Microdialysis Technique as a Method to Study the Percutaneous Penetration of Methyl Nicotinate Through Excised Human Skin, Reconstructed Epidermis, and Human Skin In Vivo

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Purpose. The aim was to assess the feasibility of cutaneous microdialysis as a method to study percutaneous penetration of methyl nicotinate through human skin in vitro and in vivo.

Methods. Microdialysis was applied in vitro in excised human skin, in isolated dermis, in reconstructed human epidermis and in vivo in the volar forearm skin of volunteers using methyl nicotinate (MN) as a model compound. After topical application of MN, aliquots of the perfusate were collected and analyzed for the presence of MN spectrophotometrically and by HPLC. In vivo, visual scoring and laser Doppler perfusion imaging (LDPI) were used to monitor the effects on skin blood flow.

Results. In vitro, MN was detected in the dialysate after a 1 min exposure of excised skin to concentrations as low as 25 mM. Higher concentrations up to 500 mM showed increased levels. Prolongation of the application time to 60 min resulted in increased levels of MN in the perfusate as the duration of application increased. Reconstructed epidermis and isolated dermis showed an almost 2- and 20-fold higher penetration compared to excised skin, respectively. In vivo, LDPI measurements showed a rapid increase in skin blood flow after application of 25 to 100 mM MN for 1 min. MN was only detectable in the microdialysate after application of 100 mM for 10 min (two of three subjects).

Conclusions. Cutaneous microdialysis may be a tool for comparative studies linking responses in human skin in vivo to in vitro data using the same technique and endpoint.

KEY WORDS: microdialysis; methyl nicotinate; LDPI; percutaneous penetration.

INTRODUCTION

Measurement of local concentrations of compounds in the skin has great value in the study of transdermal drug delivery and in the assessment of percutaneous penetration of chemicals. Most information on skin penetration characteristics of compounds has been obtained from studies using physical model systems (1,2) and diffusion cell systems (3). Detailed evaluation, however, of kinetics associated with skin absorption, retention or metabolism has been limited because drug concentrations could not easily be measured directly in the tissue. Use of techniques that have been available for assessment of local tissue events, e.g. the suction blister method (4) and the punch biopsy technique (5), is limited by their tissue destructive character and the difficulty in achieving continuous sampling.

Cutaneous microdialysis has been shown to be a promising tool for the assessment of concentrations of endogenous and exogenous compounds locally in the tissue (6). The basic principle of microdialysis is to mimic the passive function of a small blood vessel by perfusing a tubular semipermeable dialysis membrane implanted in the tissue (7). Chemical compounds can be removed from or can be added to the extracellular space by diffusion through the dialysis membrane (8). Microdialysis has been used in skin research for several years to study percutaneous absorption (9–12) and cutaneous inflammation (6,12,13). Although attention has mainly been focused on the use of microdialysis in animal and human studies, the technique can in principle also be applied in tissue preparations in vitro and may therefore be highly promising for in vitro—in vivo correlation studies.

The aim of the present study was to assess the use of the microdialysis technique in excised and reconstructed skin as well as in human skin in vivo to study percutaneous penetration of a test agent. The nicotinate ester methyl nicotinate (MN) (14–16), a water-soluble compound which elicits within minutes upon application to the skin a vasodilatory response, was chosen as a model compound. Naked eye assessment and Laser Doppler perfusion imaging (LDPI) were used in vivo to monitor the effects of MN on skin blood flow.

MATERIALS AND METHODS

Test Compound

Nicotinic acid methyl ester (methyl nicotinate; Sigma Chemical Co., St. Louis, USA) was used at concentrations from 10 to 1000 mM in phosphate buffered saline (PBS) for in vitro experiments and from 10 to 100 mM in physiological Ringer's solution for in vivo experiments.

