Cutaneous Microdialysis

Methodology and Validation

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Til mine forældre

Alt, hvad du gør, gør fuldt og helt
og ikke stykkevis og delt.

Preface

This thesis was prepared at Leo Pharmaceutical Products, Department of Dermatological Research, Ballerup, Denmark in collaboration with the Royal Danish School of Pharmacy, Department of Pharmaceutics, Copenhagen and the Academy of Technical Sciences (ATV), Copenhagen from August 1993 to July 1996. It was made to fulfil the requirements for obtaining the Ph.D. degree at the Royal Danish School of Pharmacy.

The experimental work is presented in the following papers:

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Paper II: Groth L, Jørgensen A and Serup J.
Cutaneous microdialysis in the rat: Insertion trauma and effect of anaesthesia studied by laser Doppler blood flow imaging and histamine release. Submitted.

Paper III: Groth L, Jørgensen A and Serup J.
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Paper IV: Groth L and Serup J.
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My parents for their love, trust in me and for always being there.

List of abbreviations

BrMMc	4-bromomethyl-7-methoxycoumarin
C_{MAX}	Maximum concentration
HPLC	High Pressure Liquid Chromatography
IPM	Isopropyl myristate
LDPI	Laser Doppler perfusion imaging
ng	Nanogram
SD	Standard deviation
SLS	Sodium lauryl sulphate
TTS	Transdermal therapy systems

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EXPERIMENTAL PART

1.0 Abstract

This thesis describes the methodology and validation of cutaneous microdialysis for the study of skin penetration of various topically applied substances in experimental dermatological research.

Microdialysis is a sampling technique which makes it possible to measure substances in the extracellular water space in human and animal skin *in vivo*. A microdialysis probe, i.e. a tubular semipermeable membrane connected to afferent and efferent tubings, is placed in the dermis and perfused. Substances from the extracellular space may diffuse through the pores of the membrane and be collected in the dialysate for further analysis. Glucose, sodium fusidate, betamethasone 17,21-dipropionate and calcipotriol were chosen as model substances and were investigated by *in vitro* microdialysis. The perfusion rate, the length of the membrane, stirring rate and temperature influenced recovery of the substances. Lipophilic compounds tend to have low recoveries and differ in recovery and loss.

Insertion of the microdialysis probe causes a trauma in the skin. Rat and human skin were studied *in vivo*. Increase in skin blood flow, erythema and skin thickness were demonstrated by laser Doppler perfusion imaging, Dermaspectrometer colorimetry, Minolta Chromameter colorimetry and ultrasound imaging of cross-sectional skin structure. In addition histamine was released in rat skin due to the needle insertion. An equilibration period of minimum 90 min in human skin and 30 min in rat skin after the insertion is necessary to allow the effects of trauma to diminish.

To obtain measurable concentrations in the dialysate in rats treated topically with the lipophilic drug betamethasone 17-valerate, unrealistic high doses and penetration enhancement were required. The highly protein-bound drug fusidic acid was not measurable in the dialysate after topical application, probably due to very low concentrations of free diffusible drug. Measurable concentrations were only observed after high doses of oral administrations of fusidic acid. Calcipotriol could not be detected in the dialysate.
The microdialysis technique is probably primarily useful for the study of hydrophilic substances and substances with low protein binding and low molecular weight.
However, application of cutaneous microdialysis for the study of lipophilic substances need further methodologically development.

2.0 Resumé (Summary in Danish)

I denne afhandling beskrives metodologi og valideringen af kutan microdialyse til anvendelse i experimentiel dermatologisk forskning. Formålet med dette arbejdet var, at udvikle og evaluere potentialet for kutan mikrodialyse med særlig henblik på hudpenetrationsstudier af topikalt applicerede substanser.

Mikrodialyse er en teknik, som muliggør målinger af lægemiddelstoffer og endogene stoffer i ekstracellulærvæsken i huden *in vivo*. I huden implanteres en semipermeable membran, der har form som et rør, hvortil der limes slanger i hver sin ende. Dette system benævnes en microdialyseprobe. Proben perfunderes meget langsomt med en vandig væske. Stoffer der befinder sig i huden kan diffundere gennem membranen, blive ført med væskestrømmen igennem systemet og opsamlet i dialysatet, som herefter analyseres.

In vitro mikrodialyse forsøg er udført med model stoffer, der besidder forskellige fysisk-kemiske egenskaber, glukose, natrium fusidat, betamethason 17,21-dipropionat og calcipotriol. Recovery af stofferne er afhængig af perfusionshastigheden igennem proben, længden af mikrodialysemembranen, omrøringshastigheden i væsken omkring proben og temperaturen. Recovery og loss er uafhængig af koncentrationen af stoffet i væsken omkring mikrodialyseproben, hvilket dog ikke gælder for natrium fusidat. Desto mere lipofilt stoffet er, desto mindre bliver recovery. Glukose har samme recovery og loss, men for natrium fusidat, betamethasone 17,21-dipropionat og calcipotriol er recovery og loss forskellige.
Ved at tilsætte albumin til testopløsningen, blev recovery for natrium fusidat signifikant reduceret.
En metoden til at bestemme den eksakte vævskoncentration, den såkaldte point of no-net-flux metode, måtte modificeres til studiet af stoffer, der har forskellig loss og recovery.
Mikrodialyse af stoffer, der er lipofile eller har stor affinitet til proteiner, medfører lavere recovery og er vanskeligere at arbejde med, specielt med hensyn til valg af slanger og perfusat.
Blodgennemstrømningen i huden i rotter, der er bedøvet med natrium pentobarbital er lavere, i forhold til ubedøvede rotter, men stabil. Rotter i halothan anæstesi har derimod en ustabil blodgennemstrømning.

Ved implantering af en probe i rottehud forøges blodgennemstrømningen i huden, og hudtykkelsen øges. Der frigives histamin momentant ved implanteringen. Efter indsættelse af proben er det nødvendig at vente mindst 30 minutter med opsamling af prøver, idet reaktionerne på indstikstraumaet herefter har stabiliseret sig.
Tilsvarende ses rødme, en stigning i blodgennemstrømning og hudtykkelse ved implantering i human hud. Ved lokal anæstesi før implanteringen er traumet mindre. Der var ingen sammenhæng mellem probe dybden i dermis og traumets størrelse. Huden stabiliseres ca. 90 minutter efter indsættelsen af proben.

Både i human hud og i rottehud blev proben placeret reproducerbart. Forøgelse af hudtykkelsen skyldtes en ødemdannelse omkring proben observeret såvel i human hud som i rottehud. Rotter blev behandlet topikalt med forskellige vehikler indeholdende fucidinsyre eller betamethason 17-valerat for at bestemme hudpenetrationen med mikrodialyse. Andre rotter blev behandlet peroralt med fusidinsyre, betamethasone 17-valerat eller calcipotriol.
Det var kun muligt at måle lægemiddelstoffer i huden, når ekstreme applikationer blev benyttet som f.eks. meget høje doser, brug af enhancer og beskadigelse af hudens barriere med natrium laurylsulfat. Ved realistiske og klinisk relevante applikationer kunne ingen af de nævnte stoffer detekteres i dialysatet. Dette skyldes at de testede stoffer er meget lipofile eller som natrium fusidat har en høj proteinbinding, der resulterer i meget lav recovery. Dette kræver meget følsomme analysemetoder eller anden metodeudvikling.

På nuværende tidspunkt er kutan microdialyse begrænset i anvendelse til undersøgelse af hydrofile lægemiddelstoffer og metabolitter, små molekyler og substanser med lav proteinbinding i human hud og i forsøgsdyr. Mikrodialyse er også en velegnet metode til kontinuerlige måling af mediatorer.

3.0 Introduction

In recent decades many investigators have studied mechanisms and routes by which drugs and toxic compounds may penetrate the skin. Study objects have included skin products to treat skin diseases, cosmetics to beautify and delivery systems aiming at transdermal delivery to the systemic circulation. Both *in vitro* systems and animal models have been used. The ultimate goal is to gain full knowledge of the penetration and percutaneous absorption of a compound in living human skin, qualitatively and quantitatively, in order to assess safety and efficacy of compounds and products.

Schaefer et al. (1987) addressed four main objectives of skin penetration methods used to study pharmacokinetics 1) How much drug can be delivered to organs other than the skin via topical application? 2) How can one compare the penetration of different drugs into the skin or the effects of different vehicles on the penetration of a drug? 3) How much drug enters into diseased skin compared to normal skin? 4) How much drug enters the skin after systemic application?. **Cutaneous microdialysis** has the potential to answer three of these questions, that means to compare different drugs, different vehicles, diseased and normal skin and finally to measure drug in the skin after systemic administration. In addition cutaneous microdialysis can be used to deliver drug to the skin and to study skin metabolism.

Microdialysis is a sampling technique to measure endogenous and exogenous compounds in the extracellular space, and a technique to introduce compounds into the tissue. The technique was originally developed in neurosciences because of the desire to be able to directly relate neurochemistry to behaviour. The idea to sample extracelluar fluid by dialysis was first described by Bito et al. (1966) and later by Delgado et al. (1972). In 1974 Ungerstedt and Pycock (1974) implanted a "hollow fibre" in the rat brain. Later the perfusion system was technically improved (Ungerstedt et al., 1982 ; Ungerstedt, 1984; Tossman and Ungerstedt, 1986). After further refinements, methodological studies and the coupling with appropriate analytical chemical technique like HPLC with electrochemical detection, microdialysis has become the major bioanalytical sampling tool in brain research, especially in the rat (Johnson and Justice, 1983; Sandberg and Lindström, 1983; Hamberger et al., 1985; Amberg and Lindefors, 1989; Benveniste, 1989). Recently microdialysis has been used to study the extracelluar space in many other tissues like adipose tissue (Lönnroth et al., 1987), muscle (Deguchi et al., 1991), heart (Hamberger, 1989), eye (Waga and Ehinger, 1995). The technique has been applied to various species like guinea pig, horse, rabbit, salmon, cat and dog. The first clinical study in man (adipose tissue) was published in 1991 (Ståhle et al., 1991a). Since then various studies in human skin by microdialysis have been reported (Anderson et al., 1991; Petersen et al., 1992b). The introduction of cutaneous microdialysis may allow measurements of drugs and mediators in the skin *in vivo* with a minor trauma. The technique is relatively simple to handle. It can be continuously performed for hours and days in "intact tissue" and provides protein-free samples suitable for analytical methods. These features and several others (Ungerstedt, 1991) make microdialysis unique.

The objective of this study was to refine cutaneous microdialysis, i.e. to standardize, optimize and validate microdialysis for dermatological use. The overall purpose was to develop cutaneous microdialysis into a routine sampling technique in experimental dermatology. This includes measurements of drugs, mediators and endogenous compounds in normal as well as in pathological human and animal skin and investigations on how vehicles may effect penetration of drugs into the skin.

4.0 Microdialysis

4.1 Principle of microdialysis

Dialysis (from Greek: To separate) means in the biosciences diffusion of small molecules and water through a semipermeable membrane. The basic principle of microdialysis is to mimic the passive function of a small blood vessel by perfusing a thin dialysis tube implanted into the tissue, i.e., removing chemical compounds without removing liquid and add compounds without adding any liquid. A dialysis membrane is a hollow (tubular) membrane that is permeable for small molecules and water. The dialysis membrane is continuously perfused with a perfusate, which creates a concentration gradient along the membrane. Compounds in the medium surrounding the probe, which are present in higher concentrations than in the perfusate diffuse into the dialysis membrane. Conversely, when perfusing with a higher concentration in the perfusate than in the surrounding medium, substances will leave the tubular membrane and enter the medium or the tissue. Using continuous flow through the membrane compounds in the extracellular space may diffuse into the dialysate, which is sampled for further analysis (Figure 1).

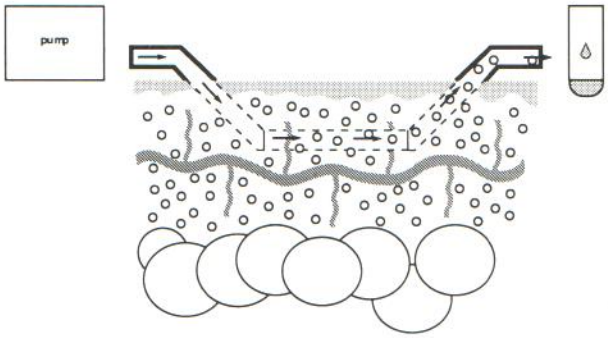

Figure 1 *A schematic drawing of cutaneous microdialysis.*

Because the flow rate of the perfusion fluid is kept very low (1-10 µl/min) ultrafiltration will be minimal because no pressure is developed, and diffusion of substances across the membrane will occur according to the principle of dialysis (Ungerstedt, 1984). Diffusion is described by Fick's law:

$$J = -D \cdot A \cdot \frac{dC}{dy} \qquad (1)$$

J is the flux, the number of molecules per unit time that cross a unit area, D is the diffusion coefficient, the parameter that determines how fast the diffusion is, A is the porosity, the fraction of the volume that is diffusible, e.g., the extracellular space (Lindefors et al., 1989). According to this law the flux, J, is proportional to the concentration gradient. The flux is directed opposite to the concentration gradient. The flux is more rapid the larger the concentration gradient (Figure 2).
It is possible by the use of membranes with a different pore size selectively to remove compounds as the pore size determines the molecular-weight limit of the compound entering the probe. Larger molecules are retained in the tissue by the membrane. Thus, the dialysate does not contain proteins, and the samples are "clean" and especially suitable for analysis.

4.2 Recovery and loss

The constant flow through the membrane results in incomplete equilibration with the surrounding medium and the concentration in the dialysate is not equal to the undisturbed concentration in the surrounding medium but lower. *Relative recovery* is defined as the ratio between the concentration in the dialysate (C_d) and the concentration in the medium surrounding the probe (C_m).

$$Recovery = \frac{C_d}{C_m} \qquad (2)$$

It is expressed either as a ratio or percentage.
Absolute recovery is referring to the total amount of a substance that is removed by the perfusate during a defined time

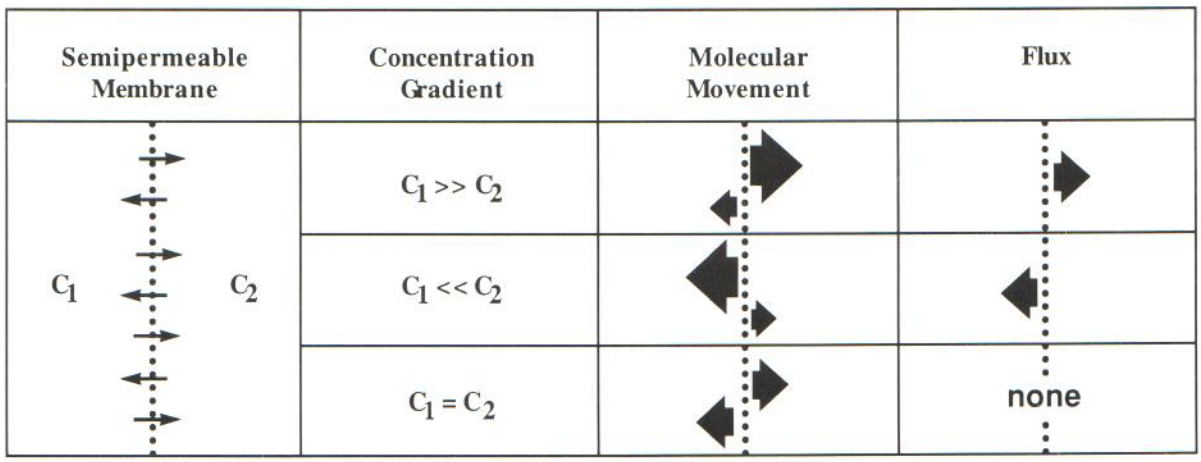

Figure 2 *The relationships between the concentration gradients and the direction of flux.*

very as referring to the relative recovery.
As diffusion across the membrane works in both directions, depending of the concentration gradient, substances can apparently be delivered to the surrounding medium. *Loss or delivery* is the ratio of the loss in concentration from the perfusate to the initial concentration in the perfusate.

$$Loss = \frac{C_p - C_d}{C_p} \qquad (3)$$

C_d is the concentration in the dialysate and C_p is the concentration in the perfusate.
Theoretically recovery and loss are equal, however, in practice, this is not always the case.

4.3 Factors affecting recovery and loss *in vitro* and *in vivo*

Several factors affect the dialysis recovery of compounds in the medium surrounding the probe, i.e., the extracellular space or a sample solution (Table 1).

Perfusion rate (flow)
Length of dialysis membrane and design
Temperature
The dialysed compound
The dialysis membrane
The perfusion medium
The tissue
Blood flow and metabolism
Time
Probe depth in dermis

Table 1 *Table 1: Factors affecting recovery and loss in vitro and in vivo.*

4.3.1 Perfusion rate (flow)
Recovery is dependent on the flow through the probe. The recovery increases when the flow declines, as low flow rates allow more time for equilibration over the membrane (Ungerstedt, 1984; Benveniste, 1989). The chosen flow rate is often a compromise between the desired level of recovery and the volume of the sample that can be critical for the analytical procedure.

