Journal of Pharmacokinetics and Pharmacodynamics, Vol. 33, No. 6, December 2006 (© 2006)
DOI: 10.1007/s10928-006-9019-z

Percutaneous Penetration of Methyl Nicotinate from Ointments Using the Laser Doppler Technique: Bioequivalence and Enhancer Effects

Yvonne Remane,[1] Claudia S. Leopold,[1,3] and Howard I. Maibach[2]

Received January 28, 2005—Final May 16, 2006—Published online September 28, 2006

Laser Doppler flowmetry (LDF) may be used to quantify erythema response as a result of an increased cutaneous microcirculation induced by methyl nicotinate (MN). Bioequivalence of a test and a standard preparation (vehicles: light mineral oil and medium chain triglycerides, respectively) was confirmed according to the pilot study of the FDA Guidance for Industry "Topical dermatologic corticosteroids: In Vivo bioequivalence" applying the staggered application and synchronized removal method for one defined concentration. Furthermore, the influence of penetration enhancers (5% w/w Dimethylsulfoxide (DMSO) and 10% w/w diethylene glycol monoethyl ether) on MN penetration was investigated. It was shown that DMSO and diethylene glycol monoethyl ether altered cutaneous microcirculation and thus MN penetration in comparison to the standard formulation. However, true penetration enhancement could only be proved with diethylene glycol monoethyl ether resulting from an improved drug solubility in the skin which was confirmed by attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR). Increased MN penetration by DMSO was only caused by thermodynamic effects, i.e. a decreased drug solubility in the vehicle.

KEY WORDS: percutaneous penetration; laser Doppler flowmetry; methyl nicotinate; dimethyl sulfoxide; diethylene glycol monoethyl ether; bioequivalence; enhancement; attenuated total reflectance fourier transform infrared spectroscopy.

INTRODUCTION

Laser Doppler flowmetry (LDF) is a widely used technique for measurement of the cutaneous microcirculation and therefore, blood flow. In the dermis, particularly in dermal papillae, an extensive capillary

[1]Institute for Pharmacy, Department of Pharmaceutical Technology, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany.
[2]Department of Dermatology, University of Califorinia San Francisco, 90 Medical Center Way, San Francisco, CA 94143, USA.
[3]To whom correspondence should be addressed. e-mail: Claudia.Leopold@uni-hamburg.de

network exists. Blood flow can be affected by pathological conditions such as arteriosclerosis, diabetes as well as Raynaud's syndrome or by application of drugs such as nicotinates (1–3). Within a few minutes of application nicotinates cause a visible erythema including a change in cutaneous blood flow due to vasodilation after prostaglandin release (4–6). Furthermore, nicotinates penetrate rapidly through the stratum corneum into the dermis. The change in blood flow depends upon the amount of nicotinates reaching the cutaneous vessels (1). Therefore, nicotinates are widely used as model drugs for quantification of cutaneous penetration (7–9). The aim of the study was the noninvasive quantification of the cutaneous methyl nicotinate (MN) penetration by pharmacodynamic measurements. First, the bioavailability of MN formulations was determined employing the LDF technique after removal of the formulations according to the FDA Guidance for corticosteroid bioequivalence testing (10). Second, the influence of penetration enhancers on drug penetration was investigated. Enhancers interact with the stratum corneum in three ways. Either they disrupt the lipid bilayer structure resulting in fluidisation, increase drug solubility in the barrier, or cause protein configuration changes, all effects assumed to be reversible (11,12). Moreover, some enhancers such as Dimethylsulfoxide (DMSO) and ethanol can cause lipid extraction, which is an irreversible effect (13). Among others, attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) may be used to analyse the mode of action of penetration enhancers (14).

MATERIALS AND METHODS

Model Drug, Vehicles and Subjects

The MN (Merck Schuchardt, Germany) was selected as model drug because of its rapid penetration via the intercellular pathway (5). DMSO 99% purity (Synopharm, Germany) and diethylene glycol monoethyl ether (DGME; Transcutol P®, Gattefossé, France) were used as penetration enhancers. This selection was due to their different modes of action. It is postulated that DMSO induces changes in the configuration of keratin from α-helix to β-sheet structure and may enhance the solvation process by hydrogen bonding (15–17). Furthermore, DMSO may interact with the stratum corneum lipids and may increase drug solubility within the barrier. The concentrations of DMSO commonly used in enhancer studies is above 50% (13). In this study it was investigated whether low concentrations of DMSO (5% w/w) affect percutaneous drug penetration (13).

The DGME leads to improved solubility of drugs in the skin without changes in the phase transition temperatures associated with stratum corneum lipids and thus penetration enhancement (18–21).

Medium chain triglycerides (MCT) were used as standard vehicle and light mineral oil (LMO) (both Synopharm, Germany) as test vehicle both containing 7.5% polypropylene (Sigma Aldrich, Germany) to achieve an ointment-like consistency. The DMSO and DGME were added to the standard vehicle in concentrations of 5–10% (w/w), respectively.

The MN solubility in the different liquid vehicles was measured spectrophotometrically (295 nm). The saturation level of MN amounted to 0.470, 0.066, 0.150, and 0.636 g/ml for MCT, LMO, MCT + 5% DMSO and MCT + 10% DGME, respectively. It was assumed that the addition of polypropylene would not affect drug solubility.

