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Differential Stripping: Determination of the Amount of Topically Applied Substances Penetrated into the Hair Follicles

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The determination of penetration pathways of topically applied substances into the skin is the subject of several investigations. Recently, follicular penetration has become a major focus of interest. To date, a direct, non-invasive quantification of the amount of topically applied substance penetrated into the follicles had not been possible. The development of such a method was the aim of this study. Therefore, the advantages of both stripping techniques, tape stripping and cyanoacrylate skin surface biopsy, were combined and evaluated. Tape stripping was used to remove the part of the stratum corneum that contained the topically applied dye. Subsequently, the follicular contents were ripped off by cyanoacrylate skin surface biopsy. The combined method termed ''differential stripping'' was evaluated *in vitro* and *in vivo*, and the amount of topically applied fluorescent dye penetrated into the hair follicles was quantified after different penetration times. After 30 min, 5% of the recovered concentration of sodium fluorescein was found in the follicular infundibula, where it was still detectable after 48 h. Altogether, the results of this investigation revealed that differential stripping is a new method that can be used to study the penetration of topically applied substances into the follicular infundibula non-invasively and selectively.

Key words: cyanoacrylate skin surface biopsy/follicular penetration/follicular reservoir/tape stripping
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Several investigations concerning the determination of penetration pathways of topically applied substances have been performed during the last decades. In the past, in particular, the lipid layers of the stratum corneum (SC) had been considered to be the major penetration pathway into and through the SC (Choi *et al*, 1999; Bouwstra *et al*, 2001; Hadgraft, 2001), whereas the skin appendages were presumed to play a subordinate role by representing only 0.1% of the total skin surface (Schaefer and Redelmeier, 1996). Although previous investigations imply that the follicles play an important role in penetration processes (Lauer *et al*, 1995; Lademann *et al*, 2001; Schaefer and Lademann, 2001; Genina *et al*, 2002; Ogiso *et al*, 2002), they, however, seem to depend on the follicle density (Maibach *et al*, 1971; Hueber *et al*, 1994; Tenjarla *et al*, 1999). Otberg *et al* (2004a) measured inter alia the follicle density and the infundibular volume of follicles on seven different body sites. They found the highest hair follicle density for the forehead region and the highest infundibular volume for the forehead and the calf region, although the latter showed the lowest hair follicle density. The calculated follicular volumes of these two skin areas were as high as the estimated reservoir of the SC. To date, different methods have been utilized to study the penetration of topically applied substances into the hair follicles. Grams *et al* (2002a) applied confocal laser scanning microscopy (CLSM) for relative quantification of a fluorophore *in vitro* on fresh human scalp skin by the determination of the degree of accumulation in different skin layers and appendageal structures. Alvarez-Román *et al* (2004) used CLSM to visualize the distribution of microparticles across the skin. The surface images revealed that the microparticles accumulated preferably in the follicular openings. Therefore, in order to acquire both the deeper layers of the skin and the follicles, another method has to be applied. Lademann *et al* (1999) and Toll *et al* (2004) used biopsies combined with histological investigations. Lademann *et al* (1999) showed that microparticles, e.g., titanium dioxide, penetrated into the follicular orifices, whereas penetration of the microparticles into viable skin tissue could not be detected. Toll *et al* (2004) determined the optimal size of microspheres for follicular targeting *in vitro*. But taking biopsies in humans and *in vivo* implies a maximal invasive method, which, in large penetration studies of topically applied substances is not justifiable for ethical reasons.

Additionally, two non-invasive methods have been described to study the penetration: cyanoacrylate skin surface biopsies (Finlay and Marks, 1982; Pagnoni *et al*, 1994) and the tape stripping technique (Weigmann *et al*, 1999).

For cyanoacrylate skin surface biopsy, a superglue is applied on the skin surface and removed after polymerization, together with an unknown amount of corneocytes and the follicular casts (Finlay and Marks, 1982). For this reason, however, a distinction between the transepidermal and the transfollicular routes of percutaneous absorption is not

Abbreviations: LSM, laser scan microscopy; SC, stratum corneum

possible (Surber *et al*, 2001). Bojar *et al* (1993) used cyanoacrylate gel to determine the concentration of azelaic acid in the follicular casts after removing the substances from the skin surface by washing with acetone.

