

The effect of irritant dermatitis on cutaneous bioavailability of a metronidazole formulation, investigated by microdialysis and dermatopharmacokinetic method

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Background: Determination of drug penetration in diseased skin represents a challenge.

Objective: To compare dermal microdialysis and tape-strip sampling of drug penetration in normal skin and skin with irritant dermatitis.

Methods: The two methodologies were employed simultaneously in 16 healthy volunteers. Samples were collected in a study of the penetration of a metronidazole cream formulation (Flagyl[®] 1%) applied to forearm skin in both areas with irritant dermatitis and normal skin. Barrier perturbation and the depth of microdialysis probes were quantified by non-invasive bioengineering methods.

Results: Microdialysis showed a significant threefold increase in metronidazole penetration in skin with irritant dermatitis compared with unmodified skin. Conversely, the concentration of metronidazole in tape-strip samples was significantly decreased in irritant dermatitis.

Conclusion: The selection of sampling methodology should be based on the skin layer of interest as well as the integrity of the skin barrier. Whenever the dermal tissue is the target for topical treatment, microdialysis sampling should be the method of choice.

Key words: irritant dermatitis; metronidazole; microdialysis; tape-strip sampling; topical penetration. © Blackwell Munksgaard, 2008.

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Dermal microdialysis (DMD) and tape-strip sampling, also called the dermatopharmacokinetic (DPK) method, are both well-established sampling techniques for studies of topical penetration of xenobiotics.

Feldman and Maibach (1) were the first to apply the DPK method for the investigation of topical penetration, and in 1991, Anderson et al. (2) reported the determination of ethanol penetration across the skin surface by microdialysis sam-

pling in the dermis. Since then, numerous studies have supported the use of both methods for sampling of exogenous substances in healthy skin. Little is known, although, about the performance of these two different techniques *in vivo* in diseased skin.

Both methodologies are robust and reliable, but they sample from two different layers of this target organ. The DPK method samples substances from the outermost layer of the skin, the stratum corneum. In contrast, microdialysis sampling is conducted in the middle of the dermis and allows a continuous real time monitoring of the events happening in this viable layer of the skin. The stratum corneum is the main limiting barrier of the skin for substances entering from the surface.

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The purpose of this study was to use microdialysis and tape stripping to investigate the topical penetration of metronidazole after application of a 1% cream formulation of it (Flagyl[®] 1%; Aventis Pharma A/S, Horsholm, Denmark) onto both normal skin and skin with irritant dermatitis. The intention was to investigate how these techniques would perform in diseased skin compared with the normal situation as well as to establish whether the results would be equivalent or otherwise correlated to each other.

Metronidazole was used as a model drug for this study as it is suitable both with regard to measurable skin penetration in the chosen formulation and to sampling by both DMD and DPK as shown in a previous study (3).

Materials and Methods

Subjects

16 healthy volunteers participated in the study: 6 women and 10 men aged 32 ± 13 years (mean $\pm$ SD). They had no allergy to metronidazole or local anaesthetics, and none of the subjects was using topical or systemic medication. The study was undertaken in accordance with the Declaration of Helsinki. All participants gave written informed consent; the study was approved by the Copenhagen County ethics committee (ref. KA 05032) and notified to the Danish Medical Agency.

Drugs and chemicals

Flagyl cream 1% (Aventis Pharma A/S) was used as received as well as Xylocaine[®] injection (lidocaine 10 mg/ml; AstraZeneca, Albertslund, Denmark) for the local anaesthesia. The perfusate consisted of sterile isotonic saline solution (sodium chloride 9 g/l, 308 mmol/l). Sodium lauryl sulfate (SLS) (>99% purity) was purchased from Sigma-Aldrich (Munich, Germany) and dissolved in distilled water.

Irritant dermatitis

2 days prior to the experiment, irritant dermatitis had been initiated on the volar aspect of the left forearm by application of 100 μ l of 1% SLS onto 2 layers of filter paper measuring 2.0×2.6 cm, kept in place on the skin by a frame of Comfeel[®] hydrocolloid dressing. Similarly, on the right arm, 50 μ l of 1% SLS was applied onto filter paper in 2 circular areas measuring 1.2 cm of diameter. The SLS was kept in place for 24 hr and removed 24 hr before the experiment. The location of the areas with irritant dermatitis was randomized separately in both arms. A typical description of the

skin with irritant dermatitis was slight-to-moderate erythema with superficial dryness.

