Systematic investigation of different formulations for drug delivery through the human nail plate *in vitro*

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Ivana Vejnović

aus Serbien

Basel, 2010

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

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Basel, den 21. September 2010

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Dekan

To my parents

Acknowledgements

I would like to express my gratitude to Dr. Gabriele Betz for giving me the opportunity to prepare my thesis at The Institute of Pharmaceutical Technology Department of Pharmacy, University of Basel, as a member of The Industrial Pharmacy Lab (IPL) research group. Her expert knowledge, positive energy, creativity, patience, and trust have been of great support throughout my work.

I am thankful to Dr. Alfredo Bruno and Dr. Christian Frei with whom this project initially started for held useful meetings, friendly communication, and easy collaboration.

I would like to thank to Dr. Susanne Drews from The Institute of Anatomy, University of Basel, for providing the nail samples and to Dr. Martin Scaal, from The Institute of Anatomy and Cell Biology, University of Freiburg for permission to collect cadaver nail samples.

I am grateful for Raman spectroscopy analysis data received from Johanna de Starke, application scientist in River Diagnostics BV, The Netherlands.

I would also like to thank Dr. Andreas Buthe and Dr. Andreas Hafner from CIBA, now part of BASF, Basel, and Guido Meurer BRAIN AG, Germany, for introducing hydrophobins and providing them to us, and for their support and professionalism.

My gratitude goes to Prof. Dr. Adel Sakr who kindly accepted to be the official reviewer of the thesis. His encouragement and support were of great help for my work.

I would specially like to thank my lab colleagues, Dr. Miriam Reiser and Elaine Darronqui, for creating a pleasant environment and for nice and joyful time spent together.

I also like to thank to all my colleagues from Pharmacenter and The Industrial Pharmacy Lab for helpful and friendly atmosphere during my years in Basel. I would particularly like to mention the great consolidation and the unique “common spirit” of IPL-research group.

My thanks go to my master students Linda Simmler and Cornelia Huonder for their enthusiasm, work engagement, and friendly relationship.

My thanks also go to Stefan Winzap, our technician, for his helpful presence and to Prof. Dr. Georgios Imanidis and Orlando Fertig for their support with HPLC.

My deepest thanks go to my parents, Tomislav and Anka Vejnović and to my sister Dubravka, for their love, trust, and support.

Contents

List of abbreviations

BA	boric acid
DMSO	dimethyl sulfoxide
DSS	docusate sodium salt
e	20% (v/v) ethanol/water
E.coli	*Escherichia coli*
FDA	The Food and Drug Administration
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
KOH	potassium hydroxide
M	methanol
NAC	N-acetyl-L-cysteine
NIR	near-infrared Raman spectroscopy
PBS	phosphate buffer saline
PC	permeability coefficient
Ph. Eur.	The European Pharmacopoeia
rpm	rounds per minute
SD	standard deviation
TEWL	transepidermal water loss
TiO2	titanium dioxide
TOWL	transonychial water loss
U	urea

UV	ultraviolet light
v/v	volume per volume
w	water
w/v	weight per volume

Summary

Human nails do not have only protective and decorative role, but can also be considered as an alternative pathway for drug delivery, especially in nail diseases such as onychomycosis or psoriasis. These nail diseases are widely spread in the population, particularly among elderly and immunocompromised patients. Oral therapies are accompanied by systemic side effects and drug interactions, while topical therapies are limited by the low permeation rate through the nail plate. For the successful treatment of nail disease the applied active drug must permeate through the dense keratinized nail plate and reach deeper layers, the nail bed and the nail matrix. Studies conducted on the human skin elucidated its structure, functions, and its permeability for some substances, but very little is known about skin derivate, the nail, and the properties of nail keratin. The purpose of this work is 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. The study was divided in two parts, which will be presented as two published articles. In the first part, caffeine has been chosen as hydrophilic model drug for permeability experiments on human cadaver nail samples. Further, a screening of possible permeation enhancers has been fulfilled and hydrophobins, small amphiphilic fungal proteins with an astonishing feature of selfassembling, have been selected as promising enhancers for drug delivery through the human nail plate. In the second part of the study, lipophilic drug terbinafine, which is applied orally in the treatment of onychomycosis, has been tested in permeability experiments from the formulations with and without hydrophobins.

Transport experiments were carried out in Franz diffusion cells across human cadaver nail samples at 32°C under an occlusive effect. Caffeine was applied in a concentration of 2% (w/v) from the water and 20% (v/v) ethanol/water solutions and it was detected by UV spectrophotometer. Duration of permeability studies with caffeine was six days. Terbinafine was applied in a concentration of 10% (w/v) from the 60% (v/v) ethanol/water solutions and it was detected by HPLC. Permeability experiments with terbinafine lasted 10 days. Characterization of the nail samples and applied formulations

was maintained throughout the study in order to illuminate examined absorption processes. To detect amount of a drug remained in the nail after experiment, milling test has been performed.

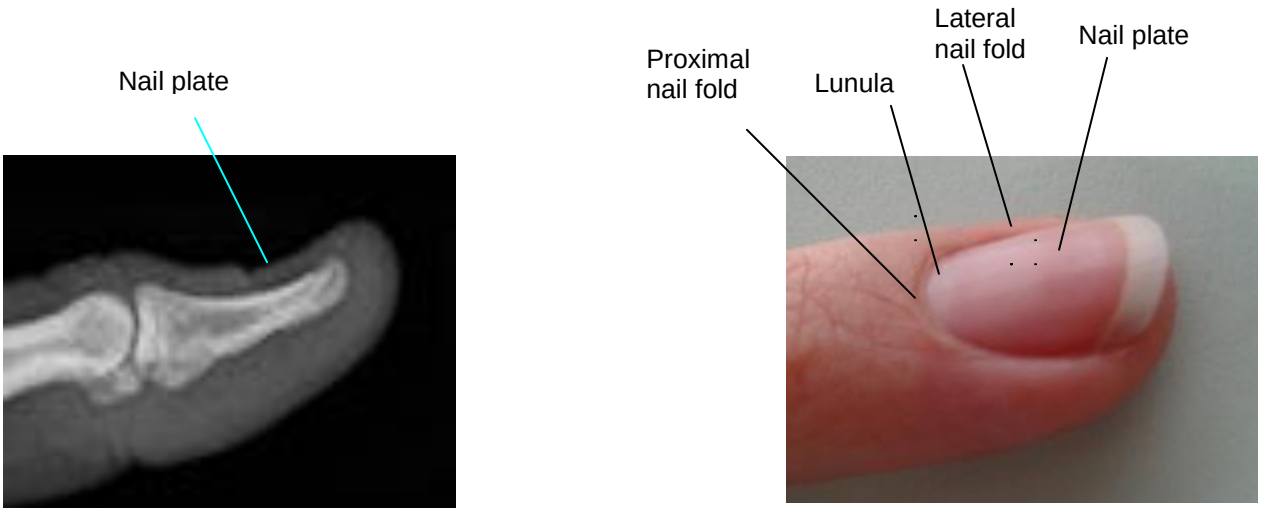
Identified potential enhancers for drug delivery through the human nail plate were methanol, dimethyl sulfoxide (DMSO), and hydrophobins. Methanol and DMSO induced irreversible structural changes in nail samples, while hydrophobins in most of the cases formed a film layer on the nail surface acting not only as enhancers but as protectors, too. Addition of 20% (v/v) ethanol in the formulations did not influence negatively the hydration of the nail and therefore the permeability coefficient. Among three different tested hydrophobins in the formulations with terbinafine, hydrophobin B increased permeation rate 13.05-fold, which assorted it in the list of substances able to augment drug delivery through the nail plate. Although a hydrophilic drug with lower molecular weight compared to terbinafine, caffeine reservoir in the nail plate samples was detected to be lower than terbinafine reservoir, which was influenced by the duration of experiment and which supported a theory that terbinafine has an affinity towards keratin in the nail plate. The question: Can we increase permeation rate even more and enable substances with different chemical and physical properties to permeate through the death keratinized cells of the nail plate, requires further investigations. Finally, an amount of drug which would be detected in the blood should be estimated *in vivo*.

A. Theoretical introduction

A.1. Structure of the human nail apparatus

The nail apparatus consists of the nail bed, nail matrix, nail folds, and nail plate (Fig. 1A and 1B). The nail bed is a thin, soft, noncornified epithelium, connected with the ventral layer of the nail plate and underlying papillary dermis. It is well perfused by blood and lymphatic vessels. The nail matrix is situated directly under the proximal nail fold. The nail plate is continually produced by the nail matrix, which consists of highly proliferating epidermal cells (Zaias and Alvarez, 1968). Cells become larger, more elongated, flatter, paler, and the nucleus disintegrates forming fragments in the horny layer. Nail growth in fingers is about 3 mm per month and in toes 1 mm per month, which means that a fingernail can be completely replaced in about 6 month and a toenail in about 12-18 month. The grow rate depends on many factors, such as health or disease state of a person or pregnancy, and usually is faster for the dominant hand, as well as for male individuals. The visible white part of the nail matrix is called lunula. The nail matrix contains melanocytes and the nail plate pigmentation varies depending on race. Melanonychia, a brown or black pigmentation of the nail unit, is common among black people and Japanese, and a prevalence increases with age. If the definition of the nail folds is given as the junctions or the borders between the nail plate and finger's epithelium, then the nail folds include lateral folds, the proximal nail fold, and hyponychium. The proximal nail fold covers aprproximately 1/4 of the nail. The stratum corneum forms the cuticle, which is a thick rim of keratinous material that borders the free margin of the proximal nail fold and is close to the emergence of the nail plate. The proximal nail fold together with lateral folds provides a physical protection against the penetration of impurities. Due to the disruption of the cuticle a free space along the folds can occur. This can lead to bacterial or fungal infection of a soft tissue around the nail plate and the nail disease called paronychia (Dawber et al., 2001; Murdan, 2002). The hyponychium is the skin under the free edge of the nail plate. It makes waterproof and protective area, similar like the proximal nail fold and the lateral folds. If it is interrupted or injured presents an open pathway for infections provocative agents. Looking at the

nail plate from above a narrow, pale band running transversely across the nail can be seen. This band usually no more than 0.5 - 1.5 mm wide is called onychodermal band and in some cases may be absent (Dawber and Baran, 1984; Lawry and Rich, 1999).



1A 1B

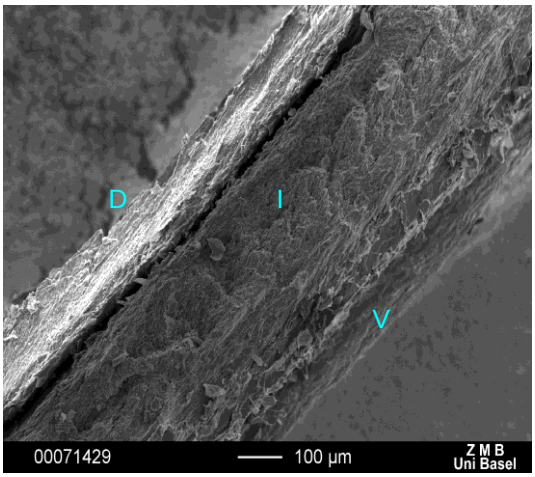
Fig. 1A Radiograph of a fingertip (Received from Huonder and Kampstra, University Hospital Zürich).

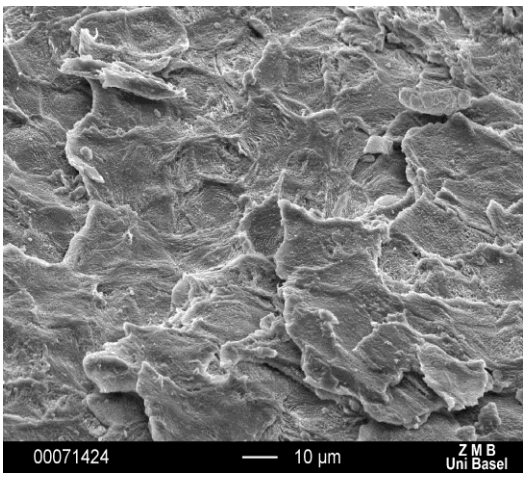
1B Photograph of a fingertip.

A.1.1. Structure of the nail plate

The nail plate consists of approximately 80-90 layers of dead, keratinized cells which are linked by desmosomal junctions and intercellular links. Cell thickness decreases with cell flattening and movement to the upper layers. Superficial cells can be about one half as thick as cells of the deepest layer in the human nail plate (Achten et al., 1991; Murdan 2008). Also, the thickness of the whole nail plate varies. The thickness increases from the proximal nail fold to the free edge of the nail plate. The nail plate can be divided into three layers: dorsal, intermediate, and ventral layer (Dawber and Baran, 1984). The thickness ratio of the dorsal:intermediate:ventral layer is 3:5:2 (Kobayashi et al., 1999). Fig. 2 shows the inner and the surface structure of the human nail plate. Cells in multiple layers are filled with keratin and two types of keratin can be distinguished in the human nail plate. The hair-like type keratin is present only in the intermediate layer and it is oriented perpendicular to the growth axis. The skin-like type keratin is found in the dorsal and ventral layers and it is oriented parallel and perpendicular to the nail

growth axis (Garson et al., 2000). The presence of the hair-like type keratin in the nail plate is four times greater than the presence of the skin-like type keratin. The high portion of cystine-rich proteins and disulphide links enable contact and relatively high physical and chemical stability of the keratin filaments. Such structure and orientation of the keratin filaments gives the nail high mechanical rigidity and hardness. Tests performed by Farren et al. (2004) have shown that the energy to cut nail longitudinally (6 kJ/m²) is 2-fold higher than to cut it transversely (3 kJ/m²). It is believed that calcium contributes little to the hardness of the nail plate, due to the finding of the same calcium-magnesium ratio (4:5:1) in the nails as in the blood. The amount of water in the nail plate is estimated to be 10-30% and it is highly dependent on the relative humidity of the environment. This amount of water is responsible for nail elasticity and flexibility. Detected amount of lipids in the nail plate varies from 0.1 to 1%. They are organized into bilayers and oriented parallel to the nail surface mostly in the dorsal and ventral layer (Dawber and Baran, 1984; Garson et al., 2000; Murdan, 2002). Kobayashi and coworkers (1999) reported that total lipid concentration in the ventral layer of the human nail plate was the highest. According to the structure of the human nail plate, a concept proposed by Walters and Flynn (1983) that the nail plate behaves like a hydrophilic gel membrane remains nowadays.





2A 2B

Fig. 2A Lateral cross section of the nail plate observed under the scanning electron microscope (SEM), University of Basel; Visible dorsal (D), intermediate (I), and ventral (V) layer.

2B Image of the nail plate surface.

A.1.2. Characterization of nail samples

A.1.2.1. Principle of Raman spectroscopy

Raman scattering has been discovered by Krishna and Raman in 1928 (McCreery, 2000). Raman spectroscopy is a non-destructive, optical, vibrational spectroscopic technique based on the scattering of light by molecules. Typically, a sample is illuminated with a monochromatic light, usually from the laser. The light from the illuminated spot is collected and sent through the rejection filter which rejects the intense Rayleigh light and allows weak Raman scattering to go through (Fig. 3). The photon gives energy to a system, which changes its energy level above or below that of the initial state. The frequency shift corresponding to the energy difference between the incident and scattered photon is termed the Raman shift. Depending on whether the system has lost or gained vibrational energy, the Raman shift occurs either as an up- or down-shift of the scattered photon frequency relative to that of the incident photon. Raman spectra are depicted by plotting the intensity of the scattered photons as a function of the frequency shift. Near-infrared Raman spectroscopy (NIR) has deeper penetration depth into the tissue and it is commonly used for biomedical applications. Raman spectra can be tools for classification and characterization of cells and tissues, including quantification of their molecular composition (Huang et al., 2003).

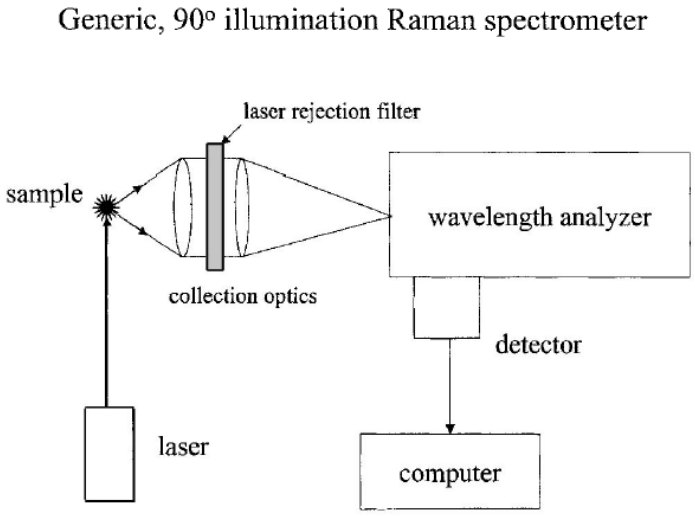


Fig. 3 Generic Raman spectrophotometer showing main components: laser, collection optics, wavelength analyzer, detector, and computer (from McCreery, 2000).

A.1.2.2. Evaluation of suitability of nail samples by Raman spectroscopy

Nail samples were placed directly on the measurement window. In order to achieve a good contact, a drop of water was put between the window and each nail sample. Also, on the top of the window additional weight increases the contact area and keeps the nail sample still. A schematic figure is given below (Fig. 4).

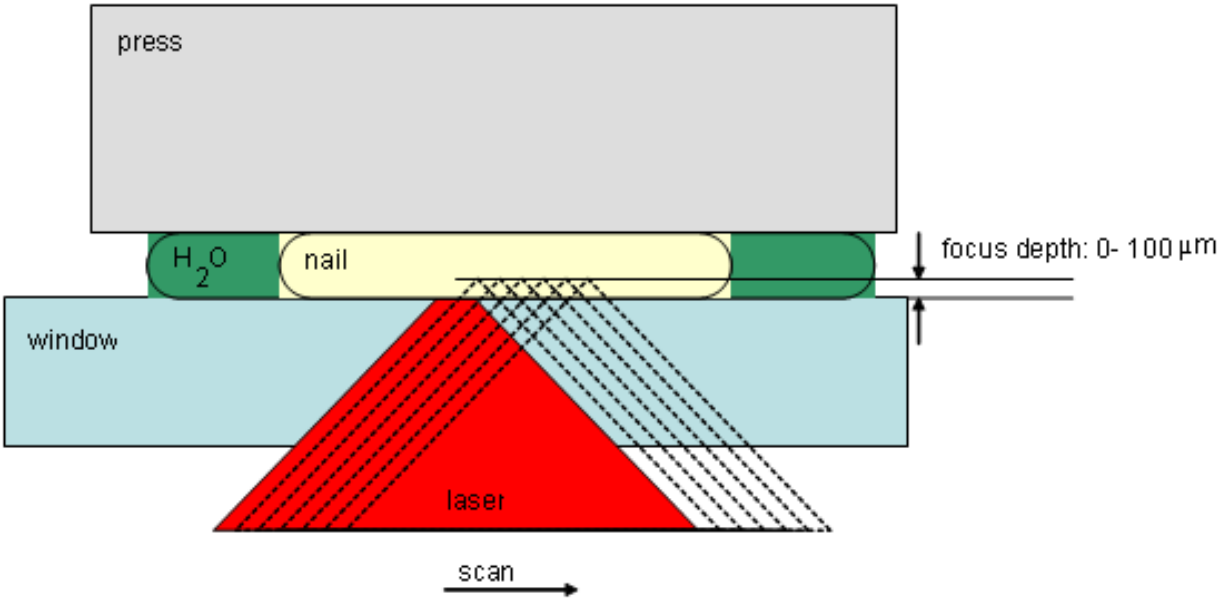


Fig. 4 A schematic view of the set up for measurements preformed on the nail samples.

