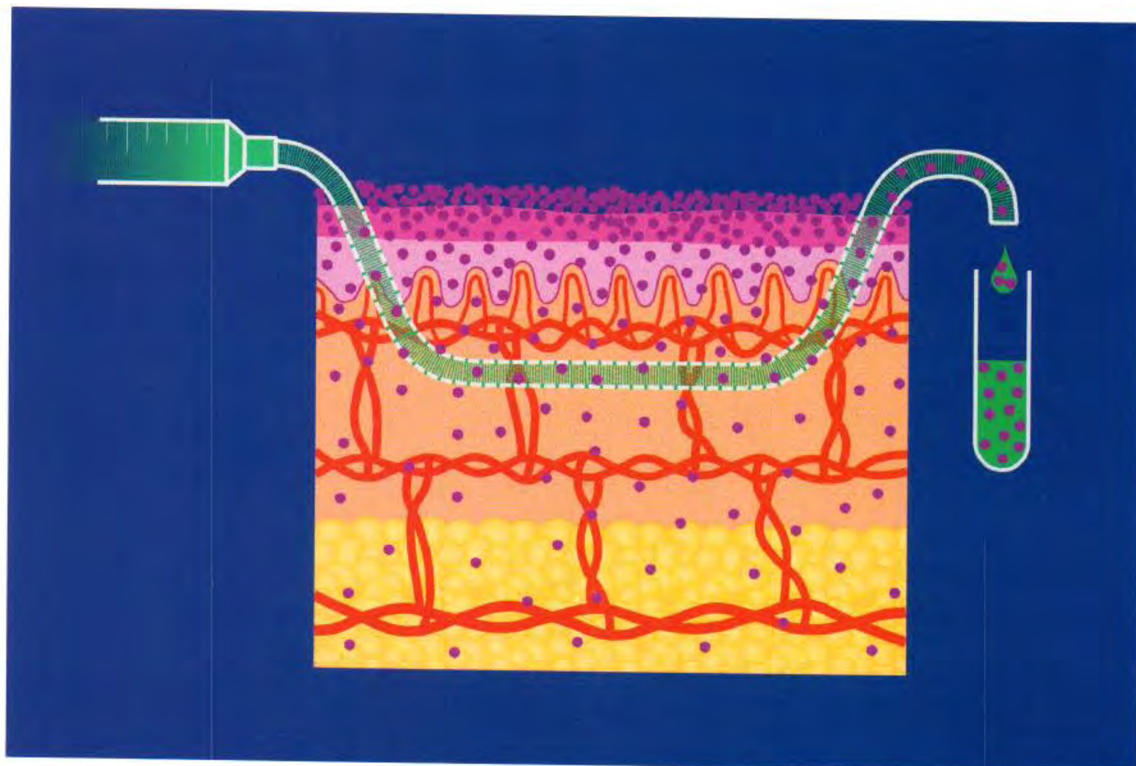
In Vivo Microdialysis for the Investigation of Drug Levels in the Dermis and the Effect of Barrier Perturbation on Cutaneous Drug Penetration

Studies in hairless rats and human subjects



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LIST OF ABBREVIATIONS

ASA	Acetylsalicylic acid, aspirin
AU	Arbitrary units
AUC	Area under the concentration-versus-time curve
B17V	Betamethasone 17-valerate
B21V	Betamethasone 21-valerate
$C_{dialysate}$	Concentration in the dialysate (after probe transit)
C_{max}	Maximum concentration
C_{medium}	Concentration in the medium (around the probe *in vitro*)
$C_{perfusate}$	Concentration in the perfusate (prior to probe entry)
C_{tissue}	Concentration in the tissue (around the probe *in vivo*)
ECF	Extracellular fluid
HPLC	High Pressure Liquid Chromatography
kDa	kiloDalton (MW)
log P	Octanol/water partition coefficient
MSA	Methyl salicylate
MW	Molecular weight
RR	Relative recovery
SA	Salicylic acid
SBF	Suction blister fluid
SC	Stratum corneum
SD	Standard deviation
SLS	Sodium lauryl sulphate
TEWL	Transepidermal water loss
$T_{½}$	Elimination half-life
T_{max}	Time to maximum concentration

PREFACE

This thesis was prepared during my research fellowship at the Department of Dermatology, Gentofte Hospital, Hellerup from 1995–1998. The fellowship was financed by The Danish Research Academy in co-operation with The Copenhagen County Research Fund and Leo Pharmaceutical Products, Denmark. The microdialysis experiments were conducted in the small animals facility and the clinical research facilities of the Department of Dermatological Research, Leo Pharmaceuticals, Denmark.

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Paper I: Benfeldt E, Groth L. Feasibility of Measuring Lipophilic or Protein-bound Drugs in the Dermis by *In Vivo* Microdialysis after Topical or Systemic Drug Administration. Acta Derm Venereol 1998; 78: 274–279.

Paper II: Benfeldt E, Serup J. Effect of barrier perturbation on cutaneous penetration of salicylic acid in hairless rats: in vivo pharmacokinetics using microdialysis and non-invasive quantification of barrier function. Arch Dermatol Res 1999; 291: 517–526.

Paper III: Benfeldt E, Serup J, Menné T. Effect of barrier perturbation on cutaneous salicylic acid penetration in human skin: *in vivo* pharmacokinetics using microdialysis and non-invasive quantification of barrier function. Br J Dermatol 1999; 140: 739–748.

Paper IV: Benfeldt E, Serup J, Menné T. Microdialysis vs. Suction Blister Technique for *In Vivo* Sampling of Pharmacokinetics in the Human Dermis. Acta Derm Venereol 1999; 79: 338–342.

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Signe Frey prepared and perfected experimental illustrations.

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INTRODUCTION

It is the aim of the present thesis to provide a full background to the reader on subjects relevant to the interpretation of the experimental work presented herein. The experimental work will be reported in detail in Chapters 8, 9 and 11, but headline results are discussed in the background review chapters when relevant.

AIMS OF THE THESIS

Our knowledge of the pharmacokinetics of drugs in the skin is sparse, despite the fact that an understanding of the bioavailability of a drug in the target organ is essential for its safe use. The cutaneous penetration of topically applied drugs is generally believed to be increased in diseased or barrier damaged skin. Most of the available data have been generated using *in vitro* or animal models or indirect methods such as tape stripping or excretion of systemically absorbed radiolabelled drug to assess the penetration. With the use of microdialysis in the dermis it is feasible to continuously monitor dermal pharmacokinetics following both topical and systemic drug administration with detailed real-time chronology.

The experiments fall in 3 different studies. The first study of topical drug administration is conducted in hairless rats. The second study regarding topical drug administration is conducted in human volunteers as is the study of systemic drug administration, comparing microdialysis and the suction blister method for sampling *in vivo* dermal drug levels in the peripheral compartment. Acetyl salicylic acid (ASA) or salicylic acid (SA) are used as model drugs.

Aims I. Barrier perturbation studies

The principal aim is to apply and evaluate the microdialysis technique for the investigation of drug transport across the living skin, and to study the physiological and pathophysiological factors that influence this process.

The following aims are addressed in either the hairless rat study or human study I, or both:

(i) To investigate the effect of 3 different methods of barrier perturbation on the cutaneous penetration of SA:
   1. Irritative dermatitis from the anionic surfactant sodium lauryl sulphate (SLS)
   2. Removal of the stratum corneum by means of repeated tape stripping
   3. Delipidization by treatment with absolute acetone.

(ii) To establish whether a correlation between non-invasive measurements of the inflicted barrier damage and the *in vivo* cutaneous penetration can be demonstrated. Quantification of barrier integrity is made by measurements of transepidermal water loss (TEWL, by evaporimetry) and erythema (by colorimetry) prior to the penetration experiment.

(iii) To evaluate whether a correlation between SA concentration and probe depth in the dermis exists. Measurements of probe depth and skin thickness are performed by 20 MHz ultrasound after the experiment.

(iv) To investigate the *in vivo* microdialysis recovery of the model substance by retrodialysis, including the possibility of tissue- or region-dependent differences in recovery.

(v) To estimate the systemic absorption of topically applied drug by sampling extracellular drug concentrations in probes placed in anatomical regions remote from the drug application site (rat study).

(vi) To provide a rough estimate of the degree of protein binding of the model drug in the extracellular fluid by performing simultaneous sampling with a microdialysis probe and an albumin-permissive ultrafiltration probe in the same skin area (rat study).

(vii) To assess if an individual 'skin reactivity' correlates with individual SA penetration measurements (human study).

(viii) To investigate if a dose-response relationship exists between the concentration of SLS used for the induction of irritant dermatitis and the cutaneous penetration of SA in the same area (human study).

(ix) To determine whether the sex of the subject has any influence on the cutaneous penetration of SA or the susceptibility to barrier damage (human study).

(x) To investigate whether the anatomical localization (proximal-distal) on the volar forearm has any influence on skin reactivity or cutaneous SA penetration (human study).

Aims II. Systemic drug administration and method comparison

The aim of human study II is:

(i) To study the relationship between pharmacokinetics in plasma and the skin, as measured by dermal microdialysis.

(ii) To study the relationship between pharmacokinetics in plasma and the skin, as measured by the suction blister method.

(iii) To establish the correlation between results obtained with the 2 sampling methods for studying skin pharmacokinetics.

Aims III. Comparisons between studies

(i) The results obtained in the hairless rat study and human study I can be compared in order to characterize the usefulness of the hairless rat as an animal model for predicting cutaneous penetration in human skin.

(ii) The results obtained in human study I and II can be compared with respect to the dermal drug levels obtained after topical and systemic drug delivery, respectively.

CHAPTER 1: MICRODIALYSIS PRINCIPLE AND THEORY

Microdialysis is a technique for *in vivo* sampling of endogenous and exogenous substances in the extracellular fluid (ECF), which represents up to 15 to 20% of the tissue volume. The principle was developed for neurotransmitter sampling in pharmaco-behavioural rat brain studies (Ungerstedt, 1984) and has now been adapted to the use in many other tissues and species. In the assessment of drug content/penetration in the skin, cutaneous microdialysis has the advantage over traditional methods (skin biopsy, stratum corneum stripping, blister fluid analysis) that continuous monitoring of drug penetration in the skin can be performed under minimally invasive conditions

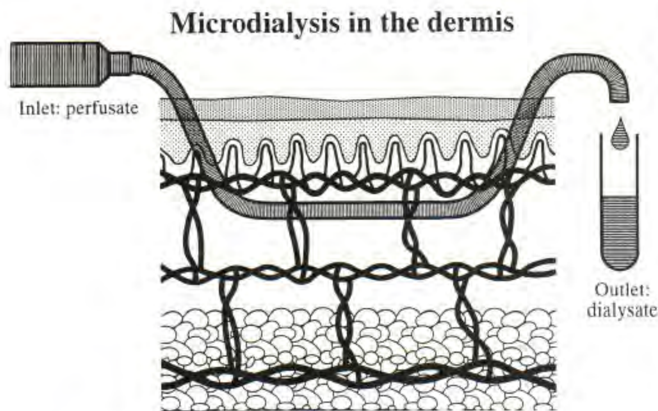


Figure 1.1. Microdialysis in the dermis.
The linear microdialysis probe, semipermeable to smaller molecules, is placed in the dermis between the superficial vascular plexus and the deep vascular plexus. The perfusate flow is controlled by the microdialysis pump. The resulting dialysate (see text) is collected at fixed time intervals.

(Surber, 1996). The technique can be applied to the study of normal, diseased and experimentally perturbed animal or human skin, and it has tremendous potential for the investigation of dermal drug distribution, cutaneous penetration, skin metabolism and inflammation – all with the advantage of sampling in the target organ, the skin.

Principle

The principle of microdialysis is that of passive diffusion. In the current thesis, microdialysis sampling is performed by placing a 0.2 mm diameter tubular microdialysis membrane in the dermis, in parallel to the skin surface (Fig 1.1).

This probe, which is permeable to water and small molecules, is continuously perfused with a physiological buffer at a low flow rate. The function of this hollow tube can be compared to that of an artificial blood vessel within the skin. The perfusate flows through the probe with no net delivery or removal of fluid from the tissue. Unbound substances present in the ECF at concentration C_{tissue} will enter the flowing perfusate in the lumen of the probe by passive diffusion according to the concentration gradient (Fick's law). The reverse process, where a substance is delivered to the tissue by passive diffusion from the perfusate, can also take place (Benveniste and Hüttemeier 1990). The entry into the lumen of the microdialysis probe is determined by the physicochemical properties of the substance such as the size and charge of the molecule and by properties of the layers through which the substance diffuses. Thus, the geometry of the extracellular space, active tissue processes such as blood flow and the effective diffusion rate in the membrane and its wall thickness will all influence the rate at which a substance enters the perfusate (Bungay et al., 1990). Molecules larger than 2 kDa, such as proteins and enzymes, cannot cross the membrane. At the outlet from the probe, the perfusate is now called the dialysate, and the ensuing concentration $C_{dialysate}$ in the dialysate can be analysed by HPLC or other very sensitive methods with little or no sample preparation as the sample has been "purified" by the protein excluding sampling process (Lunte and Lunte, 1996).

The concentration of a given compound or drug in the dialysate will reflect the ECF, but equilibration between extracellular tissue fluid and the perfusion fluid will most often be incomplete ($C_{tissue} > C_{dialysate}$). The recovery of a drug by the particular type of probe utilized in the experiment should be examined by establishing the substance-specific relative recovery (RR, see below). The dialysate data should be interpreted in connection with *in vivo* recovery characteristics of the drug or compound studied (Ståhle, 1991a) and with respect to experimental conditions influencing the recovery of the drug.

Microdialysis recovery of substances: Relative recovery (RR)

The RR is independent of the concentration of the drug/substance and is expected to be constant during experiments. It can be calculated by the equation below, with the substance of interest in known concentration in the perfusate (and for *in vitro* experimentation also in the medium). For *in vivo* experiments, the C_{medium} is substituted by C_{tissue}.

$$\text{Relative recovery (RR)} = \frac{C_{dialysate} - C_{perfusate}}{C_{medium/tissue} - C_{perfusate}} \qquad \text{(eqn. 1)}$$

Factors influencing the relative recovery

The relative recovery of a substance is influenced by a variety of factors, some of which can easily be controlled whereas others will prove difficult to standardize.

First, *the substance* itself will have inherent characteristics that will predict the ease of the process. The relative recovery is inversely proportional to the *MW* of the substance studied, i.e. smaller substances will diffuse across the membrane more easily, and the *configuration* of a given (larger) molecule is also of importance (with a spherical shape being more favourable than a Christmas tree structure). The *lipophilicity* and the *electrical charge* of a substance will also determine the affinity for the aqueous perfusate (again, depending on the composition of the perfusate) in comparison with the tissue. Some substances, in particular lipophilic drugs, display *adherence* to the plastic material used in beakers, tubing and sampling vials to a degree that even substituting all possible material with silicone-coated or non-plastic material may not solve the problem (Groth and Jørgensen, 1997). Finally, some substances are *protein bound* to an extent that reduces the unbound, diffusible fraction to a concentration below the limit of detection of most sensitive routine methods of analysis.

Second, the choice of *microdialysis instrumentation* will influence the recovery as well. Various *types of probes* (serial, concentric or other designs) are commercially available, with pore sizes in the *probe material* enabling substances of up to 100 kDa (so-called nominal cut-off of the probe) to cross the membrane. When probes are manufactured in the laboratory (as for the current thesis), the length and thus the total *probe surface area* accessible to microdialysis can be varied – the surface area is directly proportional to the relative recovery. The composition of the *perfusate*, which should be isotonic (with or without glucose, protein or lipid) will also influence recovery. The perfusion *flow rate* will be inversely connected to the relative recovery; at higher flow rates, the diffusional process has less time to equilibrate across the membrane resulting in lower relative recovery and vice versa.

Third, sampling in biological tissue *in vivo* will introduce factors that can either impair or enhance the mass transport of substance from the tissue into the lumen of the probe. One principal factor is the *tortuosity factor*, i.e. that the diffusion of most hydrophilic substances is impeded by impermeable

structures and cell membranes, thus increasing the effective path length of the molecule. In the tissue, mass transport is confined to the interstitial space, which constitutes 15–20% of the tissue only, and the microviscosity of the ECF is larger than that of water.

Furthermore, the effects of *blood flow* in the tissue, tissue *metabolism* (including intra/extracellular substance exchange) as well as the phenomenon of *tissue dependent recovery* (Ståhle, 1991b) can alter the *in vivo* recovery of the substance in question. Although mathematical models that recognize the diffusional, metabolic and microvascular effects on the microdialysis process in the tissue exist (Bungay et al., 1990; Morrison et al., 1991), the total impact and the proportional influence of each of these factors are currently neither well explained nor demonstrated *in vivo*.

Finally, as for most chemical processes, there is an effect of increasing diffusion with increasing *temperature*, and *in vitro* experiments should thus always be performed at temperatures identical to that of the tissue (Benveniste and Hüttemeier, 1990). The relative recovery is independent of the concentration level at which the dialysis process takes place.

Since many of these factors can be very difficult to either control, monitor or quantify during the course of an experiment, it can only be recommended to standardize all parameters amenable to standardization, and to aim studies at measuring relative changes in the drug/compound concentration in the skin in a semi-quantitative fashion (as in the barrier perturbation studies presented in this thesis).

Calculation of extracellular concentrations from microdialysate concentrations

If an estimate of the absolute concentration of a substance at a target organ site is calculated from microdialysis data, several approaches can be used for an in-vivo calibration of the probe used. Methods like the stop-flow method (Menacherry et al., 1992), the extrapolation to zero flow method (Jacobson et al., 1985), the point of no net flux (Lönnroth et al., 1987), retrodialysis (Ståhle et al., 1991b) or internal reference techniques (Larsson, 1991) have been developed for the quantitative estimation of unbound extracellular concentrations from dialysate concentrations. The 3 former are mainly of relevance to endogenous substances, present at a constant level at the target site, whereas the 2 latter are suitable for the monitoring of serial changes in concentrations of exogenous substances such as drugs.

The *retrodialysis method* relies on the reversibility of the passive diffusion process across the dialysis membrane. The addition of the substance of interest to the perfusate medium will enable the relative loss of substance of interest from the perfusate to the tissue studies to be calculated, and this value can be used as an estimate of *in vivo* recovery (as in the current thesis).

The *internal standard technique* consists of adding a marker molecule (which can be the substance of interest in labelled form or a compound with *in vivo* diffusion characteristics similar to the substance of interest) to the perfusate. The dialysate sample is analysed for both substances, and the relative loss of the internal standard molecule from the perfusate during the experiment can be used as a surrogate marker for the relative recovery of the substance of interest for the calculation of the tissue concentration of the substance of interest.

Attention should be paid to the fact that microdialysis samples ECF. Whereas corrections for recovery, established *in vivo*, and for protein binding, based on relevant protein binding data, can be made, other manipulations can be misleading. As an example, the calculation of an '*in vivo* recovery' by dividing the drug concentration in the dialysate with the drug concentration in a homogenized tissue specimen from the same site (as in Murakami et al., 1998a), which will be an average of intra- and extracellular concentrations, should probably not be undertaken. The error hereby inflicted can be deducted from a review concerning the concentration of various antimicrobials in suction blister fluid, intracellular fluid and tissue homogenates of 2 types of muscle (Redington et al., 1991) and from a microdialysis study of the distribution of methotrexate in rats, in which microdialysate and various tissue homogenate concentrations are compared (Ekström et al., 1995).

Insertion trauma and the invasiveness of microdialysis for dermal sampling

The insertion trauma inflicted by the placement of a microdialysis fibre in the dermis has been studied in both rats and humans. The microdialysis probe, which is thin and supple, is inserted in the skin via a guide cannula. This cannula (an ordinary venepuncture cannula) is inserted in parallel with the skin surface in the dermis, and exits with the tip at the end of the planned length of the probe in the dermis. The probe is then introduced, in the opposite direction, via the lumen of the guide cannula, which is subsequently withdrawn.

The trauma inflicted by the insertion varies with species, with the type of probe and guide cannula used and possibly with the insertion depth in the dermis. Since reliable microdialysis sampling in most instances requires that the insertion trauma has subsided and the cutaneous blood flow has stabilized, the appropriate equilibration period must be determined prior to each new type of study.

In rats, the insertion trauma has been shown to be of around 30 min duration as measured by Laser Doppler perfusion scanning and microdialysis sampling of histamine release (Groth et al., 1998a). In the same paper, the effect of halothane vs. pentobarbital anaesthesia on cutaneous blood flow during and following the insertion trauma was evaluated, and rats anaesthetized with pentobarbital were shown to have a stable skin blood flow as opposed to rats anaesthetized with halothane.

In humans, the period necessary for the skin hyperaemia to return to baseline values has been shown to be around 60 min (Anderson et al., 1994; Lindén et al., 1997). In a study comparing the effect of prior anaesthesia on the insertion trauma, as measured by skin blood flow and erythema, the use of local anaesthesia given s.c. significantly reduced the size of the trauma, and the increase in blood flow required 90-120 min to be fully normalized (Groth and Serup, 1998). Krogstad et al. found incomplete normalization of blood flow in the microdialysis area after insertion of probes in the dermis without anaesthesia, and suggested the sustained increase in blood flow to be caused by axon reflexes stimulated by manipulation of the probes in situ. They note that the 15–25% increase in blood flow did not, and was not expected to, alter neither the interstitial concentration of small substances nor microdialysis measurements. Histological examination of skin biopsies from the site of the microdialysis probes showed no signs of an inflammatory reaction (Krogstad et al., 1996).

CHAPTER 2: MATERIALS AND METHODS

Animals

All experiments were performed in female Sprague-Dawley hairless rats (OFA hr/hr) to avoid shaving/clipping and depilatories, which may alter skin barrier function. After initiation of barrier perturbation, the rats were housed in individual cages. During experiments, the animals were placed on a temperature-controlled heating pad.

Subjects

Human study I + II were conducted in healthy volunteers age 25–50 years. None of the subjects were allergic to aspirin or local anaesthetics. The volunteers had used no topical creams for 2 days prior to the experiment and took no medication. All subjects gave written informed consent and the study was approved by the Copenhagen County ethical committee (Ref. KA 97021s) and by the Danish Medicines Agency (J.nr. 5312-133-1997).

Chemicals

SLS (>99% purity) was obtained from Sigma Chemical, St. Louis, MO, USA. SA (>99% purity) was obtained from Merck, Germany.

Microdialysis sampling

Microdialysis probes were manufactured in the laboratory, using single fibres from an artificial kidney (Gambro GFE 18), dialysis fibres with an outer diameter of 216 μm, a wall thickness of 8 μm and a MW cut-off of 2 kDa. The probe was glued to nylon connection tubing using cyanoacrylate. The length of the membrane accessible to microdialysis was 3 cm in all experiments. Probes were sterilized by immersion in 70% alcohol for 20 min as standard procedure. The perfusate consisted of a sterile phosphate buffer, pH 7.4, with 2.5 mM glucose added. The perfusate flow rate was 5.0 μl/min using microinjection pumps (CMA/100 and CMA/102, CMA/Microdialysis AB, Stockholm, Sweden), and samples were collected every 20 min, giving sample sizes of 100 μl for analysis.

For the protein binding assessment study we used linear ultrafiltration probes made from plasmapheresis fibres (Asahi, Japan) with 0.4 mm diameter and a cut-off of 3000 kDa. The probes were perfused with the standard perfusate at a flow rate of 5 μl/min. These ultrafiltration probes, which permit the recovery of albumin in the ECF (Schmelz et al., 1997), were a kind gift from Dr Martin Schmelz, Department of Physiology and Experimental Pathophysiology, University of Erlangen, Germany.

In vitro microdialysis recovery

Relative recovery (RR) *in vitro* was established to ensure that the probe type used would provide reproducible sampling of SA. The *in vitro* RR is independent of the concentration of the drug/substance and is expected to be constant during experiments. *In vitro* microdialysis experiments were conducted with probes placed in beakers with SA in known concentrations of 1.0, 4.0, 10.0, 20.0 and 100.0 μg/ml (dissolved in perfusate buffer) at 35°C, stirred at 350 rpm, using the standard 3 cm probe length and perfusate flow rate of 5 μl/min (n = 19). *In vitro* loss was studied by perfusing probes with SA solutions of known concentrations of 1.0, 10.0 and 100.0 μg/ml, using the same experimental set-up (n = 7).

The relative recovery of SA was 79 ± 2% (mean ± SD, n = 19), and the relative loss of SA was 75 ± 3% (mean ± SD, n = 7). Although there is a 5% difference between these recoveries, they can be regarded as similar for practical purposes.

In vivo microdialysis recovery

As in vivo recovery may differ substantially from *in vitro* recovery (Ungerstedt, 1984; Bungay et al., 1990), an *in vivo* recovery experiment was designed to investigate the *in vivo* recovery of SA and whether it would vary with the anatomical position of the dermally implanted probe. The principle of this method relies on the assumption that the diffusion process is quantitatively equal in both directions through the semipermeable membrane (Ståhle et al, 1991b). By perfusing the dialysis probe with SA in the absence of topical or systemic drug administration, the relative loss of SA from the perfusate to the dermis can be calculated using eqn. 1. and used as an estimate of recovery. The result of *in vivo* microdialysis recovery experiments can be found in Chapter 8 (rats) and Chapter 11 (humans).

Suction blister formation

Suction blister fluid harvesting has been an established method for more than 30 years. The method principle relies on the separation of the epidermis from the dermis along the lamina lucida due to the application of prolonged suction to the skin surface. The formation of blisters can also be induced by application of irritating compounds such as cantharidine (as in Müller et al., 1998a), which results in an inflammatory exudate with a higher albumin content than suction blister fluid. The fluid drawn into this newly-formed compartment can be sampled and analysed for the content of either topically (rarely) or systemically administered drug, or for inflammatory mediators, inflammatory cells or other compounds (Kiistala, 1968).

In human study II, a disposable suction blister chamber (Dermovac® Blistering Device) was taped to the inner aspect of the upper arm and connected to controlled suction of 250 mm Hg. After 2 h of suction most subjects had developed 5 fluid-filled blisters, which were emptied using a Mantoux syringe. In some subjects, additional suction time was necessary to obtain fluid in the blisters.

Blood sampling

In human study II, blood samples were taken through an intravenous cannula, inserted in a vein on the dorsum of the hand.

Transepidermal water loss (evaporimetry)

The transepidermal water loss (TEWL) provides information on the integrity of the epidermal permeability barrier. The measurement is performed by the application of a measuring head containing 2 water vapour pressure sensors at fixed distances from the skin (3 and 6 mm) within the zone of diffusion, and the gradient between the two is computed by the apparatus,

yielding a display value of H_2O in g/m²h. 15 minutes after barrier perturbation procedures, TEWL over the demarcated areas was measured using an Evaporimeter (Servo Med® EP 1). The measurements were performed according to guidelines (Pinnagoda et al., 1990) with the arm (or the rat) placed in an incubator and recorded in triplicate. The relative humidity and the room temperature were also recorded for each experiment. The validity of TEWL measurements is influenced by many factors, particularly the measuring environment (Rogiers, 1995) but also by individual reactions such as eccrine sweating, but not by vasoconstriction or vasodilation of the cutaneous microvasculature (Pinnagoda et al., 1990).

Colorimetry

Colorimetry allows for quantitative measurements of skin colour by analysis of the light reflected from the skin surface according to the standardized CIE (Commission Internationale de l'Éclairage) system for the content of green-red (a*) and yellow-blue (b*) colour and skin brightness (L*). We used a Minolta Chromameter® CR 300 and the a* parameter, which is a measure of erythema. Erythema depends on the volume of blood under a given area of skin and not directly on blood flow in the form of moving red blood cells. Although blood flow and erythema often correlate, they represent different features of the cutaneous microvasculature and cannot be expected to correlate in all situations (Serup, 1995). The measurement is presented as a value in arbitrary units (AU). The measurements were made after calibration against a white tile and performed in triplicate, applying minimal pressure on the skin. The reproducibility and accuracy of colorimetry for the assessment of irritant dermatitis has been validated in several studies (Wilhelm et al., 1989; Agner and Serup, 1990; Agner, 1991) and although less sensitive than measurement of TEWL, the technique has the advantage of easy handling and quick measurements. In the scoring of corticosteroid-induced skin blanching, colorimetry was found to exhibit superior discrimination in comparison with ordinary visual grading of skin redness (Queille-Roussel et al., 1991).

High-frequency ultrasound imaging

At the end of each microdialysis experiment, after removal of the penetration chamber, skin thickness and probe depth in the dermis were measured in 3 separate scans along the length of the probe in situ (near probe entry, middle, and near probe exit) by 20 MHz ultrasound scanning using the Dermascan C (Serup et al., 1995), bringing the scanner head in close contact with the skin surface overlying the probes by the use of ultrasonic coupling gel. The reflection echo thus obtained is processed and presented as a colour-coded two-dimensional picture (B-scan), showing a cross-sectional image of the outermost 15–25 mm of the skin, depending on settings (see Fig. I a-b and III a-d, colour illustrations at the back). The depth of the probe was measured by application of A-mode interface, measuring the distance between the entrance echo of the epidermis and the echo peak caused by the intradermal microdialysis probe (Groth et al., 1998b). Skin thickness was measured from epidermal entry to the interface between dermis and subcutis. The accuracy of these measurements using the Dermascan C is about 0.02 mm (J Serup, personal communication). All values of probe depth and skin thickness reported are mean of 3 measurements.

Analysis of dialysates, suction blister fluid and blood

Betamethasone 17-valerate

Sample size was 80 µl from dialysate samples of 90 µl. Betamethasone 17-valerate was determined by HPLC using a Merck/Hitashi LaChrom HPLC system. A Supersphere 100 RP-18, 4 µm, 125×2 mm narrowbore column was used for separation. The mobile phase was acetonitrile/methanol/0.05M phosphoric acid 50/10/40, run at 0.3 ml/min for 3 min, then 0.6 ml/min for 5 mins. Detection was made at 245 nm and quantification by peak heights. Retention time was 3.8 min and with dialysate samples of 80 µl, the limit of quantification was 5 ng/ml.

Fusidic acid

Sample size was 80 µl from dialysate samples of 90 µl. Fusidic acid was determined by HPLC using a Merck/Hitashi LaChrom HPLC system. A Supersphere 100 RP-18, 4 µm, 125×2 mm narrowbore column was used for separation. The mobile phase was acetonitrile/methanol/0.05M phosphoric acid 50/10/40, run at 0.3 ml/min for 3 min, then 0.6 ml/min for 5 min. Detection was made at 245 nm and peak heights used for quantification. Retention time was 6.3 min and with dialysate samples of 80 µl, the limit of quantification was 10 ng/ml.

Salicylic acid (rat study and human study I)

The concentration of SA in dialysates was measured by HPLC. An 80 µl sample from the 100 µl dialysate was acidified by adding 125 µl 0.08M phosphoric acid. An aliquot of 150 µl was injected into a Merck/Hitashi LaChrom HPLC system. A LiChrosphere 100 RP-8, 5 µm, 125×4 mm i.d. column was used for separation. SA was determined at 234 nm and quantified by peak area measurements. A step gradient made up from acetonitrile/methanol/0.05M phosphoric acid was used as mobile phase. A mixture of 5/10/85 was run for 1 min at 1 ml/min, followed by 20/10/70 for 6 min at 1.5 ml/min and then 5/10/85 for 3 min at 1 ml/min. The retention time for SA was 6 minutes and the limit of quantification 10 ng/ml.

Salicylic acid and acetylsalicylic acid (human study II)

Samples were analysed as for human study I, except that isocratic elution with methanol/0.005M phosphate buffer, pH 2.2:35/65 was used. Both ASA and SA were determined at 234 nm and quantified by peak area measurements. The retention times were 4.5 and 10.5 min for ASA and SA, respectively. The limit of quantification was 10 ng/ml for both compounds. The total SA concentration in the samples was calculated by the equation [Total SA]= [ASA*0.767] + [SA].

For all microdialysis samples, detectable concentrations below the limit of quantification were arbitrarily set at 5 ng/ml.

In the barrier perturbation studies, samples displaying concentrations of SA much higher than expected (about 4 times higher than the group mean or higher) were excluded from further data analysis if technical problems such as probe or pump failure had been noted during the course of the experiment or if contamination of the sample by the SA chamber solution was likely. Less than 4% of samples were excluded.

Analysis of samples containing both SA and lignocaine showed no compounds interfering with the detection of SA. For stability assessment, SA samples were kept at –18,4, 25 and 40°C for 6 weeks and re-analysed. Optimum SA stability with negligible concentration change at 4°C was determined. Microdialysis samples were subsequently kept at 4°C and analysed within 6 weeks.

Ultrafiltration samples

The ultrafiltration samples were split into a 20 µl sample used for determination of total protein concentration by a manual biuret method (Witt and Trendelenburg, 1982), and a 50 µl sample used for HPLC analysis. The latter was added 70 µl acetonitrile for protein precipitation, followed by centrifugation. An 80 µl sample of the supernatant was subsequently analysed as described above.

Suction blister fluid

Suction blister fluid samples were treated in the same way as ultrafiltration samples with the only exception that higher sample volume allowed for triplicate HPLC analysis of both SA and ASA by the HPLC method used in human study II.

Blood sample analysis

After centrifugation, serum was pipetted into vials and frozen. After protein precipitation and centrifugation, the SA concentration in the supernatant was measured by HPLC. A 20 µl sample was injected into a TSP HPLC system with a Waters Bondapak C_{18}, 10 µm, 300×4.6 mm i.d. column for separation. SA was determined at 238 nm and quantified by peak height measurements. O-toluic acid was used as internal standard and the eluent was 42% v/v methanol in 0.005% v/v acetic acid. The limit of quantification was 3 µg/ml.

CHAPTER 3: PRINCIPLES OF CUTANEOUS DRUG PENETRATION

Since the drug present in the skin can be administered to this organ by either topical penetration or systemic distribution, the methods for measuring the concentration of a drug, particularly the indirect methods, will depend on the route of administration. In this chapter, the principles of cutaneous penetration, relevant to the conduction of *in vivo* studies of topical penetration, are presented. In Chapter 4, a summary of the most frequently used methods for measuring *in vivo* drug concentrations follows, with emphasis on the advantages and limitations of each method.

