Research paper

Non-invasive analysis of penetration and storage of Isoconazole nitrate in the stratum corneum and the hair follicles

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ABSTRACT

Bacteria and fungi are located in the stratum corneum and the hair follicles. Therefore, the development and assessment of efficient drugs requires standard in vivo investigation methods permitting a differentiation between intercellular and follicular penetration and storage of topically applied anti-microbial substances.

In the present study, the penetration and storage of Isoconazole nitrate in the stratum corneum and hair follicles was investigated by differential stripping after a 14-day topical application period and during a follow-up period of a further 21 days.

One week after the application had terminated, Isoconazole nitrate could still be detected in concentrations above the minimal inhibition concentration in the stratum corneum and the hair follicles. In some subjects, Isoconazole nitrate could even be detected 14 days after the last application. No relevant changes in TEWL values were measured, indicating that the investigated compound did not induce an impairment of the barrier function.

The study showed that differential stripping is suited to investigate the penetration and storage of topically applied substances into the stratum corneum and the hair follicles. Also, the hair follicles are a long-term reservoir for topically applied substances. This is of clinical importance, where a long-lasting therapeutic effect beyond the application time is required.

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1. Introduction

Considering the advancing age of the population in the industrial countries, fungal infections and disturbed wound healing as a result of concomitant diseases, such as diabetes mellitus are becoming increasingly important [1,2]. Consequently, there are high requirements, in particular, on treatments against bacteria and fungi affecting the skin [3]. The therapeutic effect of such anti-microbial drugs can be enhanced by combining them with anti-inflammatory substances [4,5]. The anti-microbial activity permits the reduction in bacteria and fungi on the skin, and the anti-inflammatory effect reduces the accompanying inflammatory symptoms like eczematous reactions, itching and burning [5].

Recently, Lange-Asschenfeldt et al. [6] and Ossadnik et al. [7] demonstrated that bacteria and fungi are located not only on the surface of the skin and in the upper layers of the stratum corneum, but also in hair follicles. These follicular populations of bacteria and fungi can serve as a reservoir, not only for the maintenance of the infection but also for a potential fast reinfection of the skin after skin cleaning, as conventional antiseptics are not capable of reaching the follicular reservoir efficiently in order to eradicate the follicular microbes. Therefore, modern efficient treatments must be capable of reaching the stratum corneum, as well as the microbial reservoir hair follicles as target structures. Whilst the layer thickness of the stratum corneum is 15–20 µm on average (except for the palmar and plantar regions), the terminal hair follicles extend up to 2 mm and the vellus hair follicles up to 0.7 mm deep into the skin [8]. Otberg et al. [9] showed that on the forearm, the orifices of the hair follicles represent approximately 1% of the surface, and the volume of the hair follicles is approx. $0.1\ mm^3/cm^2$ of skin surface.

Whereas various measuring methods are available to analyse the penetration of topically applied substances into the stratum corneum, both in vitro (diffusion cells) [10] and in vivo (tape stripping and in vivo Raman spectroscopy) [11,12], the analysis of the hair follicle penetration is difficult since it requires spatial resolution. So far, the hair follicle penetration could be analysed

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invasively by taking biopsies and subsequent histological analysis with the application of fluorescent dyes or radio-labelled compounds [13]. In vivo laser scanning microscopy permits, for the first time, the detection of fluorescent substances in the stratum corneum and in hair follicles of volunteers or patients, non-invasively [14,15]. However, the range of substances, which can be investigated by this method, is limited, as these must exhibit specific fluorescent properties.

The development of the differential tape stripping method represents a decisive progress in follicular penetration research [16]. This method combines conventional non-invasive tape stripping processes with cyanoacrylate skin surface biopsy, in order to differentiate between intercellular and follicular penetration, as well as storage. After topical application and penetration in accordance with the chemical substances, the stratum corneum is removed by tape stripping. The amount of stratum corneum removed by the tape strips can be determined spectroscopically, whilst chemical and analytical methods are used to determine the amount of substances penetrated into the stratum corneum. After tape stripping, a skin surface biopsy is taken from the same skin area by means of cyanoacrylate. The cyanoacrylate applied to the skin surface penetrates into the hair follicles and hardens there. When the hardened adhesive film is removed from the skin, the content of the hair follicles is also removed, since it is attached to the cyanoacrylate. The cyanoacrylate tape strips are then dissolved, and the amount of the substance that had penetrated into hair follicles is determined. In the present study, the differential tape stripping method was used for in vivo investigations of penetration and storage of Isoconazole nitrate into the stratum corneum and into the hair follicles.