As it can be seen from the Fig. 4, measurements were taken from the surface of the nail to a depth of 100 µm, with a measurement interval of 5 µm. In total, 21 measurements were taken per point. To cover the heterogeneity of the nail, five of these measurements were taken at five different points in the scan direction. Per point a measurement was taken in the highwavenumber region from 2500 to 4000 cm^{-1} using data collection time of one second and in the fingerprint area from 400 to 1800 cm^{-1} using data collection time of ten seconds.

A.1.2.3. Barrier integrity of human cadaver nail samples

Recognized methods for characterization of the barrier integrity of the skin are transepidermal water loss (TEWL), tritiated water flux, and electrical resistance (Heylings et al., 2001). The Tewameter TM 210 is based on the open chamber system with two humidity and two temperature sensors which measure evaporation gradient at the surface of the skin. The diameter of the cylinder is 10 mm which gives a surface area of 0.785 cm². This noninvasive measurement is based on Fick`s law and equation 1:

$$\frac{dm}{dt} = -D \cdot A \cdot \frac{ds}{dl}$$ Equation 1

where dm/dt presents the diffusion stream across the open chamber, expressed as transport of mass per time. The diffusion steam is proportional to the exposed area A and to the change of density per distance ds/dl. D presents the diffusion coefficient of water vapor in air. TEWL measurement depends of the body site and the environmental conditions. The same principle has been applied on the nail samples by measuring transonychial water loss (TOWL) (Murdan et al., 2008a; Dutet and Delgado-Charro, 2009).

A.1.2.4. Detection of nail surface changes

The Visioscan VC 98 has been initially developed to estimate the skin surface conditions. It has been used for the evaluation of the skin surface parameters in cosmetic treatments, as well as in the treatment of atrophic acne scars (Manuskiatti et al., 2010). The instrument is equipped with camera and the skin surface conditions are evaluated according to the graphic depiction of the skin under ultraviolet A light illumination. The analysis software evaluates black-and-white image taken by the camera comparing different gray-levels of pixels on the image. Parameters such as roughness or volume based on a virtual amount of liquid needed to fill the average of all peaks are the quantitative measures of the skin surface condition given by the profile

surface analysis. The original measuring are of 6 x 8 mm was modified to 5 x 5 mm due to the size of the nail samples. The images were taken without filter changes in the format 640 x 480 x 24b (RGB 24) and in the format 320 x 240 x 24b (RGB 24). The alterations of the surface structure were evaluated using the limited calculation area of 5 provided by software on the bigger format images.

A.2. Onychomycosis

Onychomycosis is a chronic fungal infection of the nail. It is caused mostly by dermatophytes, particularly *Trichophyton rubrum*, as well as by nondermatophyte yeasts, of which *Candida albicans* is the most common, or moulds (Debruyne and Coquerel, 2001). Prevalence is higher among elder people or one with a poor peripheral circulation, in male, diabetic and HIV positive patients, and patients who are treated by immunosuppressant drugs. Various nail diseases can be harmless or simply present only a cosmetic problem or reflect systemic ailments. However, onychomycosis can cause serious problems, especially because reported average duration of this fungal disease is 9.5 years and the recurrence rate is high. Patients with infected fingernails may experience pain or discomfort or the normal tactile function can be lost which can limit activities such as typing or playing a musical instrument. When a toenail is infected, walking, exercise, or even standing can cause pain. Additionally, patients may suffer psychological and social limitations due to their concerns about the appearance of the nails (Drake et al., 1998; Lubeck, 1998; Ghannoum et al., 2000).

The most frequently reported symptoms are discoloration, thickening, and deformity of the nails (Fig. 5). Untreated onychomycosis can cause serious complications since the surrounding skin suffers trauma, which may result in bacterial infection. Onychomycosis can be classified in several categories depending on where the infection begins (Dawber and Baran, 1984a; Lawry and Rich, 1999; Rodgers and Bassler, 2001; Murdan, 2002):

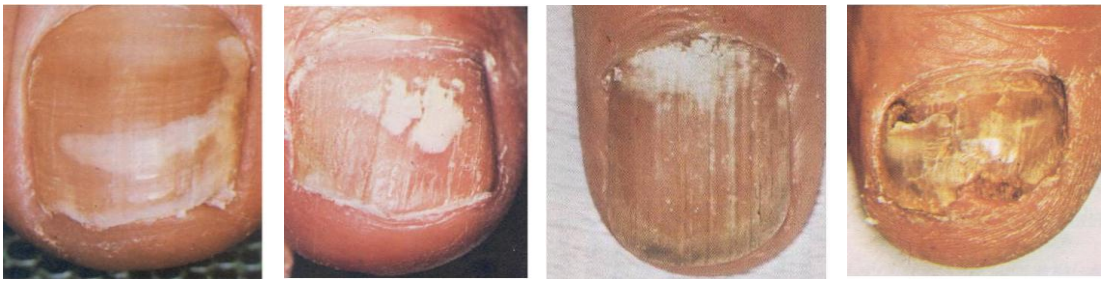
(I) *Distal and lateral subungual onychomycosis* is the most common type of onychomycosis. The organisms access to the nail unit from the hyponychium

and invade first distal nail bed, but then usually spread to proximal nail bed (Fig. 5A).

(II) *Superficial white onychomycosis* is developed when the surface of the nail plate is the initial site of invasion. Small superficial white patches with distinct edges can be distinguished in the nail plate, which can spread as the disease progresses. This type of onychomycosis can be treated with topical antifungal drugs alone (Fig. 5B).

(III) *Proximal subungual onychomycosis* starts when causative agent penetrates through the proximal nail fold, where the stratum corneum is the primary site of the fungal invasion. This type of onycomycosis is less common (Fig. 5C).

(IV) *Total dystrophic onychomycosis* is an advanced form of the previously described types. It is characterized by total destruction of the nail plate (Fig. 5D).



5A 5B 5C 5D

Fig. 5 Types of onychomycosis (from Dawber and Baran, 1984a).

Although onychomycosis is responsible for about half of all nail disorders, the diagnosis is necessary and should be made before starting the treatment with an oral antifungal drug. Usually, the specimen taken from the patient is observed under magnification with a drop of potassium hydroxide (KOH) with 20% dimethyl sulfoxide. In the case of fungal infection the branvhed hyphae can be identified. If the identification of the organism is desired or if the result of KOH is negative, fungal cultures in media should be used. Onychomycosis is excluded only when both tests are negative (Lawry and Rich, 1999; Ghannoum et al., 2000; Rodgers and Bassler, 2001).

A.2.1. Treatment of onychomycosis

There are various modalities that can be used in the treatment of onychomycosis. A diagnose in identifying a microorganism and a stage of the disease is crucial for the choice of treatment and its success. In the past, nail removal via surgical avulsion or chemical abrasion via 40% urea under the occlusion effect was used alone or as adjuvant therapy. However, avulsion is traumatic and may bring onychocryptosis (ingrown toenail). Therefore, partial avulsion is preferred over the total nail avulsion and chemical avulsion is currently used in the nail removing procedure. The other options are systemic and/or topical treatment of onychomycosis. Oral antifungals are the most effective agents available to treat onychomycosis. Some of the prescribed drugs for oral therapy are griseofulvin, itraconazole, fluconazole, ketoconazole, and terbinafine, of which griseofulvin is not currently used much. However, oral therapy is followed by some disadvantages such as drug interactions, contraindications, side effects, high cost of medication, and a long duration of treatment. Moreover, systemic use of azoles can be linked to hepatotoxicity, especially during prolonged use. Another problem which occurs is that drug resistance in fungi has been evolving and a need for novel antifungal drugs is increasing. Thus, topical therapies are more desirable. The Food and Drug Administration (FDA) approved ciclopirox nail lacquer for the treatment of mild to moderate onychomycosis caused by *T. rubrum* without involvement of the lunula, while ciclopirox and amorolfine have been approved in Europe. Due to the highly restrictive properties of the nail plate and low permeation rate of the drugs, topical therapies are usually recommended for the early stages of the disease, when one or two nails are infected, as an additional medication to the systemic medication, and when systemic therapy is contraindicated. In order to achieve a positive result by topically administered drug, prolonged and attentive compliance is needed (Rodgers and Bassler, 2001; Murdan 2002; Gupta and Tu, 2006; Gauwerky et al., 2009).

A.2.2. Terbinafine

Terbinafine is the most potent drug nowadays for the treatment of onychomycosis. It is an allylamine synthetic antifungal (Fig. 6).

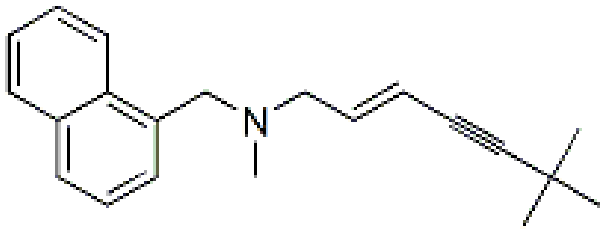


Fig. 6 Structural formula of terbinafine
IUPAC name: (2E)-N,6,6-trimethyl-N-(naphthalen-1-ylmethyl)hept-2-en-4-in-1-amine

The monograph of terbinafine hydrochloride appeared for the first time in the Supplement 5.3 of the European Pharmacopoeia implemented on 1st of January in 2006 (Supplement 5.3, 2006). Molecular weight of terbinafine hydrochloride is 327.9 g/mol. It is white fine powder, slightly soluble in water and acetone, and easily soluble in water free ethanol and methanol (Ph. Eur., 2008). The Z-isomer of terbinafine is considered inactive (Kazakov and Golosov, 2004). The drug has to be protected from light during storage.

Terbinafine is fungicidal against dermatophytes and fungistatic against some nondermatophyte molds or yeasts. It inhibits squalene epoxidase. As a result of this inhibition squalene accumulates in the cell and eventually causes cell death. The fungistatic action is thought to be due to the ergosterol deficiency, which plays a role in fungal cell growth (Gupta and Ryder, 2003).

Terbinafine is commercially available as tablets for systemic treatment of fungal infections counting onychomycosis, but it is also disposable as cream, solution, spray, or gel for the topical treatment of infected skin. At the moment, there is no approved topical

terbinafine formulation for the treatment of onychomycosis on the market. Hence, many companies are interested and compete for the predominance in this area.

For systemic application 125 mg and 185 mg terbinafine hydrochloride film-coated granules for children and 250 mg terbinafine hydrochloride tablets for adults are available. Oral granules are indicated for the treatment of tinea capitis, a dermatophyte infection of the scalp hair follicles, in patients 4 years of age and older, while tablets are indicated for the treatment of onychomycosis due to the dermatophytes (tinea unguium). The treatment is depending on the number and the stage of affected nails. One 250 mg tablet should be taken once daily for 6 weeks by patients with fingernail onychomycosis and 12 weeks by patients with toenail onychomycosis. This recommendation has been adapted in the practice and a commonly used pulse regimen 250 mg once or twice daily for 1 week each month for 3 months (De Doncker, 1999; Gupta and Ryder, 2003; Lamisil (terbinafine hydrochloride tablets) prescribing information, 2005).

As orally administered drug, terbinafine is well absorbed (>70%) and bioavailability due to the first-pass metabolism is 40%. It is nonspecifically bound to plasma proteins (>99%) and the concentration in the plasma 2 hours after oral administration of 250 mg dose ranges from 0.8 to 1.5 μg/ml. Terbinafine is a lipophilic drug with a partition coefficient (logP) of 5.9, hence it is well distributed to the sebum, skin, adipose tissue, and nails, and it has a slow elimination from those tissues. Its main metabolite is demethylterbinafine. Approximately 70% of the administered dose is eliminated in the urine and no metabolites have been identified that have antifungal activity similar to terbinafine.

For the first toenail study the mean estimated time to the overall success is approximately 10 months and for the fingernail study 4 months. In patients evaluated at least 6 months after achieving clinical cure of toenail onychomycosis and at least one year after completing therapy the relapse rate is 15%. In different comparative studies of terbinafine, ketoconazole, fluconazole, and itraconazole, terbinafine remains superior to the other oral antifungal drugs.

Besides some common adverse reactions such as diarrhea, dyspepsia, abdominal pain, nausea, headache, rashes, urticaria, there are specific side effects as taste disturbance which can result in decreased food intake leading to significant weight loss and liver failure. Reported severe adverse events are serious skin reactions, severe neutropenia and thrombocytopenia, liver failure which can lead to liver transplant or death, allergic reactions, systemic lupus erythematosus, upper respiratory tract infection, etc. Postmarketing experience includes myalgia, acute pancreatitis, reduced visual activity, and hair loss. Furthermore, drug interactions are numerous. Additionally, pre-treatment serum transaminases tests are advised. Taking into account all above mentioned, it is clear that a topical treatment of onychomycosis is desirable. Also, the fact that fungal pathogens develop resistance to terbinafine in laboratory experiments may become clinically relevant in the future (Ryder, 1992; Gupta and Ryder, 2003; Lamisil (terbinafine hydrochloride tablets) prescribing information, 2005; Gupta and Tu, 2006; Lamisil (terbinafine hydrochloride oral granules) prescribing information, 2007; DrugBank, 2009; Krishnan-Natesan, 2009; Newland and Abdel-Rahman, 2009).

A.2.3. Characterization of formulations

A.2.3.1. Viscosity measurements

Viscosity, a resistance of fluid against flow, is defined as the shear stress (τ) through the shear rate (D):

$$\eta = \frac{\tau}{D}$$ Equation 2

The rotation viscosimeter measures the shearing force between two coaxial cylinders, one containing the liquid and the other turning. The viscosity is calculated by the related software:

$$\eta = \frac{1}{\omega}\left(\frac{M}{4\pi \cdot h}\right) \cdot \left(\frac{1}{R_A^2} - \frac{1}{R_B^2}\right) = k\frac{M}{\omega}$$ Equation 3

where M is the angular momentum, h is the height of immersion which revolves in the liquid, R_A and R_B are the radii of the cylinders ($R_A < R_B$), and ω is the angular velocity. Viscosity can also depend on the constant k of the viscosimeter.

A.2.3.2. Principle of surface tension and contact angle measurements

Surface tension is a measurement of the cohesive energy present at interface. Molecules of a liquid interact with each other in an equal manner, but molecules on the surface of a liquid are in misbalance due to the border line liquid-air. These molecules interact stronger with their nearest neighbors on the surface attracting each other which is presented as surface tension of a liquid and which forces liquid surface to contract. Detecting the force needed to disrupt equilibrated stage, i.e. needed to extend the surface it is possible to calculate surface tension. Commonly applied methods are the ring method and the plate method, also called Wilhelmy plate method. Equation 4 gives the relation between surface tension and contact angle when the plate method is applied:

$$\sigma = \frac{F}{l \cdot \cos\theta}$$ Equation 4

where σ is surface tension, F is force acting on the balance, l is wetted length, which corresponds to 2a+2b of a rectangle, and θ is contact angle. If the plate used in the measurements is roughened platinum, than the contact angle is virtually 0°, i.e. cosθ is approximately 1 and the surface tension can be calculated from the measured force and the length of the plate. When the nail samples with known dimensions are used instead of the platinum plate, from the measured force and the known surface tension, contact angle can be calculated, which indicates wetting properties of the examined formulations. The lower the value for contact angle is, the better the wetting property is.

A.2.3.3. Capillary constant measurements of nail samples for different liquids

Capillary constant is a specific constant of various materials which should be predetermined in sorption measurements of tablets or bulk powders. The speed at which the liquid rises through the powder is measured by recording the increase in weight as a function of time. The principle is based on the Washburn equation:

$$\frac{l^2}{t} = \frac{\sigma_l \cdot r \cdot \cos\theta}{2\eta}$$ Equation 5

where l is flow front, t is flow time, σ_l is surface tension of the liquid, r is capillary radius, θ is advancing angle, which corresponds to the contact angle between the solid and the liquid, and η is viscosity of the liquid. Capillary radius is defined by the orientation of the micro capillaries c and the mean radius, and can be written as ($c \cdot \bar{r}$). Knowing viscosity and surface tension of the liquid, and the flow front of the sample, and taking a value for cosθ of 1, the term:

$$2\eta \frac{l^2}{\sigma_l}$$

is plotted against t (time) and a slope from linear section presents required constant ($c \cdot \bar{r}$).

A.3. Permeability of the human nail plate

On the first look, human nail plate seems to be impossible to overcome. Composed of death keratinized cells which are glued to each other the nail plate presents a solid barrier for substances. Furthermore, the lipid content is 10 times lower compared to the stratum corneum and the permeation route through the nail plate is longer due to the thickness properties of the nail as a membrane (Murdan, 2002).

However, a simple everyday demonstration of nail cutting after the shower indicates nail softening in the presents of water. Indeed, as Walters and Flynn (1983) observed the nail plate behaves like a hydrogel rather than a lipophilic membrane, such as many membranes in our body are. Besides the characteristics of the nail plate, where can be included thickness, presence of disease, level of the nail plate hydration, and presence of an intact dorsal layer, other factors influence permeation of drugs through the nail plate. Those factors are following: molecular size of substance, degree of ionization, contact time between formulation and the nail plate, ability to interact with nail constituents, and formulation effects (Walters et al., 1983; Murdan, 2002; Kobayashi et al., 2004; Rosenmayr-Templeton, 2009).

A.3.1. Permeation experiments and data analysis

Absorption process is controlled by passive diffusion and Fick`s law can be used to analyze permeation data:

$$J = \frac{dm}{dt \cdot A} = -D \cdot \frac{dc}{dx} \qquad \text{Equation 6}$$

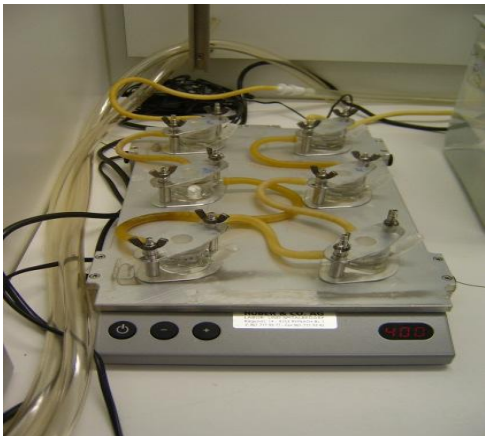
where J is diffusion flux and presents the amount of drug which permeates through the nail plate per unit time and per unit area. D is diffusion coefficient and dc/dx is the concentration gradient over a distance x. In vitro steady-state drug flux (ug/cm²·h) was calculated using the linear portion of the cumulative permeating amount versus time curve by calculation of the slope. The permeability coefficient (cm/s) was calculated from the drug flux and initial donor concentration:

$$P = \frac{J}{c} = \frac{dm}{dt} \cdot \frac{1}{A} \cdot \frac{1}{c} \qquad \text{Equation 7}$$

where dm/dt is the slope of cumulative amount versus time line and A is the diffusion area.

Permeation experiments were performed on modified Franz diffusion cells. Procedure is explained in details in original publications under the materials and methods section. Fig. 7A and 7B illustrate set-up for permeation experiments.

7A



7B

Fig. 7A Franz diffusion cells modified for nail permeation studies. The acceptor chamber with the extension for taking samples is surrounded with another chamber for floating water tempering the cell. The nail is fixed between the white plastic and the rubber ring by a metal structure.