The same thermodynamic drug activity of 0.1% of the solubility level of MN was chosen for MCT and LMO. This means, that there were equal drug escaping tendencies from both formulations into the stratum corneum (see chapter results: enhancer effects). The same thermodynamic drug activity in the formulations allows the quantification of true penetration enhancement. Formulations containing the enhancers were used with the same drug concentration as the plain standard formulation. The formulations were applied in polypropylene chambers (Hill Top®, Hill Top Research, Cincinnati, Ohio, USA) to the volar aspect of the forearm of 28 healthy caucasian volunteers of both genders with no history of skin disease (10 for the bioequivalence study, 10 for the enhancer study and 8 for the vehicle effect study). A subject number between 8 and 10 was assumed to be consistent with earlier studies (22,23). Volunteer age ranged from 18 to 58 years and the applied volume was 0.25 ml (corresponding to 0.20 mg for MCT, MCT + DMSO and MCT + DGME; 0.24 mg for LMO) per 2.5 cm^2 under occlusive conditions. Preliminary studies demonstrated that this large application volume guaranteed infinite dose conditions: it was found that after an exposure time period of 4 hours more than 95% of the applied MN concentration was still detectable in the formulation (data not published). Thus, zero order penetration kinetics (with a lag phase) could be assumed.

Occlusions conditions were a result of the polypropylene chambers. Occlusion leads to skin hydration and thus to an acceleration of percutaneous drug penetration (24).

Laser Doppler Flowmetry

The vascular response was determined with a laser Doppler DRT-4 (Moor Instruments, England) equipped with a laser and an optic fiber to

emit light at 780 nm to the skin surface. Static tissues refract the monochromatic light unchanged in frequency while moving blood cells cause a frequency shift, which is the so-called Doppler effect (4,25,26). The refracted light at the tissue surface is transmitted to photodetectors where it is analyzed. The light penetration depth is 1–1.5 mm (24,27). Before each study, the LDF system was calibrated against a standard reference (Brownian motion of polystyrene microspheres in water) provided by the manufacturer. With the LDF it is impossible to measure blood flow in absolute units (e.g., ml/min). Usually, the "flux" is analyzed, which is the product of the concentration of moving erythrocytes and the mean velocity of these red blood cells (2,6,28).

The LDF data is reproducible at temperatures between 17–28°C. Above or below this range a sudden marked increase or decrease of blood flow, respectively, can be observed (28). Therefore, conditions were adjusted to a temperature of 23 ± 2°C and a relative humidity of 45–55%. All volunteers had a 15–30 min period of adaptation in the room and were seated comfortably for the entire period of investigation. Furthermore, the volunteers were not allowed to smoke, had to avoid vasoactive substances (caffeine, acetylsalicylic acid) and mental stress was minimized (7,28).

ATR-FTIR Spectroscopy

With the attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) investigations of the quality and quantity of percutaneous drug penetration are feasible. Furthermore, the influence of penetration enhancers on the distribution of a drug in the stratum corneum can be quantified (14). The investigations were performed with the ATR-FTIR spectrometer Tensor 37 (Bruker Optics, Billerica, MA, USA) using the mid-range IR (4000–700 cm^{-1}). The spectrometer was equipped with a special kit for in vivo measurements. It contained a crystal of zinc selenide (with an angle of 60°) for the reflection of the IR beam. The forearms were horizontally placed directly on the crystal to obtain the spectrum of the skin (29, 30). The penetration depth of the IR beam into the skin was 1.5 μm. The measured data was evaluated by the spectroscopy software Opus/IR V. 5.0.53 (Bruker Optics, Ettlingen, Germany).

Study Design

The studies were approved by the Ethics Committee of the University of California, San Francisco, USA. The bioequivalence and enhancer studies were designed according to method A of the pilot study described in the FDA Guidance for corticosteriod bioequivalence testing (10), which

means staggered application with synchronized removal of the formulations. Because of the short lag time of erythema onset, exposure time periods for the MN formulations of 0.17, 0.5 and 1.5 h were chosen. In addition, a control site was treated accordingly. After removal of the formulations the blood flow renormalization was measured over one hour or until baseline values were reached. Measurements were started 5 min after formulation removal to allow the probes to adjust to the blood flow conditions. All data was baseline adjusted and untreated control-site corrected. For each exposure time period the area under the response vs. time after removal curve (AUC) were calculated starting at seven minutes to exclude the plateau phase (saturation of the flux of red blood cells and/or MN receptors) and fitted to the well known E_{max} model using the curve fitting software CurveExpert 1.37 (D. Hyans, Microsoft) (10,31). The E_{max} model describes a sigmoidal dose response relationship mathematically, where in this study the dose corresponds to the AUC and the response to the exposure time period.

For the vehicle effect study the formulations were synchronically applied to the forearms of the volunteers and removed after the exposure time periods of 0.17, 0.5, 0.75 and 1.5 h. At the beginning of each spectroscopic measurement a reference scan of the air had to be recorded to reduce influences of athmospheric absorptions of moisture and carbon dioxide on the in vivo skin spectrum (32). After removal of each formulation 30 scans per measurement time point were automatically recorded. A second spectrum was measured after stripping of the first layer of the skin with a tape to remove leftovers of the formulation. For the tape stripping D-Squame® (CuDerm, Dallas, Texas, USA) with a diameter of 25 mm was used. The strips were applied with a constant pressure of 10 kPa for 15 sec to the skin and then removed (33).

Statistical analysis was done according to the FDA Guidance (bioequivalence study) and with a two-way ANOVA with subsequent DUNCAN test (enhancer study). All requirements for the ANOVA were fulfilled.

RESULTS AND DISCUSSION

Bioavailability and Bioequivalence

Blood flow is a self-regulating physiological parameter. Therefore, a wide and variable range of the blood flow can be observed under normal circumstances (34). After penetration of MN into skin blood flow increases: It may reach values that are up to 20 times higher compared to baseline values which are between 8 and 15 arbitrary units. Blood

flow changes are measurable before any erythema is visible (35). This is in contrast to Guy and Bugatto, who found a good correlation between erythema onset and blood flow changes (8). Microcirculation renormalizes long before the erythema disappears; therefore, redness duration and increase of blood flow cannot be correlated.