The basic structure of the skin, including the keratinocyte maturation and turnover process, the SC arrangement in a "brick and mortar" structure of corneocytes separated by an intercellular mixture of lipids structured in bilayers, the possibility of transfollicular transport pathways through skin appendages and other structural/ultrastructural concepts will not be reviewed here. A comprehensive up-to-date description can be found in "Skin Barrier. Principles of Percutaneous Absorption" by Schaefer and Redelmeier (1996).

Whenever possible, examples given in the text concern acetylsalicylic acid, salicylic acid or methyl salicylate, as they are the substances of interest in the experimental part (Chapters 8–12).

Measuring topical penetration *in vivo*

The route from application to target site for topically administered drugs

The assessment of bioavailability of topical formulations is a complex process since many factors are involved. The active drug first has to be released from the preparation, and this process alone can involve several stages (determined on thermodynamic activity of the drug, microviscosity of the vehicle, steps in partitioning). At the skin surface, the drug will partition from the preparation into the predominantly lipid-rich and acidic environment of the stratum corneum. The SC acts like a multilayer membrane in intact skin, and it constitutes the main (rate-limiting) diffusional barrier against free diffusion both outwards (H_2O) and inwards (both hydrophilic and lipophilic drugs) across the epidermis. The drug will passively diffuse through the 12–15 cell layers, following a tortuous intercellular pathway diffusing through the intercellular lipid domain, and then partition into the more aqueous viable tissue. The role of drug transport through the follicular route, the transcellular route or via sweat glands is not well understood, but for most small unionised, hydrophilic drugs such as salicylic acid (SA) it can be regarded as less relevant (Barry, 1983). Following further diffusion the drug will reach the level of the dermal vasculature, where some of the drug or all of it will be removed into systemic circulation. This description is correct for the traditional definition of bioavailability in terms of measuring the systemic levels of the drug. However, since both topical treatment and some types of systemic treatment used in dermatology are meant to exert their effects at the level of the skin as the target organ, the measurement of bioavailability in the dermis provides the most relevant information (with the exemption of the monitoring of toxicological or adverse systemic effects).

The assessment of *in vivo* drug penetration in the skin has hitherto been limited to

1. Indirect methods such as detectable *effect-responses* (vasodilation induced by nicotinates, vasoconstriction caused by corticosteroids, alterations in cutaneous sensory responses caused by local anaesthetics).
2. Indirect measurements such as the classical *radiolabelled absorption technique*, introduced by Feldmann and Maibach (Feldmann and Maibach, 1965, see below). However, this technique does not discriminate between the parent drug and its metabolites, and the time resolution is bound to be coarse due to a combination of analytical sensitivity and practical factors (urinary and faecal collections). A related indirect methods is *the disappearance method*, where the concentration of drug at the site of application site in measured/monitored, and the amount of drug absorbed is calculated from the concentration of drug remaining at the application site during/after the experiment. This method has a low level of precision, since only few % of a topically applied dose will penetrate the surface of intact skin. Another indirect method is the measuring of drug content in SC, removed by *repeated application of adhesive tape* to the same skin surface area (see below) and the method of obtaining *skin surface biopsies* by applying cyanoacrylate glue to the skin surface, and subsequently removing it with the attached SC. This method also removes hair follicles.
3. The use of *animal models* (including various flap models and human skin grafted onto laboratory animals). This gives the freedom of investigating toxic or potentially hazardous substances, but has the drawback of always necessitating assumptions in order to predict the results of similar *in vivo* penetration studies in man.
4. Direct methods such as *skin biopsies*, with or without parallel slicing, mostly used with radiolabelled drug (histoautoradiography), and biopsies taken after SC removal by tape stripping. The usefulness of all the direct methods for the determination of tissue concentrations is limited because of

the tissue-destructive sampling process, the inability to provide serial sampling from the same site in the same animal/subject and the high probability of permanent scarring whenever biopsies include the lower dermis.

The *microdialysis* technique is a direct method for measuring drug levels in the skin, with the measurement in the dermis providing the relevant information for most topically applied substances, namely the drug bioavailability in the target organ. Detailed knowledge of what happens to a topical agent in the tissue compartment where it has its therapeutic activity can now be obtained, and the potential of microdialysis in bioequivalence testing is promising (FDA report on methods for measuring bioequivalence by Shah et al, 1998). The use of the method in skin research, both in the field of inflammation and allergy as well as cutaneous penetration is undergoing rapid expansion; the available studies on dermatopharmacokinetics are reviewed in Chapter 4.

Choice of species

Of the various alternatives available for the measurement of the penetration of topical drugs, studies employing *in vivo* methodology in man are most appropriate and desirable. Animal models of cutaneous penetration vary in relevance to humans, and the explanation includes differences in skin thickness, in lipid composition of the epidermis, in the response to barrier perturbation procedures or enhancers and in drug metabolism in the skin. Bartek et al. (1972) conducted an *in vivo* study comparing percutaneous penetration of 6 radiolabelled compounds in rats, minipigs, rabbits and humans, and found that the order of skin permeability was rabbit > rat > pig > man. The mouse, which is often used in studies of skin barrier function and recovery after barrier perturbation, generally has an even more permeable skin barrier (Sato et al., 1991). On the basis of the currently available data, the only animals in which permeation data are consistently quantitatively and qualitatively similar to human permeation data are the pig, the Rhesus monkey and the hairless rat (Shah et al., 1991). In the current thesis, experiments in hairless rats and humans have been carried out in a standardized fashion allowing a direct comparison of the cutaneous penetration in man and rat (Chapter 10). This comparison shows that hairless rat skin is much more permeable to penetration of SA when assessed in unmodified skin, but that the effect of barrier perturbation results in increases in drug flux over the skin in orders of magnitude not significantly different from those observed in human skin. In other words, the correlation between human and hairless rat skin is qualitatively good, although quantitatively imperfect, as barrier properties *per se* are lower in the rat than in human skin.

Factors that affect drug penetration

Drug characteristics

The percutaneous penetration of a drug is largely determined by its physicochemical properties. The penetration rate of drugs through the SC, which constitutes the main penetration barrier and can be regarded as a partition membrane, can be described by Fick's First Law of diffusion.

The drug flux, J, represents the penetrating drug mass per time and area unit. Penetration rate constant k_p and the flux are directly proportional to the drug permeability P_B of the SC barrier.

$$J = P_B * c_V = \frac{D_B * PC_{B/V}}{l} * c_V \qquad \text{(eqn. 2)}$$

When the partition coefficient of the drug between the barrier stratum corneum and the vehicle $PC_{B/V}$ is replaced by

$PC_{B/V} = \frac{c_{sB}}{c_{sV}}$, the flux can also be calculated from eqn. 3:

$$J = \frac{D_B * c_{sB}}{l} * \frac{c_V}{c_{sV}} \qquad \text{(eqn. 3)}$$

where c_V is the drug concentration in the vehicle, D_B is the effective drug diffusion coefficient in the SC, l is the thickness of the stratum corneum, c_{sB} is the drug solubility in the SC barrier, and c_{sV} is the drug solubility in the vehicle. It can be seen that the flux can be increased through increasing the drug diffusion coefficient in the barrier D_B and the drug solubility in the barrier c_{sB}, similarly the flux can be increased by increasing the drug concentration in the vehicle c_V or by decreasing the solubility c_{sV} in the vehicle (Bach and Lippold, 1998). The above equations, which can be modified further, are applicable when the penetration process is studied under steady-state conditions and interactions between drug and skin components can be regarded as insignificant.

There is a striking relationship between the hydrophobicity of compounds and their permeability coefficients (measured in cm/s) across the skin; the relationship between the structure of a compound and its permeability across the skin is the basis for predictive models of percutaneous absorption (See Roberts, 1991). For the characteristics of the model compounds used in the experimental work in this thesis, please see Table 7.1.

Vehicle and formulation

Ideally, the choice of formulation is dictated by the intended application, i.e. assessing both experimental and clinically relevant formulations and concentrations. The use of high purity compounds and the assessment of stability of the formulation are recommended. Although formulation plays a significant role, it generally does not influence the flux across the SC more than 10 to 20-fold (Schaefer and Redelmeier, 1996). The structure of the formulation, the solubility of the drug in the formulation, the thermodynamic activity of the drug therein, and the partition coefficient of the active ingredient between the vehicle and the SC should all be considered when choosing the formulation for a study. The formulation should also be nontoxic and without sensitising or irritant potential.

The vehicle in which SA is applied to the skin surface has been demonstrated to have an effect on the subsequent absorption of the drug (Hlynka et al., 1969). The *in vitro* release rate of SA and some of its ester derivatives (methyl-, ethyl-, phenyl-, and glycol-) from a variety of bases has been shown to depend on the vehicle chosen. Some of the topical formulations were used for comparative in vivo percutaneous absorption studies in rabbits, and a linear correlation between the applied SA concentration and the AUC could be shown. The time to C_{max} depended on the vehicle used, as well as the C_{max} reached in the rabbit plasma (Al-Khamis et al., 1987). In an *in vivo* study, the effect of varying pH on the percutaneous absorption of SA was demonstrated with optimum penetration at pH 2-4 (Washitake et al., 1973). The same pH effect has been shown for both human and hairless rat skin *in vitro*, with increasing penetration rate with an increase in the undissociated fraction in the SA solution and a maximum at pH 2 (Harada et al., 1993). The penetration of SA vs. its sodium salt has been investigated,

and the acid displayed faster and higher penetration rates in rabbit skin *in vivo* in a very early percutaneous absorption study (Stolar et al., 1960) and in human SC *in vitro* (Neubert et al., 1990).

Ethanol as vehicle of choice

We wished to apply the model drug in a liquid formulation after the insertion of the probes. In this way, 2 problems could be circumvented: (i) inserting the guide cannula in a skin area where a topical formulation had just been wiped off, with possible contamination of the cannula and its track in the dermis by the drug content in the superficial skin layers (as described in Benfeldt and Groth, 1998) or (ii) applying a semi-solid formulation after probe insertion with the possibility of a drug gradient developing within the vehicle, caused by diffusion of the skin-near drug content across the skin surface and limited movement of molecules in the vehicle (disturbing the principle of the "infinite dose").

Since SA does not dissolve well in water (see Table 7.1, characteristics of model drugs), an ethanol solution was chosen for the penetration studies reported in Chapters 8 and 9, as SA is easily dissolved in alcohol (1 g in 2.7 ml). Ethanol is one of the most acceptable alcohols to use in terms of toxicology (Berner and Liu, 1995). Studies of *in vitro* and *in vivo* toxicity of alcohols, applied to the skin of human volunteers for 20 h daily for 3 d or to human epitheloid cell lines in culture, have shown that ethanol was the least irritating of the 7 alcohols tested (De Haan et al., 1996).

Ethanol is known to be an enhancer (see below) of percutaneous penetration. The absorption of ethanol alone across human skin has been demonstrated in a microdialysis study (Anderson et al., 1991). Ethanol has a cosolvent function, which increases the penetration across both intact and damaged SC (Barry, 1983). The solubility of lipophilic drugs in the SC is linearly correlated with the concentration of ethanol in the SC (Berner and Liu, 1995).

The topical penetration of SA applied in ethanol in the penetration study could thus be artificially high. In hairless rats, we have previously performed the same penetration experiment, but with the application of 10% SA in petrolatum. We found steady-state SA concentrations of between 2–5 μg/ml in dialysates (hairless rats, unmodified skin, $n = 2$, data not shown). This is very similar to the SA concentrations obtained after application of 5% SA in ethanol, so the enhancer effect, at least in unmodified skin, is likely to be small.

Control of the concentration of SA in the penetration chamber was made in both hairless rat and human study. Samples were taken at the end of the experiment, showing an increase in SA concentration of $8 \pm 5\%$ (mean ± SD) in the rat study and $7 \pm 4\%$ in the human study during the 4 h experiment. This up-concentration is unlikely to influence the penetration results to the extent that a correction is necessary. The increase is due to ethanol loss by a combination of evaporation and penetration.

Enhancement of cutaneous penetration

The penetration of a drug into or across the skin can be enhanced (facilitated) in several ways: by chemical means (an enhancing adjuvant added to the vehicle), use of prodrugs or by physical means (iontophoresis, phonophoresis and occlusion, see below). The induction of barrier damage by various methods, as described in Chapter 6 and used in the 2 penetration studies in the current thesis, can also be regarded as physical or chemical enhancement.

The effect of iontophoresis on transdermal penetration of SA across excised human skin has been investigated *in vitro*, and an enhancement ratio of 75 and 50 was found in unmodified skin and tape stripped skin, respectively. The enhancement by iontophoresis was also investigated for other drugs, showing that the effect was selective for smaller, anionic compounds (Singh et al., 1995).

Occlusion

Occlusion of the skin surface leads to increased hydration and increased temperature under the occlusion, and these conditions enhance the absorption of a variety of compounds. The effect is relatively independent of the physico-chemical structure of the compound, and the molecular basis for the reduction in skin barrier function following occlusion is under debate (Schaefer and Redelmeier, 1996). Occlusion has been shown to increase the percutaneous penetration of some, but not all steroids by a factor 10–15 in one study (Feldmann and Maibach, 1965) and a factor 2-8 in another study (Bucks et al., 1988), contrasting with the finding of an effect of 100-fold increase in absorption for 3 other steroids, scored by vasoconstriction in an early study (McKenzie and Stoughton, 1962).

It should be noted that some vehicles such as petrolatum can be partially occlusive in themselves, occlusive conditions do not only occur with the use of a physical barrier such as plastic wrapping.

Furthermore, the application of an "infinite" dose as used in the experimental part of this thesis can lead to SC hydration and conditions can thus be regarded as partly occluded.

Choice of dosing regimen: single vs. multiple

Depending on the clinical purpose of a penetration study, single or multiple dosing may be most relevant. In a study of percutaneous penetration of hydrocortisone in Rhesus monkeys, the amount absorbed was shown to increase significantly between the first application (day 1) and the last (day 8) in a once daily treatment schedule, using 2 different vehicles and an appropriate placebo control (Wester et al., 1980). However, in a recent human study of multiple dosing of ^{14}C hydrocortisone, the absorption was significantly increased when applied three times daily as compared with once daily (Wester et al., 1995). The cause of this increase is not clear, but could be the phenomenon of release of drug contents in the SC reservoir by the later application of cream base only. This has been shown as rise in plasma cortisol just after application of cream base alone during a dexamethasone-suppression study in patients with atopic dermatitis or widespread dermatitis (Turpeinen, 1991; Aalto-Korte and Turpeinen, 1995a).

Temperature: heat and exercise

The temperature at which an *in vivo* penetration experiment is conducted is also a parameter to control; clinical studies of transdermal drug delivery of nicotine, nitro-glycerine, clonidine and methyl salicylate have shown significantly increased drug absorption with higher peak concentrations after letting subjects either stay in a sauna or produce heat (and vasodilation) by ergometer cycling (review by Vanakoski and Seppälä, 1998). The percutaneous penetration of methyl salicylate was also found to be significantly increased in healthy subjects either exercising, resting at 40°C, or both, the latter increasing the absorption 3-fold (Danon et al., 1986). However, it generally requires very drastic alterations in temperature, such as leav-

ing a sauna and going out in cold weather, to alter the penetration of a compound through the human skin by one order of magnitude (Barry, 1983).

Regarding *in vivo* vs. *in vitro* studies of penetration

In vitro models of cutaneous penetration are used for permeation studies and screening procedures due to the ease of experimentation. Comparisons of percutaneous absorption *in vitro* and *in vivo* generally show a reasonably good agreement, mainly for hydrophilic compounds, but care should be taken to make comparisons within the same species and the same conditions of application and formulation (Schaefer and Redelmeier, 1996). Thus, *in vitro* studies can provide relevant data for some compounds. The penetration of a compound in a static Franz diffusion cell, through a skin membrane prepared from human or animal skin, will obviously be different from the *in vivo* situation in many ways, with the 2 most striking being the effect of microcirculation and drug metabolism in the skin.

Drug metabolism in the skin

The skin is by no means a passive membrane across which drugs can traffic unaltered. The skin has significant metabolic activity and capacity, and a range of oxidative, reductive, hydrolytic and conjugative reactions take place. In other words, the skin is a source of extrahepatic metabolism of many xenobiotics and of topically applied drugs. (For a discussion of the cutaneous metabolism of betamethasone 17-valerate, see Chapter 7: Model Substances). The effect of drug metabolism can be studied *in vitro* in freshly excised skin or in tissue cultures (Kubota et al., 1994b), or *in vivo* in laboratory animals or in perfused skin flap models of human skin grafted onto e.g. athymic mice (Guzek et al., 1989). The latter study demonstrates substantially different drug distribution and metabolism of salicylate diester, which is enzymatically cleaved in the skin to a monoester and ultimately to SA, obtained *in vivo* and *in vitro*. As a result of the lack of capillary perfusion and consequent drug clearance, the SA levels in the skin were higher *in vitro*.

The microdialysis method can, by sampling ECF in the dermis, reveal pharmacological and metabolic processes previously not accessible to *in vivo* investigation. An example is the concentration of acetylsalicylic acid and its metabolite SA in the dermis after systemic drug delivery, shown in human study II of this thesis (Fig. 11.1, Chapter 11).

Cutaneous blood flow

Whereas the viable epidermis relies on diffusion of nutrients from below by passive diffusion, the dermis has an extensive vascular network (Fig. 3.1), which participates in various processes including nutrition, heat exchange, immune responses and thermal regulation.

Blood flow to the dermis follows terminal arterioles, arterial precapillaries and arterial loops, and drainage occurs via venous loops, postcapillaries and venules terminating in the skin veins. Two horisontal netlike plexuses are formed, one in the upper dermis and another in the lower dermis at the interface between dermis and subcutis; the two are connected through vertical capillaries and arterio-venous anastomoses (Yen and Braverman, 1976). The superficial system of capillary beds makes loops reaching up into the dermal papillae (60–70 per mm^2) and forms a particularly dense network around append-

Diagram: Blood vessels of the skin

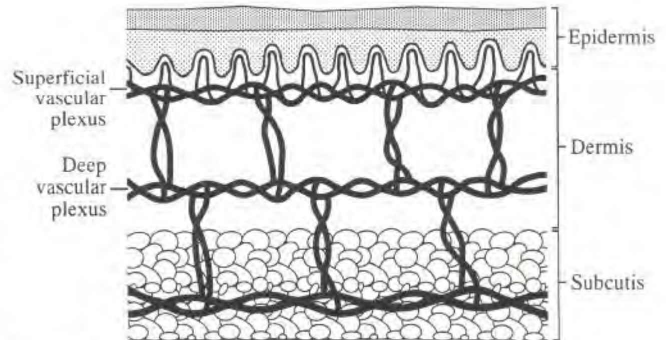


Fig. 3.1. Blood vessels of the skin.

ages. Whenever the epidermis is injured, there are immediate changes in the permeability and blood flow in the underlying capillaries. The control of cutaneous blood flow has been estimated to vary up to 100-fold in response to the environment, hormones and xenobiotics. The lymphatic system starts in the dermis as primitive lymphatic spaces, lined by primitive endothelial cells. They interconnect and ultimately form lymphatic vessels with valves. The lymphatic system is responsible for clearing protein and other substances leaking from the post-capillary venules (Ryan, 1983).

In a recent human study, microdialysis probes were used for delivery of β-adrenergic drugs to the dermis during monitoring of cutaneous blood flow by laser-Doppler flowmetry. The vasoactive effect of intradermal delivery of agonistic or antagonistic drugs was evaluated, and the significance was assessed by the application of indirect body heat, showing that the vasodilation response to heat was not affected by β-antagonist activation in the skin (Crandall et al., 1997).

The influence of cutaneous blood flow on dermal drug levels

Blood flow through the skin may influence percutaneous absorption (Riviere and Williams, 1992). This may be particularly likely whenever the barrier function of the SC is compromised or when a compound with a very high rate of penetration in uncompromised skin is applied. It can be speculated that drugs that affect the cutaneous microvasculature, such as vasoconstrictive corticosteroids or vasodilating so-called "rubefacients" may influence their own absorption.

The resorption, i.e. the uptake of compounds by the cutaneous microvasculature, is directly related to the surface area of the exchanging capillaries as well as their blood flow. For most topically applied compounds, resorption of drug in the dermis does not limit the delivery of drugs to the central compartment due to the much larger effect of resistance to diffusion present in the SC. The exception is compounds with very rapid diffusion across the SC, where the maximum rate of resorption of around 0.3 cm/h can limit the absorption into the central compartment (Kasting and Robertson, 1993). Such situations arise when the compound studied is highly lipophilic or the SC barrier function is compromised, as in the barrier perturbation studies of this thesis.

Examining the systemic levels of SA in rats (Chapter 8), it was found that the ratio of systemic uptake, calculated from SA concentrations sampled in remote sites divided by the concentration sampled at the dosed site, was statistically signifi-

cantly different after SA application onto tape stripped skin in comparison with unmodified skin. The ratio was 7.6% in unmodified skin and 0.9% in tape stripped skin, where absolute SA levels were much higher (Table 8.2). This could be taken as evidence of the formulation suggested by McNeill et al. (1992) and confirmed by Monteiro-Riviere et al. (1993) that "the cutaneous microvasculature is not simply an infinite sink for removal of topically applied drugs" and that below-chamber accumulation of SA occurred due to the dermal concentration at the dosed site, obtained after tape stripping of the SC, being in excess of the resorption capacity of the cutaneous microvasculature.

In a study of radiolabelled SA penetration in rats after dermal application (epidermis removed by use of a dermatome) for 2 h, local direct penetration was evident to a depth of 3–4 mm below the dosed site. The examination of SA concentrations in deeper tissues, contralateral tissues and plasma showed that systemic circulation of SA was responsible for equal concentrations in plasma and contralateral tissues of < 1% of the concentration in the skin at the dosed site, whereas the SA concentration in deeper tissues (4–12 mm below the application site) was higher due to a combination of direct SA penetration and systemic distribution (Singh and Roberts, 1993).

The most clear-cut experiments demonstrating the importance of dermal clearance have been conducted by Siddiqui et al. (1989). The disappearance of 7 different steroids from chambers placed on the dermal surface (epidermis removed) was compared in anaesthetized and sacrificed rats, showing a 2–5-fold higher clearance in the living rats. Singh and Roberts (1994a), using the same experimental procedure, found the clearance of 7 NSAIDS, among them SA, to be around one order of magnitude faster in living rats when compared with sacrificed rats. The difference in clearance was the largest for SA, with a 5-fold increase when cutaneous microcirculation was present.

With the use of vasoconstriction (by adding epinephrine 1:5000 to the dermal application fluid), the resulting clearance of SA in the live animal lies between the values for live and sacrificed animals, with enhanced local tissue drug levels down to 6 mm below the site of application (Singh and Roberts, 1994b).

In conclusion, vasoconstrictors that decrease blood flow to the skin will lead to an increased diffusion of drug into the underlying tissue following topical application, and there is no doubt that cutaneous blood flow influences both dermal drug levels, the depth of deep penetration below the site of application and systemic distribution.

Regarding blood flow and erythema in the barrier perturbation studies of this thesis

The systemic absorption ratio for the rat study and the impact of tape stripping on systemic distribution has been mentioned above, but the impact of cutaneous blood flow on the absorption rate has not been discussed. Whereas cutaneous blood flow is probably increased following tape stripping of the SC, no firm statement can be made about the blood flow in the barrier perturbation experiments in this thesis. We measured erythema by colorimetry, and the registration of colour shades by the colorimeter is not a measurement of blood flow velocity in the skin. There is a relationship between the superficial skin blood flow and the colour, whenever the two are part of the same inflammatory process as in e.g. irritant dermatitis. From the studies by Agner and Serup (1990) and Wilhelm et al. (1989), where SLS-induced irritant reactions are simultaneously quantified by several non-invasive techniques, the non-linear correlation between the two parameters can be seen from data presented in the former (with readings at 24 and 48 h), whereas a correlation coefficient of 0.83 between individual measurements of the two can be found in the latter. Parallel measurements of skin colour by colorimetry (a*) and skin blood flow by laser Doppler perfusion imaging were undertaken in a human study investigating the insertion trauma caused by placing a microdialysis probe in normal skin. In this study, the similarities in skin blood flow and erythema regarding response to cutaneous trauma and pattern of normalization can be seen (Groth and Serup, 1998).

CHAPTER 4: *IN VIVO* METHODS FOR MEASURING DRUG LEVELS IN THE SKIN OR PERCUTANEOUS PENETRATION

The indirect radiochemical method

This is the classical method, introduced by Feldmann and Maibach (Feldmann and Maibach, 1965;1967;1970) by which most human *in vivo* percutaneous penetration experiments have been performed. Typically, a known quantity of a ^{14}C labelled substance is applied topically in a volatile solvent vehicle and penetration is evaluated from the excretion of the ^{14}C radiolabel over the next 5–10 days. A correction for incomplete elimination is made by performing an identical protocol after intravenous administration of the same ^{14}C labelled material. The approach has some limitations, since it does not discriminate between the parent drug and its metabolites, and the time resolution is bound to be coarse due to a combination of analytical sensitivity and practical factors (urinary and faecal collections). Furthermore, the elimination process following topical and intravenous drug administration must be assumed to be identical. It has been suggested to add tape stripping of the application site to the protocol in order to obtain an improved mass balance, i.e. account for all radiolabelled drug (Bucks et al., 1988). The method is not applicable in humans for compounds, which are toxic or either poorly absorbed or excreted, and it is relatively time-consuming, expensive and unsuitable for routine studies (Schaefer and Redelmeier, 1996).

Skin stripping method

Tape stripping of the human SC is widely used for studying the kinetics and penetration depth/absorption of topically applied drugs (Dupuis et al., 1984; Rougier et al., 1986; Rougier et al., 1987a; Pershing et al., 1992; Lotte et al., 1993). The method determines the concentration of chemical/drug in the SC at the end of a short application period (30 min) and by linear extrapolation, the percutaneous absorption of the compound over the following days is predicted. The method consists of applying the drug in a suitable topical formulation, and after 30 min adhesive tape is applied to the skin surface with gentle pressure and subsequently removed with the adhering SC cells. Layers of SC cells are removed by successive tape applications and removals, and the SC material on the tape strips is finally analysed for drug content.

In a comparative human study of the traditional total absorption method by measuring the amount of radiolabelled drug

excreted vs. cellophane tape stripping of stratum corneum 30 min after drug application and measurement of radiolabelled drug on the strips, a linear correlation ($r = 0.97$, $p < 0.001$) existed between the amount of drug present in the stratum corneum strips and the total amount absorbed and excreted over 4 days. Thus, for the 4 compounds studied (benzoic acid, benzoic acid sodium salt, caffeine and acetylsalicylic acid) the percutaneous penetration could be predicted by the stripping method (Rougier et al., 1987a). The authors rightly highlight the advantage of the skin stripping method: since a relatively large amount of drug/substance is present in the horny layer 30 min after application, the method could be used with traditional nonradioactive analytical techniques. The collection of excreta can be abandoned. Furthermore, if substances had to be radiolabelled, the contact time would be short.

Another comparative human study concerned the correlation between the traditional skin blanching assay, which is not always conducted in a standardized fashion, and the skin stripping method for measuring bioavailability of topical betamethasone dipropionate in 4 different formulations. In this study, where the drug exposure time was 24 h, a close correlation could be demonstrated between the amount of drug in the stratum corneum strips and the skin blanching ($r = 0.994$). The amount of SC removed by the stripping procedure was quantified *in vitro* and *in vivo* and found to be > 90% of the total SC (Pershing et al., 1992).

The limitation of this method is that the quantity of SC cells removed by tape stripping is not linearly proportional with the number of tape strips, and several factors can influence the quantity of SC removed by each piece of tape, i.e. the manner of tape stripping, the hydration of the skin, anatomical site and interindividual variation (Marttin et al., 1996). Recent papers using electron microscopy and X-ray microanalysis techniques have disclosed that skin stripping yields SC cell layers that originate from varying depth in the skin due to the macroscopic furrows running in parallel to the skin surface. Thus, tape stripping will remove incomplete cell layers, i.e. the "hills", whereas the cells in the "valleys" will not be accessible to removal by the adhesive tape (Pflücker et al., 1997; van der Molen et al., 1997). The findings in these studies must lead to the recommendation that the interpretation of the skin strip method should be made with caution, and that its validity in determining drug contents in various layers of the SC can be questioned. It should also be noted that employing the skin stripping technique in patients with diseased skin is likely to introduce additional variability (Shah et al., 1998) and that no standardization for the use of the method in diseased skin exists.

Suction blister method

For a description of the method principle, see Chapter 2. The drug can be administered before (Korting et al., 1982; Bruun et al., 1991) or after the blister production (most other studies). Occasionally, the technique has been used for the assessment of drug levels in the dermis after topical drug administration (Averbeck et al, 1989; Surber et al., 1993) but the technique is generally used with systemic dosing.

In addition to the blister fluid, the epidermal blister roof can be sampled, homogenized and analysed for drug concentration. The suction blister fluid (SBF) has been found to correspond to average interstitial tissue fluid of the dermis. The method allows measurement of molecules of all molecular weights, but preserves the vascular effect of "molecular sieving", i.e. the larger the MW, the lower the passage into the SBF (Rossing and Worm, 1981). The protein content has been found to be 29% of the content in plasma (Vermeer et al., 1979), but a 24% mean increase in protein content in SBF during 12 h has been found (Bruun et al., 1991).

Suction blisters are mainly used for the evaluation of peripheral compartment kinetics of drugs used in infectious or inflammatory diseases, since knowledge of drug distribution, tissue penetration and bioavailability is particularly relevant to this group of drugs. Studies have concerned the distribution of beta-lactam drug in patients undergoing abdominal surgery (Mazzei et al., 1994), sulfonamide and trimethoprim pharmacokinetics after oral and intravenous administration (Bruun et al., 1991), netilmicin pharmacokinetics in small vs. large suction blisters (Blaser et al., 1991) metronidazole and metabolites (Klimowicz et al., 1996) and fusidic acid pharmacokinetics after repeated administration of two dosages (Vaillant et al., 1992). Most studies show that drug levels in the blisters are characterized by a delayed peak concentration and a prolonged elimination time, occasionally with SBF levels exceeding those of plasma after some time (Hoffstedt et al., 1982). In the latter study, tissue concentrations of 2 antibiotics were evaluated by SBF technique and compared with a technique using "implanted cotton threads", inserted in the subcutaneous tissue and successively pulled out 2 cms, cut off and subsequently analysed for drug content. The thread method, sampling extracellular fluid in the tissue by simply absorbing it, can be considered a primitive forerunner for the microdialysis technique in some respects.

Suction blister studies of the psoralen group of drugs are particularly relevant, since this drug is administered prior to UVA-irradiation as a part of photochemotherapy (so-called PUVA treatment) of skin diseases such as psoriasis or atopic dermatitis (Parrish et al., 1974). Since the UVA exposure should preferentially coincide with the maximum concentration in the skin for maximum efficacy, knowledge of the pharmacokinetics in the skin is particularly relevant for this drug. The 8-methoxy-psoralen level in plasma and in suction blisters, raised after drug administration, has been found to correlate closely, and the possibility of calculating skin levels from plasma samples has been suggested (Korting et al, 1982). Using a biological assay, the content of psoralen in SBF after topical and systemic administration has been evaluated, and the effect of altering the vehicle in topical preparations on cutaneous penetration and thus psoralen content in the SBF has also been demonstrated (Averbeck et al., 1989).

Each blister results in a small erosion, which heals over 5–10 days, often with post-inflammatory hyperpigmentation during some months, so the number of blisters that can be raised in one subject is limited. In diseased skin, particularly if epidermal changes are pronounced, blisters can often not be raised and the technique has not been standardized for use in diseased skin.

For a comparison of dermal drug levels sampled by microdialysis and SBF methodology, see Chapter 11.

Microdialysis for pharmacological studies

The principle and theory of the microdialysis method can be found in Chapter 1.

For the first many years of microdialysis experimentation it was utilized solely in animals, and most studies were conducted in the rat brain in neurophysiological and neurophysiochemical studies of neurotransmitter release, sampled in awake rats. The first report on microdialysis in humans came from Lönnroth et

al. (1987), who measured glucose in the subcutaneous fat of the abdominal wall. Since then a whole field of research in glucose and lipid metabolism, using microdialysis to study the effect of exercise, hormones and other factors on the concentration of endogenous compounds, has been established.

The development of microdialysis for drug sampling and pharmacokinetic analyses began in the late 80'ies and the first clinical study in humans, concerning the concentration of caffeine in adipose tissue, was published in 1991 (Ståhle et al., 1991a). Microdialysis can be, and has been, performed in almost every organ or tissue of the body: muscle, liver, heart, subcutaneous tissue, systemic blood circulation, skin, eye, cortical bone and even in the brain of patients undergoing epilepsy surgery or in intensive care following stroke or severe head injury. For a survey of microdialysis in pharmacokinetic studies, see review by Elmquist and Sawchuk (1997).

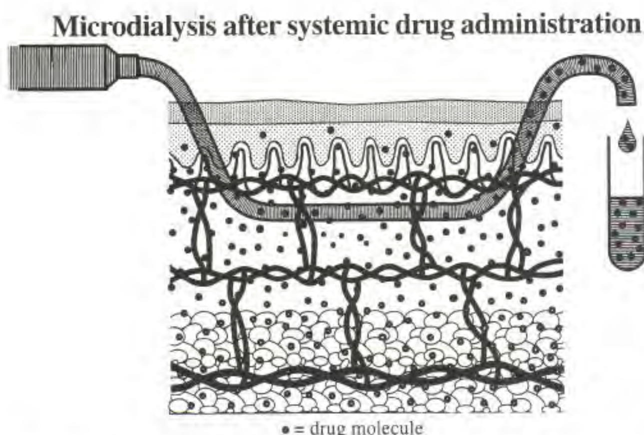


Fig. 4.1. Microdialysis in the dermis after systemic drug administration.

In human drug studies to determine pharmacokinetics of propranolol (Lönnroth et al., 1991), paracetamol (Müller 1995a) and theophylline (Müller 1995b), different classes of antibiotics (Müller et al., 1995a, 1996, 1997a; Lorentzen et al., 1996) sampled in muscle and subcutaneous tissues. The technique has also been assessed for monitoring free valproic acid s.c. in ambulatory epileptic patients for up to 3 days (Ståhle et al., 1996; Lindberger et al., 1998).

Recent interesting applications of microdialysis include the monitoring of drug interactions e.g. rat studies showing a doubling of methotrexate bioavailability in the presence of the NSAID naproxen, probably through a combination of competition for protein binding sites on albumin and possibly also altered renal excretion of methotrexate – a drug-drug interaction of relevance to the clinical situation (Ekström et al., 1997).