4.3.2 Dialysis membrane length and design.
Recovery is directly proportional to the area of the membrane. Efficiency of dialysis increases with increasing active dialysis area, which includes increasing length of the membrane (Hamberger et al., 1983; Parsons and Justice, 1994). The design of the probe will influence the speed at which the perfusate passes the membrane and thereby recovery (Zhao et al., 1995).

4.3.3 Temperature
Recovery will increase at higher temperature (Wages et al., 1986) since the diffusion coefficient increases 1-2%/C° (Bard and Faulkner, 1980)

4.3.4 The properties of the dialysed compound
There exists an inverse relationship between recovery and molecular weight (Kendrick, 1988). This can be explained by the fact that the diffusion coefficient of a given compound is inversely proportional to the solute radius and obviously the molecular weight. The electrical charge and size of the hydrated ion affect recovery (Ungerstedt, 1984; Sandberg and Lindström, 1983). Finally the lipophilicity and protein binding of the drug may influence recovery. This results in varied recovery for different substances.

4.3.5 The dialysis membrane
Recovery of a particular compound varied when the compound was dialysed by different type of membranes (Hsiao et al., 1990). The pore size of the membrane determines which compounds that are allowed to pass the membrane, expressed by the molecular weight cutoff. The dialysis membrane can interact physically with the dialysed compounds. The membrane may be able to capture the compound or the compound may adsorb to the membrane resulting in different values for recovery and loss, where loss is higher than recovery (Amberg and Lindefors, 1989). Yang et al. (1993) found a difference in recovery and loss for cyclosporin A. They concluded that the important factor was a nonsaturable membrane capture component.

4.3.6 The perfusion medium
The perfusion medium should resemble and ideally be identical to the medium surrounding the probe in composition and tonicity, i.e. *in vivo* the extracellular fluid, since substances in the extracellular fluid otherwise would be dialysed. Drainage can change the physiological conditions and recovery (Ungerstedt, 1984; Benveniste, 1989). If the perfusate lacks a component present in the extracellular fluid the substance is drained within a radius of 1 mm from the probe (Benveniste et al., 1989). However, this issue is less important in the skin as compared to the brain, since neuroactivity is sensitive to different concentrations of ions in the brain interstitial space (Osborne et al., 1991). Drainage of glucose from adipose tissue was found unless glucose was added to the perfusate. The drainage disturbed local glucose metabolism (Jansson et al., 1990). In contrast, no drainage of glucose was apparent in the skin during perfusion when no glucose was added to the perfusate (Petersen et al., 1992a). Obviously the perfusion fluid must be free of air bubbles and particles, which can disturb the flow and stick to the membrane and decrease the surface area.

4.3.7 The tissue, blood flow and metabolism
Recovery of a certain compound is different in different tissues (Ståhle, 1991). This shows that the mass transport between the membrane and the tissue is tissue dependent. Blood flow and metabolism can alter the tissue concentration. This may not affect the recovery, but can make the interpretation of the results more difficult.

4.3.8 Time dependence of recovery and loss
Initially after the insertion of the probe *in vivo* the recovery is high caused by a traumatic tissue response, but decreases ra-

pidly (Benveniste and Hansen, 1991). However, time dependence of recovery is also reported *in vitro*, because of an initially steep concentration gradient across the membrane (Amberg and Lindefors, 1989).

4.3.9 Concentration dependence
Recovery is independent of the concentration in the medium surrounding the dialysis membrane. When changes in the outer medium occur, the concentration gradient changes correspondingly, thus keeping recovery constant. This fact makes it possible to determine the concentration in the extracellular space (Johnson and Justice, 1983). However, recovery can sometimes be dependent on the outer concentration because of interaction between the substance and the dialysis membrane.

4.3.10 Dependence on probe depth in dermis
In cutaneous microdialysis the probe depth, i.e., the distance from the surface of the skin to the microdialysis membrane inserted in the dermis may affect recovery (Andersson et al., 1995a) (See later).

The factors which have been described in this section should all be taken into account when designing microdialysis experiments.

4.4 *In vitro* recovery versus *in vivo* recovery

In vitro and *in vivo* recoveries are not equal, because of the complex nature of tissues, consisting of the intracellular, the interstitial and the vascular compartment as compared with a simple aqueous solution used for *in vitro* studies. The *in vivo* recovery is lower than the *in vitro* recovery (Benveniste, 1989). The mass transport or flux in a tissue is lower than in a liquid. The diffusion is lower in tissues because of the tortuosity, the rate limiting factor for diffusion (Nicholson et al., 1979), and the limited volume fraction of the extracellular space. The tortuosity of the tissue arises from impediments by impermeable cell membranes making the diffusion pathway longer and hence the transverse diffusion slower. The volume fraction of the extracellular space in the tissue, where diffusion is possible, is lower than the total volume of the tissue. This reduces flux in the tissue as compared to flux in bulk fluid, for the same concentration gradient. Furthermore, the extracellular matrix has a gelatinous character due to glycoproteins and other large molecules which increase the viscosity as compared to a simple solution. The dermis contains an amorphous ground substance of glycosaminproteoglycans, collagen fibres and elastic fibres which all may reduce the diffusion.
In equation (1) both the diffusion coefficient D, and A, the porosity, is lower *in vivo* than *in vitro* resulting in a decrease in flux and an apparent decrease in *in vivo* recovery (Lindefors et al., 1989). *In vitro* the main factor for limited mass transfer is the membrane resistance to diffusion. The rate-limiting step *in vivo* is the tissue, and not the molecular exchange between the dialysis membrane and the tissue (Benveniste et al., 1989). This indicates that the choice of material of the semipermeable membrane may have a relatively minor effect on the *in vivo* microdialysis results (Hsiao et al., 1990). Metabolism can also affect the *in vivo* recovery. Recovery *in vitro* has been reported to be lower than *in vivo* recovery for neurotransmitters, indicating that active processes are involved in the *in vivo* recovery of these compounds (Parsons and Justice, 1992).
In vitro recovery is used to evaluate the degree of recovery of new substances, reproducibility, similarities of loss and recovery and to compare the probe performance before and after an *in vivo* experiment.

4.5 The true extracellular concentration

As described above the dialysate concentration is not equal to the extracellular concentration in the tissue. Anatomical and analytical limitations do not allow use of membranes so long and flows so low that it would be possible to reach 100% recovery. Therefore several methods, theoretical and experimental approaches, have been developed for quantitative estimation of the unbound extracellular concentrations from dialysate concentrations (Table 2).

Mathematical models
In vitro recovery
Flow rate method
Slow flow rate method
Difference method
Reference methods

Table 2 *Methods used for estimation of the true tissue concentration.*

The first attempt was to use *in vitro* recovery in calculation of the extracellular concentration which as stated above lead to underestimation of the concentration in the tissues (Zetterström et al., 1983). This approach has now been abandoned. Mathematical models and theoretical principles have been used to describe diffusion in the extracellular space in order to estimate extracellular concentrations (Benveniste et al., 1989; Lindefors and Amberg, 1989; Benveniste et al., 1991; Bungay et al., 1990; Morrison et al., 1991). These models, which are based on unavailable constants like diffusion coefficients and tortuosity factors, are too approximative and not suitable for routine applications. Therefore, other groups have developed more empirical methods to estimate recovery and the true extracellular concentration. The *flow rate method* is based on extrapolation to a condition of zero perfusate flow leading to 100% recovery and the exact extracellular concentration. Various forms of the method have been described, which all involve a mathematical description of the relationship between extracellular concentration and the flow rate using non-linear regression (Jacobson et al., 1985; Lerma et al., 1986; Ekblom et al., 1992). The method demands a long experimental time because measurements with various flow rates must be conduced. A second method based on the fact that low perfusion rate results in a dialysate concentration close to the tissue concentration is the *slow flow method* (Wages et al., 1986).

The disadvantage of this method is very small samples and problems keeping a constant low flow rate such as 0.057 µl/min. The latest developed method based on different flow rates is a modification where the perfusion is stopped for a fixed period (Merlo-Pich et al., 1993).

With the *calibration method* also named the *difference method* or the *point of no-net-flux* method the compound of interest is added to the perfusate in lower and higher concentration than the expected extracellular concentration. There exists a point where the concentrations inside and outside the membrane are equal, and no net diffusion occurs because no concentration gradient exists. This point, the point of no-net-flux represents the extracellular concentration (Lönnroth et al., 1987). The method is time consuming and requires steady-state conditions in the tissue. However, a modification of the method is developed for transient conditions (Olson and Justice, 1993).
In the *reference methods* the idea is to find an internal marker for *in vivo* recovery, which serves as a reference for calculations of extracellular concentrations of drugs or endogenous compounds. Two approaches have been used. Systemically introduced markers (Deguchi et al., 1991; Teraksaki et al., 1992) and locally introduced markers (Larsson, 1991; Wong et al., 1992). The *in vivo* loss of the locally introduced marker is assumed to be the recovery for the compound of interest. It is thus critical that loss and recovery are really equal for the used compounds, both *in vitro* and *in vivo*. Thus, the marker must be chemically and biologically similar to the substance of interest (Kehr, 1993). *Retrodialysis* is also based on the idea that *in vivo* loss represent the *in vivo* recovery, as used with locally introduced markers. But in this method the loss of the compound of interest is used to estimate the recovery of the compound itself, and it is therefore mainly used for exogenous substances (Wang et al., 1993)
A review of the different methods is provided by Kehr (1993) and by Parsons and Justice (1994).

Several papers have been published with more or less successful outcome using and comparing these methods in *in vivo* and *in vitro* microdialysis studies for estimating the true extracellular concentrations (Ståhle et al., 1991b; Menacherry et al., 1992; Eisenberg and Eickhoff, 1993; Van Belle et al., 1993).
In the pharmacokinetics perspective it is the unbound concentration of the drug in the extracellular fluid surrounding the target tissue that is most important, since the free drug concentrations determine the pharmacological effect. Microdialysis is therefore very relevant and suitable for pharmacokinetic studies to estimate parameters like elimination constants and Area Under the Curve in order to assess or understand pharmacodynamic effects (Ståhle, 1993; Müller et al., 1995a; Müller et al., 1995b). Microdialysis in the target organ, like cutaneous microdialysis after topical application, is an excellent alternative to the indirect measurement of total plasma drugs levels.

4.6 The microdialysis probe

A dialysis membrane connected to an inlet and an outlet tube is termed a **microdialysis probe**. There are two main groups of dialysis probes. One dialysis membrane is a hollow fibre, where the in- and outlet tubes are in a straight and serial arrangement, named *microdialysis probe per se*, *serial probe* or *single lumen probe* (Figure 3) (Lönnroth et al., 1987). These probes are simple and very cheap to make.

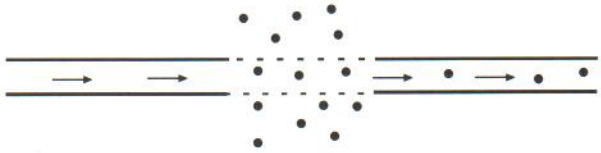

Figure 3 *Single lumen dialysis probe used in the present study.*

The other group include those where the tubes are positioned in parallel as a *loop probe* (Zetterström et al., 1983), the *side by side probe* (Sandberg et al., 1986) or a *concentric probe* (Figure 4) (Tossman and Ungerstedt, 1986).
Any type of microdialysis probe has some advantages and disadvantages (Ungerstedt, 1984).
Different membrane material can be used in the probes. The molecular weight cutoff varies with the different materials. Most membranes have cutoff values less than 20.000 Da, but 100.000 Da membranes exist. The membrane should be biocompatible and no interaction between the membrane material and the dialysed compound should occur.

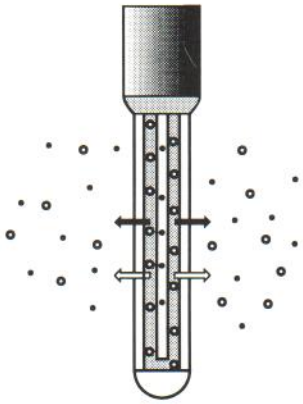

Figure 4 *The concentric microdialysis probe.*

4.7 Microdialysis experiments and instruments

The probe is immersed into an aqueous sample solution *in vitro* or implanted *in vivo* in the tissue of interest using a guide cannula. The inlet tube of the microdialysis probe is connected to a pump, which delivers the perfusion fluid. High technical demands of the pump are required since very low flow and small volumes are essential prerequisites of microdialysis. Various precision pumps have been developed specifically for microdialysis. The dialysate from the outlet tube is collected in individual vials either manually or by an automatic collector. The dialysate is then analyzed.

4.8 Trauma after insertion of a microdialysis probe

Insertion of microdialysis probes, obviously, creates tissue damage and thus, for a time, disrupts the extracelluar environment. This transient state of the tissue surrounding the probe after implantation can change diffusion characteristics and thus alter recovery. It is therefore most important to decide the appropriate time to start the microdialysis experiment, i.e., decided on *an equilibration period.*

The implantation of a microdialysis probe in the brain causes severe disturbance in the tissue metabolism and increase in the extracellular concentration of neurotransmitters and other intracellular neurochemicals, but this can be neglected at 24 hours after implantation (Ungerstedt, 1991). Local cerebral blood flow decreased 50% the first hour after implantation of a horizontal probe into rat hippocampi. In contrast a vertical probe did not result in changes in local blood flow (Benveniste and Hansen, 1991). The most convenient time for using *in vivo* microdialysis in the rat brain is 8-24 h after implantation which, however, not always is practicable (Parsons and Justice, 1994).

Oedema in the brain developed after implantation of a probe (Dykstra et al., 1992). Oedema increases the volume fraction of the extracellular space, thereby increasing the aqueous surface area of the dialysis membrane available for diffusion of drugs towards the membrane, and then recovery increases. The same phenomena was seen in human skin. Recovery of glucose was increased during the implantation trauma phase and after challenged of the skin with histamine which induced a wheal (Petersen et al., 1992a).

Recently the insertion trauma in human skin has been studied. Anderson et al. (1994) observed increased skin blood perfusion, which diminished 15 minutes after insertion, and by 60 minutes the skin perfusion was close to normal. One subject anaesthetized with a local anaesthetic showed a minor reaction than two subjects without local anaesthesia. In another study Anderson et al. (1992) found that the probe (a commercial concentric probe) insertion caused histamine release. It was concluded that 40 minutes is a suitable period of equilibration (time required for histamine levels to return to normal). Petersen et al. (1992a), who uses a serial probe, registered traumatic hyperaemia 90-135 minutes after implantation in human skin. Petersen et al. (1992a) suggested a 90 minutes stabilization period. In contrast Krogstad et al. (1996) did not find complete normalization of skin perfusion in the microdialysis area after insertion of a serial probe.

Histological examination of biopsies from human skin demonstrated small areas of bleeding due to probe insertion. This did not alter the microdialysis results (Krogstad et al., 1996). Histology studies were performed in rat skin after probe implantation. Immediately after probe insertion substantial tissue change were not evident. Acute oedema is difficult to assess in histology sections. After six hours infiltration with lymphocytes was observed, but this did not change the performance of the probe. Elongation of the connective tissue surrounding the probe started 32 hours after implantation. It was concluded that the implantation did not cause significant physical damage to the skin (Ault et al., 1994).

In ovalbumin-sensitized guinea pigs the release of histamine returned to normal endogenous level within 30 minutes (Okahara et al., 1995).

Implantation of a small dialysis tubing does not cause any major oedema or bleeding in the adipose tissue (Lönnroth and Smith, 1990).

For each microdialysis experiment, with different design and substances, the optimal equilibration period must be considered, since the traumatic reaction after implantation of the probe apparently varies with species, tissue, type of probe and possibly probe depth.

4.9 Analysis of microdialysis samples

Another challenge in microdialysis is the chemical analysis of the microdialysis samples. The concentrations in the dialysate are usually low and the volumes of the samples are small. Fortunately, the dialysate samples are free from protein, and there is no need for a sample cleanup, and no enzymatic degradation occurs once the compound has crossed the dialysis membrane. The dialysate can be directly injected into an HPLC. The limits of microdialysis experiments are often set by the sensitivity of the analytical technique. It is possible to compensate for inadequate sensitivity by using long membranes, long sampling times, and a low flow rate. However, a compromise between these experimental variables may also be used. HPLC coupled with electrochemical-, fluorescence-, UV- and conductance detectors or RIA are the most widely used techniques for analysis of microdialysis samples.