In this study, infinite dose conditions were chosen to guarantee the same available drug quantity over the entire time course of the experiment. Therefore, the measured flux of red blood cells only depends on the thermodynamic drug activity level in the formulation and on the exposure time period. The flux of erythrocytes is displayed in Fig. 1 as a function of the time after removal of the formulation for different durations of exposure of skin to MN. The profiles are characterised by a first-order elimination process as the corresponding semilogarithmic plots are linear. Therefore, an open one-compartment model with the drug receptors representing the main compartment may be assumed (33). This was expected because MN is a hydrophilic substance and should be released quickly into the underlying tissues. With increasing exposure time periods the flux curves approach each other due to the zero order drug input kinetic. Zero order penetration kinetics lead to a logarithmic curve shape of the drug concentration at the receptor site vs. time profiles. Therefore, with increasing exposure time periods the drug concentration in the main compartment, does not increase in a linear manner, which explains the decrease in the distances between the flux curves observed here (Fig. 1). No significant difference in the flux vs. time curves was found between 0.5 and 1.5 h duration of exposure suggesting that the maximum flux of erythrocytes has been reached within this time frame. The flux plateau observed right after the removal of the formulations supports this hypothesis.

In Fig. 2 the area under the flux vs. exposure time curves are displayed. The exposure time period corresponding to 50% of the maximum area value, and thus to the traditional ED_{50} value, was estimated to be 0.45 h with both formulations using the E_{max} model (10). No significant difference between MCT and LMO formulations was found as a result of the same thermodynamic drug activity of MN in both ointments and negligible penetration enhancing effects of the vehicles. This indicates the same bioavailability of the two investigated formulations and thus bioequivalence. However, according to the bioequivalence test of the FDA Guidance, the lower value of the confidence interval (56%) was not within the Westlake limits, probably because of the smaller number of 8 subjects compared to 12 subjects recommended in the FDA guidance.

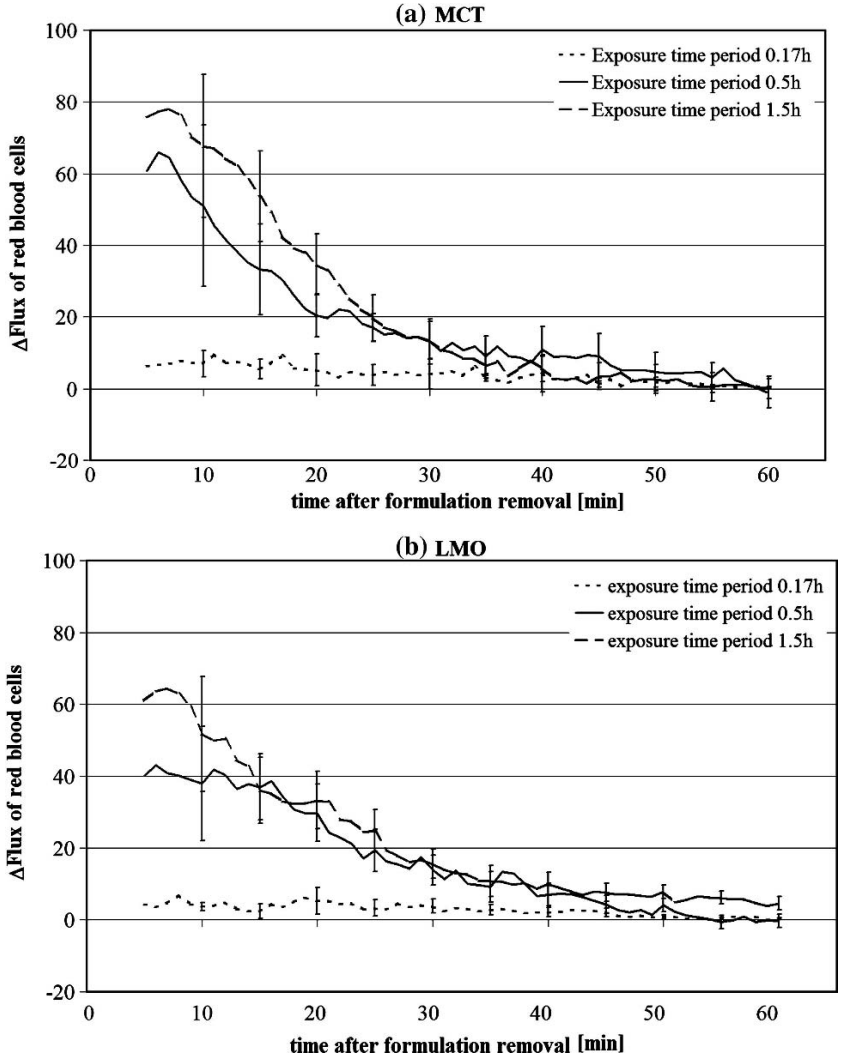

Fig. 1. Δ Flux of blood flow vs. time after formulation removal (a) MCT and (b) LMO; means ± SD, $n = 10$ subjects, measurement interval 1 min.

Enhancer Effects

Drug penetration into skin can be enhanced either by physical techniques (such as iontophoresis, electroporation or ultrasound) or by various chemical substances (36–38). An enhancement of percutaneous skin penetration using chemicals can be achieved by increasing the thermodynamic

