	25 °C e	32 °C e
Blank	1.10 ± 0.11	1.01 ± 0.09	1.71 ± 0.06	1.42 ± 0.10
Reference	1.05 ± 0.06	1.00 ± 0.10	1.79 ± 0.09	1.39 ± 0.09
BA	1.19 ± 0.10	1.11 ± 0.05	1.77 ± 0.04	1.46 ± 0.01
DSS	1.10 ± 0.12	0.95 ± 0.09	1.82 ± 0.09	1.43 ± 0.20
M	1.19 ± 0.08	1.02 ± 0.02	1.90 ± 0.18	1.47 ± 0.11
NAC	1.19 ± 0.11	1.07 ± 0.02	1.84 ± 0.06	1.57 ± 0.07
U	1.16 ± 0.10	1.15 ± 0.04	1.78 ± 0.03	1.45 ± 0.04
DMSO	1.20 ± 0.06	1.14 ± 0.13	1.77 ± 0.03	1.52 ± 0.06

boric acid (BA); dimethyl sulfoxide (DMSO); docusate sodium salt (DSS); methanol (M); N-acetyl-L-cysteine (NAC); urea (U); water as solvent (w); 20% ethanol/water as solvent (e).

Table 3
Capillary constants ($n = 6$) with standard deviations (SD).

Liquid	Nail surface (mm^2)	Capillary constant	SD
n-Hexane	18.58	2.67E−10	9.21E−11
Ethanol	19.03	2.03E−10	2.52E−11
Ethanol/water	18.10	8.34E−11	3.86E−11
Water	18.30	6.19E−11	5.44E−11

stable. However, instability of DSS in water and methanol in water was detected when formulations were stored in the refrigerator at 8 °C. In these formulations caffeine was stable after seven days of storage, but not after 32 days. This can be due to incompletely dissolved caffeine crystals, which were formed during increased storage time in the refrigerator at 8 °C. Considering the fact that the decrease of caffeine concentration in all formulations was not more than 2 mg/ml, we concluded that these formulations could be stored in the refrigerator for up to 90 days.

Capillary constant is a specific constant for various materials. It is applied for sorption measurement of tablets or bulk powders in a sample tube. Usually it is determined by a non-polar liquid with a small contact angle. In most cases hexane is suitable and applied. In the present work nail samples were considered as solid porous systems, such as tablets. The influence of weight and surface area of nail samples was analyzed by statistical spss software (one-way ANOVA, post-hoc test Tukey, $p > 0.05$) and no significant difference was found. Therefore, measurements could be performed with prepared nail samples, handled and stored in the same way as for contact angle measurements. Liquids with known properties used for the measurements were n-hexane, distilled water, 96% ethanol, and 10% (w/v) ethanol/water. Table 3 presents the obtained results.

The data expressed as capillary constants could be divided into two groups. Hexane and ethanol showed capillary constants in the range of 2–2.7E−10 and ethanol/water and water in the range of 6.2–8.3E−10. The difference was found to be significant between the groups, but not between the group members (one-way ANOVA, post-hoc test Tukey, $p < 0.05$). Organic solvents showed higher capillary constant values, not only because they have good wettability properties, but also due to their ability to solubilize a small amount of lipids in the dorsal nail layer. Characteristics of extraction should be attributed to the hexane solvent. On the other hand, water caused swelling of the nail samples and formed H bonds with amino acids from keratin. Thus, water speed through the "capillaries" decreased. Ethanol in comparison with water did not interfere with keratin in such a manner.