Another promising use for microdialysis sampling is in investigations of the distribution of antineoplastic drugs into tumour tissue. Distribution of platinum into malignant melanoma tissue and 5-fluorouracil in breast carcinoma has been studied, and in the latter study the authors relate the tumour-to-plasma AUC ratio to the clinical outcome of chemotherapy in each patient. This approach undoubtedly has a great potential for optimising treatment schedules, for identifying so-called poor responders early, and for aiding the selection of novel antineoplastic compounds with favourable tumour penetration characteristics (Blöchl-Daum et al., 1996; Müller et al., 1997b).

Transdermal penetration and regional effect of topically applied formulations can also be studied by microdialysis sampling. Using probes in skeletal muscle below the site of twice daily application of a diclofenac foam formulation, an over-the-counter topical formulations used for arthritic or muscular symptoms, Müller et al. (1998b) have demonstrated direct drug penetration into the muscle tissue below the site of repeated application with significantly higher regional drug concentrations than measured in plasma.

Finally, the possibility of delivering drugs into the tissue by reverse microdialysis with simultaneous monitoring of drug responses in the target tissue has been demonstrated. e.g. the increase in muscle cAMP in response to local milrinone administration as well as dose dependent histamine release in response to compound 48/80 administration in the dermis (Müller et al., 1997d).

Microdialysis in the dermis

The 2 first report of microdialysis in the human dermis concerned the measurement of ethanol absorption across the normal skin (Anderson et al., 1991) and the quantitative measurement of cutaneous glucose concentrations (Petersen et al., 1992). The 2 research groups mentioned here have, as for the glucose/lipolysis area, started a field of research in allergic skin reactions, urticaria and immune mediators, based on the easy and reproducible sampling of the small, hydrophilic histamine molecule at the site of release in the dermis.

Microdialysis in the dermis has only been used for the investigation of pharmacokinetics of systemically administered drugs in few studies. As a part of a larger study, Müller et al. (1996) compared the pharmacokinetics of phenoxymethylpenicillin in inflamed and non-inflamed dermis of patients with cellulitis, following an oral dose, and found no differences. In human study II of this thesis, the dermal pharmacokinetics of ASA and SA were sampled by microdialysis following an oral aspirin dose, and the intraindividual variation was studied by using 4 probes in parallel for sampling (Benfeldt et al., 1999b).

Microdialysis for cutaneous penetration studies

The first reports regarding the use of microdialysis for dermal drug sampling came from Ault et al. (1994), who investigated 5-fluorouracil penetration in the dermis of rat skin *in vitro* (with the probe inserted in the skin in situ in a Franz cell) and *in vivo* in anaesthetized rats. They found a 40-fold higher drug concentration in the *in vitro* excised skin than in the *in vivo* intact skin, thus emphasising the effect of cutaneous blood flow. Histological examination of the tissue around the probes was made at regular intervals and showed a slight cellular infiltrate after 6 h and apparent fibroblast activity around the probe after 32 h. Other studies of drug penetration in animals include the investigation of betamethasone 17-valerate and fusidic acid (Benfeldt and Groth, 1998), salicylic acid (Benfeldt and Serup 1999) and of methotrexate (Matsuyama et al., 1994a) and valproate (Matsuyama et al., 1994b) in rats. In the 2 latter studies, the effect of adding the enhancer HPE 101 to the medium was a 4-500-fold and an 80-fold increase in transdermal penetration, respectively. There is an increasing number of microdialysis studies of transdermal formulations and chemical enhancer effects, measured in rats (Nakashima et al., 1996; Murakami et al., 1998a,b; Hashimoto et al, 1998).

Microdialysis for pharmacokinetic studies of cutaneous penetration in the dermis of humans has recently been reviewed in

a book chapter by Groth (1998), and in theses by Andersson (1995) and Groth (1996).

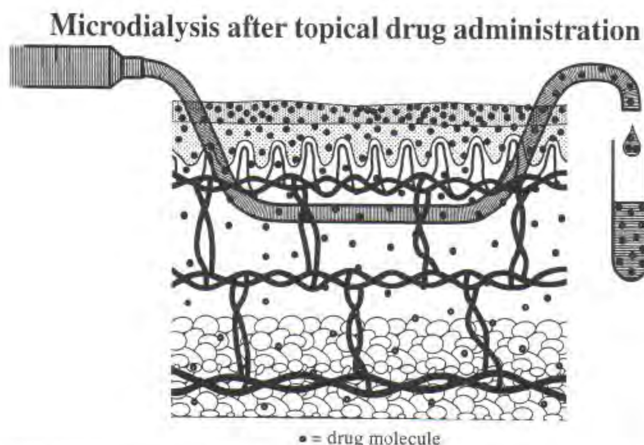


Fig. 4.2. Microdialysis in the dermis after topical drug application.

Although transdermal administration aims at systemic drug distribution, studies of drug levels under the site of administration can be considered to reflect topical pharmacokinetics. Intradermal microdialysis has successfully been used to describe the rising levels of nicotine under a nicotine patch for transdermal administration (Müller et al., 1995c; Hegemann et al., 1995). In the former, an association between nicotine steady-state levels, and also nicotine AUC, and probe depth in the dermal/subcutaneous tissue was found. In the latter study, neither values of TEWL nor probe depth in the dermis showed any correlation with nicotine kinetics. In the hairless rat study, we could demonstrate a correlation between probe depth and drug concentration, but not in the human study. In both studies, we found highly significant correlations between barrier perturbation, as quantified by TEWL and erythema measurements, and the cutaneous penetration of SA across the skin (Papers II and III).

It will probably be necessary to deliberately vary probe implantation depth (with some intentionally placed subcutaneously) if the relationship between probe depth and drug penetration is to be studied, particularly since with practise, the variation in probe implantation depth becomes rather small (e.g. SD of ± 0.16 mm in the rat study and 0.17 mm in human study II). On the other hand, in a human study of transdermal diclofenac penetration after a single application, employing the use of a superficial (3 mm below skin surface) and a deep (9 mm below surface) probe, no correlation between the AUC of diclofenac sampled and the depth of the probe could be demonstrated (Müller et al., 1997c). Whether the cause was interindividual differences in epidermal penetration, in drug absorption and subsequent removal by the cutaneous vasculature, or both, was not determined.

The cutaneous penetration of topical lidocaine and prilocaine in EMLA® cream has been shown in a preliminary report (Anderson et al., 1997). The mixture of anaesthetics, formulated in a cream base, was applied to the intact skin and dermal levels were detectable after 60 min.

Advantages of microdialysis

When listing the advantageous features of microdialysis (also reviewed by Ungerstedt, 1991), it should be highlighted that

(i) it samples in a defined compartment within the tissue, namely the ECF, as opposed to whole tissue sampling by biopsies and other types of tissue removal.

(ii) it can be performed in any organ or tissue of the body, including the circulating blood.

(iii) it causes minimal damage to the tissues.

(iv) it has a much higher temporal resolution than other methods.

(v) following the insertion of a probe, continuous (repeated) sampling for hours, days or even weeks is feasible, particularly since portable pumps with all necessary control features are becoming available.

(vi) monitoring of pharmacokinetics at multiple sites in a single animal/subject simultaneously can be made and as a result, overall fewer experiments and fewer animals/subjects are used. Variability can be reduced, as intraanimal/subject variation is smaller than interanimal/subject variation.

(vii) it can be used both for sampling and for the delivery of drugs to the tissue via the probe, enabling the study of local effect responses.

(viii) the exclusion of protein prevents enzymatic degradation of samples and no sample preparation is necessary prior to analysis.

In volunteers and patients alike, the tolerability of microdialysis sampling is high. Pharmacokinetic studies of up to 3 days duration have been performed in ambulatory patients (Lindberger et al., 1998), and glucose monitoring has been performed in healthy volunteers for up to 3 weeks (Wientjes et al., 1998).

Limitations of microdialysis

One of the limitations of microdialysis is that, in most instances, the subject studied must rest in the supine position for the duration of the experiment, particularly if the experiment concerns cutaneous penetration or combines microdialysis with other methods such as blood flow measurements. An initial investment in precision pumps has to be made, investigator has to acquire the skills for the *in vivo* probe insertion procedure, and reproducible manufacturing of probes in the laboratory has to be established (investigator or laboratory technician). Microdialysis requires access to sensitive methods of analysis and often to further method development, if traditional methods of analysis are used. As suggested by Ståhle (1991a), microdialysis sampling of compounds becomes increasingly difficult as hydrophobicity increases. Furthermore, the accessibility of very heavily protein-bound drugs has proved to be limited, see below.

Drug-protein binding problems

We have reported (Benfeldt and Groth, 1998) reduced *in vivo* recovery of a heavily protein-bound drug, fusidic acid, both after topical and systemic drug delivery (see table 7.1 for characteristics). *In vitro* RR over a wide concentration range was 44%. In human experiments, twice daily application of the commercially available ointment formulation of 2% fusidic acid onto normal skin and skin with irritant dermatitis was made for 48 h. No fusidic acid could be detected in the dialysate, using 2 probes in the dermis of each area. In hairless rats, the application of the commercial 2% ointment was again without detectable dialysate levels. Topical treatment with either 2% ointment, applied onto irritant dermatitis, or 10% fusidic acid in

100% ethanol for 72 h under occlusion did not result in measurable levels in the dialysates. Systemic dosing in clinically relevant doses were without detectable levels of fusidic acid, and only after stepwise increasing the dose to 312.5 mg/kg was fusidic acid measurable in dialysates in concentrations of 10-90 ng/ml (Benfeldt and Groth, 1998).

The albumin concentration in the ECF has been studied in the (undisrupted) human skin *in vitro* and showed a concentration of albumin in the interstitial fluid of 0.44–0.93 of the albumin concentration in plasma with a mean of 0.68, n = 4 (Bert et al., 1986).

For the solution of the protein-binding problem, it will probably be necessary to introduce a competitive protein content in the perfusate (with the shifting of problems to the analytical side). A confirmation of this idea can be found in a study of the protein binding effect of SA in an isolated perfused rat limb model, where radiolabelled SA was applied in a chamber placed directly onto the exposed dermis (epidermis removed) and the concentration of SA in the perfusate, running through the limb, was measured. A significantly increased concentration of SA in the perfusate was observed when 4% albumin was added to the perfusate (Cross et al., 1996). The experiment has similarities to the principle of adding albumin to a microdialysis perfusate in the investigation of cutaneous (full-thickness) penetration.

Lipophilicity problems

Very lipophilic drugs are not easily sampled by microdialysis due to the aqueous nature of the perfusate and frequently encountered problems with drug adherence to tubing and plastic vials (Groth and Jørgensen, 1997). We have described the problem of reduced *in vivo* recovery of the lipophilic corticosteroid betamethasone 17-valerate (B17V, see table 7.1 for characteristics). *In vitro* microdialysis of B17V showed a RR of 38% over a wide concentration range. In human experiments, twice daily application of the commercially available ointment formulation of 0.1% B17V onto normal skin and skin with irritant dermatitis was made for 48 h. No betamethasone could be detected in the dialysate, using 2 probes in the dermis. In hairless rats, the application of the commercial 0.1% ointment was again without detectable dialysate levels. It required topical treatment with 4% betamethasone 17-valerate in 100% ethanol for 72 h under extreme conditions, such as provocation of severe irritant dermatitis prior to application or the use of continuous occlusion, to obtain measurable drug levels (11–45 ng/ml) in the dialysates. Systemic dosing in clinically relevant doses was without detectable levels of B17V in the dialysate, and only after stepwise increasing the dose to 158 mg/kg was B17V measurable in concentrations of 25–44 ng/ml in the dialysates (Benfeldt and Groth, 1998).

In a human study of transdermal delivery of estradiol, high dermal drug levels could be expected to provide some feasibility of sampling this very lipophilic hormone. The *in vivo* recovery of estradiol by the retrodialysis technique was 2%, and in the penetration experiment, dialysate concentrations, sampled in the subcutis below the patch, proved below the limit of detection of analysis in 8 of 10 subjects. Estradiol was thus characterized as 'non-dialysable' (Müller et al., 1995c).

In an *in vitro* study of 3 psoralens, the inherent difficulty in using microdialysis methodology for the investigation of these lipophilic drugs (with log P values of 1.9, 2.0 and 3.1) became evident. The most lipophilic of the compounds, trimethylpsoralen, was not detected in the dialysate at all, using 2 different types of probes and an *in vitro* concentration of 1 µg/ml and an aqueous buffer as perfusate (Mary et al, 1998). This study also demonstrated the discriminatory capacity of the microdialysis process: although the 2 remaining psoralens, 5-methoxypsoralen and 8-methoxypsoralen, have identical molecular weights and almost identical log P values, the relative recovery of 5-methoxypsoralen was consistently twice as high as that of 8-methoxypsoralen (Mary et al., 1998).

The possible solution to the decreased recovery of lipophilic compounds has been investigated by Carneheim and Ståhle (1991), who improved *in vitro* recovery of an extremely lipophilic compound, oleate, by adding albumin or a physiological lipid emulsion (for intravenous nutrition) to the perfusate. Recently, adding lipid to the perfusate has been investigated further in an *in vitro* study, where the use of lipid emulsion as perfusate led to greatly enhanced (2-fold up to 390-fold) *in vitro* recoveries of the 4 alkylparabens used as model lipophilic substances. A log-linear relationship for increasing enhancement by lipid in the perfusate and the lipophilicity of the model drugs was also shown (Kurosaki et al., 1998).

Microdialysis for *in vivo* sampling in diseased human skin

It has been demonstrated in this thesis (papers I and III) that microdialysis sampling can be conducted in different types of barrier perturbed skin. This posed no technical problem. The use of microdialysis in patients with psoriasis, inserting the probe in the psoriatic plaque or in the unaffected skin, has recently been described in 2 studies. The first study demonstrated that histamine release in the psoriatic plaque is 10-fold elevated in comparison with non-lesional skin (and values in healthy volunteers) by calculation of histamine release from information obtained by microdialysis sampling in the skin, ^{133}Xe determination of blood flow and histamine concentration in plasma, using Fick's law (Krogstad et al., 1997). In the second study local anaesthesia, administered in the form of topical application of EMLA® cream under occlusion, was shown to have no effect on the release of histamine in psoriasis, but to cause a 30% reduction in cutaneous blood flow as measured by Laser Doppler perfusion imaging and ^{133}Xe washout method. Using iontophoresis of adrenaline into the lesional skin in 3 patients, the effect of vasoconstriction was an increase in histamine concentration in the psoriatic tissue, confirming the relationship between the two (Krogstad et al., 1998).

CHAPTER 5: THE BARRIER FUNCTION OF NORMAL HUMAN SKIN

Various factors influence the barrier function of the unmodified skin in humans:

Age

The effect of age on the function of the skin as a barrier is surprisingly small.

The epidermal development in utero is complete at 34 weeks gestation, and babies born at 30–32 weeks gestational age seem to have a competent barrier, comparable with that found in adults, according to a recent study of barrier function in pre-

mature infants (Kalia et al., 1998). In an *in vitro* permeation study of skin samples from newborn and preterm infants, the permeation of sodium salicylate was 10^2–10^3 times greater in infants of 30 weeks gestation or less compared with babies born at term (Barker et al., 1987).

Baseline TEWL is mainly independent of age in the remaining range, with the exception of the just significantly decreased TEWL in the elderly (Pinnagoda et al., 1990). In a study comparing barrier function in young (20–30 years) versus aged (>80 years) humans, the barrier was more easily perturbed with tape stripping or acetone, and barrier function recovered more slowly in the elderly (Ghadially et al., 1995). The reason for this could be the significantly decreased level of all lipid classes, found with increasing age in a human study by Rogers et al. (1996), comparing the SC lipid content and composition in tape strippings from females of all ages.

The percutaneous penetration of 6 different radiolabelled compounds (testosterone, estradiol, hydrocortisone, benzoic acid, acetylsalicylic acid and caffeine) has been compared in young (18–40) and old (>65 years) human subjects, and the absorption of the 4 latter compounds was significantly reduced in the older group. Testosterone and estradiol, on the other hand, showed similar penetration in both groups. The authors speculate that the diminished surface lipid content of "old" skin has a more pronounced impact on the dissolution of hydrophobic compounds into the SC, whereas the highly lipid-soluble compounds testosterone and estradiol are not affected by this physiological change (Roskos et al., 1989).

Gender

In most studies, sex as a factor has no apparent effect on baseline TEWL nor on susceptibility to irritants (Pinnagoda et al., 1990). However, in one study of irritant response to SLS in males and females, the mean TEWL increase was larger in women, and it was suggested that female skin is more prone to irritation (Goh and Chia, 1988). In the human barrier perturbation study presented in the current thesis (human study I, Chapter 9), no difference between sexes was found as regards irritability/reactivity or skin penetration, but the number of subjects (n = 16) is relatively small. However, an individual-specific overall reactivity index for each subject was found to correlate with the cumulated SA penetration in the same subject. This positive correlation was strongest in women (n.s. when analysed for men only) and has not been demonstrated before.

Race

Microscopic examination of skin from different races show no substantial differences, and baseline TEWL has found to be without differences between human races (Pinnagoda et al., 1990). In a study of the effect of tape stripping on TEWL increase, a more resistant barrier was found in darkly pigmented persons than in individuals with lighter complexion, regardless of race as such (Reed et al., 1995). The penetration of 3 compounds (caffeine, acetylsalicylic acid and benzoic acid) was compared in black, Caucasian and Asian subjects. No significant difference in penetration was found between races, but the number of subjects was relatively small (6–9 in each group) and the model substances for penetration were not very different (Lotte et al., 1993). Generally, studies of percutaneous penetration show different patterns of penetration depending on the molecule tested, and although the majority of studies that find any difference in penetration favour a lower penetration in black skin, no firm conclusion can be deducted from a review of the relevant literature (Berardesca and Maibach, 1996).

The human subjects in Human study I were Caucasians with skin types I–III.

Anatomical site

The skin from different regions of the body shows pronounced differences with respect to skin structure, the thickness of the horny layer and the regional distribution of hair follicles and eccrine sweat glands. Furthermore, different anatomical regions are variably exposed to sunlight and the effects of tanning. It is therefore not surprising that baseline TEWL has been shown to vary, with the following rank order: palm > sole > forehead = postauricular skin = nail = dorsum of the hand > forearm = upper arm = thigh = chest = abdomen = back (Pinnagoda et al., 1990).

In the first study of regional variation by Feldmann and Maibach (1967), the absorption of ^{14}C hydrocortisone was assessed, and a rank order of scrotal skin > jaw angle > forehead > axilla > back > forearm > palm > planta pedis was found, with the absorption through the scrotal skin being 42 x that of the forearm. In a study comparing the excretion method with the skin strip method for 4 compounds, the effect of changing the anatomical site was also incorporated. For the 4 compounds studied (benzoic acid, benzoic acid sodium salt, caffeine and acetylsalicylic acid) the penetration showed a rank order of arm = abdomen < postauricular < forehead, with the skin of the forehead being twice as permeable as the arm or abdomen (Rougier et al., 1987a). Using the same study, now reported with additional information about TEWL measurements, a direct correlation between TEWL (measured over the contralateral site) and percutaneous penetration was found, demonstrating that every anatomic site has its specific features with regard to the outward movement of water and the inward uptake of molecules (Lotte et al., 1987).

In a study of methyl salicylate penetration, measured as SA excretion in urine, the application of 25% methyl salicylate to 50 cm^2 skin under an occlusive dressing was studied in 4 subjects. The rank order here was planta pedis < heel < instep < forearm < abdomen, with a four-fold increase in percutaneous absorption from planta to abdomen (Roberts et al., 1982).

One of the main factors influencing the rate of permeation across the human SC barrier at various sites has been found to be the regional variation in the content and composition of the intercellular lipid (Elias et al., 1981).

Even intraregional variation in skin characteristics such as vasoconstriction response to corticosteroid, topical penetration and susceptibility to injury exists. A discussion of the intraregional variation of the volar forearm region, relevant to the human experiment in this thesis, can be found in Chapter 9.

Other factors that may influence skin barrier function

In a recent investigation of skin barrier function in normal skin during two 24-h cycles, a *circadian rhythm* in TEWL, surface pH and skin temperature was found, suggesting that skin permeability is higher in the evening and night than in the morning (Yosipovitch et al., 1998). This variation may reflect alterations in sweat gland activity, accompanying the known diurnal variation in core temperature. In a study by Pershing et al. (1994), who investigated the vasoconstriction response to

topical application of betamethasone dipropionate in healthy volunteers, a more extensive and prolonged response was seen following application at 1600 h in comparison with application at 0900 h. This could indicate a parallel pattern in skin barrier function and corticosteroid penetration.

Seasonal variation in skin reactivity has been demonstrated by comparing SLS patch testing in 18 volunteers in the winter and summer period, respectively, with increased reactivity in the winter months as measured by TEWL (Agner and Serup, 1989), possibly explained by the decreased lipid content in the SC in the winter (Rogers et al., 1996). Studies of SLS-responses have also shown a small variation in skin susceptibility relating to the *menstrual cycle*, with increased reactivity on day 1 of the cycle in comparison with day 9–11 (Agner et al., 1991). Whereas the 3 former studies may be of some importance to studies of topical penetration, the latter is more likely to reflect variations in inflammatory reactivity, rather than alterations in skin barrier function. This is supported by the fact that pre-test TEWL measurements showed no difference relating to the menstrual cycle.

CHAPTER 6: PATHOPHYSIOLOGY OF THE SKIN BARRIER

Diseased skin

The presence of skin disease modifies pharmacokinetics in the dermis in many ways. Changes in blood flow and vascular permeability may alter the drug distribution to the target tissue and influence the elimination from the site of action as well. Changes in the thickness, structure or lipid composition of the SC will induce changes in barrier function, influencing the topical drug penetration and the subsequent bioavailability of a drug (Schalla et al., 1989).

Ichtyosis

The ichtyoses comprise a group of inherited and acquired dermatoses, characterized by a thickened and scaly SC, so-called hyperkeratosis. Although the molecular basis of these disorders of keratinisation is different, a slight reduction in barrier function is the most common finding. The TEWL, measured over the volar forearm, was significantly increased in patients with 3 types of heritable ichtyosis as was the vascular response to topical hexyl nicotinate (Lavrijsen et al., 1993).

Atopic dermatitis

Atopic dermatitis is a familiar skin disease associated with mucosal atopy and asthma. In eczematous skin of adults and children alike, the TEWL value is higher and the SC hydration lower than in uninvolved skin. The apparently uninvolved skin has been shown to be different from control subjects, with increased TEWL values, decreased hydration values and impaired water retention capacity, altogether demonstrating that the clinically uninvolved skin of patients with atopic dermatitis is functionally abnormal. Furthermore, the presence of active eczema determines the degree of impairment of the barrier function in uninvolved skin, even in sites far from the eczematous skin (Werner and Lindberg, 1985; Seidenari and Giusti, 1995). An increased susceptibility with a more intense response to topical application of irritants, as measured by either TEWL increase, visual scoring or ultrasound measurement of skin thickness increase, has been demonstrated in patients with atopic dermatitis, even in a quiescent phase (van der Valk et al., 1985; Agner 1990). This increased susceptibility is likely to be caused by a constitutionally impaired skin barrier function and not by general atopic hyperreactivity, as reactivity to SLS patch testing in patients with respiratory atopy (in the absence of dermatitis) has been found to be no different from healthy controls (Seidenari et al., 1996).

In systematic studies of percutaneous penetration of hydrocortisone, measured as a rise in plasma cortisol during oral dexamethasone suppression of endogenous cortisol synthesis, the following conclusions have been obtained: The percutaneous absorption in patients with atopic dermatitis is significantly higher during exacerbation than in remission in both adults (Turpeinen et al., 1988a) and children (Turpeinen et al., 1988b). In children, peak cortisol plasma levels (resulting from systemic hydrocortisone absorption) during exacerbation equalled levels seen in ACTH testing, where the adrenals are stimulated to maximum endogenous production. A strong correlation between the impairment of barrier function, as measured by TEWL, and the percutaneous absorption of hydrocortisone has been shown (Aalto-Korte and Turpeinen, 1993). Longitudinal investigation shows that both TEWL and percutaneous absorption of hydrocortisone normalizes over 5–7 days of treatment, and that individual reductions in TEWL correlates with changes in systemic hydrocortisone absorption (Aalto-Korte, 1995). In a study of topical hydrocortisone absorption in patients with erythroderma, in 6 of 7 patients on a basis of atopic dermatitis, the absorption was quantified by a modification of the radiolabel method. Systemic absorption of 19-93 mg, corresponding to 4-19% of the topical dose of 500 mg hydrocortisone, was found (Aalto-Korte and Turpeinen, 1995b).

Psoriasis

Psoriasis is an inherited skin disease characterized by dermal inflammation and epidermal hyperproliferation with abnormal keratinisation. It is a reference skin disease for the assessment of efficacy and potency of topical steroids, as the effect of treatment of a psoriatic plaque can be quantified (erythema, scaling and thickness) and compared with vehicle treated control plaques.

The barrier function of the skin is reduced in psoriasis. The SC has reduced water binding capacity and the TEWL is increased. Gradual normalization of TEWL occurs during remission, whether spontaneous or treatment mediated (Marks et al., 1981). The reduced barrier function has also been shown to result in increased percutaneous penetration in some (but not all) studies, depending on method, vehicle, pre-treatment and use of occlusion (see Wester and Maibach, 1992). From the elevated TEWL value, an impaired barrier function would seem likely, but the presence of thick, hyperkeratotic scales may distort drug penetration measurements and possibly alter reservoir capacity.

The microvasculature in the upper dermis is altered in psoriasis, with elongated, tortuous and dilated skin capillaries and an increased skin blood flow. Using Laser Doppler flowmetry cutaneous blood flow in psoriatic plaques has been shown to be 9 times that of normal skin, and the cutaneous blood flow approaches uninvolved skin during 3-4 weeks of treatment (Staberg and Klemp, 1984). The erythema of the psoriatic plaque is difficult to quantify reliably with instruments such as the dermatospectrophotometer or colorimeter as the thick scales disturb measurements (Serup, 1995).

Barrier perturbed skin

The following methods all lead to a decreased barrier function as determined by TEWL, and the recovery phase relies on replenishment of the intercellular lipid domain by immediate secretion of lamellar bodies and increased lipid and DNA synthesis for the restoration of skin barrier function (Menon et al., 1992, Proksch et al., 1993).

The results obtained in the present thesis are discussed in the context of existing data on drug penetration in barrier damaged skin, summarized in Table 6.1. A comment regarding the comparison of penetration studies can be found at the end of the chapter.

Irritant dermatitis

Sodium lauryl sulphate (SLS) is an anionic surfactant (detergent) frequently used for the induction of experimental irritant dermatitis in animals and humans (Agner, 1990,1991; Agner and Serup, 1989,1990; Tupker et al, 1997). The reaction consists of erythema with or without infiltration and epithelial changes depending on the concentration used and is typically produced by 24 h exposure in a chamber on the forearm, in the case of human experiments, or on the side or back of experimental animals. SLS induces a dose-related reaction (Agner and Serup, 1990), which can be monitored by TEWL measurements (van der Valk et al., 1984).

Ultrastructurally, in lower concentrations (0.5–1%) SLS penetrates to the nucleated layers of the epidermis and disturbs lamellar body processing and epidermal cell differentiation, resulting in prolonged barrier perturbation as demonstrated by increased TEWL values for 10–12 days (Fartasch et al., 1998). In higher concentrations (5%), the microscopic picture consists of parakeratosis, spongiosis, cytoplasmic and intranuclear vacuolation and, in severe reactions, necrolysis of the basal cell layer and loss of the dermo-epidermal junction (Willis et al., 1989).

We have found an *83-fold increase* in penetration in irritant dermatitis in hairless rats and a *46-fold increase* in penetration in mild irritant dermatitis and a *146-fold increase* in severe irritant dermatitis in humans. This is considerably higher than the 2-4-fold increases found in other studies (Moon et al., 1990; Wilhelm et al., 1991). See Table 6.1.

One factor to take into account is the timing of the removal of the SLS. Comparing the results from the hairless rats, where the SLS patch was removed 18 h prior to the experiment (n = 3), with a similar experiment in which the SLS-pre-treatment was removed 1 h prior to the penetration experiment after the same dose and exposure time (n = 3, data not shown), the SA concentrations measured in the latter were approximately 50% of the former. The TEWL values measured in the late-removal group were correspondingly lower as well, demonstrating that the barrier impairment reaches its maximum some time after the removal of the patch test as also reported in humans by Fartasch et al. (1998). With the only difference between the two experiments being the timing of the SLS exposure in relation to the experiment, this also illustrates the impact of small alterations in experimental procedure.

It has previously been shown that a positive relationship exists between the SLS concentration used in irritant patch testing and the barrier disruption detected by TEWL measurement (Agner, 1990) and that a positive correlation exists between TEWL measurements over normal human skin in changing anatomical sites and the percutaneous penetration of 4 organic compounds (caffeine, benzoic acid, acetylsalicylic acid and benzoic acid salt) tested in the same sites (Lotte et al., 1987). In a guinea pig study it has been shown that SLS-induced irritant dermatitis increases TEWL measurements as well as the percutaneous penetration of hydrocortisone, indomethacin and ibuprofen but not acitretin (Wilhelm et al., 1991).

We have found a *positive dose-response relationship* for the percutaneous penetration of SA in response to increasing SLS concentrations as can be seen in Fig. 9.9. This linear dose-response relationship of increasing penetration in response to an increased barrier insult has not, to our knowledge, been demonstrated *in vivo* in humans before.

We have also found that an *individual-specific overall reactivity index* for a subject correlates with the cumulated SA penetration in the same subject and that this positive correlation is strongest in women (n.s. when analysed for men only). A discussion of skin reactivity in humans is found in Chapter 9.

Delipidization

The removal of lipids from the SC barrier by treatment with apolar solvents results in perturbed barrier function with an increase in TEWL (Menczel, 1995). Ultrastructurally, the extraction of lipids from the SC has been shown to be the mechanism of barrier disruption (Yang et al., 1995; Fartasch 1997), and barrier recovery seems to be initiated by a rapid onset upregulation in lipid production and DNA synthesis (Proksch et al., 1993).

In a human study, Bucks et al. (1983) found no effect of delipidization with 1:1:1 trichlorethane on the percutaneous penetration of ^{14}C hydrocortisone, however, the penetration site used was the palm of the hand (in order to make the result relevant to occupational exposure) – which is quite different from the remaining integument regarding the thickness of the SC, absence of hair follicles etc. In a hairless guinea pig study, penetration through skin delipidized by an aggressive chloroform/methanol mixture was found to be up to 5-fold increased (Moon et al., 1990).

We have used absolute acetone, which is most often used in delipidization models and selectively removes glycerolipids and sterols from the skin, for a delipidization effect in both penetration studies reported. Even prolonged acetone exposure (1-3 h) makes little morphological change to the human skin (Fartasch, 1997) and it is a weak delipidization procedure compared with the above-mentioned treatment, which resulted in deep erythema and oedema after the delipidization. Nevertheless, acetone treatment resulted in a significant *2.2-fold increase* in SA penetration in humans, whereas a non-significant decrease in penetration was seen in hairless rats.

In the human study, the fact that neither TEWL measurements nor erythema scores were significantly different from untreated skin shows that microdialysis sampling of SA penetration is superior in sensitivity to the non-invasive measuring techniques in detecting this discrete barrier perturbation.

Tape stripping

Physical stripping of the stratum corneum using adhesive tape leads to a sequential increase in TEWL as the SC is progressively removed, demonstrating that barrier of the skin is provided by the whole of the SC (van der Valk and Maibach, 1990). Measuring the pH value of the skin between consecutive tape strippings, a gradient from the initial surface pH of 4.5–5 to a

Table 6.1. In vivo drug penetration studies in barrier perturbed skin.

Barrier perturbation	Species	Drug	Penetration ratio	
None			1	
Tape stripping	human	hydrocortisone	4	Feldmann and Maibach, 1965*
	hairless guinea pig	hydrocortisone	3	Moon et al., 1990*
	hairless guinea pig	benzoic acid	2.1	Moon et al., 1990*
	rat	salicylic acid	0.8– 46	Murakami et al., 1998a
	human	methylpred. aceponate	91.5	Günther et al., 1998*
	hairless rat	salicylic acid	180	Benfeldt and Serup 1999 (II)
	human	salicylic acid	157	Benfeldt et al. 1999a (III)
Irritant dermatitis	hairless guinea pig	hydrocortisone	4	Moon et al., 1990*
	hairless guinea pig	benzoic acid	2.1	Moon et al., 1990*
	hairless guinea pig	hydrocortisone	2.6	Wilhelm et al., 1991*
	hairless guinea pig	indomethacin	1.6	Wilhelm et al., 1991*
	hairless guinea pig	ibuprofen	1.9	Wilhelm et al., 1991*
	hairless guinea pig	acitretin	1.4	Wilhelm et al., 1991*
	hairless rat	salicylic acid	83	Benfeldt and Serup 1999 (II)
	human (1% SLS)	salicylic acid	46	Benfeldt et al. 1999a (III)
	human (2% SLS)	salicylic acid	146	Benfeldt et al. 1999a (III)
Delipidization	hairless guinea pig	hydrocortisone	5.2	Moon et al., 1990*
	hairless guinea pig	benzoic acid	2.7	Moon et al., 1990*
	hairless rat	salicylic acid	0.6	Benfeldt and Serup 1999 (II)
	human	salicylic acid	2.2	Benfeldt et al. 1999a (III)
Stripping+ occlusion	human	hydrocortisone	32	Feldmann and Maibach, 1965*

* denotes studies using traditional radiolabelled drug and 98 h excreta collection for absorption calculation.

pH of 6.9 in the deepest layers of the SC has been demonstrated in humans (Öhman and Vahlquist, 1994). Usually, not all of the SC is removed by tape stripping as 10–30% remains, depending on protocol and species. This subtotal removal of the SC increases water or solute permeability by approximately 10 to 20-fold, but the skin still retains substantial barrier properties even after extensive barrier perturbation (Schaefer and Redelmeier, 1996). There is some evidence that the cutaneous reservoir function of the SC, initially proposed by Vickers (1963), is largely removed by tape stripping. Generally, tape stripping is a very useful scientific tool for evaluation of the maximum amount of percutaneous absorption that can be achieved from a topical formulation (Schaefer and Redelmeier, 1996). Going through the literature, the enhancement of penetration induced by tape stripping has been very variable:

In a study of the effect of tape-stripping on *in vivo* TEWL measurements in (haired) rats, followed by studying the *in vitro* permeability of tritiated water in the same skin area, Scott et al. (1986) found a 50-fold *in vivo* increase in TEWL and a 36-fold increase in *in vitro* permeability after tape-stripping. The percutaneous absorption of SA was showed to be 10-fold increased in tape stripped guinea pigs in a study where drug absorption was calculated by the disappearance method (Washitake et al., 1973). In a hairless guinea pig study, tape stripping increased penetration of hydrocortisone and benzoic acid 2-3-fold only (Moon et al., 1990). In a recent microdialysis study by Murakami et al. (1998a), the topical and transdermal delivery of SA in 5 different ointment bases was studied in unmodified and tape-stripped (haired) rat skin. They found a highly variable effect (from slightly reduced to 46-fold increased) of tape-stripping on the dermal SA concentration, with large variations in systemic absorption as well.