7B Franz diffusion cells on a magnetic stirrer plate during the experiment.

A.3.2. Factors which influence drug delivery through the human nail plate

Thickness of the nail is a path through which diffusing molecules permeate. The ticker the nail is, the more difficult it will be for the drugs to reach the nail bed (See Equation 6).

Presence of disease can alter the properties of nail plate, such as nail thickness. Kobayashi et al. (2004) detected fluxes of 5-fluorouracil through fungal nail plates from eight patients and compared them with fluxes through nail plates from healthy volunteers. They concluded that there is no significant difference and thus the fungal nail permeability can be estimated from the healthy nail permeability data with an exception

of very heavy fungal nail plates, where the flux is thought to be higher due to the nail destruction by fungi.

Hydration of the nail plate is an important factor which influences drug permeability. With increasing hydration rate of the nail plate, an increase in drug permeability can be observed (Gunt and Kasting, 2007). The fact that water uptake was used as a marker for pre-formulation screening of potential enhancers indicates the importance of the nail swelling on drug permeability (Khengar et al., 2007).

The dorsal layer of the nail plate is the main barrier to drug permeation process, as it was suggested in the work of Kobayashi et al. (1999), which was later confirmed by Nair et al. (2009). Thus, many techniques have been used in order to remove or damage the dorsal nail layer, not only to influence the main permeability barrier, but in the same time to reduce the thickness of the nail plate. The PathFormer device is approved by the Food and Drug Administration (FDA) for controlled nail trephination, i.e. generation of microscopic holes in the nail plate (Boker and Bruks, 2007). Other successful attempts of partial or total dorsal layer removal and increase of permeability were sanding of the nail plate or abrasion by dentist's drills (Di Chiacchio et al., 2004), use of keratinase enzyme in hoof membranes (Mohorčič, et al., 2007), or use of 10% phosphoric acid gel on the nail plate surface (Repka et al., 2004).

Molecular size of diffusing molecule has an inverse relationship with permeation into the nail plate. The smaller the diffusing molecule and the less branched it is, the faster diffusion through the "pores" of the membrane takes place. Kobayashi and coworkers (2004) found a linear relationship ($r = -0.860$) between the permeability coefficient and the molecular weight.

Degree of ionization of diffusing molecule plays an important role in permeation through the human nail plate. In the same work of Kobayashi et al. (2004) it has been demonstrated that the nail permeability of an ionic drug is significantly lower than that of

a non-ionic drug. They suggested that the decrease in permeability was caused by a decrease in diffusivity due to ion hydration.

Applied formulation can influence drug delivery through the human nail plate from many aspects such as hydration of the nail plate, drug solubility, contact time between formulation and the nail plate, and ability to interact with nail constituents. Aqueous based formulations are suitable for increasing hydration rate of the nail plate which leads to higher permeability of the nail. Therefore, many researchers apply solutions, suspensions, or gels (Kobayashi et al., 1999; Malhotra and Zatz, 2001; Brown et al., 2009). In practice, aqueous based formulations are less suitable than lipophilic vehicles due to their easy removal from the nail plate and thus short term contact with the nail surface. It is thought that although lipophilic vehicles such as lacquers do not cause hydration of the nail plate till maximum degree, they leave a hydrophobic film on the nail surface which limits transonychial water loss and at the same time, after the evaporation of the solvent, cause higher concentration of the drug in the film than from original applied formulations (Flagothier, 2005). On the market there are lacquers, e.g. Loceryl® (amorolfine) or Penlac® (ciclopirox), which are used in topical treatment of onychomycosis. Hui and coworkers (2004) demonstrated that the marketed gel with 0.77% ciclopirox deliver much more drug than nail lacquer with 8% ciclopirox. They concluded that the concentration of the drug was not as important factor as the nature of the vehicle and that antifungal drug delivery can be altered with formulation and delivery enhancers.

A.3.3. Enhancing drug delivery through the human nail plate

Physical techniques, usually applied prior to the formulation containing the drug, alter the physical properties of the membrane, the nail plate. In most of the cases this change is irreversible. Removal of the dorsal nail layer by filing or microporation of the nail plate leads to the improvement of drug permeability coefficient. The same effect has been recorded when a low-frequency ultrasound technique was used as a pretreatment in permeability experiments (Torkar et al., 2007). Another approach is iontophoretic drug

delivery. The increase of the amount of drug loaded into the nail using iontophoresis was demonstrated by Nair et al. (2009a). They suggested that iontophoresis can increase the permeation of terbinafine hydrochloride through the nail as well as form a drug depot in the nail which could lead to a prolonged therapeutic effect. The use of plasma treatment before the application of nail lacquer results in an improved adhesion of nail lacquer and reduced drying time by 78% (Kaemling et al., 2005).

Chemical enhancers are substances able to break chemical and physical bonds responsible for the stability of nail keratin. Targets for such substances are disulphide bridges, peptide, hydrogen, and polar bonds (Murdan, 2008). In the work of van Hoogdalem et al. (1997) on six healthy volunteers was demonstrated that acetylcysteine increases drug uptake in the upper nail layer, while in the lower layers this effect is not observed. N-acetyl-L-cysteine and 2-mercaptoethanol increase drug permeation through the human nail plate, but the penetration enhancing effect of 5-fluorouracil by N-acetyl-L-cysteine depends on the swelling and softening of the nail pieces (Kobayashi et al., 1998). Sodium sulphite is another example of compound which cleaves the disulphide bond and there are indications of its enhancing effect in drug delivery through the nail plate (Murdan, 2008, unpublished results). Brown and coworkers (2009) used two types of possible enhancers, tioglycolic acid as reducing agent and urea hydrogen peroxide as oxiding agent. It was shown that the steady-state flux of terbinafine was enhanced to the greatest extent following nail pretreatment with tioglycolic acid followed by urea hydrogen peroxide. The authors explained this result by the expanded, “swollen” keratin network and open pore state of the nail induced by penetration enhancer pretreatment. Approach with keratinolytic enzymes which hydrolyzes nail keratins acting on the intercellular matrix by separating corneocytes on the dorsal surface from one another was successful in increasing metformin flux through hoof membranes (Mohorčič, et al., 2007; Murdan, 2008).

A.4. Hydrophobins

Hydrophobins are relatively small proteins secreted by fungi and typically constituted of 100-150 amino acids. Their discovery and name are linked to the researchers Dons and Wessels who were studying fungus *Schizophyllum commune* in the eighties (Wessels, 2000; Linder et al., 2005; Sunde et al., 2008). Eight cysteine residues can be found along the chain of amino acids. They are divided into two classes, class I and class II (Wessels, 2000). Class I aggregates only dissolve in strong acids such as trifluoro acetic acid, while class II aggregates can be dissolved using aqueous dilutions of organic solvents. Class I hydrophobins have been found in Basidiomycetes, while class I and class II have been found in Ascomycetes (Linder et al., 2005; Linder, 2009). Hydrophobins have a special role in the life of fungi. They are secreted by fungi and generally known to occur on fungal spores. It is assumed that hydrophobins have a function in keeping the spores dry and prevent clumping, allowing dispersal of the spores by wind. While the hydrophobin hydrophobic layer prevents water penetration, at the same time it is highly permeable to gas exchange. Acting as natural surfactants, they reduce surface tension of the growing medium by self-assembling, thus allowing fungi to overcome medium-air interface and to produce hyphae into the air. Disulfide bridges in the hydrophobin monomer prevent the protein from aggregating spontaneously in the absence of a hydrophilic-hydrophobic interface, but in the same time the protein still has a significant degree of plasticity. Since the same fungus can contain different hydrophobin genes, it is thought that each of them has a specialized function in the life of fungus. Some of the hydrophobins are involved in interactions with other living organisms which can be developed in symbiotic relationships (Wessels, 2000; Sunde et al., 2008).

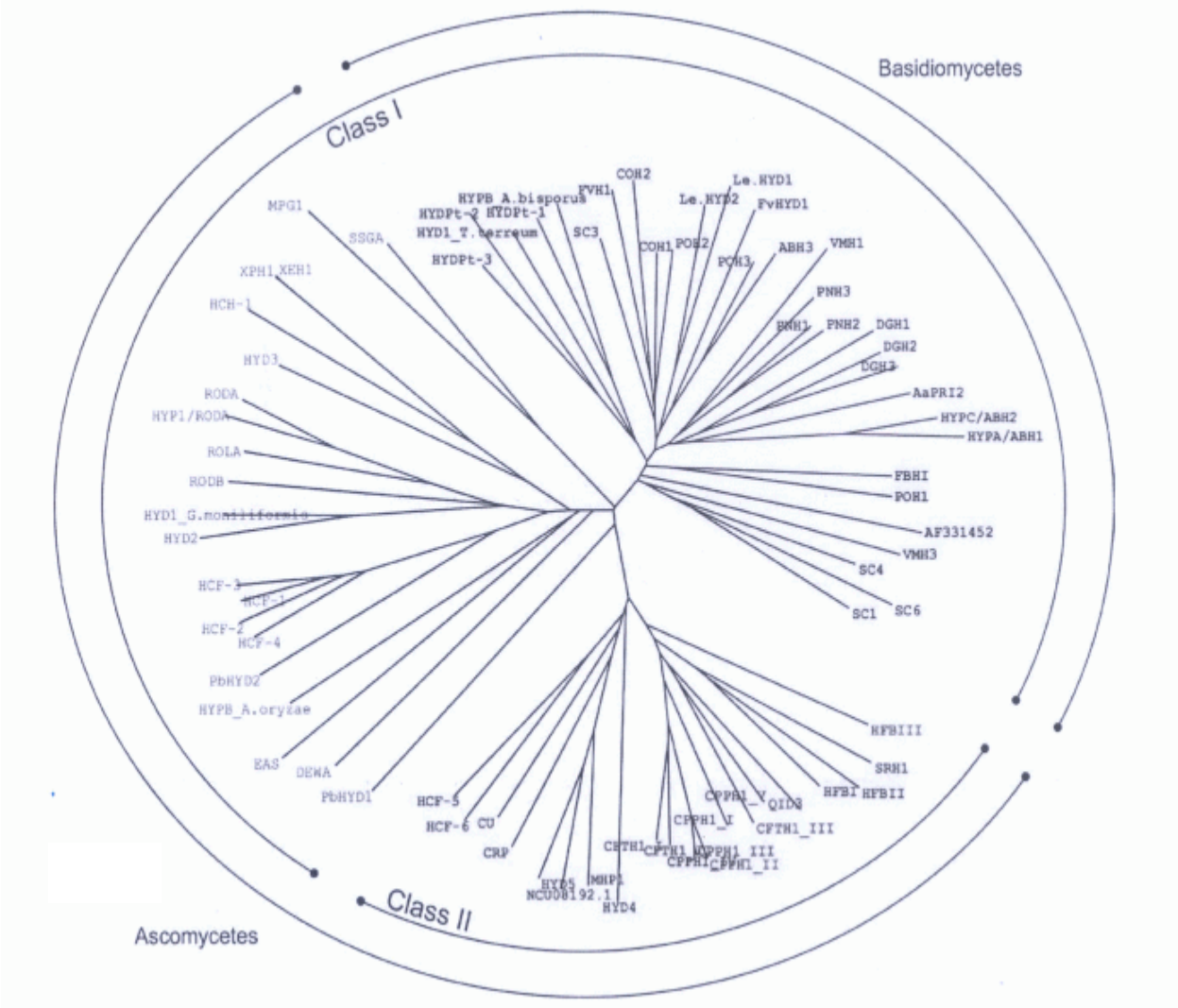


Fig. 8 An unrooted phylogenetic tree of the deduced hydrophobin protein sequences deposited in databases (from Linder et al., 2005).

A high variety of hydrophobin genes resulted in numerous proteins (Fig. 8). The most studied hydrophobins are CS3 from *Schizophyllum commune* and EAS from *Neurospora crassa* which belong to class I, and HFBI and HFBII from *Trichoderma reesei* which belong to class II hydrophobins. Hydrophobins from both classes are very soluble in water. The common amphiphilic property of hydrophobins is shown as a tendency to migrate to interfaces such as air-water interface and the ability to encapsulate and dissolve hydrophobic molecules into aqueous media. The form of assembled class I hydrophobins is the insoluble rodlet layer which can occur after a drop of dilute protein

solution dries on a solid base and which is the final stage from other intermediate assembled structures. In the case of class II various aggregates have been reported as needles or fibrils or hexagonal repeating pattern, but their properties in size and solubility differ. Thus, class I members adhere strongly to surfaces, while class II members dissociate more easily (Linder et al., 2005; Sunde et al., 2008; Linder, 2009).

Proposed applications of hydrophobins due to their amphiphilic nature and self-assembly properties are as surfactants, in formation of coatings, as emulsifiers in food industry, as stabilizers of foams, in removal of oil from contaminated water, as modifiers of surfaces such as electrodes, in immobilization purposes such as immobilization of enzymes, antibodies, or cells, etc (Linder et al., 2005; Linder, 2009).

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B. Objective

Nail diseases are not only a cosmetic problem, but can cause pain and seriously affect the quality of life. The most common nail disease responsible for approximately 50% of all nail disorders is onychomycosis (Ghannoum et al., 2000). This fungal infection can be treated using various modalities: nail removal via surgical or mechanical procedures, oral antimycotic agent, topical lacquer, or combination of these therapeutic options depending of the severity of the onychomycosis, number of nails affected, location of the disease, and patient/physician preference (Gupta and Tu, 2006). Debridement of the infective nail or surgical nail avulsion can bring a relief to the patients in terms of pain and further trauma, but it is likely to be able to cure onychomycosis when used as monotherapy. Additionally, a risk of subsequent nail ingrowth and bacterial infection remains. Some of the medicines prescribed for oral therapy are griseofulvin, itraconazole, ketoconazole, and terbinafine, of which terbinafine is the most effective oral agent for treatment of dermatophyte onychomycosis nowadays. Griseofulvin became an obsolete drug in economically developed countries. As a chronic and persistent disease, onychomycosis requires long duration of treatment, which is a negative factor for the selection of the oral therapy due to its unavoidable side effects. On the other hand, topical therapies are facing highly restrictive barrier properties of the human nail plate. An antifungal agent has to permeate nail plate layers and reach the nail bed and the nail matrix in defined minimum inhibitory concentration or higher, in order to obtain a positive cure rate. Although leading antifungal drug, up to now terbinafine is not available for topical application in the treatment of onychomycosis.

Pharmaceutical formulation plays an important role in drug permeation through the nail. One of the biggest challenges faced by pharmaceutical scientist is to design an optimal pharmaceutical dosage form. This study has been conducted due to the existing knowledge gap in the area of nail structure, nail properties, nail permeation, and development of suitable and effective topically applied pharmaceutical formulations for treatment of onychomycosis. The aim of the work was to screen various potential enhancers with different modes of action in order to successfully overcome nail plate

barrier and deliver active drug in situ. The work was divided into two parts. In the first part possible enhancers were tested from the liquid formulations containing the model drug caffeine. Also, the human cadaver nails suitability as samples for the permeation experiments has been evaluated by Raman spectroscopy analysis. A method for collecting human cadaver nails from human corpuses did not exist and needed to be developed in the present work. Contact angle measurements have been fulfilled on the nail samples in order to classify wettability properties of the tested formulations. In the second part hydrophobins as identified possible enhancers were incorporated into the formulations containing active drug terbinafine. Since the non uniform conclusions in the literature were deduced from the studies on the nail plate, a special attention was given to the nail properties under the experimental conditions and tested formulations. Parameters as weight, thickness, transonychial water loss (TOWL), state of the nail surface, and influence of ethanol portion in the formulation on the nail swelling and its correlation with nail permeability properties, allowed better insight into the nail behavior. Further, the drug reservoir in the nail plates after the permeability experiments was measured and a possible correlation with the obtained permeability coefficients was investigated. Moreover, a comparison between the terbinafine concentrations rates in the systemic circulation after an oral administration and terbinafine concentration in the acceptor chamber after the permeation experiments has been performed. Also, a need for topical treatment of onychomycosis is discussed and an overview of current status of products in clinical development is given in the following chapter.

C. Original publications

C.1. Investigation of different formulations for drug delivery through the nail plate

C.1.1. 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 to 30%, and simultaneously ten times lower lipid content comparing with the stratum corneum of the skin, 0.1 to 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 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 to 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.

C.1.2. Materials and methods

C.1.2.1. 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).

C.1.2.2. Collection, evaluation, and characterization of nail samples

C.1.2.2.1. 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.

C.1.2.2.2. 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% to 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% to 93.3% and temperature of 23.3°C. Again, after one day, weight and thickness were noted and increase in weight was compared.

C.1.2.2.3. 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 16mm in diameter and afterwards thickness and weight were measured. Nail rests were collected, left over

night, and weighed ($m_{whole\ nail} - m_{nail\ rest} = m_{nail\ in\ experiment}$). 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 hours.

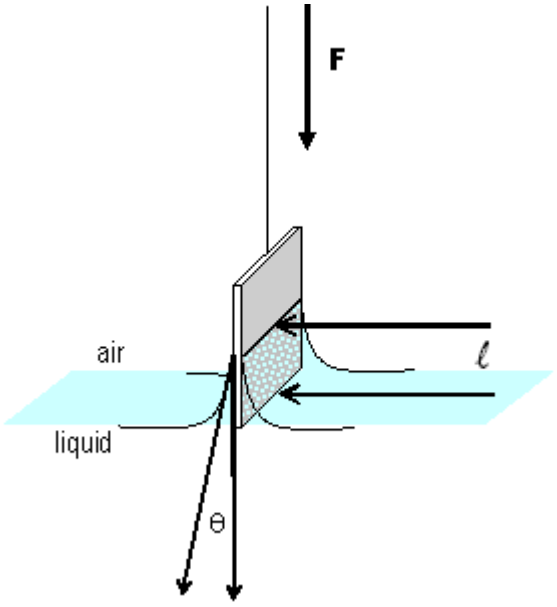
C.1.2.3. Preparation and characterization of the formulations

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), 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.

C.1.2.3.1. 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 minutes 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 to 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 mm to 4 mm.



$$\sigma = F/(l \times \cos\theta)$$

σ	surface tension
F	force
l	wetted length
θ	contact angle

Fig. 1 Wilhelmy plate method.

C.1.2.4. 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 273nm 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.

C.1.2.5. 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 minutes and then milled for 2 minutes. 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 minutes. 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.

C.1.3. Results and discussion

C.1.3.1. 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 (Fig. 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 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.