In Vitro Studies

Microdialysis Technique. The microdialysis system consisted of a CMA/102 Microdialysis pump (CMA Microdialysis Research AB, Stockholm, Sweden) and linear AN67HF acrylonitrile/sodium methallyl sulphonate copolymer membranes with a diameter of 0.2 mm and a molecular weight cutoff of 20 kD (Hospal Filtral, Meyzieu, France). The fibers were glued to afferent tubings to make linear microdialysis probes and inserted into the skin specimens using a 23-gauge needle as a guide. Experiments were performed with full-thickness excised human skin and reconstructed human epidermis at the Department of Dermatology, Leiden University Medical Center. Human breast skin, obtained from plastic surgery, was used within 24 h of surgery. Small pieces were placed epidermal side up onto underlays prewetted with PBS. Probes were inserted superficially at a length of about 1.5 cm in the specimens. MN was applied at

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ABBREVIATIONS: HPLC, high performance liquid chromatography; LDPI, laser Doppler perfusion imaging; MN, methyl nicotinate; PBS, phosphate buffered saline.

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concentrations of 10 to 1000 mM using a patch (11 mm diameter; Scanpor[R] for Finn Chamber). After each experiment, the skin specimens were processed for histological examination and hematoxylin/eosin stained paraffin sections were prepared to estimate probe depths. For experiments in which the influence of dermal layers on penetration was investigated, full-thickness skin was placed on a heatblock (50°C) between two small glass plates for 1 min in a drop of PBS followed by removal of the epidermis from the underlying dermis. Reconstructed epidermis were generated by growing human adult keratinocytes on filter inserts (Costar, Cambridge, USA) for 14 days at the air-liquid interface as described earlier (17). On the day of the experiment, the epidermis was carefully removed from the insert and a microdialysis membrane was placed underneath.

Experimental Design. An equilibration time of 50 min was used prior to 1 min patch application of MN. The probes were perfused with PBS at room temperature. After treatment, the skin surface was wiped with a tissue to remove excess liquid. A perfusion rate of 5 μl/min was used and 50 μl samples were collected during the equilibration period and 20 μl samples were collected every 4 minutes following application. Samples were stored at −20°C until analysis. All experiments were performed in triplicate.

In Vivo Studies

Subjects. Healthy human volunteers participated in the study after giving their informed consent. The study was approved by the Regional Ethics Committee for Human Research, University Hospital, Linköping and conducted at the Department of Dermatology (University Hospital, Linköping).

Microdialysis Technique. The microdialysis system consisted of a CMA/100 high precision pump, a CMA/140 microfraction collector and CMA/70 microdialysis catheters with a shaft length of 60 mm and a membrane length of 10 mm (CMA Microdialysis Research AB, Stockholm Sweden). The shaft of the probe has an outer diameter of 0.6 mm. The diameter of the polyamide dialysis membrane was 0.5 mm with a molecular weight cutoff of 20 kD. Probes were inserted in the ventral forearm skin. In some experiments the point of insertion was anesthesized with an intradermal injection of Xylocain[R] (10 mg/ml). An apheresis needle (Venflon2 1.4 × 45 mm, Ohmeda AB, Helsingborg, Sweden) was used as a guide which was inserted subcutaneously for the first 1.5 cm and then intradermally for the last centimeter to position the dialysis probe membrane as superficially as possible. The probe was inserted through the guide, after which the guide was withdrawn. Ultrasound measurements (Dermascan A, Sonotron AB, Sweden) were performed to estimate the probe depth. The probes were in place for 2.5–5 h.

Experimental Design. An equilibration time of 60 min was observed prior to MN application as based on LDPI measurements monitoring when skin blood flow returned to basal levels after insertion of the probes. 100 mM MN in physiological Ringer's solution was applied on blotting paper in an aluminium chamber patch application (diameter 10 mm, volume 50 μl) for 1 or 10 minutes. The probes were perfused with physiological Ringer's solution at room temperature. In experiments in which patches were applied for 1 min, a perfusion rate of 5 μl/min was used and 10 μl samples were collected every 2 minutes. For 10 min patch application, a perfusion rate of 1 μl/min was used and 6 μl samples were collected every 6 minutes. Samples were stored at −20°C until analysis. Room temperature was kept constant at 20–22°C and humidity was 40%.