In humans, hydrocortisone absorption through tape stripped skin has been found to be only twice the absorption in unmodified skin, but the procedure for tape stripping and thus the degree of SC removal was not specified. However, the effect of both tape stripping and occlusion was a 32-fold increase in absorption as measured by the excretion method over 10 days (Feldmann and Maibach, 1965). In a semi-quantitative human study, the amount of methylprednisolone aceponate (applied to a 100 cm^2 area on the back under an occlusive dressing) absorbed through the skin increased from less than 0.5% to 15% (i.e. a 30-fold increase) when tape stripping was used (Günther et al., 1998).

In the current thesis, the procedure of tape stripping resulted in a *180-fold increase* in dermal drug penetration in hairless rats and a *157-fold increase* in penetration in the human study.

Regarding comparisons between penetration studies

It is a simplified approach to compare the effect of any kind of barrier perturbation between studies without considering the experimental conditions. The effect is usually calculated by, for each study, merely dividing the total amount of drug absorbed in barrier modified skin by the amount absorbed in unmodified skin, or, in the case of cutaneous microdialysis data, dividing the Area Under the concentration-versus-time Curve (AUC) in e.g. tape stripped skin by that obtained in unmodified skin.

First, the topical dose administered may be a single application in a volatile vehicle or an "infinite dose", present at the epidermal side of the barrier throughout the experiment (as for the microdialysis experiments in this thesis). Second, the physico-chemical characteristics of the drug, and the vehicle chosen, will both have a (most probably large) effect of their own, with the penetration of some drugs being more facilitated

by the alterations in the skin barrier than others. Third, the time span studied, ranging from the observation of excretion of the total absorbed dose over 10 days to the monitoring of dermal drug levels for 4 h, will obviously be comparing different pharmacokinetic phenomena.

Whether measured at the level of the skin as the target organ or as systemic absorption and excretion, not many *in vivo* studies with quantitative data on the effect of barrier perturbation on drug penetration exist. In general, the entire complex of factors, which determine penetration rates in damaged or diseased skin, requires further study. In the opinion of this author, the opportunity of measuring the effect of alterations in the epidermal barrier function by microdialysis sampling just 1 mm below the skin surface in the dermis, obtaining detailed real-time chronology of penetration kinetics, is going to provide a leap forward in skin barrier and drug penetration research. Microdialysis has some inherent method weaknesses (see discussion in Chapter 4), and much work in the field of standardization has to be done before the method has found its place in basic research and in clinical and toxicological studies. The advantages, e.g. the ease of experimentation, the tolerability and safety in human experiments, the feasibility of application in diseased skin too, and the independence of the use of radiolabelled material for penetration makes it a technique of the future.

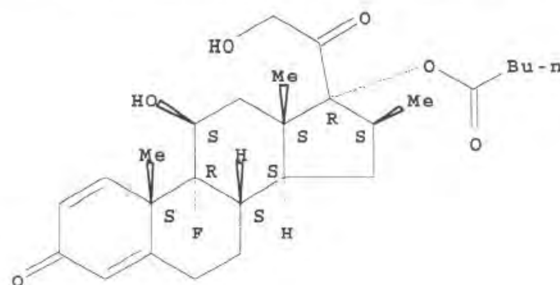
For a discussion of the clinical relevance of the results obtained in the barrier perturbation studies, see Conclusions (p 47).

CHAPTER 7: MODEL SUBSTANCES

Betamethasone 17-valerate

Betamethasone 17-valerate is a corticosteroid introduced for topical use as a 0.1% cream in 1967. It was considered a major breakthrough in topical corticosteroid therapy, as clinicians depended on the administration of parenterally administered steroids to treat difficult dermatoses, since the few available topical formulations (hydrocortisone, dexamethasone) did not possess adequate anti-inflammatory effect. The parent compound, betamethasone, had been identified and introduced for systemic use in 1961.

In the search for a more potent topical agent, derivatives of betamethasone were synthesized and screened by the Mackenzie-Stoughton test, which correlates vasoconstrictor activity and clinical efficacy (McKenzie and Stoughton, 1962). The most promising derivatives were tested in double-blind studies of treatment of psoriasis under occlusion for 10–14 days, and



Betamethasone 17-valerate

betamethasone 17-valerate was found to be comparable with the standard fluocinolone acetonide ointment (Samson et al., 1992).

Percutaneous penetration of betamethasone 17-valerate is less well described than the cutaneous vasoconstrictive effect. In a study comparing plasma levels of betamethasone after oral administration of 0.6 mg betamethasone vs. topical application of 4 mg betamethasone 17-valerate (applied in a 100 cm^2 adhesive formulation for 28 h), plasma peak levels were 20 times higher and the mean AUC 10 times higher after oral administration. However, the topical administration displayed a marked depot function, with a $T_{½}$ twice as long as after oral administration (Kubota et al., 1994a).

Problems with betamethasone 17-valerate: in vivo and in vitro metabolization

During the initial phase of our experimental work with betamethasone 17-valerate (B17V), we discovered that establishing the *in vitro* recovery of the drug posed a problem. Conducting *in vitro* microdialysis in beakers with B17V in stirred aqueous solutions of varying concentrations at 37° C, the resulting samples contained not only B17V, but also betamethasone and betamethasone 21-valerate (B21V). The conversion of 17-valerate to the 21-valerate isomer takes place *in vivo* and *in vitro*, when pH is > 5. It does not require the presence of enzymes, and as the reaction is faster than the next step in B17V metabolism, accumulation of B21V occurs. The next step, where B21V is converted to betamethasone, is accelerated in the presence of esterases, found in viable skin. The $T_{½}$ of the initial conversion has been found to be just over 8 h, whereas the degradation to betamethasone has been shown to have a $T_{½}$ of 0.87 h in the presence of skin homogenate and 3.89 h in the absence of skin esterases (Kubota et al., 1994b). *In vitro* permeation of B17V through human skin has been found to give

Table 7.1. Characteristics of model drugs.

Drug	MW (Da)	Protein binding	pKa	Charge at pH 7.4	Solubility (mg/ml water)	Log P[a]
Betamethasone 17-valerate	476.6	low[b]	–	neutral	0.0093	3.5
Fusidic acid	516.7	97%	5.35	anion	0.30	2.68
Salicylic acid	138.12	concentration dependent	2.97	anion	2.17	2.25
Acetylsalicylic acid	180.2	concentration dependent	3.49	anion	3.33	1.26

[a] Octanol/water partition coefficient.
[b] Siddiqui et al., 1989.

concentration ratios of B17V: B21V: betamethasone of 1:0.6:0.18 in the receptor fluid (Ademola and Maibach, 1993).

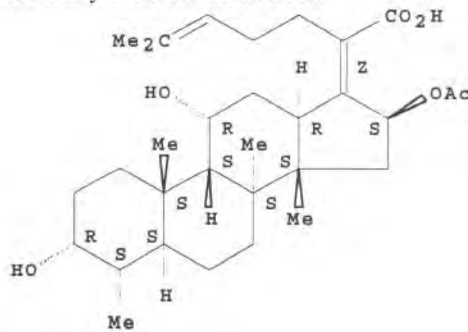
We conducted an *in vitro* experiment, where B17V (1 mg/ml in aqueous buffer) was kept at 25°C and samples were taken for 3 h, showing a conversion of B17V to B21V of 11% in 3 h. We left the solution at room temperature for a week, and re-sampling showed that 85% had now been converted to B21V.

Several *in vitro* microdialysis experiments, assessing the recovery of each of the 3 molecules, were performed using a 1:1:1 mixture of B17V:B21V:betamethasone in concentrations from 0.1/0.25/0.5/1.0/2.5 μg/ml. The results showed that the *in vitro* recovery rate (RR) was 75% for betamethasone, 36% for B17V and 33% for B21V. The differences in recovery can probably be explained by the difference in lipophilicity, with betamethasone being the least lipophilic of the 3.

At this stage, the negative results of the initial human experiments with topical B17V (described in Paper I) in combination with the obstacles involved in sampling 3 drugs with different recoveries and measuring 3 rather than 1 compound in each sample led to the conclusion that B17V was not a good candidate for further microdialysis research. It was thus abandoned.

Fusidic acid

Fusidic acid is an antibiotic widely used for both topical and systemic treatment of staphylococcal skin infections. The chemical structure resembles that of corticosteroids, but it does not possess any corticosteroid effect.



Fusidic acid

In vitro studies of the permeation of fusidic acid in human skin have shown that 2% of the fusidic acid applied in an ethanol solution permeates through the intact skin, and that fusidic acid remains in the epidermis for a long time after *in vivo* application, suggesting a depot function in the skin (Vickers, 1969). When the horny layer is damaged, permeation through as well as drug levels in the skin increases, whereas the permeation through normal skin is very low (Stüttgen and Bauer, 1988).

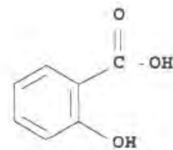
Fusidic acid is 97% protein bound (Reeves, 1987). The fusidic acid concentration has been measured in human skin blister fluid after oral administration of 250 or 500 mg fusidic acid twice daily for 6 days, resulting in blister fluid concentrations of 21 ± 5 μg/ml and 79 ± 11 μg/ml (Vaillant et al., 1992). Blister fluid contains both protein-bound and unbound drug fractions in contrast to samples obtained by microdialysis. Fusidic acid has an *in vitro* microdialysis recovery of 44%, but was expected to display lower values *in vivo* due to the high protein binding. Furthermore, topical penetration in intact skin *in vivo* had not previously been demonstrated.

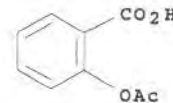
The negative results of the initial human experiments with topical fusidic acid (described in Paper I), in combination with the difficulties involved in creating experimental conditions where cutaneous penetration of fusidic acid could be measured in humans *in vivo*, led to the conclusion that fusidic acid was going to pose a wide range of methodological problems if used for further microdialysis research, and it was thus also abandoned.

Salicylic acid and acetylsalicylic acid

Historical pharmacology: Acetylsalicylic acid 100 years

One hundred years ago, the German chemist Felix Hoffmann synthesized acetylsalicylic acid (ASA) in a chemically pure form from salicylic acid and acetic acid for the first time. It proved to become the most widely used and tested drug worldwide during the next century (Weissmann, 1991).





Salicylic acid   Acetylsalicylic acid

The first records of the use of salicylate-containing remedies date back to 1500 BC. The glycoside salicin can be found in many plants, e.g. the willow tree (Salix alba) and in meadowsweet (Spiræa ulmaria). Thus, the analgesic, anti-inflammatory and antipyretic effects of extracts of either the bark, leaves or flowers of the willow tree were known by ancient civilisations with references dating back to Egyptian papyrus and clay tables from Assyria and Babylon. Hippocrates (460-377 BC), Dioscorides (40–80 AC) and Galenos (130–200 AC) were aware of the effect of remedies containing salicylates, and in the year 30 AC, Celsius described the classical signs of inflammation: *rubor, dolor, calor et tumor* and used an extract of bark from the willow tree to treat the symptoms.

In the Middle Ages, the use of the bark from the willow tree was gradually forgotten, and it was rediscovered by the reverend Edward Stone in 1763. He successfully conducted a series of well-planned experiments in which bark from the willow tree was used to treat patients with fever. His prescription was 20 gran (approx. 1 g) of pulverised willow bark in a drink every 4 hours. This was the first step towards evidence-based medicine through clinical trial of effect.

In 1838 the Italian chemist Raffaele Piria from Pisa demonstrated that salicin was a glycoside, and he named it *l'acide salicylique*, or salicylic acid (SA). I 1860 professor Herman Kolbe at Marburg University synthesized SA and its sodium salt from phenol, carbon dioxide and sodium. In 1874, the first factory for salicylate synthesis was established, and the improved availability of SA at a steadily cheaper price facilitated the spread of its use. In 1876, the first 2 reports of the successful treatment of acute rheumatic fever with salicylates at doses of 5–6 g per day were published. The SA did not have the curative effect that had been anticipated, and tolerability was low due to en extremely unpleasant taste and gastric irritation.

Felix Hoffmann, an assistant chemist at Bayer Laboratories, had a father with arthritis who couldn't use sodium salicylate because of stomach irritation. This prompted Hoffmann junior to search for a less acidic derivative of SA, which he achieved

by acetylation of the hydroxyl group of the benzene ring by letting SA react with acetic acid. The new drug was given the name aspirin, the "a" from *acetyl* and "spirin" from the German *Spirsaüre*. After testing in arthritic patients, where the effects were found to be slightly less than the SA, but with greatly reduced gastric side effects, the drug was marketed – initially as a powder in 1899, and in tablet form the following year.

Not until the early 1970s did biologists find a hypothesis to explain the action of aspirin. The pharmacologist John Vane explored the relationship between prostaglandins in the tissues and the activation of pain perception. Through experiments with and without ASA medication of volunteers, he was able to demonstrate the inactivation of cyclooxygenase by ASA and the subsequent blockade of the arachidonic acid cascade – a discovery for which he received the Nobel Prize in 1982.

The antipyretic effect of ASA is caused by the blockade of the effect of the fever-promoting interleukin-1, released by activated macrophages in inflamed and infected tissue, on the temperature-regulating centre in the hypothalamus. The anti-inflammatory effect is promoted through inhibition of the 2 isoforms of the cyclooxygenase enzyme, COX 1 & COX 2. In low ASA doses, COX 1 is inhibited with reduced platelet aggregation as a result, and in higher doses of ASA, COX 2 is inhibited with analgesic, anti-inflammatory and antipyretic effects as a result.

The present-day indications for ASA are multiple, with large clinical trials documenting the effect of small doses of ASA on survival after acute myocardial infarction, effect on disease progression in angina pectoris, reduced risk of new insult in transitory ischaemic attacks and stroke. Large trials to evaluate the indication for low-dose ASA in arteriosclerosis and in uncomplicated hypertension are underway (Kampmann and Knudsen, 1998). This confirms that we can still find use for the same drugs as used in ancient Egypt, and that not all drug substances are outdated with the expiry of their patent.

Dermatopharmacology of salicylic acid

The topical application of salicylic acid in the form of plant extracts has been used for many hundred years, and it was a known antiseptic in 1874. SA itself is pluripotent in effect when applied in the form of the free acid, whereas many of its esters and salts provide a regional counterirritant effect due to efficient percutaneous penetration. SA is present in a wide range of wart and callus remedies, often in combination with other keratolytic agents such as lactic acid. It is found in many shampoos and in acne preparations. SA has be shown to have at least 7 different effects on the skin after topical application (modified after Weirich, 1975 and Lin and Nakatsui, 1998), namely:

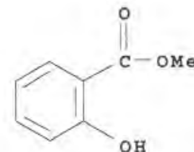
(i) A *germistatic effect* on gram-positive and gram-negative bacteria and on pathogenic yeasts, dermatophytes, moulds etc. This effect can be seen in concentrations as low as 0.3% w/w SA, becomes germicidal in higher concentrations and can be used for topical antisepsis.
(ii) In concentrations of 0.1% w/w and above, SA has an *acidifying effect* on the skin surface.
(iii) In the same concentration, SA has a *photoprotective effect* in the UVB range, corresponding to 60% of the absorbancy of para-aminobenzoic acid (and thus, topical SA can interfere with UVB phototherapy for psoriasis).
(iv) SA has a superficial *astringent action*, also in low concentrations, which can be used in cosmetic formulations (so-called "wrinkle creams"; SA is a β-hydroxy acid).
(v) When applied in a suitable form and concentration, SA has an *anti-inflammatory* effect. Tested in guinea-pigs with UV light-induced dermatitis, the topical anti-inflammatory effect of SA was equal to 66% of that of indomethacin, approximately 77% of that of ASA and 82% of that of hydrocortisone.
(vi) In concentrations of 5% or above, SA exerts a potent and rapid deep *"keratolytic" effect* on the stratum corneum. The terms keratolytic is a misnomer, as it has been shown to be a decrease in corneocyte cohesion, and not the breakdown (lysis) of keratin, which is responsible for the effect (Huber and Christophers 1977; Roberts et al., 1980). In a human *in vivo* study, where upper arm skin was treated with 2% SA or vehicle alone for 6 h, significantly more stratum corneum was removed by standardized tape stripping of the SA treated area than from the vehicle treated area (Lodén et al., 1995)
(vii) SA has been reported to be an *enhancer* of topical corticosteroid penetration, but this has not been confirmed by *in vivo* studies.

Recent new applications include the use of 30% SA for superficial chemical peels of facial skin as a cosmetic treatment of the effect of photoageing (Kligman and Kligman, 1998).

Methyl salicylate

Methyl salicylate (MSA) a mild counter-irritant, often used in combination with menthol or camphor as an over-the-counter remedy for topical treatment of aching muscles and joints. MSA is only used topically, since oral administration leads to gastric irritation and systemic toxicity. The absorption of topically applied MSA has been reported to result in salicylism, and measurable plasma concentrations can be detected 1 h after the application of 5 g 12.5% MSA to intact skin (Morra et al., 1996).

In the initial experimental phase of this thesis, a new model drug for penetration experiments had to be chosen. MSA was thought to be more suitable than SA due to the higher lipophilicity of the drug (log P value of 2.46) and the presumed higher topical penetration (Pratzel et al., 1990).



Methyl salicylate

Preliminary *in vivo* experiments in hairless rats (data not shown) had shown that 20 min after topical application of MSA, microdialysates contained both MSA and SA, the latter in concentrations about 6–9 times higher due to metabolization of MSA to SA en route. A similar result has been reported by Megwa et al. (1995), who studied absorption of MSA-containing commercial formulations in rats. Since we aimed at analysing one compound only, and the fraction of MSA was too large to be ignored, further work was based on the application of SA.

Recent studies using dermal and subcutaneous microdialysis sampling in humans after topical MSA application have shown that penetration can be detected after 30–60 min, and

that the first-pass metabolism of MSA to SA in human skin is extensive, with no detectable MSA in the dialysates. The tissue concentrations found were indicative of direct tissue penetration rather than absorption and redistribution by the systemic blood supply as expected. *In vitro* permeation of MSA across human skin showed a MSA:SA ratio of 3:1 in the receptor phase, but the skin had been frozen with possible alteration in esterase activity after thawing (Cross et al., 1998).

Salicylate metabolism in blood and tissues

ASA is rapidly hydrolysed to SA by ubiquitous esterases, present in blood and tissues. SA is removed from the body by 5 parallel and competing pathways; renal excretion and the formation of 4 metabolites: conjugation with glycine to form salicyluric acid (SU); conjugation with glucuronic acid to form two glucuronides: salicyl acyl glucuronide (SAG) and salicyl phenolic glucuronide (SPG); and oxidation to form gentisic acid (GA). The elimination pathways over SU and SPG are saturable and display Michaëlis-Menten kinetics, whereas the other pathways exhibit linear first-order kinetics. In the blood, the hydrolysis of ASA to SA is a rapid, dose-dependent process with a $T_{½}$ of less than 10 min; the $T_{½biol}$ of ASA is only 15–20 min in man following an intravenous or rapidly absorbed oral dose. The $T_{½}$ of SA varies between 2.4 and 19 h because of 2 easily saturable SA biotransformation pathways.

The protein binding of both SA and ASA is concentration dependent. The binding site can be assumed to be albumin only, and it has been demonstrated to be saturable in man (Shen et al., 1991).

Percutaneous salicylate absorption and intoxication

Feldmann and Maibach (1970) studied the absorption of SA and ASA among 19 other compounds in a human study of the percutaneous absorption and excretion of ^{14}C substances (See Chapter 4). SA and ASA showed similar absorption patterns with absorption of 22% of the dose applied, with absorption rates of 0.14%/h (ASA) and 0.12%/h (SA) during the initial 12 h after application. The 2 compounds were considered well absorbed, judged by their position as no 5 and 6 in a field of 21 highly different compounds.

Systemic toxicity from percutaneous absorption of SA is a rare but serious event, which still occurs with three cases reported in 1994. Intoxication causes nausea, vomiting and confusion proceeding to encephalopathy, all through CNS toxicity. Salicylate-induced tinnitus, hearing loss and induction of hyperventilation with respiratory alkalosis can occur, as can severe metabolic acidosis, particularly in neonates and young children (Galea and Goel, 1990). Another symptom of systemic SA absorption can be severe hypoglycemia (Raschke et al., 1991).

Deaths from topical SA have occurred in at least 15 cases of which 10 were children. The patients had been treated for psoriasis, scabies, dermatitis, lupus vulgaris and ichtyosiform erythroderma (von Weiss and Lever, 1964; Lindsey, 1968; Anderson and Ead, 1979).

The lack of a normal epidermal barrier in psoriasis greatly enhances the absorption of topical salicylate, and in 3 patients with extensive psoriasis, treated with 3% or 6% SA ointment up to 6 times daily, salicylism occurred after 2–4 days of treatment (von Weiss and Lever, 1964). The application of 6% SA to psoriatic skin under an occlusive dressing for 10 h has resulted in absorption of 60–80% of the drug applied. Measurable plasma concentrations of SA occurred without symptoms of salicylism, which occurs at plasma levels of SA of 0.3–0.4 mg/ml (Taylor and Halprin, 1975). However, studies of cutaneous or percutaneous penetration of SA have demonstrated that the intact human stratum corneum offers excellent resistance to the penetration of SA. No detectable SA blood levels could be demonstrated in four volunteers after application of SA to the entire forearm skin, neither when applied a PEG-based vehicle nor applied in hydrophilic ointment under occlusion. The same experiment with application on tape-stripped skin resulted in peak salicylate blood levels of 800 μg/ml (Birmingham et al., 1979). In rheumatological treatment, patients can be treated by means of baths containing methylsalicylate or SA in the water. In a study of percutaneous absorption after bath treatment, SA blood levels of only 10 ng/ml was measured for 24 h after letting healthy volunteers bathe for 20 min in bathing water containing 0.33 g/l SA (Pratzel et al., 1990).

Predisposing factors favouring the development of SA toxicity are the lack of a normal epidermal barrier (particularly psoriasis or ichtyoses), renal failure (with associated decreased SA plasma protein binding and reduced renal excretion capacity), and an increased surface-to-volume ratio as seen in children and in dehydrated patients. Obviously, factors such as the area of skin treated, the concentration of SA applied (and the vehicle), the frequency of application and the use of occlusive dressings will all influence the potential for intoxication.

Topical or transdermal ASA: New indications ahead?

The possibility of obtaining *pain relief from topically applied SA, ASA* and indomethacin has been investigated in an experimental human pain model (Steen et al., 1995). Cutaneous pain was induced by continuous slow infusion of an acidic buffer into the dermis on the volar aspect of the forearm. The study compared subjects' pain scores on a visual analogue scale before onset of treatment and following the application of either placebo or saturated solutions of the drugs dissolved in diethyl ether. Both ASA, SA and indomethacin provided potent analgesia with 92–96% pain reduction during the 25–30 min observation period. In a follow-up study by the same group (Steen et al., 1996) topical application of SA and ASA in ointment formulations (a mixed base of white vaseline and liquid paraffin) was investigated in a placebo-controlled, double-blind cross-over study using the same pain model, demonstrating dose-dependency for both ASA and SA responses. Pain suppression was higher (95%) with ASA than with SA (80%). Comparison of the 3% ASA formulation with commercially available benzocaine and ibuprofen cream formulations showed equally effective analgesia (with benzocaine causing loss of touch sensation as well). The authors suggest that the clinical relevance of the effect of topical SA and ASA could be in the treatment of cutaneous pain in e.g. burn injury, sunburn, cutaneous viral infections or vasculitic pain.

The topical application of crushed aspirin tablets, dissolved in chloroform, was reported to provide pain relief to patients with herpetic and post-herpetic cutaneous pain by King (King, 1988), and was followed by a double-blind crossover placebo-controlled clinical study of *topical treatment of acute herpetic neuralgia and postherpetic neuralgia* with the application of 1 g crushed aspirin, dissolved in diethyl ether. The ASA was applied by daubing the solution onto the skin of the painful dermatome, and proved to give significant pain relief. The pain relief obtained by the application of indomethacin and

diclofenac, prepared in the same fashion, was not significantly different from placebo (De Benedittis and Lorenzetti, 1996).

The sensation of pain and the sensation of itch are both conducted by the fine unmyelinated C-fibres via the lateral spinothalamic tract to the thalamus. The idea of using *ASA for the relief of itch* has been tried over the years in studies of varying validity. An initial study used experimentally elicited itch, induced by histamine and trypsin prick-testing. Systemic premedication with ASA was found not to be helpful as regards the duration of the itch, but to increase the size of the flare reaction (Hägermark, 1973). In a study of itchy patients, 900 mg ASA was administered at bedtime to a heterogeneous group of patients as an open-label trial. Itch was scored on a visual scale and sought monitored by a "limb-movement meter", activated by the patients' movements at night. No effect of ASA was found in this study (Daly and Shuster, 1986). Recently, in an experimental study of histamine-induced itch, a significant effect of topical application of 3% aspirin dissolved in dichloromethane on itch severity and duration was demonstrated (Yosipovitch et al., 1997). The effect was present 25 min after ASA application, indicating rapid cutaneous penetration of the drug in the chosen vehicle.

Since even low-dose aspirin, taken for thromboembolic prophylaxis, causes GI bleeds and toxicity in some patients, the use of the *transdermal route for continuous low-dose administration of ASA* has been tried in healthy volunteers (Keimowitz et al., 1993). ASA was dissolved in isopropyl alcohol or ethanol/propylene glycol, and applied to the surface of the forearm and the upper arm once daily for 10 days. A marked and selective inhibition of platelet cyclooxygenase could be demonstrated along with measurable plasma levels of both ASA and SA following each treatment session. The feasibility of transdermal patch system delivery of ASA was explored further by the same group (McAdam et al., 1996), who found that daily application of a 50 cm^2 patch containing 120 mg ASA resulted in 60-80% reduction in platelet cyclooxygenase. Marked hydrolysis of ASA to SA, which is inactive with respect to platelet inhibition, was noted along with the development of erythematous skin reactions in most volunteers.

The therapeutic use of topical ASA in the management of itch or pain or in thromboprophylaxis, with the application of the drug to large areas of broken or inflamed skin, could be increasing.

CHAPTER 8: HAIRLESS RAT STUDY

Abstract

The penetration of topically applied drugs is altered in diseased or barrier damaged skin. We used microdialysis in the dermis for measuring salicylic acid (SA) penetration in hairless rats following application onto normal (unmodified) skin ($n = 11$) or skin with perturbed barrier function from 1. tape-stripping ($n = 5$), 2. sodium lauryl sulphate (SLS) 2% for 24 h ($n = 3$) or 3. delipidization by acetone ($n = 4$). Prior to the experiment, transepidermal water loss (TEWL) and erythema were measured. Two microdialysis probes were inserted in the dermis on the side of the trunk and 5% SA in ethanol was applied in a chamber overlying the probes. Microdialysis sampling was continued for 4 h, followed by measurements of probe depth by ultrasound scanning. SA was detectable in all samples and rapidly increasing up to 130 min. Microdialysates collected between 80–200 min showed mean SA concentrations of 3 µg/ml in unmodified and acetone-treated skin, whereas mean SA concentrations were 280 µg/ml in SLS pre-treated skin and 530 µg/ml in tape stripped skin ($p < 0.001$). The penetration of SA correlated with barrier perturbation measured by TEWL ($p < 0.001$) and erythema ($p < 0.001$). A correlation between dermal probe depth and SA concentration was found in unmodified skin ($p = 0.04$). Microdialysis sampling in anatomical regions remote from the dosed site excluded that SA levels measured were due to systemic absorption ($n = 5$). Microdialysis sampling of cutaneous penetration was highly reproducible. Impaired barrier function, caused by irritant dermatitis or tape-stripping, resulted in an 80 to 170-fold increase in the drug level in the dermis. This dramatic increase in drug penetration could be relevant to humans, in particular to topical treatment of skin diseases and to occupational toxicology.

The aim of the study

The aim of the present study was to investigate the physiological and pathophysiological factors that affect SA transport across the living skin. SA acid was chosen as the model compound as it is extensively used in topical preparations and has a high ability to penetrate the skin barrier. A principal barrier perturbation study and 3 supporting control studies were performed.

Materials and methods

Details regarding materials and methods can be found in Chapter 2 and in Paper II.

In vivo microdialysis experiments: Barrier perturbation study

Prior to starting the experiment with barrier perturbation procedures, the rats were anaesthetized with pentobarbital sodium 50 mg/kg given as intraperitoneal injection with supplementary injections of 10 mg/kg every 90 min. Prior to probe insertion, quantification of the barrier perturbation was made by non-invasive measurements of TEWL and erythema as outlined in Chapter 2.

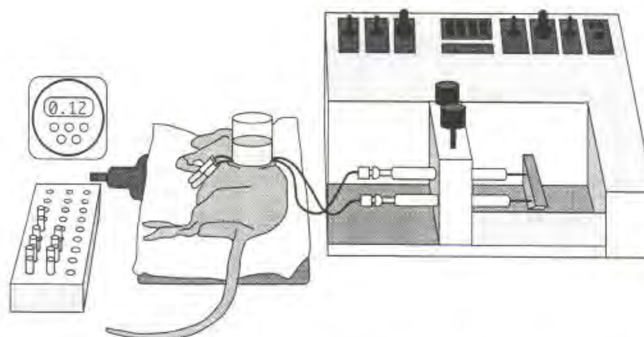


Fig. 8.1. Experimental set-up for the rat study. The hairless rat is anaesthetized by intraperitoneal pentobarbital and placed on a temperature-controlled heating pad. 2 probes have been inserted in the dermis and are perfused at a flow of 5 µl/min by the microdialysis pump. The plastic chamber is glued onto the skin overlying the probes and filled with a 5% w/v salicylic acid solution. The dialysate is collected in capped vials to minimize evaporation.

Following an equilibration period of 1 h after probe insertion to allow insertion trauma to subside (as shown necessary by Groth et al. 1998a,b), 5 ml 5% w/v solution of SA in ethanol were added to the chamber and sampling was started. Perfusion of the probes was continued for 4 h. At the end of the experiment the chamber was removed, and probe depth and skin thickness were measured by 20 MHz ultrasound. The rats were killed by a pentobarbital overdose.

Barrier perturbation procedures

The 4 groups of rats, grouped after barrier perturbation modality, were:

1. *No treatment* (unmodified skin, n = 11).
2. *Tape stripped skin*. 1 hour before the experiment, stripping of the stratum corneum (SC) was done by applying 5×5 cm squares of Transpore® tape to the left side of the trunk and subsequently tearing it off. This procedure was repeated 10 times (n = 5).
3. *Irritant dermatitis*. 2 days prior to the experiment, irritant dermatitis was induced on the left side of the trunk by application of 600 µl 2% SLS onto 3 layers of filter paper measuring 2.5×3.0 cm, kept in place by a frame of Comfeel®. The area was sealed by impermeable tape. After 24 h the SLS was washed off and the area left unoccluded until the experiment 18 h later (n = 3).
4. *Acetone-treated skin*. 1 hour before the experiment, the skin on the left side of the trunk was treated by gently wiping the skin 20 times with a cotton ball soaked in 100% acetone (n = 4).

In vivo microdialysis recovery study

As *in vivo* recovery may differ substantially from *in vitro* recovery (Ungerstedt 1984; Bungay et al. 1990), an *in vivo* recovery experiment was designed to investigate the *in vivo* recovery of SA and whether it would vary with the anatomical position of the dermally implanted probe. The principle of this method relies on the assumption that the diffusion process is quantitatively equal in both directions through the semipermeable membrane (Ståhle 1991b). By perfusing the dialysis probe with SA in rats not treated with topical application of SA, the relative loss of SA from the perfusate to the dermis could be calculated using eqn. 1. and used as an estimate of recovery. Rats (n = 3) were anaesthetized as above, and microdialysis probes (3–5 per rat, n = 11) were inserted on the left side of the rat. Probes were placed in the dermis of the anterior trunk, the thorax, the abdomen, the back and the hind leg. The probes were perfused for 4 h with the usual perfusate with 10 µg/ml SA added.

Systemic absorption study

An evaluation of the systemic absorption of the topically applied SA was studied by sampling from additional probes in other anatomical regions in rats with unmodified or tape-stripped skin. Rats (n = 5) were anaesthetized and 3 of the rats were tape-stripped on the left side of the trunk as described above (group 2). Microdialysis probes (3–5 per rat, total n = 21) were inserted on the left side of the rat. Probes were placed in the dermis, in subcutaneous tissue or in the underlying muscle of the anterior trunk, the thorax, the abdomen, the back and the hind leg. As in the barrier perturbation study, a plastic chamber was glued to the skin surface on the trunk, and 5 ml of 5% w/v SA in ethanol were added. Sampling from all probes, including a reference probe in the dermis under the chamber as in the barrier study, was continued for 4 h.

Protein binding assessment study

The degree of protein binding of SA in the ECF was studied by performing simultaneous *in vivo* sampling with a microdialysis probe and an albumin-permissive ultrafiltration probe in the skin under the penetration chamber. Rats (n = 3) were anaesthetized and 1 of the rats was tape-stripped on the left side of the trunk as described above (group 2). One microdialysis probe and one ultrafiltration probe were inserted in the dermis on the left side of each rat. A plastic chamber was glued to the skin surface and 5 ml of 5% w/v SA in ethanol were added. Sampling from both probes was continued for 4 h.