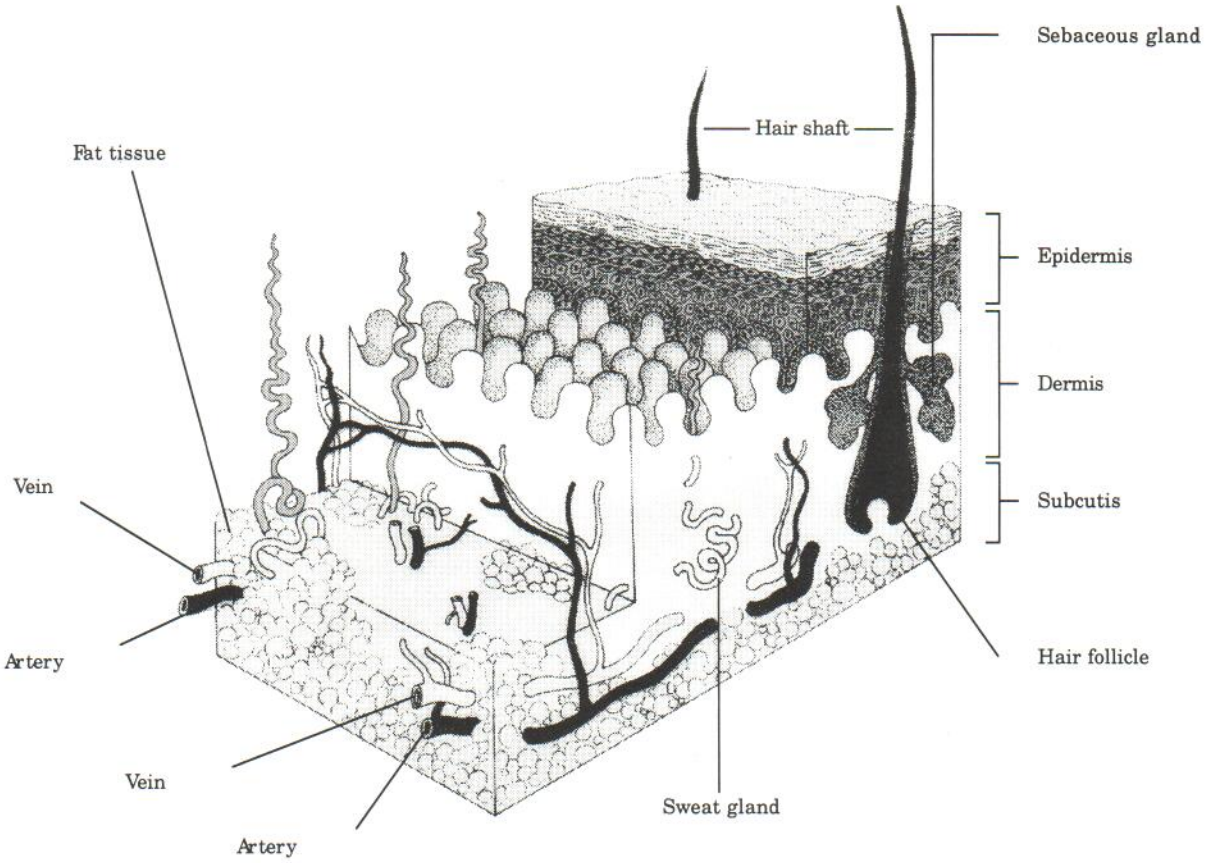

Figure 5 *Schematic section of the skin consisting of the epidermis and dermis with the underlying subcutis, the pilosebaceous unit, the vascular supply and eccrine sweat gland.*

5.0 The skin

5.1 Structure of the skin

The skin is the heaviest and largest single organ of the body. The skin of an average adult possesses a surface area of 2 m^2. Together with the mucosal linings of the respiratory tract, digestive, and urogenital tracts the skin forms a barrier between the internal body and the external environment. The skin consist of two different layers, the epidermis and the underlying dermis, a dense connective tissue which contributes to the principal mass of the skin. Beneath the dermis is the fatty subcutaneous layer (Figure 5).

5.1.1 The epidermis

The thickness of the epidermis varies with cell size and the number of cell layers, depending of the body region. The thickness becomes maximal on the palm and sole and minimal on the eyelids, e.g. ranging from 0.8 mm to 0.06 mm. There are two main parts of epidermis, i.e. the stratum corneum, a dry keratinized layer of dead cells, and the viable stratum germinativum. The epidermis may be further separated into the basal layer, the stratum spinosum, the stratum granulosum, and the overlying stratum corneum as mentioned above (Figure 6).

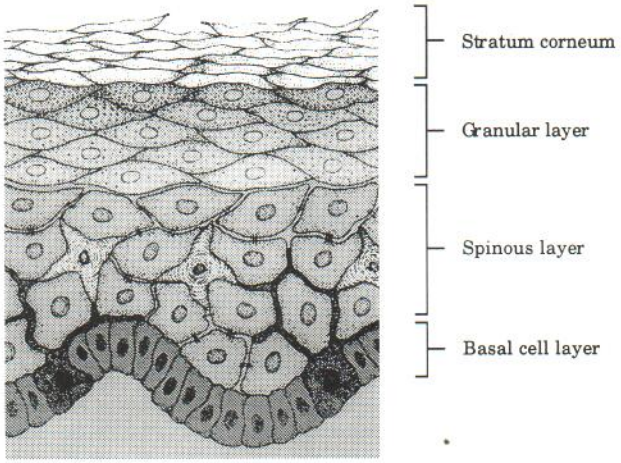

Figure 6 *Enlargement of the epidermis.*

The epidermis is in a constant state of turnover and outward migration. The basal layer is the proliferating layer of keratinocytes. Keratinocytes differentiate and move successively towards the surface to end up in the stratum corneum compartment and finally desquamate invisibly as new keratinocytes are formed in the basal layer. The cells make a journey and trans-

fer from being metabolically active and dividing basal cells to dense, dead keratinized cells at the surface. This journey takes 26 - 42 days in normal skin. Finally the outermost cornified cells desquamate as loose scales. The normal turnover time in the stratum corneum is appraise 14 days.

The keratinocytes are attached one another by special anchoring structures, i.e. desmosomes. The desmosomes alter in structure during the transition from stratum granulosum to stratum corneum, where there are degraded before desquamation.

As the keratinocytes approach the surface they alter morphologically and histochemically. The cells flatten and their nuclei shrink. Early in the differentiation lamellar granules are extruded from the cytoplasm into the intercellular compartment. Their lipid components are rearranged into lipid bilayers between the keratinocytes. This bilayer of intercellular cement is considered of major importance for the stratum corneum barrier function. In the final stage of differentiation the degradation of mitochondria, ribosomes and nuclei occur. The cytoplasm changes into keratohyalin masses and filaments and the plasma membrane form a thick envelope of cornified cells. The stratum corneum has 15-20 layers of flat keratinized dead cells, which are vertically stacked in a highly organized tissue structure. The hexagonally cells lie tangential to the skin surface and interdigitate with each other to form a cohesive lamina only disrupted by glandular openings and hairs. The dry stratum corneum is approximately 10 μm thick, but can be hydrated and swell with up to 75% of its weight.

This horny layer provides an almost impermeable layer which controls the percutaneous absorption of compounds.

Besides the keratinocytes there are three dendritic cell types, e.g. the melanocytes, which produce the pigment melanin, the Lagerhans cells, assumed to be involved in the immune response, and the Merkels cells, which are attached to keratinocytes by desmosomes and located in the vicinity of nerve terminals and therefore believed to be associated with the sensation of touch.

The dermis-epidermis interface is a wavy structure known as the dermoepidermal junction. This junction with its basement membrane gives mechanical support for the epidermis and controls the passage of cells and some large molecules. The dermoepidermal junction is moreover, the interface between diffusional nutrition in the epidermis and vascular nutritional supply in the outer dermis determined by the vasculature organized in a superficial plexus underneath the basement membrane zone.

5.1.2 The dermis

The dermis is a moderately dense connective tissue composed of collagen fibres and elastic fibres embedded in an amorphous ground substance of glycosaminoglycans or GAGs (hyaluronates, chrondroitin-4-sulfate, dermatansulfat), salt and water. The dermis consists of an outer dermis folded in papillae and grooves replicating the lower surface of the epidermis, and a thick underlying reticular dermis. The papillary dermis contains elastic and collagen fibres and fibrils, ground substance and connective tissue cells. The reticular layer provides structural support and therefore has extensive collagen and elastin networks. The principal cells of the dermis are fibroblasts. The interstitial fluid forms a gel. GAG is a continuous water binding network in the concentrations present in the interstitium, e.g. 0.55 g/100 ml (Aukland and Nicholaysen, 1981). Additionally mast cells and macrophages are found in the dermis.

5.1.3 The vasculature

The vascular supply is located in the dermis. A deep dermal plexus lies in the interface to the subcutaneous tissue and sends out branches to the hair follicles and the sweat glands. Other branches ascend toward the skin surface, and a superficial papillary plexus is formed parallel to the skin surface as mentioned above. The vasculature does not progress into the epidermis. The cutaneous circulation regulates body temperature and blood pressure besides its local function with delivery of nutrients to the skin and removal of waste products.

The lymphatics of the dermis serves for central transport of plasma proteins, particules and liquid constitutes of the extracellular compartment of the dermis.

5.1.4 Neural supply

The nerve supply in the skin depict touch, pain, itch, tickle, heat and cold. A motor innervation of the skin by sympathetic fibres of the autonomic nervous system supplies the sweat glands, pilomotor apparatus, the muscle and the microvasculature. These fibres follow the distribution of blood vessels in the dermis.

5.1.5 The subcutaneous tissue

The subcutaneous fat, the hypodermis, is dominated by fat lobules with fibrous connective tissue. Its thickness varies with age, sex and nutritional status of the individual. The main function of this layer is to store lipids and provide a flexible layer between the skin and the underlying muscle and bone. The subcutis works as a thermal barrier and a mechanical cushion.

5.1.6 The skin appendages

Epidermis and dermis contain a number of different appendages.

The eccrine sweat glands are distributed over almost the whole body surface. The glandular part is a secretory coil structure in the lower dermis with a duct leading through the dermis to the surface of the skin. They play a role as part of the thermoregulatory function of the skin. Under normal conditions the more than 3 million glands deliver ½-1 litre of sweat per day to the surface. The secretion is a hypotonic solution with a pH of about 5.

The apocrine sweat glands are found in the axillae, the perianal, urogenital skin and the areola of the breast. Apocrine glands are lager than eccrine glands and extend to the subcutaneous tissue. The sweat produced by apocrine glands is an oily fluid which contains lipids, proteins, lipoproteins and saccharides. Bacteria of the skin metabolize the secretion and produce body odour.

Hairs are keratinous fibres growing out from epithelial surfaces. Hair originate from follicles in the skin. A special pilomotor muscle is attached to the follicle together with the seba-

ceous gland and in some regions also an apocrine gland. This forms the *pilosebaceous unit*. Hair follicles are distributed over the entire skin surface except for the palms, soles and lips. Two types of hair are classified, i.e. terminal hairs, which mainly grows on the scalp and vellus hairs which are fine body hairs that rarely get more than 1 cm long. The growth of hair varies with body region, sex, age and species.

Sebaceous glands vary in size and number depending on the body region. They are numerous on the forehead, face, anogenital surfaces and the midline of the back. The duct of a gland is often associated with a hair follicle. The secretion, sebum is a mixture of lipids, i.e. glycerides, wax esters, squalene, cholesterol and cholesterol esters. This composition varies from species to species. Their function is not quite clear, but it could be controlling of water loss and protection of the skin from infections. Sebaceous glands are involved in comedones and acne.

Nails are plates of hard keratin, a special modification of the stratum corneum. Nails grow continuously, about 0.1 mm per day.

5.1.7 The functions of the skin

The skin has several vital functions. The most important are to keep survival in dry environments and to protect from hazardous external influences. The skin forms a barrier against microorganisms, chemicals, radiation, heat, mechanical trauma and electricity. The nerve supply makes the skin able to communicate with the outside environment and sense touch, pain and heat. Regulation of body temperature is another unique function of the skin. Finally the skin identifies and expresses the characteristics of the individual with respects to skin colour, hair, odour and evoke emotional state like the redness of anger, the pallor of fear and the sweat of anxiety.

5.2 Skin penetration and absorption of substances

Percutaneous absorption is the passage of substances trough the cutaneous layers into the systemic circulation. The absorption is divided into three phases (Schaefer and Jamoulle, 1988). The *penetration phase* is the entry of a substance into the stratum corneum. The *permeation phase* is the penetration through the viable epidermis into the dermis. The *resorption phase* is the final step with uptake of the substance through the vascular system.

Before passing this series of barriers, the drug has to escape from its vehicle and enter the stratum corneum. All the barriers mentioned influence the absorption of a drug. However, stratum corneum is the rate limiting barrier to penetration of most topically applied drugs, especially the water soluble substances. It has been shown that the skin is highly permeable when stratum corneum is artificially removed and the isolated stratum corneum is nearly as impermeable as the entire skin (Monash and Blank, 1958). Furthermore, drug passage through the skin *in vitro* is nearly the same as through in living skin in situ (Feldmann and Maibach, 1974). This indicates that the underlying tissue offers little resistance to drug transport. However, one must not disregard the influence of blood flow and metabolism in the living tissues (see later). Additional evidence for the postulate that stratum corneum possesses the barrier to penetration is found in the inverse penetration model. By applying Desoximetasone® ointment to the lower surface of the dermis and allowing the drug to penetrate inversely into the skin, 75-90% of the applied quantity were found in dermis, whereas only 2.5-20% permeated when the formulation was applied on the stratum corneum surface (Schaefer et al., 1982).

There are three possible routes for penetration in the skin: 1) through the pilosebaceous unit, i.e. hair follicles with their associated sebaceous glands, 2) via the sweat ducts or 3) across the intact stratum corneum (Figure 7) (Barry, 1991a).

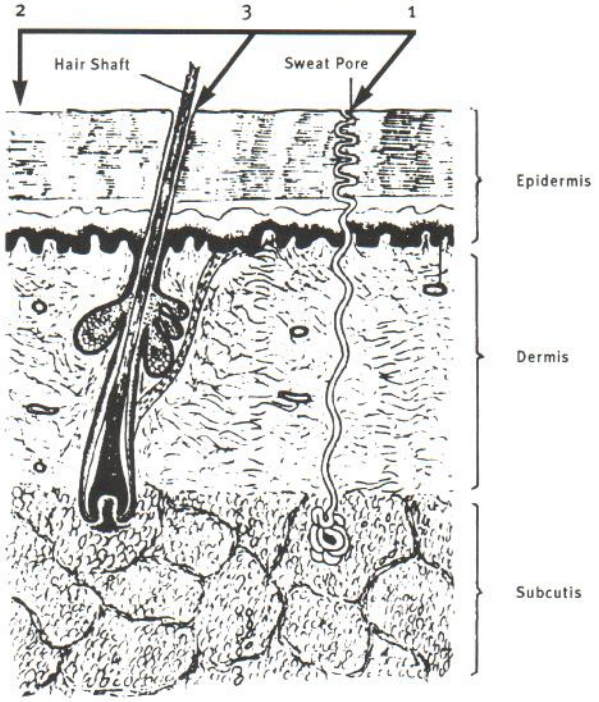

Figure 7 *The three possible routes for penetration in the skin: 1) via sweat ducts; 2) across the intact stratum corneum; 3) or through the hair follicles.*

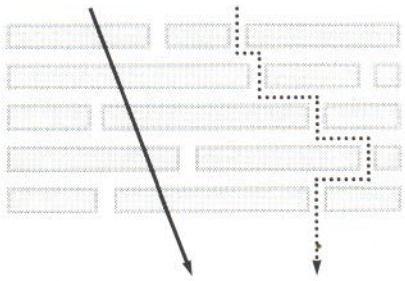

Figure 8 *Schematic section of stratum corneum showing the intracellular and the intercellular pathways for diffusion.*

Penetration via the skin appendages, i.e. the shunt route, is limited by the small area the shunts occupy. Approximately 0.1% is available for absorption variably distributed over the body surface. The permeability of these shunts can neverthe-

less be high for some drugs like ions and large polar molecules (Barry, 1983).

In stratum corneum there are two possible pathways for diffusion, i.e. between or across the cells, corresponding to intercellular and intracellular pathways, respectively (Figure 8).

The stratum corneum is described as a brick-and-mortar model with very flattened keratin filled corneocytes embedded in a lipid cement (Barry, 1991a). The intercellular pathway is along the lamellar lipids between the cells. As the epidermal cells differentiate the extruded lipids change in character from polar to neutral (Lampe et al., 1983). The complex lipid mixture forms bilayers. These bilayers are not solid, but dependent on temperature, the presence of other chemicals and conformation of the chains. The lipid chains can be packed in a variety of ways leading to for instance crystalline structures, gel structures or liquid chain domains which are more or less rigid. Normal components of the intercellular domains are fatty acids, ceramides (sphingolipids, 35-40%), triglycerides, sterols esters, cholesterol, cholesteryl sulphate and alkanes. Besides these different lipids proteins such as enzymes have been found in the bilayer. Proteins may be inserted within the lipid, either extrinsically, intrinsically or both. Gaps in the lamella are observed by electron microscopy. Molecules, typically lipophilic drugs, that are more soluble in the intercellular phase than in the keratinized cells are believed to follow the intercellular route.

The intracellular pathway is an aqueous pore route. There is some evidence that an aqueous pore route exists, since ionic forms would otherwise be excluded from partition into the stratum corneum. Hydrophilic molecules probably diffuse through corneocytes and the presence of a lacunae in the intercellular space would help the passage to the next corneocyte (Barry, 1991b).