Isoconazole nitrate is an anti-fungal drug from the azole family with excellent antimycotic and antibacterial (gram-positives) properties [17–19]. In previous investigations, the radio-labelled Isoconazole nitrate equivalents (representing drug and metabolites) were determined in skin compartments from biopsies after topical application [20]. After a single application of Isoconazole cream, approximately 10–15% of the dose could be detected in the stratum corneum layer corresponding to Isoconazole concentrations of about 3–4 mg/mL (approx. 8 mmol). Taeuber [20] described an elimination half-life of 1.4 days for radio-labelled Isoconazole-equivalents from the stratum corneum layer. Thus, five half-lives would have been passed within 1 week. Based on this half-life, it can be expected that drug levels in stratum corneum would decrease to 3% ($1/2^5$) of the original concentration within 1 week.

The aim of the present study was to investigate the feasibility of the differential tape stripping method with a model substance (Isoconazole nitrate) without using radio-labelled compound. Thereby, the extraction of Isoconazole nitrate from the cyanoacrylate skin surface biopsies represents an analytical challenge as cyanoacrylate in higher concentrations causes signal depression. Therefore, new analytical techniques have to be developed. However, the realization would allow a drug-specific analysis in these skin compartments to be established providing an accurate and precise bioanalytical assay with appropriate sensitivity and selectivity. Application of this methodology would allow knowledge to be gained, as to whether relevant drug concentrations can be detected in the stratum corneum and the hair follicles, even after termination of the topical application, and to detect a potential storage as a long-term reservoir.

2. Material and methods

2.1. Volunteers

The investigations were performed on 10 healthy volunteers with skin type II, according to the Fitzpatrick classification [21],

of both genders (five men and five women), aged between 20 and 31 years (mean age 26 years). The volunteers were examined dermatologically prior to the start of the study to ensure that they were not suffering from skin irritations or skin diseases. One female volunteer dropped out of the study due to an adverse event with an elevated body temperature during the product application phase, which was unrelated to the study drug. The study was continued with nine volunteers. The study was conducted in accordance with the Declaration of Helsinki. Approval of the Ethics Committee of the Charité had been obtained for the investigations and all volunteers had given their written informed consent.

2.2. Applied substances and application protocol

The topical combination drug, commercialized as Travocort® cream, was applied onto the forearms of the volunteers twice daily on an area of 8 × 12 cm², for a period of 14 days. Travocort® cream is composed of an antimycotic compound Isoconazole nitrate 1%, formulated in a vehicle containing white soft paraffin, liquid paraffin, cetostearyl alcohol, Polysorbate 60, sorbitan stearate, disodium edentate, purified water and the anti-inflammatory compound, corticosteriod diflucortolone valerate 0.1%. The cream was applied at a concentration of 2 mg/cm² resulting in an area dose of 20 μg Isoconazole nitrate per cm². The volunteers received pre-weighed doses within a syringe and applied the cream independently. The sample area was selected so that tape strips could be removed from four different skin areas without affecting the adjacent areas. After an application period of 14 days, 10 tape strips were removed from the first skin area 6 h after the last dose, followed by a cyanoacrylate skin surface biopsy. The remaining strips were removed and biopsies taken, respectively, 7, 14 and 21 days post end of treatment.

2.3. Tape stripping

The tape stripping method has been described in detail by Weigmann et al. [22]. Here, adhesive films (tesa film No. 5529, Beiersdorf AG, Hamburg, Germany) were pressed onto the treated skin areas by a roller with a weight of 300 g and then removed again. This process was repeated 10 times. Each strip contained approximately one layer of corneocytes and amounts of the topically applied substance, which penetrated into the respective skin area down to the depth from which the tape strip had been taken. Pre-investigations proved that the removal of 10 tape strips is sufficient to remove the complete Isoconazole nitrate from the stratum corneum.