Control experiments

Vehicle control experiments: Rats (n = 2) were prepared with 2 probes in the dermis of unmodified skin (as in group 1 above). The chamber was filled with 5 ml of 100% ethanol and sampling performed as usual. The samples contained no compounds interfering with the HPLC analysis of SA.

Results

Highly differentiated penetration of SA in barrier perturbed skin

SA was detected in all samples collected from areas to which SA had been applied. In all experiments the concentration was steadily rising throughout the experiment, with an initial steep rise followed by an incomplete steady state phase after 80–90 min (raw data, Fig. 8.2 a-d, next page).

The intraanimal variation in SA concentration (mean of SD of SA concentrations sampled by 2 probes in one animal) was smaller than the interanimal variation in SA concentration (mean of SD of SA concentrations sampled in the whole group). The variability (intraanimal in% of interanimal variation) for each group was: 1. unmodified skin: 22%; 2. tape-stripped skin: 39%; 3. irritant dermatitis: 44%; 4. acetone-treated skin: 53%.

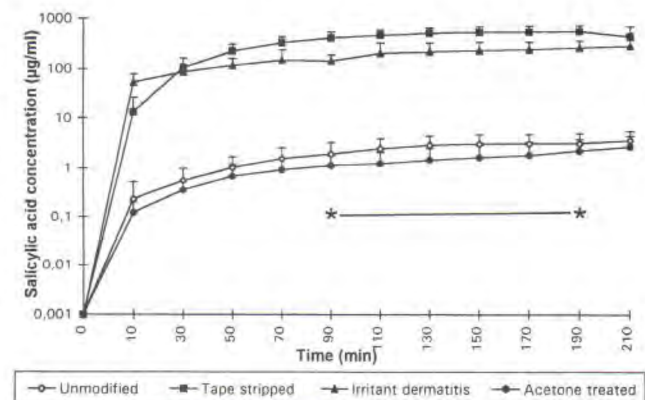


Fig. 8.3. Pharmacokinetic differences in salicylic acid penetration. * = Statistically significant ($p < 0.01$) difference between the mean SA concentration levels in the interval 80–200 min for groups 1, 2 & 3, whereas group 1 and 4 are not significantly different.

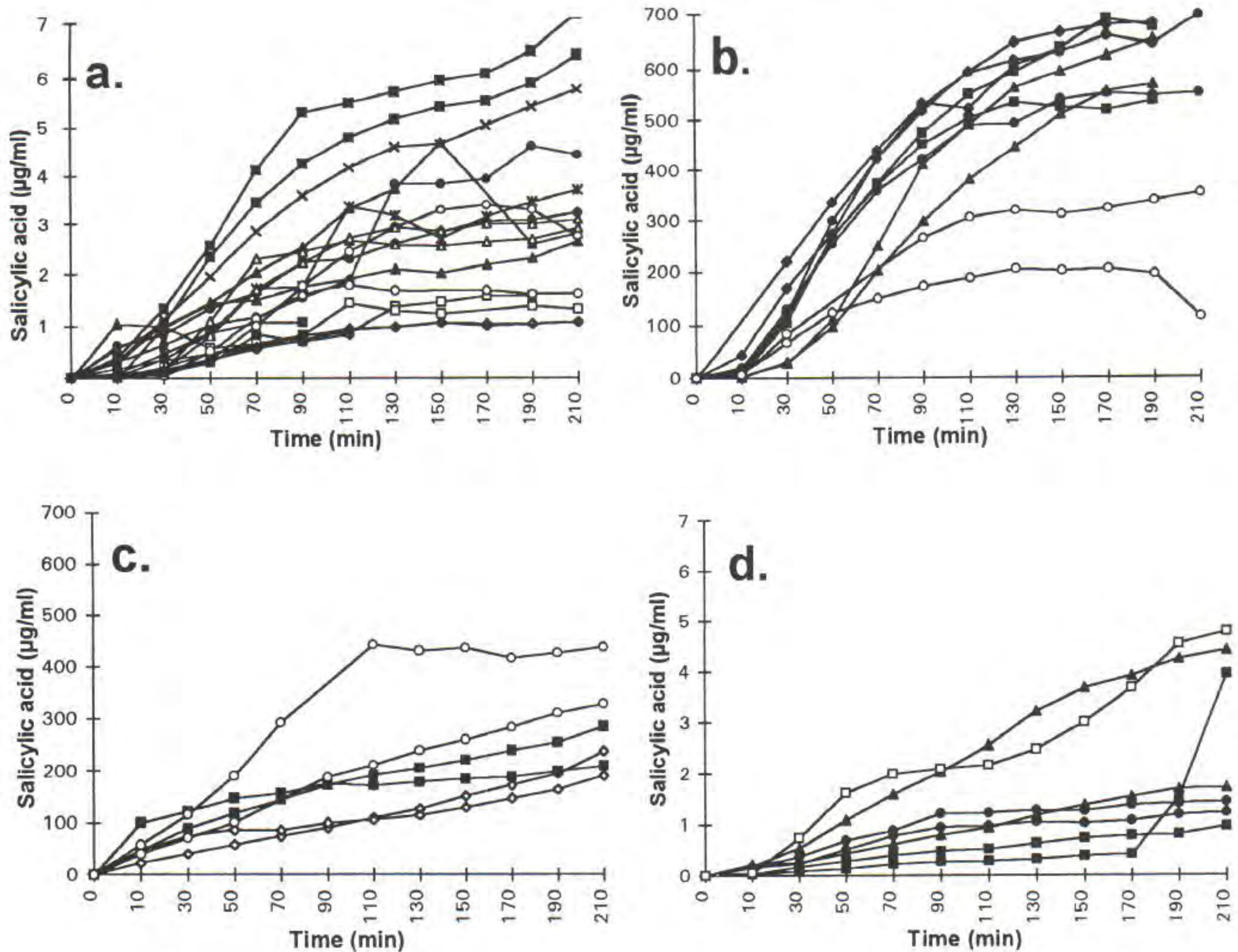


Fig. 8.2. Salicylic acid concentration in dialysates.
Note the different y-axis scales. Penetration through unmodified skin (a, n = 11), tape-stripped skin (b, n = 5), skin with irritant dermatitis (c, n = 3) and acetone-treated skin (d, n = 4). The concentrations in dialysates sampled by one probe are depicted as one curve. The 2 probes in the same animal have been given the same symbol to illustrate the smaller intraanimal variation as compared with interanimal variation (see results).

For unmodified and acetone-treated skin, the mean SA concentration (Fig. 8.3) in samples taken between 80–200 min was around 3 µg/ml, whereas the mean concentration in the SLS pre-treated group was around 280 µg/ml, in the tape-stripped group 530 µg/ml.

When calculated as the Area Under the concentration-vs-time Curve (AUC) from 0–200 min, the increase in penetration over the skin surface was 83-fold in irritant dermatitis and 172-fold in tape-stripped skin. For acetone-treated skin, a non-significant decrease to 60% of the penetration in unmodified skin was seen.

As standard deviation increased proportionally with the mean concentration in the 4 groups, the statistical analysis was performed using logarithmically transformed SA concentration data. An analysis of variance between the logarithmically transformed SA concentration data showed a significant difference ($p < 0.001$) in microdialysates sampled from 80–200 min between the levels for group 1, 2 and 3, whereas group 4 (acetone-treated skin) was not significantly different from group 1 (unmodified skin).

Differentiation of barrier perturbation by non-invasive methods

Besides the measurement of barrier perturbation, a clinical description of the barrier damage inflicted was noted for each rat. The typical description for tape-stripped skin was: moderate erythema and a glistening surface due to total/subtotal removal of the SC; for irritant dermatitis: moderate to deep erythema with crusting and dryness; for acetone-treated skin: minimal or no erythema, slight superficial dryness.

The non-invasive measurements of barrier perturbation are shown in Table 8.1.

Analysis of variance showed an overall difference in ΔTEWL and ΔErythema between the groups, only group 1 and 4 did not differ significantly with respect to these 2 parameters. While the concentration of SA was highest in tape-stripped rats, SLS-treated rats had the largest increase in erythema. The rank order of TEWL increases was the same as for SA concentrations.

Table 8.1. Differentiation of barrier perturbation and salicylic acid penetration.

Barrier perturbation	No. of rats	No. of probes	Δ TEWL g/m²h	Δ Erythema AU	Probe depth mm	AUC μg/ml*min
Unmodified	11	19	0	0	0.88 ± 0.16[c]	402 ± 214[d]
Tape stripped	5	10	69 ± 14[a]	2.41 ± 0.87[b]	0.94 ± 0.15	69.500 ± 21.100
Irritant dermatitis	3	6	41 ± 18	3.93 ± 1.26	1.07 ± 0.19	33.100 ± 12.900
Acetone treated	4	7	6 ± 3	0.95 ± 1.66	0.89 ± 0.12	231 ± 159

[a]Measurement of TEWL over the barrier perturbed skin area minus the value measured over the untreated side. Mean ± SD. [b]Measurement of erythema (a*, in arbitrary units) over the barrier perturbed skin area minus the value measured over the untreated side. Mean ± SD. [c]Mean ± SD. [d]Mean ± SD. 0–200 min of sampling used for calculations.

Correlation between SA penetration and quantification of barrier perturbation

Regression analysis (not divided by group, but entering rats individually) showed that the concentration of SA increased significantly ($p < 0.001$) with increased TEWL and erythema (Fig. 8.4 a-b).

The correlations can be expressed with the equations:

$$\text{SA concentration} = 0.08*\Delta\text{TEWL} + 7.13$$
$$\text{SA concentration} = 1.24*\Delta\text{Erythema} + 7.35$$

The increase in TEWL/erythema and the SA concentration are all dependent on/caused by the barrier perturbation of the skin prior to the experiment. It would therefore be erroneous to describe the increased TEWL/erythema measurements as the cause of the increase in SA concentration. The interdependence structure between SA concentration, TEWL and erythema (measured in the same animal, n = 23) was investigated by principal component analysis. The first principal component accounts for 88% of the variation between the 3 variables. Therefore almost all variation can be explained by the simple weighted average of the 3 variables:

0.59*SA concentration + 0.59*ΔTEWL + 0.56*ΔErythema, where the coefficients are the correlation coefficients of the first principal component. The coefficients express the relative importance of each of the 3 variables in the description of the effect of skin barrier damage.

Ultrasound measurements of probe implantation depth

The mean probe implantation depth (Table 8.1) in the untreated group was 0.88 ± 0.16 mm (mean ± SD, n = 19). No significant differences in probe depth were found between treatment groups ($p = 0.12$), but the depth was influenced by the degree of swelling of the skin during the experiment (see Fig. I a-b, colour illustrations p. 55). According to the ultrasound scans (measured in 3 cross-sectional scans over the first, middle and last third of the probe length), all probes were confined to the dermis, except at the points of entry and exit.

Salicylic acid concentration correlation with probe depth in the dermis

For the study group with unmodified skin (group 1), a correlation between the depth of a probe in the dermis (in mm) and the SA concentration sampled by that probe was found (Fig. 8.5). The more superficially the probe was placed, the higher were both the initial SA measurements but also the mean SA concentration sampled by that probe (in accordance with the initial flux of drug across the skin surface and into the deeper tissues).

Taking into account that the 2 observations in one animal are not independent and thus entering the mean probe depth for each animal, regression analysis showed a statistically significant correlation ($p = 0.04$).

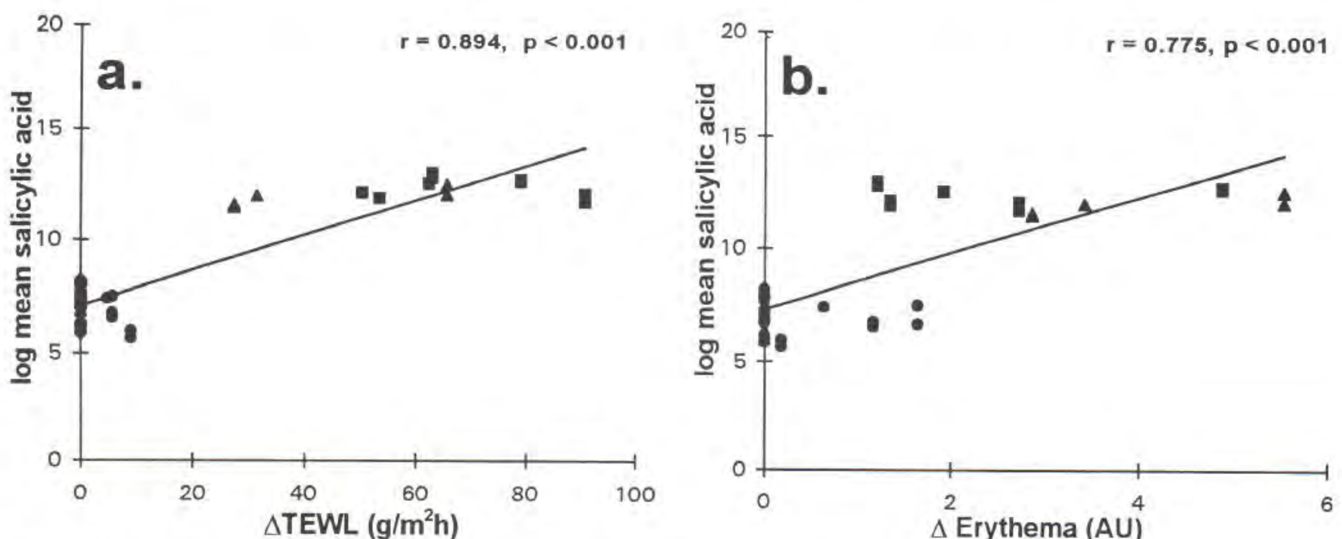


Fig. 8.4. Correlation between salicylic acid penetration and non-invasive quantification of barrier perturbation.
a. TEWL as measured by evaporimetry. b. Erythema as measured by colorimetry. Each point represents data from one probe, shown by symbols according to the type of barrier perturbation used: unmodified (diamond), acetone treated (circle), irritant dermatis (triangle) and tape stripped (square). ΔTEWL is the measurement over the treated site minus the measurement over the contralateral (untreated) site. Unmodified skin thus has a ΔTEWL value of 0, similarly for ΔErythema.

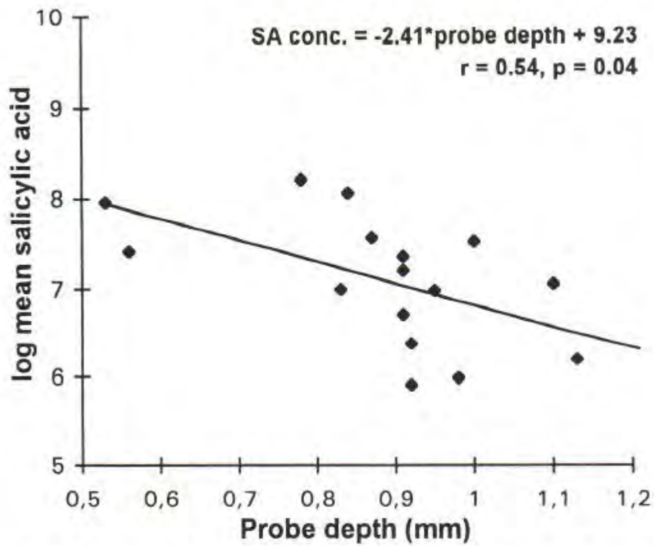


Fig. 8.5. Correlation between salicylic acid penetration and probe depth in the dermis.
Data shown for group 1, unmodified skin. Each point represents data from one probe.

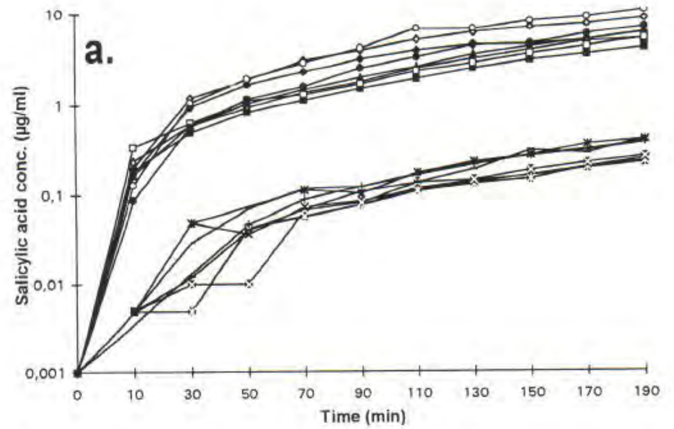


Figure 8.6 Ratio of systemic absorption of salicylic acid measured by microdialysis in remote anatomical regions/tissues.
R4, R5, R6 are tape-stripped rats (n = 3). R7, R8 are rats with unmodified skin (n = 2). a. Salicylic acid concentration in dialysates. Note the logarithmic y-axis scale. The concentrations in dialysates sampled by one probe are depicted as one curve. The 2 probes in the same animal have been given the same symbol to show the smaller intraanimal variation in comparison with interanimal variation. b. The ratio of systemic absorption of salicylic acid. The systemic SA concentration (AUC_{0-200}), measured in a remote probe, in % of the SA concentration (AUC_{0-200}) measured at the dosed site in the same rat.

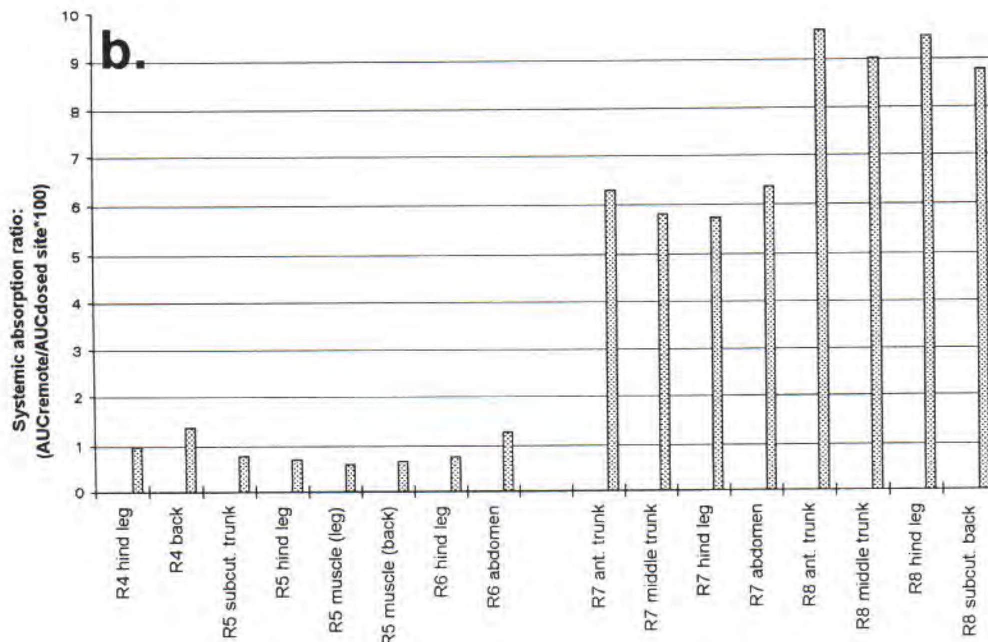


Table 8.2. Systemic absorption: Salicylic acid concentration sampled in remote sites.

Barrier perturbation	No. of rats	No. of probes	AUC_{remote} µg/ml*min	Variability %	Ratio $AUC_{remote}/AUC_{dosed\ site}$ %
Unmodified	2	10	26 ± 6[a]	18[b]	7.6 ± 1.7[c]
Tape stripped	3	11	632 ± 219	92	0.9 ± 0.3

[a]Mean ± SD. 0–200 min of sampling used for calculations. The difference in mean AUC for the 2 treatment groups is statistically significant (p = 0.02). [b]Mean ± SD. The variabilities are calculated as intraanimal in % of interanimal variation. [c]Mean ± SD.

In vivo microdialysis recovery study

Analysis of dialysates from probes in the dermis perfused with 10 µg/ml SA showed a relative loss of 29 ± 4% (mean ± SD, n = 11) using eqn 1. for calculation. There were no differences in relative loss with regard to the anatomical region of the dermally placed probe.

Systemic absorption of SA: Different absorption patterns

In all experiments comparing microdialysis sampling below the chamber (dosed site) and sampling in other anatomical regions/tissues (remote sites), the SA concentration in remote sites rose in parallel with the concentration at the dosed site. A definite systemic absorption of SA was seen in all animals as can be seen in Table 8.2.

Comparing the mean systemic absorption ratios for the 2 treatment groups, taking into account that each rat contributes with several non-independent measurements, the difference is statistically significant ($p = 0.02$, analysis of variance). Fig. 8.6a (previous page) shows the absolute levels of SA in dialysates from remote probes in tape stripped rats (top group) and unmodified rats (bottom group). Fig. 8.6b shows the pattern of the relative AUC ratios (remote/dosed site).

In this study, ultrasound scanning of the probes confirmed that all probes were in dermis, subcutaneous tissue or muscle, as intended.

Estimates of protein binding

In two rats without barrier perturbation and one tape-stripped rat, the SA concentrations in samples obtained by simultaneous microdialysis and ultrafiltration rose in parallel as can be seen in Fig. 8.7. The concentration in the albumin-free microdialysate was between 60 and 100% of the SA concentration in the ultrafiltrates.

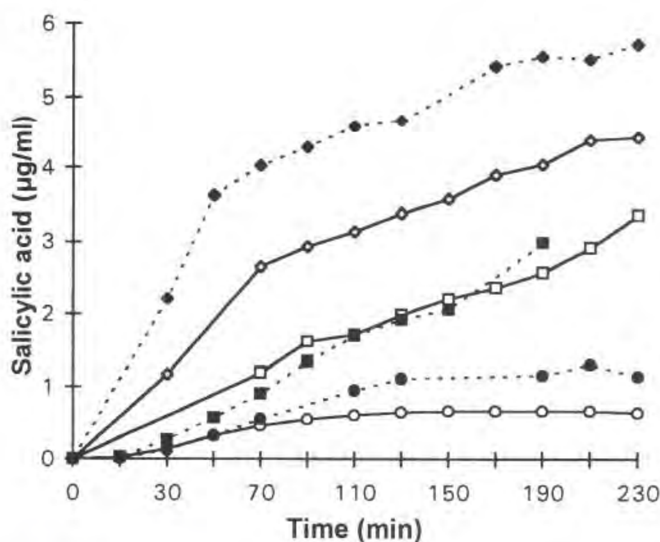


Fig. 8.7. Evaluating protein binding by simultaneous microdialysis and ultrafiltration sampling.
Salicylic acid concentration in microdialysates (open) and ultrafiltrates (solid) from simultaneous sampling with the 2 different types of probes in two rats with unmodified skin (◆ and ■) and one rat with tape stripped skin (●). The SA concentrations sampled in the skin of the tape-stripped rat are measured in mg/ml, but have been included here 1:1000 for illustration.

Discussion

It has been shown in 7 *in vivo* studies that barrier perturbation results in increased percutaneous penetration compared with penetration in normal, untreated skin. The order of magnitude varies among drugs and species investigated (Table 6.1, Chapter 6). Furthermore, most of the studies (marked *) have used the traditional radiolabelling technique, where the penetration has been measured as total drug absorption over 4-10 days. In contrast, the present study has a time resolution of 20 min over the 4 h of penetration studied. The dramatic increase in cutaneous penetration in barrier damaged skin, demonstrated in the current study, is only partly explained by the use of a model with sustained drug administration (since the chamber is left in situ for the entire experiment) and by the choice of ethanol as vehicle/solvent for SA.

For the discussion of the results regarding the effect of barrier perturbation on SA penetration, please see Chapter 6.

Systemic absorption and different absorption patterns for high and low penetration

We have evaluated the systemic absorption and distribution of SA by sampling ECF from probes in the dermis of other anatomical regions and in deeper tissues. After SA application onto intact skin, dermal concentrations sampled in remote regions were 7.6% of the dermal concentration sampled at the dosed site. After SA application onto tape-stripped skin, remote sampling in skin and deeper tissues gave SA levels of only 0.9% of the dosed site.

In a study of SA penetration in anaesthetized rats after dermal application (epidermis removed using a dermatome) for 2 h, local direct penetration was evident to a depth of 3-4 mm below the dosed site. The examination of SA concentrations in deeper tissues, contralateral tissues and plasma showed that systemic circulation of SA was responsible for equal concentrations in plasma and contralateral tissues of < 1% of the concentration in the skin at the dosed site, whereas the SA concentration in deeper tissues (4–12 mm below the application site) was higher due to a combination of direct SA penetration and systemic distribution (Singh and Roberts, 1993). This occurrence of high local drug concentration in tissues subjacent to the site of application has been described in other *in vivo* studies (McNeill et al., 1992), where the cause was suggested to be that the cutaneous microvasculature is not simply an "infinite sink" for removal of topically applied drugs. This theory could explain the much lower systemic absorption ratio observed in the present study for the tape-stripped rats, where the cutaneous microvasculature may have failed to completely resorb and subsequently distribute the very high topical SA concentration. The other conclusion is that systemic absorption is not a likely cause of error in the barrier perturbation study, as the systemic concentrations were low (always below 10% of the concentrations measured at the dosed site).

The effect of protein binding

SA in blood and tissue is protein-bound, mainly to albumin. In the present study SA concentrations of 1–700 µg/ml were sampled from the ECF. We established an *in vivo* recovery of SA of 29%. From this, we would expect the free ECF concentration of SA to be between 3–2400 µg/ml. In an *in vitro* study using microdialysis in plasma, the protein binding of SA in the concentration range relevant to the present study varied from

70% in the low SA range to 30% in the high range (Herrera et al., 1990). Looking at our *in vivo* experiments comparing protein-free and protein-permissive sampling (Fig. 8.7) it can be estimated that protein binding in the ECF is probably responsible for a reduction of 30–40% of the SA concentration in the samples. These experiments, which are insufficient to allow conclusions in themselves, were performed to estimate the *in vivo* effect of SA protein binding in the tissue on microdialysis sampling of SA. However, the effect of this concentration-dependent protein binding of SA on the present study is likely to be very limited. SA binding to albumin is a reversible and rapid reaction; when diffusion reaches a steady state (which is the case in our experiments), the dermal free concentration is in equilibrium with the bound concentration and neither change with time. In this situation, the diffusion of SA across the microdialysis membrane is unaltered by protein binding (see Cussler, 1997).

Correlation between measurements of barrier damage and cutaneous penetration of SA

The measurements of TEWL is presently regarded as the technique of choice for standardized skin barrier function evaluation (Schaefer and Redelmeier, 1996). We have found significant correlation ($p < 0.001$) between non-invasive measurements of barrier perturbation by TEWL and colorimetry (a*) and the *in vivo* penetration of SA. In a guinea pig study it has been shown that SLS-induced irritant dermatitis increases TEWL measurements as well as the percutaneous penetration of hydrocortisone, indomethacin and ibuprofen but not acitretin (Table 6.1, Wilhelm et al., 1991). In a human study in patients with widespread (endogenous) dermatitis, the measurement of reduced barrier function by TEWL was found to correlate with the percutaneous absorption of topically applied 1% hydrocortisone across affected skin (indirectly measured as the increment in plasma cortisol during dexamethasone suppression) (Aalto-Korte and Turpeinen, 1993). For the correlation between erythema and penetration in the current study it should be specified that colorimetry measures colour and not blood flow. Whereas erythema and blood flow will be very similar in many situations, no firm statements on the blood flow in the barrier perturbed skin areas in this study can be made.

Correlation between probe depth in the dermis and salicylic acid concentration

In the rats with unmodified skin, we found a significant correlation between probe depth in the dermis and the SA concentration sampled by that probe ($p = 0.04$). A similar correlation could not be shown for the other treatment groups, probably due to fewer observations. In human penetration studies using microdialysis, a correlation between probe depth and drug concentration has been demonstrated in one study of transdermal nicotine, where probes were placed both in dermis and subcutis (Müller et al., 1995c), but not in a similar study with probes in the dermis only (Hegemann et al., 1995). In a human study of topically applied diclofenac, sampling in tissue layers 4 and 9 mm from the skin surface (i.e. subcutis), no correlation between the AUC sampled in a defined layer and the depth of the microdialysis probe in the tissue could be demonstrated (Müller et al., 1997c).

In conclusion, the present study demonstrates the usefulness of microdialysis in the investigation of cutaneous drug penetration, measured in the target organ. The penetration of SA could be reproducibly sampled with short time resolution and small intra- and interanimal variability. Cutaneous drug penetration correlated with barrier perturbation quantified by TEWL and erythema measurements. Impaired barrier function, caused by irritant dermatitis or tape-stripping, resulted in an 80 to 170-fold increase of the drug level in the dermis. A correlation between dermal probe depth and SA concentration was found in unmodified skin. Systemic absorption of the drug applied was investigated and excluded that SA levels measured were due to systemic absorption. Microdialysis sampling in different tissues showed no tissue dependent differences in drug recovery.

CHAPTER 9: HUMAN STUDY I

Abstract

We have used microdialysis in the dermis for assessing penetration kinetics of salicylic acid (SA) in healthy volunteers ($n = 18$), following application on the volar aspect of the left forearm. Penetration was monitored in 4 locations, in normal (unmodified) skin and in skin with perturbed barrier function from (i) repeated tape stripping, (ii) irritant dermatitis from 1 or 2% sodium lauryl sulphate (SLS) for 24 h or (iii) delipidization by acetone. The order of the treatments was randomized according to a latin square design. Epidermal barrier function and skin irritation were assessed in each location using evaporimetry and colorimetry. Transepidermal water loss values confirmed that both mild (acetone), moderate (1% SLS) and severe barrier damage (tape stripping and 2% SLS) had been created.

Microdialysis sampling with 2 parallel probes in the dermis was performed in each of the 4 treatment areas in every subject. 5% SA in ethanol was applied in a chamber glued to the skin overlying the microdialysis probes and sampling was continued for 4 h.

SA was detectable in all samples and measurable in all samples from penetration through perturbed skin. Comparing the SA penetration in barrier perturbed skin with the penetration in unmodified skin in the same subject, the mean SA penetration increase was 2.2-fold in acetone-treated skin ($p = 0.012$), 46-fold increased in mild dermatitis and 146-fold and 157-fold increased in severe dermatitis and tape stripped skin, respectively ($p < 0.001$).

The penetration of SA significantly correlated with the measurements of barrier perturbation by TEWL ($p = 0.01$) and erythema ($p = 0.02$) for each individual. Microdialysis sampling of SA penetration was more sensitive than non-invasive measuring techniques in detecting significant barrier perturbation in acetone-treated skin. A positive dose-response relationship for the percutaneous penetration of SA in response to increasing SLS pre-treatment concentrations and thus the degree of irritant dermatitis was found.

When analysing data by location on the forearm, a tendency towards an intraregional variation in the reactivity to barrier damage was found, with the most proximal location displaying higher reactivity scores than the most distal location in response to the same barrier perturbation procedures. The penetration of SA was not significantly different between locations.

In conclusion, using microdialysis in the dermis to obtain real-time dermal pharmacokinetics in the target organ, this study demonstrates highly increased and differentiated cutaneous penetration of SA in barrier perturbed skin. The measured drug

penetration could be demonstrated to correlate with non-invasive quantification of barrier damage.

The aim of the study

The aim of the present study was to investigate the physiological and pathophysiological factors that affect drug transport across the living human skin. In the study design were 4 objectives:

I. To evaluate the effect of 3 different methods of barrier perturbation on the cutaneous penetration of SA: (i) irritative dermatitis from the anionic surfactant sodium lauryl sulphate (SLS), (ii) removal of the stratum corneum by means of repeated tape stripping and (iii) delipidization by treatment with absolute acetone.
II. To establish whether a correlation between non-invasive measurements of the inflicted barrier damage and the *in vivo* cutaneous penetration could be demonstrated. Quantification of barrier integrity was made by transepidermal water loss (TEWL) and erythema measurements by colorimetry prior to the penetration experiment.
III. We wished to determine if an individual 'skin reactivity' would correlate with individual SA penetration. Furthermore, we wanted to determine whether the sex of the subject had any influence on the cutaneous penetration of SA or the susceptibility to barrier damage.
IV. To investigate whether the localization (proximal-distal) on the volar forearm had any influence on skin reactivity or cutaneous SA penetration.

Materials and methods

Subjects

We investigated 18 healthy volunteers: seven women and nine men, aged 28 ± 3 years (mean ± SD). No subjects were allergic to aspirin or local anaesthetics. None of the subjects used any medication and all refrained from applying any topical formulations to the left forearm during the study period. All subjects gave written informed consent and the study was approved by the Copenhagen County ethical committee (Ref. KA 97021s).

Study design

The study was planned as a latin square, where the order of the 4 modalities of barrier perturbation was randomized between locations 1–4 on the forearm (Fig. 9.1). The design required data from 16 experiments. A total of 18 subjects were enrolled as data from 2 experiments were disqualified due to a combination of analytical and experimental problems. Experiments were conducted in April, May and June 1997.

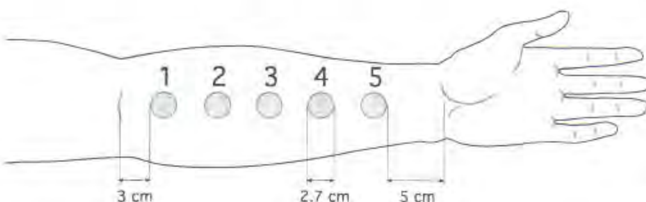


Fig. 9.1. Experimental design for human study I.
Locations 1–4 were randomized to the 4 treatments according to a latin square design. Location 5 was included as vehicle control in subjects with forearms long enough to accommodate a fifth chamber.

Experimental protocol

Day 1: Enrolment, application of SLS patch on left forearm (location randomized according to latin square design).
Day 3: Penetration experiment.

Methods of barrier perturbation

Barrier perturbation procedures were standardized as follows:

1. *No treatment* (unmodified skin).
2. *Tape stripped skin.* Partial removal of the stratum corneum (SC) was made by applying 2.5×5 cm pieces of tape (Transpore®, 3M) with firm pressure and subsequently tearing it off, repeating the procedure 20 times.
3. *Irritant dermatitis.* 2 days prior to the experiment, irritant dermatitis had been initiated by application of 400 μl 1 or 2% SLS onto 3 layers of filter paper measuring 2.5×3.0 cm, kept in place on the skin by a frame of Comfeel®. The area was covered by an adhesive dressing. After 24 h the SLS was washed off and the area left unoccluded until the experiment 18 h later.
4. *Acetone-treated skin.* The skin was treated by gentle wiping with large cotton buds soaked in 100% acetone for 3 min.