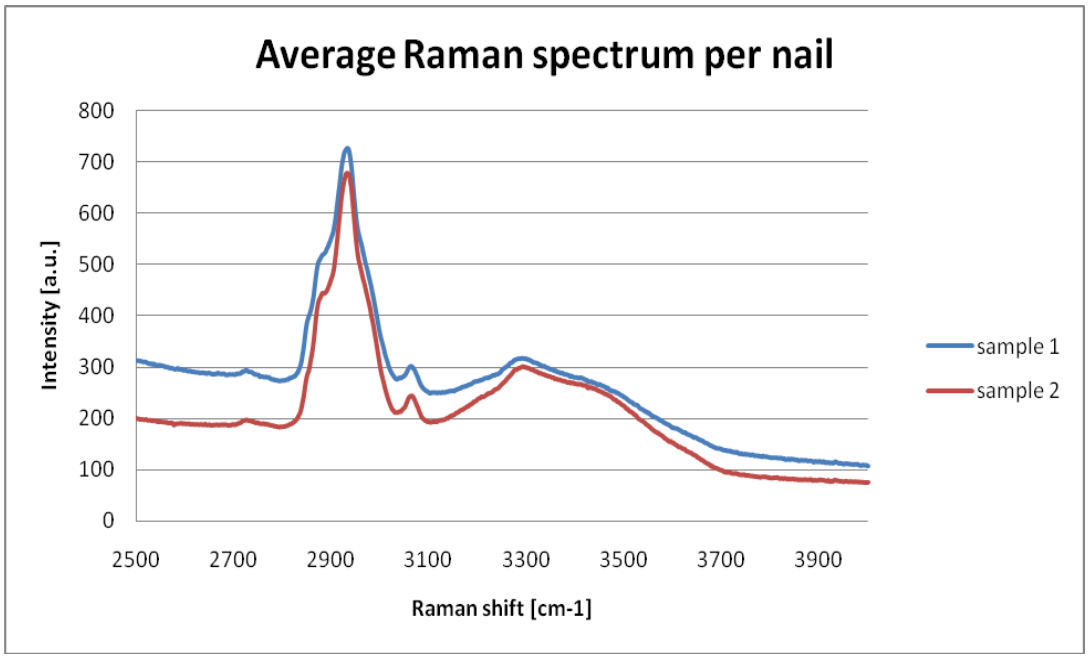


Fig. 2 Average spectrum of 105 measurements taken in the high wavenumber region.

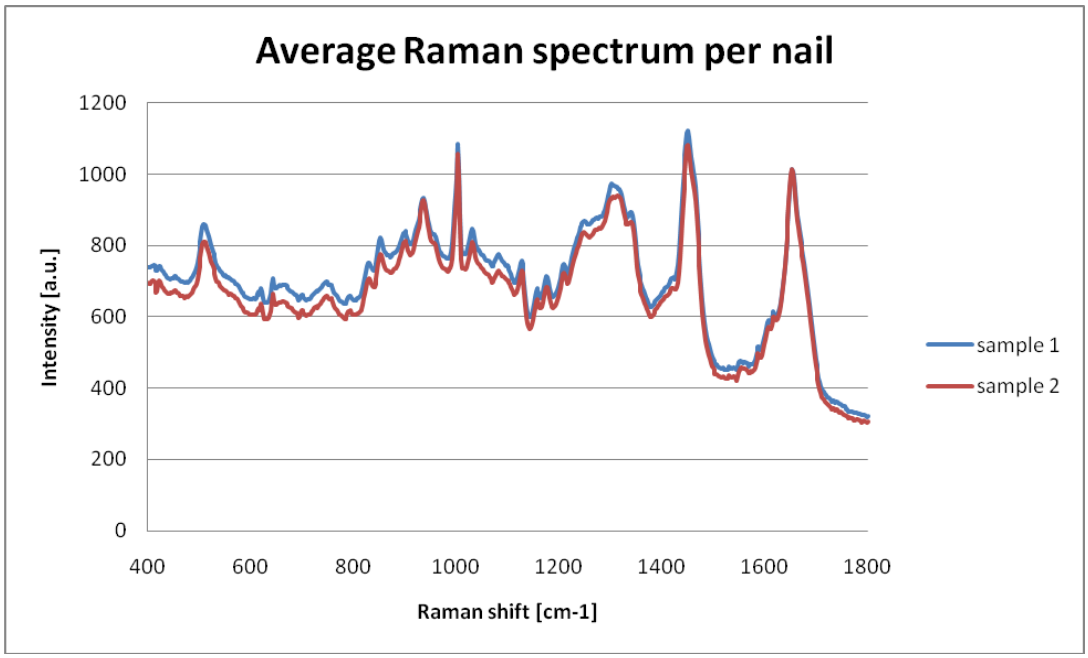


Fig. 3 Average spectrum of 105 measurements taken in the fingerprint region.

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 to 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 microns 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 (dorsal : 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.

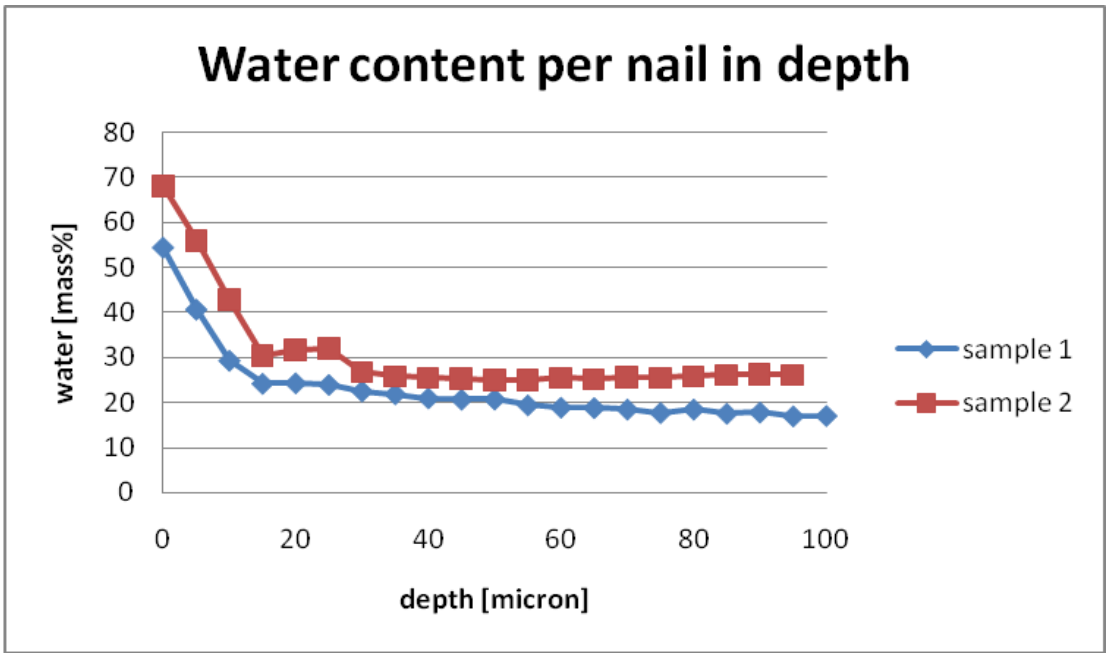


Fig. 4 Average over five measurements of water content per sample per depth.

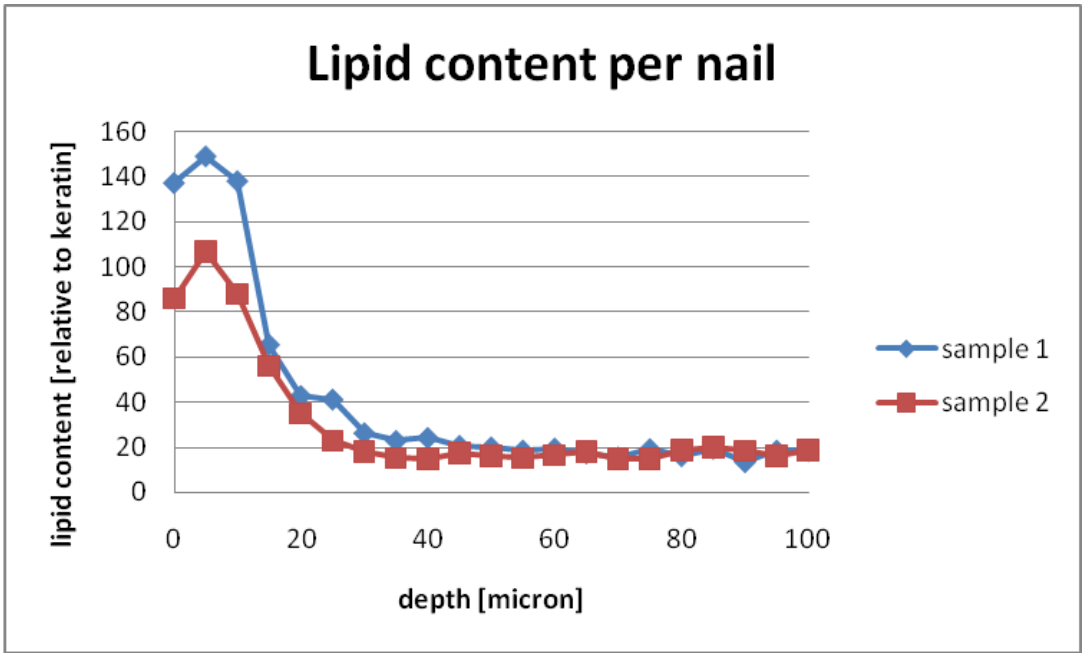


Fig. 5 Average over five measurements of lipid content per sample per depth.

Increase in weight for female samples was determined to be 21±1%, for male 17±1%, and for cadaver's 22±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.

C.1.3.2. 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±10%. This correlates very well with 22±8%, the result presented in Section C.1.3.1. 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/hm²) before		TOWL (g/hm²) after	
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

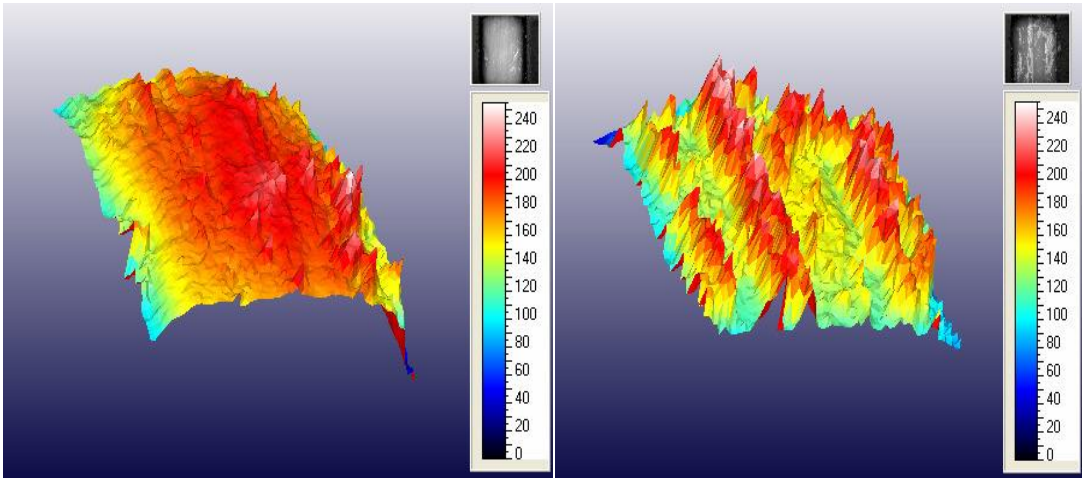


Fig. 6 Changes in the nail surface structure illustrated on the 3D graph. On the left image is dry nail before and on the right after the experiment with methanol in 20% (v/v) ethanol/water formulation.

(1) (2) (3)

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 24h after the experiment with class I hydrophobin.

C.1.3.3. Characterization of the formulations

All formulations had pH values in the range of 5 to 7.5, except the formulations containing NAC and BA. They were acidic with pH in the range of 1.8 to 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 to 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.

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)

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 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.

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

The data expressed as capillary constants could be divided into two groups. Hexane and ethanol showed capillary constants in the range of 2 to 2.7E-10 and ethanol/water and water in the range of 6.2 to 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.

C.1.3.3.1. 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.

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)

C.1.3.4. 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 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.

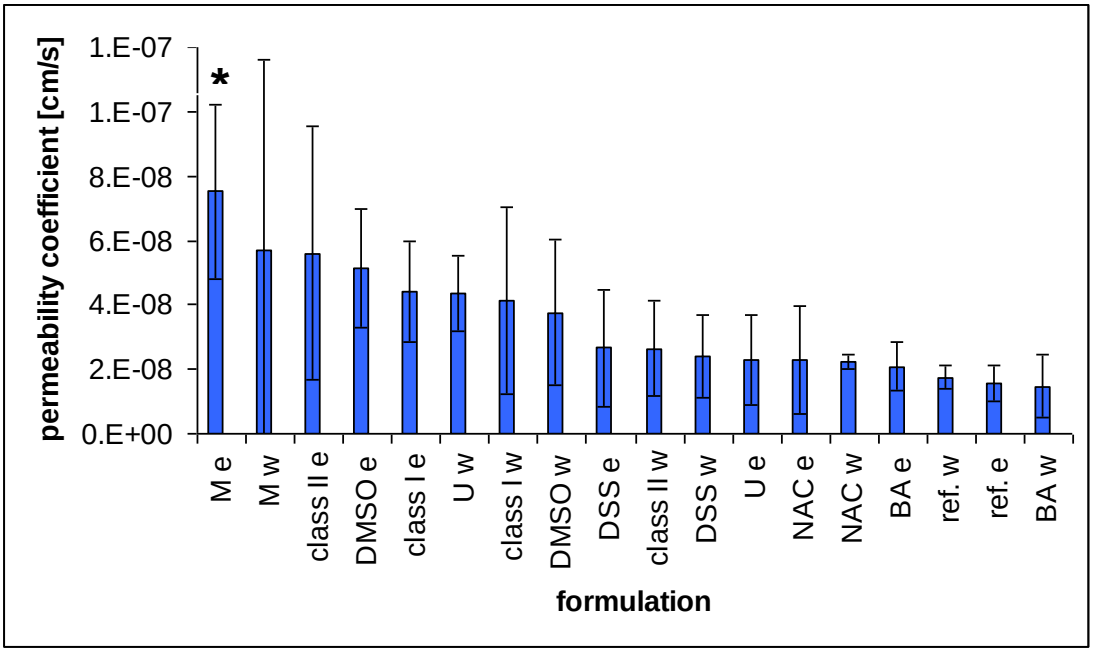


Fig. 8 Permeability coefficients with standard deviations (n=3). Asterisk indicates significant difference.

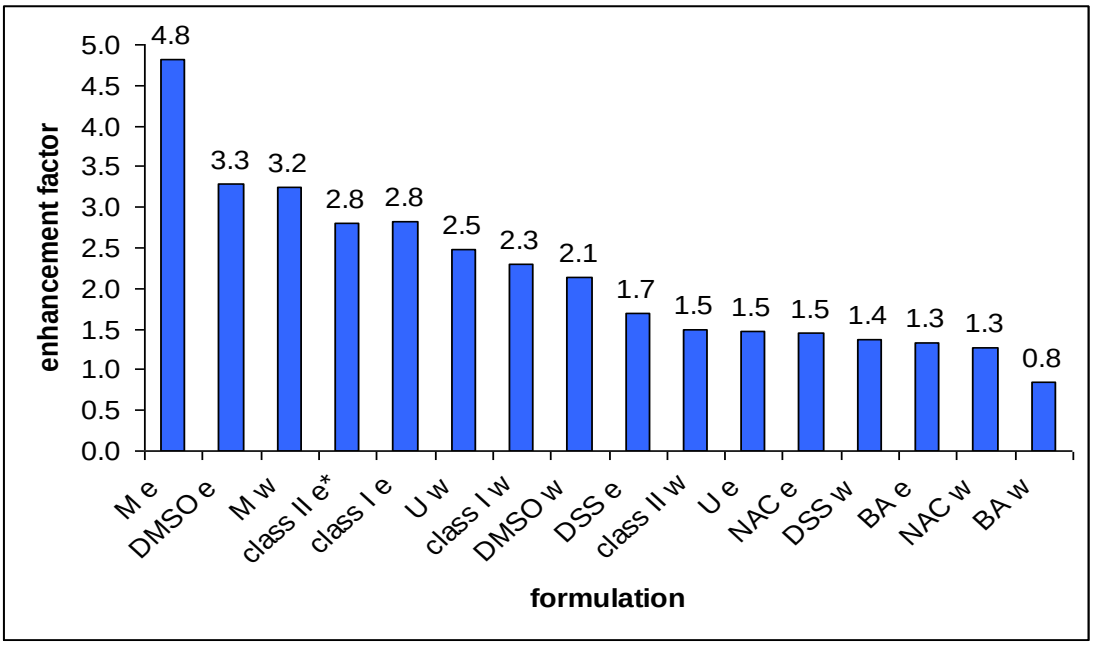


Fig. 9 Enhancement factors calculated to the caffeine formulations without enhancer (references); class II e* (n=6).

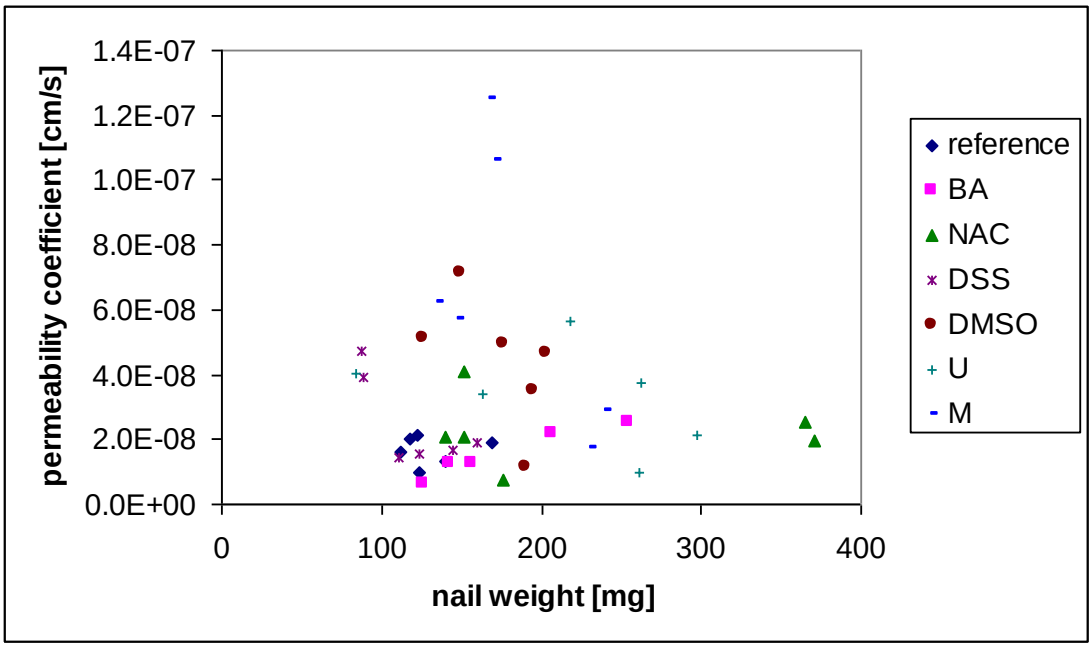


Fig. 10 Correlation between permeability coefficient and nail weight of dry nails before experiments.