3.3.1. Surface tension and contact angle measurements

Table 4 shows the results obtained from surface tension and contact angle measurements. The results for the contact angles in the second column of the table were strongly dependent on the surface tension results presented in the first column. The smaller the value for contact angle was, the better the wetting properties were. All formulations showed improvement in wetting properties compared with pure water (see Table 4). The lowest values standing for better wetting properties were obtained for the formulations containing methanol and DSS. It is well known that surfactants, as DSS, can reduce surface tension and alter the wetting behavior of materials. Although DSS influenced the contact angle significantly, it did not increase the permeability coefficient of caffeine to a greater extent. Most probably, it was due to the molecular size of DSS and its disability to penetrate into the nail. The contact angle result of the formulation with methanol in 20% ethanol/water, marked in bold in Table 4, was an outlier, suggested due to the evaporation of methanol and ethanol. This hypothesis is confirmed in the work of Fang et al., 2005. The validation of Hildebrand's solubility parameter using different solvents showed that methanol evaporation breaks up the equilibrium conditions. For the plate method one of the requirements is that both sides of solid must have the same properties, i.e., roughness and impurities can affect the measurements, which in the case of biological material, such as human cadaver nails, was difficult to achieve and it is present as an error.

3.4. Permeation studies

Fig. 8 illustrates permeability coefficients of applied formulations and Fig. 9 shows the classification of formulations by their enhancement factor. The permeability coefficient of caffeine in ethanol/water solution was determined to be 1.56E−08 cm/s. Addition of DMSO improved it to 5.12E−08 cm/s, which was an increase of 3.3-fold. Moreover, an improvement in permeability rate was found in water formulations with enhancers and the best enhancement factor among them was determined with methanol in water formulation of 3.2. No correlation between permeability coefficient and nail weight before experiment was found (Fig. 10), thus it was suggested that the main influence on the permeability rate was derived from the formulation. Fig. 11 shows that correlation between permeability coefficient and TOWL after experiment was not established, although the formulation containing methanol, with the highest permeability rate among the tested formulations, had the highest values for TOWL. The formulation with methanol as enhancer in 20% ethanol/water solvent provided the highest permeability coefficient and it was the only

Table 4
Surface tension and contact angle measurements.

Formulation	Surface tension (mN/m) ($n = 3$)	Advancing contact angle, θ (°) ($n = 3$)	Regression	%RSD
Blank	69.6	72.29	0.92	0.63
Reference w	61.5	71.44	0.96	3.09
U w	48.7	69.54	0.94	1.78
NAC w	44.0	69.33	0.94	0.95
BA w	43.8	68.71	0.90	1.29
Blank	42.1	66.74	0.88	1.77
Reference e	42.0	66.27	0.85	3.05
NAC e	41.4	61.87	0.87	0.92
U e	41.4	60.69	0.91	1.65
BA e	40.4	58.39	0.88	3.14
DMSO w	40.0	53.74	0.92	0.83
DMSO e	39.1	55.24	0.94	5.82
M e	36.9	**39.83**	0.95	1.58
M w	36.2	48.26	0.96	1.86
DSS e	29.1	16.95	0.90	56.43
DSS w	27.7	2.73	0.95	50.65

Boric acid (BA); dimethyl sulfoxide (DMSO); docusate sodium salt (DSS); methanol (M); N-acetyl-L-cysteine (NAC); urea (U); water as solvent (w); 20% ethanol/water as solvent (e).

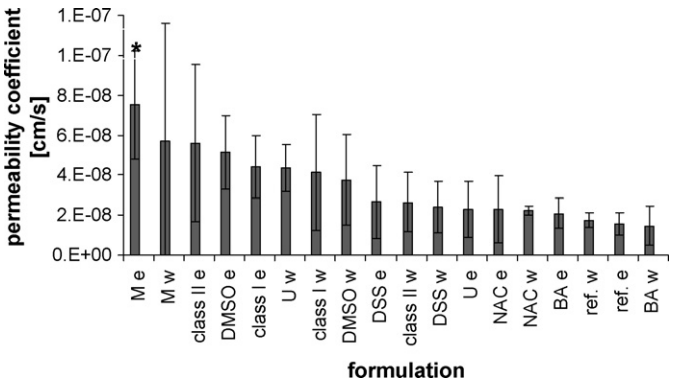

Fig. 8. Permeability coefficients with standard deviations ($n = 3$). Asterisk indicates significant difference.