In vivo microdialysis experiments

A photographic illustration of the procedure for the human microdialysis experiments can be found at the back of this thesis, Fig. II a-d. After barrier perturbation and a 15 min rest, non-invasive measurements of barrier function were performed, followed by injection of local anaesthesia (1% Xylocaine®) in the dermis/s.c. tissue around (but not into) the demarcated areas (volume < 20 ml). In all experiments, 2 probes were inserted in parallel in the demarcated/barrier perturbed areas 1–4 on the volar aspect of the left forearm. The probes were inserted in parallel to the skin surface in the dermis by means of a 21 G guide cannula and the outlet placed in a 300 μl glass vial through the pierced lid. Perfusion was started at low flow and a plastic chamber (made from a transected 50 ml syringe) was glued to the skin over the microdialysis fibres using cyanoacrylate glue. Following an equilibration period of 1 h after probe insertion to allow insertion trauma to subside (Groth and Serup, 1998), 5 ml 5% w/v solution of SA in ethanol were added to the chamber. A plastic lid was glued over the top opening to prevent spills and evaporation and sampling was started. Perfusion of the probes was continued for 4 h.

Quantification of barrier perturbation by non-invasive measurements

15 minutes after barrier perturbation procedures, the TEWL over the demarcated locations 1–4 was measured using an Evaporimeter and skin colour was measured using a Minolta Chromameter® CR 300 (a* parameter).

Ultrasound measurement of probe depth and skin thickness

At the end of each microdialysis experiment, after removal of the penetration chamber, skin thickness and probe depth in the dermis were measured by 20 MHz ultrasound scanning (see Fig. III a-d, colour illustrations at the back).

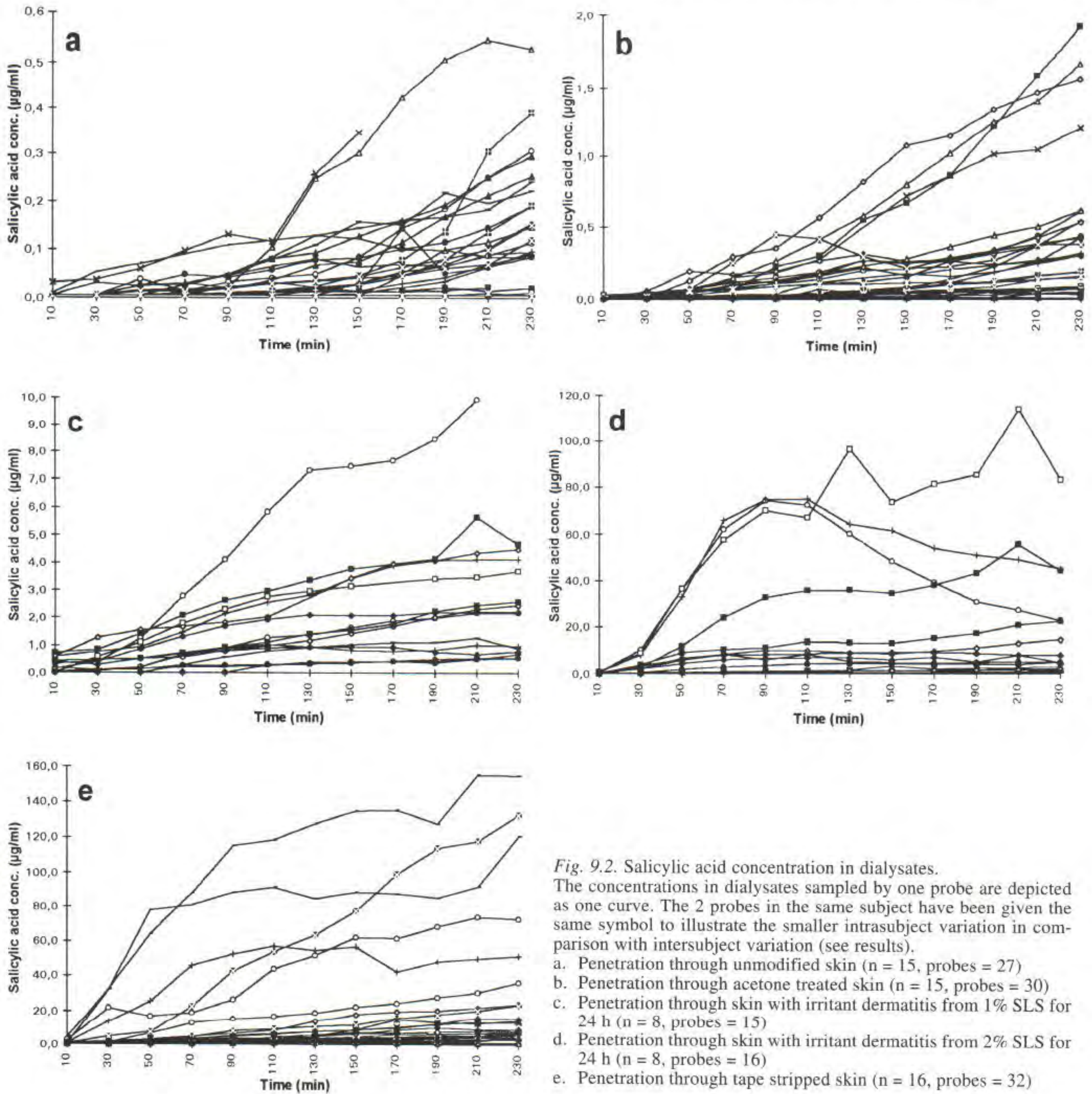


Fig. 9.2. Salicylic acid concentration in dialysates.
The concentrations in dialysates sampled by one probe are depicted as one curve. The 2 probes in the same subject have been given the same symbol to illustrate the smaller intrasubject variation in comparison with intersubject variation (see results).

a. Penetration through unmodified skin (n = 15, probes = 27)
b. Penetration through acetone treated skin (n = 15, probes = 30)
c. Penetration through skin with irritant dermatitis from 1% SLS for 24 h (n = 8, probes = 15)
d. Penetration through skin with irritant dermatitis from 2% SLS for 24 h (n = 8, probes = 16)
e. Penetration through tape stripped skin (n = 16, probes = 32)

Control experiments

Vehicle control: This location (area 5) was included in subjects whose forearms were long enough to accommodate one more chamber (n = 7). The vehicle control was always placed nearest the wrist as this area was not included in the latin square design. 2 probes were inserted in the dermis and perfused as usual. The chamber overlying the probes was filled with 100% ethanol only.

Drug concentration control: In all experiments, a sample of the SA chamber solution was analysed and the initial concentration was 48.9 ± 2.9 mg/ml (mean ± SD, n = 18).

Statistical analysis

All data are presented as means ± SD. The statistical significance of differences between barrier perturbed and unmodified skin was analysed using one-way analysis of variance followed by Dunnett's multiple comparisons. Significance of differences between treatment groups was calculated by analysis of variance followed by pair-wise comparisons. Linear regression was evaluated by analysis of variance. $P < 0.05$ was considered significant.

For pharmacokinetic analysis of SA data, the area under the concentration-versus-time curve (AUC) was used. As standard deviation increased proportionally with the mean concentration in the 4 groups, further analyses were performed using

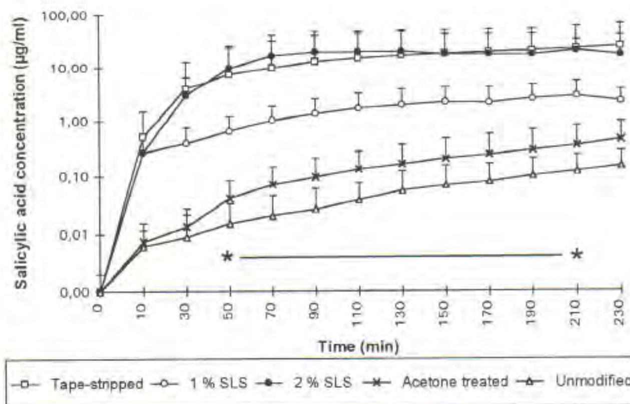


Fig. 9.3. Pharmacodynamic differences in dermal salicylic acid penetration.
Note the logarithmic y-axis scale. The curves show the mean SA concentration sampled by microdialysis probes, inserted in each of the 4 barrier perturbed skin areas, during the 4 h experiment (n = 16). Error bars are SD. In the interval from 50–210 min, there is a statistically significant difference in the mean SA concentration level ($p < 0.05$) between all treatment groups, except between tape stripped and 2% SLS pre-treated skin.

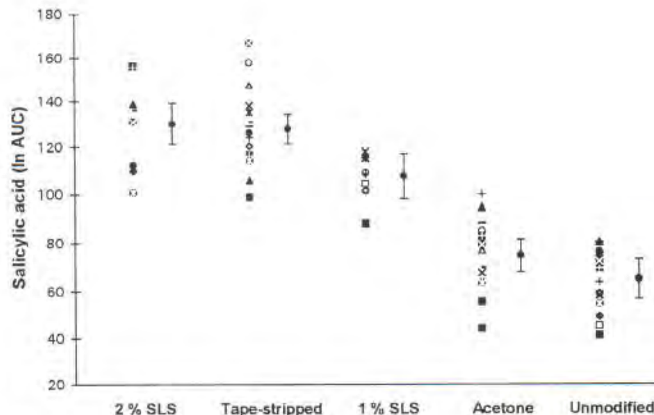


Fig. 9.4. Penetration of SA in barrier perturbed skin shown by individual AUCs.
Individual values of SA penetration over 200 min (ln AUC, mean of the 2 probes in one individual) are shown for all barrier perturbation types. For clarity, geometric mean values with 95% confidence intervals are shown alongside.

logarithmically transformed SA concentration data in the form of ln AUC:

$$lnAUC = lnAUC_1 + lnAUC_2 + lnAUC_3... = \sum_{n=1}^{n=13} ln\ AUC$$

Results

Analysis of SA pharmacokinetics by barrier perturbation treatment

SA was detectable in all samples collected from areas to which SA had been applied and measurable in all samples from penetration through perturbed skin (raw data, Fig. 9.2 a-e). Concentrations increased rapidly up to 70 min, and highly differentiated penetration kinetics between treatment groups were evident (Fig. 9.3).

Comparing the areas under the curve (AUC) from 0-200 min for each perturbation modality with the penetration in unmodified skin, the penetration of SA was 2.2-fold increased in acetone-treated skin, 45-fold increased in mild dermatitis, and 146-fold and 157-fold increased in severe dermatitis and tape stripped skin, respectively (Table 9.1). In all perturbation modalities, the penetration was significantly different from unmodified skin.

In Fig. 9.4, the log transformed AUC values from each barrier perturbation modality in each subject are shown, illustrating the variability in SA penetration. The variation (in ln AUC values) within subjects (mean of SD between 2 probes in one treated area) was about 40% of the between subject variation (mean of SD between probes in each treatment group). An analysis of variance showed that the sex of the subject had no influence on SA penetration.

Differentiation of barrier perturbation by non-invasive methods

The non-invasive measurements of barrier perturbation are shown in Table 9.1. As it can be seen both mild, moderate and severe barrier damage has been created by the procedures utilized.

Beside the measurement of barrier perturbation, a clinical description of the barrier damage inflicted was noted for each

Table 9.1. Quantification of barrier perturbation and cutaneous salicylic acid penetration.

Barrier perturbation	None	Tape stripping	SLS 1%	SLS 2%	Acetone treatment
No. of subjects	15	16	8	8	15
No. of probes	27	32	15	16	30
TEWL (g/m^2h)	4.3 ± 2.2	30.6 ± 22.2	19.5 ± 6.8	30.1 ± 12.5	9.1 ± 7.5
Erythema (AU)	8.7 ± 1.6	11.7 ± 2.8	11.6 ± 2.1	14.3 ± 2.6	9.2 ± 1.5
Skin thickness (mm)	1.72 ± 0.35	1.90 ± 0.30	1.85 ± 0.19	2.14 ± 0.41	1.75 ± 0.24
Probe depth (mm)	1.00 ± 0.31	1.21 ± 0.32	1.06 ± 0.28	1.41 ± 0.50	0.98 ± 0.18
AUC[a] (µg/ml*min)	6.3	1022	262	1099	15.6
95% CI (µg/ml*min)	3.3–12.0	486–2150	124–552	262–4615	7.6–31.7
Penetration ratio[b]	1	157	46	146	2.2
P-value[c]		<0.001	<0.001	<0.001	0.012

For each perturbation modality mean values ± SD of non-invasive measurements are given. Measurements of TEWL and erythema were performed prior to SA application, skin thickness and probe depth were measured after the penetration experiment. [a]Calculated as geometric mean of AUCs. [b]Calculated as mean of individual increases compared with unmodified skin in the same subject. [c]Compared with penetration in unmodified skin by t-test.

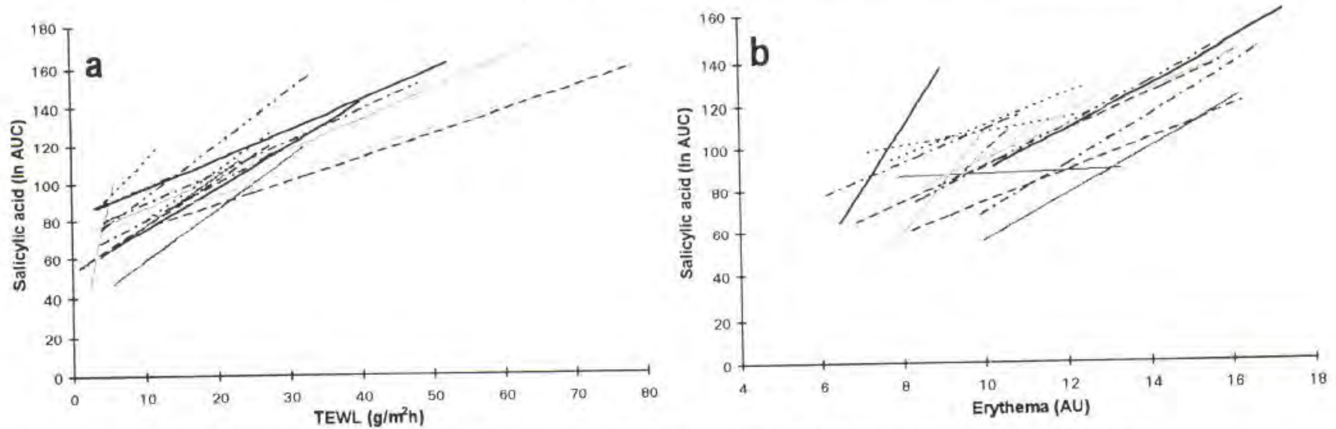


Fig. 9.5. Correlation between non-invasive measurements of barrier perturbation and salicylic acid penetration (all data).
The regression lines between the 8 observations for each subject (4 areas with 2 probes in each area) are shown for all subjects (n = 16)
a. Measurements of SA concentration vs. TEWL as measured by evaporimetry.
b. Measurements of SA concentration vs. erythema as measured by colorimetry.

treated area prior to the penetration experiment. The typical description for tape stripped skin was: moderate erythema and a slightly glistening surface due to subtotal removal of the SC; for irritant dermatitis: light to moderate erythema with superficial dryness; for acetone-treated skin: minimal or no erythema, slight superficial dryness.

Analysis by subject/individual results

Merely analysing for a correlation between the measured TEWL or erythema value and the SA penetration in the same skin area is not feasible, since observations are not independent (several observations in one subject). However, in a graphic presentation of non-invasive measurements vs. SA penetration, in which each set of data from one subject is shown as one line of regression between points of measurements (giving a total of 8 points for each line)(Fig 9.5 a-b), it is evident that a positive relationship between SA penetration and barrier damage as measured by TEWL and erythema, respectively, exists.

Correlation between the measured barrier perturbation and SA penetration

The relationship between the non-invasive measurements of barrier perturbation and AUC was investigated by regression analysis, using the cumulated measurements of either TEWL or erythema vs. the cumulated penetration of SA (AUC) for each individual. For both parameters a positive correlation could be shown (Fig. 9.6 a-b). The regression equations were:

$ln\ AUC = 1.1 * TEWL + 586$ (regression analysis, $p = 0.02$).
$ln\ AUC = 5.1 * erythema + 319$ (regression analysis, $p = 0.01$).

The increase in TEWL/erythema and the SA concentration all depend on the barrier perturbation of the skin prior to the experiment. This interdependence structure between SA concentration, TEWL and erythema was investigated by principal component analysis. The first principal component accounts for 71% of the variation between the 3 variables. Therefore, the largest proportion of the variation can be explained by the simple weighted average of the 3 variables:

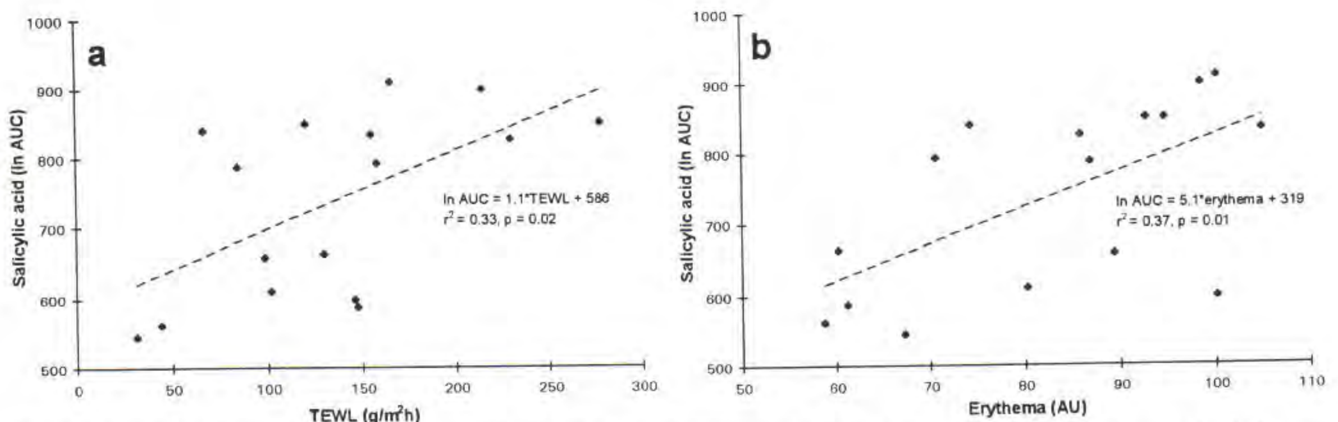


Fig. 9.6. Correlation between non-invasive measurements of barrier perturbation and salicylic acid penetration (cumulated individual measurements).
Each point represents cumulated data from one subject (Σ TEWL/erythema plotted against Σ AUC, entering data from all locations in that subject).
a. TEWL as measured by evaporimetry.
b. Erythema as measured by colorimetry.

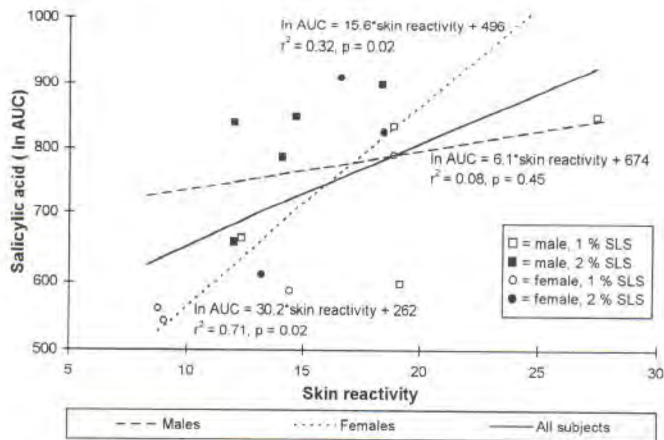


Fig. 9.7. Individual skin reactivity and salicylic acid penetration. For the calculation of skin reactivity, see results.

$0.60*SA\ ln\ AUC + 0.56*\Delta TEWL + 0.57*\Delta erythema$,

where the coefficients are the correlation coefficients of the first principal component. The coefficients express the relative importance of each of the 3 variables in the description of the effect of skin barrier damage.

Reactivity to barrier damage and SA penetration kinetics

An individual-specific overall reactivity index was calculated for each subject by dividing each of the 4 TEWL measurements (one from each treated area) in a subject with the mean TEWL value for the same treatment modality (thereby obtaining a unit-free measure) and subsequently adding all the TEWL proportions from that subject. The same was done for the erythema measurements, and the skin reactivity was the sum of the 2 cumulated scores (TEWL and erythema). This reactivity index was positively correlated with the cumulated SA penetration in the same subject as can be seen in Fig. 9.7.

Formally, the equation was:

$ln\ AUC = 15.6*skin\ reactivity + 496$
(regression analysis, $p = 0.02$).

When analysing subjects divided by sex, this correlation continued to be significant in women only with the equation:

$ln\ AUC = 30.2*skin\ reactivity + 262$
(regression analysis, $p = 0.02$).

Analysis of intraregional differences on the forearm

Analysing data by location on the forearm, a tendency towards an intraregional variation in reactivity to barrier damage was found with the most proximal location displaying higher reactivity scores than the most distal location in response to the same barrier perturbation procedures.

From Fig. 9.8 it can bee seen that the skin reactivity does show a pattern of reducing reactivity going from the antecubital fossa to the most distal area near, but always > 5 cms from, the wrist. Due to the large variation in skin reactivity scores and the limited number of participants, this decline is not significant. The penetration of SA was not significantly different between the 4 locations on the forearm.

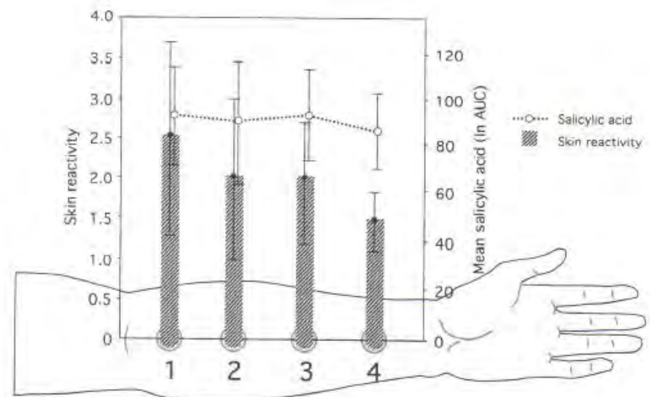


Fig. 9.8. Intraregional variation in skin reactivity and salicylic acid penetration.
Error bars are SD. Skin reactivity was calculated for each location by dividing each TEWL measurement in a location with the mean TEWL value for all locations (thereby obtaining a unit-free measure) and subsequently adding all the TEWL proportions from that location. Similarly for the erythema measurements, and the skin reactivity is the sum of the 2 cumulated scores (TEWL and erythema). A tendency towards a decline in reactivity from the antecubital fossa to the wrist can be seen. The penetration of SA is not significantly different between the 4 locations on the forearm.

Skin thickness and skin reactions

As can be seen in Table 9.1, the measured skin thickness increases with the severity of barrier damage. The increase in skin thickness is an artefact caused by swelling of the skin during the experiment. This effect was most pronounced in the skin areas pre-treated with 2% SLS, where the skin was significantly increased in thickness compared with unmodified skin ($p < 0.01$). The skin thickness in the vehicle control group (data not shown) was no different from the unmodified skin thickness at the end of the experiment, so the swelling artefact is probably caused by a combination of barrier damage and the ensuing higher influx of SA/ethanol. In 4 of the 8 subjects pretreated with 2% SLS, intraepidermal vesicles developed in this skin area during the experiment. This adverse effect is most likely to be caused by the combination of SLS and SA/ethanol exposure, as no signs of vesiculation were observed in the tape stripped skin areas, where the measured SA concentration was similar to the concentration in 2% SLS pre-treated skin.

We have found descriptions of similar reactions in a paper describing the attempt at developing an "aspirin patch", where the authors describe that many of the volunteers developed mild, self-limiting skin reactions characterized by varying degrees of erythema and pruritus, occurring in the treatment group only. The cause of the contact reaction was either ASA or possibly the metabolite SA (McAdam et al., 1996). In a study of topical 25% MSA, all subjects experienced pain and redness at the site of application (Roberts et al., 1982).

Implantation depth of the microdialysis probes

According to the ultrasound scans (measured in 3 cross-sectional scans over the first, middle and last third of the probe length), all probes were confined to the dermis, except at the points of entry and exit. The mean probe implantation depth (Table 9.1) in unmodified skin was 1.00 ± 0.31 mm (mean ± SD, $n = 27$). The increase in probe depth seen in 2% SLS-pretreated skin is, again, caused by the above-mentioned swelling artefact during the course of the experiment.

Probe depth and salicylic acid concentration

Regression analysis of AUC of SA sampled by a probe and the probe depth in the dermis showed no correlation. The reason why a correlation could not be shown is probably the above-mentioned swelling artefact, which alters skin thickness and thus also probe depth during the course of the experiment. Correcting the probe depth for the swelling effect by the factor $\frac{\text{skin thickness}_{\text{unmodified skin}}}{\text{skin thickness}_{\text{perturbed skin}}}$ did not alter the result.

Environmental measurements

The room temperature during experiments was 23.6 ± 1.1°C, and the relative humidity was 43 ± 10% (mean ± SD).

Control experiments

Vehicle control experiment: microdialysis in the dermis without SA in the penetration chamber (n = 5, probes = 10) showed occasional SA concentrations of 5-10 ng/ml in 8 of the probes.

Discussion

Barrier perturbation and penetration

For the discussion of the effect of barrier perturbation on SA penetration, please see Chapter 6 (Table 6.1).

Dose-response relationship between penetration and SLS concentration

It has previously been shown that a positive relationship exists between TEWL values and the SLS concentration used in irritant patch testing (Agner and Serup, 1990) and that a positive correlation exists between TEWL measurements over normal human skin in different anatomical sites and the percutaneous penetration of 4 organic compounds (caffeine, benzoic acid, acetylsalicylic acid and benzoic acid salt) tested in the same sites (Lotte et al., 1987). In a guinea pig study it has been shown that SLS-induced irritant dermatitis increases TEWL measurements as well as the percutaneous penetration of hydrocortisone, indomethacin and ibuprofen but not acitretin (Wilhelm et al., 1991).

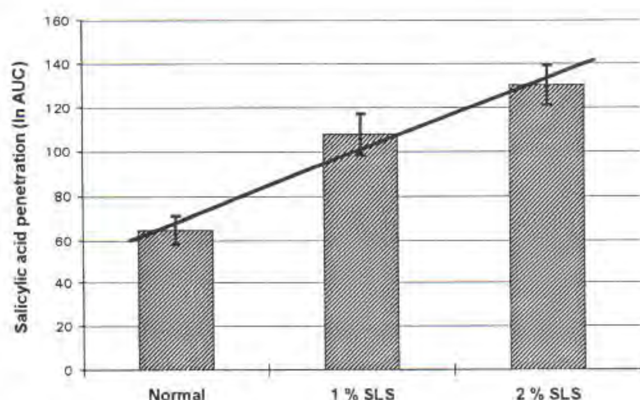


Fig. 9.9. Dose-response relationship for penetration and SLS concentration.
The relationship between percutaneous penetration of SA in response to increasing SLS concentrations is demonstrated (n = 16 in unmodified skin, n = 8 in SLS-pre-treated skin).

We have found a positive dose-response relationship for the percutaneous penetration of SA in response to increasing SLS concentrations as can be seen in Fig. 9.9.

This linear dose-response relationship for increasing penetration in response to an increased barrier insult has not, to our knowledge, been demonstrated *in vivo* in humans before.

Intraregional variation in skin reactivity and permeability on the volar forearm

We found a tendency towards an intraregional variation in reactivity with higher reactivity scores proximally than distally, whereas the penetration of SA was no different between the 4 locations. Studies of TEWL, measured in unmodified skin on the forearm, have shown that the area nearest the wrist has significantly higher values than the other sites (van der Valk and Maibach 1989; Panisset et al., 1992), but a clear-cut TEWL gradient along the long axis of the volar forearm has not been shown. Seidenari and Di Nardo (1992) have shown an increase in reactivity from the antecubital fossa to the wrist, measured as echographic pixel and thickness increase in response to nickel sulphate patch testing at 4 different sites on the volar forearm in nickel-sensitive women. Van der Valk and Maibach (1989) found that the potential for SLS-induced irritation, measured by TEWL, increases from the wrist to the antecubital fossa - which is the opposite gradient. In a study of skin thickness before and after anthralin inflammation on the forearm, increased pre-treatment skin thickness was measured distally, whereas the thickness increase caused by the inflammation was larger proximally (Lawrence et al., 1986).

Using chromametry, comparisons of basal redness (a*) along the proximal-distal axis of the ventral forearm showed a hammock-shaped pattern with significantly higher values nearest the antecubital fossa and the wrist (Ale et al., 1996). In a study of microvascular skin blood flow of the forearm skin, baseline flux was similar along the proximal-distal gradient, whereas the responsiveness to transdermal acetylcholine was lower in the distal measuring site. A possible gender difference with more pronounced vasoreactivity in women was also seen (Gardner-Medwin et al., 1997). Regarding the penetration of substances on the forearm, the percutaneous penetration of benzoic acid has been measured in 3 sites on the volar forearm of young males, showing increasing penetration going from the antecubital fossa to the wrist (Rougier et al., 1989). In 2 other studies, the application of topical corticosteroids (Kirsch et al., 1982) and of methyl nicotinate (Tur et al., 1985) have been found to elicit stronger vasoconstriction or vasodilation, respectively, going from the wrist to the antecubital fossa. However, these pharmacodynamic measurements could reflect the above described intraregional difference in vasoreactivity rather than differences in drug penetration. In conclusion, the factors determining reactivity and penetration differences on the forearm are difficult to predict. Symmetrical sites and randomized treatment orders should be used in studies of inflammation and/or penetration when using the volar aspect of the forearm.

Skin reactivity

We have found that an individual-specific overall reactivity index for a subject correlates with the cumulated SA penetration in the same subject (Fig. 9.7) and that this positive correlation is strongest in women (n.s. when analysed for men only).

For surfactant-induced irritant dermatitis, it has been suggested that each individual has his own pattern of susceptibil-

ity as measured by evaporimetry and scored visually (van der Valk et al., 1984). An increased susceptibility in fair subjects (Agner, 1991) and a seasonal variation (Agner and Serup, 1989) have been found. In a study of the relationship between biophysical skin properties and the susceptibility to SLS-induced dermatitis, the only significant parameter for the prediction of susceptibility was the surface pH of the skin (Wilhelm and Maibach, 1991), the importance of which is unclear. Most other host-related factors, including the influence of gender, have been reviewed and showed conflicting results in the literature (Lee and Maibach, 1995). In a study of the effect of tape stripping on TEWL increase, a more resistant barrier was found in darkly pigmented persons (Reed et al., 1995). Our subjects were Caucasians with skin types I-III and all experiments were conducted during the same season. This has probably supported our chances of demonstrating the reactivity/penetration correlation.

Relevance to diseased skin and clinical dermatology

We have found a significant correlation between non-invasive measurements of barrier perturbation, measured by TEWL and colorimetry (a*), and the *in vivo* penetration of SA. Thus, the more impaired the barrier function of the skin, the higher the penetration of topically applied SA. In a study in patients with widespread (endogenous) dermatitis, the measurement of reduced barrier function by TEWL could be shown to correlate with the percutaneous absorption of topically applied 1% hydrocortisone across affected skin (indirectly measured as the increment in plasma cortisol during dexamethasone suppression) (Aalto-Korte and Turpeinen, 1993).

The lack of a normal epidermal barrier in psoriasis greatly enhances the absorption of topical salicylate (von Weiss and Lever, 1964), and the application of 6% SA to psoriatic skin under an occlusive dressing for 10 h has been shown to result in the absorption of 60% of the drug applied (Taylor and Halprin, 1975). Percutaneous salicylate intoxication, both fatal and non-fatal, has been reported after topical treatment of widespread skin disorders (Anderson and Ead, 1979). The topical application of SA and its derivatives has recently been investigated for the treatment of acute herpetic neuralgia and postherpetic neuralgia (De Benedittis and Lorenzetti, 1996), and topically applied acetylsalicylic acid (aspirin) has been shown to significantly reduce experimentally induced itch (Yosipovitch et al., 1997). Thus, the therapeutic use of topical SA/ASA in the management of itch or pain, with the application of the drug to large areas of broken or inflamed skin, might be increasing.

The results found in this experimental study, where a pronounced effect of epidermal barrier damage on the cutaneous penetration of SA was demonstrated, are highly relevant to clinical dermatology. It can be speculated that the epidermal barrier, which is most often very impaired at the time of onset of topical therapy in e.g. psoriasis or recurrent eczema, will initially permit drug penetration rather freely, but penetration will be reduced as the barrier capacity of the SC recovers during (successful) treatment. Barrier function in the form of TEWL has been measured in psoriatic plaques in study of anthralin vs. PUVA treatment of chronic plaque psoriasis, demonstrating the above barrier function recovery during 4 weeks of treatment (Marks et al., 1981). This alteration in barrier capacity could be the reason why complete clearance of chronic skin diseases can be difficult to achieve, even when topical treatment is applied as prescribed throughout. A study of cutaneous penetration and barrier function, measured repeatedly over time in spontaneous or treatment-mediated recovery of endogenous skin disease, could clarify this issue further with potential therapeutic innovations as a result.

CHAPTER 10: COMPARISON OF HUMAN AND HAIRLESS RAT SKIN

The use of animal models will probably continue to be an important alternative for investigations of cutaneous drug penetration, as it is not feasible to study all aspects of penetration either *in vitro* or in human *in vivo* studies. Animal models of cutaneous penetration vary in their relevance to humans, and the explanation is found in differences in skin thickness, in lipid composition of the epidermis, in response to barrier perturbation procedures or enhancers and in metabolization of drugs either in the skin or systemically.

Scott et al. (1986), on the basis of their results, suggested that rat skin could be regarded as a good model for assessing absorption of small polar nonelectrolyte molecules such as water *in vivo* and *in vitro*. In a report by Shah et al. (1991) regarding models for the prediction of percutaneous penetration in humans, the conclusion was that the animals in which permeation data were consistently quantitatively and qualitatively similar to human permeation data were the pig, the Rhesus monkey and the hairless rat.