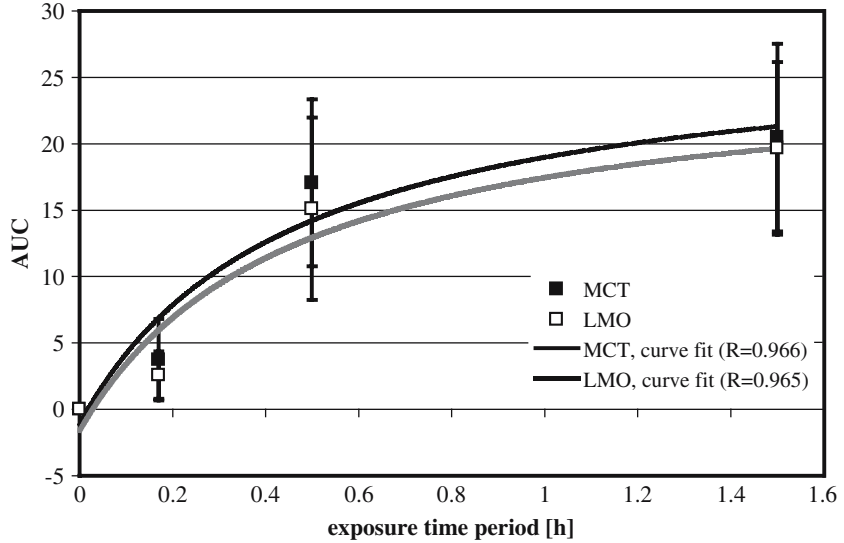

Fig. 2. Log AUC of the curves displayed in Fig. 1 vs. exposure time periods, means ± SD, $n = 10$ subjects.

drug activity. This can be achieved by using inactive prodrugs, that are converted to the active drug within the skin or by structural changes of the stratum corneum (13,15,39). The latter can either be the result of a change in configuration of keratin, lipid fluidisation or effects of improved solvation and therefore increased drug solubility in the skin (15,17,19). DMSO enhances permeation of many drugs having a molecular weight under 3000 Da at concentrations over 50% (w/w) (13,18,40). However, in the present study DMSO concentrations of only 5% (w/w) had to be used because of the limited miscibility of DMSO with lipophilic vehicles (MCT). According to the literature only high concentrations of DMSO significantly enhance cutaneous drug penetration, but, in turn, this causes irritations (13). In contrast to DMSO the commonly used concentration of DGME is between 10 and 50% (w/w) (40,41). Therefore, in the present study a concentration of 10% (w/w) was selected. Flux of red blood cells vs. the time after formulation removal profiles depend on the drug concentration and the exposure time period (Fig. 3). Again, an exponential decrease of the flux of red blood cells was measured. Maximum flux values reached were highest with DGME followed by DMSO. Interestingly, the flux curves of the penetration enhancers do not differ significantly at exposure time periods of 0.5–1.5 h. This may be explained by the nonlinear drug concentration increase in the receptor compartment approaching

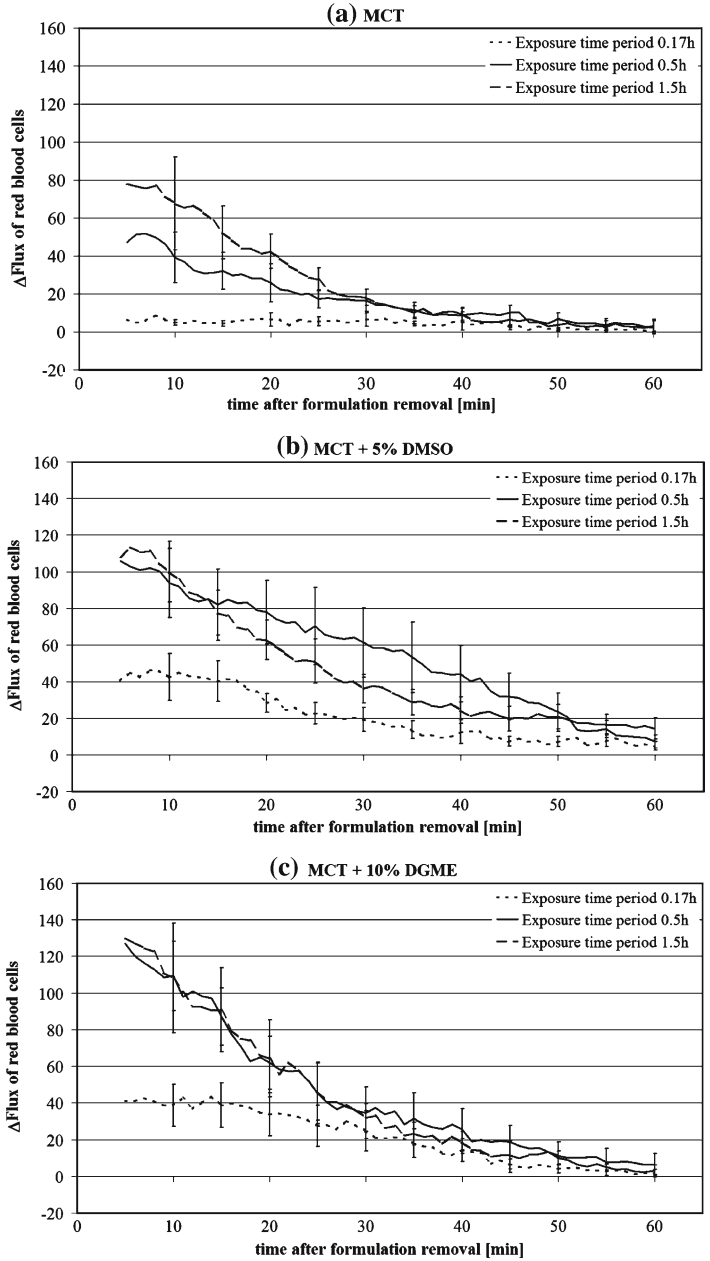

Fig. 3. Δ Flux of blood flow vs. time after formulation removal (a) MCT, (b) MCT + 5% DMSO and (c) MCT + 10% DGME, means ± SD, $n = 10$ subjects; measurement interval 1 min.

a plateau at long exposure time periods but also by reaching the maximum possible blood flow resulting in a limitation in erythema and microcirculatory response because of saturation of receptors at the capillary wall (8).

Calculation of the elimination rate constants from the flux curves led to the data shown in Table I. The increase of the elimination rate constants with increasing exposure time periods may be explained by a more rapid elimination of MN as a result of increased blood flow (4).