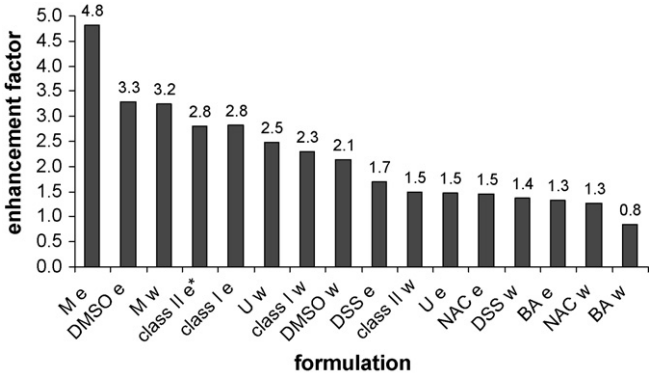

Fig. 9. Enhancement factors calculated to the caffeine formulations without enhancer (references); class II e* ($n = 6$).

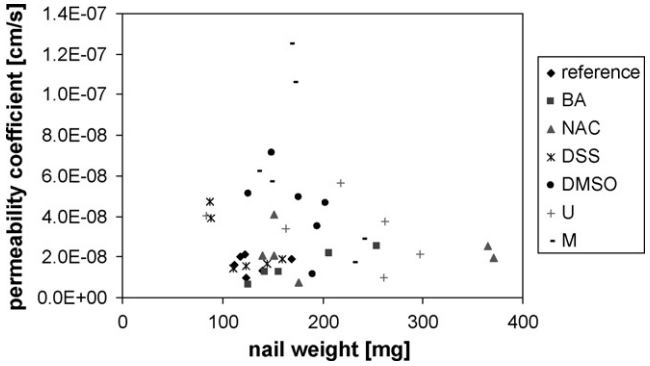

Fig. 10. Correlation between permeability coefficient and nail weight of dry nails before experiments.

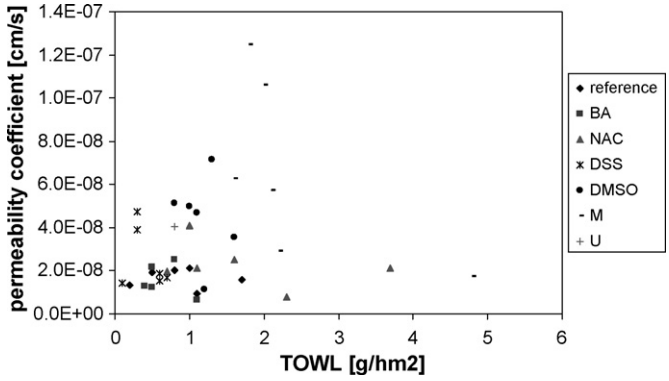

Fig. 11. Correlation between permeability coefficient and TOWL of dry nails 24 h after experiments.

one that showed statistically significant difference compared with the reference (one-way ANOVA, post-hoc test Tukey, $p < 0.05$). Ethanol/water formulation with class II hydrophobin showed a permeability coefficient of 5.6E−08 cm/s. Thereby, hydrophobin from class II could be classified in the group of efficient enhancers together with methanol and DMSO. The great effect of methanol was attributed to its small molecular weight and damage caused on the keratin network and decrease in lipid content in the dorsal nail layer, confirmed visually by naked eye and by visioscan. This act loosened the nail structure, allowing caffeine to penetrate easier. Methanol showed the best enhancer potential and it is a good solvent for lipophilic drugs, but its toxicity limits its use.