The hairless rat is not, as the name would suggest, at all hairless. It is covered with very fine, downy, white hairs that do not disturb the appearance of being hairless. Generally, rat skin has more hair follicles (289 follicles/cm^2) than human skin (11 follicles/cm^2). The number of hair follicles has been found to be no different in haired and hairless rats of the same type and age (Iwamoto et al., 1998). Rat skin is somewhat thinner than human skin with a SC thickness of 10–15 μm in comparison with human SC of typically 15–20 μm. The SC consists of 7–8 cell layers in the rat, 15–20 in humans (Bronaugh et al., 1982).

In the 2 previous chapters, the studies of SA penetration in hairless rat and human skin have been reported. Both studies employ exactly the same techniques, and the concentration of SA in the chamber, the composition of the perfusate, perfusate flow rate, probe material, sampling intervals and analysis procedure are all identical in the 2 studies. The only differences were in the barrier perturbation procedures:

1. Tape stripping with Transpore tape: 10 times in rats, 20 times in humans. The procedures both resulted in the appearance of a glistening layer.
2. SLS-pre-treatment: 600 μl 2% SLS sealed by impermeable tape in rats, 400 μl 2% SLS under a non-occlusive dressing in humans.
3. Acetone treatment: the skin was wiped 20 times with a cotton ball soaked in 100% acetone in rats, in humans gentle wiping with large cotton buds soaked in 100% acetone was done for 3 min.

Thus, a direct comparison of the cutaneous penetration of SA in the 2 species can be conducted without any data correction. It can be speculated that alterations in cutaneous blood flow, either caused by the anaesthesia, by the insertion of a microdialysis probe, or both, will influence the *in vivo* measurements of cutaneous penetration. The cutaneous blood flow in rats under pentobarbital anaesthesia has been evaluated and was shown to be stable, once the insertion trauma has subsided after 30 min (Groth et al., 1998a). In humans, the cutaneous blood flow

Table 10.1. Comparing the effects of barrier perturbation in human and hairless rat skin.

Barrier perturbation	Parameter	Humans	Hairless rats	P value[a]
None	AUC	4997	263470	$p < 0.001$
Tape stripping	exp(ratio)[b]	167	241	$p = 0.55$
2% SLS	exp(ratio)	161	123	$p = 0.71$
Acetone	exp(ratio)	2.2	0.91	$p = 0.14$

[a]t-tests of geometric mean values. [b]Ratio = $\log(AUC_{<treat>}/AUC_{<normal>})$

has been shown to (i) have stabilized around 60-90 min after the insertion trauma and (ii) display a diminished insertion trauma response when local anaesthesia is used (Groth and Serup, 1998). The only assumption necessary is thus that the degree of systemic absorption in rats does not contribute to the measurements of cutaneous drug levels to a degree that warrants correction (as discussed in Chapter 8).

When making the rat-human comparison, the absolute levels of drug penetration are highly different as can be seen (Table 10.1) ($AUC_{0\text{-}210}$ in unmodified skin). The following comparison of the effect of barrier perturbation on SA penetration in the 2 species has therefore been conducted by comparing the unit-free penetration increase ratio (perturbed/unmodified) and analysing whether the increase ratio is different (or comparable), taking the number of animals/subjects in each group into account (Table 10.1).

This comparison shows that hairless rat skin is much more permeable to penetration of salicylic acid when assessed in unmodified skin, with 53 times as much SA penetrated and sampled in the 210 min period we compare. The effect of barrier perturbation, however, results in increases in drug flux over the skin in orders of magnitude not significantly different from the effect in human skin. In other words, the correlation between human and hairless rat skin is qualitatively very good, but quantitatively not perfect, as the inherent barrier properties are much lower in the rat than in human skin.

The *in vitro* permeation of acetylsalicylic acid, in a petrolatum vehicle, was found to be little different in human, pig and rat skin by Bronaugh et al. (1982). Similarly, in an *in vitro* comparison of the effect of barrier perturbation in the form of abrasions with a hypodermic needle on the permeation of 7 very different compounds, (haired) rat skin served as a reasonable model for the permeation through human skin both with and without barrier damage, and the resulting enhancement ratio (abraded/unmodified) in percutaneous permeation was largely similar to that in human skin (Bronaugh and Stewart, 1985). Conducting an *in vitro* study of the permeation of SA in skin from humans, Wistar rats, hairless rats, nude mice and shed snake skin, Harada et al. (1993) found very similar penetration rates in hairless rat skin, human breast skin and shed snake skin from *Python reticularis*. Thus, the hairless rat seems to have provided permeation data more similar to human data in these *in vitro* studies than in the above *in vivo* study.

A comparison of *in vivo* percutaneous absorption of ^{14}C benzoic acid in human and hairless rats has been made, showing that both the SC reservoir and the percutaneous absorption was twice that of human skin, tested over a dose range (Dupuis et al., 1984).

In a later comparative study of the percutaneous absorption of 4 radiolabelled compounds in humans and hairless rats, Rougier et al. (1987b) found the same rank order in total penetration (% of total dose) of benzoic acid < caffeine < benzoic acid < acetylsalicylic acid in man and rat. The penetration of all 4 drugs was investigated in different anatomical regions in humans, and the penetration rates in hairless rats resembled human upper, outer arm and abdominal skin the most.

In conclusion, the hairless rat can be regarded as an appropriate animal of choice for predictive studies, when also considering features such as cost, availability, and ease of handling. In the interpretation of *in vivo* results, attention should be paid to the limitations in direct rat-human comparisons and extrapolations.

CHAPTER 11: HUMAN STUDY II

Abstract

Our aim was to simultaneously investigate 2 techniques for *in vivo* sampling of peripheral compartment pharmacokinetics after systemic administration of acetylsalicylic acid.

10 volunteers were given 2 g acetylsalicylic acid orally. Blood samples and dialysates from 4 microdialysis probes inserted in the dermis of the forearm were collected for 5 h and suction blisters were raised 1–3 h after dosing.

In microdialysates, both acetylsalicylic acid and the metabolite salicylic acid were measurable in the absence of hydrolysing enzymes. The mean C_{max} (maximum concentration) of total, unbound salicylic acid was 9.5 µg/ml in microdialysates, 13.2 µg/ml in suction blister fluid and 56.5 µg/ml in plasma. Mean T_{max} (time to C_{max}) for salicylic acid was 188 and 161 min in plasma and microdialysates, respectively. The dermis-to-plasma C_{max} ratio was 0.16 ± 0.04 by microdialysis sampling and 0.25 ± 0.09 by the suction blister fluid method.

Close correlations ($p < 0.01$) were found between C_{max} of salicylic acid in microdialysates and plasma, and between C_{max} of SA in suction blister fluid and plasma. The 2 techniques were in excellent accordance with even closer correlation between maximum concentrations obtained by microdialysis and suction blister fluid sampling ($p < 0.001$). However, comparing the tolerability of the sampling procedure, ease of analysis, and detail in chronology, microdialysis is superior for sampling *in vivo* pharmacokinetics in the dermis.

The aim of the study

The aim of the present study was threefold:

I. To study the relationship between pharmacokinetics in plasma and the skin, as measured by dermal microdialysis.
II. To study the relationship between pharmacokinetics in plasma and the skin, as measured by the suction blister method.
III. To establish the correlation between results obtained with the 2 sampling methods for studying skin pharmacokinetics.

Both techniques are used for the determination of *in vivo* dermal drug levels by sampling at the level of the target organ. As a model drug we chose acetylsalicylic acid (ASA, aspirin), which is the most widely used systemic drug today (Weissmann, 1991). After oral administration, ASA is rapidly hydrolysed to salicylic acid (SA) by ubiquitous esterases, also present in the blood.

Materials and methods

Chemicals

Acetylsalicylic acid tablets (Albyl®) 500 mg were obtained from Leo Pharmaceutical Products Ltd. SA (>99% purity) was obtained from Merck, Germany.

Subjects

We investigated 10 healthy volunteers: 5 women and 5 men, age 25-50 years. None of the subjects were allergic to aspirin or local anaesthetics. The volunteers had used no topical creams for 2 days prior to the experiment and took no medication. All subjects gave written informed consent and the study was approved by the Copenhagen County ethical committee (Ref. KA 97021s) and by the Danish Medicines Agency (J.nr. 5312-133-1997).

In vivo microdialysis recovery study

A description of the equipment used can be found in Chapter 2.

As *in vivo* recovery may differ substantially from *in vitro* recovery, *in vivo* recovery of SA was established. By perfusing the dialysis probe with a known concentration of SA, the relative *in vivo* loss of SA from the perfusate to the dermis can be calculated using eqn. 1. and used as an estimate of *in vivo* recovery. 2 subjects had microdialysis probes (2 each, total n = 4) inserted in the dermis of the left volar forearm. The probes were perfused for 4 h with the usual perfusate with 10 µg/ml SA added.

Experimental protocol

Local anaesthesia (1% Xylocaine) was injected in the dermis/s.c. tissue around (but not into) a demarcated area on the volar aspect of the left forearm (volume < 10 ml). 4 probes were inserted in parallel in the anaesthetized skin by means of a 21 G guide cannula. Perfusion was started at low flow for a period of 1 h after probe insertion to allow the insertion trauma to subside. An intravenous cannula (18 G Venflon®) was inserted in a hand vein and a baseline blood sample was taken. The subject was given 2 g ASA with a glass of water (t = 0) and microdialysis and blood sampling was started. Blood samples were drawn at the mid-point of the microdialysis sampling interval. After 1 h, a disposable suction blister chamber (Dermovac® Blistering Device) was taped to the inner aspect of the upper arm (also left arm) and connected to controlled suction of 250 mm Hg. After 2 h of suction most subjects had developed 5 fluid-filled blisters, which were emptied using a Mantoux syringe. In some subjects, additional suction time was necessary to obtain fluid in the blisters. Blood- and microdialysis sampling was continued for 5 h.

Ultrasound measurements

At the end of the experiment, skin thickness and probe depth in the dermis were measured in triplicate by 20 MHz ultrasound scanning.

Results

In vivo microdialysis recovery study

In vivo microdialysis of SA gave a relative loss (delivery to the tissue from the perfusate) of 24 ± 4% (mean ± SD, n = 4) using eqn 1. for calculation. The *in vivo* loss, which is an indirect measure of the *in vivo* microdialysis recovery of SA when using the same probe type in the same tissue, was constant during the 4 h of the experiment The relative recovery value was subsequently used for calculating the absolute concentration of SA in the dermal interstitial fluid from the SA concentration in the microdialysis samples.

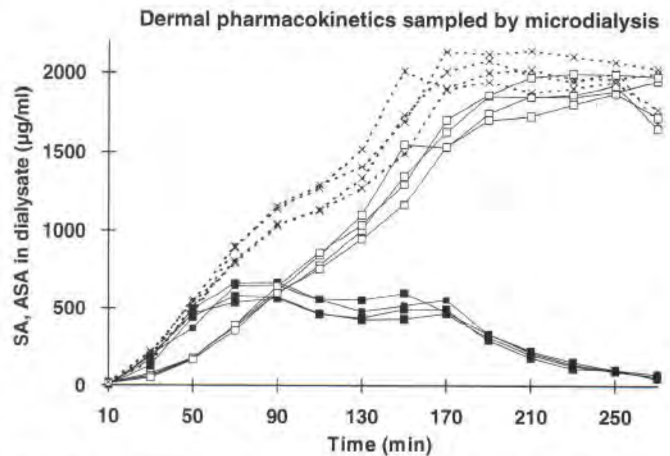


Fig. 11.1. Dermal pharmacokinetics sampled by microdialysis. Time-concentration curves, showing the dialysate concentrations of SA (□), ASA (■), and the total salicylate content (×), sampled simultaneously by the 4 microdialysis probes in one individual.

Pharmacokinetics study: Microdialysates

In microdialysis samples, the concentration of both acetylsalicylic acid (ASA) and its metabolite salicylic acid were measurable due to the absence of hydrolysing enzymes in the sample. Once the microdialysis sample has been collected, no further hydrolysis occurs (as shown by Steele et al., 1991), and the sample is amenable to direct injection onto the chromatographic column. A representative time-concentration curve, showing the concentration of SA and ASA in the 4 probes in one individual, is shown in Fig. 11.1.

In one subject, the HPLC analysis of microdialysates displayed high concentrations of a non-identifiable compound, which interfered with SA/ASA quantification. The mean C_{max} (maximum concentration) of unbound SA was 8.3 ± 2.7 µg/ml in microdialysates, and C_{max} of ASA was 5.5 ± 2.8 µg/ml (n = 9, total no of probes = 36). Mean T_{max} (time to C_{max}) for ASA and SA was 87 ± 48 and 205 ± 52 min in microdialysates. The total SA concentration in microdialysates showed a C_{max} of 9.5 ± 3.1 µg/ml and a T_{max} of 161 ± 50 min. The intrasubject variability in total SA concentration measured by microdialysis sampling was 10%, which was a third of the intersubject microdialysis variability of 30%.

Pharmacokinetics study: Plasma samples and suction blister fluid

In the blood, the hydrolysis of ASA to SA is a rapid, dose-dependent process with a half-life of less than 10 min. Thus, in the plasma samples only SA could be detected, and in suction blisters only a non-quantifiable concentration of ASA could be detected (limit of quantification 10 ng/ml).

In order to obtain comparable levels of unbound SA, measurements of SA concentrations in plasma and SBF in the present

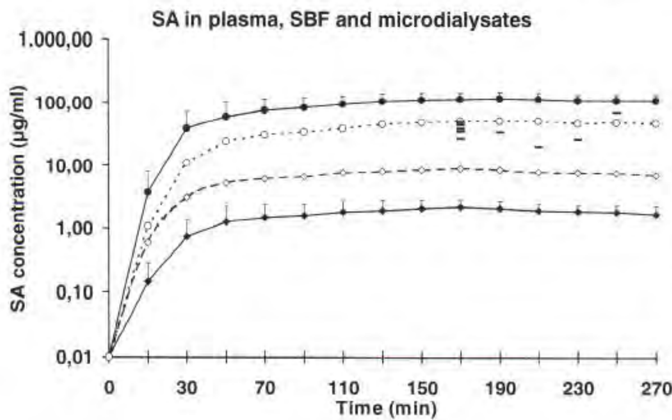


Fig. 11.2. Time-log concentration curves for mean total SA concentration in plasma (●), suction blister fluid (-) and microdialysis samples (◆), following oral administration of 2 g ASA to healthy volunteers at t = 0 (n = 10). Error bars are SD of original measurements. Microdialysate concentrations are also shown corrected for *in vivo* recovery of SA (--), as is the unbound drug concentration in plasma (...). Suction blister fluid concentrations are uncorrected, total SA concentrations.

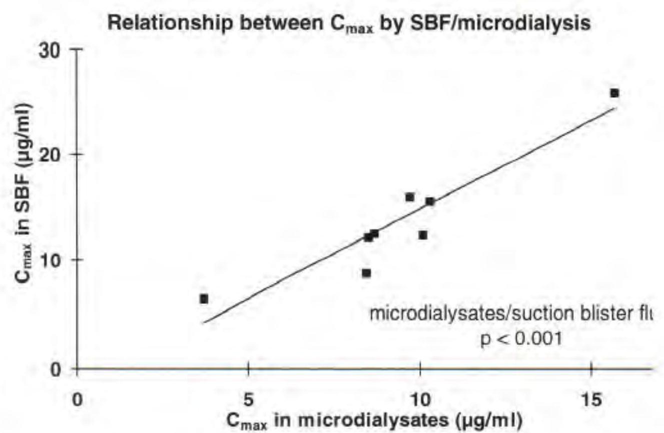


Fig. 11.4. Relationship between C_{max} by SBF and microdialysis. Correlation between maximum unbound SA concentrations measured by microdialysis and by suction blister fluid method. Each point represents data from one individual.

study were corrected to unbound SA levels using the degree of binding applicable to the SA concentration in each sample. Data regarding the plasma protein binding of ASA and SA were obtained from 2 *in vitro* studies of SA/ASA plasma protein binding (Herrera et al., 1990; Steele et al., 1991), in which the binding has been determined both by ultrafiltration and microdialysis. In the present study, plasma and SBF concentrations of SA were between 2 and 166 µg/ml, and in this concentration range SA is between 50 and 70% protein bound (the higher the SA concentration, the lower the protein binding). The unbound SA concentration in SBF has been calculated using the same protein binding data as for plasma samples.

The C_{max} of SA was 13.2 ± 5.7 µg/ml in SBF and 56.5 ± 18.9 µg/ml in plasma. Mean T_{max} for salicylic acid was 188 ± 52 min in plasma. Fig. 11.2 shows the mean time-log concentration curves for SA in blood and microdialysis samples over the 5 h.

SBF concentrations have been plotted at the time of actual sampling, which was delayed in some subjects due to slow blister formation (n = 9; in one subject suction blisters did not form). The protein content in SBF was 25.4 ± 3.9 mg/ml, and there was no correlation between protein content and SA concentration in SBF, nor was there any difference in SA concentration in SBF or in SBF/plasma ratio between normal and slow blistering subjects ($p > 0.10$).

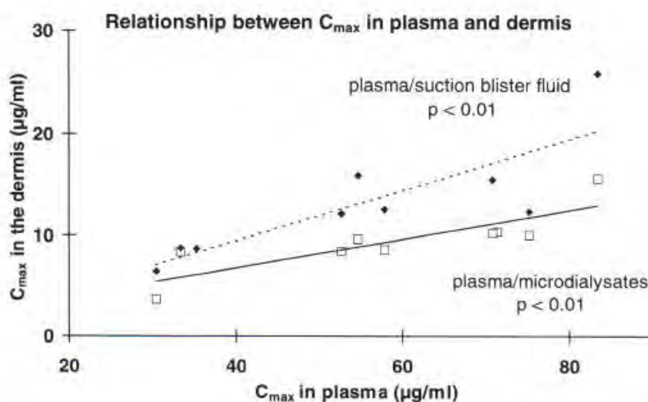


Fig. 11.3. Correlation between maximum unbound SA concentration in plasma versus maximum unbound SA concentration measured by microdialysis (□) and suction blister fluid method (◆). Each point represents data from 1 individual. Regression lines (best fit) are shown for microdialysates (full drawn line) and suction blister fluid (dotted line).

Comparison of the 2 methods for sampling drug concentrations in the skin in vivo

The dermis-to-plasma C_{max} ratio was 0.16 ± 0.04 by microdialysis sampling and 0.25 ± 0.09 by the suction blister fluid method. The positive correlation between C_{max} of unbound SA in plasma and unbound SA measured by microdialysis and SBF, respectively, is shown in Fig. 11.3.

The highly significant correlation between C_{max} of unbound SA in microdialysates and in SBF is shown in Fig. 11.4.

No interferences were found in the blank samples taken at t = 0. The ultrasound scans of the probes inserted confirmed that all probes were situated in the dermis.

Discussion

Whereas both *in vitro* (see Chapter 2) and *in vivo* recovery of SA have been determined in the present study, the recovery of ASA has not been established. However, since the *in vitro* relative recoveries of both SA and ASA have been determined in another study (Steele et al., 1991) and found to be 11.3 (SA) and 12.5 (ASA), the recoveries can be assumed to be largely similar in this study (although not performed *per se*).

We have used protein binding data from the literature for estimating free drug concentrations in plasma and SBF (taking the different protein content into account). It has been demonstrated in a pharmacokinetic study of bendroflumethiazide, which is also essentially solely bound to albumin, that the protein binding is not very different (84 vs. 76%) in plasma and SBF (Schäfer-Korting, 1985).

In the present study, the production of suction blisters caused moderate discomfort with slight pain and itching, usually perceived at the peak time of blister formation. The insertion of microdialysis probes was not perceived as painful due to the

use of local anaesthesia. Microdialysis probes can be inserted in the dermis without anaesthesia, and the discomfort is similar to what is perceived during ordinary blood sampling (Groth and Serup 1998; Lindberger et al., 1998).

In drug distribution studies, suction blisters may be raised before (Schäfer-Korting 1985; Müller et al., 1998a; Brunner et al., 1998) or after (Korting et al., 1982) the drug administration, and single or multiple dosing may be used. In a recent study by Müller et al., peripheral tissue kinetics of theophylline was followed in cantharidine-induced skin blisters, saliva and microdialysis samples from subcutaneous and muscle tissue following a single i.v. dose to healthy volunteers (Müller et al., 1998a). Subcutaneous and muscle tissue drug levels, sampled by microdialysis, closely correlated with unbound plasma levels, whereas the data from saliva and blister fluid did not correlate with free plasma values of theophylline. It was concluded that microdialysis is a reliable technique for sampling of unbound peripheral compartment concentrations, superior to saliva and skin blister concentration measurements in this regard, and it was suggested that skin blisters should not be employed for this purpose. Using the same design, the peripheral pharmacokinetics of paracetamol following an oral dose has also been studied, showing close correlations between pharmacokinetics in serum and both blister fluid and microdialysates, whereas saliva sampling was less useful (Brunner et al., 1998). Paracetamol is a drug with a low protein binding, whereas theophylline displays a protein binding of around 60%. The usefulness of any method for sampling pharmacokinetics in the peripheral compartment will depend on the pharmacokinetic properties of the drug, including the protein binding in the blood and tissues. For SA, we have been able to demonstrate very close correlations between concentrations in the dermis and plasma by both methods, although the calculated absolute free drug concentrations were very different in plasma and dermis (as can be seen in Fig. 11.2).

The correlation found between drug concentrations in SBF and plasma in the present study has most likely been improved by the timing of the suction blister formation, planned to coincide with the peak salicylate concentration in plasma. The highly significant correlation between the two peripheral compartment sampling methods employed in this study demonstrates the potential usefulness of both techniques. However, we find microdialysis sampling superior to suction blisters for obtaining dermal pharmacokinetics, when factors such as the tolerability of the sampling procedure, the ease of analysis, and the detail in chronology and reproducibility are considered.

CHAPTER 12: DRUG LEVELS IN THE SKIN AFTER TOPICAL VS. SYSTEMIC DRUG ADMINISTRATION

In human study I the dermal concentration of SA was measured by microdialysis sampling after topical administration onto either normal (unmodified) or barrier perturbed skin, and in human study II the dermal concentration of both ASA and SA was measured after systemic (oral) drug administration.

Without any correction for recovery, protein binding or other processing of the raw data, they can be compared as they have been sampled in the same tissue, in the same species under the same conditions.

Mean steady-state levels in the dermis following *topical administration* were as follows:

normal (unmodified) skin	100 ng/ml
tape stripped skin	18 μg/ml
mild irritant dermatitis	2 μg/ml
moderate-severe irritant dermatitis	18 μg/ml
acetone treated skin	300 ng/ml

Mean steady-state (during the long elimination phase of SA) level after *oral administration* of 2 g ASA:

normal skin	2 μg/ml
normal skin (by SBF*)	13 μg/ml

Thus, the drug level in the dermis after a large oral dose is comparable with the level obtained after topical drug application onto skin with a compromised barrier function in the form of mild irritant dermatitis.

A comparison of the concentration of acitretin (a synthetic retinoid) in the skin after oral (multiple dose) and topical dosing has been made, using both punch and shave biopsy and analysis of suction blister fluid and blister roof tissue (Surber et al., 1993; Laugier et al., 1994). The following results were obtained:

	oral administration	*topical administration*
punch biopsy	275 ng/mg	160 ng/mg
shave biopsy	160 ng/mg	360 ng/mg
suction blister fluid	200 ng/mg	90 ng/mg
suction blister skin	460 ng/mg	3800 ng/mg

Thus, the skin level after multiple oral doses was comparable with the level after a single topical application of a saturated acitretin/isopropyl myristate solution (with isopropyl myristate being a fatty acid ester with pronounced enhancer effect).

Recently, the distribution of penciclovir, the active component in antiviral treatment with famciclovir, has been investigated. The penciclovir concentration in the dermis, as measured by microdialysis, and in suction blister fluid and plasma was investigated in healthy volunteers (Borg et al., 1999). Pharmacodynamic differences in plasma and skin were found, with the T_{max} being 90 min in plasma and 150 min in microdialysates and SBF. The $T_{1/2}$ was 1.8 h in plasma and 2.6 h in the skin, suggesting a depot function of the skin. SBF levels were generally twice as high as microdialysate levels.

In this study, manipulations of local blood flow in the skin by adding adrenaline to the perfusate or by cooling the skin by local chilling, both producing vasoconstriction, were used. The possibility of investigating the influence of cutaneous microcirculation on drug levels in the skin, which can be done after both topical and systemic drug delivery, in a minimally invasive human *in vivo* model has thus been demonstrated for the first time.

* For the sake of completeness the level of unbound SA in suction blister fluid has also been shown. The higher value does not reflect a difference in dermal concentration, but a difference in sampling methodology. The microdialysis steady-state concentration is 8 μg/ml when correction for *in vivo* recovery of SA has been undertaken (see Chapter 11).

CONCLUSIONS

In vivo investigations and results obtained in human volunteers or patients represent the "gold standard" for skin penetration investigations. With the development of microdialysis sampling in the dermis, drug pharmacokinetics and drug metabolism can now be studied with improved and detailed real-time chronology *in vivo* in the target organ, the skin.

Microdialysis compares favourably with traditional methods for dermatopharmacological investigations such as measuring drug concentrations in skin biopsies or blister fluid, or measuring systemic drug absorption by radiolabel excretion or quantifying the amount of drug in the horny layer. The microdialysis technique offers features such as non-radioactive analysis, minimum trauma or disturbance of the living tissue where sampling takes place and high tolerability with the possibility of parallel sampling in one subject and even prolonged sampling over several days, if desired. These conditions are part of the ethical considerations relevant to the conduction of human pharmacokinetic studies in healthy volunteers and patients alike.

Like any other method, microdialysis has some inherent method weaknesses, and much work in the field of standardization has to be done before the method has found its place in basic research and in clinical and toxicological studies. The limitation of this new method consists of the impact of drug-protein binding on the accessibility of drug for sampling and the small sample volumes of often ultra-low drug concentration necessitating very sensitive methods of analysis. In cutaneous penetration studies of dermatological drugs and formulations, the drugs of interest will often be very hydrophobic as they have been developed with the aim of maximum SC penetration. Sampling very hydrophobic drugs by microdialysis is difficult, and despite the appropriate development of perfusates and analytic sensitivity it will probably continue to prove difficult to sample cutaneous drug levels of these drugs after application onto unmodified human skin.

Nevertheless, microdialysis will no doubt prove useful as a tool for the development of transdermal drug delivery systems, and the technique is likely to be suitable for safety assessment of cosmetics and risk assessment of occupational or toxicological hazards as well.

Barrier perturbation and topical drug penetration

Whether measured at the level of the skin as the target organ or as systemic absorption and excretion, not many *in vivo* studies with quantitative data on the effect of barrier perturbation on drug penetration exist. In general, the entire complex of factors which determine penetration rates in damaged or diseased skin requires further study.

In the studies of skin barrier function and cutaneous drug penetration of SA presented in this thesis, the use of microdialysis has been demonstrated to be both useful, dynamic, reproducible and sensitive. In fact, in one instance, microdialysis sampling of cutaneous penetration was more sensitive than TEWL measurement in the detection of discrete barrier perturbation in human skin. The increases in drug penetration in barrier damaged skin, measured *in vivo* in the experimental part of this thesis, are considerably higher than found in other studies, where indirect methods have been used (quantifying systemic absorption more than cutaneous drug concentrations). The penetration results obtained can only partly be explained by the experimental conditions, which were aimed at maximum drug penetration.

Future studies of the effect of barrier perturbation on drug penetration could include experiments where the conditions are modified to simulate the clinical situation better. The barrier perturbation procedures should be reduced in impact and applied one at a time in each subject, exploring the dose-effect relationship of each type of barrier damage by creating several degrees of barrier perturbation, including very mild or subclinical reactions. The drug could be applied in a cream formulation instead of using ethanol as a solvent (with the possible risk of not being able to detect drug penetration in unperturbed skin). The recovery phase after barrier injury could be explored by prolonged or repeated assessments of drug penetration over time in the same subject.

Relevance to the clinical situation and future studies

We have found a significant correlation between non-invasive measurements of barrier perturbation, measured by TEWL and colorimetry, and the *in vivo* penetration of SA. Thus, the more impaired the barrier function of the skin, the higher the penetration of topically applied SA. The pronounced effect of epidermal barrier damage on the cutaneous penetration of SA demonstrated in the current thesis is highly relevant to clinical dermatology. Using microdialysis in the diseased skin, the impact of endogenous dermatoses on cutaneous drug penetration can be investigated. The feasibility of such a study can be seen from the studies presented in this thesis.

In the treatment of skin diseases, topical treatment is the first choice in most disorders. The epidermal barrier, which is most often very impaired at the time of onset of topical therapy in e.g. psoriasis or recurrent eczema, will initially permit drug penetration rather freely, but penetration will be reduced as the barrier capacity of the SC recovers during (successful) treatment. This alteration in barrier capacity could be the cause of the frequently encountered problems seen in the clinic: clearance of chronic skin diseases can be difficult to achieve, even when topical treatment is applied as prescribed throughout. A study of cutaneous penetration and barrier function, measured repeatedly over time in spontaneous or treatment-mediated recovery of endogenous skin disease, could clarify this issue further with potential therapeutic innovations as a result.

Finally, the unresolved questions regarding the impact of alterations in cutaneous blood flow on the cutaneous drug penetration and the drug levels obtained in the dermis should be further investigated. These studies could include the use of either topical application or intraprobe delivery of vasoactive drugs with simultaneous sampling of drug penetration and monitoring of cutaneous blood perfusion in the same skin area, again ideally employing dose-effect methodology to characterize the effect of vasoconstriction or vasodilatation on cutaneous drug levels.

SUMMARY IN ENGLISH

The thesis opens with review chapters concerning theoretical and practical aspects of the investigation of drug contents in the skin. A discussion of the advantages and limitations of the established methods as well as the relatively new sampling method of microdialysis, which is employed in the experimental section, is given. Factors influencing the barrier function of the normal human skin are described as are the alterations in skin barrier function found in diseased and experimentally barrier perturbed skin.

The microdialysis technique consists of introducing an ultra thin, semipermeable tube, a so-called probe, in the dermis. The tube is connected to a precision pump, which provides a steady flow of a tissue-compatible fluid through the probe at a very low flow. Smaller molecules in the tissue, among them the non-protein bound fraction of the drug content in the extracellular fluid, will passively diffuse across the surface of the membrane and thus enter the flow of the perfusate, which is sampled at regular intervals and analysed. Microdialysis is used for the determination of drug levels in the skin after topical as well as systemic drug delivery in the experimental part of the thesis. The method is not applicable to the investigation of all drugs or compounds, as we have shown that it is not feasible to sample highly protein-bound drugs or very lipophilic drugs by microdialysis without further development of the method.

The investigation of topical drug administration consists of 2 studies of cutaneous penetration of a model drug, salicylic acid, initially investigated in hairless rats and subsequently in human volunteers. In both studies, barrier perturbation of the skin was undertaken by physical (removal of the stratum corneum by repeated tape stripping) or chemical (treatment with acetone) methods or by provocation of irritative dermatitis (by application of sodium lauryl sulphate, a detergent). Prior to the penetration experiment, the barrier damage inflicted was quantified by non-invasive measurements of transepidermal water loss and erythema. The penetration of salicylic acid, applied in an ethanol solution in chambers glued to the skin in the barrier perturbed areas, was measured by microdialysis sampling of the drug level in the underlying dermis. At the end of the experiment, probe depth in the dermis and skin thickness were measured by ultrasound scanning.

In humans and hairless rats alike, the cutaneous drug penetration was highly increased in tape stripped skin (157- and 170-fold increased, respectively, in comparison to the penetration in unmodified skin) and in skin with irritative dermatitis (46- and 80-fold increased). Delipidization by acetone led to a doubling of the penetration in humans but had no effect on penetration in hairless rats.

In both studies a close correlation between the measurements of barrier perturbation by non-invasive methods and the cutaneous drug penetration in the same area was found. In the human study, the barrier perturbation in the acetone treated area was not measurable by non-invasive methods, whereas drug penetration, measured by microdialysis sampling, was significantly increased, indicating that the microdialysis method possesses high sensitivity in the detection and quantification of perturbed skin barrier function.

In the human study, a dose-response relationship between the concentration of detergent used for the induction of irritant dermatitis and the ensuing increase in drug penetration across the skin could be demonstrated.

In the hairless rat study a correlation between probe depth in the dermis and drug penetration was found, demonstrating that the more superficially a probe was placed, the earlier it would be reached by the influx of drug across the skin.

Systemic drug distribution was studied in healthy volunteers following oral administration of 2 g acetylsalicylic acid. Drug levels in the dermis were simultaneously investigated by microdialysis sampling and by sampling blister fluid in subepidermal blisters produced by the application of gentle suction for 2 h. A comparison of the 2 methods showed a very good correlation between the free drug concentration in plasma, suction blister fluid and microdialysate, respectively, and an even closer correlation between results obtained by the 2 methods for sampling in the peripheral compartment. A comparison of method tolerability, tissue destruction, detail in pharmacodynamics and time resolution is made, concluding that microdialysis sampling in the dermis is preferable to suction blister technique for dermal drug studies of drugs available to microdialysis sampling.

Finally, the barrier function of the skin, including the effects of barrier perturbation procedures, is compared in humans and hairless rats based on the experimental results. Likewise, on the basis of experimental results a comparison of drug levels obtained in the skin after topical vs. systemic drug administration in humans is made.

In conclusion, this thesis presents the development and employment of the microdialysis technique for *in vivo* sampling of dermal pharmacokinetics and for investigations into the effect of skin barrier impairment on cutaneous drug penetration.

SUMMARY IN DANISH (DANSK RESUMÉ)

Afhandlingen indledes med oversigtskapitler omhandlende teoretiske og praktiske aspekter af undersøgelse af lægemidlers koncentration i huden. Der gives en gennemgang af fordele og ulemper ved såvel etablerede metoder som den relativt nye opsamlingsteknik, mikrodialyse, som er anvendt i den eksperimentelle del. Faktorer, som påvirker den humane huds normale barriérefunktion, gennemgås, ligesom barriérefunktionen i hhv. syg og eksperimentelt beskadiget hud beskrives.