Topical treatment

Flagyl cream was applied simultaneously at $t = 0$ to the 6 investigational areas on the volar aspect of both forearms using pre-weighed glass spatulas with a pre-weighed (Mettler Toledo PB303) amount of formulation 3 mg in excess of the intended dose; a dose of 4 mg/cm² was applied (as per American Association of Pharmaceutical Scientists (AAPS)/ Food and Drug Administration (FDA) Workshop Report, 1998) (4) directly onto the skin surface and with no occlusion.

On the left arm, the formulation was applied onto 2 areas (1 with normal skin and 1 with irritant dermatitis) measuring 2.6×2.0 cm. On the right forearm, the cream was applied onto 4 circular areas, 2 with normal skin and 2 with irritant dermatitis (for stripping after 30 and 120 min, respectively, in each situation).

In vivo microdialysis

After non-invasive measurements of skin barrier function, a gentle wash of both forearms was followed by demarcation of the penetration areas on the skin surface with a permanent marker pen. Local anaesthesia (1% Xylocaine) was injected in the dermis/subcutaneous tissue around the demarcated areas (volume <20 ml). In each area, 2 probes were inserted in parallel to each other and to the skin surface using a 22 G guide cannula and the outlet placed in a 300- μ l glass vial. Perfusion was then started at a low flow by ultra precise syringe drivers (CMA100). After an equilibration period of 1 hr following probe insertion (5), microdialysis sampling was started at a flow rate of 1.174 μ l/min. After 20 min (the blank sample), the formulation was applied to the surface of the skin above the probes. The length of the membrane accessible to microdialysis was 3 cm in all experiments. The samples were collected every 20 min for 3 hr, giving sample sizes of 23.46 μ l for analysis.

Relative recovery was calculated by *in vivo* retrodialysis for an earlier study, and given that the experimental set-up was the same as in the present study, we assumed that the same value (34%) would be valid for this investigation.

In vivo tape-stripping experiments

5 investigational areas were outlined on the volar aspect of the right forearm, 2 areas with unmodified skin and 2 areas where a well-demarcated

irritant dermatitis had been generated. D-squame[®] tapes with a diameter of 22 mm (CuDerm[®]; CuDerm Corp., Dallas, TX, USA) were used. Harvesting was performed 30 and 120 min after cream application, respectively. The first 2 discs were discarded and discs 3–12 stacked and placed in an airtight container and stored at -35°C until analysis.

A control area just above the wrist was stripped at the beginning of the experiment to establish a baseline of drug content in the skin. The concentration was below the limit of detection (0.2 ng/ml of the final sample solution) in all tape-strip control samples.

Quantification of barrier perturbation by non-invasive measurements

At the beginning of each experiment, the integrity of the skin barrier on the areas with both unmodified skin and skin with barrier perturbation inflicted by the SLS provocation was quantified in triplicate by non-invasive measurements of erythema and transepidermal water loss (TEWL), using a Minolta Chromameter[®] CR 300 (a* parameter) and a evaporimeter (Dermalab[®]; Cortex Technology, Hadsund, Denmark).

Ultrasound measurement of probe depth and skin thickness

At the end of each microdialysis experiment and with the probes still lying in the skin, probe depth and skin thickness were measured by 20 MHz ultrasound scanning (Dermascan-C; Cortex, Hadsund, Denmark). Skin thickness was measured in triplicate (medial, middle, and lateral) on each investigational area and between the areas to have a picture of the thickness of the skin not altered by the presence of the probes.

Analysis of metronidazole in microdialysates and tape strips

The microdialysates were analysed without any pretreatment. The liquid chromatography-mass spectrometry (LC-MS) system used consisted of an 1100 series chromatograph of Agilent Technologies (Palo Alto, CA, USA) equipped with a Quat-pump, a degasser, column oven (colcomp), an autosampler (ALS), and Mass Selective Detector (MSD). Data were collected using the HP CHEM-STATION software, version 6.03 (HP, Palo Alto, CA, USA).