A more appropriate technique to remove and determine a substance from the skin surface and, particularly, from the SC, both *in vivo* and *in vitro*, is tape stripping (Surber *et al*, 2001). Here, the transepidermal route of percutaneous absorption can be studied by removing the SC partially or completely (Surber *et al*, 2001; Jacobi *et al*, 2003). In general, the highest concentrations of topically applied substances were detected on the skin surface and within the superficial layers (Cambon *et al*, 2001; Weigmann *et al*, 2001; Jacobi *et al*, 2003, 2004).

The aim of this study was the development of a selective, non-invasive, and quantitative method to determine the amount of topically applied substances penetrated into the hair follicles. Therefore, the advantages of both non-invasive stripping techniques were combined. The amount of topically applied substance that penetrated into the SC was removed using tape stripping. Subsequently, the follicular contents were removed by cyanoacrylate skin surface biopsy. This combined method was primarily proven *in vitro* on biopsies punched out from porcine skin, after which the developed standard protocol was used *in vivo* to quantify the follicular penetration of sodium fluorescein at different penetration times.

Results and Discussion

In the last decades, several investigations concerning the determination of penetration pathways of topically applied substances have been performed; yet, the role of the single pathways has not been clarified in detail. Recent studies implicate that the follicles play an important part in penetration and reservoir processes (Ogiso *et al*, 2002; Grams *et al*, 2004; Otberg *et al*, 2004a; Toll *et al*, 2004). But a study design that allows a direct and non-invasive quantitative determination of the amount of topically applied substance penetrated in the hair follicle has not been developed yet. In fact, previous investigations have compared the penetration of topically applied substances through normal, follicle-containing skin with skin devoid of follicles. In this context, different *in vivo* and *in vitro* study designs have been developed (Hueber *et al*, 1992, 1994; Tenjarla *et al*, 1999). The *in vivo* investigation compared the penetration of normal skin with the penetration of scarred skin (Hueber *et al*, 1992) or skin of newly born rats devoid of follicles (Hueber *et al*, 1994). All studies revealed a surprisingly high influence of the follicles on the penetration process; however, this did not permit a direct and quantitative determination of the penetration into the hair follicles, particularly, as the structure of scarred skin and the skin of newly born rats are not one-to-one comparable with healthy and mature skin.

The aim of this investigation was to develop a method that allows the selective determination of the amount of topically applied substance into the hair follicles by the combination of both well-known stripping techniques: tape stripping (Weigmann *et al*, 1999; Surber *et al*, 2001) and cyanoacrylate skin surface biopsy (Pagnoni *et al*, 1994). As a first step, the combination of both stripping techniques was evaluated histologically on porcine skin.

Histological evaluation of both stripping techniques on porcine skin (study design A) Biopsies were punched out from intact porcine skin after the removal of 100 tape strips and the removal of 100 tape strips and a cyanoacrylate skin surface biopsy, respectively. Intact porcine ear skin shows a SC (Fig 1*A*), viable epidermis with rete ridges and a dermis. Slices of hair follicles show a hair shaft surrounded by layers of corneocytes (Fig 1*B*).

In the past, the influence of the furrows of the skin on the tape stripping procedure was discussed controversially (Van der Molen *et al*, 1997). Recently, Lademann *et al* (2002)[1] have demonstrated that the effect of the furrows is negligible when a roller is used to stretch the skin and to press the adhesive tape onto the skin. This could be confirmed by the results of this investigation. The skin surface and the furrows were completely free of corneocytes after the removal of 100 tape strips (Fig 1*C*), whereas the corneocytes inside the hair follicles remained unaffected (Fig 1*D*).

After the application of both stripping methods, however, the corneocytes of both the SC and the hair follicles were eliminated (Fig 1*E* and *F*). This effect was demonstrated on six porcine samples. The application of the cyanoacrylate skin surface biopsy led to the removal of the infundibular content of the hair follicle as described previously (Finlay and Marks, 1982). Furthermore, a histological investigation revealed that if the removal of 100 tape strips is followed by a cyanoacrylate skin surface biopsy, distortions inside the viable epidermis can be observed (see Fig 1*E*). Previous investigations have shown that, in general, the highest concentrations of topically applied substances can be detected on the skin surface and within the superficial layers of the SC (Cambon *et al*, 2001; Weigmann *et al*, 2001; Jacobi *et al*, 2003, 2004). Therefore, study design B was developed to determine the number of tape strips that are necessary to remove the dye-containing SC, because, if the number of tape strips is reduced, distortion of the viable epidermis can be prevented.

