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Bioequivalence of Topical Formulations in Humans: Evaluation by Dermal Microdialysis Sampling and the Dermatopharmacokinetic Method

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The aim of this study was to evaluate the relationship between dermal microdialysis (DMD) sampling and the dermatopharmacokinetic method when employed simultaneously for bioequivalence (BE) investigations of topical formulations. Topical lidocaine cream and ointment (both 5%) was investigated in eight healthy human volunteers (four male, four female). On one forearm, four microdialysis probes in two penetration areas sampled for 5 hours, and on the other arm, tape stripping was performed 30 and 120 minutes after product application. Lidocaine content in samples was analyzed by HPLC–mass spectrometry. The two methods were in agreement showing 3- to 5-fold higher lidocaine penetration from cream formulation than from ointment. A rank-order correlation between the two methods was demonstrated for lidocaine contents in microdialysates *versus* tape strip at 120 minutes, significant for the ointment formulation and for both formulations analyzed together. Analysis of variance demonstrated reproducible lidocaine concentrations in microdialysates with an intrasubject variability of 19% between probes and 20% between the two penetration areas. Thus, intersubject variability accounted for 61% of the variance. DMD sampling proved effective and variability analyses demonstrated the feasibility of BE studies in as little as 18 subjects.

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INTRODUCTION

The dermatopharmacokinetic (DPK) principle for bioequivalence (BE) determination of topical drug products has come under criticism that the measurement of drug represents the concentration in ''dead tissue''. The primary aim of this research was to evaluate the relationship between DPK and dermal microdialysis (DMD) methodology, where the latter represents the drug concentration in the living tissue.

We employ the DPK method (Shah, 2001), where tape-stripping harvesting of stratum corneum (SC) samples the outermost layers of the skin, with simultaneous sampling in the dermis by microdialysis sampling. The sampling matrix is thus essentially different, and the overall evaluation concerns the relationship between the results obtained with the two methodologies.

Second, we wished to evaluate the usefulness of DMD for topical BE studies in human volunteers, and in particular to identify and quantify the variability components.

Third, based on the results, we wished to estimate the number of subjects or patients necessary in future microdialysis protocols investigating BE of topical formulations in healthy skin.

Background

In the determination of BE of topical products, the method of choice is the tape-stripping technique, also called the DPK method (Shah *et al.*, 1998; Food and Drug Cosmetics Act, 2002). The method consists of a standardized protocol of repeated applications and removal of adhesive tape on the skin surface, whereby consecutive layers of SC cells are sampled. Using analysis of each tape strip, it has been shown that the drug concentration in the SC decreases loglinearly, and that about 90% of the concentration is found in the first 10 strips. The next 10 strips contribute less than 5% (Caron *et al.*, 1990).

Auxiliary techniques can be used to further determine the mass of SC cells or thickness removed by the sampling procedure (Weigmann *et al.*, 1999a, b), or by quantification of SC mass by protein analysis of strips (Dreher *et al.*, 2005).

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Abbreviations: AUC, area under the curve; BE, bioequivalence; CI, confidence interval; DMD, dermal microdialysis; DPK, dermatopharmacokinetics; SC, stratum corneum; CV, coefficient of variation

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In the application of the method for assessment of drug penetration deeper than the cell layers of the SC, the predictive value of the method relies on the studies by Rougier *et al*. (1987). For compounds with different physicochemical characteristics, Rougier *et al*. have shown a good correlation between the concentration in SC at 30 minutes and their systemic absorption over 4 days (using radiolabelled compounds and excretion collection and analysis).

This is particularly relevant for drugs aiming at an effect at a deeper level in the epidermis or in the dermis as a target organ. The DPK method has not been standardized for use in diseased skin. The weakness of the DPK approach is the endpoint nature of the data obtained and the lack of studies confirming the correlation between DPK data and clinical efficacy.

Microdialysis technique has been introduced for studies of dermal drug levels after topical drug administration in the last decade (Ault *et al*., 1994; Groth, 1996; Cross *et al*., 1998; Benfeldt, 1999). The method consists of placing an ultrathin, semipermeable hollow fiber structure in the dermis and perfusing this fiber, called a probe, with a tissue-compatible sterile buffer at a very low rate by means of a microdialysis pump (a very precise syringe driver). The probe will function as an ''artificial vessel'' in the dermis and thus exchange small, diffusible molecules from the probe to the tissue and *vice versa* according to laws of simple diffusion (Ungerstedt, 1984; Benveniste and Huttemeier, 1990). The recovery of a given compound closely reflects the concentration of unbound, that is, pharmacologically active, compound in the intercellular fluid of the tissue surrounding the probe. For a review of microdialysis in drug research, see Joukhadar and Muller (2005). For the set-up in the current study, see Figure 1.