According to Fig. 4 percutaneous penetration of MN appears to be enhanced by DMSO and DGME. Analysis of these curves show that the exposure time period, where AUC is 50%, was calculated again to 0.45 h for the plain standard formulation and to 0.17 h for formulations containing enhancers. Thus, test formulations reach $t_{AUC50\%}$ 2.6 times earlier than the standard. This indicates enhanced drug penetration.

To compare drug penetration from the standard ointment with that from formulations containing enhancers, enhancement factors (EF) were calculated according to Eq. 1.

$$EF = \frac{f}{\gamma T/ST}. \tag{1}$$

The enhancement factor is the quotient of the relative bioavailability factor (f) and the relative effective activity coefficient of a test and a standard formulation (γT/ST) (Table II).

The relative effective activity coefficients $\gamma_{T/ST}$ were calculated as the ratio of the vehicle/10% sodium chloride solution partition coefficient of the standard vehicle ($PC_{ST/NaCl}$) and the test vehicles ($PC_{T/NaCl}$):

$$\gamma_{T/ST} = \frac{PC_{ST/NaCl}}{PC_{T/NaCl}} = PC_{ST/T}. \tag{2}$$

The aqueous phase consisted of a 10% (w/v) sodium chloride solution to lower the high water solubility of MN (42).

Table I. Elimination Rate Constants Estimated With the Curve Fitting Software CurveExpert 1.37 (D. Hyans, Microsoft)

Exposure time [h]	k_e MCT [h^{-1}]	k_e MCT + 5% DMSO [h^{-1}]	k_e MCT + 10% DGME [h^{-1}]
0.17	n. d.[a]	3.860 ($R = 0.980$)	3.396 ($R = 0.951$)
0.5	4.453 ($R = 0.989$)	2.730 ($R = 0.967$)	4.619 ($R = 0.993$)
1.5	5.630 ($R = 0.993$)	3.867 ($R = 0.996$)	5.003 ($R = 0.996$)

[a] Nondeterminable.

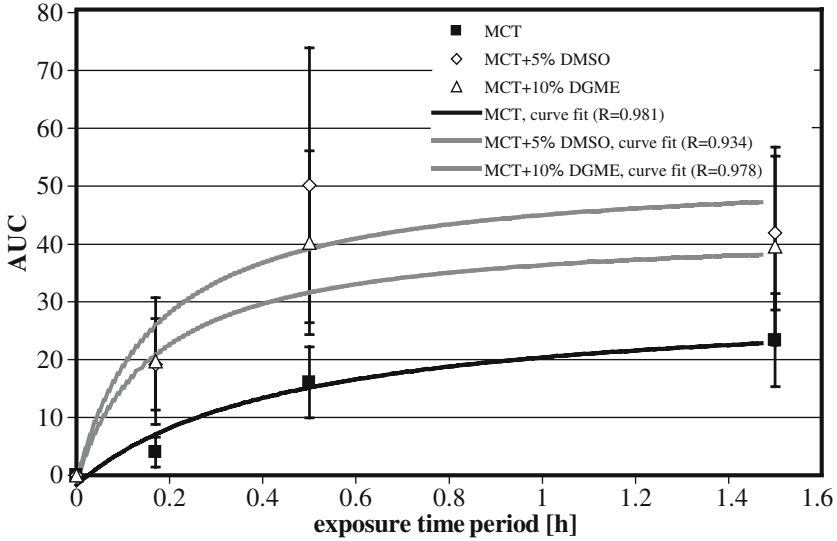

Fig. 4. Log AUC of the curves displayed in Fig. 3 vs. exposure time periods (■) MCT, (◇) MCT + 5% DMSO; (△) MCT + 10% DGME; means ± SD, $n = 10$ subjects.

Table II. Partition Coefficient Vehicle/10% Sodium Chloride Solution, Relative Effective Activity Coefficient and Bioavailability Factor of MCT + 5% DMSO and MCT + 10% DGME

Vehicle	Partition coefficient $PC_{V/10\%\ NaCl\ solution}$	$\gamma_{T/ST}$[a]	Bioavailability factor (f)
MCT (standard)	7.447	–	–
MCT + 5% DMSO (test)	2.637	2.824	2.847
MCT + 10% DGME (test)	11.25	0.662	3.050

[a]Calculated according to Eq. (2).

For calculation of the relative bioavailability factors the AUC of test and standard formulation at the exposure time period of 0.5 h were used because at longer exposure time periods saturation of the receptors in the main compartment is expected (Fig. 4). Calculation of the EF resulted in values of 1.133 ± 0.387 for DMSO and 4.607 ± 2.679 for DGME. Therefore, cutaneous MN penetration was significantly enhanced by only DGME (ANOVA, Duncan test, $p \leq 1\%$).

Interestingly, solubility data showed a pronounced decrease of the saturation level of MN in MCT containing 5% (w/w) DMSO (approximately three times). Thus, an increase of the thermodynamic drug activity occurs.

Therefore, the higher bioavailability observed with MCT + 5% DMSO is mainly caused by penetration enhancement resulting from thermodynamic effects. With regard to penetration enhancement low DMSO concentrations may be subject to type 2 errors. Higher subject numbers are needed to eliminate this error. DMSO is well known as a good solubilizer and penetration enhancer. An explanation for the decreased MN solubility might be the relatively high solution capacity of MCT for lipophilic as well as hydrophilic compounds compared to water.

Vehicle Effects

Occlusion conditions lead to a hydration of the stratum corneum by reducing the transepidermal water loss. Under these conditions the FTIR spectrum of the skin shows additional bands between 3000 and 3600 cm^{-1}, representing the hydroxyl (OH) resonance vibration of water (Fig. 5) (43).