2.4. Cyanoacrylate skin surface biopsy

After the 10 tape strips had been removed, the Isoconazole nitrate was completely removed from the stratum corneum. Afterwards, one drop of cyanoacrylate (UHU GmbH & Co. KG, Germany) was applied onto the same skin area, covered by an adhesive film and pressed onto the skin by a roller with a weight of 300 g. After 10 min of hardening, the adhesive film with the dried cyanoacrylate adherent thereto was removed from the skin. Therefore, the cyanoacrylate skin surface biopsies only contained parts of the stratum corneum without Isoconazole nitrate plus the follicular contents containing the follicular percentage of Isoconazole nitrate.

2.5. Determination of the Isoconazole nitrate content in the samples

An isocratic high performance liquid chromatography (HPLC) electrospray ionization mass spectrometry (ESI-MSMS) method for qualitative and quantitative determination of Isoconazole in

Table 1
Isoconazole nitrate equivalents (ng/cm^2) in the stratum corneum and the pilosebaceous unit (PSU) up to 2 weeks post last application of Travocort® cream on the forearm of 10 volunteers.

Volunteers	Concentration of Isoconazole nitrate (ng/cm^2)					
	6 h post last application		1 week post last application		2 weeks post last application	
	Stratum corneum	PSU	Stratum corneum	PSU[b]	Stratum corneum	PSU
1	[a]	[a]	[a]	[a]	[a]	[a]
2	2297	1381	0	N.a.	5	[c]
3	8991	4774	24	58	6	[c]
4	2757	546	11	77	0	[c]
5	3106	447	102	63	0	[c]
6	3658	678	23	66	0	[c]
7	597	210	98	60	0	[c]
8	8449	3678	123	64	6	[c]
9	4773	1378	5	45	0	[c]
10	1549	924	0	57	0	[c]
Mean ± SD abs.	4020 ± 2921	1557 ± 1587	43 ± 50	61 ± 9	2 ± 3	[c]
Ratio (%)	72	28	41	59	[c]	[c]

[a] No Isoconazole detected, as this volunteer prematurely stopped treatment (excluded).
[b] To be considered as rough estimates only due to variability of analyte recovery during workup of cyanoacrylate samples.
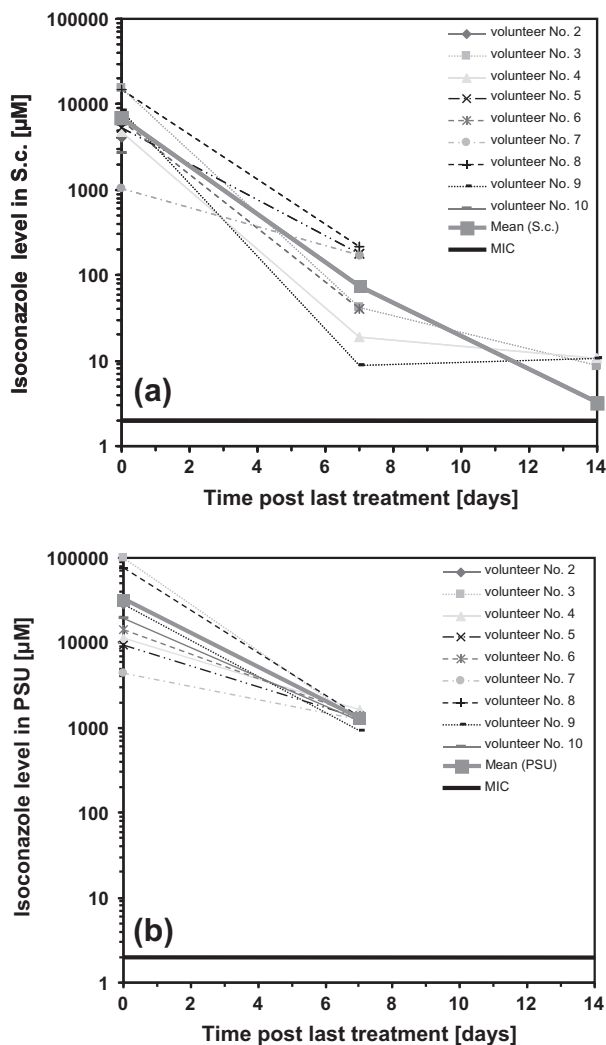[c] Not analysed/not calculated.