The analytical column was a reversed phase Phenomenex Aqua C18, 3 μm (Rockland Technologies, Newport, DE, USA) column (2 mm internal

diameter $\times$ 75 mm), and the column temperature was 30°C .

A mobile phase consisting of 0.1% formic acid in acetonitrile:water (6.5:93.5 v/v) was used at a flow rate of 0.2 ml/min.

The MSD detector was equipped with an electrospray interface and used in positive mode for SIM detection of the mass 172.0 using the following settings: fragmentor voltage, 65 V and capillary voltage, 1600 V. The temperature of the nitrogen drying gas was set to 350°C with a flow rate of 13 l/min. The nebulizer gas pressure was 30 psig.

The lower limit of quantification of the assay was 0.5 ng/ml of metronidazole in the microdialysate, and the coefficient of variation (CV) was less than 10% for metronidazole tested at all 3 levels of concentration (1, 5, and 20 ng/ml).

To the tape strips (10 pieces on top of one another), 10.0 ml of acetone was added. After treatment in an ultrasonic bath for 30 min, the mixture was left overnight. After a further 30-min treatment in the ultrasound bath, it was confirmed that the tape strips had fallen apart. Then, 15.0 ml of water was added and after mixing the mixture was centrifuged for 5 min at 16 000 *g*. About 500 μl was diluted to 50.0 ml with water and 10 μl of the supernatant was analysed by LC-MS as described. The recovery of metronidazole from the tape strips was found to be 100%.

Statistical analysis

As standard deviation (SD) increased proportionally with the mean concentration in both microdialysis areas of all experiments, log-transformed (natural logarithm transformation) data were used for further analysis. All data are presented as mean $\pm$ SD or standard error of the mean (SEM). The statistical significance of differences between barrier perturbed and unmodified skin was analysed using one-way analysis of variance (ANOVA) followed by pairwise comparisons. Correlations between both methods as well as linear regression were evaluated by ANOVA. $P < 0.05$ was considered significant.

For pharmacokinetic analysis of metronidazole data obtained by microdialysis, the area under the concentration versus time curve (AUC) was used.

Results

Pharmacokinetics by microdialysis

Metronidazole was detectable in all samples after topical application and measurable in all samples from penetration through perturbed skin (Fig. 1; Table 1).

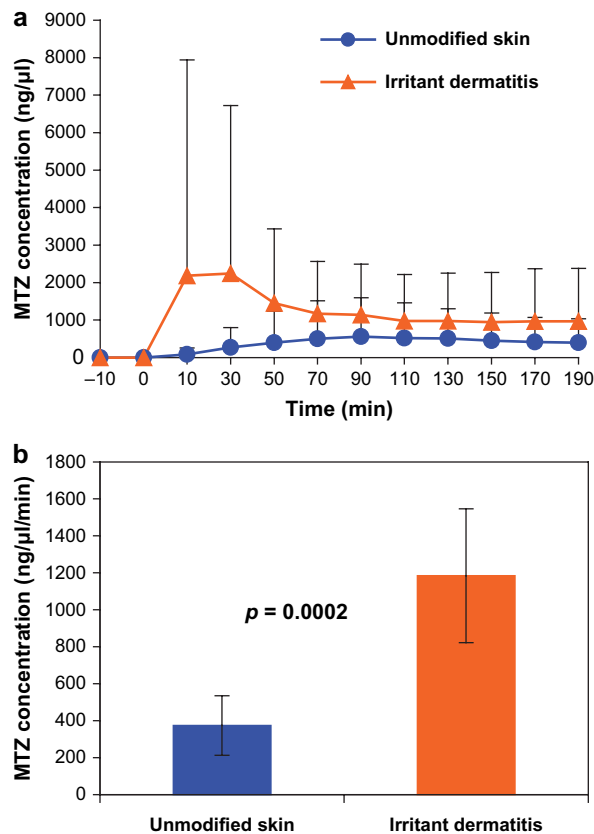


Fig. 1. (a) Microdialysis. MTZ concentration versus time in unmodified skin and skin with irritant dermatitis of healthy volunteers. Error bars are given as SEM. (b) MTZ concentration in unmodified skin and skin with irritant dermatitis by dermal microdialysis. Error bars are given as SEM. MTZ, metronidazole; SEM, standard error of the mean.