Contradictory results about the effect of DMSO in permeation studies were reported in the literature (Murdan, 2002). In the present study, the established skin enhancer, DMSO, facilitated caffeine permeation and there is a possible explanation for its mode of action. The permeability enhancing mechanism for DMSO in skin is suggested to act in two ways: (I) DMSO can weaken the bilayers in the stratum corneum consisting of a high amount of ceramides 2 and (II) DMSO can induce the transition of ceramide bilayers from the gel phase to the liquid crystalline phase (Notman et al., 2007). The phenomenon is concentration and temperature dependent. Also, it was suggested that DMSO may interact with membrane proteins (Notman et al., 2007). Anigbogu et al. (1995) reported that DMSO disturbs lipid bilayers and induces changes in stratum corneum keratin fibers from α-helical to a β-sheet conformation by substituting or displacing bound water. Considering the structure of the nail plate, substances which influence keratin have a high possibility to be potential permeation enhancers. If we accept Kobayashi's et al. (1999) conclusion that the low drug diffusivity in the upper layer is the main barrier to drug permeation, then DMSO, by altering the lipids in the dorsal layer, allows easier and faster permeation of the drug to deeper keratin network. We assume that DMSO alters the concentration of lipids present in the nail plate and causes conformational changes in the keratin structure. Therefore, DMSO can be assorted as promising permeation enhancer for drug delivery through the nail plate. An excellent enhancer effect was shown by hydrophobins, from class II in particular, better than DMSO, although applied in a concentration which was 50 times less. Those water soluble proteins with an ability to change physical properties of the surface in the way that hydrophobic surface becomes hydrophilic and vice versa, are suggested to be used in the treatment of nail diseases as stabilizers and enhancers. Their positive effect can be due to their ability to reduce the surface tension of water by self-assembling in the solution. Different results from different classes seemed to be owing to the differences in their structure and self-assembling in the solution. Class I does not contain α-helix, but contains two large disordered regions, i.e., many hydrophobic residues comparing with class II. Oligomerization was proposed for the mechanism of how hydrohobins remain soluble despite the amphiphilicity of the molecules (Kallio et al., 2007). Further, Kallio et al. (2007) observed rodlet-like surface pattern for class I, while for class II hydrophobin surfaces of the layer appeared to be smooth, but with a closer examination by atomic force microscopy, it had an ordered pattern of small pores. These porous structure of assembled hydrophobin layers matched with the findings that the layers are permeable for small molecules. Kisko et al. (2008) reported that ethanol breaks oligomers into monomers in a concentration dependent manner. They concluded that hydrophobins act as surfactants, but in a very different way than any other surfactants that have been described earlier. From the experiments with class II hydrophobins in ethanol/water, an enhancement factor of 2.8 has been obtained (Fig. 9). Future studies are necessary to elucidate whether hydrophobins can be used as enhancers for biomaterials and medical application to the nail plate.

Around two times higher enhancement factor compared with the reference in water was found for urea, hydrophobin from class I, and DMSO formulations. Urea is a frequently discussed substance in the literature regarding the studies on nails. It expressed moderate consequence in the concentration of 5%, which was insufficient for its keratolytic effect. This was in agreement with the observation that urea hydrates and softens the nail, rather than acts as permeation enhancer (Murdan, 2002).

It was surprising that NAC did not augment the permeability coefficient of caffeine at all. The reaction with disulphide bonds of keratin in the nail plate failed, because of the NAC instability in the formulation. Boric acid seemed not to interfere with proteins in the nail and did not show any enhancing effect. The lack of positive influence on permeability through the nail was seen in formulations with DSS. Geometry of DSS and relatively high molecular weight was suggested to hinder its penetration.

No statistically significant difference was found between particular formulations with different solvents (*t*-test, $p > 0.05$). It was concluded that ethanol presence in the formulation did not influence negatively the hydration of the nail during six days of experiment and therefore the permeability coefficient. This finding can be utilized for dissolving lipophilic drugs used in medicines for treatment of nail diseases. It could not be concluded so far to what extent water needs to be present in the formulation. Wessel et al. (1999) confirmed changes in water content and protein structure during nail moisturizing in the Raman spectra. Conformation shifts of heavily folded α-helix keratin in the nails, induced by water, seemed to have a crucial role in drug permeability through the nail plate. Whether the new chemical enhancers and optimized formulations containing some portion of water, biological material as hydrophobins, or future development of the substance similar to the one in desert beetle, which collects water from fog-laden wind (Parker and Lawrence, 2001), will facilitate drug delivery through the nail, it remains to be seen.