Laser Doppler Perfusion Imaging (LDPI)

Skin blood perfusion was mapped using in initial experiments a standard LDPI system (PIM 1.0, Lisca Development AB, Linköping, Sweden) in which a low power He-Ne laser (1 mW, 632 nm), moved by a step motor, scans the skin surface. Doppler shifts in the backscattered light are detected and processed to generate an output signal which is linearly proportional to tissue blood perfusion in the upper 200–300 μm of the skin (18). In later experiments a high resolution (HR) LDPI system was introduced with a 4 times higher resolution than the standard system due to a step length of about 0.3 mm instead of 1.5 mm (19–20). This gave a scan area of 6 cm × 6 cm with a distance of about 1.5 mm between each measurement site for the standard system, whereas the HR-LDPI created a scan area of 1.5 cm × 1.5 cm. During the LDPI measurements the scanner head was positioned at a distance of 15 cm above the selected test area on the volar forearm and the orientation of scanning was parallel to the arm. One hour after probe insertion and directly after removal of the patch, the treated skin area was scanned with LDPI. For the standard system, measurements were repeated in 3 to 5 min intervals for 1 h, and in 10 min intervals for a total of two hours for the HR-LDPI. The average blood perfusion was calculated from the captured perfusion values for each of the measurement sites in the recorded area. It should be noted that due to the introduction of HR-LDPI during the course of the experiments, the voltage levels in Figures 4 and 5 were not directly comparable. Data analysis was performed using the manufacturer's software. Erythema and edema diameters at the site of MN provocation were scored visually.

Relative Recovery

Relative recovery (dialysate/surrounding fluid exchange ratio) was determined by placement of the probes in a vial containing 0.5 mM MN in PBS. The perfusion rates were varied from 1.0 to 10 μl/min and the amount of MN in the samples was determined to estimate the recovery from the surrounding fluid. In both in vivo and in vitro experiments a perfusion rate of 5 μl/min was used. In the in vivo experiments in which 100 μM MN was applied for 10 min a perfusion rate of 1 μl/min was used. Data were corrected for membrane length and diameter by dividing the relative recovery values by the length at which the membrane was immersed in the surrounding fluid and the diameter of the membrane.

Sample Analyses

Samples were analyzed spectrophotometrically at a wavelength of 263 nm or by high performance liquid chromatography (HPLC) using reversed-phase chromatography with 30%

water - 35% ethanol - 35% PBS as mobile phase and a 150 mm × 4.6 mm Alltima C18 5 μm column (Alltech Associates Inc., Deerfield, IL, USA). A flow rate of 0.8 ml/min was used and the injection volume was 20 μl (in vitro samples) and 12 μl (in vivo samples). Peak detection of samples and standards was performed at a wavelength of 263 nm. Standards of MN were run for quantitation.

RESULTS

Relative Recovery

Figure 1 shows the in vitro relative recovery from a solution of 0.5 mM MN in PBS using a linear AN69HF probe and from 0.5 mM MN in physiological Ringer's solution using a CMA/70 microdialysis catheter. Relative recovery at a perfusion rate of 5 μl/min was 25.8% and 23.8% for the CMA/70 catheter and the linear AN69HF probe respectively after correction of data for membrane length and diameter. For the CMA/70 catheter relative recovery was 65.2% at a perfusion rate of 1 μl/min.

In Vitro Studies

MN at concentrations reported to induce vasodilatation in vivo (10, 25, 50 and 100 mM), was applied epicutaneously on full-thickness excised human skin for 1 min. After exposure to 10 mM MN, no MN was detected in the perfusate at any time point (data not shown). For 25 and 50 mM MN, maximal levels of 17.5 ± 11.9 μM and 44.0 ± 4.1 μM, respectively, were measured in the first sample, i.e. 4 min after application. The concentration was, however, low and had fallen below the detection limit 20 min after removal of the patch. Exposure to 100 mM yielded a maximal amount of 57.9 ± 0.64 μM which was reached at 10 min after patch removal (Fig. 2A).