Visual evaluation of both stripping techniques *in vivo* on human skin (study design B) An o/w emulsion containing 0.1% of the hydrophilic dye, patent blue V, was applied topically *in vivo* on the flexor forearm of a human volunteer to determine the number of tape strips that are necessary to remove the part of the SC containing the dye. In this case, 40 tape strips were necessary to remove the dye-containing SC. Subsequently, the hair follicles comprised the dye, selectively (Fig 2), and the corneocytes were free. When the tape stripping was followed by a cyanoacrylate skin surface biopsy, the follicular contents were also removed and the skin surface was completely dye free.

But the number of tape strips necessary for each topically applied substance will have to be predetermined, as

[1]Lademann J, Weigmann HJ, Lindemann U *et al*: Investigations on the influences of furrows and wrinkles when quantifying penetration of drugs and cosmetics by tape stripping. In: Brain KR, Walters KA (eds). Perspectives in Percutaneous Penetration, Vol 8a. Cardiff: STS, 2002; p 49 (abstr).

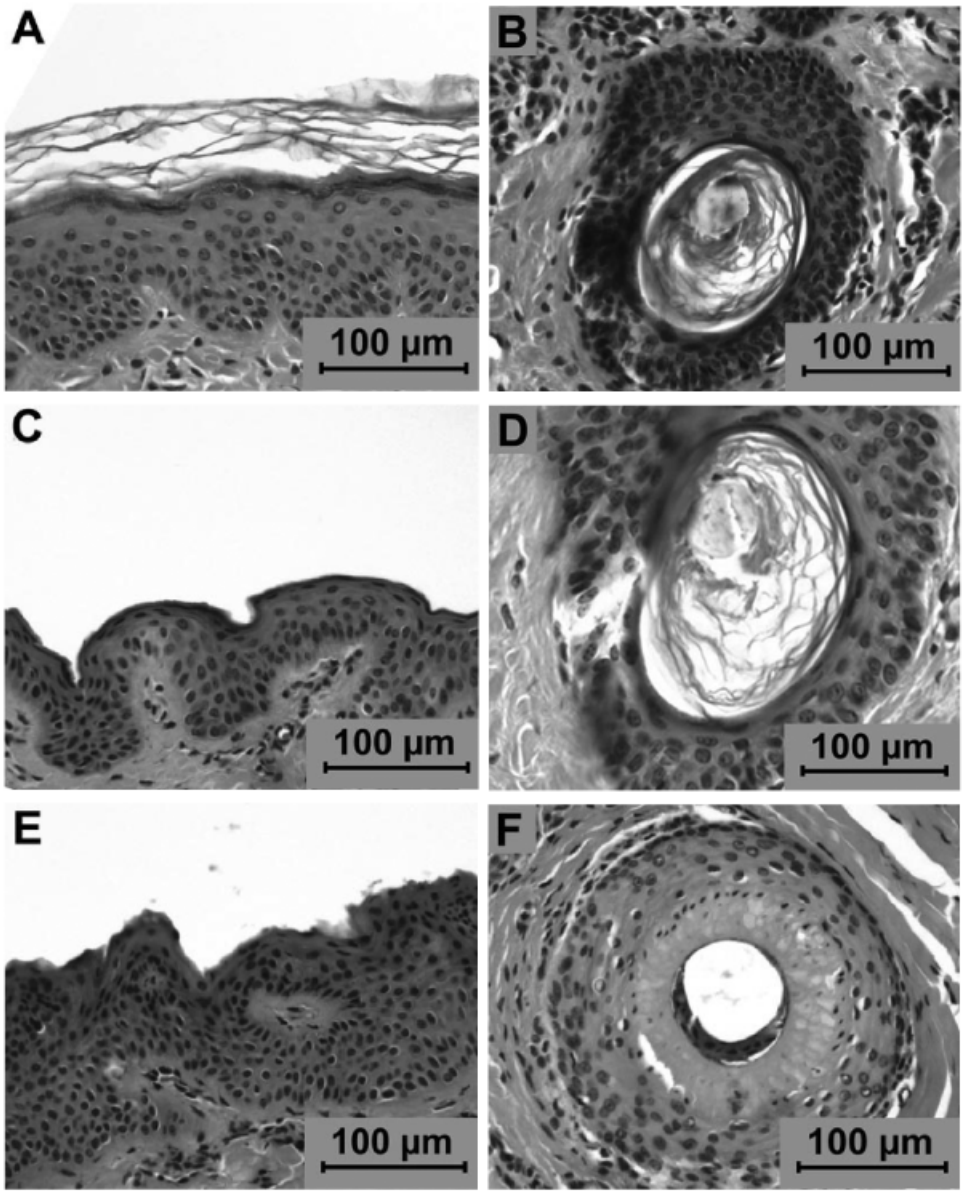

Figure 1
Histological sections of porcine ear skin before and after stripping. (*A*) Histological slice of porcine ear skin before tape stripping. Intact stratum corneum. (*B*) Histological slice of a follicle before tape stripping. Hair shaft is surrounded by layers of corneocytes. (*C*) Histological slice of porcine ear skin after the removal of 100 tape strips. The stratum corneum has been removed completely. (*D*) Histological slice of a follicle after the removal of 100 tape strips. Hair shaft is surrounded by layers of corneocytes. (*E*) Histological slice of porcine ear skin after the removal of 100 tape strips and a cyanoacrylate skin surface biopsy. The stratum corneum has been removed completely and distortions inside the viable epidermis can be observed. (*F*) Histological slice of a follicle after the removal of 100 tape strips and a cyanoacrylate skin surface biopsy. The corneocytes surrounding the hair shaft have been removed.