The spectrum of the stratum corneum shows two amide bands at 1645 and 1545 cm^{-1}. In the presence of water the amide band II (NH-vibration) at 1545 cm^{-1} is not significantly changed. However, the intensity of amide band I (carbonyl stretch vibration in the CO-NH group) at 1645 cm^{-1} is increased (Fig. 5) (44,45). The degree of hydration of the stratum corneum can be determined using the area under the water absorption band as well as the quotient of the areas of both amide bands (Figs. 6 and 7) (45,46). With plain MCT an increase of the intensity of

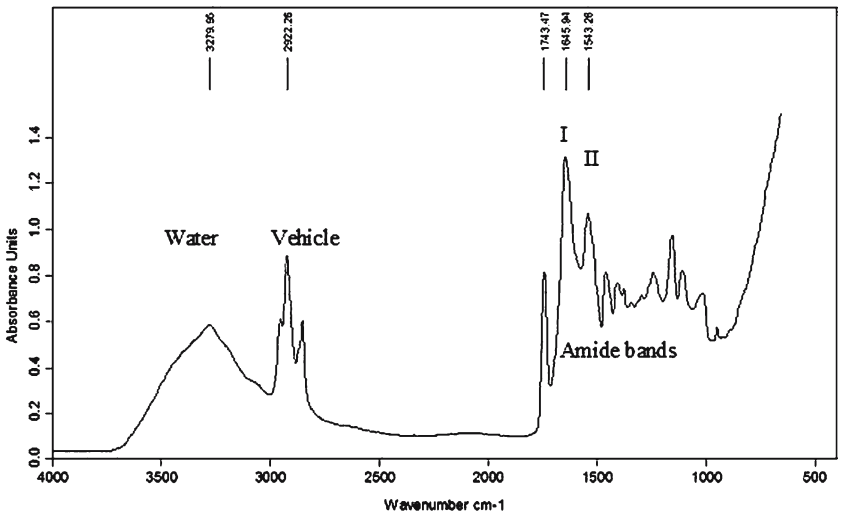

Fig. 5. Spectrum of hydrated stratum corneum after a 90 min application of MCT + 5% DMSO.

the amide bands could be observed while the OH resonance vibrations remained unchanged. Therefore, no effect on the stratum corneum can be postulated with regard to protein interactions. The addition of the penetration enhancers DMSO and DGME to MCT led to a slightly increased water binding capacity. The DMSO has hygroscopic properties and the ability to hydrogen bonding. Therefore, it builds up a strong hydration sheath with water because of hydrogen bonding and dipol-dipol interactions (46). These DMSO-water-bondings are 1.33 times stronger than water-water-bondings (13). Furthermore, DMSO binds to the polar head groups of the lipids of the stratum corneum and replaces water molecules (46). The described changes in the configuration of proteins could not be confirmed, probably because of the low DMSO concentration used. However, it may act as a drug solubilizer in the stratum corneum.

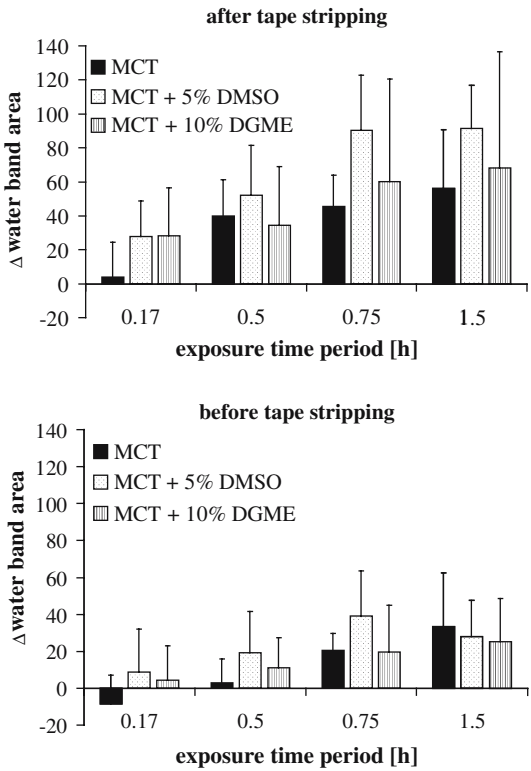

Fig. 6. Δ Water band areas at 2963,7–3801,25 cm^{-1} before and after tape stripping. Means ± SD, $n = 8$.

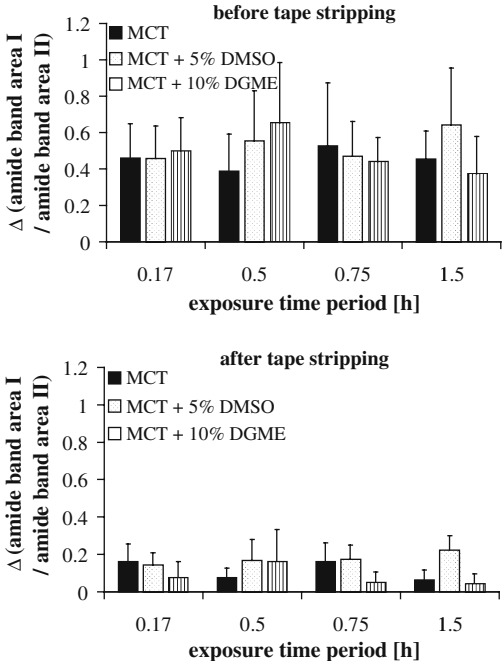

Fig. 7. Δ (Amide band area I/amide band area II) as a function of time at 1770–1613 and at 1613–1477 cm^{-1}. Means ± SD, $n = 8$.

The DGME, similar to DMSO, has hygroscopic properties and enhances the transport of water through the skin (19,20), which is assumed in this study, too. Furthermore, it was shown, that after the removal of the first skin layer, the process of hydration was quickly reversible, as can be seen by the lower quotient of the areas of the amide bands (Fig. 7).