Fig. 1. Isoconazole nitrate concentration in stratum corneum and hair follicle. Isoconazole nitrate concentrations (μmol) in stratum corneum (S.c.) (a) and the pilosebaceous unit (PSU) (b) up to 2 weeks post last application of Travocort® cream on the forearm of 9 evaluable volunteers. (MIC: minimum inhibition concentration for growth inhibition of bacteria and fungi (according to Kessler et al. [18]); mean (S.c.): $n = 9$ at day 0, $n = 7$ at day 7, $n = 3$ at day 14; mean (PSU): $n = 9$ at day 0, $n = 8$ at day 7.)
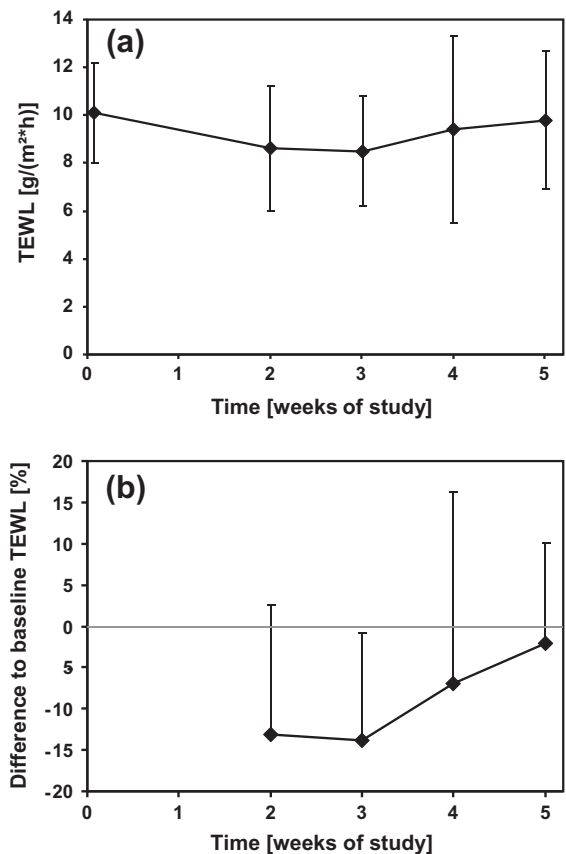

Fig. 2. Time course of TEWL. Time course of mean transepidermal water loss (TEWL) of the skin up to 3 weeks after end of a 2-week treatment with Travocort® cream on the forearm of nine evaluable volunteers; (a) absolute TEWL values, mean ± SD and (b) change to pre-treatment baseline, mean ± SD.

human stratum corneum was developed. The sample strips were extracted in a water/acetonitrile solution, which contains a specific amount of reserpine as an internal standard for the SIM-Modus (selective ion monitoring) or econazole as an internal standard for the SRM-Modus (selective reaction monitoring). A separation of reserpine and Isoconazole could be realized with a Phenomenex Luna C18 column (3 μ, 150 × 2 mm) and a mobile phase,

containing 70% acetonitril and 30% 5 mM aqueous ammonium acetate (SIM). The separation of econazole and Isoconazole is not required for the SRM. Isoconzole and econazole were identified and quantitatively analysed by their MS characteristics, that is, molecular ions and three specific segments in a positive ion mode. This method afforded a good linearity, selectivity, precision and recovery in the 100–2000 ng/mL (SIM) and 25–500 ng/mL (SRM) range with a short analysis time, respectively.

In case of the cyanoacrylate strips, the samples were placed into acetonitrile containing the internal standard. An aliquot of the acetonitrile was mixed with methanol at 5 °C, resulting in precipitation of the cyanoacrylate. Following centrifugation, the surplus was taken and subsequently subjected to MS analysis. The dilution step in acetonitrile resulted in a lower sensitivity of the analytical method as compared to the analysis of stratum corneum sample strips.

2.6. Calculation of concentrations in stratum corneum and pilosebaceous unit

Isoconazole nitrate was used to prepare the calibration curve for bioanalysis. As a correction factor was not applied to convert Isoconazole nitrate to free Isoconazole levels, original bioanalytical results are given in Isoconazole nitrate equivalents.