The dermal metronidazole concentration sampled by microdialysis expressed as the AUC was threefold increased in the area with irritant dermatitis [1184.3 ± 362.5 ng/μl (mean $\pm$ SEM), 95% CI: 401.3–1967.3 ng/μl] compared with the results obtained in the unmodified skin (373.6 ± 161.3 ng/μl, 95% CI: 25.2–722 ng/μl). This dif-

ference was tested significant by a paired *t*-test ($P < 0.001$).

Variability of metronidazole penetration by DMD. The intraindividual CV found in microdialysate concentrations sampled in normal skin was 72.8% and 49.0% in samples from skin with irritant dermatitis.

The interindividual CV was considerably higher (194% in normal skin and 115% in the irritant condition) because of the natural biological variation between subjects of the same species.

Pharmacokinetics by DPK

The concentration of metronidazole in the stratum corneum, sampled by tape stripping (TS) (Fig. 2; Table 2) was significantly higher in normal skin [TS30 min: 48 ± 9.6 μg/cm² (mean $\pm$ SD) and TS120 min: 47.5 ± 8 μg/cm²] than in skin with irritant dermatitis (TS30 min: 40 ± 10 μg/cm² and TS120 min: 36 ± 15 μg/cm²), with *P* values of 0.003 after 30 min and 0.012 after 120 min.

Variability of metronidazole penetration sampled by DPK. As there was only one measurement per subject in each area for tape stripping harvesting of the skin after 30 and 120 min, it was not possible to calculate the intraindividual CV. The interindividual variation of tape-stripping results was 18% for normal skin and 34% for skin with irritant dermatitis.

Non-invasive bioengineering methods

Transepidermal water loss. TEWL confirmed that moderate barrier damage had occurred. In both arms, significantly higher TEWL values were measured in areas with irritant dermatitis compared with normal skin ($P = 0.007$). Results are shown in Tables 1 and 2.

Minolta Chromameter. The degree of redness of the barrier-damaged skin was also significantly

Table 1. Quantification of metronidazole penetration in normal skin and skin with irritant dermatitis by dermal microdialysis*

Barrier perturbation	Unmodified	Irritant dermatitis (SLS 1%)	<i>P</i> value
Number of subjects	16	16	
Number of probes	32	31	
TEWL (g/m ² ·h)†	5.7 ± 3.3	17.1 ± 15.6	0.007
Erythema†	5.1 ± 2.0	6.5 ± 2.7	0.04
Skin thickness (mm)†	1.53 ± 0.12	1.56 ± 0.18	0.5
Probe depth (mm)†	0.7 ± 0.15	0.6 ± 0.14	0.2
AUC (ng/μl·min)‡	373.4 ± 161.3	1184.2 ± 362.45	<0.001
95% CI (ng/μl/min)	(25.0–721.8)	(401.3–1967.1)	
Coefficient of variation (%)	72.8	49.0	

AUC, area under the concentration versus time curve; TEWL, transepidermal water loss.

*Results from microdialysis were log transformed before further statistical analysis.

†Results are expressed as mean $\pm$ SD.

‡Results are expressed as mean $\pm$ SEM.

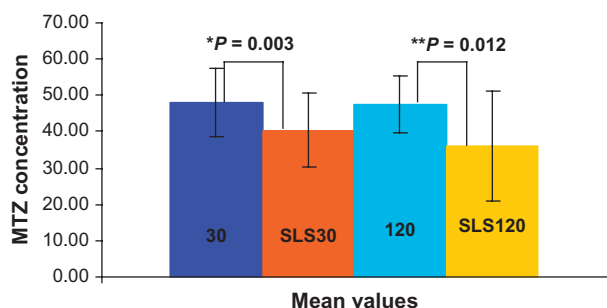


Fig. 2. Tape stripping. Concentration of MTZ obtained by tape stripping after 30 and 120 min, respectively, in both normal skin and skin with irritant dermatitis (marked S30 and S120). **P* value for the difference of MTZ concentration between normal and perturbed skin after 30 min. ***P* value for the difference after 120 min. MTZ, metronidazole; SLS, sodium lauryl sulfate.

increased compared with normal skin, with *P* levels of 0.04 on the microdialysis arm and 0.03 on tape-strip locations (Tables 1 and 2).