CONCLUSION

The LDF technique can be used for bioequivalence testing of different formulations using nicotinates as model drugs, because of an increase of cutaneous microcirculation caused by these compounds. Method A of the FDA Guidance for corticosteroid bioequivalence testing cannot only be used for chromametric measurements but also for the determination of cutaneous blood flow using the LDF technique. In this study bioequivalence of test LMO and standard MCT formulation was shown using the same thermodynamic drug activity level of MN. The DGME in

concentrations of 10% (w/w) enhances percutaneous drug penetration significantly (ANOVA, Duncan test, $P \leq 1\%$) by increasing the water content in the stratum corneum. In contrast to DGME, DMSO decreases the solubility of MN in MCT, which results in an increase of the thermodynamic drug activity and explains the increased drug penetration from this vehicle. The effects of DMSO are concentration dependent and the low concentrations used in this study (<50%) do not influence drug penetration significantly, but lead to a slight increase in the water binding capacity of the skin. Only DMSO concentrations of 50% and above act primarly as drug penetration enhancer and change the configuration of stratum corneum proteins (13). However, these high DMSO concentrations may lead to an increase of unwanted irritation, which in turn increases cutaneous microcirculation and thus influences blood flow measurements (13).

ACKNOWLEDGMENT

We thank all volunteers.

REFERENCES

1. E. K. Chan, and J. A. Pearce. Visualization of dynamic subcutaneous vasomotor response by computer-assisted thermography. *IEEE Trans. Biomed. Eng.* **37**:786–795 (1990).
2. A. M. Seifalian, G. Stansby, A. Jackson, K. Howell, and G. Hamilton. Comparison of laser Doppler perfusion imaging, laser Doppler flowmetry, and thermographic imaging for assessment of blood flow in human skin. *Eur. J. Vasc. Surg.* **8**:65–66 (1994).
3. S. Clark, G. Dunn, T. Moore, M. Jayson IV, T. A. King, and A. L. Herrick. Comparison of thermography and laser Doppler imaging in the assessment of Raynaud's phenomenon. *Mircrovasc. Res.* **66**:73–77 (2003).
4. P. Elsner, and H. I. Maibach. Cutaneous responses to methyl nicotinate in human forearm and vulvar skin. *J. Derm. Sci.* **2**:341–345 (1991).
5. J. Wilkin, G. Fortner, and L. A. Reinhardt. Prostaglandins and nicotinate-provoked increase on cutaneous blood flow. *Clin. Pharmacol. Ther.* **38**: 273–277 (1985).
6. H. A. E. Benson, J. C. McElnay, R. Harland, and J. Hadgraft. Influence of ultrasound on the percutaneous absorption of nicotinate esters. *Pharm. Res.* **8**:204–209 (1991).
7. R. H. Guy, R. C. Wester, E. Tur, and H. I. Maibach. Noninvasive assessments of the percutaneous absorption of methyl nicotinate in humans. *J. Pharm. Sci.* **72**:1077–1079 (1983).
8. R. H. Guy, and B. Bugatto B. Pharmacodynamic measurements of methyl nicotinate percutaneous absorption. *Pharm. Res.* **1**:76–81 (1984).
9. C. Duval, M. Lindberg, A. Boman, S. Johnsson, F. Edlund, and M. Loden. Differences among moisturizers in affecting skin susceptibility to hexyl nicotinate, measured as time to increase skin blood flow. *Skin Res. Technol.* **9**:59–63 (2003).
10. FDA Guidance for Industry. Topical dermatologic corticosteroids: in vivo bioequivalence (1997).
11. J. Hadgraft. Modulation of the barrier function of the skin. *Skin Pharmacol. Appl. Skin Physiol.* **14**:72–81 (2001).

12. H. Loth. Vehicular influence on transdermal drug penetration. *Int. J. Pharm.* **68**:1–10 (1991).
13. T. J. Franz, P. A. Lehman, and M. K. Kagy. Dimethylsulfoxide. In E. W. Smith and H. I. Maibach, (eds). Percutaneous Penetration Enhancers. CRC Press, Boca Raton, 1995, pp. 115–127.
14. J. Hadgraft. Skin deep. *Eur. J. Pharm. Biopharm.* **58**:291–299 (2004).
15. B. W. Barry. Mode of action of penetration enhancers in human skin. *J. Contr. Rel.* **6**:85–97 (1987).
16. A. N. C. Anigbogu, A. C. Williams, B. W. Barry, and H. G. M. Edwards. Fourier transform raman spectroscopy of interactions between the penetration enhancer dimethyl sulfoxide and human stratum corneum. *Int. J. Pharm.* **125**:265–282 (1995).
17. B. J. Aungst, N. J. Rogers, and E. Shefter. Enhancement of naloxone penetration through human skin in vitro using fatty acids, fatty alcohols, surfactants, sulphoxides and amides. *Int. J. Pharm.* **33**:225–234 (1986).
18. K. Moser, K. Kriwet, A. Naik, Y. N. Kalia, and R. H. Guy. Passive skin penetration enhancement and its quantification in vitro. *Eur. J. Pharm. Biopharm.* **52**:103–112 (2001).
19. J. E. Harrison, A. C. Watkinson, D. M. Green, J. Hadgraft, and K. R. Brain. The relative effect of azone® and transcutol® on permeant diffusivity and solubility in human stratum corneum. *Pharm. Res.* **13**:542–546 (1996).
20. A. Ganem-Quintanar, C. Lafforgue, F. Falson–Rieg, and P. Buri. Evaluation of the transepidermal permeation of diethylene glycol monoethyl ether and skin water loss. *Int. J. Pharm.* **147**:165–171 (1997).
21. M. Dias, S. L. Raghavan, and J. Hadgraft. ATR-FTIR spectroscopic investigation on the effect of solvents on the permeation of benzoic acid and salicylic acid through silicone membranes. *Int. J. Pharm.* **216**:51–59 (2001).
22. M. Bach and B. C. Lippold. Influence of penetration enhancers on the blanching intensity of betamethasone 17-benzoate. *Int. J. Pharm.* **168**:97–108 (1998).
23. B. C. Lippold, and H. Reimann. Wirkungsbeeinflussung bei Lösungssalben durch Vehikel am Beispiel von Methylnicotinat Teil II Beziehung zwischen relativer thermodynamischer Aktivität, Bioverfügbarkeit: Penetrationsbeschleunigung und Entleerungseffekt. *Acta Pharm. Technol.* **35**:136–142 (1989).
24. K. S. Ryatt, M. Mobayen, J. M. Stevenson, H. I. Maibach, and R. H. Guy. Methodology to measure the transient effect of occlusion on skin penetration and stratum corneum hydration in vivo. *Br. J. Dermatol.* **119**:307–312 (1988).
25. H. Tanojo, E. Boelsma, H. E. Junginger, M. Ponec, and H. E. Boddé. *In vivo* human skin permeability enhancement by oleic acid: a laser Doppler velocimetry study. *J. Contr. Rel.* **58**:97–104 (1999).
26. N. Aspres, I. B. Egerton, A. C. Lim, and S. P. Shumack. Imaging the skin. *Australas. J. Dermatol.* **44**:19–27 (2003).
27. E. Berardesca, and H. I. Maibach. Bioengineering and the patch test. *Contact Dermatitis.* **18**:3–9 (1988).
28. A. Kistler, C. Mariauzouls, and K. van Berlepsch. Fingertip temperature as an indicator for sympathetic responses. *Int. J. Psychophys.* **29**:35–41 (1998).
29. D. B. Bommannan, R. O. Potts, and R. H. Guy. Examination of stratum corneum barrier function in vivo by infrared spectroscopy. *J. Invest. Dermatol.* **95**:403–408 (1990).
30. H. M. Klimisch and G. Chandra. Use of Fourier transform infrared spectroscopy with attenuated total reflectance for in vivo quantitation of polydimethylsiloxanes on human skin. *J. Soc. Cosmet. Chem.* **37**:73–87 (1986).
31. Y. Remane, and C. S. Leopold. Quantification of the erythema and temperature response induced by cutaneously applied methyl nicotinate ointments according to the FDA Guidance for bioequivalence testing. *Proc. Int. Meeting Pharm. Biopharm. Pharm. Technol.* Nuremberg (2004).