Barry (1988; 1991b) studied the structure of the horny layer and phase transition within the epidermal membranes modified by enhancers according to the *lipid protein partitioning theory* using differential scanning calorimetry. The results of such studies are taken as evidence that enhancers may increase the permeation by changing the nature and conformation of the barrier. Many enhancers increase intercellular fluidity due to disordering of lipid packing and thereby facilitate the diffusion of lipid drugs. Other enhancers interact with the polar region of the bilayer and increase the water volume between the lipid layers or interact with the intracellular protein and their associated water forming pore routes and so promote the diffusion of polar drugs. Drug absorption is promoted by any enhancer which increase the water content, since skin hydration decreases the diffusional resistance.

Magee (1995) describes a protein domain which is a tandem pathway for polar and moderately lipophilic compounds and a lipid domain for more lipophilic compounds. In the protein domain there is free access of water and other polar substances, despite the lipid barrier. Moreover, the protein domain can absorb water until the skin is fully hydrated. The cellular material of the protein domain contains organized keratin filaments which possess regions for nonspecific binding of polar drugs like hydrogen binding.

Other authors state that there is no critical need for an aqueous pore route (Potts et al., 1991). They supposes that the permeation of hydrophilic and lipophilic compounds is due to free-volume fluctuations in the lipid alkyl chains. This model neglects the idea of pore pathways since the intercellular lipid domain can account for the permeation of a wide range of hydrophilic/lipophilic compounds. The lipids of the stratum corneum alone can account adequately for all the permeability properties. This is in agreement with the statement that the degree of percutaneous penetration in various body areas is inversely related to the percentage of lipids (w/w) in the respective stratum corneum tissues (Elias et al., 1981). The removal of the lipid components of the cutaneous barrier by extraction with non polar organic solvents results in perturbation of the barrier function with increase in transepidermal water loss (Menzel, 1995). This perturbation stimulates biosynthetic processes with accelerated formation of lipids and DNA (Proksch et al., 1993). The delipidization has a more pronounced effect on the percutaneous penetration of substances of low lipophilicity as compared with highly lipophilic chemicals (Menzel, 1995).

Forslind (1994) has formulated a Domain mosaic model for the skin barrier. The intercellular stratum corneum lipids are expected to be in a crystalline state surrounded by domains of lipids in liquid crystalline state, i.e. lipid units are allowed to diffuse freely in the plane of the bilayer. Transport over the barrier bilayer only occurs in the liquid crystalline state regions. Transcellular transport seems unlikely or minimal. Structural changes in the bilayer create pores resulting in aqueous or lipid pathways or both. Water soluble molecules will diffuse horizontally in the aqueous compartment of the bilayer until they find a pore where they can cross the bilayer. The domain mosaic model provides an explanation of a watertight enclosure with control of hydration of the corneocytes, restricted penetration to the extracellular compartment and to some degree mechanical resistance (Forslind, 1996). Despite the debate on whether there are one common or two distinct pathways for penetrating the stratum corneum, one for lipid compounds and one for hydrophilic compounds, it is generally believed that the intercellular route is the major absorption pathway for lipophilic drugs.

A very hydrophobic drug will move easily into the stratum corneum and pass the horny layer. At the interface with the more aqueous epidermis in which it is poorly soluble, the partition into the viable tissue drops resulting in decreased flux. In this case the rate-limiting step becomes the viable tissue instead of the stratum corneum. In contrast, a very hydrophillic drug can hardly move into the stratum corneum. Topical drugs need to have a balanced hydrophobic/hydrophilic character (Washington and Washington, 1989).

Topically applied drugs may form a reservoir or a depot within the stratum corneum. Especially steroids are able to accumulate in the horny layer. The reservoir effect was observed when blanching from steroids could be demonstrated 7-10 days after topical administration by reoccluding the test site (Rieger, 1993). Substances forming reservoirs are characterized by restricted ability to diffuse in stratum corneum, an octa-

nol/water partition coefficient larger than 1 and a high affinity for protein (Rieger, 1993). Griseofulvin, sodium fusidate and fusidic acid form depots in a way comparable to steroids (Barry, 1983, Vickers, 1969).

Penetration is a passive process and the transport into the skin follows the laws of diffusion. Based on Fick's first and second laws and the assumption that skin is a composite membrane, a law for diffusion at steady-state can be elaborated (Schaefer and Jamoulle, 1988) :

$$J = \frac{K_m D C}{d} = k_p \Delta C \qquad (4)$$

$$k_p = \frac{K_m D}{d}$$

J is the flux of the drug (quantity of drug absorbed per unit of area and unit of time), K_m is the partition coefficient between the skin membrane and the vehicle, D is the diffusion constant, C is the drug concentration in the vehicle considered constant, Kp is the permeability coefficient and d the thickness of the skin membrane. D is the rate of migration through the skin membrane, which is dependent on the characteristics of the drug and the medium. The partition coefficient reflects the solubility characteristics of a substance relative to its ability to penetrate the skin, since stratum corneum exhibits the characteristics of a lipophilic structure. If for instance K_m is high the drug will accumulate in the stratum corneum.

This is an oversimplification and the heterogenous multilayer tissue structure of the skin is obviously a very complex barrier. Each layer, stratum corneum, epidermis and dermis contribute to diffusional resistance as barriers in series. On the other hand diffusion through different pathways like shunts and follicular pores are parallel. A resistant phase with a much greater resistance than the other layers and routes determines the barrier properties. (Barry, 1991a)

5.3 Factors affecting skin penetration and absorbtion of substances

When a topical formulation is applied to the skin three main components are involved: the drug, the vehicle and the skin. The drug has to be released from the formulation and penetrate through the skin barriers to exert the desired pharmacological effect on the receptors within the skin. These processes are very complex as stated above and can be influenced by several biological factors and physico-chemical factors resulting in variations in skin penetration and therapeutic efficacy.

5.3.1 Biological factors

Essentially, the skin functions as a barrier only if the stratum corneum is intact. Removal of the stratum corneum by adhesive tape stripping or alteration in the structure of the epidermis due to skin diseases or chemical exposure may enhance penetration into the skin (Moon and Maibach, 1991; Wester and Maibach, 1992). There are regional variations in percutaneous absorption parallel to the varied thickness of the stratum corneum and the number of skin appendages (Rougier et al., 1989; Wester and Maibach, 1989). Dermal blood supply plays an important role in clearance of topically applied compounds (Roberts, 1991). Decreased cutaneous blood flow may influence the drug absorption by decreasing the absorption into the capillaries and thereby deposit the drug in situ. Thermoregulation of the skin can affect the blood flow and in this way influence absorption (Riviere, 1993). Increased skin temperature also increases the penetration rate because of increase of the diffusion coefficient.

At the surface, skin is least moist (10-25%), but the lower epidermis contains up to 70% water. The pH is 4.5 (men) and 5.3 (women) at the surface. The pH gradually increases in the stratum corneum to the lower epidermis, i.e. 6.9 (men) and 6.8 (women) (öhman and Valhquist, 1994). Drugs may alter charge or stability during diffusion into the skin because of these pH alterations.

Skin metabolism can give a major contribution to variation in skin penetration. Unstable drugs may be biotransformed as they pass the enzymes of the viable epidermis and dermis (Williams, 1991). Skin metabolism may reduce the pharmacological response and transform the drug into inactive metabolites. However, the reverse can also occur, a concept used in the *prodrug approach*. The skin possesses many of the same enzymes as the liver. The viable epidermis has the highest activity. It is, since the cutaneous metabolic activity is high, possible that a first pass metabolic effect can occur in the skin with chemical change of the topical drug applied. If drugs pass the skin slowly the metabolism may substantially reduce the amount of active drug. On the other hand fast penetrating drugs may saturate the enzymes and the degree of metabolism is then minimal (Noonan and Wester, 1989). Skin metabolism may or may not be relatively important depending on the drug. Transdermal delivery is often used to avoid the first-pass metabolism by the liver, but this is only rational if cutaneous first-pass effect is insignificant. Variation of biotransformation activity within and between species and in normal and pathological skin can influence the outcome of drug penetration studies. Different species have different skin penetration of the same drug. Laboratory animals have more hair follicles, may lack sweat glands and may differ in thickness of the epidermis, which obviously can affect the route and resistance to penetration. Some considerations must be taken into account when choosing an animal as a pharmacological model in dermatological research. Humans and minipigs have many similarities with respect to microscopic skin structure, whereas rabbit and mice resembles each other, but are different from man. The rat is different from the other rodents and has a markedly developed stratum granulosum suggesting a different epidermal differentiation in the rat (Bouclier et al., 1990). Selection of an acceptable and relevant species should implicate comparable skin permeabilities and the ability to produce a given pharmacological response which is similar to that obtained in human skin (Bouclier et al., 1990). Pig skin seems to be the best choice since it has biochemical, physiological and morpho-

logical similarities with human skin. However, the size of the pig makes it less attractive to work with. Parameters like strain, age, sex and reproductive cycle can also influence the structure of the epidermis, affect hair growth and density of hair follicles. The hairless species are often recommended because shaving can damage the stratum corneum leading to permeability variation. *In vitro* rabbit, mouse, rat and guinea pig skin are more permeable than human skin. Skin of pig and monkey approximate the properties of human skin the best.
Variation in the skin among races of man has been reported. It seems that negro skin has a greater density and more cell layers than caucasian skin (Barry, 1983). The parameters age and sex have not been investigated much. Without much evidence it is usually assumed that the skin of the fetus, the young and the elderly is more permeable than skin of adults.

5.3.2 Physico-chemical factors
Physico-chemical properties may influence drug penetration in the skin due to interactions between drug and skin, drug and vehicle and skin and vehicle.

Drug and skin interactions
Hydration of the skin is a major factor affecting the rate of absorbtion. When skin absorbs water the tissue swells and softens and the permeability of the skin increases probably due to an increase in diffusitivity of the penetrating molecule. Hydration of the skin is normally achieved by using an occlusive dressing over the topically applied formulation, which prevents the spontaneous surface evaporation of endogenous water. Moisturizers like urea also modifies the hydration state of the horny layer and can be administrated together with the drug (Feldmann and Maibach, 1974). The hydration effect is usually more important for nonpolar drugs than for polar drugs (Behl et al., 1980).
Drugs may adsorb to the stratum corneum membrane. The binding to proteins in the skin can range from weak to strong chemical binding. Bound drug acts as a local reservoir controlled by the equilibration between the bound and the free fraction of the drug.

Vehicle and skin interactions
Vehicles applied on the skin such as creams, solutions, ointments, gels and powders can change the skin permeability. Some vehicles can either increase or decrease the humidity of the stratum corneum. Ointments may be occlusive. The limited permeability of the skin often makes it necessary to improve or enhance the permeation properties of the drug of interest. A number of chemicals have been applied to the skin in order to temporarily open the barrier of the skin. These are named *enhancers* and should be pharmacologically inactive and non toxic, and their effect on the barrier reversible. They increase the permeability of the stratum corneum either in the lipid or the keratinized protein regions or modify the thermodynamic properties of the drug. Most enhancers change the lipid structure in the stratum corneum and increase fluidity in the lipid phase. This group include DMSO (dimethyl sulphoxide), azone and dimethylformamide. Propylene glycol and ethanol assist other enhancers to enter the skin as solubilizing agents or function as cosolvents to produce saturated solutions and thereby maximize the thermodynamic activity of the penetrant. The surfactants may alter the physical state of water in the skin and affect the keratinized protein regions in the stratum corneum. These compounds promote penetration of hydrophilic drugs. The most powerful surfactants, such as sodium dodecyl sulphate and the laurate ion, interfere with the keratin protein and decrease the number of charged groups on the surface of the helix available to interact and slow down the diffusion of hydrophilic drugs. Many enhancers have the drawback to provoke irritant reactions because of their quality to alter the barrier. More information is found in Smith and Maibach (1995). Iontophoresis (Behl et al., 1989) and electroporation (Prausnitz et al., 1993) are physical approaches to enhance drug penetration

Drug and vehicle interactions
It is the thermodynamic activity of the drug which determines the biological activity. The maximum thermodynamic activity is obtained in a saturated solution. If the solubility of the drug is increased in the vehicle by a cosolvent then the partition coefficient towards the skin is reduced. On the other hand a higher concentration of the drug in the vehicle is an advantage, since the concentration gradient is increased with increase of flux. Only compounds in the solute state penetrate the stratum corneum. The solubility of the drug in a vehicle determines the concentration for the diffusional processes. Suspended drug particles do not increase the penetration rate. The vehicles only affect penetration if the release rate of the drug from the vehicle is the rate-limited step. This may occur in the clinic if the horny layer is damaged or absent. The release from different vehicles is expressed mathematically by Higuchi (1960) which can be used to optimize the vehicle composition. Interaction between drugs and adjuvants in the vehicle may modify the release rate.
An important issue which was only little investigated is the question if the composition of the vehicle remains constant after application to the skin. Alterations in the formulation will occur as the patient rubs the vehicle onto the skin. Surface materials from diseased skin may mix with the vehicle, and components of the vehicle can evaporate after application. These events alter the physico-chemical milieu in the vehicle and may influence the penetration rate.
The diffusion constant of the drug in the skin is determined by the molecular size, shape and charge, the binding capacity to the tissue and the lipophilicity of the drug. The partition coefficient will be determined both by the drug and the vehicle. The partition coefficient should not favour neither the lipid nor the aqueous phase.
In most situations the horny layer is the rate limiting step in the penetration process, and drug-vehicle-skin interaction determines the penetration rate.

6.0 Cutaneous Microdialysis

In 1958 the first attempt to investigate the extracelluar fluid in the skin was made. In order to measure bradykinin Fox and Hilton (1958) subcutaneously inserted two parallel needles with holes drilled along the shaft. Later Søndergaard and Greaves (1971) tried to measure histamine during cutaneous inflammatory responses using a similar technique. However, in 1991 a major progress was made when an improved microdialysis probe was inserted in human skin to study ethanol absorption (Anderson et al., 1991). Since then a number of studies where microdialysis is used in dermatological research have been performed. During the same period more sensitive analytical techniques have been developed as a basis for advanced study of the skin including mediators and drug penetration. A range of techniques for the study of skin penetration other than microdialysis have been developed. These will be described in the later session.

6.1 Insertion of the microdialysis probe in the skin

The microdialysis probe is inserted in the skin using a guide cannula. The guide is inserted horizontally in the dermis, and the probe is thereafter pushed through the guide which is then withdrawn (Figure 9).

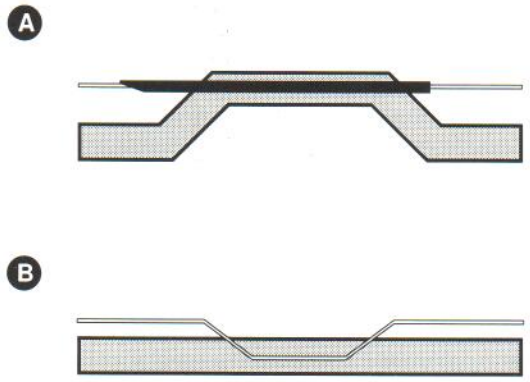

Figure 9 *Insertion of the microdialysis probe in the skin. A) Insertion of the guide cannula in the skin with the microdialysis membrane inside the cannula. B) After withdrawal of the cannula, the microdialysis membrane is placed inside the dermis. Thereafter afferent and efferent tubings are glued to the membrane forming a microdialysis probe.*

With a single lumen probe entry and exit punctures are made in contrast to the concentric probe which only needs one puncture. The size of the guide depends on the microdialysis probe. The insertion of the guide is not painless due to the neural supply in the dermis. An anaesthetic creme (EMLA®, Astra, Sweden) or lidocaine injection is used to avoid discomfort during insertion. It is important to evaluate if the anaesthetic interferes with the experiment. As described above the insertion results in a minor trauma with increase in skin perfusion and histamine release. In some papers it has been reported that the insertion caused no or slight pain and was well tolerated, even without anaesthesia (Anderson et al., 1994).

6.2 Endogenous substances in the skin

Until now most of the published papers on cutaneous microdialysis are dealing with endogenous substances in the skin and especially the inflammatory mediator histamine. Histamine levels have been widely investigated in normal skin and skin challenged with allergens or nonimmunological stimulation. Petersen et al. studied histamine release induced by substance-P (Petersen et al., 1994), codeine (Petersen et al., 1995) and Timothy grass pollen (Petersen at al., 1996) by a single lumen microdialysis probe. These authors studied the dose-response relationship and the clinical observation of wheal and flare. A comparison of histamine release caused by codeine given by intraprobe delivery and intradermal injection was performed (Petersen et al., 1995). Furthermore histamine elimination was studied. Similar studies with the commercial concentric probe were performed in 10 atopic patients (Horsmanheimo et al., 1996), in healthy volunteers (Huttunen et al., 1996) and in ovalbumin-sensitized guinea pigs (Okahara et al., 1995). Anderson et al. (1992) used the concentric microdialysis probe in human skin and made provocation with compound 48/80. Based on histamine release they concluded that 40 min was a suitable period of equilibration. Andersson et al. (1996) showed that histamine release measured by microdialysis was not dependent on the probe depth as studied in atopic and non-topic subjects. The same group (Andersson et al., 1995b) used patients with cold induced urticaria to follow histamine release. Provocation was made with ice. Simultaneously they used laser Doppler perfusion imaging to monitor skin perfusion response and found that cutaneous microdialysis and Laser Doppler imaging offer new possibilities for the chronological multi parameterassesment of inflammatory skin disorders in vivo.