2.7. Tape strip sample analysis

A tape area of about 3.5 cm^2 was analysed. A volume of 4.75 mL acetonitrile/ammonium acetate (pH 4.0) and 0.25 mL internal standard solution were added. After a 30-min ultrasonification, an aliquot of the solution was transferred into a 2 mL vial, and 20 μL was subjected to the LC–MS system. Thus, the concentration of the sample (ng/mL) was multiplied with the sample volume (mL) in order to obtain the amount of drug in the tape sample (ng). The amount per tape sample was divided by the tape area analysed, in order to obtain Isoconazole nitrate equivalent levels in ng/cm^2 skin. For calculation of molar concentrations, the result in ng/cm^2 was divided by the molecular weight of Isoconazole nitrate (i.e. 0.47915 μg/mL) and the assumed thickness of the stratum corneum. Pre-investigations could demonstrate that the layer thickness of the stratum corneum taken by means of the standardized tape stripping method corresponds to 60% of the thickness of the stratum corneum, that is, 12 μm for the volar forearm.

2.8. Analysis of cyanoacrylate skin surface biopsies

In analogue to the evaluation of the tape strip samples, the area of the cyanoacrylate biopsies was measured. Defined volumes of solvent and internal standard solution were added. Thus, the concentration of the sample (ng/mL) was multiplied with the sample volume, in order to obtain the amount of drug in the sample (ng). The amount per sample was divided by the cyanoacrylate biopsy area analysed, so as to obtain Isoconazole nitrate equivalent levels in ng/cm^2 skin. For calculation of molar concentrations, the result in ng/cm^2 was divided by the molecular weight of Isoconazole nitrate (i.e. 0.47915 μg/mL) and the assumed volume of the pilosebaceous unit. It was assumed that the hair follicles contribute to a volume of approximately 0.1 mm^3 (0.1 μL) per cm^2 of skin [9].

2.9. Determination of the epidermal barrier function as transepidermal water loss (TEWL) values

The TEWL values were determined after 20 min of equilibration to the surrounding conditions under controlled environmental conditions at baseline before treatment and at days 14, 21, 28 and 35 using the Tewameter TM 210 (Courage + Khazaka electronic GmbH, Cologne, Germany). The measurement was carried out on the same skin site for at least 20 s and was stopped when the standard deviation reached ⩽0.15 g m^{-2} h^{-1}. Previous experiences demonstrated that a difference in TEWL values before and after the treatments of ⩽8% could be neglected [23].

3. Results

The results of the Isoconazole nitrate analysis of the tape strips and the cyanoacrylate surface biopsies of each volunteer are shown in Table 1 for the measurements upon termination of the application (day 14) and after 1 week (day 21) and 2 weeks (day 28), thereafter. The Isoconazole nitrate concentrations in the samples taken 3 weeks after the last application did not exceed the detection limit and were, therefore, not included in the table. In three of nine subjects, Isoconazole nitrate could even be detected in stratum corneum, 2 weeks after the last topical application.

Table 1 reveals that approximately 30% of the drug amount applied at the last treatment was recovered from the stratum corneum and the pilosebaceous unit at 6 h after end of treatment. The major part thereof, on average 72%, was allocated to the stratum corneum and the minor part of 28% to the hair follicles. Within 1 week after the end of the application, Isoconazole levels decreased by about two orders of magnitude in stratum corneum as well as in the pilosebaceous unit. At this point in time, amounts recovered from the stratum corneum and the pilosebaceous unit were similar (approx. 1:1 ratio).

3.1. Impact assessment

Based on the research results achieved by Kessler [18], the minimum concentration of the substance, which is necessary to inhibit growth of gram-positive bacteria and fungi (minimum inhibitory concentration, MIC), amounts to 2 μmol corresponding to 0.83 μg/mL. Fig. 1 shows the Isoconazole nitrate concentrations in Table 1 converted to μmol for stratum corneum (Fig. 1a) and pilosebaceous unit (PSU) (Fig. 1b). On average, mean Isoconazole nitrate concentrations exceeded the MIC at least for 2 weeks after end of treatment. Although drug levels in the pilosebaceous unit (PSU) substantially exceeded the Isoconazole nitrate levels in the stratum corneum during the first week after end of treatment, no drug levels were obtained in the PSU at 2 weeks after end of treatment. Although higher levels can be expected to occur in the PSU as compared to the stratum corneum also at that later point in time, the lower sensitivity of the analytical method for the content in the PSU did not allow for quantification of Isoconazole nitrate. The reason for the lower sensitivity was due to extraction and dilution of the cyanoacrylate adhesive sample prior to injection into the mass spectrometer in order to avoid contamination of the instrument.