32. N. A. Puttnam. Attenuated total reflectance studies of the skin. *J. Soc. Cosmet. Chem.* **23**:209–226 (1972).
33. S. J. Bashir, A. L. Chew, A. N. C. Anigbogu, F. Dreher, and H. I. Maibach. Physical and physiological effects of stratum corneum tape stripping. *Skin Res. Technol.* **7**:40–48 (2001).
34. A. Bircher, E. M. De Boer, T. Agner, J. E. Wahlberg, and J. Serup. Guidelines for measurement of cutaneous blood flow by laser Doppler flowmetry. *Contact Dermatitis.* **30**:65–72 (1994).
35. E. Oestmann, A. P. M. Lavrijsen, J. Hermans, and M. Ponec. Skin barrier function in healthy volunteers as assessed by transepidermal water loss and vascular response to hexyl nicotinate: intra- and inter-individual variability. *Br. J. Dermatol.* **128**:130–136 (1993).
36. C. Curdy, Y. N. Kalia, and R. H. Guy. Post-iontophoresis recovery of human skin impedance in vivo. *Eur. J. Pharm. Biopharm.* **53**:15–21 (2002).
37. D. A. Edwards, M. R. Prausnitz, R. Langer, and J. C. Weaver. Analysis of enhanced transdermal transport by skin electroporation. *J. Contr. Rel.* **34**:211–221 (1995).
38. J. Y. Fang, T. L. Hwang, Y. B. Huang, and Y. H. Tsai. Transdermal iontophoresis of sodium nonivamide acetate. V. Combined effect of physical enhancement methods. *Int. J. Pharm.* **235**:95–105 (2002).
39. T. M. Suhonen, J. C. Bouwstra, and A. Urtti. Chemical enhancement of percutaneous absorption in relation to stratum corneum structural alterations. *J. Contr. Rel.* **59**:149–161 (1999).
40. R. Panchagnula, and W. A. Ritschel. Development and evaluation of an intracutaneous depot formulation of corticosteroids using transcutol as a cosolvent: in-vitro, ex-vitro and in-vivo rat studies. *J. Pharm. Pharmacol.* **43**:609–614 (1991).
41. S. O. Williams, S. Long, J. Allen, and M. L. Wells. Scale-up of an oil/water cream containing 40% diethylene glycol monoethyl ether. *Drug Dev. Ind. Pharm.* **26**:71–77 (2000).
42. C. S. Leopold and B. C. Lippold. Enhancing effects of lipophilic vehicles on skin penetration of methyl nicotinate in vivo. *J. Pharm. Sci.* **84**:195–198 (1995).
43. R. E. Baier. Noninvasive, rapid characterization of human skin chemistry in situ. *J. Cosmet. Chem.* **29**:283–306 (1978).
44. M. Gloor, G. Hirsch, and U. Willebrandt. On the use of infrared spectroscopy for the in vivo measurement of the water content of the horny layer after application of dermatologic oinments. *Arch. Derm. Res.* **271**:305–313 (1981).
45. A. Jadoul, J. Doucet, D. Durand, and V. Preat. Modifications induced on stratum corneum structure after in vitro iontophoresis: ATR-FTIR and X-ray scattering studies. *J. Contr. Rel.* **42**:165–173 (1996).
46. P. J. Caspers, A. C. Williams, E. A. Carter, H. G. M. Edwards, B. W. Barry, H. A. Bruining, and G. J. Puppels. Monitoring the penetration enhancer dimethyl sulfoxide in human stratum corneum in vivo by confocal raman spectroscopy. *Pharm. Res.* **19**:1577–1580 (2002).