Cytokine IL-6 was dialysed in nickel and SLS reactions with a new dialysis membrane, which is permeable to molecules up to 100 KDa (Anderson et al., 1995). Higher levels than those previously reported in peripheral blood in healthy subjects were found.

Prostaglandin E_2, a mediator in the inflammatory processes, was followed after dermal application of croton oil and SLS to rabbit ear (Agut et al., 1994). Microdialysis of 5-S-Cysteinyl-dopa a tumor marker, in human melanoma, was performed to study pigment synthesis (Kågedal, 1991). Glucose was measured in intact human skin to evaluate the microdialysis technique and estimate the glucose concentration in the skin (Petersen et al., 1992a). A highly significant correlation between skin glucose concentrations and plasma glucose concentrations during steadystate was found. Dermal interstitial pyruvate and lactate were measured using the calibration method (Krogstad et al., 1996). In the same study the retrodialysis method was used. This allowed accurate recordings of dermal glucose concentrations.

The general conclusion from the described papers is that the microdialysis technique is a promising method which opens

new possibilities for quantitative and chronological studies of inflammatory mediators and other endogenous substances in intact and diseased human skin. It offers the opportunity to monitor biochemical events in parallel with clinical observations, either by the naked eye or non-invasive methods.

6.3 Drugs in the skin after application of TTS

Transdermal devices deliver drugs through the skin into the systemic circulation. In two studies of human skin nicotine levels were measured after application of a nicotine delivery system using a commercial probe (Müller et al., 1995c; Hegemann et al., 1995). In both studies it was concluded that it is feasible to study drug kinetics by microdialysis after transdermal delivery of nicotine, since high dialysate concentrations were measured. Nicotine was detected in the dialysate approximately 60 min after application of the patch, and concentrations thereafter increased and reached a steadystate level. The Authors also reported on inter-individual and intra-individual variations of the steadystate concentrations. Müller et al. (1995c) stated that the intra-individual variation was due to probe depth variations and found a linear relationship between the probe depth and log AUC (r=0.90). After application of an estradiol delivery system Müller et al. (1995c) did not detect any estradiol in the dialysate in eight individuals. Even with 7% albumin added to the perfusate only 2% estradiol was recovered *in vitro*. Estradiol is a highly lipophilic compound with a low water solubility as compared to nicotine. Müller et al. (1995c) concluded that microdialysis is limited to the study of hydrophilic drugs.
Thus, it is possible to study drugs in the skin by cutaneous microdialysis after transdermal delivery.

6.4 Drugs in the skin after topical application

Topical application relies on the local penetration of drugs into the skin in therapeutically active concentrations with a minimum of systemic absorption. Obviously microdialysis is a qualified tool for the study of drug levels in the dermis after penetration and percutaneous absorption. Anderson et al. (1991) have tested the potential of microdialysis to study ethanol absorption across human skin and shown that microdialysis can be used in human skin to study transcutaneous absorption kinetics. Andersson et al. (1995a) used microdialysis to demonstrate the absorption kinetics of the two hydrophilic solvents ethanol and isopropanol in human volunteers. Either ethanol or isopropanol were filled in a glass reservoir and applied to the skin. Ethanol and isopropanol were detected in the dialysate within 20 minutes after application. Maximum levels were reached at 100-105 minutes. Recoveries for ethanol and isopropanol were similar *in vitro*, however *in vivo* isopropanol recovery was lower than that of ethanol. Thus, there must be some difference in diffusion and clearance characteristic between ethanol and isopropanol. Most experiments were performed with ethanol and a conspicuous variation in the level of absorption was shown. The probe depth, measured by ultrasound, was one major variable. The more superficially the probe was inserted, the higher the ethanol maximum. Other variables might be the blood flow, the dermal metabolism of ethanol and variation in barrier function among humans.
The anaesthetic cream EMLA which is a eutectic mixture of lidocaine and prilocaine, was investigated by microdialysis. The active agents were detectable 60 minutes after application and increased in concentration the following 60 minutes (Andersson, 1995).
Matsuyama et al. (1994a) could detect the lipophilic drug valproate *in vitro* with a recovery of 80%. They suggested that there was no interaction between the drug and the membrane. A marked increase in transdermal absorption of valproate formulated with 3% HPE-101, a penetration enhancer, as compared to the absorption without enhancer was observed in abdominal skin of the rat. Penetration enhancement by HPE-101 was also found for methotrexate in rats. Three percent HPE-101 increased dermal methotrexate permeability and raised the concentrations in the skin (Matsuyama et al., 1994b). In both studies commercial probes were perfused with a flow of 1 µl/min resulting in few sampling points. Matsuyama et al. (1994b) concluded that microdialysis is useful for assessing *in vivo* transdermal drug absorption.
Ault et al. (1992) compared the *in vitro* penetration method of Franz and microdialysis to study dermal drug transport. A single-lumen probe was implanted in a piece of excised rat skin. The skin was then mounted in the diffusion cell, centering the probe. A cream containing 5-fluorouracil (Efudex®) was applied to the skin. Samples from the receptor phase and the dialysate were measured. In the dialysate a rapid increase in drug concentration was measured followed by a steadystate. In the receptor phase an initial lag time was observed followed by an increase in concentration. Increase in receptor drug concentration was seen after insertion of a guide, a probe (guide + probe) and after puncturing of the skin. The guide cannula promotes the flux of drug into the receptor phase of the Franz diffusion cell. This effect can be diminished by a reduction in the dimensions of the cannula. Besides this effect the microdialysis provides reasonable time concentration profiles in a Franz diffusion experiment. Later Ault et al. (1994) used a linear microdialysis probe, and this probe did not affect the flux. The same study included *in vivo* microdialysis in rats following topical application of Efudex® cream. A steadystate level was monitored in the skin *in vivo*, although larger variations and a 40-fold lower level was found as compared to the Franz diffusion cell study. This is supposed to be due to clearance of the drug because of vascularisation.

6.5 Drugs in the skin after systemic administration

Oral or other systemic route of administration may be required for treatment of skin diseases, when local application is ineffective. Simultaneous sampling from the skin and the jugular vein by microdialysis was performed in a rat following intraperitoneal administration of lomefloxacin. In this way the relationship between the plasma concentration and the skin concentration was established (Dicken et al., 1993).

6.6 Transcutaneous microdialysis

A transcutaneous microdialysis probe applicable to the skin surface was developed for continuous on-line monitoring of glucose and ethanol in adult volunteers (Boer et al., 1993) and glucose in newborn infants (Boer et al., 1994). The probe is a 42 °C thermoregulated element with a cellulose membrane, which is perfused with physiological saline. Before attaching the probe to the skin surface the stratum corneum was partially removed by tape stripping to impair the barrier function of the skin (Figure 10).

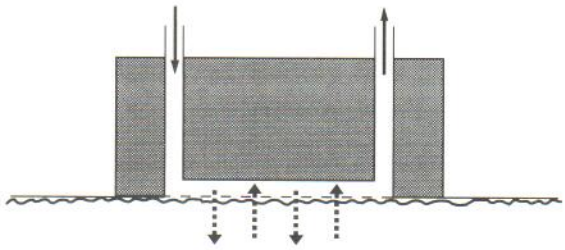

Figure 10 *The transcutaneous microdialysis probe.*

Subjects were given either glucose or ethanol and blood concentrations were measured simultaneously with performing transcutaneous microdialysis. The *in vivo* recovery of glucose and ethanol was defined as the dialysate concentration expressed as a percentage of the blood concentration. Dialysate and blood concentrations of both ethanol and glucose correlated linearly. However, the dialysate to blood ratio varied among subjects. It was not possible directly to compare the dialysate concentration with the blood level without any calibration. This makes the method difficult to use as a routine. The variation in *in vivo* recovery was large. This might be due to the skin stripping procedure and variations in effective contact area of the probe. The transcutaneous microdialysis system has the advantage of not being invasive. But slow diffusion through the stratum corneum results in very low recoveries and highly sensitive analytical techniques are therefore necessary.

6.7 Probe depth influence on dialysate levels

In several of the papers mentioned above variations in dialysate levels were observed. The variation was due to variations in probe depth (Müller et al., 1995c; Hegemann et al., 1995; Andersson et al., 1995a). It is preferable to insert the probe as superficial as possible in the dermis. Practical experience in insertion of the probe would be expected to reduce variation in the probe depth, but the exact position in the dermis cannot be fully controlled by the manual probe insertion procedure. Therefore it is important to measure the probe depth ideally in every cutaneous microdialysis study. The probe depth can quite easily be measured by ultrasound examination (Serup et al., 1995). An ultima goal is to be able to correct for differences in probe depth in the interpretation of results (Andersson et al., 1995a). Then the relationship between drug levels and probe depth must be consistent and known. Histamine values were shown to be independent of probe depth, however, large inter-individual and intra-individual variations in histamine release may conceal the relation to probe depth (Andersson et al., 1996). Probe depth was linearly correlated with interstitial lactate and puryvate. Concentrations in the dermis may indicate a difference in production or passive diffusion of the substances from the non-vascularized epidermal region (Kogstadt et al., 1996).

In conclusion, cutaneous microdialysis can provide continuous *in vivo* dermal levels of endogenous substances as well as exogenous compounds. The successful experiments described above listed papers that involved either hydrophillic drugs or small compounds. Valproate was used as a lipophilic model drug for transdermal absorption (Matsuyama et al., 1994a). This is rather misleading, however, valporate is ionized at the pH of the perfusate and therefore behaves as a hydrophilic compound. Müller et al. (1995c) found that estradiol, a highly lipophilic compound, was non-dialysable, even *in vitro* with albumin in the perfusate. This indicates that dialysis of lipophilic drugs may require some alternatives with regard to perfusate, membrane materials and tubings. One major problem with lipophilic compounds is that they adsorb to membrane and tubings.

Within these limits cutaneous microdialysis can be a powerful method in dermatological research and cutaneous pharmacology.

7.0 Other techniques for the study of cutaneous penetration and percutaneous absorption

7.1 *In vitro* methods

Diffusion cells are commonly used *in vitro* methods. According to guidelines of the Food and Drug Adminstration (FDA) (Skelly et al., 1987) *in vitro* permeation studies can be used for documentation and as a quality control procedure to study the release and permeation rate during product development, assuring lot to lot bioavailability equivalence of topical formulations and to enable minor reformulations of already existing topical formulations. In addition *in vitro* methods may be the only possible way to investigate permeation for highly toxic compounds. Excised skin samples are mounted in glass diffusion chambers. Drug is applied on the stratum corneum side, i.e. the donor side. In the chamber beneath the skin, i.e. the receptor side, the receptor fluid is installed. Receptor fluid is withdrawn at regular intervals and analyzed, and the rate of permeation is determined. A commonly used type of diffusion cell is the one-chamber cell described by Franz (Franz, 1975). Provided the rate-limiting barrier of percutaneous absorption is the dead horny layer, the *in vitro* methods should correlate reasonably well with *in vivo* studies. However, the excised tissue is not a living tissue with respect to blood supply and metabolism.

Using membranes such as the cellulose acetate membrane, which does not constitute a rate-limiting barrier, the release of drug from the vehicle can be studied. When excised skin is used, the penetration and/or the percutaneous absorption may be studied. Human skin is the preferred membrane, but this can be difficult to obtain. Substitutes like for instance polydimethylsiloxane or animal skin have been used. It must be taken into account that differences in structure between human and animal skin may result in different permeabilities. The treatment of the excised skin during and after harvesting and the skin storage conditions before use can be critical, since the barrier properties can be impaired under these processes. Bronaugh (1989) has measured tritium permeability constants to verify the integrity of the barrier.

Full-thickness skin or sections of skin made by a dermatome can be used.

There are two modes of application of drugs on the stratum corneum, the finite dose and the infinite dose methods Franz (1978). In the *finite dose method* a small volume of drug is applied onto the skin. This dose will mimic clinical conditions the most. In the *infinite dose method* a large and excessive dose is applied onto the skin. The amount of drug that penetrates into the skin is negligible compared to the dose and a constant donor concentration is maintained. With sink conditions in the receptor phase a constant flux should be obtained. The flux of the drug can be calculated under these steadystate conditions.

An important issue is that the barrier of human skin is variable and therefore several experiments should be performed for each formulation (Skelly et al., 1987). *In vitro* experiments can be done using animal skin, but great variability is possible among species and results may not apply to human skin and may thus be different from compound to compound (Bronaugh et al., 1982).

7.2 *In vivo* methods

7.2.1 Skin blisters

Suction blisters are located subepidermally and the blister fluid can be taken as representative for the interstitial fluid containing proteins and lipids. The blisters can be raised by mild suction using a special dome-shaped Dermovac® cap with several small holes, each 6 mm in diameter (Kiistala et al., 1968) or a plane block with holes of similar diameter (Hellum et al., 1978). A typical blister is formed after 2-3 h suction with a vacuum of 200 mmHg. Normally the blister contains 50-150 µl fluid. The concentration of electrolytes in suction blister fluid is essentially the same as in serum. The albumin concentration is 30-42% of the respective serum value in humans and 23-31% in blisters from rats using the Dermovac®. Blisters induced by the plane block contain 60-70% albumin of the corresponding serum value (Schäfer-Korting and Korting, 1989). The blister fluid is harvested by puncture of the blister roof with a thin needle and syringe. In addition to the collection of blister fluid the drug concentration can be measured in the blister roof (epidermis) and the base (dermis). The technique is difficult to use in diseased skin, since the blister cannot be raised properly or ruptures.

For pharmacokinetic studies, 10 or more blisters are formed. Drugs are applied topically or systemically before, during or after the blisters are raised. The blister fluid is collected at various times for further analysis.

Until now the influence on drug penetration into blister fluid of protein bound drugs is uncertain. It is suggested that drug transfer into the blister is not impaired by protein binding, but the transfer may be delayed. The hydrophobicity of the drug may also influence the rate of penetration into the blister. The suction blister method has been used to investigate drug levels in skin blister fluid and serum after oral fusidic acid administration, 250 or 500 mg twice daily (Vaillant et al., 1992). The suction blister concentration of fusidic acid was 21 ± 5 and 79.2 ± 11.2 mg/l after the 250 and 500 mg doses, respectively. The blister fluid concentration was lower than the serum levels and the blister fluid peaked 2-10 h later than the serum did. Thus, diffusion of fusidic acid is slow, but it is not prevented by the high protein binding of fusidic acid (Vaillant et al., 1992). Besides pharmacokinetic studies the suction blister technique has also been used for pharmacodynamic studies. Photobiological activity and bioavillability were evaluated after oral administration of 8-methoxypsoralen and topical application of 5-methoxypsoralen (Averbeck et al., 1989). Skin blisters were also raised to assess the relationship between skin glucose and blood glucose in diabetic patients (Jensen at al., 1995). The blister glucose concentration paralleled plasma glucose, although the glucose concentration in blisters was slightly lower than the plasma concentration. A noticeable difference in skin blister suction time was seen among the patients probably due to differences in skin thickness or mechanical resistance of the epidermal-dermal junction (Jensen et al., 1995). An alternative blister technique is the cantharidin blister method. An intraepidermally located blister is produced after skin exposure to cantharidin, a substance from the Spanish fly. The blisters can only be developed in man. The can-

tharidin blister fluid is an inflammatory exudate which contains 650-12,700 white cells/μl. The albumin concentration exceeds that of suction blister fluid, i.e. 70-80% of the respective serum level (Surber, 1995). The drug transfer into suction blister fluid seems more rapid as compared to cantharidin induced blisters (Schäfer-Korting and Korting, 1989).

7.2.2 Tape stripping.

When topical formulations are applied to the skin, some of the drug penetrates into the stratum corneum. For a period the drug will stay in the stratum corneum, depending on lipophilicity and diffusion coefficient among others, before further diffusion into the viable epidermis takes place. It is possible to remove the stratum corneum before the drug diffuses to the lower layer and thereby estimate the absorption. By repeated application of adhesive tape to the skin surface, the stratum corneum can be successively removed. This *tape stripping method* is used for evaluation of the structure of the stratum corneum, evaluation of the dynamic of the stratum corneum renewal (scaliness), *in vivo* evaluation of efficacy of antimicrobials and determination of endogenous and exogenous chemical components of the barrier. Drug concentrations can be measured in the stratum corneum after topical application as well as systemic administration under *in vitro* and *in vivo* conditions. When measuring the drug amount in a single tape, each concentration profile within the horny layer is obtained. The stripping of the superficial part of the stratum corneum can be performed by sticky slides, adhesive tape and adhesive-coated discs like the D-Squame® (Miller DL, 1995). Cyanoacrylate skin surface stripping or biopsy is an alternative to the tapes (Piérard and Piérard-Franchimont, 1996). The time of application, hydration state and scaliness of the skin, the tape used and dirt on the adhesive surface will influence the amount of horny layer removed. Each strip removes a different amount of skin. The first contains the largest amount because squamous cells are loosely packed. To determine drug concentration of a penetrant it is necessary to quantify the amount of removed stratum corneum, which is normally done by weighing, before and after stripping.