3.5. Milling test

The values of penetrated caffeine into the nail layers are shown in Table 5. The amount of caffeine was presented as a percentage of the dry nail weight before the milling test. The high standard deviation resulted due to the used biological material and variations among nail samples and the fact that not all nail powder could be washed out totally of the mill tube. The greatest caffeine reservoir was formed in the nails with the formulation containing methanol as enhancer, followed by DMSO and urea. This was the same order in which the permeability coefficient decreased. The logical conclusion was that if more caffeine permeated the nail, more caffeine was inside the nail after the experiment and thus one more proof for the steady state permeation after a certain lag time. The same logic cannot be applied for formulations with hydrophobins and NAC. Possible explanation for formulations containing NAC can be that positive effect of NAC on loosening keratin network started, but did not last long enough to ensure caffeine penetration through the nail in the higher amount, which resulted low permeability coefficient of caffeine. Correlation between enhancement factor and remaining caffeine in nail is shown in Fig. 12. Good correlation was found among previously mentioned formulations with methanol, DMSO, and urea, as well as, among formulations with BA.

Table 5
Remaining caffeine in nail after experiment with standard deviation (SD) and relative standard deviation (%RSD).

Formulation	Remaining caffeine (%) ($n = 3$)	SD	%RSD
M e	0.61	0.14	22
DMSO e	0.56	0.14	24
U w	0.56	0.01	2
M w	0.53	0.11	20
NAC w	0.51	0.28	55
NAC e	0.47	0.19	40
Class II w	0.41	0.15	37
U e	0.41	0.12	28
Class II e	0.40	0.17	42
DMSO w	0.39	0.15	39
DSS w	0.37	0.17	46
Reference e	0.36	0.09	24
DSS e	0.35	0.24	68
Reference w	0.33	0.16	47
BA e	0.26	0.07	28
BA w	0.20	0.04	22

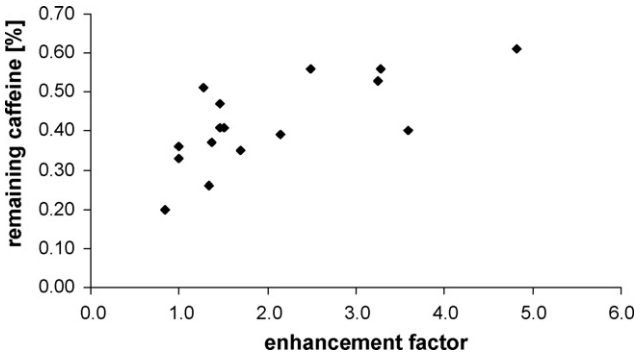

Fig. 12. Correlation between enhancement factor and remaining caffeine in nail ($n = 3$).

4. Conclusions

Human cadaver nails are a suitable model for permeation studies. No formaldehyde was found in the nails after collection and hence its interference was excluded. Correlation between TOWL and weight or thickness of the cadaver nail samples was not found. It is known and confirmed in this study that caffeine is a stable drug. Promising enhancers for drug delivery through the nail plate were methanol, DMSO, and fungal proteins such as hydrophobins. Each of them had a different mechanism of action. Although water plays a crucial role in drug permeation through the nail plate due to its ability to hydrate the nail and to induce conformational changes of α-helical keratin fibers, formulations with 20% ethanol did not significantly influence permeability coefficients in comparison with pure water formulations. An effective enhancer, which can facilitate drug permeation through the keratinized nail membrane, could find application not only in the treatment of nail diseases, but also in the treatment of neighboring target sites, for example in the therapy of rheumatoid arthritis of hands and feet, if systemic circulation is reached.

Acknowledgments

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References

Achten, G., Andre, J., Laporte, M., 1991. Nails in light and electron microscopy. Semin. Dermatol. 10, 54–64.