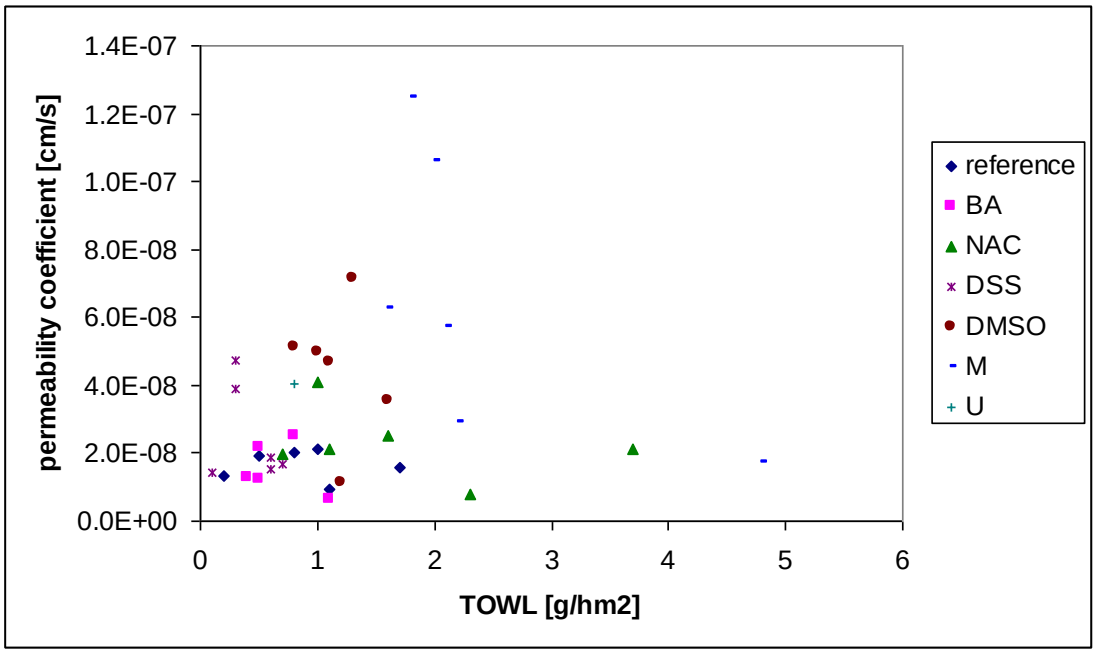


Fig. 11 Correlation between permeability coefficient and TOWL of dry nails 24h after experiments.

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 (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. Kisco 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.

C.1.3.5. 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 from 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

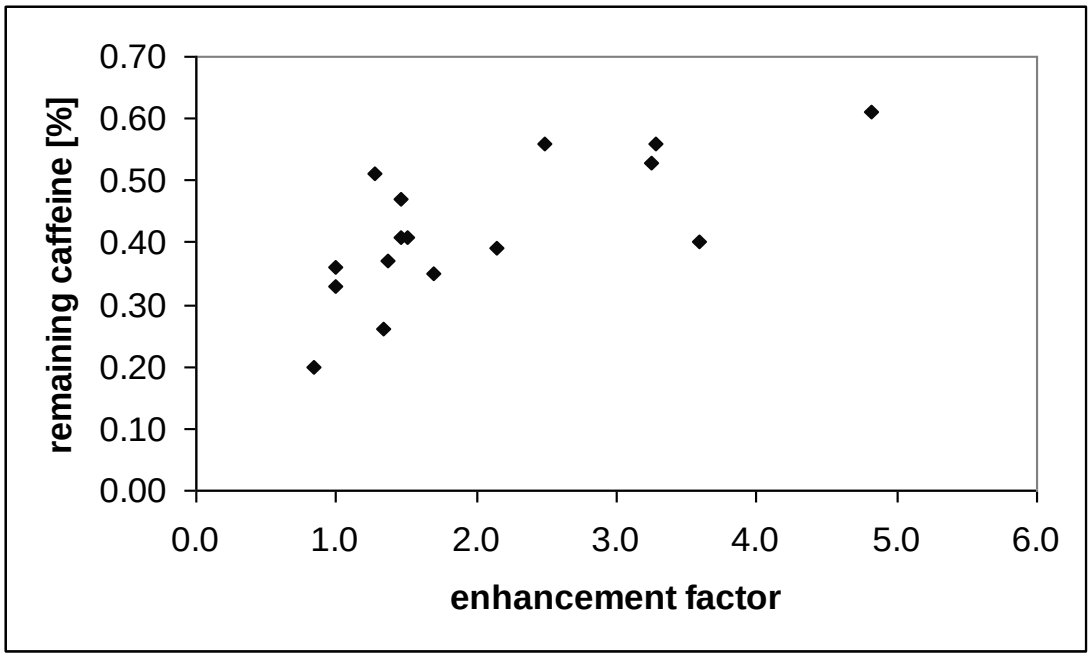


Fig. 12 Correlation between enhancement factor and remaining caffeine in nail (n=3).

C.1.4. 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.

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C.2. Permeation studies of novel terbinafine formulations containing hydrophobins through human nails *in vitro*

C.2.1. 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/m²) is only half that is needed to cut them longitudinally (6 kJ/m²), 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 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 III 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), (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.

C.2.2. Materials and methods

C.2.2.1. 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.

C.2.2.2. 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 hours.

C.2.2.3. 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 one hour 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 three 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.

C.2.2.4. Influence of ethanol content in the formulation 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 one hour, 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 one hour 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 minutes 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 one hour, 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.

C.2.2.5. 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 hours. Results for coefficient of variation were in the range of 3.99 to 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.

C.2.2.6. 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 six 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 reference, a preliminary test for the equality of variances was performed prior t-test.

C.2.2.7. 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.

C.2.3. Results and discussion

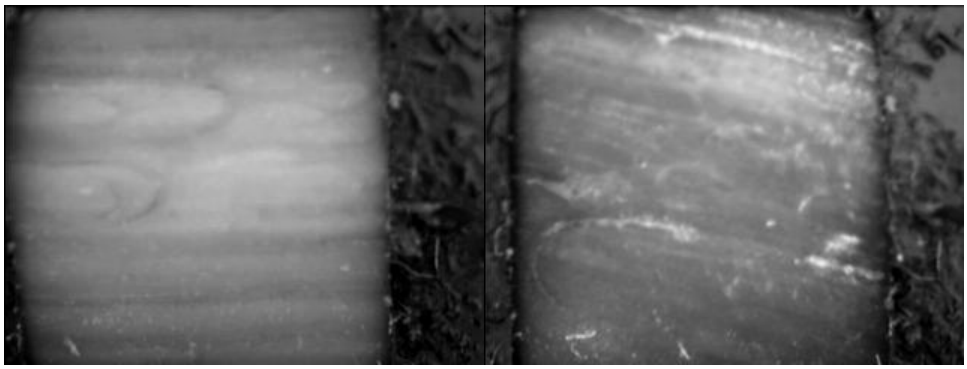
C.2.3.1. Characterization of the nail samples

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 one hour, 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

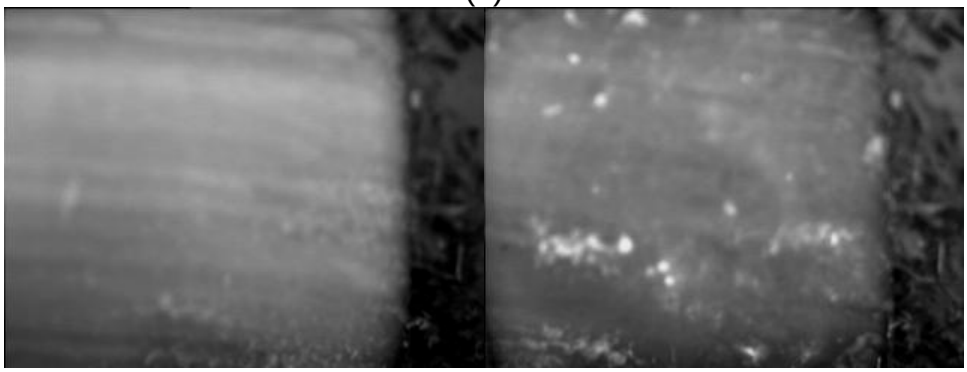
plates. This slight increase of weight and decrease or no change of TOWL values in more than half of the samples could be due to the adsorption of hydrophobins on the nail surface. If this hypothesis can be confirmed then hydrophobins could act as enhancers and as protectors at the same time. Visioscan 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.

Table 1 TOWL of dry nails before and after permeation experiments

TOWL (g/hm²) before experiment		TOWL (g/hm²) 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



(1)



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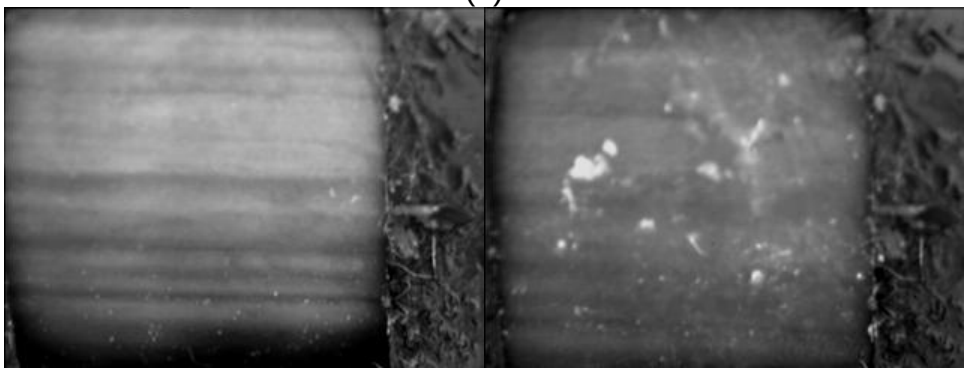

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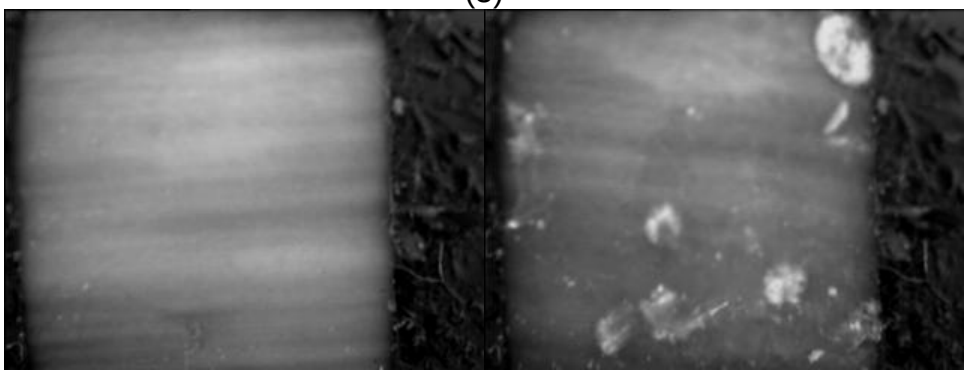
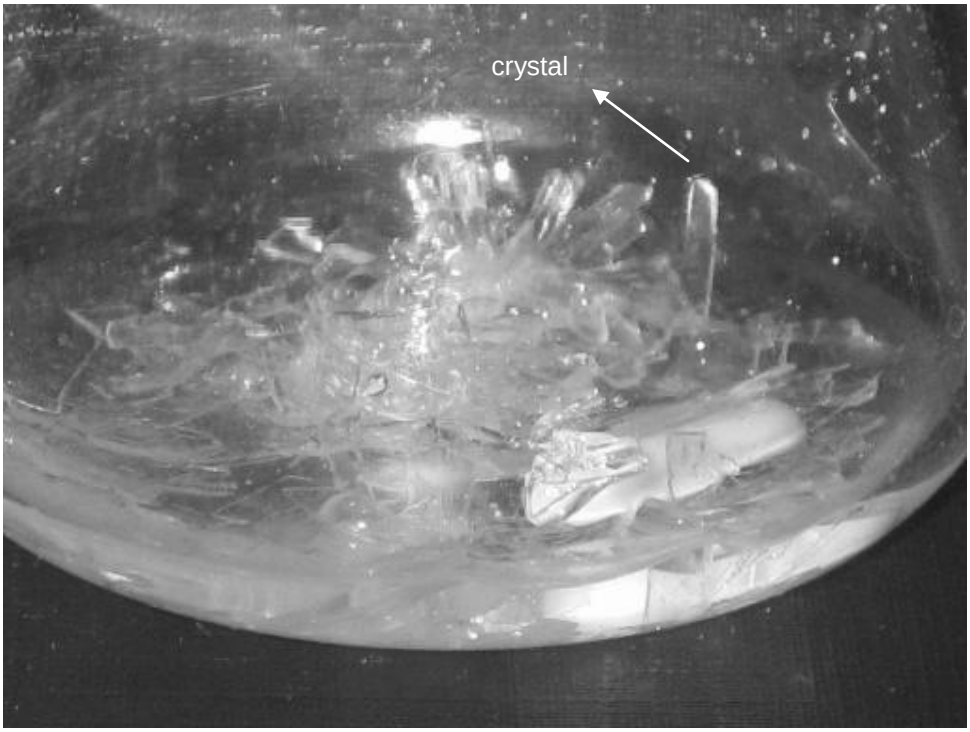


(4)

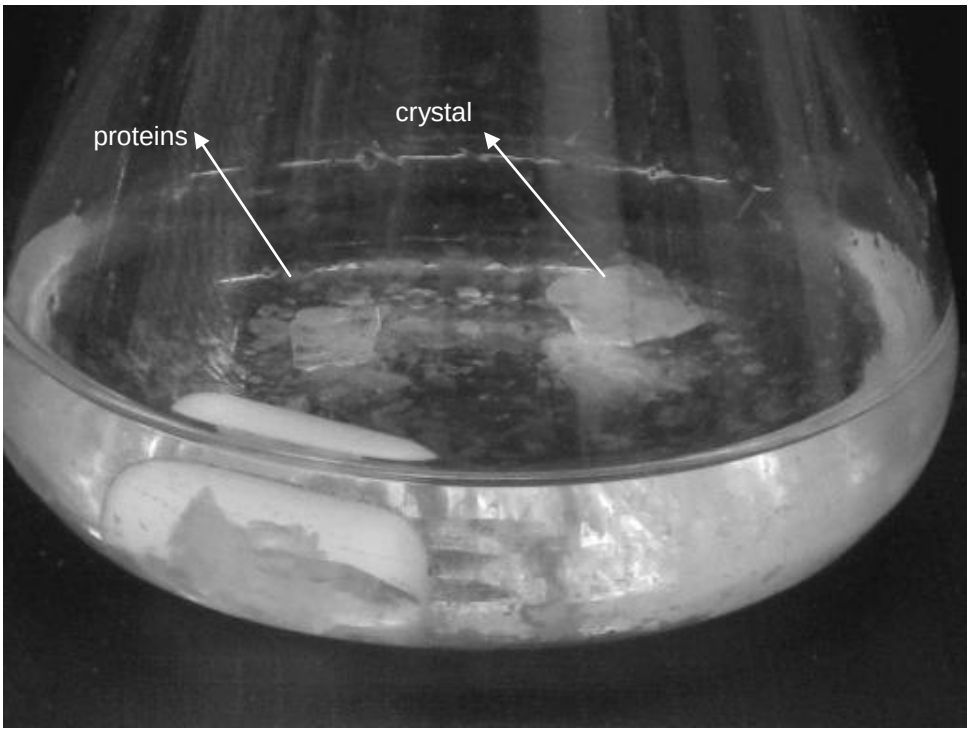
Fig. 1 Visioscan images of dry nail surfaces before (left) and 24 h after (right) the experiment (1) with reference, (2) with hydrophobin A, (3) with hydrophobin B, (4) with hydrophobin C.

C.2.3.2. 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 two 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 six 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 two to six 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.



(1)



(2)

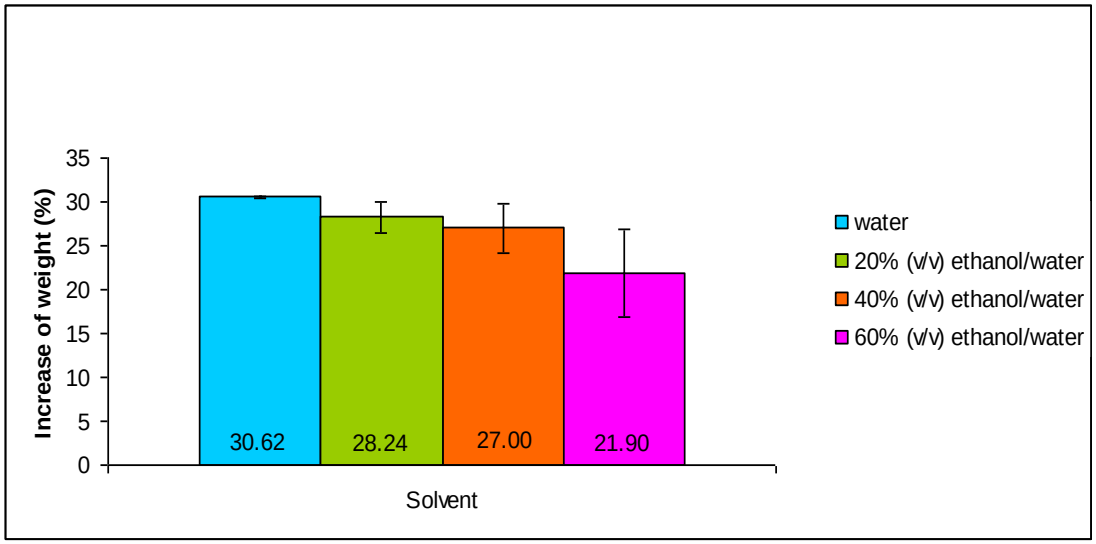
Fig. 2 Formulations after 6 months of storage at room temperature in a dark place (1) reference, (2) with hydrophobin A.

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 hours. 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 applied vehicles onto the skin of volunteers at a concentration equal to ¼ 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 C.2.3.4.).

C.2.3.3. 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 C.2.3.1. This could be due to the number of samples investigated 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 one hour 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 six 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 one hour. The drying process was fast and 50% of all absorbed solutions evaporated between 10 and 30 minutes (marked in Fig. 5). Interesting was that after 24 hours 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 minutes, but then the process slowed down. This finding illuminated the data from Section C.2.3.1 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 one hour 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 hours 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 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 hours of incubation in water of 26.9 ± 4.8% and in 70% (v/v) ethanol of 22.2 ± 2.5% (Khengar et al., 2007). Increase in weight (n=3) after 24 hours of incubation in water was found to be 32.01 ± 1.88% and in 60% (v/v) ethanol/water 19.86 ± 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.



(1)

Fig. 3 (1) Average (n=3) of the nail weight increase (%) with standard deviations after immersion in various solvents for one hour in the first set of exp.

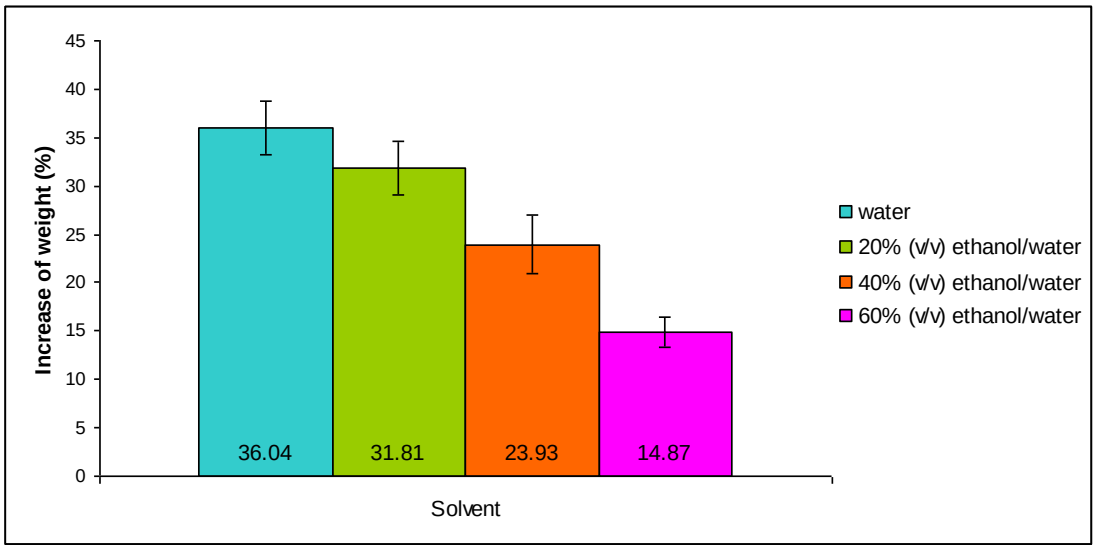


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Fig. 3 (2) Average (n=3) of the nail weight increase (%) with standard deviations after immersion in various solvents for one hour in the second set of exp.