After the strips are peeled off the skin, the compound of interest is extracted from the tape-strips by an organic solvent and analyzed by a suitable method.

The method depends on a correlation between short-time uptake by stratum corneum and total percutaneous absorption, which only has been established for some drugs and formulations. Rougiers and Lotte (1993) have found a linear relationship between the stratum corneum reservoir 30 minutes after application of a test drug and *in vivo* percutaneous absorption using the standard urinary excretion method. They investigated the influence of applied dose, vehicle and anatomical site on the absorption and found relationship to the drug content in the tape-strips. Pershing et al. (1992) studied the bioavailability of five commercial betamethasone dipropionate formulations in human skin using visual skin blanching, chromameter measurements and tape-stripping. A similar rank order of potency of the formulations was found for the visual score, the a* scale of the chromameter and tape strips concentrations. The rank correlation between the tape strip concentrations and the visual skin blanching assay was moderate, maybe because of inter-subject variability of more than 70%. Drug metabolites of cytosol were present in stratum corneum and was removed by tape stripping (Wiechers et al., 1989). Biotransformation occurs in the viable epidermis and dermis only. Occurrence of the metabolites in the stratum corneum is explained by outward migration of the metabolites formed in the viable epidermis and dermis. The majority of metabolites are expected to move inwards and the stripping technique therefore underestimates metabolism.

Unlike blood sampling that can be collected over time after a dose, only one stratum corneum sample can be obtained from one site of application. Multiple sites of application are therefore required to perform pharmacokinetic studies, which will increase the variability. Stratum corneum concentrations according to tape stripping and relationship to clinical activity is an open question.

The skin stripping technique can be used to imitate a disturbed or damaged horny layer in the investigation of penetration rates in diseased skin. This is highly relevant since topical formulation will often be applied to damaged skin.

7.2.3 Skin biopsy

A classical method to access skin compartments is by excision of the skin under local anaesthesia. A punch biopsy will contain epidermis, dermis and maybe subcutaneous fat. A shave biopsy will mainly contain epidermis and some dermis. These methods are invasive and traumatic, but allow direct admission to the tissue of interest. Drug concentrations in human or animal skin can be studied after oral or topical dosing. After a penetration period following topical application the stratum corneum is partly removed by skin stripping. Biopsies are taken and frozen. The biopsy can be sectioned parallel to the skin surface and divided into epidermis, dermis and subcutaneous tissue. The skin sections are analyzed separately and a concentration gradient from the skin surface to the subcutis can be obtained. Skin biopsies can provide information on drug distribution within the skin both *in vivo* and *in vitro* (Surber, 1995).

7.2.4 Measurements of radioactivity in excreta and blood

The percutaneous absorption can be determined indirectly by measuring radioactivity in excreta (urine or urine plus faeces) after topical application of a labelled compound. The amount of radioactivity retained in the body or excreted by some other route is corrected for by determination of the amount of radioactivity excreted after parenteral administration. The percutaneous absorption is determined as a ratio between the total radioactivity excreted after topical administration and the total radioactivity excreted after parenteral administration (Feldmann and Maibach, 1969). This method does not account for metabolism in the skin, since the radioactivity in the excreta originates from both parent compound and metabolites. Percutaneous absorption can also be determined as the area under the plasma concentration curve following topical and intravenous administration of a labelled compound. The absolute topical bioavailability is only obtained by measuring the compound of interest by specific analysis. However, this can be very diffi-

cult since plasma concentrations after topical administration are often very low.

7.2.5 Other methods

In skin flap models (e.g. the rat-human skin flap system and the isolated perfused porcine skin flap) tissue is surgically prepared so that vessels draining the skin can be directly accessed. Human skin is in some skin flap models grafted onto immunosuppressed rodent hosts. The technique is technically demanding, but useful for bioavailability and toxicological studies. (Riviere, 1993).

An alternative but indirect method for estimation of absorption is the use of pharmacological response. This method is limited to drugs that perform responses which can be measured. A classical example is the vasoconstrictor assay, where the blanching of the skin is an estimate of steroid penetration (McKenzie and Stoughton, 1962).

Mathematical models of percutaneous penetration have been utilized to simulate release from the formulation, penetration into the skin, systemic distribution and metabolism (Hadgraft, 1990).

Non-invasive methods are used for examination of the structure and function of the skin. These methods include ultrasound imaging, laser Doppler image scanning, transepidermal water loss (TEWL) and erythema measurements.

Additional information about methods for investigating the penetration in the skin, like surface disappearance, surface recovery and autoradiography can be found in Shah et al. (1991), Wester and Maibach (1993a) and Surber (1995).

7.3 Animal models

The ultimate way to obtain knowledge of the penetration of a drug in human skin is to study in man, since this is directly relevant to the clinical situation. However, *in vivo* penetration studies in man are often not possible because of high toxicity or for ethical reasons. Therefore, many studies on penetration, mechanisms of action, factors affecting penetration/absorption and estimation of toxicity must be performed in animals. Many species have been used over time like monkeys, pigs, rabbits, guinea pigs, rats, mice, cats, dogs and snakes. Rats, mice, guinea pigs and dogs are available in hairless strains. As described above the animals differ significantly from man with regard to thickness and nature of the stratum corneum, lipid content, the density of hair follicles and sweat glands, the blood supply and biochemical events in the skin. Generally the monkey and the pig are the more relevant animal models. Animals usually have higher penetration/absorption as compared to humans. Especially the rabbit and the mouse exceed man. Thus, several studies were used to predict absorption in humans by using animals as for instance the rhesus monkey (Wester and Maibach, 1975) and the hairless rat (Rougier et al., 1987). Humans and animals do have regional variation in common. Bronaugh et al. (1983) reported that the absorption was greater from the abdomen than from the back of male rats. The monkey shows similar regional variations as humans. The same anatomical site (ventral forearm) can be used in comparative studies in man and monkey (Wester and Maibach, 1993b). Besides regional variation, sex difference was also found in the rat (Bronaugh et al., 1983).

Gummer and Maibach (1986) showed that various precautions should be taken when rodents are used as model. Contamination of the metabolic cage from an uncovered site caused by rubbing, scratching and simple desquamation can influence the drug level found in the excreta.

The animals are also able to ingest the applied dose from the skin by licking, which obviously change the experimental conditions.

Methodology may have an influence on data comparing results from man and animals. Factors like surface area treated, dose, vehicle, method to remove hair and application time must be carefully controlled during *in vivo* experiments. Care must be taken when comparing results from different publications.

8.0 Cutaneous microdialysis as a method in cutaneous penetration studies in comparison with other techniques

None of the techniques except cutaneous microdialysis are able to follow drug transport in the skin and metabolism continuously on the same test area during penetration. Methods using suction blisters and skin biopsies represent a physiological endpoint. Unlike a bioequivalence study where many blood samples are collected over time after application of the dose, only one sample can be obtained from the site of application at a given time after dosing. Therefore multiple sites of drug application are required, where each site represent a single test. It is possible to obtain multiple sample points by microdialysis and this makes the method very suitable in pharmacokinetic studies. The number of samples is determined by the flow, the recovery and the analytical method.

Some of the methods mentioned such as skin biopsies are also widely invasive and may interfere with the obtained results. It can be complicated to use a traumatic technique on diseased skin, and it is not possible to raise skin blisters in damaged skin. Cutaneous microdialysis implies a minor reversible trauma and the experiment is not started until the trauma has vanished.

It is supposed that microdialysis can be used both on normal and pathological skin. In pharmacokinetic studies the unbound drug concentration at the target tissue is important, since it is the unbound fraction that is accessible for the receptors and thus determines the pharmacological responds. The microdialysis technique provides the unbound concentration directly because the protein-bound drug cannot pass the membrane. The other methods determine the total concentration in the skin, both the unbound and the bound drug, and such methods are therefore less specific, since the extraction procedures with organic solvents release drug from binding sites. However, it can be laborious to determine the free drug concentration in the tissue by microdialysis as described above.

In several studies excessive drug is removed from the skin surface by a washing procedure. Materials used for the washing can influence the penetration and the drug concentration in the skin. The residual amount washed from the skin and the amount found in the skin by stripping or by continuous radioisotopic monitoring (used when radiolabelled isotopes are applied on the skin) represents the remaining amount of the drug in the skin (the massbalance technique and the surface disappearance method, Guy et al., 1987). This amount is used for determination of the amount absorbed by subtracting of the amount applied from the remaining amount. For poorly penetrating drugs, this procedure is dubious, since the subtraction is between two nearly equal numbers.

The skin flap model and skin graft technique are rather labour intensive. In the rat-human skin flap system the skin metabolism can be assessed, but the rats require cyclosporine to prevent rejection of the skin grafts, which appears to enhance absorption of agents applied to rat skin (Pershing and Kreuger, 1987).

Since plasma concentrations are extremely low after topical application and because the local dose is distributed throughout the whole body the use of radiolabelled compounds or high doses of low clinical relevance have been necessary in *in vivo* percutaneous absorption studies. A disadvantage of using the tracer technique is the poor specificity. Impurities and degradation products will be counted as the parent compound which can result in misleading data. Degradation of the labelled compound can occur during preparation of topical formulations and during studies. Degradation can lead to more permeable products and give an overestimation of the penetration rate (Sznitowska, 1996).

It is obvious to use the microdialysis technique in combination with other methods, especially the non-invasive methods which measure biological responds. In this way pharmacokinetics and pharmacodynamics evaluation are combined. It is evident that cutaneous microdialysis presents advantages as compared to other established techniques. However, more studies are needed to clarify of its precise potential in dermatological research.

Experimental part

9.0 Aims

The aims of the study were to standardize, optimize and validate cutaneous microdialysis in experimental dermatological research, and to elucidate applications and limitations of microdialysis for the study of skin penetration.

The specific objectives were:

- to establish an experimental setup for in vitro microdialysis,
- to determine recovery and loss for drugs with different physico-chemical characteristics,
- to determine experimental parameters and their influence on *in vitro* recovery,
- to investigate effects of anaesthesia on cutaneous skin perfusion in hairless rats,
- to investigate effects of trauma due to insertion of a microdialysis probe in rat and human skin,
- to investigate the potential of cutaneous microdialysis as a model for the study of drug penetration *in vivo* in hairless rats.

10.0 Materials and Methods

10.1 Model substances

The model drugs are selected to represent a range of hydrophilic and lipophilic properties, and a spectrum of indications such as diseases like infections, dermatitis and psoriasis (Figure 11).

10.1.1 Glucose monohydrate

$C_6H_{12}O_6$, H_2O, molecular weight 198.18. Solubility in water = 1000 mg/ml.

Glucose was selected because it is a small hydrophilic molecule that equilibrates rapidly in its distribution volume.

10.1.2 Sodium fusidate

$C_{31}H_{47}NaO_6$, molecular weight 516.72, pK_a = 5.35. Solubility in water = 300 µg/ml (pH 7.00). Log P = 2.68 (at pH 7.48). 97% protein bound (Güttler and Tybring, 1971)

Sodium fusidate and fusidic acid are both used in Fucidin® products which are highly active against Staphylococcus aureus, a causative organism in many skin infections. Fucidin exert antibacterial effects by interfering with the protein synthesis of the bacteria leading to the breakdown of their cell walls. Fucidin® is used to treat patients with a variety of skin infections, e.g. impetigo, folliculitis and infected wounds and burns. Several studies have shown a high cure rate of skin infections (Baldwin and Cranfield, 1981; Soeby, 1966)

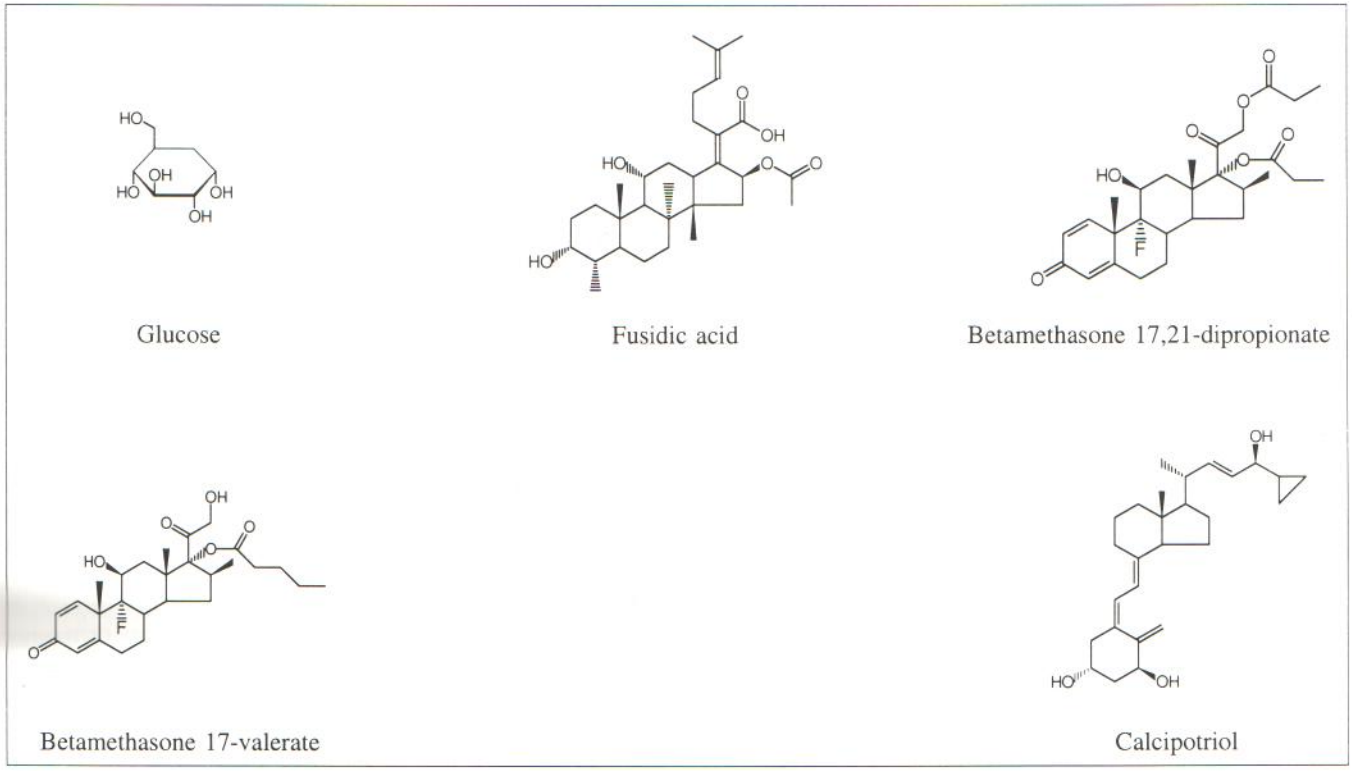

Figure 11 *Drugs used in this study.*

10.1.3 Betamethasone 17,21-dipropionate and Betamethasone 17-valerate

Betamethasone 17,21-dipropionate
$C_{28}H_{37}FO_7$, molecular weight 504.60.
Solubility in water < 0.04 mg/ml. Log P = 3.24

Betamethasone 17-valerate
$C_{27}H_{37}FO_6$, molecular weight 476.60.
Solubility in water = 0.0093 mg/ml. Log P = 3.5.

Betamethasone dipropionate and betamethasone 17-valerate are corticosteroids with potent anti-inflammatory effects. Topically applied corticosteroids have a good effect on inflammatory diseases of the skin like atopic dermatitis, contact dermatitis and psoriasis. Betamethasone dipropionate has a higher anti-inflammatory activity than betamethasone 17-valerate. However, the vehicle is very important in influencing clinical potency of corticosteroids due to different release rates of the active drug from different vehicles (Maibach and Surber, 1992).

10.1.4 Calcipotriol
$C_{27}H_{40}O_3$, molecular weight 412.6. Solubility in alcohol = 330 mg/ml and in water = 0.6 µg/ml. Log P = 4.40
Calcipotriol is a vitamin D analogue. Calcipotriol is a potent regulator of cell differentiation and an inhibitor of cell proliferation in human keratinocytes. Calcipotriol is used in the treatment of psoriasis, a hyperproliferative state. It was shown that calcipotriol has a dose-dependent anti-psoriatic effect (Kragballe et al., 1988).

All the drugs used were obtained from LEO Pharmaceutical Products. Glucose monohydrate was purchased from Cerestare (Holte, Denmark).

10.2 Sample analysis

10.2.1 The HPLC system
In vitro dialysis samples of sodium fusidate, betamethasone dipropionate and calcipotriol were analyzed by isocratic reversed-phase high performance liquid chromatography. The chromatographic system (Merck, Darmstadt, Germany) consisted of an AS-4000A intelligent autosampler, a L-6200A intelligent pump, a L-5025 column thermostat, a L-4250 UV-VIS detector and a D-6000 interface. The system was controlled by an HPLC manager software (HPLC Manager, D-6000).
The dialysate samples from the in vivo microdialysis studies a Merck LaChrom HPLC system consisting of an programmable autosampler (L-7250), a pump system (L-7100), a column oven (L-7300), a fluorescence detector (L-7480), a UV-VIS detector (L-7400) and an interface (D-7000) were used. The system was controlled by an HPLC manager software (D-7000) UV detector.
Quantitations of the compounds were done by measurements of the peak heights in relation to those of standards chromatographed under the same conditions.