Ultrasound scanning. Probes were reproducibly placed at a depth of 0.68 ± 0.11 mm in the dermis. No significant difference in probe depth was found between normal and damaged skin (*P* = 0.2). The ultrasound scans showed that all probes were confined to the dermis, except at the point of entry and exit.

No significant difference in the thickness of the skin between normal [1.53 ± 0.12 mm (mean $\pm$ SD)] and perturbed skin (1.56 ± 0.18 mm) was found (*P* = 0.5).

Correlation between DMD results and non-invasive bioengineering methods

In diseased skin, a strong positive correlation between the penetration of metronidazole and ery-

thema ($r = 0.7$ and *P* = 0.003) for each individual was found (Table 3). The correlation found between metronidazole penetration and TEWL values was also positive but not strong enough to be significant ($r = 0.5$ and *P* = 0.06). Interestingly, in unmodified skin, a strong positive correlation between metronidazole levels in the skin and TEWL measurements was found too ($r = 0.7$ and *P* = 0.002) as well as positive but not significant correlation between metronidazole concentration and erythema ($r = 0.5$ and *P* = 0.06).

Between unmodified skin and skin with irritant dermatitis, no significant differences in either the skin thickness of the volunteers or the depth of the probes were found. Consequently, there was no significant relationship between the concentration of metronidazole sampled by DMD and results from the ultrasound scanning (Table 1).

Correlation between DPK and non-invasive bioengineering methods

In unmodified skin, no significant correlation between metronidazole concentrations and values from TEWL and erythema measurements could be shown. In the results from the irritant dermatitis, a positive correlation between metronidazole concentrations in the stratum corneum after both 30 and 120 min and the degree of barrier impairment measured by TEWL ($r = 0.4$, *P* = 0.01 and $r = 0.6$, *P* < 0.001, respectively) was found in both locations (Table 3).

Discussion

In the present study, DMD and DPK have been used for the determination of cutaneous penetration

Table 2. Quantification of metronidazole penetration in normal skin and skin with irritant dermatitis by DPK

Barrier perturbation	Unmodified skin		Irritant dermatitis (SLS 1%)	
	TS30 min	TS120 min	TS30 min	TS120 min
Number of subjects	16	16	16	16
TEWL (g/m ² /h)*	5.5 $\pm$ 2.6	5.9 $\pm$ 3.5	15.8 $\pm$ 10.4	15.2 $\pm$ 8.2
Erythema*	5.1 $\pm$ 1.6	5.1 $\pm$ 1.9	6.6 $\pm$ 2.5	6.1 $\pm$ 2.0
Concentration (μ g/cm ²)*	48.1 $\pm$ 9.6	47.5 $\pm$ 7.9	40.4 $\pm$ 10.2	35.9 $\pm$ 15.3
95% CI (μ g/cm ²)	(42.9; 53.3)	(43.2; 51.8)	(34.9; 45.9)	(27.6; 44.2)
Coefficient of variation (%)		18.2		34.0
<i>P</i> values				
Areas of TS30 min				
MTZ concentration			0.003	
TEWL			<0.001	
Erythema			0.03	
Areas of TS120 min				
MTZ concentration			0.012	
TEWL			<0.001	
Erythema			0.03	

MTZ, metronidazole; DPK, dermatopharmacokinetic; TEWL, transepidermal water loss; TS, tape stripping.

*Results are expressed as mean $\pm$ SD.

of a topical formulation in normal skin and skin with irritant dermatitis.