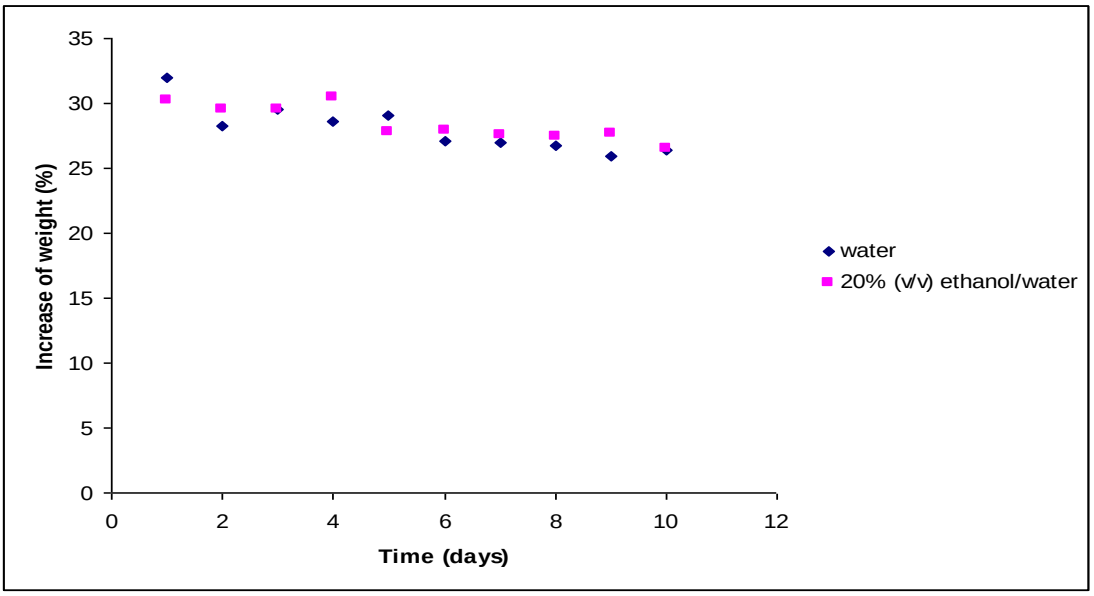

Fig. 4 Average (n=3) of the nail weight increase (%) in water and 20% (v/v) ethanol/water during 10 days.

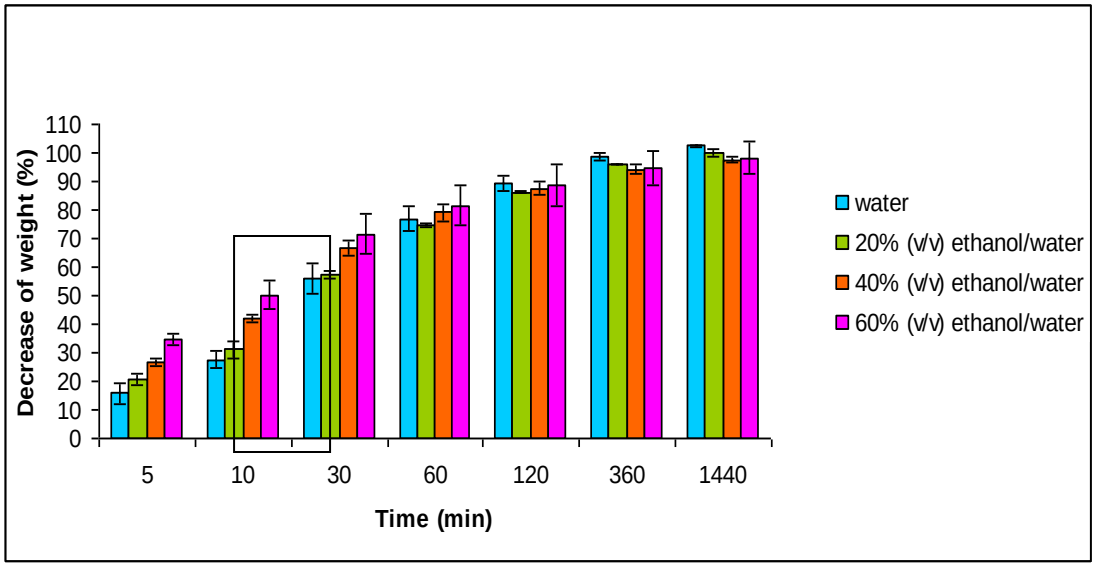

Fig. 5 Average (n=3) of the nail weight decrease (%) with standard deviations during the drying period.

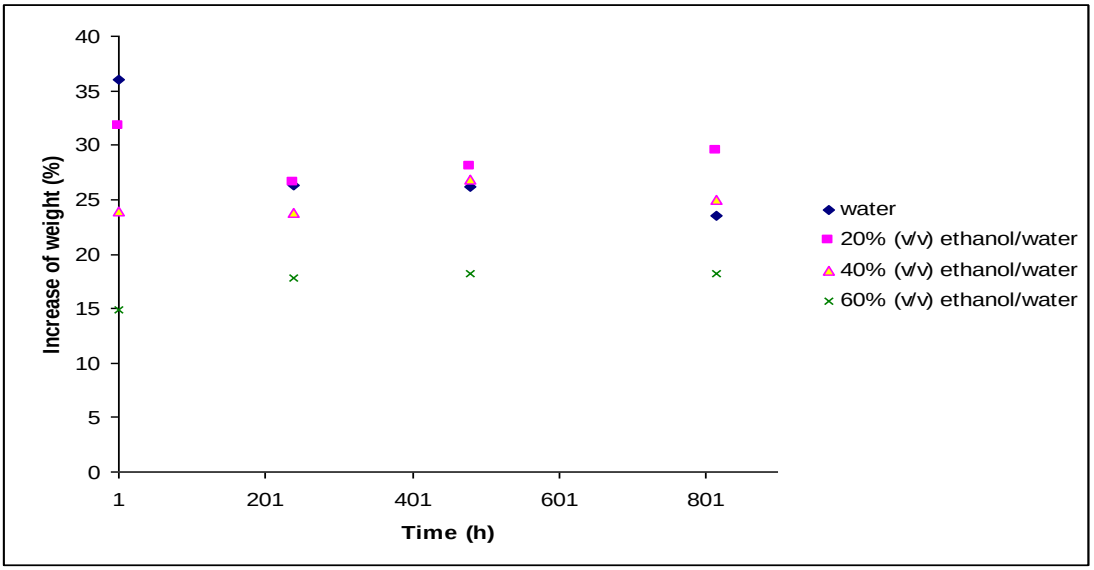

Fig. 6 Average (n=3) of the nail weight increase (%) in various solvents at four time points: 1, 240, 480, and 816 hours.

C.2.3.4. 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, 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.

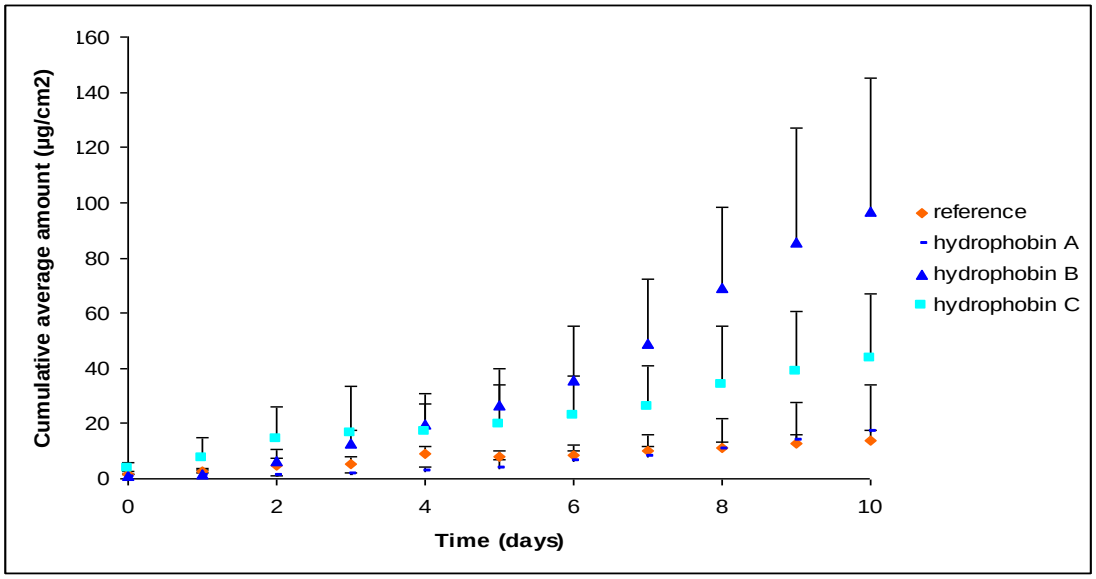

Fig. 7 Permeation profiles of terbinafine formulations (n=3) with standard deviations.

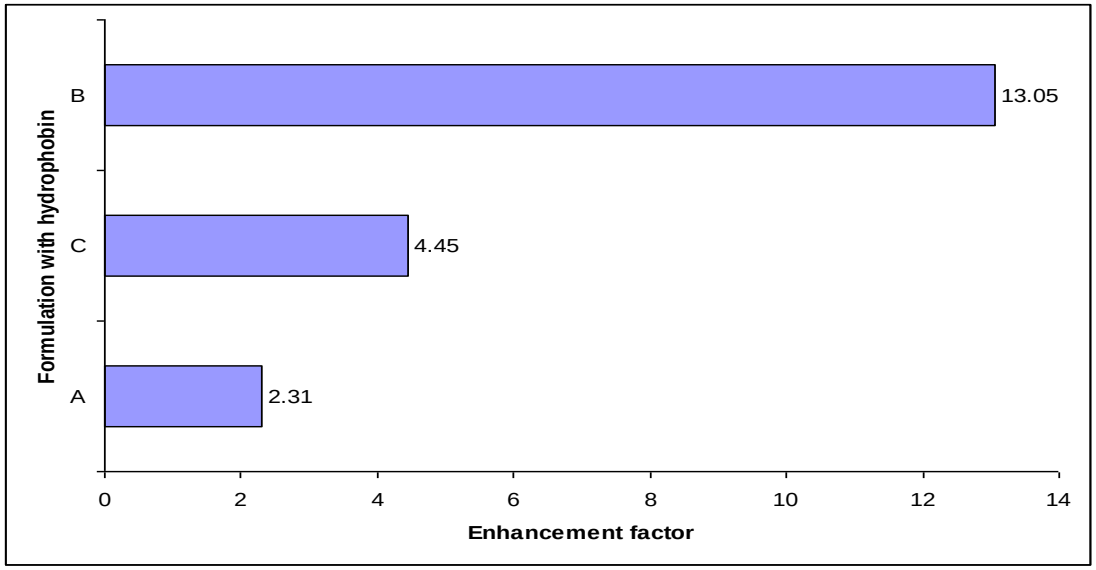

Fig. 8 Enhancement factors calculated to the reference, where the formulation with hydrophobin B shows the highest enhancement factor of 13.05 and formulation with hydrophobin A the lowest enhancement factor of 2.31.

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.

C.2.3.5. 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 four 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 (SD)

Formulation	Remaining terbinafine (%)	SD
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

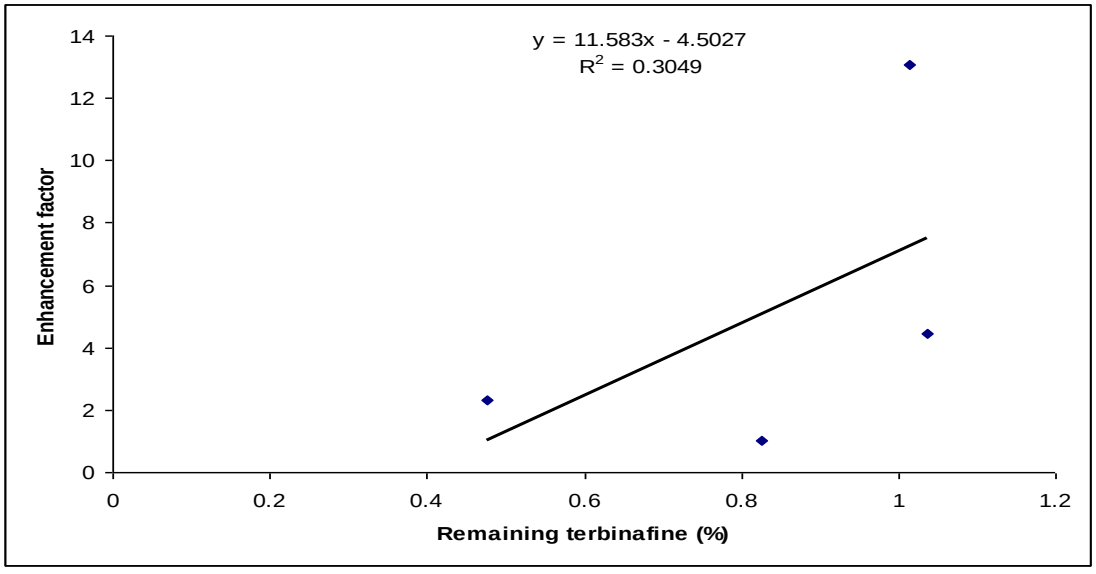

Fig. 9 Correlation between enhancement factor and remaining terbinafine in the nail, where 1 is attributed to the enhancement factor of reference solution.

C.2.3.6. Toxicity issue 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 (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.

C.2.3.7. 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.

C.2.4. 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 hours. 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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D. Conclusion and future perspectives

During this study, some new properties of the nails and used substances have been illuminated or known confirmed. Nails` affinity towards absorption of water or the stability of caffeine molecule was expected. The stability tests showed that the formulations were stable or negligibly instable. It was determined that chemical enhancing substances which partially destroyed the nail plate, such as methanol or DMSO, increased caffeine flux. Although NAC theoretically should cleavage disulfide bridges in keratin, it failed due to instability in the formulation. Hydrophobins were identified as transungual enhancers with a unique property to augment permeation of hydrophilic as well as hydrophobic drugs. Keratin from the nail plate accepts water and swells, but no significant difference was found in swelling behavior when nail samples were immersed in 20% (v/v) ethanol/water solution. The nail samples in mixtures with higher portion of ethanol needed a longer time to achieve full hydration status in a defined mixture compared to water.

According to the findings from this study and the properties of the nails, future vision how to increase permeability coefficient of drugs through the nail plate may go in several directions. Physical technique which consists of creation of cavities in the nail plate generated by laser showed promising enhancement (data not shown). Another physical method could be the use of transparent silicon patches on the finger tips which would provide occlusion effect and therefore limit evaporation of water in the nail resulting in nail softening and loosening its barrier properties. Chemical enhancers such as hydrophobins with a nondestructive influence on the nail plate may be used in a development of various pharmaceutical forms for application on the nail. A creation of novel materials which would imitate the water-capturing surface of the desert beetle could contribute to hydration of the nail plate, since the nail plate has ability to intake water from its gas state at high relative humidity (experiment in exsiccator). The superhydrophilic property of titanium dioxide (TiO_2), i.e., the formation of nearly 0° contact angle between the water drop and TiO_2 surface irradiated by the sun light could be utilized for the development of novel formulations for topical application on human nails. Finally, a combination of proposed modalities would have a greater impact in

overcoming the nail plate barrier and make it accessible to drugs which final destination is systemic circulation.

E. Appendix

Additional validation procedures, experiments, and data of the published work in Section C. are presented in this section.

This Section E. is divided into two parts, E.1. and E.2.

Part E.1. contains additional data regarding the first publication in 2010: Investigation of different formulations for drug delivery through the nail plate. Int. J. Pharm. 386, 185-194.

Part E.2. contains additional data regarding the second publication in 2010: Permeation studies of novel terbinafine formulations containing hydrophobins through human nails *in vitro*. Int. J. Pharm. 397, 67-76.

E.1. Part I

E.1.1. Preparation procedure of corpuses

The standard procedure for preparation of corpuses at The Institute of Anatomy and Cell Biology, Freiburg is as follows:

A conservation solution (3% formaldehyde, thymol, and glycerin) is given with a pressure of 1.5 bar into A.femuralis, usually 20 L inside the corpus lying on the table, but it depends of the weight of the body. After the fixation time, corpus is put into preservation object. Inside, the bodies are sprinkled by the same solution without thymol and glycerin. After 3 months period the corpuses are ready for preparation for students' anatomy courses.

E.1.2. Raman spectrum of 4% formaldehyde solution

The spectrum in Fig. 1 is added to a fitting library to calculate the content as described by Caspers et al. (2001).

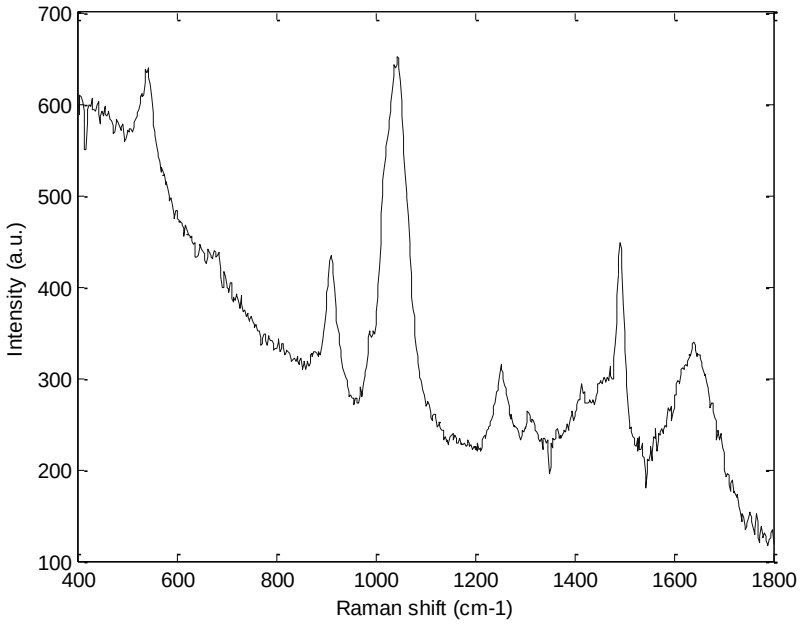


Fig. 1. A Raman spectrum taken of a 4% formaldehyde solution, with a typical peak at 1041 cm^{-1} and 1492 cm^{-1}.

E.1.3. Characterization of nail samples

E.1.3.1. Transonychial water loss (TOWL)

Transonychial water loss (TOWL), also called transungual water loss (TUWL), was measured by Tewameter TM 210. Measurements were performed before and after experiments at constant distance of 7.78 mm (Table 1) between the nail samples and measuring sonde, which presents the thickness of gum ring. A covering box from plexiglass was used to avoid the influence of environment since it is known that measurement can be affected by ambient air movements, room temperature, and humidity (Murdan et al., 2008). The duration of each measurement was 5 minutes, because during that time constant conditions were obtained. The values for temperature

(T°C), relative humidity (%), and water vapor partial pressure (mbar) under the sensors were recorded 2 minutes after the beginning of each measurement.