The DMD method can provide very detailed chronological pharmacokinetic data and several sampling sites can be studied simultaneously in the same volunteer. The method is currently undergoing rapid development in topical drug penetration research (for a review of microdialysis methodology in skin research, see Groth *et al*., 2006).

The method can be challenging when used for sampling very lipophilic or very highly protein-bound drugs due to low recoveries of these compounds (Benfeldt and Groth, 1998), and it requires training of laboratory personnel to obtain the low variability demonstrated in some studies (Benfeldt, 1999). DMD is comparatively more invasive than DPK. DMD can be performed in barrier perturbed or diseased skin.

Kreilgaard *et al*. (2001) published the first human study demonstrating the potential of DMD for BE studies of topical formulations in 2001.

Similar to DPK, for BE studies DMD also allows the testing of both T (test) and R (reference) product at the same time in the same individuals.

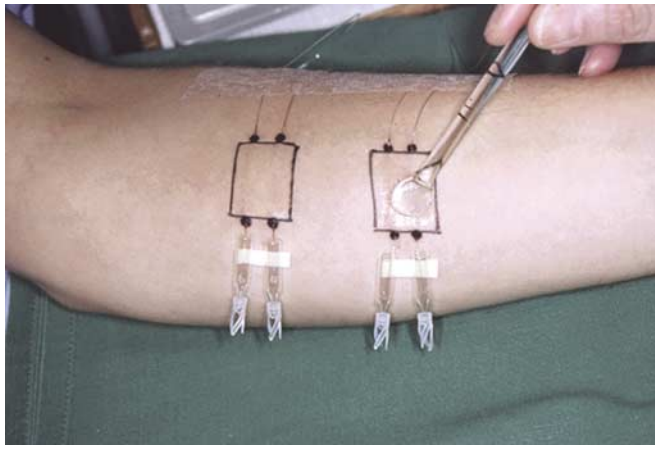

Figure 1. Linear microdialysis probes *in situ* in the dermis. The length accessible to microdialysis sampling is 3 cm. A microdialysis pump provides the perfusate flow of 1.25 μl/minute. Samples of 25 μl are collected every 20 minutes for 5 hours. At $t = 0$, the topical formulation is applied in a dose of 4 mg/cm^2 and left throughout the experiment. Markings for insertion of the guide cannula are circles in order to avoid the introduction of a tattoo.

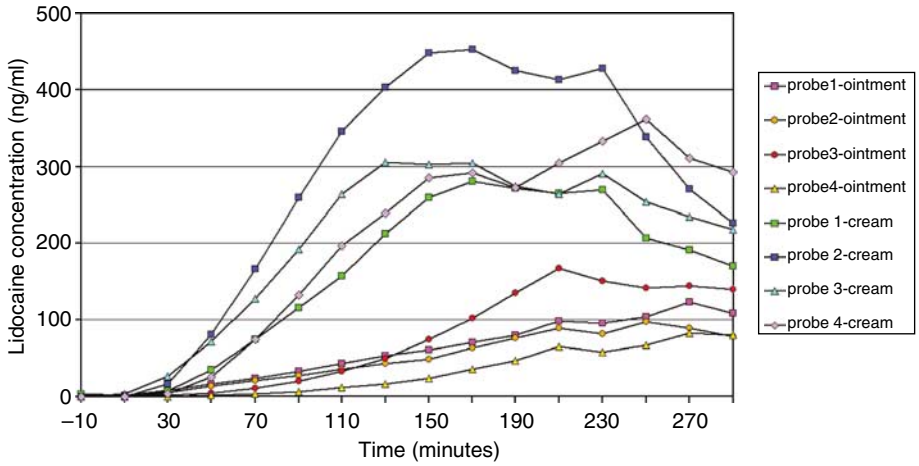

Figure 2. Raw microdialysate concentration data from the two separate experiments conducted in one volunteer. In blue–green, the lidocaine concentration in dialysates from the four probes following topical application of the 5% cream formulation. In orange, the lidocaine concentration in the dialysates from the four probes sampling topical penetration form the 5% ointment formulation. The sample concentration is plotted at mid-interval by convention.

In order to evaluate the relationship between the two methodologies, two formulations known to have different drug penetration profiles (cream and ointment with the same 5% concentration of the active compound) were selected.

RESULTS

Pharmacokinetics obtained by DMD sampling

Both cream and ointment formulation provided measurable dermal concentrations of lidocaine in the dialysates; the result from one subject is shown in Figure 2. Following topical drug application at $t=0$, very rapid dermal drug delivery is seen, providing lidocaine concentrations above the limit of detection sampled only 20 minutes after application of the formulation in most microdialysates.