previous investigations have shown that the vehicle influences the amount of SC removed by tape stripping (Schwarb *et al*, 1999; Jacobi *et al*, 2003). Furthermore, in general, the penetration behavior of topically applied substances differs depending on physicochemical properties such as size, charge, and formulation,[2] that might influence the penetration depth. Based on these preliminary results, a quantitative determination of the amount of topically applied substance penetrated into the hair follicles could be carried out (study design C).

Quantitative determination of the amount of topically applied substance into the hair follicles at different penetration times (study design C) For the quantitative *in vivo* determination of topically applied sodium fluorescein within the hair follicles at different penetration times, cyanoacrylate skin surface biopsies were taken from previously stripped skin areas. Afterwards, the biopsies were analyzed by LSM to ensure that the removal of the tape strips led to the complete removal of sodium fluorescein from the SC. In Fig 3, a fluorescence image of a cyanoacrylate skin surface biopsy is presented, which demonstrates that the fluorescent dye is only located in the follicular casts and not in further areas of the biopsy.

Subsequent to the LSM investigation, the tape strips and the biopsies were extracted in ethanol and the concentrations of the sodium fluorescein on the tape strips, representing the amount in the SC, and in the biopsies, which corresponds with the concentration of sodium fluorescein in the follicular infundibula, were determined quantitatively using fluorescence measurements. After a 30 min penetration time, 1.1 ± 0.2 µg per cm^2 of sodium fluorescein was recovered from the follicular infundibula after applying in *Eucerin cum Aqua* as vehicle which corresponds with $5\% \pm 0.9\%$ of the totally recovered sodium fluorescein (volunteer nos 2–6); $95\% \pm 14\%$ were recovered from the

[2]Lieb LM, Liimatta AP, Bryan RN, Krueger GG: Description and definition of a transfollicular route of permeation for topically applied agents to human scalp skin. J Invest Dermatol 104:655, 1995 (abstr).

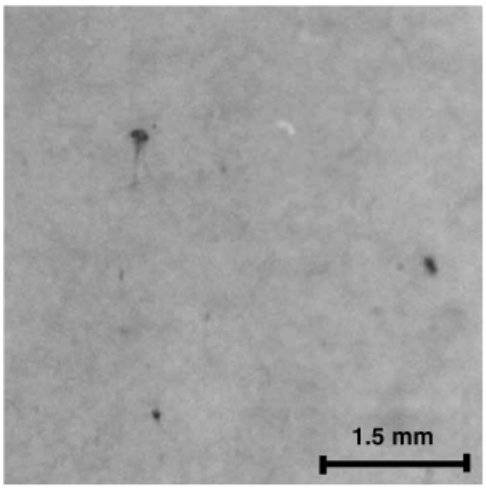

Figure 2
Image of human skin after the removal of 40 tape strips (volunteer no. 1). In the present case, 40 tape strips were necessary to remove the dye-containing stratum corneum. Then, the follicles comprised the dye, selectively, the skin surface was free.