Anigbogu, A.N.C., Williams, A.C., Barry, B.W., Edwards, H.G.M., 1995. Fourier transform Raman spectroscopy of interactions between the penetration enhancer dimethyl sulfoxide and human stratum corneum. Int. J. Pharm. 125, 265–282.

Benson, H.A.E., 2005. Transdermal drug delivery: penetration enhancement techniques. Curr. Drug Deliv. 2, 23–33.
Boker, A., 2007. Can a new trephination device help treat nail conditions? Pod. Today 11, 34–36.
Clarke, E.G.C., 1986. Clarke's isolation and identification of drugs in pharmaceuticals, body fluids, and post-mortem material, second ed. The Pharmaceutical Press, London, 420–421.
De Stefano, L., Rea, I., Armenante, A., Giardina, P., Giocondo, M., Rendina, I., 2007. Self-assembled biofilm of hydrophobins protects the silicon surface in the KOH wet etch process. Langmuir 23, 7920–7922.
Debruyne, D., Coquerel, A., 2001. Pharmacokinetics of antifungal agents in onychomycoses. Clin. Pharmacokinet. 40, 441–472.
Di Chiacchio, N., Kaduc, B.V., De Almeida, A.R.T., Madeira, C.L., 2004. Nail abrasion. J. Cosmet. Dermatol. 2, 150–152.
Elewski, B.E., 1998. Onychomycosis: pathogenesis, diagnosis, and management. Clin. Microbiol. Rev. 11, 415–429.
Fang, X., Li, B., Sokolov, J.C., Rafailovich, M.H., 2005. Hildebrand solubility parameters measurement via sessile drops evaporation. Appl. Phys. Lett. 87, 094103.
Farren, L., Shayler, S., Ennos, A.R., 2004. The fracture properties and mechanical design of human fingernails. J. Exp. Biol. 207, 735–741.
Garson, J.C., Baltenneck, F., Leroy, F., Riekel, C., Müller, M., 2000. Histological structure of human nails as studied by synchrotron X-ray microdiffraction. Cell. Moll. Biol. 46 (6), 1025–1034.
Gauwerky, K., Borelli, C., Korting, H.C., 2009. Targeting virulence: a new paradigm for antifungals. Drug Discov. Today 14, 214–222.
Ghannoum, M.A., Hajjeh, R.A., Scher, R., Konnikov, N., Gupta, A.K., Summerbell, R., Sullivan, S., Daniel, R., Krusinski, P., Fleckman, P., Rich, P., Odom, R., Aly, R., Pariser, D., Zaiac, M., Rebell, G., Lesher, J., Gerlach, B., Ponce-de-Leon, G.F., Ghannoum, A., Warner, J., Isham, N., Elwski, B., 2000. A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns. J. Am. Acad. Dermatol. 43, 641–648.
Gupta, A.K., Tu, L.Q., 2006. Therapies for onychomycosis: a review. Dermatol. Clin. 24, 375–379.
Hao, J., Li, S.K., 2008. Transungual iontophoretic transport of polar neutral and positively charged model permeants: effects of electrophoresis and electroosmosis. J. Pharm. Sci. 97, 893–905.
Hui, X., Baker, S.J., Wester, R.C., Barbadillo, S., Cashmore, A.K., Sanders, V., Hold, K.M., Akama, T., Zhang, Y., Plattner, J.J., Maibach, H.I., 2007. *In vitro* penetration of a novel oxaborole antifungal (AN2690) into the human plate. J. Pharm. Sci. 96, 2622–2631.
Kallio, J.M., Linder, M.B., Rouvinen, J., 2007. Crystal structures of hydrophobin HFBII in the presence of detergent implicate the formation of fibrils and monolayer films. J. Biol. Chem. 282, 28733–28739.
Keck, C., Kruschel, A., Böttcher, S., Müller, R., 2005. Formulation of caffeine for dermal application. In: Presented Abstract at Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Nashville, 05.11–10.11 2005.