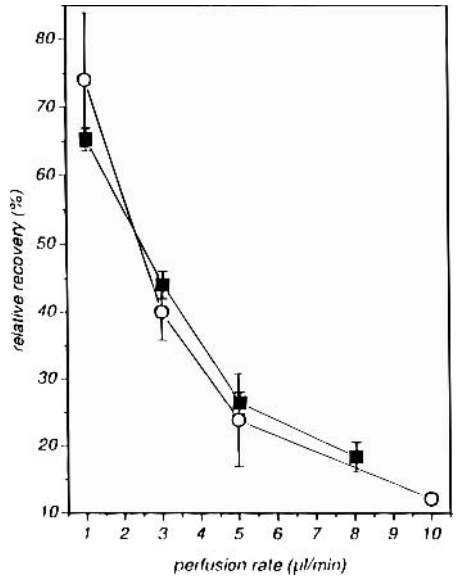

Fig. 1. MN recovery as a function of perfusion rate. Linear AN69HF probes (○) and CMA/70 microdialysis catheters (■) were used to determine relative recovery of MN at a concentration of 0.5 mM in PBS and physiological Ringer's solution resp. at perfusion rates varying from 1.0 to 10 μl/min. The concentration of MN in the collected samples was assessed by means of HPLC. All values presented are means ± SD from five individual measurements.

In order to increase MN penetration through the skin, thereby providing more ideal conditions for evaluation of the microdialysis technique, higher doses of the compound were applied on the skin surface in the same fashion and for the same time period. Concentrations of 500 and 1000 mM MN gave a dose-dependent increase of MN penetration in the dialysate as measured by HPLC analysis; 500 mM resulted in a maximal penetration of 212.0 ± 56.7 μM at 15 min after application, and 1000 mM gave a maximal penetration of 287.0 ± 50.6 μM at 25 min after patch removal (Fig. 2A). Thus, when the amount of MN applied on the skin surface increased, the time to maximal penetration also increased.

Removal of the epidermis from the underlying dermis and subsequent application of 100 mM MN on the isolated dermis for 1 min, resulted in an almost 20-fold higher maximal penetration compared to full-thickness excised skin (Fig. 2B).

The possibility to further examine differences in the penetration of MN through dermis, epidermis and full-thickness skin using microdialysis, the membranes were inserted directly underneath reconstructed epidermis. Reconstructed epidermis was shown to be only slightly more permeable for MN than full-thickness excised skin in vitro; treatment with 100 or 500 mM MN for 1 min resulted in a dose-dependent increase of MN in the dialysate reaching maximal levels of 109.1 ± 20.1 μM and 360.9 ± 45.5 μM, respectively, at 10 min after treatment (Figure 2C).

To investigate whether levels of MN in the perfusate were increased by increasing the application time, 100 and 500 mM MN were applied to the skin surface for 10 min. Figure 3A shows a maximum penetration of 116.3 ± 54.8 μM at 20 minutes after start of the application of 100 mM MN and 1087.0 ± 185.3 μM at 20 minutes after starting treatment with 500 mM MN. After a 10 min patch application, maximally reached values were approximately 2- respectively 5-fold higher compared to a 1 min patch application. When excised skin was exposed to 100 mM MN for 10, 30 and 60 min, the amount of MN recovered from the skin increased with increasing the duration of application (Fig. 3B). It should be noted that the amount of MN in the perfusate decreased rapidly after removal of the substance from the skin surface.

In Vivo Studies

LDPI Measurements. DPI measurements after 1 min patch application of 10, 25, 50 and 100 mM MN on the volar forearm skin showed a rapid increase in skin blood flow within minutes for all concentrations studied (Fig. 4). Visually, erythema was visible extending beyond the area of application quite considerably when the concentration was increased up to 100 mM. In some cases also edema was evident. For 10, 25 and 50 mM MN, blood flow reached maximal values at 10–15 min with a gradual decrease to baseline levels at 60 min. After a 1 min provocation with 100 mM MN, blood flow was already increased at 2 min after removal of the patch and reached a maximum at about 5 min. Application of 100 mM MN for 10 min resulted in vasodilatation, edema and erythema directly after removal of the patch. Since edema in the center of the reaction area was so pronounced, edema and erythema diameters were scored separately. Skin blood flow was measured in