The cream formulation provided rapid penetration with an almost 5-fold higher area under the curve (AUC) than the ointment formulation. For the whole group of subjects, the pharmacokinetics are shown for both formulations in Figure 3 and summarized in Table 1.

Table 1. Pharmacokinetics by dermal microdialysis

	AUC (ng/ml min)	C_{max} (ng/ml)	T_{max} (min)	Lag time (min)
Cream				
Mean	15,983	112	243	26.0
CV (%)	41	41	5	18
95% CI	6,317–444,835	44–3,132	214–272	20.2–31.9
Ointment				
Mean	3,309	27.5	275	45.6
CV (%)	42	41	2	27
95% CI	1,271–8,612	11–71	260–290	26.6–64.6
Paired *t*-test *P*-value	0.018	0.030	0.006	0.060

AUC, area under the curve; CV, coefficient of variation; CI, confidence interval.
Number of subjects: 8.
AUC and C_{max} were log transformed and geometric means are shown.

T_{max} has a higher value and smaller variance for ointment than for cream formulation. Conversely, for the cream formulation, the variance for lag time is large, as lag time is generally shorter for cream formulation and values thus lower.

No significant differences in AUC were present between the four probes or the two areas.

Analysis of possible co-variables

Measurements of *skin thickness and probe depth* (Figure 4) did not show any significant correlation with microdialysis kinetics. A difference in skin thickness between the cream group (1.39 ± 0.01 mm; mean $\pm$ SEM) and the ointment group (1.48 ± 0.01 mm) could be attributed to the hydrating effect of the occlusive ointment formulation. The measurement was performed at the end of the experiment.

Measurements of *probe depth* demonstrated highly accurate placement horizontally within the dermis at a depth of 0.91 ± 0.02 mm in the cream series, 0.83 ± 0.01 mm (mean $\pm$ SEM) for the ointment series. This slight difference in mean depths between the two groups, which we attribute to a time effect of increased routine in probe insertion during the series of experiments, had no significant effect on lidocaine measurements.

Analyses of the influence of *remaining co-variables* (actual dose applied, gender, age, room temperature, and humidity; 70 possible correlations) were without significant findings of any effect on lidocaine kinetics, sampled by microdialysis.

The *prilocaine concentration* in the perfusate, added as a calibrator for estimation of lidocaine recovery by the probe, proved to be of no advantage owing to an inexpedient procedure during the initiation of the experiment. Owing to inadequate flushing out of the dead space in the inlet tubing of the probe, the prilocaine concentration in the initial microdialysis samples was very variable and reflected variable calibrator concentration in the perfusate and not loss of calibrator as intended. Hence, we chose not to use prilocaine data to correct lidocaine recovery data.

The average recovery by loss of prilocaine between 110 and 290 minutes, once the dead space was cleared, was 70%. The intraindividual coefficient of variation (CV) for prilocaine

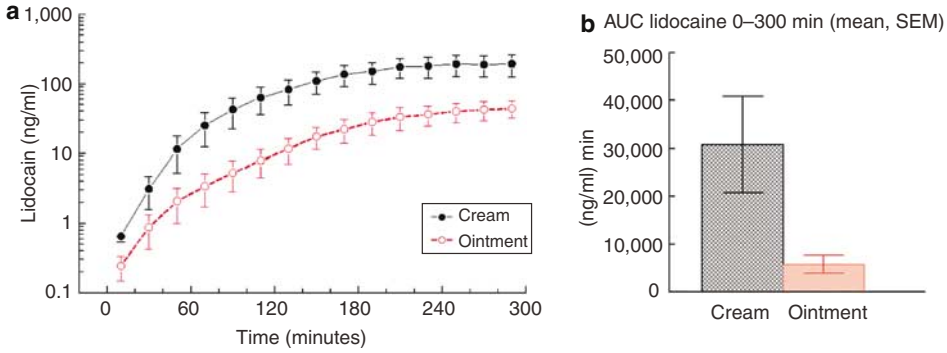

Figure 3. Different penetration from the two formulations: DMD methodology. (**a**) Pharmacokinetics obtained by microdialysis, showing the dermal lidocaine penetration from the cream (solid black) and ointment (red line) for all volunteers ($n=8$). Means with SEM in log-scale. (**b**) Mean AUC with SEM for cream (black) and ointment (red) formulation, sampled by microdialysis.

recovery by loss was 13% and the interindividual CV was 16%.

Pharmacokinetics by the DPK method

The tape-stripping procedure provides two end-point measurements for each formulation, but robustly discriminates (Figure 5) between cream and ointment formulations. For cream formulation, the penetration measured by the DPK method at 30 minutes was $96 \pm 8.4\ \mu g$ (mean $\pm$ SEM), which is significantly different from ointment formulation: $35 \pm 1.5\ \mu g$ ($P < 0.0001$).