Table 1 Thickness of the gum ring at extension

Thickness (mm)					Average (mm)
7.89	7.85	7.72	7.63	7.81	7.78

E.1.3.1.1. Evaluation of the reproducibility

Each time before the measurements of TOWL microenvironment, which presents the conditions inside the covering box, was recorded in triplicate. If the mean value is within the limits of the calibration status of the instrument, measurement can be carried out. Otherwise, the instrument has to be calibrated.

E.1.3.2. Determination of weight and thickness of cadaver nail samples

The nail samples were characterized by measuring their weight (Analitical balance, type AT261, Mettlero Toledo, Switzerland) and thickness (Digit cal SI, TESA S.A., Renens, Switzerland) during the preparation process prior and after the permeation experiments. The wet nail samples dry fast, which can reflect as differences in their weight. Therefore, the first value after the stabilization of the balance was taken. Thickness of the nail samples before cutting was measured in triplicate for each edge and a mean value calculated. Thickness of cut, round nails was recorded as a mean value of four measurements at different points. It was observed that measurement of the thickness particularly of the wet nails depends on applied force, length of area implied by micrometer, and the researcher who performs measurement. Thus, the thickness is considered as less reliable parameter in the characterization of the nail samples.

E.1.3.3. Correlation between nail weight and thickness

It was found linear correlation between the weight and the thickness of nail samples (evaluation exclude experiments with hydrophobins), where correlation coefficient indicates linearity.

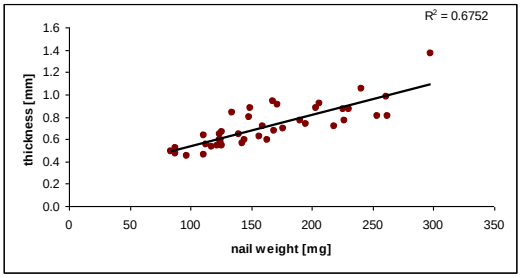


Fig.2

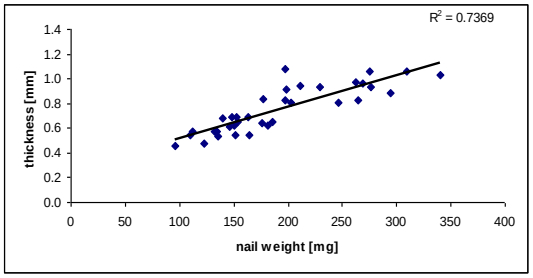


Fig.3

Fig. 2 Correlation between weight and thickness of dry nail samples before the permeation experiment.

Fig. 3 Correlation between weight and thickness of wet nail samples before the permeation experiment.

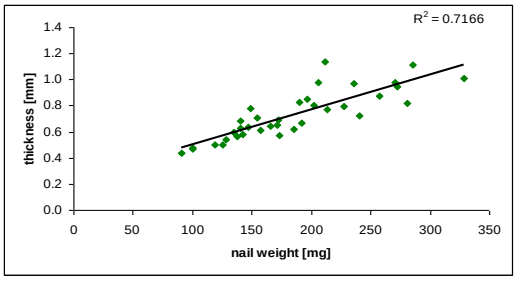


Fig.4

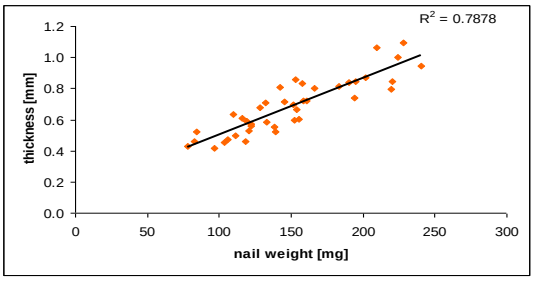


Fig.5

Fig. 4 Correlation between weight and thickness of wet nail samples after the permeation experiment.

Fig. 5 Correlation between weight and thickness of dry nail samples after the permeation experiment.

E.1.3.4. Correlation between TOWL and weight or thickness of nail samples

Reported data suggest a correlation between nail plate thickness and TOWL in individuals during *in vivo* measurements. Murdan et al. (2008) noticed strong correlation (-0.83 and -0.92) for two individuals, while Dutet and Delgado-Charro (2009) reported relationship between two variables for each volunteer of 0.55, 0.53, and 0.63. Also, TOWL values for the same digits of the opposite hands of the same individual are slightly different, which brings a conclusion that the opposite digits should not be used as controls for one another and that other factors such as the composition of the nail plate influences TOWL (Murdan et al., 2008).

The correlation between TOWL and weight or thickness of nail samples has been investigated in four different states during the characterization process of the cadaver nail samples: dry and wet before experiment, and wet and dry after the permeation experiment. The observed population of nail samples does not include the nail samples from the tests with hydrophobins and the test with urea as possible enhancer. No correlation between TOWL and weight or thickness of samples was found.

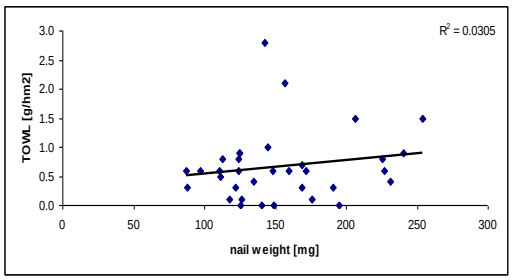


Fig. 6

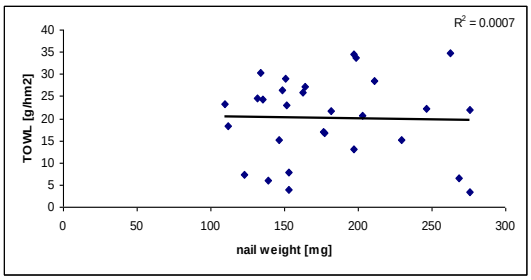


Fig. 7

Fig. 6 Correlation between weight and TOWL of dry nail samples before the permeation experiment.

Fig. 7 Correlation between weight and TOWL of wet nail samples before the permeation experiment.

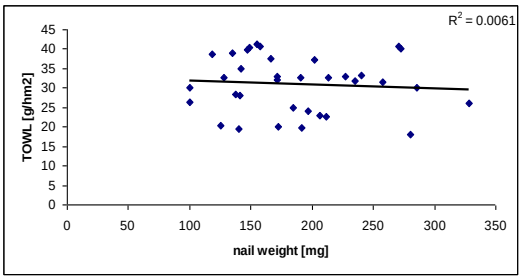


Fig. 8

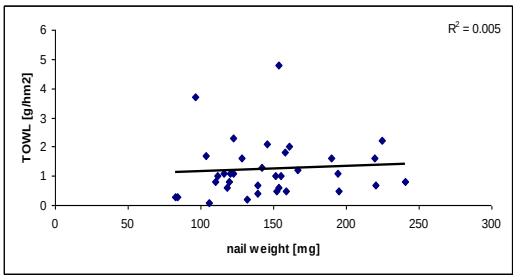


Fig. 9

Fig. 8 Correlation between weight and TOWL of wet nail samples after the permeation experiment.

Fig. 9 Correlation between weight and TOWL of dry nail samples after the permeation experiment.

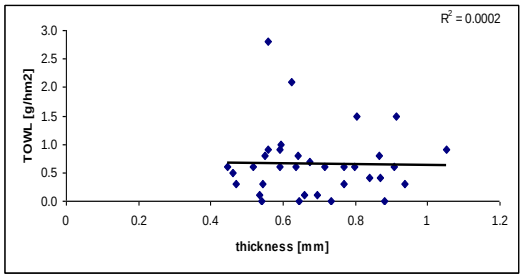


Fig. 10

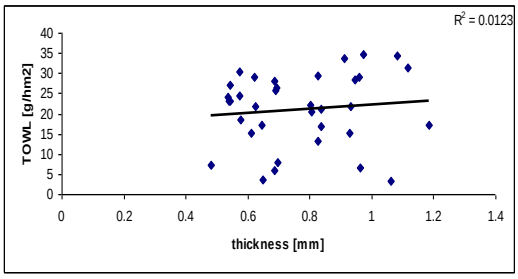


Fig. 11

Fig. 10 Correlation between thickness and TOWL of dry nail samples before the permeation experiment.

Fig. 11 Correlation between thickness and TOWL of wet nail samples before the permeation experiment.

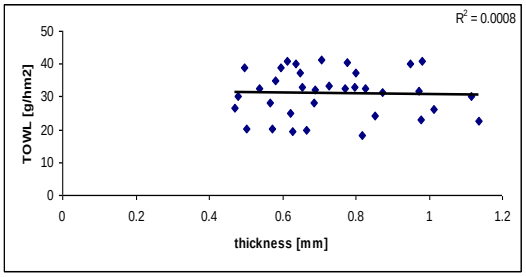


Fig. 12

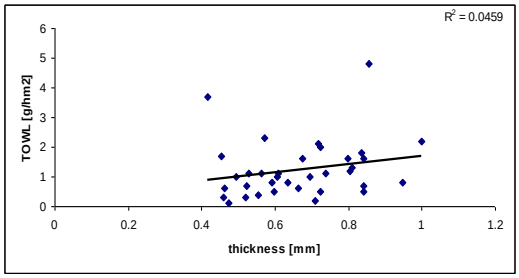


Fig. 13

Fig. 12 Correlation between thickness and TOWL of wet nail samples after the permeation experiment.

Fig. 13 Correlation between thickness and TOWL of dry nail samples after the permeation experiment.

E.1.4. Characterization of the formulations

E.1.4.1. Preparation of the formulations

Formulations were prepared in double distilled and filtrated water and in 20% (v/v) ethanol/water at room temperature. All formulations contained caffeine in the concentration of 2% (w/v), since the saturated caffeine concentration in PBS is 21.6 mg/ml at room temperature (Khengar et al., 2007). Possible enhancers were added in the concentration of 5% (w/v) or (v/v) in the case of liquids. Exceptions were DSS and hydrophobins which were added in the concentrations 1% (w/v) and 0.1% (w/v), respectively.

E.1.4.2. Viscosity measurements

Viscosity of the formulations (except the formulations containing hydrophobins) was measured at 25°C and at 32°C in triplicate. The temperature was kept in the range of ±1°C by circulated water from the water bath connected to the measuring cylinder head. The used sensor was NV and the torque constants were set for the measurement of very low viscosity. The mean values of viscosity above shear rate of 1,500 1/s in ramp curve were used for the evaluation. The ramp curve gives an insight in flow behavior of

the material by increasing and afterwards decreasing continuously shear rate during the measurement. Here, the tests were performed from 0 to 3,000 1/s during 5 minutes in overall measured cycle. A control measurement was done in time curve module, where the shear rate was kept constant at 2,500 1/s.

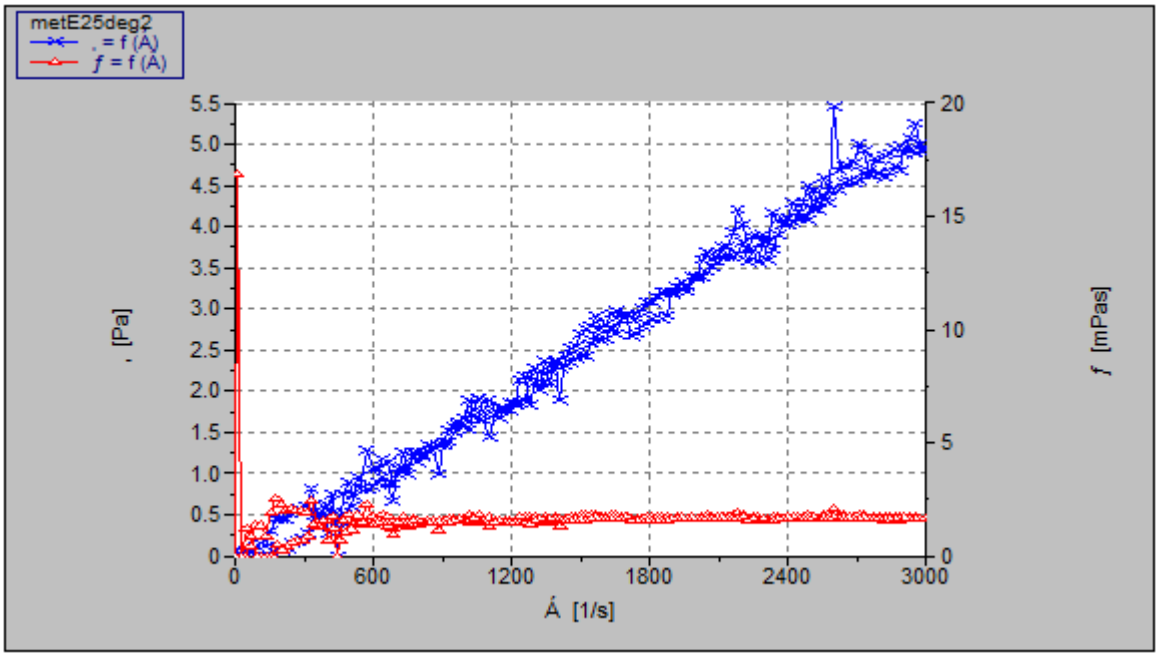


Fig. 14 Ramp curve of the formulation containing 5% (v/v) methanol in water obtained at 25°C. The viscosity is independent of the shear rate (x-axis) and the rate of the deformation is in proportion to the applied force (y-axis), which indicates Newtonian behavior of the formulation (Markesich et al., 1995).

E.1.4.3. Surface tension and contact angle measurements

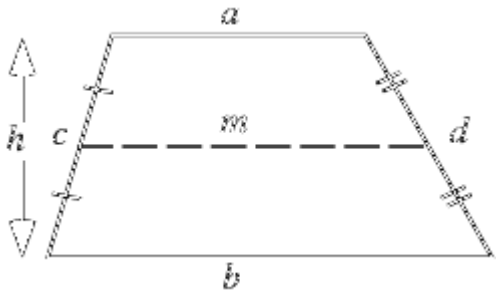
In order to fulfill contact angle measurements it was necessary to determine the surface tension of solutions. For the measurement of surface tension of the formulations (except formulations containing hydrophobins) Krüss standard platinum plate was used with following specifications: width 19.9 mm, thickness 0.2 mm, and height 10 mm. The measurement procedure included immersion of the platinum plate with detection speed of 6 mm/min, detection sensitivity of 0.01 g, and maximum measured time of 120s. The

glass vessel was filed with the tested formulation and surface tension was recorded in triplicate as a function of time.

The nail samples were left over night at room temperature in a room with a controlled temperature of 22°C. Hereafter, they were cleaned and cut in the shape of rectangle by scissors. Length, height, and thickness at five various points were measured, because it is required that sample has constant perimeter over a portion of its immersion depth. The wetted length presents perimeter of a sample given as double length plus double average thickness. No difference between wetted lengths was found (analyzed by ANOVA and post-hoc Tukey test in SPSS Statistics 17.0 software). The samples were connected to the sensitive balance and immersed in the direction of nail growth. The measuring procedure included detection speed of 6 mm/min, detection sensitivity of 0.01 g, measuring speed of 3 mm/min, maximum immersion depth of 4 mm, minimum immersion depth of 1 mm, with a delay at maximum and minimum immersion of 1 s. Regression was calculated from 1.9 mm to 4 mm length by choosing the values nearest to 1.

E.1.4.4. Capillary constant measurements

Immersing the nail samples in different liquids three main processes occur: wetting of the sample, which depends of the wetting properties of a liquid, absorption of a liquid by sample, and extraction of nails` constituents. In order to allow all processes to occur, the capillary constant measurement on Krüss tensiometer K100 was performed and no-unit values given as a regression on a graph, which indicates weight increase of the sample as a function of time, were recorded. Prior the measurements two things are taken into account: 1) better wetting properties of a liquid would influence higher values and would be the main driving force of a liquid, 2) exposed surface area of samples should be as constant as possible. Therefore, the samples were cut by scissors in a square shape, but due to the difficulties in achieving equal sides by cutting, a surface area of trapezoid was taken for comparison and was calculated as follows:



$$P = m \cdot h = \frac{a+b}{2} \cdot h$$ Equation 1

Fig. 15 Trapezoid.

The ventral side of prepared samples was glued to metal pin which was connected to the sensitive balance and the dorsal side was exposed to the liquids. Measuring procedure was set at detection speed of 6 mm/min, detection sensitivity of 0.001 g, maximum measuring time of 300 s, and linear acquisition.

E.1.4.5. Stability studies

The stability of caffeine within the formulations (excluding formulations with hydrophobins) was investigated. The caffeine concentrations were observed over a longer period of time under different storage conditions. Additionally, pH was measured parallel to the concentration measurements. In order to investigate precision of pH measurements, pH was determined in a randomly chosen formulation with 5% (w/v) urea in water and standard deviation (SD) was calculated (Table 2).

Table 2 pH measurements

Measurement	pH	Temperature (°C)
1	7.20	25.1
2	6.73	25.0
3	6.59	25.0
4	6.33	25.0
5	6.39	25.1
6	6.33	25.1
7	6.34	25.1
8	6.26	25.1
9	7.80	24.6
10	7.09	24.9
11	6.76	24.9
12	6.49	24.9
Mean	6.69	
SD	0.49	

The formulations were stored in a plastic tube at room temperature (24°C) in a dark place and in the fridge (8°C) up to 90 days, while concentrations and pH were measured at time zero, after 7, 30, and 90 days. Parallel, the formulations were stored in glass vessels at 32°C using a water bath, while concentrations and pH were measured at time zero, after 24 hours (41hours for pH) and after 6 days. Therefore, the conditions during the permeation tests were imitated.

For all pH measurements, a standard mistake of ± 0.49 was established. For the concentration measurements it was defined that instability of caffeine occurred when the concentration of caffeine after 90 days (6 days for storage at 32°C) differ more than ± 1.2 mg/ml from the concentration at time zero. In order to perform measurements, one dilution was prepared, which was measured three times.

Table 3 Results of the stability of caffeine in different formulations after 6 days of storage at 32°C

Formulation	Outcome
Reference w	stable
Reference e	stable
BA w	stable
BA e	instable
DMSO w	stable
DMSO e	stable
DSS w	stable
DSS e	stable
M w	instable
M e	stable
NAC w	stable
NAC e	stable
U w	instable
U e	stable

E.1.5. Permeation studies

E.1.5.1. Franz diffusion cell

Permeability experiments on nail samples are either performed on nail pieces with a small diffusion area of approximately 0.05 cm² (Kobayashi et al., 1998; Kobayashi et al., 1999; Kobayashi et al., 2004; Brown et al., 2009) or on whole cadaver nail samples with the exposed dosing area of 0.78 cm² (Hui et al. 2007). Some of these are performed on standard Franz diffusion cell which consists of a donor and a receiver compartment, filled with an acceptor solution, separated by the membrane, in this case nail sample. Others are performed according to a methodology of a small cotton ball under the nail plate wetted with normal saline to provide moisture to the nail plate, as it was showed in the work of Hui and coworkers (2007).

A set of 6 Franz diffusion cells, together with a magnetic plate and 6 magnetic stirrers was received from the TLT company with which this project initially started. The cells were designed to enable assembly of whole human cadaver nails between the donor and acceptor compartment, to ensure uniformity of acceptor solution by mixing it with magnetic stirrer, and to maintained constant temperature during the permeation experiment.