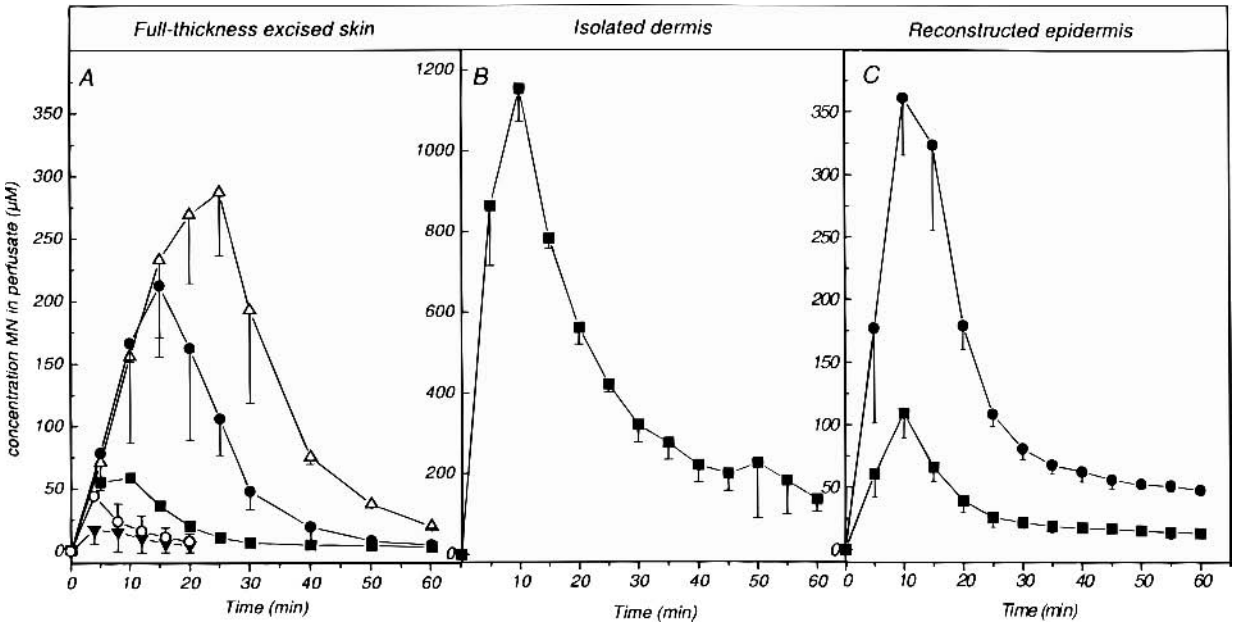

Fig. 2. Percutaneous penetration of MN through excised skin and reconstructed epidermis increases with increasing applied dose and after removal of the epidermal barrier. Time course of MN levels in the perfusate after 1 min patch application of 25 mM MN (▼), 50 mM (○), 100 mM (■), 500 mM (●), and 1000 mM (△) on (A) full-thickness excised skin, (B) isolated dermis, and (C) reconstructed epidermis. Samples were analyzed by HPLC. Data are presented as mean ± SD from three individual experiments. Probes lay about 200 μm beneath the skin surface.

the outer erythematous area and after disappearance of the swelling, blood flow was presented as the mean value of the center and the frame area. It should be noted that skin blood flow was still increased at 2 h after application (Fig. 5).

Microdialysis

Parallel to measurement of erythema and edema diameters, microdialysis samples were collected from the tissue at the site