3.2. Epidermal barrier function (TEWL values)

The results are presented in Fig. 2. The values were reduced 6 h after the last topical application (day 14) indicating a small improvement in epidermal barrier function or a minor occlusive effect produced by a 2-week application of the cream formulation. The minimally lower values were also seen after 1 week and then increased to baseline values.

4. Discussion

Kessler [18] was able to show that Isoconazole nitrate has excellent antimycotic and antibacterial (against gram-positive

bacteria) properties. The dermatopharmacokinetics of Isoconazole nitrate had earlier been investigated using radio-labelled substances determined in the skin compartments and in the urine. In these studies, Isoconazole could be detected above the minimal inhibition concentration in the skin biopsies at least 10 days after the end of topical application. [20]. However, these studies could not differentiate between drug and metabolites as well as the different compartments in the skin.

The results of the present study showed that the Isoconazole nitrate could be detected in the stratum corneum of some subjects even 2 weeks after the last topical application. Also, the hair follicles represent excellent long-term reservoirs for topically applied Isoconazole nitrate. The ratio between the Isoconazole nitrate concentration in the stratum corneum and in the hair follicle was 7:3 at 6 h after end of treatment. Within 1 week, Isoconazole nitrate levels decreased by about two orders of magnitude in stratum corneum as well as in the pilosebaceous unit. After a period of 1 week post the last application, approximately equal amounts of the substance were recovered from the hair follicle and from the stratum corneum indicating a shift in the distribution of the drug within the skin. A reason could be that the hair follicle represents a protected reservoir, not being influenced by textile contact, washing and desquamation. Only slow processes such as sebum excretion and hair growth are able to drain the follicular reservoir [7]. In consequence, desquamation of stratum corneum layers might have resulted in a faster elimination from the stratum corneum compartment as compared to the PSU. These results are in concordance with previous studies demonstrating the long-term storage of topically applied substances for more than 10 days [24]. As a consequence, the differential stripping method used in the present study is better suited to analyse the penetration and storage of the drugs in the target structures (stratum corneum and hair follicle) in vivo in humans than the method of taking tissue biopsies and/or urine samples as the procedure is less invasive and avoids the use of radiolabel.

As aforementioned, Ruppert [25] was able to show that the hair follicles contain a high concentration of bacteria. For fungi, similar results could be demonstrated by Ossadnik et al. [7]. Contrary to the skin surface, it is somewhat difficult to remove or reduce bacteria and fungi from the hair follicles by washing, mechanical abrasion or disinfection measures. Consequently, the anti-microbial or anti-fungal substances must efficiently penetrate into the stratum corneum and hair follicles, to be stored there over an extended period in order to inhibit the growth of bacteria and fungi and possibly also prevent the skin from re-infection.

The results of the present study showed that Isoconazole nitrate penetrates into the stratum corneum and also into the hair follicles and is stored there very efficiently. During the study, relevant concentrations were detected in both the stratum corneum and in the hair follicles well above the minimum inhibition concentration for Isonconazole, which should in clinical use result in an effective inhibition of the growth of fungi and bacteria (Fig. 1). Under the given study conditions, the test product did not induce relevant changes in transepidermal water loss values as a measure for epidermal barrier function. All TEWL values were within the range of normal barrier function meaning that the corticosteroid compound of the drug applied did not induce any impairment of barrier function.

In diseased-skin conditions such as infected eczema or inflamed fungal infections, the epidermal barrier function is impaired [26]. Thus, Isoconazole is likely to penetrate in higher amounts than shown in the present study.

In summary, it can be stated that the differential tape stripping method is well suited to investigate and differentiate the penetration and storage of topically applied substances into the stratum corneum and the hair follicles. The model substance Isoconazole nitrate investigated in this study could still be detected in the stratum corneum and the hair follicles, in relevant concentrations, up to 2 weeks after the topical application had terminated. Also, the hair follicles represent a long-term reservoir for topically applied substances. This is of clinical importance, where a long-lasting therapeutic effect beyond the application time is required, for example, for prevention of re-infection.

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