The limit of quatitation was determined as follows:
A standard solution of low concentration was injected 20 times and mean and SD were calculated. The limit of quantitation was determined as 10X SD.

10.2.2 Analysis of sodium fusidate, betamethasone dipropionate and calcipotriol in in vitro studies
Sodium fusidate
The detector was operated at UV-235 nm. The mobil phase was 60% (v/v) acetonitrile, 10% (v/v) methanol and 30% (v/v) 0.05 M phosphoric acid. Flow rate was 1.2 ml/min. The separation was achieved using a Lichrospher 100 RP-18, 5 µm column (125 X 4 mm) (Merck, Germany). Column oven temperature was 35 °C.

Betamethasone dipropionate
The detector was used at UV-240 nm. The mobile phase was 50% (v/v) acetonitrile and 50% (v/v) 0.05 M phosphoric acid. Flow rate was 1.5 ml/min. The separation was achieved using Lichrospher 100 RP-18, 5 µm column (125 X 4 mm) (Merck, Germany). Column oven temperature was 35 °C.

Calcipotriol
The detector was operated at UV-265 nm. The mobile phase was 50% (v/v) acetonitrile, 20% (v/v) methanol and 30% (v/v) 0.01 M diammonium hydrogen phosphate buffer, pH = 8.0. Flow rate was 1.0 ml/min. A Superspher 100, RP-18, 4 µm column (125 X 4 mm) (Merck, Germany) was used at 35 C°.

10.2.3 Analysis of sodium fusidate and betamethasone 17-valerate in in vivo studies
Sodium fusidate and betamethasone 17-valerate were analyzed by the same HPLC-method using a narrowbore column. A detector wavelength of UV-245 nm was used for both sodium fusidate and betamethasone 17-valerate. The mobile phase was 50% (v/v) acetonitrile, 10% (v/v) methanol and 40% (v/v) 0.05 M phosphoric acid. The sample analysis time was 8 minutes. Flow rate was 0.3 ml/min for the initial 3 minutes followed by 0.6 ml/min for 5 min. The separation was performed on a Superspher 100 RP-18 column, 4 µm particle sizes (125 X 2 mm) (Merck, Germany). Column oven temperature was 35 °C. The retentions time were 3.5 min and 5.5 min for betamethasone 17-valerate and fusidic acid, respectively. The limit of quantitation was 5 ng/ml for betamethasone 17-valerate and 50 ng/ml for fusidic acid, when a 150 µl sample of an 80 + 125 diluted dialysate was injected on the column.

10.2.4 Analysis of calcipotriol in in vivo studies
Calcipotriol was eluted by a gradient mobil phase. Two mobile phase compositions, A and B, were used, both made up from acetonitrile/methanol/ 0.01 M diammonium hydrogen phosphate buffer, pH = 8.0. Mobile phase A had the composition ratio 50/20/30 (v/v), whereas the ratio of B was 20/10/70 (v/v). Each chromatographic run was started with 100% B for 0.5 min followed by a gradient to give 20% B and 80% A at 1.5 min and a further gradient to give 100% A at 14 min. The composition was returned to 100% during the period 14 to 15 min and B then stayed on for the remaining part of the 18 min

run. Flow rates were 0.4 ml/min for the first 1.5 min, then 0.5 ml/min between 1.5 and 14 minutes followed by 0.4 ml/min for the rest of the chromatographic run. Calcipotriol was detected at 265 nm and peak heights were used for quantitation. The retention time was 9.3 min and the limit of quantitation was 5 ng/ml, with injection volume and dilution as for sodium fusidate and betamethasone 17-valerate.

10.2.5 Analysis of glucose in in vitro studies

Glucose was analyzed by an enzymatic method using glucose oxidase (Trinder, 1969).

10.2.6 Analysis of histamine in in vivo studies

Six µl samples of dialysate were collected into microtiter wells coated with a glass fiber matrix used for determination of histamine by a spectrofluorometric assay (Skov et al., 1984). The limit of detection of this assay is approximately 5 ng/ml. Codeine did not interfere with the histamine analysis (Petersen et al., 1995).

10.2.7 Development of an HPLC method for fusidic acid

It was expected that the dialysate samples from *in vivo* cutaneous microdialysis would contain very low concentrations of the drugs of interest. At the time when the *in vivo* microdialysis with topically applied fusidic acid was started, the available HPLC method was estimated to fail in sensitivity for the dialysate samples. The method was originally developed for determination of fusidic acid in serum, with a detection limit of approximately 1 µg/ml, based on a signal-to-noise ratio of 3 and 100 µl samples (Sørensen, 1988). Thus, a more sensitive analytical method for fusidic acid was needed. A way to improve the detection of fusidic acid is to couple fusidic acid with a fluorophore label, since fluorescence detection often yields higher sensitivity than UV-detection. Fusidic acid is not fluorescent in itself.

The method is based on a procedure described by Dünges et al. (1977), Lam and Grushka (1978) and Horst et al. (1988a; 1988b; 1990). The acid group of fusidic acid was reacted with 4-bromomethyl-7-methoxycoumarin (BrMMC) to form a fluorescence derivative. The reaction was investigated both in an aqueous micellar system and in an acetone solution.

Derivatization procedure in an aqueous micellar system

Often is derivatization of acids incompatible with the presence of water. This implies that the compound to be analyzed must be extracted from the aqueous matrix into a suitable organic solvent. Extraction is hardly possible in microdialysis due to the very small sample volume. By a pre-column derivatization in an aqueous micellar system, this extraction is avoided. The surfactants Triton-X 100 and Arkopal N-130 are forming the micellar system. 4-bromomethyl-7-methoxycoumarin is the fluorescent label and tetrahexylammonium bromide is a ion-pairing agent taking the acid into the micelles. The mechanism of the derivatization is based on phase-transfer catalysis (Horst et al., 1988b).

Arkopal N-130, 1.68 g, (kindly donated from Hoechst, Rødovre, Denmark) and tetrahexylammonium bromide, 625.8 mg (THxABr, Sigma, St. Louis, MO, USA) were dissolved in 20.00 ml acetone. BrMMC, 80 mg (Fluka Chemie, Buchs, Switzerland) was dissolved in 10.00 ml acetone in an amber-coloured flask. The Arkopal N-130 and THxABr solution (240 µl) and BrMMC solution (90 µl) were transferred into an Eppendorf vial (amber-coloured, 1.5 ml Safe-Lock, Eppendorf, Hamburg, Germany) and the acetone was evaporated under a stream of nitrogen with exclusion of light. The incubation vials were stored at -20 °C until use.

Standard solutions, 0.5 - 1.5 µg/ml, of sodium fusidate, 500 µl, were placed in the incubation vial and vortexed for 5 s. The vial was placed at 70 °C for 40 min in a waterbath for the reaction to take place. Thereafter 500 µl acetonitrile was added to the vial.

The samples were injected into a Merck La Chrom HPLC system (Darmstadt, Germany). Four hundred µl samples were injected. The detector was operated at excitation and emission wavelengths of 320 and 391 nm, respectively. The mobile phase was 80:20 (v/v) methanol:water from 0 to 3 min, followed by a linear gradient up to 100% methanol during 6 min. Hundred per cent methanol was continued for 4 min and the mobile phase was then returned to 80:20 (v/v) methanol:water during 2 min. The flow rate was 1 ml/min. A Lichrospher 100 RP-18, 5 µm (125 X 4 mm) column (Merck, Darmstadt, Germany) was used.

Derivatization procedure in acetone

To 45 µl sodium fusidate standard in an isotonic phosphate buffer, pH = 7.4, in an amber-coloured Eppendorf vessel, 20 µl 10% acetic acid was added to form the fusidic acid. The standard was freeze dried for 16 hours to remove the aqueous solvent.

The reagent BrMMC and the catalyst 18-crown-6 ether (Fluka Chemie, Buchs, Switzerland) was used in the following standard solutions: 18-crown-6 ether in acetone, 2.40 mg/ml (HPLC grade, Merck, Darmstadt, Germany) and 0.90 mg/ml 4-bromomethyl-7-methoxycoumarin in acetone.

To the freeze dried product 5 mg potassium carbonate (Merck, Darmstadt, Germany), was added as well as 245 µl acetone and 30 µl 18-crown-6 ether solution. The mixture was vortexed for 5 s. The derivatization was started by addition of 25 µl of the BrMMC solution with exclusion of light. After vortexing for 5 s the samples were incubated at 60 °C for 30 minutes.

To 250 µl of the reaction mixture 250 µl water was added and the sample was analyzed as described above.

The method was validated for linearity, precision and evaluation of blanks.

Linearity

Six standards, 0.00 - 1.5 µg/ml, of sodium fusidate were injected. The relationship between the concentration of standards and peak heights was studied for 3 sets of standards on three different days.

Precision

Six standards of 0.25 µg/ml and six of 1.00 µg/ml were injected on three different days. The relative standard deviation of

peak heights was calculated.

Blanks value
Twelve samples of the 0.00 µg/ml standard blanks were injected.

10.3 *In vitro* microdialysis (Paper I)

10.3.1 Standard solutions
An isotonic 0.05 M phosphate buffer, pH 7.4 was used as a standard solvent. To the solutions of betamethasone dipropionate and calcipotriol 2% ethanol was added. The concentration of the standard solutions is found in table 3.

Substance	Standard concentration	Log P
Glucose	5.2 mM	<< 1
Sodium fusidate	50 µg/ml	2.68
Betamethasone dipropionate	300 ng/ml 2% ethanol	3.24
Calcipotriol	250 ng/ml 2% ethanol	4.40

Table 4 *Standard concentrations and log P value of the substances used in the in vitro microdialysis study.*

10.3.2 In vitro microdialysis experiments
A 4 cm microdialysis tubular membrane of Cuprophan®, obtained from an artificial kidney (Gambro GFE 18, Gambro Dialysaten AG, Hechingen, Germany, outer diameter 216 µm, wall thickness 8 µm, 2,000 Da molecular weight cutoff) was glued to afferent and efferent tubings representing a linear microdialysis probe. The probe was mounted in a polypropylene beaker and connected to a microinjection pump (CMA/100, CMA/microdialysis, Stockholm, Sweden). The probe was perfused at 3 µl/min. Test solutions were filled into the beaker, in a volume that allowed the probes to be covered with solution. The solution was stirred using a stirplate (Variomag Electronicrührer, Mono, Oberscheissheim, Germany) placed under the beaker. Dialysates were collected in 300 µl glass test tubes (Figure 12, colour insert, rear inside cover).
For glucose and sodium fusidate a standard nylon tube (Portex Limited, Hythe, Kent, England, inner diameter 0.50 mm, outer diameter 0.63) was used with approximately 15 cm in- and outlet tubing. In the experiments with betamethasone dipropionate and calcipotriol a 2.5 cm PTFE tubing (Bolinder Labor-geräete, Lauder, Köningshofen, Germany) was first glued to the microdialysis membrane and then approximately 10 cm peek tubings (MF5366, CMA/microdialysis, Stockholm, Sweden) were coupled to the PTFE tubings on each side.
One *in vitro* microdialysis experiment included 3 microdialysis probes used under the same test conditions. At least four samples were collected from each probe.

10.3.3 Determination of recovery
A standard solution with the compound of interest was filled into the beaker. The probes were perfused with phosphate solution or a 2% ethanolic phosphate solution (betamethasone dipropionate and calcipotriol). Recovery (in percentage) was calculated as the ratio between the concentration in the dialysate and the concentration of the standard solution in the beaker. Samples from the beaker were withdrawn at the beginning and at the end of the experiment to determine the concentration in the beaker.

10.3.4 Determination of loss
A perfusate solution with the substance of interest was used. The beaker contained phosphate buffer or 2% ethanolic phosphate buffer (betamethasone dipropionate and calcipotriol). Percent loss or delivery was calculated as the ratio of the loss from the perfusate concentration to the initial concentration in the perfusate. The perfusate concentration was determined from samples of the syringe in the pump before and at the end of the experiment.

10.3.5 Parameters affecting recovery and loss
Stirring rate
Recovery of glucose was determined with different stirring rates to study the influence of stirring rate on recovery.

Temperature
A waterbath was connected to control the temperature in the medium surrounding the probes. The relationship between temperature and recovery of glucose was studied.

Flow rate
Different flow rates of the perfusate through the probes were used to study recovery of glucose and sodium fusidate.

Concentration
Different concentrations of glucose, sodium fusidate, betamethasone dipropionate and calcipotriol were used to study the influence of concentration on recovery and loss. Recovery was found as the mean recovery as the slope of the regression line when dialysate concentrations were plotted as a function of the concentration in the medium. Mean loss was defined as the slope of the regression line when the difference between the concentration in the perfusate and dialysate was plotted as a function of the perfusate concentration.

Dialysis membrane length
Recovery of glucose and sodium fusidate were determined using different lengths of the dialysis membrane.

Effect of protein in the surrounding medium on recovery
To evaluate if protein binding influences recovery of sodium fusidate bovine serum albumin (Sigma chemicals, St. Louis, MO, USA) was added to the medium.

10.3.6 Point of no-net-flux
The point of no-net-flux method was performed with glucose and sodium fusidate. Microdialysis probes were perfused with

different concentrations of the compound of interest, higher and lower than the concentration in the medium, which was kept constant. The difference between the concentration in the dialysate and the perfusate was plotted as a function of the concentration in the perfusate. In this way a linear relationship is established with recovery as the slope and the concentration in the medium as the intercept with the x-axis.

10.3.7 Statistical analysis
A two-sample t-test was used to analyze the difference between recoveries for the different drugs and recovery and loss for a single drug. The stirring rate, the temperature and the concentration dependency was evaluated by a one-way analysis of variance. Probabilities less than 0.05 were considered significant. Values were given as mean ± SD.

10.4 Trauma studies

10.4.1 Laser Doppler Flowmeter
In laser Doppler measurements a Doppler shifted laser light is used to generate an output proportional to the red blood cell movement through the skin vessels under investigation. A commercial laser Doppler flowmeter (LDF) (Periflux, pf2B, Perimed, Sweden) with a He-Ne light source was used to measure skin blood flow. The laser detects small changes in the wavelength of the laser light as a result of red blood cell movements. The laser light is guided through an optical fiber to the probe head (probe 309) in contact with the skin surface. A probe holder is taped to the skin (Micropore, surgical tape, 3M Health Care, St. Paul, MN, USA). The depth of penetration of the light in the skin is about 1 mm (Belcaro and Nicolaides, 1995). LDF is non-invasive and does not interfere with the microcirculation when measuring local blood flow. LDF provides a continuous measurement of the blood flow in a limited skin area.
The instrument was adjusted to a gain at 3 (12 Kh) and a response at 1.5. The laser Doppler flowmeter was connected to a plotter which recorded the flow output. Results are expressed in arbitrary units.

10.4.2 Laser Doppler perfusion imaging
The blood flow in the skin was imaged and measured by Laser Doppler Perfusing imaging, LDPI (PIM 1.0, Lisca Development AB, Linköping, Sweden). PIM 1.0 is a computer controlled system that performs two-dimensional scanning of the tissue using a low power He-Ne laser, without touching the skin. A scan covers an area up to 12 cm times 12 cm and includes a total of 4,096 individual measuring sites. At each measuring site, moving red blood corpuscles cause Doppler shifts in the backscattered light. The Doppler-shifted light is detected and converted to an electrical signal (range 0-10 Volt) which is linearly proportional to tissue blood perfusion (Wårdell, 1992). The measurements of the test site provide a colour code image on the display. Data analysis can be performed using the manufacturer's software.
The scan head was positioned 16.5 cm (rats studies) or 17.5 cm (human studies) above the test area. The resolution was set at high and the background threshold at 6.1.

10.4.3 Ultrasound imaging
Skin thickness was measured by 20 MHz ultrasound scanning using the Dermascan-C (Cortex Technology, Hadsund, Denmark). High-frequency ultrasound is a non-invasive method for *in vivo* imaging of the skin.
The instrument consists of three main parts: The C-probe, the elaboration and visualization system and the memorizing and data-storing system. The probe is surrounded by water, and sealed at the point of contact with an ultra-thin plastic diaphragm. The intensity of the reflection echo is evaluated by the microprocessor and is visualized as a colour coded two-dimensional B-mode image. A-mode interfaces are shown as well defined peaks. The accuracy of this method for skin thickness measurements is about 0.1 mm (Serup, 1984).

10.4.4 Minolta Chromameter
Skin colour and erythema was measured by the Minolta Chromameter CR 200® (Osaka, Japan). The light source is a high power xenon arc lamp. The Minolta measures skin colour by tristimulus analysis of reflected light according to the CIE system (Commision Internationale de l'Eclairage). The colour is expressed in a tri-dimensional system with green-red (a*), yellow-blue (b*) and L* axis where L* expresses brightness. Measurements are based on the parameter a* which is a measure of erythema. Readings are presented digitally on a liquid-crystal display. The apparatus provides good accuracy and reproducibility (Westerhof, 1995). Before measurement on a subject the Minolta Chromameter was calibrated using a white calibration tile.