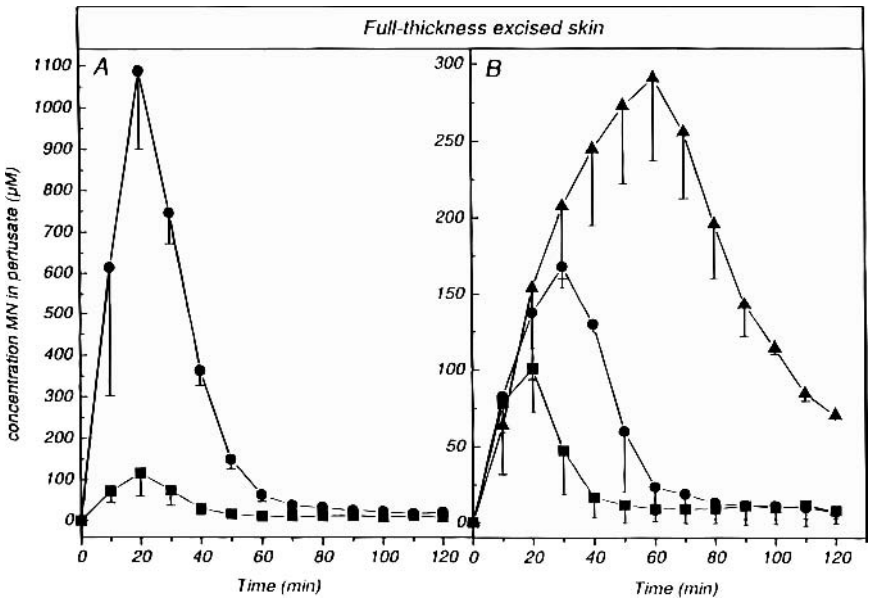

Fig. 3. Percutaneous penetration of MN through excised skin increases with prolongation of application time. Time course of MN levels in the perfusate after (A) a 10 min patch application of 100 mM MN (■) and 500 mM (●) on excised skin and after (B) application of 100 mM for 10 min (■), 30 min (●), and 60 min (▲) analyzed by HPLC. Data are presented as mean ± SD from three individual experiments.

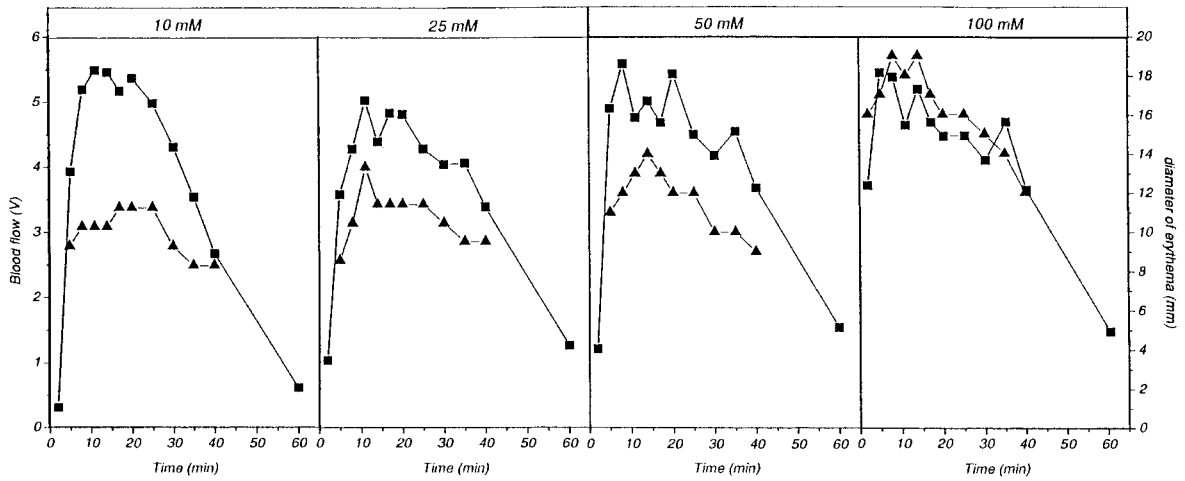

Fig. 4. In vivo effects following topical application of MN can be monitored using blood flow and erythema measurements. LDPI measurements of cutaneous blood flow (Volt) (■) and the diameters of erythema (mm) (▲) are shown after a 1 min patch application of 10, 25, 50 and 100 mM MN on the volar forearm skin in a single subject on the left and the right scale respectively.

of application. No MN could be detected in the perfusate after a 1 min application of 100 mM MN, neither by spectrophotometric nor by HPLC analysis in any of the subjects studied (n = 5). After treatment of the skin with 100 mM MN for 10 min, MN was detected in the perfusate in two of three subjects. Microdialysate values returned to near detection threshold levels more rapidly than in the in vitro studies. The subject in whom no MN was detected in the dialysate, developed a severe erythema and edema (Fig. 5).