Similarly for 120 minutes, cream formulation $102 \pm 9.1\ \mu g$ was significantly different from ointment $41 \pm 2.9\ \mu g$ ($P = 0.0004$).

Correlation between the two methods used

Both methods provided the same overall result, with the 5% cream formulation delivering 2.5- to 3-fold more lidocaine to the skin when investigated by the DPK method (significant difference; $P \leqslant 0.0001$) and a 4.8-fold difference when investigated by microdialysis methodology at the same time in the same volunteers (significant difference; $P = 0.018$ and 0.030 for AUC and C_{max}, respectively).

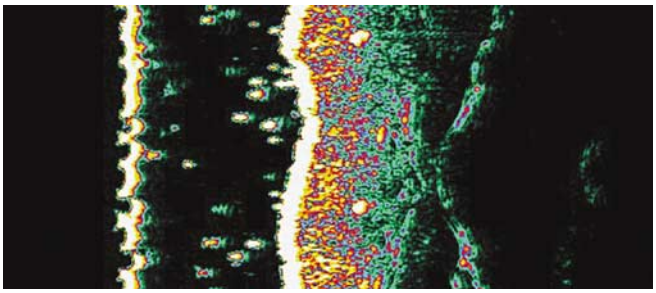

Figure 4. Ultrasound scanning image of two probes in the dermis in one treatment area. The depth of the probes, measured from the entrance echo of the epidermis, is 0.75 mm (upper) and 0.74 mm (lower). The thickness of the skin (dermis and epidermis) is 1.5 mm.

Thus, correlations between the two methods were expected to exist, and pharmacokinetic parameters from DMD were tested against DPK results for both 30- and 120-minute samples (Table 2). Significant correlations were present only with ointment formulation tape strip 120 minutes and AUC, C_{max}, or their logarithms. For dermal microdialysate levels *versus* tape-strip samples, both at 120 minutes, a significant correlation could be seen for cream and ointment data (Figure 6). The correlation is weaker for the cream formulation, for which a higher variability was found by both DMD and DPK methodologies.

When analyzing the correlations using either individual MD sampling points or MD $AUC_{(0-t)}$ *versus* DPK results, these correlations were significant for all time points for ointment, whereas correlations were weaker for the two formulations analyzed together, and absent for cream formulation alone (for details, see Table S1).

BE evaluation by the two methods

Cream and ointment formulations are not qualitatively the same, and therefore the two formulations should not be tested for BE as such. However, ruling out that they are BE can demonstrate the applicability of both DMD and DPK methodologies in BE evaluation. For the two formulations to be BE, the 90% confidence interval (CI) for the ratio between the AUCs must be between the boundaries of 80 and 125% (Schuirmann, 1987).

For microdialysis methodology

The two formulations were tested on two separate occasions in the same eight individuals.

For each person, the difference in log AUC between cream and ointment was calculated. The mean difference in log AUC was 1.575 ± 0.515 (mean $\pm$ SEM, 90% CI 0.599–2.551), and the mean ratio for all subjects was 4.83.

The 90% CI for the ratio cream/ointment is the antilog of the previous interval 1.82–12.82. Thus, the bioavailability of lidocaine from the cream formulation, relative to the ointment, is 483% with a 90% CI of 182–1,282%. The two

Table 2. Correlations between microdialysis and tape-strip data

	Cream formulation				Ointment formulation			
	Tape strip 30		Tape strip 120		Tape strip 30		Tape strip 120	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
AUC	−0.12	>0.50	0.23	>0.50	0.45	0.27	0.80	**0.016**
log AUC	−0.21	>0.50	0.22	>0.50	0.18	>0.50	0.85	**0.0072**
C_{max}	−0.27	>0.50	0.11	>0.50	0.33	0.42	0.80	**0.016**
log C_{max}	−0.27	>0.50	0.13	>0.50	0.11	>0.50	0.84	**0.0087**
T_{max}	−0.80	**0.017**	−0.39	0.35	−0.24	>0.50	−0.51	0.19
Lag time	−0.07	>0.50	−0.27	>0.50	−0.13	> 0.50	−0.64	0.086
TS30	—	—	0.29	0.49	—	—	0.38	0.35

AUC, area under the curve.
The values in bold are significant correlations, the remaining are not.