Mikrodialyseteknikken består i at indføre et ultratyndt, semipermeabelt rør, en såkaldt probe, i dermis. Røret forbindes til en præcisionspumpe, som sender en vævsneutral væske gennem proben i huden med et langsomt flow. Mindre molekyler i vævet, herunder den ikke-proteinbundne fraktion af lægemiddelindholdet i hudens extracellulærvæske, vil diffundere passivt over membranens overflade og føres med den konstante væskestrøm ud til opsamling med regelmæssige intervaller og efterfølgende analyse. Metoden, som i den eksperimentelle del er anvendt til undersøgelse af koncentrationen af såvel lokalt som systemisk administreret lægemiddel, er ikke egnet til undersøgelse af alle stoffer eller lægemidler. Vi har vist, at mikrodialyse ikke er velegnet til opsamling af stoffer med enten en meget høj grad af proteinbinding i vævet eller meget lipofile egenskaber.

Den eksperimentelle undersøgelse af lokal lægemiddeladministration består af 2 studier af kutan penetration af et modellægemiddel, salicylsyre, initielt undersøgt i et studie af hårløse rotter og efterfølgende i frivillige, raske forsøgspersoner. I begge studier er hudens barriérefunktion beskadiget hhv. fysisk (fjernelse af stratum corneums øverste lag med gentagen tape-stripping), kemisk (affedtning med acetone) og ved fremkaldelse af irritativt kontakteksem (med applikation af et

hudirriterende sæbestof, natrium lauryl sulfat). Den fremkaldte barriéreskade er forud for undersøgelsen af lægemiddelpenetrationen i samme hudområde kvantiteret med non-invasive målinger af transepidermalt vandtab over hudoverfladen og måling af hudens rødmegrad. Penetrationen af salicylsyre, påført huden i en alkoholopløsning i kamre påklistret hudoverfladen over de barriéremodificerede områder, er herefter undersøgt med mikrodialyseopsamling af lægemiddelkoncentrationen i dermis. Afsluttende er probens beliggenhed i huden undersøgt med ultralydsscanning, hvorunder hudens tykkelse og probens dybde i dermis måles.

For både hårløse rotter og mennesker fandtes en stærkt forøget lægemiddelpenetration i tape-strippet hud (hhv. 170 og 157 gange højere end penetrationen i ubehandlet hud) og i hud med irritativt kontakteksem (hhv. 80 og 46 gange forhøjet). Acetoneaffedtningen bevirkede en fordobling af lægemiddelpenetrationen i mennesker, men ikke i hårløse rotter.

I begge studier fandtes en tæt korrelation mellem graden af barriéreskade, målt med non-invasive måleteknikker, og penetrationen af lægemiddel i det samme hudområde. I menneskestudiet var barriérebeskadigelsen i det acetonebehandlede hudområde ikke målelig med de non-invasive målemetoder, hvorimod lægemiddelpenetrationen i området, målt med mikrodialyse, var fordoblet, hvilket tages som udtryk for at mikrodialysemetoden besidder stor følsomhed i detektionen og kvantiteringen af barriéreskade.

I det humane studie kunne en dosis-response effekt mellem koncentrationen af sæbestof, anvendt til at fremkalde irritativt kontakteksem, og den resulterende stigning i lægemiddelkoncentration over hudoverfladen, påvises.

I rottestudiet kunne en sammenhæng mellem probedybden i dermis og de målte lægemiddelkoncentrationer påvises som udtryk for at jo mere superficielt beliggende en probe er, desto tidligere vil den kunne opsamle lægemiddelfluxen over hudoverfladen.

Systemisk lægemiddeladministration blev undersøgt i et studie af frivillige, raske forsøgspersoner efter oral indtagelse af 2 g acetylsalicylsyre. Lægemiddelindholdet i dermis blev bestemt med samtidig opsamling af mikrodialysat og væske i subepidermale blærer, fremkaldt på hudens overflade ved applikation af et let undertryk i 2 timer. Sammenligningen af de 2 metoder viste, at der er en meget god overensstemmelse mellem indhold af frit lægemiddel i hhv. plasma, sugeblærevæske og mikrodialysat, samt at de 2 metoder til opsamling af lægemiddelstoffer i dermis indbyrdes korrelerer tæt. Der gives en sammenligning af metodernes tolerabilitet, grad af vævsbeskadigelse, opnåelig farmakodynamik og tidsopløsning, konkluderende at mikrodialyse er at foretrække til måling af lægemidler i dermis for de lægemidler, som er tilgængelige for dialyseprocessen.

Afslutningsvis er hudens barriérefunktion i mennesker og hårløse rotter, inklusive effekten af barriérebeskadigende påvirkninger, sammenlignet på basis af de gennemførte forsøg. Ligeledes er lægemiddelkoncentrationen i human hud efter hhv. lokal og systemisk lægemiddeladministration beskrevet udfra de eksperimentelt bestemte koncentrationer.

Sammenfattende omhandler afhandlingen udvikling og anvendelse af mikrodialysemetoden til *in vivo* studier af farmakokinetik i huden og til undersøgelse af effekten af en beskadiget hudbarriere på lægemidlers hudpenetration.

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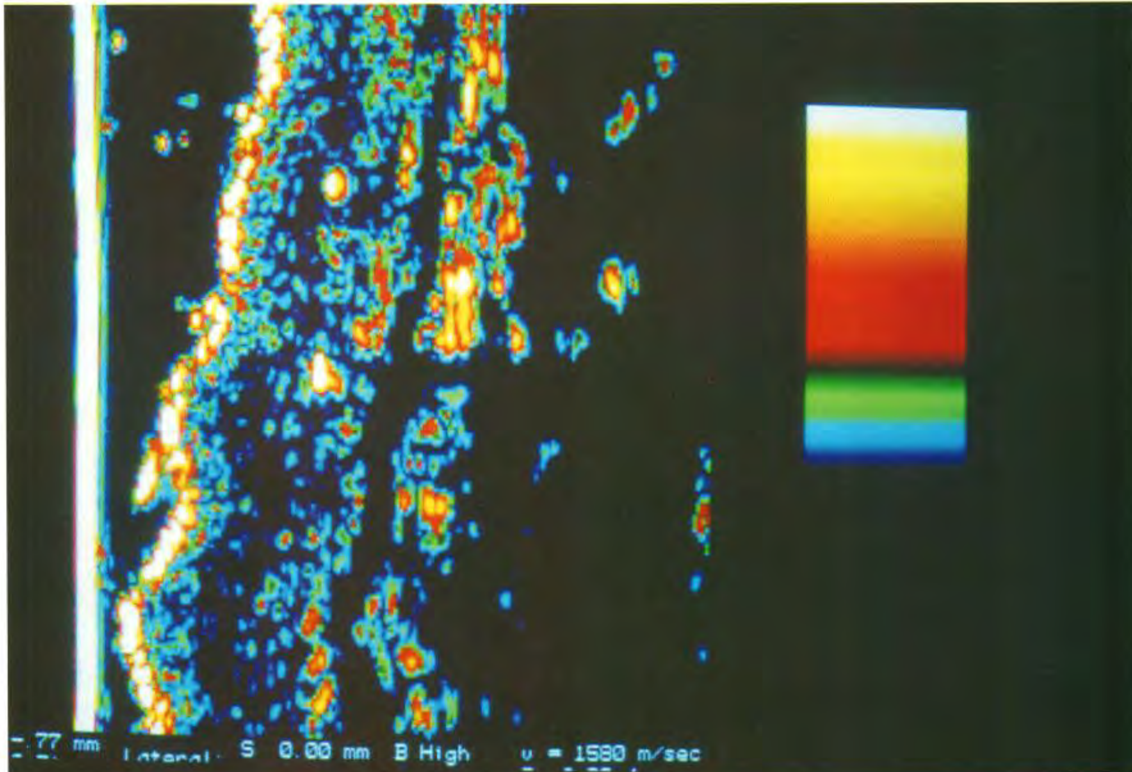
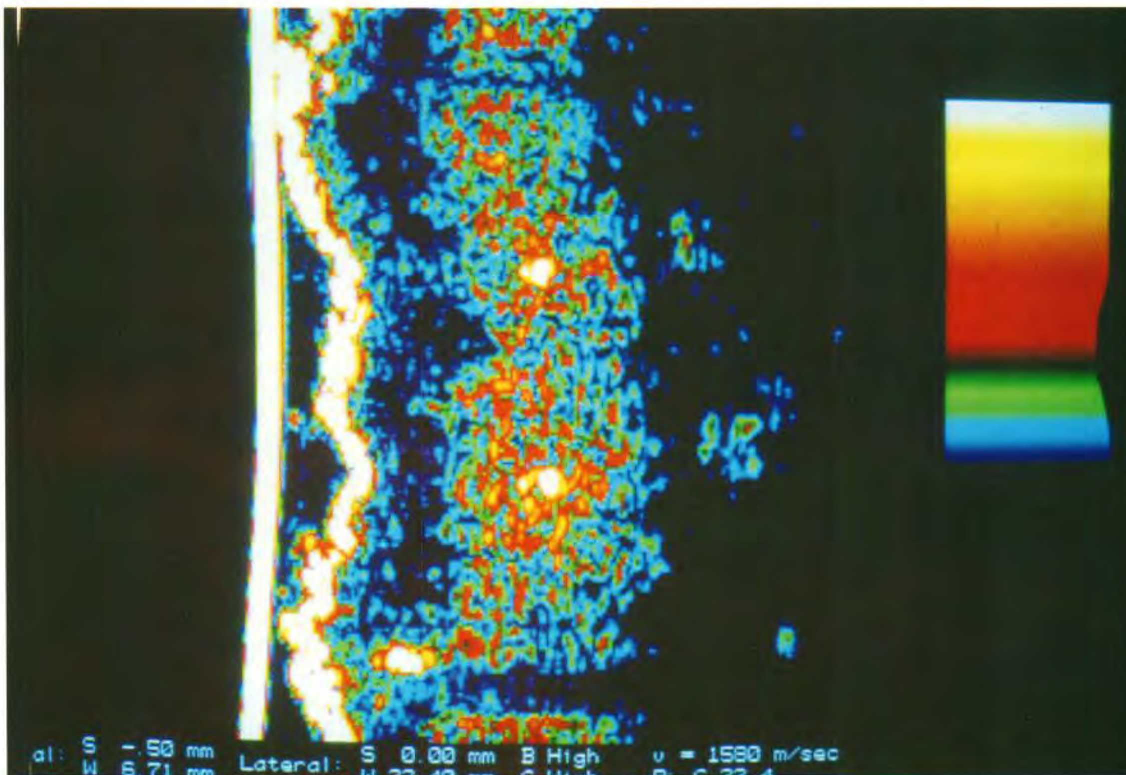
COLOUR ILLUSTRATIONS

I. Hairless rat study. B-mode ultrasound scanning.

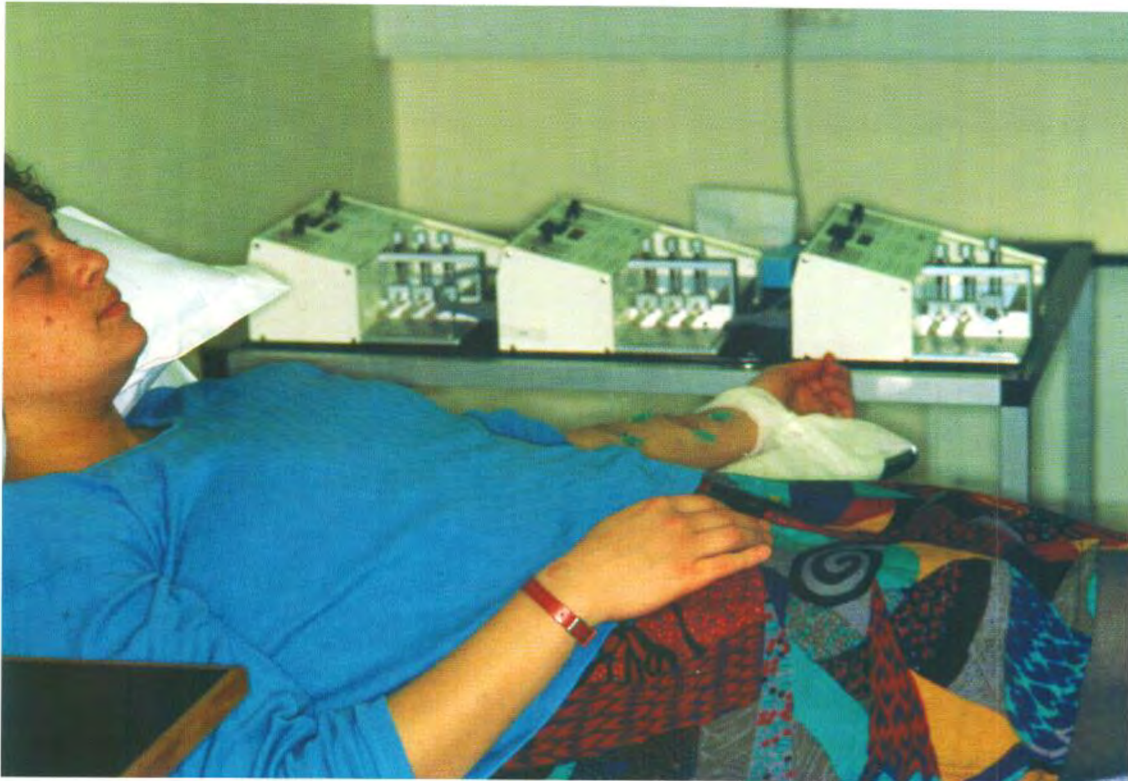


a. Cross-sectional scan of unmodified skin. The two microdialysis probes can be seen as round white structures in the middle part of the dermis. The epidermis is seen as a white line (left).

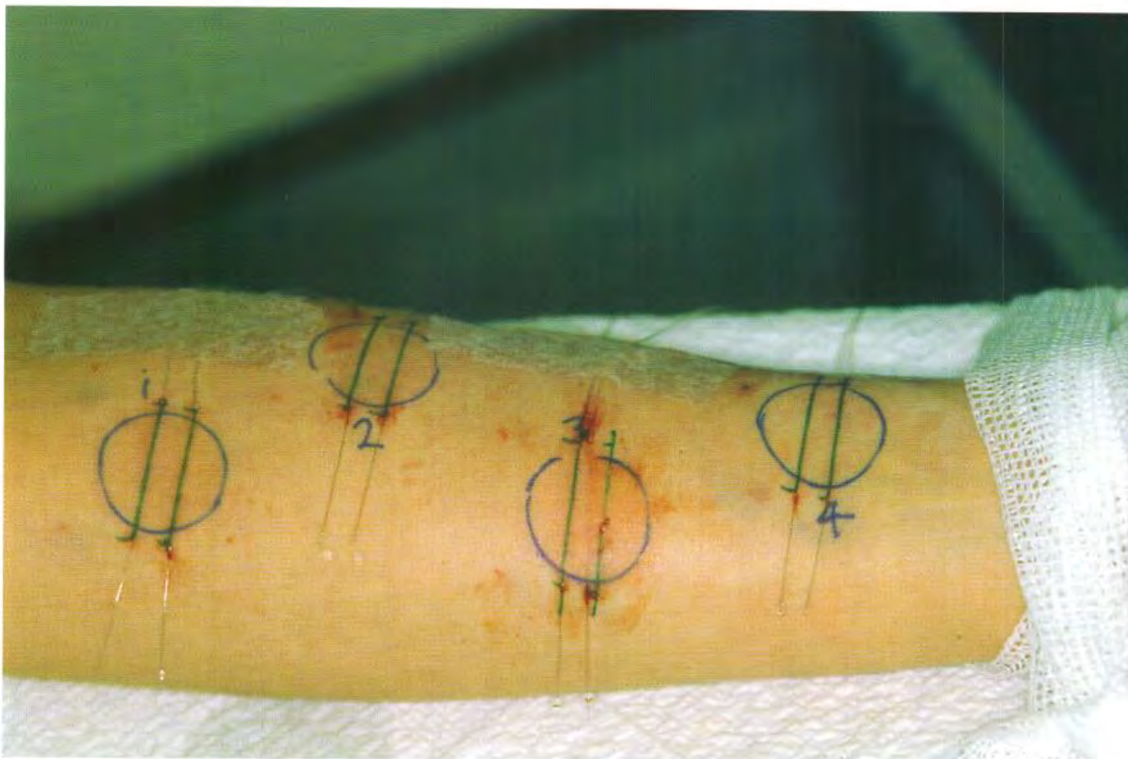


b. Irritant dermatitis. Two microdialysis probes in the mid-dermis of SLS-pre-treated skin. Note the increased skin thickness due to increased water content (water appears black).

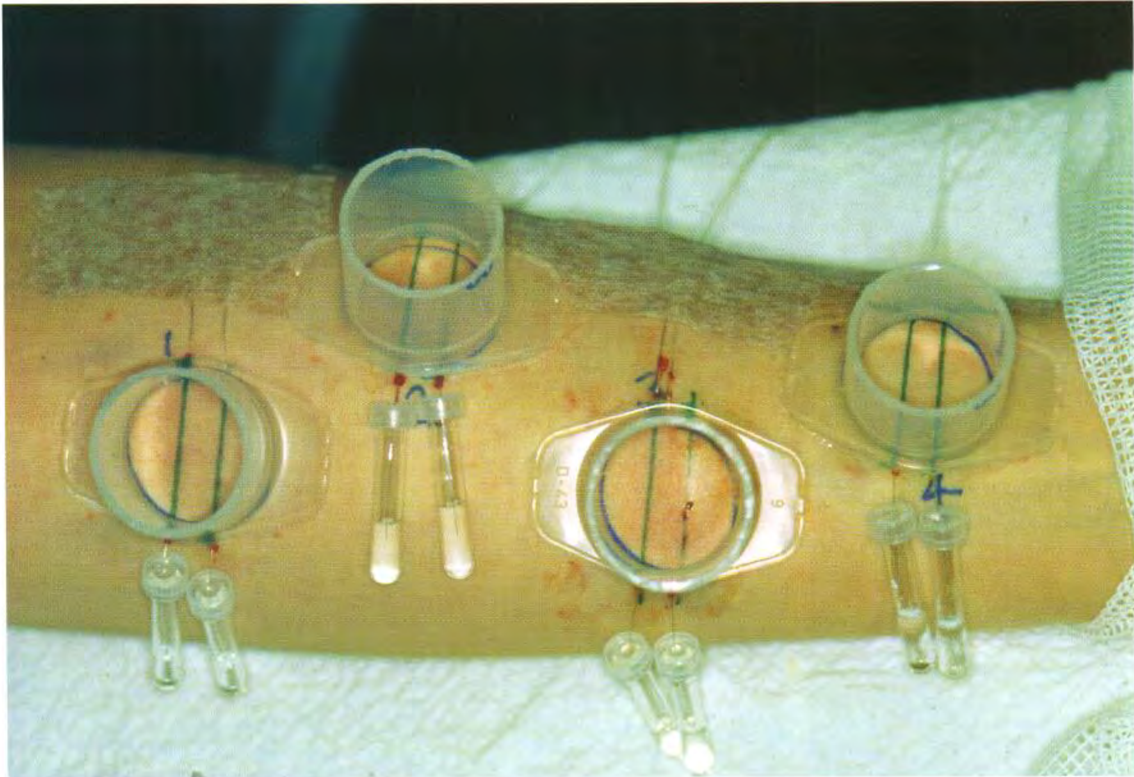
II. Human study I. *In vivo* microdialysis penetration experiment.



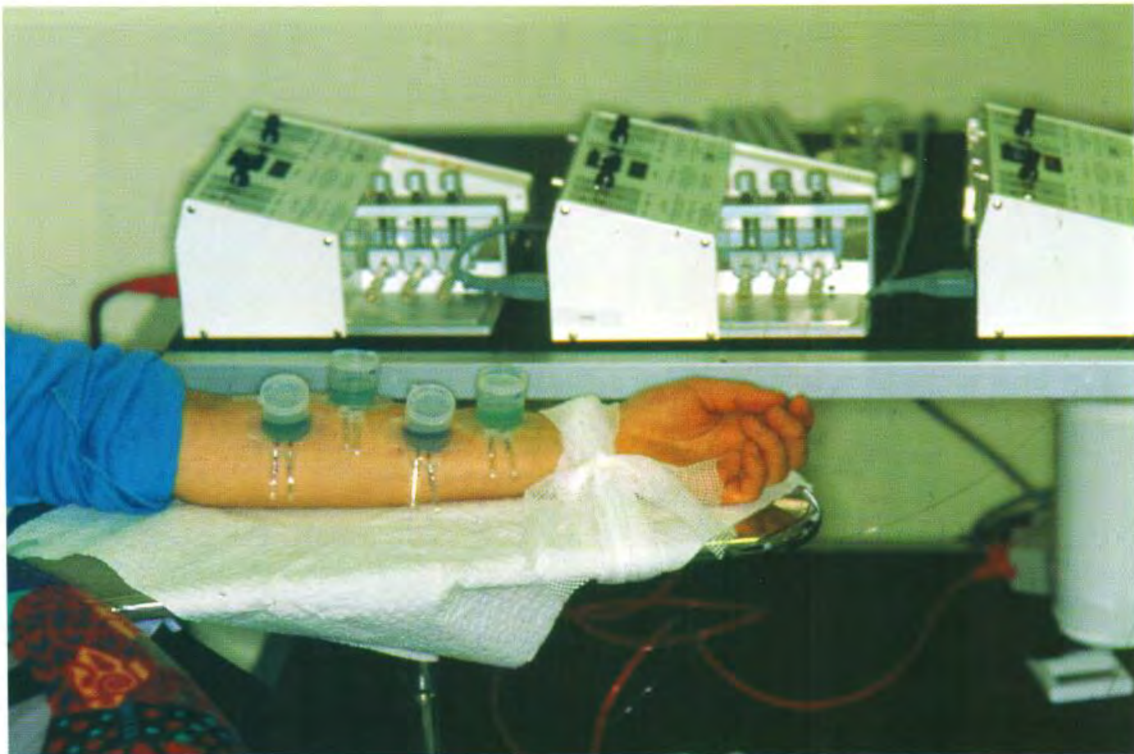
a. The subject is resting with the left arm fixed in a horisontal position. Following barrier perturbation procedures, measurements of TEWL and erythema and s.c. injections of a local anaesthetic, the guide cannulae have been inserted in the dermis.



b. The left forearm after insertion of 8 microdialysis probes, 2 in each barrier perturbed area. During the equilibration period after the insertion trauma, the probes are perfused at a low flow in order to minimally disturb the tissue and prevent the wash-out of endogenous substances in the dermis.



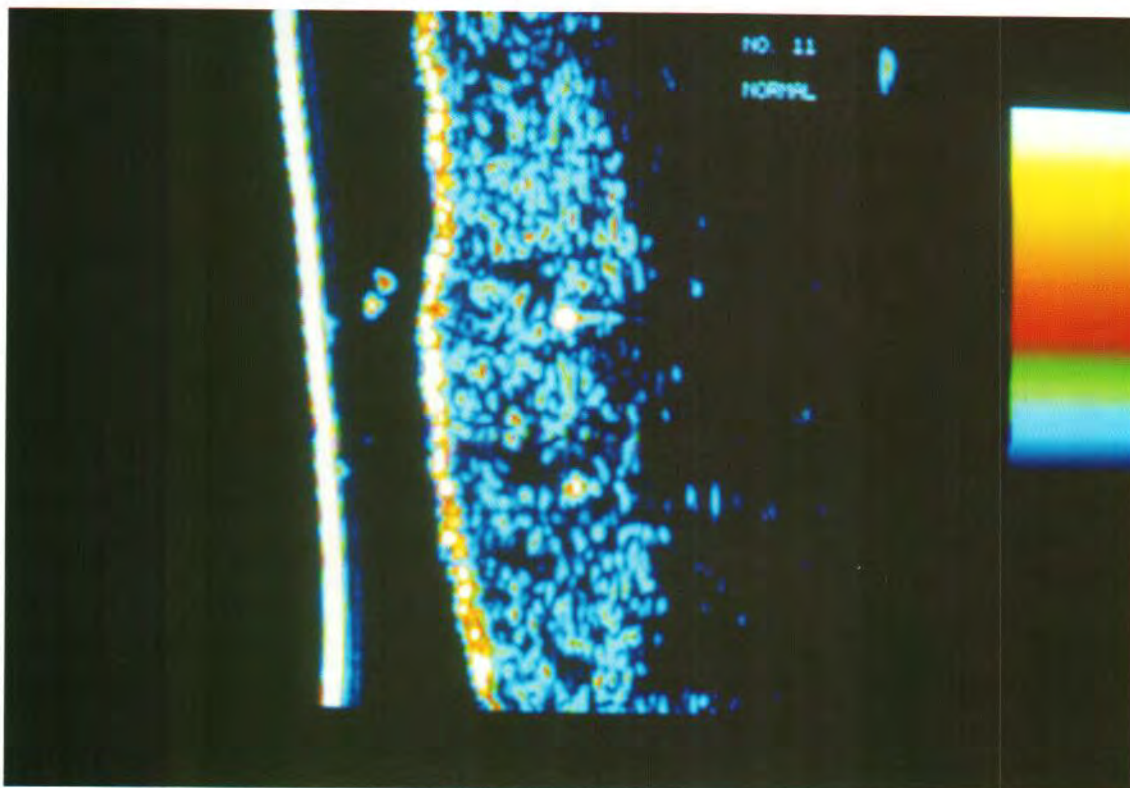
c. The penetration chambers have been glued to the skin overlying the probes, paying attention to cover both entry and exit sites of the probe (thus preventing the artefact caused by the solution in the chamber tracking down the probe canal).



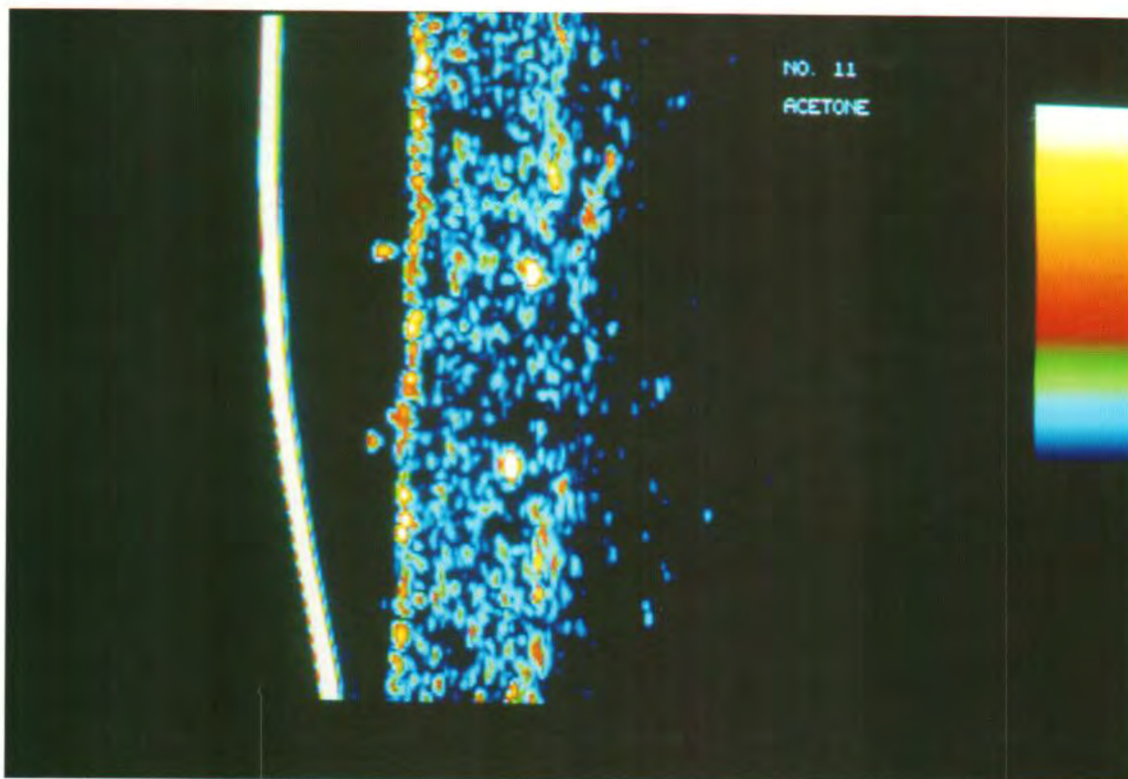
d. The salicylic acid solution has been added to the chambers and the microdialysates are collected every 20 min. The perfusate flow rate of 5 µl/min through the probes is provided by the highly accurate precision pumps in the background. The dialysates are collected in capped vials to minimize evaporation.

III. Human study I. B-mode ultrasound scanning.

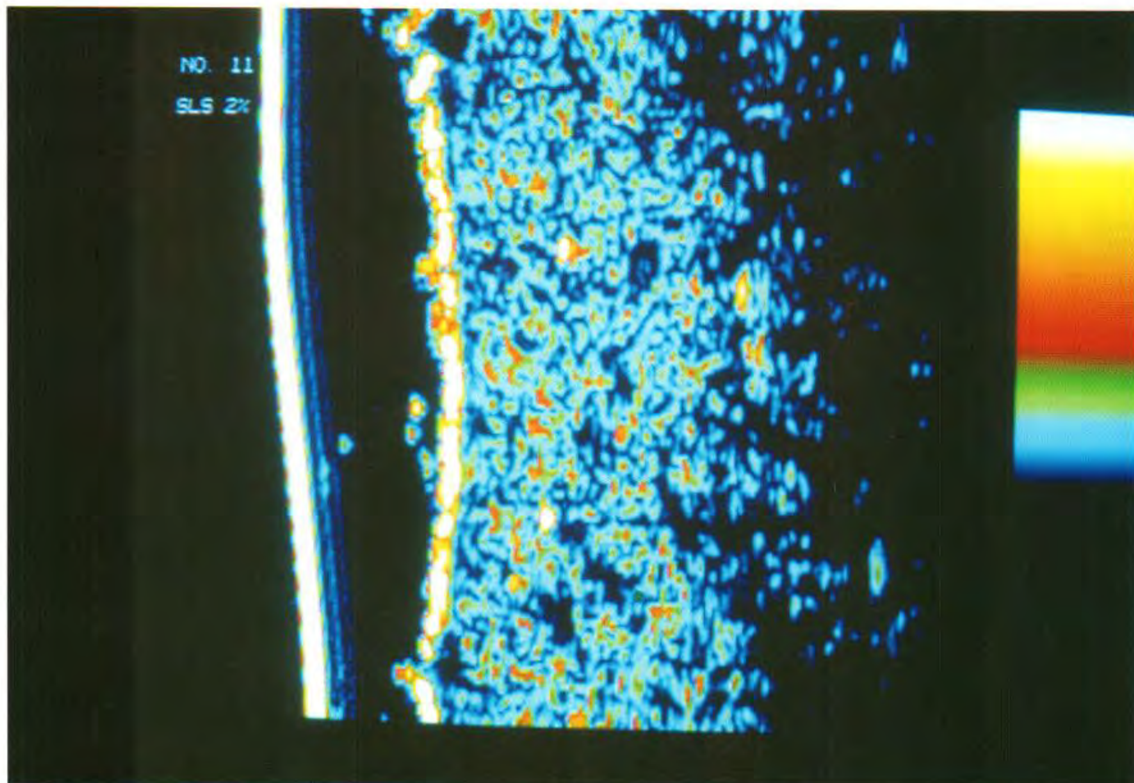
The scanning pictures below show scans from the same subject in order to demonstrate the morphological skin changes with as little variation as possible.



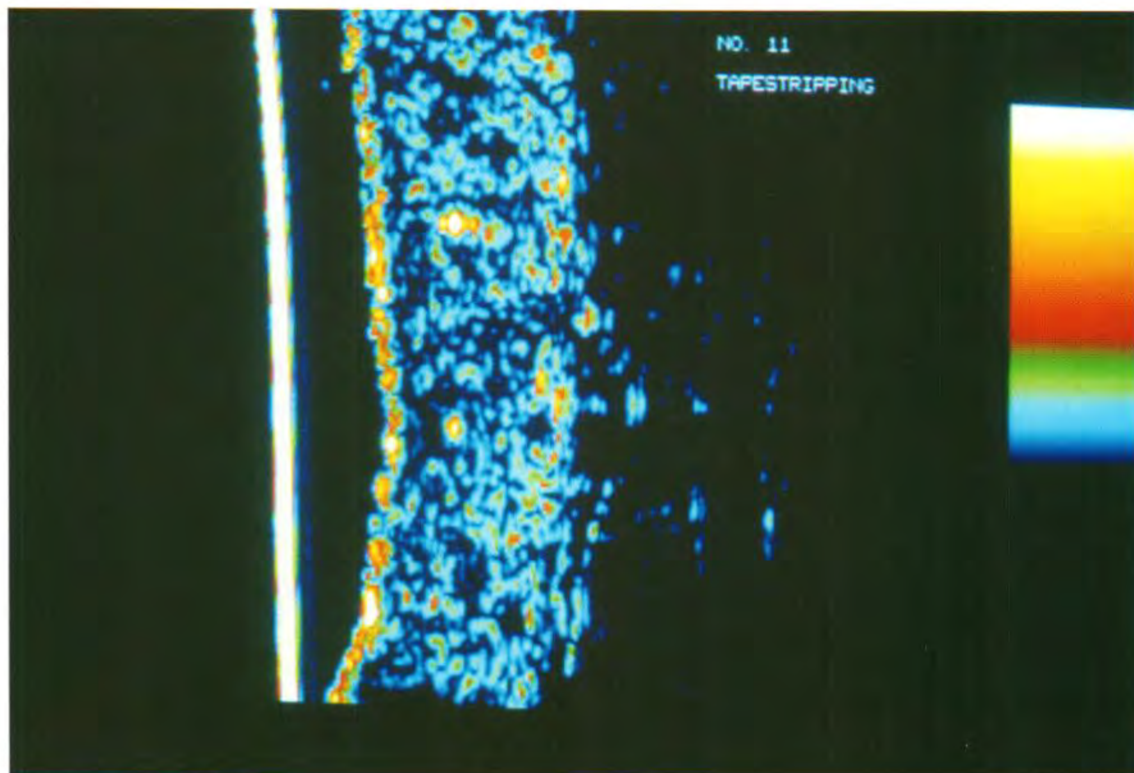
a. Unmodified skin.



b. Acetone treated skin.



c. Irritative dermatitis from 2% SLS. Note the increase in skin thickness.



d. Tape stripped skin.
The scans were undertaken at the end of the penetration experiment. Regarding the swelling of the skin during experimentation, see Chapter 9.