DISCUSSION

In this study concerning the feasibility of using microdialysis for in vitro - in vivo correlation studies of percutaneous penetration, MN was selected as a model compound on the basis of its widespread use (15,21) and the availability of in vivo data on its clinical effects (14,16,22). The vasodilatory effect of the compound has been used as an endpoint for penetration, by visually noting erythema or instrumentally using techniques such as laser Doppler velocimetry and photoplethysmography (14,16,23). Penetration of the compound itself across human skin in vivo has only previously been demonstrated by detection of radioisotopes in urine (15). In the present paper, the penetration of MN through living human skin has been demonstrated directly and without the use of radioactive isotopes, albeit under conditions of maximal provocation as regards dose and duration of application. In the in vitro (excised skin and reconstructed skin) studies reported here, the advantages of the use of microdialysis are considerably more clearcut and of immediate applicability; microdialysate values increased with increasing concentrations and duration of application for MN, and in addition increased after measures to decrease the skin barrier function.

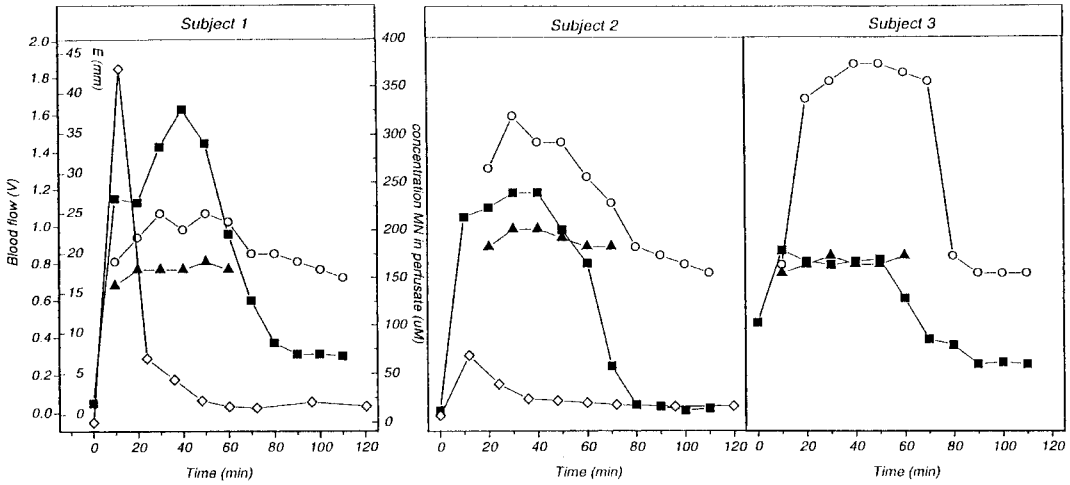

Fig. 5. In vivo effects following topical application of MN can be monitored by direct measurement of MN in the skin. LDPI measurements of cutaneous blood flow (Volt) (■) and HPLC analysis of MN concentrations in the perfusate (μM) (◇) after 10 min patch application of 100 mM MN on the volar forearm skin in three subjects. Erythema (○) and edema (▲) diameters (mm) were assessed visually. Note that in two of three subjects MN was detected in the perfusate. The mean probe depth was 0.9 mm (range 0.63-1.1 mm).

In vitro studies using diffusion cells have shown that a large amount of the applied dose of MN was immediately present in the skin, suggesting a very rapid penetration into the tissue in vitro which was only to a small extent limited by the presence of the stratum corneum barrier (15). This is in contrast to our findings that penetration through isolated dermis is much higher than through full-thickness excised skin. In addition, the fact that in vitro there is hardly any time delay between removal of MN from the surface and a fall in microdialysate levels implies the absence of a reservoir formation in the upper skin layers. Moreover, compared to full-thickness skin, recovery of MN in the perfusate was only increased about twofold when only epidermis was used as a membrane whereas a much higher amount of MN was recovered when isolated dermis was used. This suggests that the epidermis may indeed be the limiting factor for MN diffusion in vitro. In addition, the finding that penetration of MN through reconstructed epidermis closely approached that through native skin is in agreement with previous studies showing that reconstructed epidermis contain a stratum corneum exhibiting the same a barrier function as native skin as judged from the structure, organization and composition of stratum corneum lipids (17).