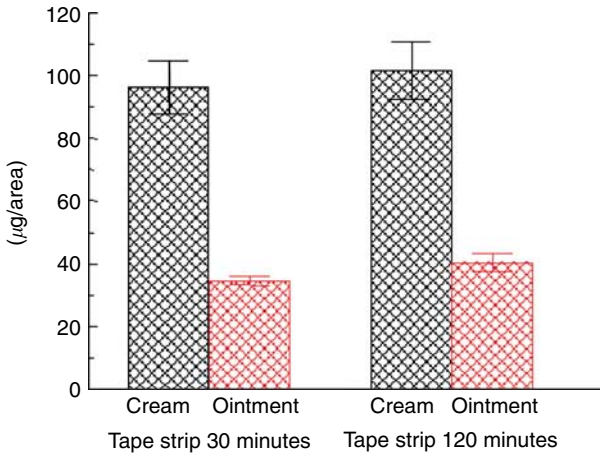

Figure 5. Different penetration from the two formulations: DPK methodology. Pharmacokinetics by tape-stripping methodology. The mean lidocaine content in cumulated tape strips is shown for cream (black) and ointment (red) formulation ($n = 8$), sampled at $t = 30$ and 120 minutes. Error bars are SEM.

formulations are therefore not BE, and cannot be regarded as interchangeable.

For the DPK method

In a similar fashion, the DPK data obtained for the two formulations can be compared with BE evaluation methodology in order to reject BE.

For DPK 30 minutes: Mean ratio for cream/ointment is 2.72, the CI for the difference is 0.849–1.150, and the interval for the ratio is the antilog: 234–316%. This is outside of the 80–125% BE criteria.

For DPK 120 minutes: Mean ratio for cream/ointment is 2.48, the CI for the difference is 0.671–1.146, and the interval for the ratio 196–315%.

Imagining that the two formulations had indeed been similar, and the ratio had been 1, then the above intervals for the ratio would have been 86–116 and 79–127%, which would mean fulfillment of the BE criteria for DPK 30 minutes, but just not fulfillment for DPK 120 minutes. As cream and ointment are two different types of formulations, they should not be tested for BE. The above calculation just illustrates that the two formulations are not BE.

Analysis of variability components in microdialysis sampling

The design of the study facilitated identification of the various components that are integrated in the overall variability of microdialysis sampling of topical drug penetration. The analysis by the variance component model demonstrates the intrasubject variability of 19% between probes and 20% between the two penetration areas. Thus, the intersubject variability accounted for the remaining 61% of the variance observed.

Calculations of sample size for topical BE studies by microdialysis sampling

Based on the analysis of variance above, the number of subjects necessary for a BE study of two formulations by microdialysis can be calculated (Table 3). The huge difference in number of subjects needed, depending on whether the formulations are tested one at a time or simultaneously in the same subject, reflects the impact of the interindividual differences in topical drug penetration, probably founded in differences in the skin barrier function.

The calculated number of 18 subjects for a BE study with three probes in each test area is based on the variability observed in the current study (ointment data set).

DISCUSSION

The result obtained by DPK, shown in Figure 5, demonstrates that there is no significant difference in SC concentration of lidocaine after 30 and 120 minutes. The result also shows that there is a significant difference in SC content of lidocaine between cream and ointment, around 2.5-fold.

The AUC measured by DMD represents the total amount of drug that has penetrated through epidermis and diffused into the dermis over the 5-hour period. The AUCs for 5% lidocaine cream and ointment are significantly different, with the AUC for the cream being almost 5-fold higher than for the ointment (Figure 3).

Thus, an excellent rank-order correlation is found between the DPK result and the DMD result in this paper, which is state-of-the-art in BE of topical products.

These results provide an answer to the DPK critiques. In May 2002, the FDA Draft Guidance Document on ''Pharmacokinetics Applied to Drug Concentration Measurements in Stratum Corneum (SC)'' was withdrawn. This was in part owing to lack of correlation between the drug concentration in SC (by DPK) and clinical efficacy and partly owing to the finding of different DPK results from lab to lab. This latter issue has recently been attributed to the lack of standardization of procedures (Shah, 2005).

The DPK variability in the present study was low (CVs between 12–25%, lowest for ointment). The variability for DPK methodology can be around 50% (Pershing *et al.*, 2003).

The DMD variability, expressed as CV for AUC, in studies of topical drug penetration has previously been found to be 56–61% (Kreilgaard *et al.*, 2001), and the variability has been demonstrated to be introduced by individual drug penetration properties (variability in the skin barrier) in a DMD study in hairless rats (Simonsen *et al.*, 2003).

For the parameters AUC and C_{max}, we have found CVs around 41% (Table 1), and the study design allowed for very accurate analysis of each variability component.

A clear conclusion from the present study is that individual differences in skin barrier function have a large impact (61% of the overall CV) on the variability in topical drug penetration. In order to further characterize the cause(s) of this variability, auxiliary non-invasive measurements of skin temperature, trans-epidermal water loss, colorimetry, blood flow by laser-Doppler perfusion imaging, or even evaluation of SC thickness by tape stripping (Kalia *et al.*, 2001) could be undertaken before or preferably during the DMD experiments.