DMD showed a significant threefold higher dermal concentration of metronidazole in the presence of skin barrier damage than in unmodified skin. This is concordant with the results reported by Benfeldt et al. (6), who found a 46-fold increase in the dermal concentration of salicylic acid, sampled by microdialysis, in irritant dermatitis provoked by 1% SLS. A few studies have previously reported similar results; thus, Moon (7) performed an *in vivo* animal investigation in hairless guinea-pigs, demonstrating a two- to fourfold increased transcutaneous absorption of hydrocortisone and benzoic acid after inducing barrier perturbation by 2% SLS, and similar to the human experiments, Benfeldt et al. (8) demonstrated a 90-fold increase in the dermal concentration of salicylic acid after perturbation with 2% SLS in an *in vivo* hairless rat study. The order of magnitude of this increase was closely related to the vehicle used. Whereas the current study investigates a commercially available formulation, the salicylic acid studies (6, 8) employed continuous drug administration (infinite dose) of salicylic acid dissolved in 100% ethanol as vehicle. This vehicle is known to markedly enhance percutaneous penetration across the skin barrier.

Controversially, in 1991, Wilhelm et al. (9) reported that hydrocortisone penetration in the dermis and epidermis decreased by 70% after barrier perturbation by 0.5% SLS for 24 hr, whereas the penetration of concentration of indomethacin, ibuprofen, and acitretin was unaffected by the presence of irritant dermatitis. However, the systemic absorption of these 3 last drugs was significantly increased, showing a higher penetration through the skin in absence of barrier integrity. Hence, the results from these different investigations support what was found by microdialysis sampling in the present study, namely that in the absence of barrier integrity, the cutaneous drug penetration increases.

In contrast to the above, the concentration of metronidazole in the stratum corneum, sampled by DPK, was significantly lower in skin with irritant dermatitis compared with unmodified skin. Jakasa et al. (10) have recently showed a threefold increase in the permeability coefficient of polyethylene glycols in skin after barrier perturbation by 5% SLS 24 hr prior to the investigation compared with normal skin by tape stripping. In this investigation, 5% SLS was applied and the experiments were conducted the day after, which is somewhat different from the procedure we have used in the present study.

Thus, considering the findings of this recent publication as well as the consistency of the results from investigations earlier referred, where dermal and systemic drug concentrations have increased in the presence of skin barrier perturbation by SLS, it seems plausible that a faster initial drug penetration into and through the epidermis can account for the lower concentrations found in the stratum corneum. Furthermore, this explanation is corroborated by the dermal pharmacokinetics of metronidazole penetration as sampled by microdialysis. In Fig. 1a, the difference between the metronidazole penetration in irritant dermatitis and unmodified skin (i.e. the initial flux of metronidazole) sampled at around 1 mm depth in the skin during the first 20 min following application of the formulation can be observed. Although this is 'direct evidence' of a faster metronidazole penetration in irritant dermatitis, we admit that a follow-up experiment where tape stripping was conducted at e.g. 5, 10, 15, and 20 min following drug application would clarify our finding of reduced metronidazole content in tape-strip samples from SLS-pretreated skin further.

The intraindividual variation of DMD results was higher (72.8% in unmodified skin and 49% in skin with irritant dermatitis) than in our previous investigation, where the bioequivalence of 3 marketed metronidazole cream formulations in healthy skin were studied and were found to have a within-subject variability of 34% (3). In general, the intraindividual variability of the DMD studies of topical penetration presented in the literature ranges between 30% and 90%; for an overview over the coefficients of variation in 8 different bioequivalence studies by microdialysis, see McCleverty et al. (11). Biological differences in the skin barrier function of subjects of the same species can account for the variability found in the unmodified skin. This is graphically represented in Fig. 3, where the concentration versus time curve for metronidazole penetration in unmodified skin of every subject is shown. Here, it is possible to appreciate the spread of the normality range of cutaneous penetration of metronidazole and consequently the normal biological variation. In the present study, microdialysis results were less variable in skin with irritant dermatitis than in unmodified skin. Hence, it can be thought that, in irritant dermatitis, the function of the stratum corneum is altered and that this modification consequently reduces group diversity, resulting in a more uniform penetration and lower variability.