SC. The overall recovery rate of sodium fluorescein was 54% $\pm$ 8.1% relating to the applied amount of sodium fluorescein. This is in concordance with previous investigations (data not published yet). For the non-recovered part of sodium fluorescein, absorption is presumed. For other vehicles, the penetration rate into the hair follicles and their reservoir may be larger or even smaller dependent on their physicochemical properties. It has already been described that percutaneous absorption, in general, and local delivery to the hair follicle, in particular, depend to a large extent on drug and vehicle physicochemical properties, such as size, charge, and lipophilicity (Lauer *et al* 1995; Illel, 1997; Grams *et al*, 2002b). For more lipophilic substances, the penetration rate into the hair follicles will be presumably enlarged.

Otberg *et al* (2004a) already assumed the existence of a follicular reservoir. This follicular volume, however, is not completely available for the penetration of topically applied substances, as the follicles cannot be seen as cavities but as structures filled with corneocytes, sebum, hairs, etc.

Otberg *et al* (2004a) measured the infundibula volume of hair follicles on different body sites. The potential follicular volume of the back was determined at 0.11 mm^3 per cm^2 skin surface. For the SC, a reservoir size of 1 mm^3 per cm^2 was assumed. In this investigation, 5% of sodium fluorescein was recovered from the follicular infundibula of the back and 95% from the SC. This means that the obtained results are in good correlation with the relation of the volumina of the SC and the hair follicles determined by Otberg *et al* (2004a) when considering the fact that the SC and the follicles are not empty cavities. Additionally, differences in dissolubility within the SC and the follicles, differences in penetration speed, anterograde, and retrograde transfollicular penetration, etc., have to be taken into consideration. Furthermore, Lademann *et al* (2001) ascertained the phenomenon of active and inactive hair follicles. Active in contrast to inactive signifies that topically applied substances penetrate into the hair follicles. The follicles are active if hair growth and sebum production are detected. As a reason for inactive follicles, Otberg *et al* (2004b) observed a follicular cover that consists of desquamated corneocytes and dry sebum. Closed follicles can be opened artificially by mechanical peeling. Depending on the anatomical site, usually more than 70% of the hair follicles are active (Otberg *et al*, 2004b). Therefore, differences in follicular physiology affect the extent of follicular penetration and reservoir.

In addition, the results indicate that a long-term reservoir of the hair follicles also exists. Figure 4 presents the concentrations of sodium fluorescein penetrated into the hair follicles at different penetration times. After a 30 min penetration time, the highest concentration (1.3 $\pm$ 0.12 μg per cm^2) of sodium fluorescein was detected in the follicular infundibula. After a 24 and 48 h penetration time, the

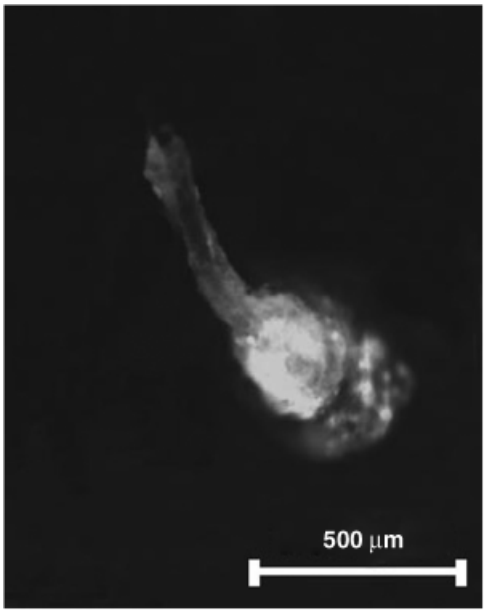

Figure 3
Fluorescence image of a cyanoacrylate skin surface biopsy. After the removal of tape strips to clean the skin surface of the topically applied fluorescent substance, cyanoacrylate skin surface biopsies were taken to rip off the follicular contents. The image shows the fluorescent follicular content, whereas, the stratum corneum shows no fluorescence.