We investigated two formulations containing a small, relatively hydrophilic compound of interest. When closely

studied, we found significant correlations between the two methods employed for the ointment formulation separately and for the two formulations analyzed together, but no significant correlations for the cream data set (apart from the overall agreement in BE evaluation of the formulations).

In general, only high correlations ($r > 0.8$) can be shown with as little as eight pairs of observations. With a larger study, more correlations may be detectable. Drugs that form a reservoir in the SC, or that only penetrate the epidermis to a small degree, will not exhibit the correlation shown in this study. Again, the two methods sample in different ''compartments'' and are not likely to be interchangeable for any given compound.

DMD is a step closer in providing drug concentrations sampled at the site of action for topical drugs. In the FDA criteria for evaluations of bioavailability (Food and Drug Cosmetics Act, 2002), it is stated that for drugs not intended to be absorbed into the bloodstream, measurements should reflect the rate and extent to which the active molecule becomes available *at the site of action*. The microdialysis technique makes it possible to do exactly this, to sample in the target organ, with real-time chronology.

This approach is far better than expensive clinical trials currently required for generic drug approvals. To gain adequate statistical power, they may require as many as 300 patients (Shah *et al.*, 1998). Employing the DPK method, it has been estimated that between 40 and 50 subjects are needed for a BE study (Pershing *et al.*, 2003). Statistical calculations indicate that with DMD methodology, BE studies with 90% CI and 80–125% BE limits can be conducted in 27 subjects, using two probes in each test area, or 18 subjects using three probes per formulation application site.

The DMD and DPK method resulted in similar rank-order correlation for the two products studied. This provided a much-needed answer to DPK critiques for ''measurements in dead skin'', as in the current study we establish that SC concentration was predictive of the drug concentration in the living part of the skin.

Future studies will be aiming at confirming this for other formulations and other substances, and at exploring the correlation between the two sampling methodologies further.

Table 3. BE study size estimates

Probability (%)	Limits of variation (%)[1]	Two probes per area	Three probes per area
BE study with two formulations in each subject			
80	<25 (80–125%)	20	14
80	<33	13	9
80	<50	8	6
90	<25	27	18
90	<33	17	12
90	<50	10	7
95	<25	33	23
95	<33	21	15
95	<50	12	8
BE study with one formulation in each subject			
80	<25 (80–125)	711	695
80	<33	427	417
80	<50	216	211
90	<25	985	962
90	<33	591	577
90	<50	299	292
95	<25	1,244	1,215
95	<33	746	729
95	<50	378	369

BE, bioequivalence.
Number of subjects required for BE determination of topical formulations in healthy human volunteers, based on intraindividual (upper) and interindividual (lower) variabilities.
[1]The limit of variation for equivalency determination described as ''<25%'' means less than factor 1.25 above and below the mean, thus between 125% and 100/1.25=80%. The fourth row, with 90% probability and limitation of variability to between 80 and 125%, corresponds to the current FDA criteria for BE determination.

MATERIALS AND METHODS

Materials

Xylocaine cream and Xylocaine ointment (both 5% lidocaine) formulations (AstraZeneca, Sweden) were used for topical application.

Sterile perfusate (phosphate-buffered isotonic saline, pH 6.5, with added prilocaine 10 mg/l) was prepared by the Copenhagen County Hospital Pharmacy in sealed 20 ml glass vials (D-Squame Standard discs, 22 mm = 3.8 cm^2 (Cuderm Corp., Dallas, TX)).

Study population

The study was performed in eight healthy human volunteers (four male, mean age 34 years (range 29–37) and four female, mean age 49 years (range 46–55)). All subjects participated on two occasions, where either cream or ointment was investigated, with a minimum recovery period of 6 days. Subjects were free from skin disease, took no regular medication, and refrained from using any topical formulations the 3 days preceding an experiment.

Subjects were given a detailed description of the study and written consent was obtained. The study was approved by the Copenhagen County Ethical Committee (KA 02076gs) and the Danish National Drug Review Board (j. no. 2612-2054), and it was conducted according to the Declaration of Helsinki Principles and in accordance with the Guidelines for Good Clinical Practice.

Microdialysis equipment

Linear microdialysis probes were manufactured in the laboratory employing single fibers from hemodialysis cylinders (Hemophane, Gambro GFS 16+, Hechingen GmBH, Germany), outer diameter 216 μm, molecular weight cutoff 2 kDa, inserted in Portex flexible grade nylon tubing (Astra Tech, Denmark) and fixed with cyanoacrylate glue (Loctite). The probe was stabilized by an internal stainless steel guide wire (0.10 mm diameter, Sandvik Steel, Norway) and prepared 1–3 days before the experiment. (Regarding the effect of an internal wire, see (Klimowicz *et al.*, 2004.)