E.1.5.2. Determination of diffusion area

The diameter of three gum rings from the experiment set up was measured at five different points and area of the circle was calculated as follows:

$$P = \left(\frac{R}{2}\right)^2 \pi \qquad \text{Equation 2}$$

Table 4 Measurements of diameter of gum rings from the side which comes in the contact with a nail sample

$R1$ (mm)	$R2$ (mm)	$R3$ (mm)	$R4$ (mm)	$R5$ (mm)	$\overline{R}$ (mm)	P (cm²)
9.98	10.00	10.05	10.04	10.02	10.02	0.788
10.00	10.04	10.00	9.99	9.98	10.00	0.785
9.98	10.01	10.01	10.05	10.05	10.00	0.785

E.1.5.3. Composition of buffer solution

In order to mimic physiological conditions, phosphate buffer saline (PBS) was chosen for buffer system in the acceptor cells. Measured osmolality and pH of PBS had values of 293±3 mOsm/kg (Osmomat 030 cryoscopic osmometer) and pH=7.4 (Metrohm 827 pH-meter), which corresponds to physiological range from 282 to 303 mOsm/kg (Prakash et al., 2003).

Table 5 Composition of phosphate buffer saline (PBS)

Supstance	Weight (g)	Function
NaCl	8.00	isotonicity
KCl	0.20	isotonicity
Na_2HPO_4 x $12H_2O$	3.63	buffer system
KH_2PO_4	0.24	buffer system
NaN_3	0.10	preservative

The substances were dissolved in double distilled and filtrated water and pH was adjusted to 7.4 with 1M HCl or 1M NaOH.

E.1.5.4. UV analysis

UV spectrophotometer measures the concentration of a substance according to the Lambert-Beer law:

$$A = \log\left(\frac{I_0}{I}\right) = \alpha \cdot l \cdot c \qquad \text{Equation 3}$$

where A is absorbance, I_0 is intensity of the incident light, I is intensity of the light after passing through the sample in the cuvette, α represents molar absorptivity, l is width of the cuvette, and c presents concentration of a substance. Possible interference of the substances used as enhancers with caffeine has been checked in Clarke's index (1986), as well as the wavelength for the highest absorbance (λ_{max}) of caffeine. With a wavelength scan the λ_{max} of 273 nm has been confirmed and used in all absorption measurements. PBS has been used as blank and for dilutions. One standard curve has been used during the whole study (Fig.16).

Table 6 Standard curve measurements

Concentration (µg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Mean absorbance	SD
44.73	2.056	2.055	2.055	2.055	0.001
39.76	1.890	1.893	1.890	1.891	0.002
19.88	0.990	0.986	0.993	0.990	0.004
14.91	0.732	0.735	0.729	0.732	0.003
7.46	0.373	0.358	0.373	0.368	0.009
4.97	0.264	0.253	0.250	0.256	0.007
2.49	0.125	0.117	0.108	0.117	0.009
0.99	0.045	0.034	0.032	0.037	0.007

$$y = 0.0465x + 0.0196$$ Equation 4

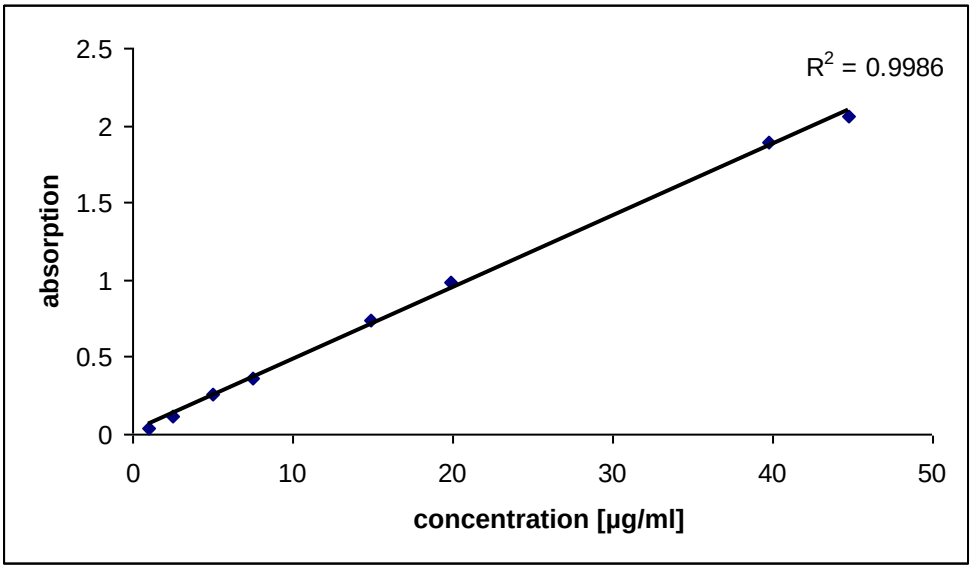


Fig. 16 Standard curve for caffeine created in Excel.

E.1.5. 5. Evaluation of ethanol influence on UV measurements

To evaluate possible influence of ethanol on UV measurements a cross-study was performed. Standard curves of caffeine in PBS and caffeine in 20% (v/v) ethanol/PBS solution were measured with PBS and 20% (v/v) ethanol/PBS solution as blank. Afterwards, obtained four different equations of standard curve were implemented on three randomly chosen permeation experiments for calculation of permeability coefficient, i.e. on experiments with dimethyl sulfoxide, urea, and N-acetyl-L-cysteine as possible enhancers. The difference between permeation coefficients were analyzed by one-way ANOVA statistical software spss and no difference was found. Thus, it was concluded that the ethanol portion in the formulations did not influence the absorbance. This conclusion is applicable for the measurements in stability studies and in permeation studies, as well.

E.1.6. Pulverization of nail samples

A Freezer/Mill 6750 was used for pulverization of the nail samples in order to detect remaining caffeine in the samples after the permeation studies. It operates on the principle of cooling a sample by liquid nitrogen and in such a way making it suitable for grind process. A nail sample was placed in a plastic tube together with a magnetic coil which pulverizes the frozen nail by moving it back and forth at high speed. The settings were adjusted to:

P Cool T3 (freezing time)	10 min
Run T1 (milling time)	2 min
Cycles	1

The powder was dispersed in the phosphate buffer and transferred in a graduated flask of 25 ml and filled with the buffer till mark. The caffeine was extracted using an ultrasonic bath for 5 min. Afterwards, powder separation from the solution was achieved by centrifuge operating at room temperature for 5 min at 10,000 min^{-1}. The supernatants were measured by UV spectrophotometer.

E.2. Part II

E.1.1. Preparation of the formulations

The choice of the solvent mixture was performed through series of separated tests. The aim was to find the lowest amount of ethanol needed to dissolve the whole 10% (w/v) portion of terbinafine hydrochloride and therefore achieve a stable formulation. The amount of 1g of terbinafine was weighted in a flask and 4ml of ethanol added. The flask was sealed and covered with aluminium foil, avoiding influence of light. After waiting time of 10 min and 10 min shaking by hand, it was applied ultrasonic bath for 2 min and clear solution appeared. Subsequently 6 ml of water was added and at the junction of the two solvents a milky transition was observed. After 1h the formulation remained milky. It was treated by ultrasonic bath in order to improve solubility of the drug, but precipitation increased. In the trial with 50% (v/v) ethanol/water mixture, although shaking by hand was applied, after 3 days of storage crystals appeared (Figs. 17 and 18).

Fig.17

Fig.18

Fig. 17 10% (w/v) terbinafine hydrochloride in 40% (v/v) ethanol/water (left) and 10% (w/v) terbinafine hydrochloride in 50% (v/v) ethanol/water (right) after 1 day of storage at room temperature in a dark place.

Fig. 18 Appearance of 10% terbinafine hydrochloride in 50% (v/v) ethanol/water solution after 3 days of storage at room temperature in a dark place.

Therefore, the solvent mixture of 60% (v/v) ethanol/water was chosen. The drug and hydrophobins in the concentration of 0.1% (w/v) were weighted in the glass flask covered with aluminium foil prior to the solvents addition and minimum five hours before the experiment, and were stirred by magnetic stirrer.

E.2.2. Solubility of terbinafine

In order to determine saturation concentration of terbinafine in 60% (v/v) ethanol/water mixture 7.00195 g of terbinafine hydrochloride was added in 10ml 60% (v/v) ethanol/water mixture in glass vial covered by aluminium foil and closed with a gum lid to prevent evaporation. Saturated solution was prepared by stirring an excess of the drug at 400 rpm for 48 hours at room temperature of 25°C. It was taken 200 µl of the sample and centrifuged at 10,000 min^{-1} for 5 min and afterwards 20 µl was diluted in 200 ml of 60% ethanol/water solution which was analyzed by HPLC. The average of last three points resulted in saturation concentration of 191.63 mg/ml.

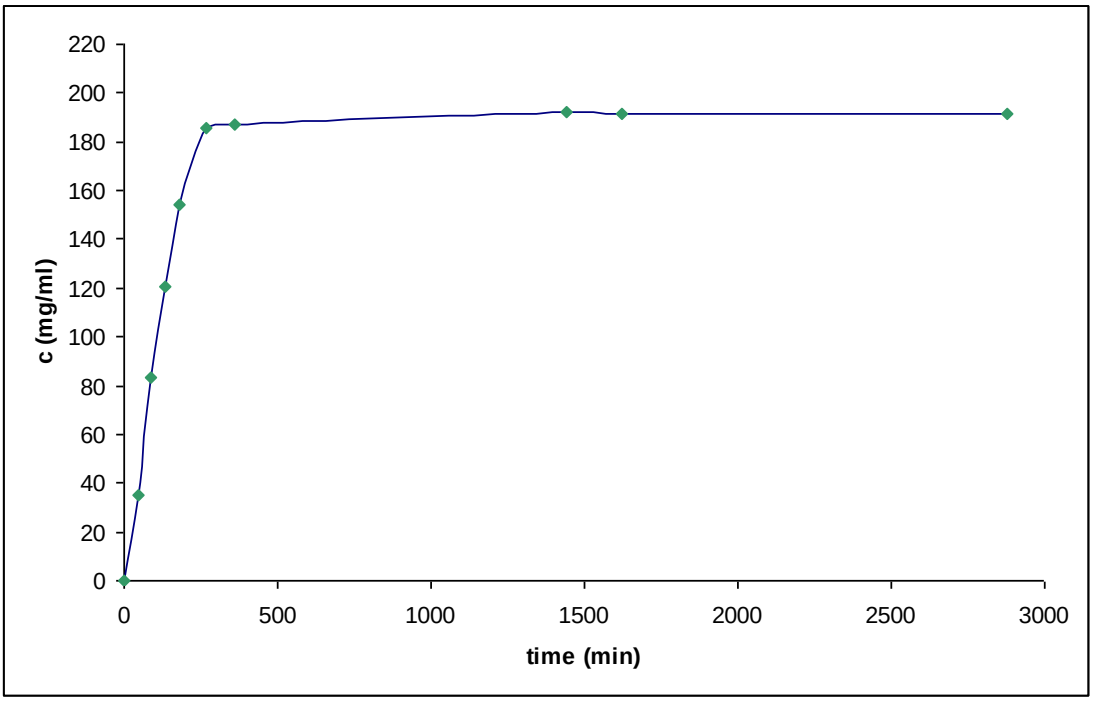


Fig. 19 Terbinafine solubility in 60% (v/v) ethanol/water solution.

E.2.3. Validation of HPLC analytical method

The mother solution was prepared in dimethyl sulfoxide and contained terbinafine in the same concentration of 10% (w/v) as in the tested formulations. The amount of 10.12 mg of terbinafine was weighted in eppendorf tube and 1 ml of DMSO added. After 1 hour of dissolving time, standards were prepared in 60% (v/v) ethanol/water and three runs were done during the night. The formula from the second run was chosen as a standard curve throughout the study (Fig.20). Maintenance of the method was checked by sporadically measured standards during the analysis of the samples, which were served as internal standards. Table 7 shows results for retention time (Rt) and area under the curve (AUC) during the measurements of the standards.

Table 7 The second run of standards

C (µg/ml)	Rt (min)	AUC (mAU*s)
20.24000	20.417	17168.60000
15.18000	20.516	12940.80000
10.12000	20.508	8529.81055
5.06000	20.546	4366.20361
1.01200	20.606	884.58234
0.10120	20.611	123.32228
0.05060	20.596	50.40585
0.01012	20.505	17.42642

$$y = 847.72x + 23.909$$ Equation 5

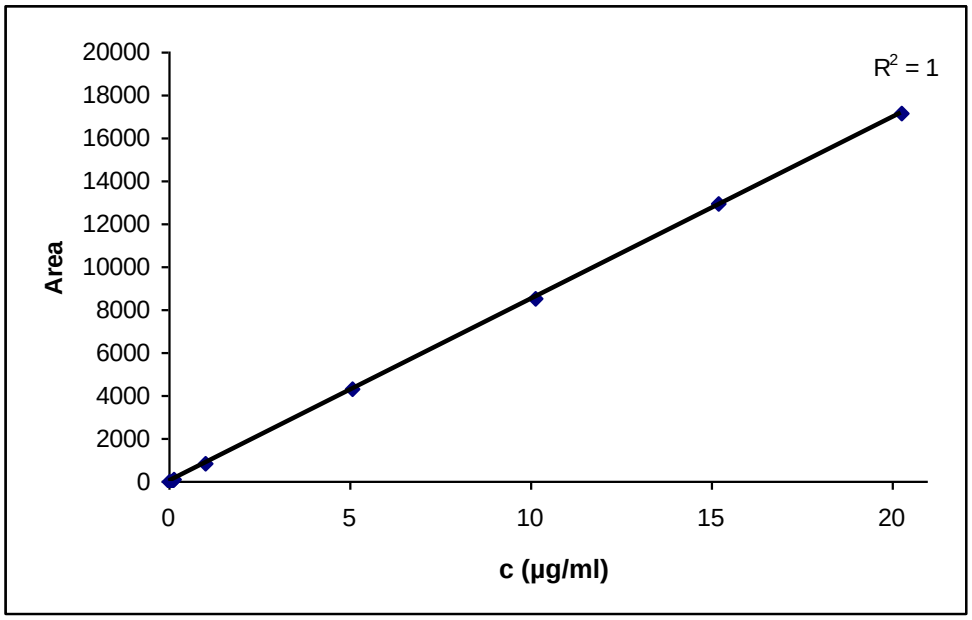


Fig. 20 Standard curve for terbinafine created in Excel.

E.2.3.1. Specificity

For the specificity of the procedure it is required that peak of the substance to be determined is sufficiently well resolved from other peaks, such as peaks of the impurities, degradation products, system components (solvents), etc (Epshtein, 2004). This was confirmed by the similar chromatograms, well separated peaks, and the retention time range from 16.60 to 22.12 min which depended mainly on the pressure in the system.

E.2.3.2. Linearity and stability

The method achieved high linearity with correlation coefficient of >0.999. The stability of standard solutions was confirmed within 28 days by evaluating the linearity of measurements. The measurements were performed after 5, 14, and 28 days with correlation coefficients of 0.9999, 0.9996, and 0.9998, respectively.

E.2.3.3. Sensitivity

From prepared standard solutions in the range from 0.0002 to 30.36 µg/ml the sensitivity test was performed. In three continuous measurements the minimum detectable concentration was 0.01012 µg/ml which remained detectable after 28 days of storage. On 55th day of storage at room temperature in shadowy place this concentration was not detectable anymore. This did not influence evaluation since the samples were measured within 28 days.

E.2.3.4. Precision

The precision of the terbinafine determination method is described by the coefficient of variation (CV), i.e. relative standard deviation (Epshtein, 2004):

$$CV(\%) = \frac{SD}{\bar{x}} \cdot 100 \qquad \text{Equation 6}$$

where SD is standard deviation and $\bar{x}$ is mean value. Three different formulations in 60% (v/v) ethanol/water containing 10% (w/v) terbinafine, 1% (w/v) terbinafine with 0.1% (w/v) hydrophobin C, and 1% (w/v) terbinafine were prepared. Samples taken from the formulations were diluted and analyzed by HPLC five times within 24 hours.

Table 8 Precision

Terbinafine c (µg/ml) in formulation	$\bar{x}$ ±SD	CV(%)
10.0099	9.50±0.71	7.48
1.0006	0.98±0.05	4.81
1.0038	0.99±0.04	3.99

E.2.3.5. Accuracy

“The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as conventional true value or an accepted reference value and the value found. This is sometimes termed trueness” (ICH Q2(R1), 2005). In order to evaluate accuracy, precision, linearity, and specificity have to be established. Accuracy was calculated according to formula:

$$R(\%) = \frac{c_{found}}{c_{introduced}} \cdot 100$$ Equation 7

The same formulations used for the evaluation of precision were analyzed for accuracy and presented as average accuracy (R) ± confidence interval (ΔR).

Table 9 Accuracy

Terbinafine c (µg/ml) in formulation	R (%) ± ΔR
10.0099	94.93±6.22
1.0006	97.93±4.13
1.0038	98.45±3.44

E.3. List of instruments

Analytical Balance AT261, No. P27846	Mettler Toledo AG, Switzerland
Balance PM400	Mettler Toledo AG, Switzerland
Centrifuge 5415C	Dr. Vaudaux AG, Switzerland
Destillator F285	Büchi Laboratoriums Technik, Switzerland
Filtercandle 250 ml AD8003-9	Hirt Walther AG, Riedikon, Switzerland
Filter MF^{TM} - Membranefilters, 0.45 µm HA	Filters made in Ireland
Franz diffusion cells (set 1)	TLT Medical, Reinach, Switzerland
Franz diffusion cells (set 2)	Glastechnik Rahm, Muttenz, Switzerland
Freezer tempered at -20°C	Electrolux
Freezer/Mill 6750-230, S/N 98060	SpexCertiPrep, Metuchen, New Jersey, U.S.A.
Haake Viscotester 550	Tracomme AG, Adliswil, Switzerland
HPLC 1100 Series	Agilent, Santa Clara, U.S.A.
HPLC column EC 250/4.6 Nucleodur 100-5 C18 ec	Marcherey-Nagel, Önsingen, Switzerland
Heating plate RCT basic No. 98 11 00 03	IKA Labortechnik, Staufen, Switzerland
Magnetic stirrers (for Franz cells)	Huber+Co. AG, Reinach, Switzerland
Magnetic stirrer Telemodul 40C	Sterico, Wangen, Switzerland
Magnetic stirrer plate Variomag Telesystem Komet	Sterico, Wangen, Switzerland
Micrometer	Tesa S.A., Renens, Switzerland

Osmometer Osmomat 030 No. 07 04 15	Gonotec Haslab GmbH, Ostermundigen, Switzerland
pH-meter Metrohm 827 pH lab No. 1827001008376	Metrohm Herisau, Switzerland
Tensiometer K100 No. 20012403	Krüss GmbH, Hamburg, Germany
Tewameter TM210 No. 94 28 0023	Courage+Khazaka electronic GmbH, Köln, Germany
Ultrasoundbath No. 1021892N	Property of the University of Basel
UV-spectrophotometer DU720 General Purpose UV/Vis Spect.	Beckman Coulter
Visioscan VC98 S/N 07362387	Courage+Khazaka electronic GmbH, Köln, Germany
Water bath Thermomix 1460 Type 850062, No. 001775	B.Braun Melsungen AG, Germany
Water bath Julabo EC	Labortechnik GmbH, Seelbach, Germany
Water bath	Salvis, Brendale Queensland, Australia

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