The variability found in DPK results (18% in unmodified skin and 34% in the diseased skin) was approximately of the same magnitude as those observed in the previous bioequivalence

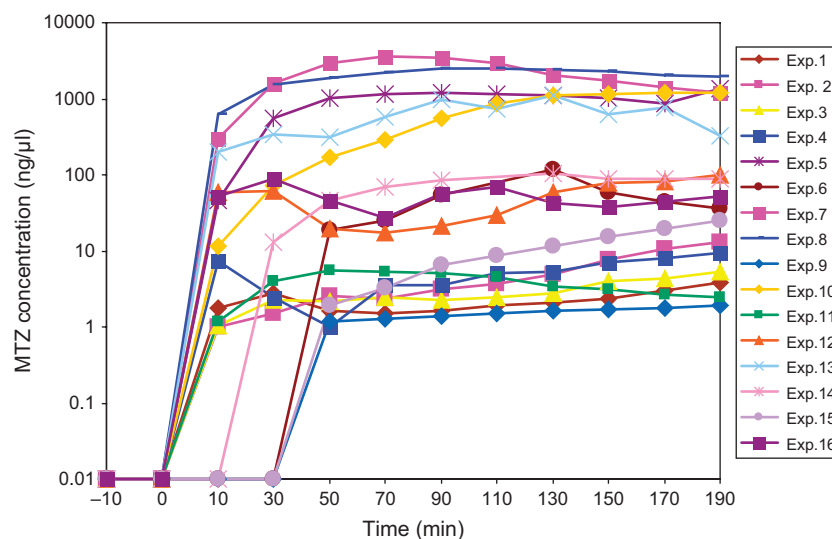


Fig. 3. Microdialysis. Concentration versus time curves for MTZ penetration in unmodified skin measured by dermal microdialysis for 3 hr ($n = 16$, mean of results from 2 probes in each volunteer). Note the log-scale of the y-axis. MTZ, metronidazole.

study (26.5%), and although the variability is increased in irritant dermatitis, the overall results support the robustness of this sampling method.

No significant differences in the thickness of the skin between normal and perturbed skin were found ($P = 0.5$), in contrast to the positive correlation shown by Benfeldt et al. (12) between the thickness of the skin and the severity of barrier damage.

This can be explained by the fact that the irritant reaction evoked in the present study was very mild and resulted in modest erythema and surface dryness.

We have used SLS to induce a significant but controlled irritant dermatitis as a model for skin disease. SLS is an anionic surfactant that concentrates at oil–water interfaces and holds both cleansing and emulsifying properties. The skin toxicity of SLS arises from its damaging influence on the stratum corneum, leading to impaired barrier function and inflammation. In the present investigation, the irritant dermatitis induced by 1% SLS was very modest, which may be the reason for the inconclusive results obtained by the

study of correlations between non-invasive measurements of skin barrier function and metronidazole penetration.

The observations derived from the present study have a notable importance for the development of new dermatological products. New dermatological formulations are currently evaluated in healthy subjects, despite their future clinical use in application onto diseased skin. Both DMD and tape stripping are shown to be applicable for sampling of drug concentrations in the dermis and stratum corneum, respectively, after topical administration in healthy and damaged skin. However, the results are not the same. Here, we have demonstrated that a modest irritant dermatitis results in a threefold increase in dermal drug concentration as measured by microdialysis, whereas DPK did not disclose this increase in penetration. Hence, whenever the dermis is the target organ, DMD sampling is the most relevant method for studies of topical penetration. Furthermore, microdialysis methodology should be preferred when the integrity of the skin barrier in altered or skin disease affecting the barrier function is present.

Table 3. Correlation coefficients between tape strip data and microdialysis concentrations versus values from non-invasive measuring methods of the skin*

	Dermal microdialysis MTZ concentration		Dermatopharmacokinetic MTZ concentration			
	Unmodified skin	Irritant dermatitis	Unmodified skin		Irritant dermatitis	
			30 min	120 min	30 min	120 min
Transepidermal water loss	0.7	0.5	0.2	0.2	0.4	0.6
Erythema	0.5	0.7	0.04	0.02	0.07	0.2

MTZ, metronidazole.

*Values in bold are significant correlations ($P < 0.05$).

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