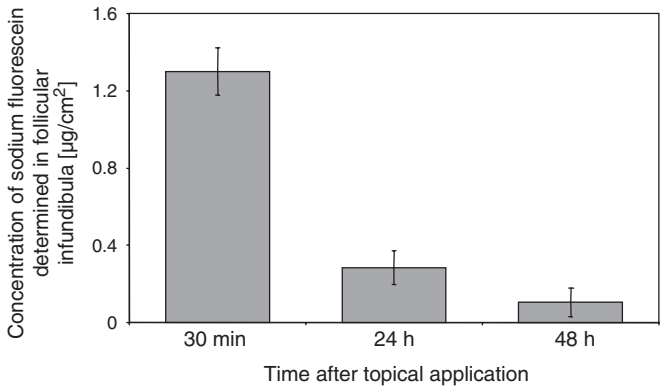

Figure 4
Quantitative determination of sodium fluorescein in the hair follicles by cyanoacrylate skin surface biopsy 30 min, 24, and 48 h after the application of 40 μg per cm^2 of sodium fluorescein and after the removal of the stratum corneum (SC) by tape stripping (back of volunteers nos. 7–9).

recovered concentration of sodium fluorescein decreased continuously.

Therefore, transfollicular penetration and retrograde penetration, accompanying the sebum flow, should be taken into consideration. Using histological investigations, Lademann *et al* (1999) showed that microparticles, which penetrated into the follicular orifices, did not reach viable skin tissue. For other substances, however, this might be different, as a penetration through the hair follicles seems possible.

Altogether, the results of this investigation revealed that, by means of differential stripping, a method has been developed to quantify the penetration of topically applied substances into the follicular infundibula. Differential stripping provides several opportunities in dermatologic science, such as the characterization of the long-term reservoir of different topically applied substances exhibiting varying lipophilicities and formulations, which will be the topic of further investigations in our laboratory. This leads to a better understanding concerning pharmacokinetics of topically applied substances. The kinetics of follicular penetration should be determined using this technique in combination with the determination of sebum excretion.

Materials and Methods

Materials The o/w emulsion containing 0.1% of the dye patent blue V (Byk Gulden, Lomberg Chemische Fabrik GmbH, Konstanz, Germany) and the w/o formulation *Eucerin cum Aqua* with 2% sodium fluorescein ($C_{20}H_{10}Na_2O_5$) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were prepared by the Pharmacy of the Charité (Berlin, Germany).

Sodium fluorescein and patent blue V are both hydrophilic dyes. Sodium fluorescein has a molecular weight of 376.3 g per mol and a log P (octanol/water partition coefficient) of -0.67. Patent blue V has a molecular weight of 582.7 g per mol and a log P of -5.34.

Pre-treatment of volunteers/porcine skin The study was performed *in vivo* on nine healthy volunteers, four males and five females, mean age 23.1 ± 2.7 y, and *in vitro* on ear skin of six freshly slaughtered pigs (6-mo-old, German domestic pigs). Porcine skin is known to represent a suitable model for human skin (Simon and Maibach, 2000), showing a similar penetration for topically applied substances (Benech-Kieffer *et al*, 2000; Schmook *et al*, 2001). Approval had been obtained from the Ethics Committee of the Charité and from the Veterinary Board of Control, Berlin Treptow-Köpenick. The study was conducted according to the ethical rules stated in the Declaration of Helsinki Principles. The volunteers participating in the study had given their written consent.

The flexor forearms and the backs, respectively, of the volunteers and the ear skin of the pigs were prepared by washing, drying with a soft tissue, and abscising the hairs; defined skin areas of 5 cm $\times$ 12 cm were demarcated using a permanent marker.

Study designs

Study design A Histological evaluation of both stripping techniques, tape stripping and cyanoacrylate skin surface biopsy (application *in vitro* on porcine skin).

Study design B Visual evaluation of both stripping techniques (application *in vivo*, on the flexor forearm of a volunteer).

Study design C Application of the combined method of both stripping techniques to quantify the amount of topically applied substances penetrated into the follicular infundibula (application *in vivo*, on the back of eight volunteers).

Application of topically applied substances According to the different study designs B and C, different formulations were applied within the demarcated areas.

Study design B Two milligrams per centimeter square of an oil-in-water (o/w) emulsion containing 0.1% of the hydrophilic dye patent blue V (Byk Gulden, Lomberg Chemische Fabrik GmbH) was applied homogeneously in order to evaluate both stripping techniques visually.