The mechanism of MN-induced vasodilatation has been assumed to be direct action of the compound on smooth muscle cells (24) and/or by involvement of a mediator (25), but no clear evidence exists about the actual mechanism of action of nicotinates. Naked eye and LDPI assessments of the effects of MN application showed a comparable time frame at early time points compared to microdialysis measurements. LDPI demonstrated a higher sensitivity to detect the effects of low concentrations of MN compared to microdialysis measurements, but it was more diffucult to delineate the effects of different concentrations. Our study demonstrated the presence of MN in the tissue within minutes after topical application supporting a direct effect on the vasculature. This does not, however, exclude the possibility of an indirect effect of MN via keratinocytes as has been suggested for prostaglandin release. In particular prostaglandin D_2 has been implicated as a mediator possibly responsible for vasodilatation as provocation of the skin by topical MN in vivo has been shown to evoke the release of large quantities of this substance from the site of application (22,26).

Although our data showed the presence of MN in the skin and a rapid penetration of MN in vitro as well as a rapid onset of the physiological response, the collection of data from in vivo experiments was much more difficult due a number of factors. One important factor was the low concentration of MN in the perfusate, which places great demands on analytical sensitivity. Transport possibilities by blood and lymph vessels in vivo can be expected to cause a rapid clearance of MN from the tissue. Also for other compounds the high clearance and intact vasculature in vivo have been thought to account for the discrepancy between in vivo and in vitro data and may explain the lower concentrations recovered in vivo (27,28). With increase in the penetration rate of MN in an in vivo study, the blood stream at the site of application will increase and the concentration of MN recovered in the perfusate will decrease, because MN is taken up rapidly by the increased blood stream as observed in subject 3 (Fig. 5). This indicates that the concentration of MN recovered in the perfusate in vivo may not reflect the physiological activity of MN. In this case the severity of the reaction may well have increased clearance as well as introducing an artefact in the LDPI registration (edema reduces the LDPI signal).

Other subject-related differences have been suggested to exist in response to topical MN, such as racial differences, appendageal density at the site of application, and sensitivity of the skin (29,30). Although these factors may not have been of major importance in our study, they illustrate that such individual differences may strongly affect the degree of penetration.

For large screening studies of percutaneous absorption, often easy analysis methods, such as spectrophotometry, are preferred. In the present study spectrophotometry was used in parallel to HPLC to assess concentrations of MN in the perfusate (data not included in this paper). Although measurement of the samples by spectrophotometry and HPLC yielded comparable data when epidermis or dermis were used separately, spectrophotometric analysis was nevertheless less accurate resulting in high inter-experimental variations. In addition, perfusate recovered directly from the tissue gave positive background levels which were probably caused by unknown substances present in the skin. The presence of other compounds absorbing at a wavelength of 263 nm was even more clear in samples obtained from full-thickness skin in vitro showing high positive background levels and high standard deviations. It should, however, be mentioned that in vivo spectrophotometric results (data not included in this paper) demonstrated also lower background values compared to in vitro data obtained from excised skin, suggesting that the interfering components may have come from some aspect of the surgical procedure.

Although HPLC has been frequently used for analysis of microdialysis samples, the lowest detectable amount of the compound is one of the major concerns in microdialysis research. Our study clearly demonstrated this problem as MN could only be measured in the perfusate after increasing the application time and the use of very low perfusion rates. For characterization of fast penetrating substances which require short sampling times and low perfusion rates, sensitivity of the analyzing technique may be the limiting factor in microdialysis research. On the other hand, developments in analytical techniques can be expected.

In conclusion, the work presented here demonstrates the application of the microdialysis technique to study percutaneous penetration in vitro in excised human skin and in reconstructed epidermis. Use of the same technique in vivo, although limited in the present study by high analytical thresholds, allowed a multiple parameter observation (naked eye, LDPI and microdialysis) of the process of percutaneous penetration of MN and its physiological effects.

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