Khengar, R.H., Jones, S.A., Turner, R.B., Forbes, B., Brown, M.B., 2007. Nail swelling as a pre-formulation screen for the selection and optimisation of ungual penetration enhancers. Pharm. Res. 24, 2207–2212.
Kisko, K., Szilvay, G.R., Vainio, U., Linder, M.B., Serimaa, R., 2008. Interactions of hydrophobin proteins in solution studied by small-angle X-Ray scattering. Biophys. J. 94, 198–206.
Kobayashi, Y., Miyamoto, M., Sugibayashi, K., Morimoto, Y., 1998. Enhancing effect of N-acetyl-L-cysteine or 2-mercaptoethanol on the *in vitro* permeation of 5-fluorouracil or tolnaftate through the human nail plate. Chem. Pharm. Bull. 46, 1797–1802.
Kobayashi, Y., Miyamoto, M., Sugibayashi, K., Morimoto, Y., 1999. Drug permeation through the three layers of the human nail plate. J. Pharm. Pharmacol. 51, 271–278.
Kobayashi, Y., Komatsu, T., Sumi, M., Numajiri, S., Miyamoto, M., Kobayashi, D., Sugibayashi, K., Morimoto, Y., 2004. In Vitro permeation of several drugs through the human nail plate: relationship between physicochemical properties and nail permeability of drugs. Eur. J. Pharm. Sci. 21, 471–477.
Lubeck, D.P., 1998. Measuring health-related quality of life in onychomycosis. J. Am. Acad. Dermatol. 38, 64–68.
Marty, J.L., 1995. Amorolfine nail lacquer: a novel formulation. J. Eur. Acad. Dermatol. Venereol. 4, 17–21.
Mohorčič, M., Torkar, A., Friedrich, J., Kristl, J., Murdan, S., 2007. An investigation into keratinolytic enzymes to enhance ungual drug delivery. Int. J. Pharm. 332, 196–201.
Murdan, S., 2002. Drug delivery to the nail following topical application. Int. J. Pharm. 236, 1–26.
Murdan, S., 2008. Enhancing the nail permeability of topically applied drugs. Expert Opin. Drug Deliv. 5 (11), 1267–1282.
Murdan, S., Hinsu, D., Guimier, M., 2008. A new aspects of transonychial water loss (TOWL): inter-individual, and intra-individual inter-finger, inter-hand and inter-day variabilities, and the influence of nail plate hydration, filing and varnish. Eur. J. Pharm. Biopharm. 70, 684–689.
Murthy, S.N., Wiskirchen, D.E., Bowers, C.P., 2007. Iontophoretic drug delivery across human nail. J. Pharm. Sci. 96, 305–311.
Notman, R., Den Otter, W.K., Noro, M.G., Briels, W.J., Anwar, J., 2007. The permeability enhancing mechanism of DMSO in ceramide bilayers simulated by molecular dynamics. Biophys. J. 93, 2056–2068.
Parker, A.R., Lawrence, C.R., 2001. Water capture by a desert beetle. Nature 414, 33–34.
Repka, M.A., Mididoddi, P.K., Stodghill, S.P., 2004. Influence of human nail etching for the assessment of topical onychomycosis therapies. Int. J. Pharm. 282, 95–106.
Scher, R.K., 1994. Onychomycosis is more than a cosmetic problem. Br. J. Dermatol. 130 (Suppl. 43), 15.
Walters, K.A., Flynn, G.L., 1983. Permeability characteristics of the human nail plate. Int. J. Cosmet. Sci. 5, 231–246.
Wessel, S., Gniadecka, M., Jemec, G.B.E., Wulf, H.C., 1999. Hydration of human nails investigated by NIR-FT-Raman spectroscopy. Biochim. Biophys. Acta 1433, 210–216.
Zhai, H., Barbadillo, S., Hui, X., Maibach, H.I., 2007. In vitro model for decontamination of human skin: formaldehyde. Food Chem. Toxicol. 45, 618–621.