Study design C Two milligrams per centimeter square of a formulation (*Eucerin cum Aqua*, Pharmacy of the Charité) containing 2% of the fluorescent dye sodium fluorescein (Sigma-Aldrich Chemie GmbH) was applied homogeneously within the demarcated skin areas in order to determine the follicular penetration quantitatively.

Tape stripping procedure The tape stripping procedure was performed, as described previously (Weigmann *et al*, 1999), after different penetration times of the topically applied substances (study design B: 30 min; study design C: 30 min, 24, and 48 h), whereby, the skin was stripped using an adhesive tape (*tesa* No 5529, Beiersdorf, Hamburg, Germany). The tape strips were pressed onto the skin using a roller that stretches the skin surface and brings the tape strip in contact with the entire flat skin surface, which is normally structured by wrinkles und furrows. Then, the tape strips were removed until the blue dye (study design B) and the fluorescent dye (study design C), respectively, were completely eliminated from the SC. This was detected visually in the case of the patent blue and by a disappearing fluorescent signal using LSM (LSM 2000, Carl Zeiss, Jena, Germany) in the case of the sodium fluorescein. Concerning study design A, the complete SC was removed by 100 tape strips. This is concordant with previous investigations (Öhman *et al*, 1994).

Cyanoacrylate skin surface biopsy Subsequent to the removal of the necessary tape strips (study designs A–C), a drop of superglue (UHU GmbH, Brühl, Germany) was placed on the stripped skin areas. Afterwards, the glue was covered with a glass slide under slight pressure, as described previously (Otberg *et al*, 2004a). After 5 min, the cyanoacrylate polymerized and the glass slide was removed with one quick movement, whereas the follicular casts and corneocytes remained on the slide.

Analysis of the cyanoacrylate skin surface biopsy by LSM The distribution of the topically applied sodium fluorescein in the cyanoacrylate skin surface biopsies was analyzed by LSM (LSM 2000, Carl Zeiss). Ar^+-laser radiation at 488 nm was used to excite the dye. The fluorescent signal was detected at a wavelength $\geqslant$560 nm.

Histological investigations (study design A) Biopsies (8 mm in diameter) were sampled from the porcine skin after the tape stripping procedure and after both the tape stripping and cyanoacrylate surface biopsy procedure had been performed. The samples were fixed in formaldehyde solution and embedded in paraffin. Sections of 4 μm thickness were cut vertical to the surface using a microtome (paraffin microtome Reichert-Jung HN 40, Optische Werke AG, Vienna, Austria). The sections were stained with hematoxylin/eosin (H/E) following histological standard procedures. Images were taken using a microscope (System microscope BX60, Olympus Optical, Hamburg, Germany) equipped with a digital camera (SIS Color View 12, Soft Imaging System GmbH, Münster, Germany).

Quantitative analysis of the topically applied substance penetrated into the hair follicles (study design C) After removal of the tape strips and the cyanoacrylate surface biopsies, the samples were punched to a constant size of 15 mm diameter. Afterwards, the samples were extracted in ethanol (Uvasol, Merck, Darmstadt, Germany) using ultrasound (Sonorex Super RK102H, Bandelin Electronic, Berlin, Germany) and centrifugation (at rcf $=$ 1487, 36 $\times$ g for 10 min at 20°C, Centrifuge MR1812, Jouan GmbH, Unterhaching, Germany), followed by the determination of

the concentration of the sodium fluorescein using fluorescence measurements (Luminescent LS 50B, Perkin Elmer, Überlingen, Germany). The fluorescence signal was detected in the spectral range from 480 to 600 nm. The maximum fluorescence intensity was detected at a wavelength of 510 nm. For quantitative determination of the sodium fluorescein, a calibration curve of sodium fluorescein extracted in ethanol was established taking into account cyanoacrylate and untreated skin.

Statistical analysis For statistical analysis of the data, we utilized the Kolmogorov–Smirnov test (SPSS 11.0) to demonstrate that the obtained data were not not-normal distributed ($p > 0.05$). Then, we calculated the mean values and standard deviations (SPSS 11.0) of the recovered concentrations of the sodium fluorescein in the SC, follicular infundibula, and at different penetration times.

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