The perfusate flow was provided from disposable syringes (2 ml) placed in the microdialysis pumps (CMA/100, CMA/Microdialysis AB, Stockholm, Sweden) and connected to the probes by Perifix connectors (B Braun Medical, Germany).

For both *in vitro* and *in vivo* experiments, probes were sterilized by immersion in 70% ethanol/water for 20 minutes and subsequently used before drying.

Dialysate samples were collected in glass vials, capped immediately after sampling, and stored frozen at −35°C.

In vitro microdialysis recovery and loss

Relative recovery *in vitro* was established experimentally to ensure reproducible and concentration-independent sampling of both lidocaine and prilocaine (prilocaine to be employed as calibrator during the *in vivo* experiments) (Larsson, 1991; Kreilgaard *et al.*, 2001).

In brief, these experiments were conducted at room temperature, employing magnetic stirrers (350 r.p.m.) in the plastic beakers with lidocaine content in the medium and prilocaine in the dialysate. The probes were prepared with 30 mm accessible dialysis membrane length and a fixed perfusate flow rate of 1.25 μl/minute was employed, both as used later for *in vivo* experiments.

Values obtained by *in vitro* microdialysis

Lidocaine: Relative recovery 81 ± 6% (two probes per concentration, range 1–5–10–20–50 μg/ml). *Prilocaine*: Relative recovery by loss 21 ± 5% (two probes per concentration, range 1–5–10–20 μg/ml).

There was no sign of either time or concentration dependence and no evidence of interaction between lidocaine recovery and prilocaine loss.

Experimental procedure

Following a standardized wash of the underarms with a drop of mild liquid soap and drying the underarms without rubbing, the subject lies in the supine position with the left arm placed on an armrest. Line drawings on a transparent sheet, demarcating the placement of the penetration areas on the forearms on Day 1, were used for placing the areas well outside of these areas on Day 2.

Left arm: The 2.0 × 2.6 cm areas for topical drug application were drawn on the skin with a felt tip pen and the entrance and exit points for guide cannula insertion marked by circles (see Figure 1). Employing sterile technique, the guide cannulae (22 G Microlance, Becton Dickinson) were placed transversally across the volar forearm in parallel pairs, 0.6 cm apart, in the two areas. Care was taken to obtain a superficial and horizontal placement in the dermis. The probes were taken from the ethanol bath and introduced in the opposite direction through the guide cannulae, which were subsequently withdrawn. Intradermal probe length after insertion was 3.0 cm. After testing probe function, the entry and exit puncture sites were sealed with a drop of cyanoacrylate glue (Sicomet Tixotropic, Kemitura, DK) in order to prevent contamination from topical drug formulation (see later). Perfusion of the probes was started at a low flow rate of 0.2 μl/minute during the 1-hour recovery period (Groth and Serup, 1998).

Before application of the formulation, the perfusate flow was increased to 1.25 μl/minute and microdialysis sampling was started.

Right arm: The three areas for topical drug application were marked on the skin by drawing around large Finn Chamber discs with a felt tip pen and marked A, B, and C going distally on the forearm. Areas A and B were randomized to 30/120 minutes. The C area for control (blank) tape stripping nearest the wrist was sampled (procedure, see below).

Application of formulation (t = 0): At $t = 0$, the formulation was applied simultaneously to all test areas. Using pre-weighed glass spatulas with a pre-weighed amount of formulation 3 mg in excess of the intended dose (Mettler Toledo PB303), a dose of 4 mg/cm^2 was applied (12 mg to areas for tape stripping and 20 mg to microdialysis areas).

Right arm: A fenestrated eye shield (ref. 0002, Aaron Medical, St Petersburg, FL) was placed over the area due to be sampled at 120 minutes in order to protect against contamination. At 30 and 120 minutes, the tape-strip areas on the right arm were sampled.

Tape-strip procedure: Skin surface was wiped twice with cotton gauze to remove residual formulation. Tape discs were applied and removed by pincers, using gentle pressure with the blunt end after application to assure good skin contact, and alternating strip removal directions (N, S, E, and W). The first two discs were discarded and discs 3–12 stacked and placed in an airtight glass container and stored at −35°C.

Left arm: Continuous microdialysis sampling was started at a perfusion flow rate of 1.25 μl/minute and samples collected every 20 minutes for 5 hours.

At the end of the experiment, the probes were disconnected from the pumps, ultrasound scanning was performed, probes were removed, and a small bandage applied to protect the insertion areas the remainder of the day.

Room temperature and humidity were recorded at the start and end of the experiment.

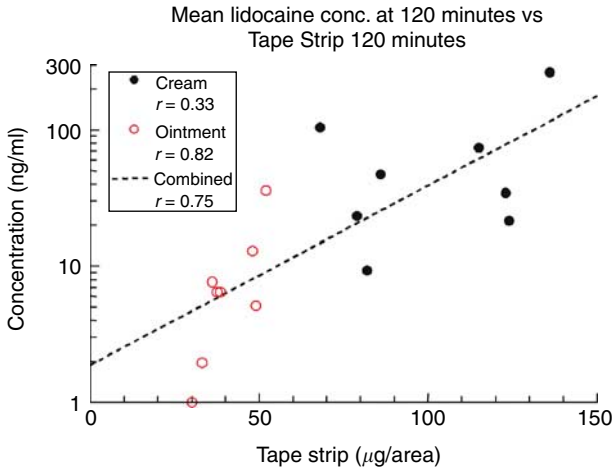

Figure 6. Correlation between DMD and DPK methodologies. Correlation between drug content in tape-strips, sampled at 120 minutes, *versus* dermal drug levels, sampled by microdialysis, at 120 minutes. Solid black, cream formulation ($r = 0.33$, $P = 0.42$), red circles, ointment formulation, ($r = 0.82$, $P = 0.013$) and both combined ($r = 0.75$, $P = 0.0007$).

Ultrasound scanning

High-frequency B-mode ultrasound scanning images using the Dermascan C (Cortex, Denmark) (Serup *et al.*, 1995) were obtained at the end of the experiment (Figure 4). As described previously (Benfeldt *et al.*, 1999), using the A-mode interface, measurements of probe depth and skin thickness were performed in triplicate over

each probe (over the first, middle, and last third of the probe length in the skin). All values concerning probe depth and skin thickness are thus mean of three measurements.

Analysis of samples

Chemicals: Acetonitrile, ammonium acetate, acetone, and formic acid were obtained from Merck (Darmstadt, Germany) and were of analytical reagent grade.

Liquid chromatography–mass spectrometry method: Apparatus: Agilent 1100 HPLC system equipped with a diode array detector and a single quadrupole mass spectrometer.

Column: Phenomenex Aqua C18, 3 μm, 100 × 4.6 mm. Mobile phase: acetonitrile + 25 mM ammonium acetate (40:60 v/v). Flow rate: 0.5 ml/minute. Column temperature: 40°C. UV detection: 250 nm.

The mass spectrometer was operated in the positive electro spay mode with single ion monitoring at 235.1 mass units. Further settings: fragmentor: 70; Vcap: 1200; drying gas: 10 l/minute; nebulizer: 40.

Sample preparation: Microdialysates were collected in the micro-autosampler vials used in the HPLC autosampler and 10 μl dialysate was injected onto the HPLC column.

Limit of detection: 0.1 ng/ml. *Limit of quantification*: 0.5 ng/ml.

Analysis of tape-strip samples: 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 minutes, the mixture was left overnight and re-treated in the ultrasonic bath for 30 minutes. Then, 15.0 ml of 0.1% formic acid was added and after mixing the mixture was centrifuged for 5 minutes at 16,000 × *g*. A 10 μl portion of the supernatant was injected onto the HPLC column.

Statistical analyses

For microdialysis pharmacokinetic data, the area under time *versus* concentration curve (AUC) was calculated by the trapezoidal method and the lag time was the time when the concentration exceeded the lower limit of quantification (0.5 ng/ml). T_{max} was the time corresponding to the single highest microdialysate lidocaine concentration observed.

Preliminary analysis showed that the SD increased proportionally with the mean for all parameters except T_{max} and lag time. These parameters were thus log-transformed to achieve variance homogeneity, which is required for the analysis of variance and calculation of variance components within and between subjects. For log-transformed data, CVs were calculated as $CV(\%) = 100\sqrt{\exp(SD^2) - 1}$, where SD^2 is the variance of the log-transformed observations. Geometric means of log-transformed data were calculated as antilog of the means of the log-transformed, and confidence limits were likewise calculated as antilog of the confidence limits of the log-transformed data. Paired *t*-tests were used for comparison of cream and ointment results, and correlations were analyzed by regression methods. *P*-values >0.05 were considered insignificant. Statgraphics version 4 (Maneugistics, Rockville, MD) was used for the statistical analyses.

CONFLICT OF INTEREST

Financial support for study leave (E.B.) was received from Pfizer Ltd, Sandwich, UK.

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SUPPLEMENTARY MATERIAL

Table S1. Correlation coefficients between tape-strip data 120 minutes *versus* microdialysis concentrations and microdialysis $AUC_{(0\text{-}t)}$ for cream and ointment formulation separately and combined.

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