10.4.5 Dermaspectrometer
Erythema was also measured by the Dermaspectrometer® (Cortex Technology, Hadsund, Denmark). Erythema and melanin indices are derived from the reflectance of the object in a selected band of the spectrum. Light-emitting diodes emit narrow-band radiation, and the reflectance of the skin within the narrow bands of the spectra corresponding to green and red light are measured.
The erythema index is expected to have a roughly linear relationship with red blood cells in the upper dermis (Takiwaki and Serup, 1995). The erythema index is displayed on a digital panel meter. Before measurement on a subject calibration of the dermaspectrometer was preformed with a white standard surface.

10.4.6 Transepidermal Water Loss
The Transepidermal Water Loss (TEWL) measurement is a non-invasive method for assessing the efficiency of the skin as a protective water barrier. TEWL provides information of the integrity of the epidermis in normal, irritant and diseased skin and how chemicals affect the water barrier (Pinnagoda and Tupker, 1995; Barel and Clarys, 1995). TEWL is measured by the Evaporimeter EP1 (ServoMed, Stockholm, Sweden) which has a measuring probe connected to a processing unit. The evaporimeter was turned on for 30 minutes and the instrument was adjusted manually to zero TEWL before measuring. The

probe head is placed on the skin and stabilization is usually reached 45 s after the start of measurement. The operator was wearing an insulating glove during the measurement. Measurements were performed in an incubator to avoid variations due to temperature changes and air convection. The values of TEWL are g/m^2 h.

10.4.7 Insertion of the microdialysis probe.
A microdialysis membrane obtained from an "artificial" kidney (Gambro GFE 12, Gambro Dialysaten AG, Hechingen, Germany, outer diameter 216 μm, wall thickness 8 μm) was inserted horizontally (intradermal) within the dermis using a guide cannula, 21-Gauge, i.d. 0.80 mm, length 40.0 mm. The guide was then withdrawn leaving the membrane horizontally within the dermis. The length of the membrane was 3.0 or 4.0 cm.

10.4.8 Animals
Hairless female rats were used, 200g ± 20g (OFA-hr/hr, IFFA CREDO, France).
The animal experiments were performed at constant room temperature of 25 °C. After anaesthesia the rats were placed on a temperature controller (CMA/150, CMA/Microdialysis, Stockholm, Sweden).

10.4.9 Subjects
Healthy volunteers (N=28) without atopic dermatitis, allergy or asthma were studied. Subjects were excluded if they had any skin disease or any suspicion about allergic reaction towards lidocaine. The subjects were not allowed to use any local or systemic medication, including oral contraception, and no skin care products for 24 hours prior to the investigation. All subjects gave their informed consent. The study was approved by the regional ethics committee

10.5 Effects of insertion trauma and anaesthesia on rat skin

10.5.1 Anaesthesia of the hairless rat
Halothane anaesthesia
Rats were anaesthetized with halothane (Halothan®, "Halocarbon", Halocarbon Laboratories, North Augusta, SC, USA) evaporated by a vaporizer (PPV sigma, Penlon LTD., Abingdon, England) connected to a closed plexiglass container or to an open mask. Oxygen and nitrous oxide were delivered with halothane at a total flow rate of 700 ml/min (1:1). The rat was placed in a closed plexiglass container and anaesthesia was induced by 4-5% halothane. After induction 2% halothane was continuously administered using an open mask. Subsequently the concentration was adjusted approximately every 30 minutes depending on depth of anaesthesia.

Pentobarbital sodium anaesthesia
Rats were anaesthetized using 50 mg/kg pentobarbital sodium injected in the peritoneal cavity.

10.5.2 Skin blood flow in awake and anaesthetized rats
The skin blood flow in awake and anaesthetized rats were measured to investigate the influence of anaesthesia on skin blood flow as compared to normal blood flow. A pen-recorder was recording the skin blood flow measured by the laser Doppler flowmeter. Stable skin blood flow values were determined. A minimum of 5 minutes with the same blood flow value was considered as a stable period. The mean of the values in the stable periods were used.

Halothane anaesthesia
A laser Doppler flowmeter probe was placed on the back on 5 awake rats. The probe was kept in place by a net bandage Brend-a-rete (Figure 13, colour insert, rear inside cover) (no.2, Artsana, Como, Italy). The rat was placed in a plastic bowl (CMA/Microdialysis AB, Stockholm, Sweden). The rat was left undisturbed and the blood flow was measured for an hour or more. Only values obtained when the rats were asleep or just relaxing were used, since the blood flow was only stable during these periods.
The 5 rats (used in the study of awake rats) were anaesthetized with halothane the blood flow was measured on the back of the rat similar to the measurement of the awake rats. The rats was measured for at least one hour.

Sodium pentobarbital
The same experimental procedure as described above was used when the skin blood flow was measured in awake and anaesthetized rats. Six rats were studied in the awake state and subsequently the same six rats were anaesthetized with pentobarbital sodium.

10.5.3 Basal skin blood flow (Paper II)
Eight rats were anaesthetized with halothane and four rats with pentobarbital sodium and the skin blood flow was measured by LDPI.

Basal skin blood flow measurements
In rats anaesthetized with halothane LDPI measurements were performed at 3, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min after introduction of anaesthesia. In rats anaesthetized with pentobarbital sodium measurements were performed with the same intervals, but only for 120 min. A test site area of 4.5 cm X 3.5 cm was marked on the back of the rats, allowing a scan test area of 64 X 50 measuring points. Every time, three scans each lasting approximately three minutes were taken and the mean of the three measurements were used. An analysis area of 45 X 30 measurering points in the centre of the 64 X 50 points test area was used to record the average blood flow in each scan.

10.5.4 Effect of insertion trauma on skin blood flow (Paper II)
Effect of insertion of a microdialysis probe on skin blood flow was measured in rats by LDPI. Measurements were performed before, during and after needle insertion. The rats were anaesthetized with halothane or pentobarbital sodium. Sixteen insertions were performed in eight hairless rats anaesthetized with halothane, one insertion on each side of the back. Insertions in the right and left side were performed with an interval of three days as a minimum. Insertions were executed on the right side of four rats anaesthetized with pentobarbital sodium. The probe

was inserted in a length of 3 cm.

Skin blood flow measurements

A test site of 2.5 X 3.5 cm was marked with blue ink on the side of the back of the rats corresponding to an area of 64 X 30 measuring points. LDPI scans were performed prior to, during and immediately after needle insertion, followed by measurements at 10, 20, 30, 40, 50, 60, 90 min and 24 hours after insertion. Every time, three scans were carried out each lasting approximately two min. The colour code image was separated into two squares, forming the largest squares that did not overlap. A square correlates to an analysis area of 14 X 25 measurering points. Each square was positioned with its center at the insertion and exit of the cannula, respectively. The point of cannula insertion was on the upper part of the back and the outlet point was on the lower part of the back. The blue ink position marks were seen as grey points in the colour code image. They were not included in the calculation.

Statistical analysis

Increase in skin blood flow after cannula insertion was evaluated as the difference between skin blood flow before and after cannula insertion. A paired t-test was used to analyze the increase in skin blood flow. $P < 0.05$ was considered significant.

10.5.5 Effect of insertion trauma on histamine release (Paper II)

Effect of insertion of a microdialysis probe on histamine release in the skin was evaluated using cutaneous microdialysis in rats. The time course of histamine release and elimination was monitored immediately after needle insertion. Codeine phosphate was used to further investigate histamine release after intradermal provocation. As anaesthetic either halothane or pentobarbital sodium was used.

Trauma induced histamine release

The microdialysis probe was inserted on the side of the back of the rat. Four rats anaesthetized with halothane and four rats anaesthetized with pentobarbital sodium were studied. A nylon inlet tubing was glued to the dialysis membrane and connected to a microinjection pump (CMA/100, CMA/Microdialysis, Stockholm, Sweden). The probe was perfused with isotonic phosphate buffer 0.05 M, pH 7.4, at a rate of 3 µl/min. The length of the probe was 30 mm. Sampling of dialysate was started immediately after needle insertion. Six µl samples of dialysate were collected.

Codeine phosphate induced histamine release

As a positive histamine release control, a skin prick test was performed with codeine phosphate, a mast cell degranulating agent (Goldberg et al., 1990). Two rats anaesthetized with halothane and two rats anaesthetized with pentobarbital sodium were used. After the trauma induced experiment, a period of 15 minutes was allowed between the trauma experiment and the codeine provocation. Fifty µl 1 mg/ml codeine phosphate was intradermally injected (Insupak; ASIK A/S, Rødby, Denmark, 27-gauge) above the microdialysis probe, taking care not to ruin the microdialysis membrane. In all cases the injection-induced wheal covered the probe area. Six µl samples were collected during 24 minutes and analyzed for histamine content.

10.5.6 Effect of insertion trauma on skin thickness (Paper III)

Groups of rats anaesthetized with halothane and pentobarbital sodium were studied. Seven rats were anaesthetized with halothane and two microdialysis probes were inserted into the skin of each rat, one probe on each side of the back.

Six rats were anaesthetized with pentobarbital sodium. One probe was inserted on the right side of the back. The probes were inserted in a length of 4 cm.

Skin thickness measurements

Skin thickness was measured prior to and immediately after needle insertion, followed by measurements at 15, 30, 45, 60, 90 and 120 minutes. Three B-scans were taken every time at different positions along the intradermal probe. The Dermascan-C was operated with a linearly increasing gain, adjusted for each rat in order to individualize and optimize the operation of the instrument. A typical gain curve was 25 dB to 55 dB. The velocity of ultrasound in the skin was set at 1580 m/s. Ultrasonic coupling gel was applied to the test area. The ultrasound probe was oriented so that the scan line was 90 degrees to the tubular dialysis membrane. A-mode scanning of the microdialysis probe was used to measure skin thickness, defined as the distance between the epidermis entrance echo and the interface between dermis and subcutis (Figure 14, colour insert, rear inside cover). Ultrasound image analysis of scans was performed using the ROI function and the inbuilt software of the Dermascan C. The individual scans were stored on floppy disks. All assessments were based on the mean of three recordings of any inserted probe.

Probe depth measurements

The probe depth, i.e., the distance from the skin surface to the dialysis membrane was measured on the scans also used for total skin thickness measurements. The microdialysis membrane is echodense in structure and can easily be visualized by ultrasound. The microdialysis membrane appears as a white hyperreflecting dot. In A-mode scan, probe depth corresponds to the vertical distance between the epidermis entrance echo and the echo of the microdialysis membrane. Triple measurements along every probe at any time served to asses the variation in probe depth, i.e. intra-probe variation in depth.

Statistical analysis

Increase in skin thickness after cannula insertion was evaluated as the difference between skin thickness before and after cannula insertion. Additionally the difference between the skin thickness before and 15 minutes after insertion was calculated. A paired t-test was used to analyze the increase in skin thickness. $P < 0.05$ was considered significant. Finally, to elucidate if skin thickness reached the pre-insertion value over time, the difference between skin thickness before and at 120 minutes after insertion was calculated.

10.6 Effects of insertion trauma and anaesthesia on human skin (Paper IV)

The study was separated into three parts:

10.6.1 Effect of insertion trauma on skin blood flow and erythema with prior anaesthesia, ***part 1***

Twelve healthy volunteers were bilaterally anaesthetized with 3 ml subcutaneously injected lidocaine (Xylocain, 10 mg/ml, Astra, Sweden) in the dorsal forearm of both arms. The lidocaine was dispersed in marked areas of 3 X 4 cm using 3 injections. In one of the arms a microdialysis probe was inserted in the middle of the test area in a length of 3 cm. The opposite arm served as a control arm to establish the effect of the lidocaine. Insertions were randomly performed in right or left arm.

Non-invasive measurements were performed before and after anaesthesia and after needle insertion on both arms in parallel. After injection of the lidocaine 10 minutes elapsed before the probe was inserted, allowing the anaesthetic effect to develop. After the experiment the depth of the probe was measured by ultrasound imaging, see below.

Skin blood flow measurements

Skin blood flow was measured by the LDPI in normal skin (before any treatment), after the lidocaine was injected and immediately after the cannula insertion, followed by measurements at 10, 20, 30, 40, 50, 60, 80, 100 and 120 minutes after insertion. The one arm that was only anaesthetized was measured at the same time as the arm with the dialysis membrane inserted. The test area was 22 X 44 measuring points, which equals the analysis area. The site of insertion and exit of the cannula were marked with blue ink. They were seen as grey points in the colour coded image and they were not included in the calculation.

Erythema measurements

Erythema was measured by the Minolta Chromameter and the Dermaspectrometer. Erythema was measured immediately after blood flow measurements, first with Minolta Chromameter next with the Dermaspectrometer. This order was kept throughout the study. Three measurements were carried out at any time. The mean values were used.

10.6.2 Effect of insertion trauma on the skin thickness, ***part 2***

Twelve healthy volunteers were anaesthetized with 3 ml subcutaneously injected lidocaine (Xylocain, 10 mg/ml, Astra, Sweden) in both forearms. The lidocaine was dispersed in marked areas of 3 X 4 cm using 3 injections. In one of the arms a microdialysis probe was inserted in the middle of the test area in a length of 3 cm. The other arm served as a control arm to determine the effect of lidocaine. The needle insertions were randomised as regards to right and left arm. Skin thickness was measured on both arms in parallel. After injection of lidocaine 10 minutes elapsed before the probe was inserted, allowing the anaesthesia to develop. The probe depth was also determined.

Skin thickness measurement

Skin thickness was measured before anaesthesia, after lidocaine was injected and immediately after the insertion of the probe, followed by measurements at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after insertion. The arm that was only anaesthetized was measured at the same time. Prior to the skin thickness measurements an ultrasonic coupling gel was applied to the test area. Three B-scans were taken every time from different positions along the intradermal probe. The Dermascan-C was operated with a linearly increasing gain, adjusted for each subject in order to individualize and optimize the operation of the instrument. A typical gain curve was 15 dB to 30 dB. The probe head of the Dermascan-C was oriented so that the scan line was 90 degrees to the dialysis membrane. A mode scanning of the microdialysis probe was used to measure skin thickness.

Probe depth

The probe depth, i.e., the distance from the skin surface to the dialysis membrane, was measured on the scans also used for total skin thickness measurements. In A-mode scan probe depth corresponds to the vertical distance between the epidermis entrance echo and the echo of the microdialysis membrane. The three scans at every time point were used to determine the mean probe depth.

10.6.3 Effect of insertion trauma on skin blood flow and erythema without prior anaesthesia, ***part 3***

In 4 subjects a microdialysis probe was inserted in the skin of the dorsal forearm. The probe was placed in the center of a 3 X 4 cm area. Left or right arm were used randomly. Non-invasive measurements were performed before and after needle insertion. After the experiment the depth of the probe was measured by ultrasound imaging.

Blood flow measurements

LDPI scans (22 X 44 measuring points) were performed prior to and immediately after needle insertion, followed by measurements at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after insertion. The site of insertion and exit of the probe were marked with blue ink. The analysis area was 22 X 44 measuring points.

Erythema measurements

Erythema was measured before and after cannula insertion by the Minolta Chromameter and Dermaspectrometer following LDPI scans using as described above, i.e. first Minolta and next the Dermaspectrometer. Three measurements were carried out at any time. The mean values were used.

10.6.4 Statistical analysis

Part 1 and part 2:

The values on left and right forearm were compared at each time by a paired t-test, and sites with and without needle inserted were analyzed. The changes were calculated as the difference between baseline (baseline after local anaesthesia in the arm only anaesthetized) and the responds in the opposite arm with needle inserted (skin blood flow, skin colour, erythema and skin thickness).

Part 3:
The changes induced by cannula insertion were calculated as the differences before and after insertion and analyzed by a paired t-test (skin blood flow, skin colour and erythema).
Paired t-test was used to evaluate the changes in skin blood flow, skin colour, erythema and skin thickness before and after local anaesthesia established in the control arm where no probe was inserted in order to assess the effect of the local anaesthesia itself.
In addition within each study, a t-test was performed in order to test whether probe depth had any influence on the measured responds to needle insertion (skin blood flow, skin colour, erythema and skin thickness).

10.7 *In vivo* microdialysis

Hairless rats were used to study the penetration of betamethasone 17-valerate from different vehicles by *in vivo* cutaneous microdialysis (Figure 15, colour insert, rear inside cover).
Furthermore, a few studies of cutaneous microdialysis in rats *in vivo* after oral administration of fusidic acid and betamethasone 17-valerate were performed.
The rats were anaesthetized with pentobarbital sodium 50 mg/kg injected into the peritoneal cavity. Supplementary injections of pentobarbital sodium (10 mg/kg) were given every 90 minutes. A 3 cm long microdialysis membrane (Gambro GFE 18, Gambro Dialysaten AG, Hechingen, Germany, outer diameter 216 µm, wall thickness 8 µm, 2,000 Da molecular weight cutoff) was inserted in the rat skin. Tubings were glued to the dialysis membrane and connected to a microinjection pump (CMA/100, CMA/Microdialysis, Stockholm, Sweden). The probe was perfused with Ringer isotonic solution (SAD, Denmark). Sampling of dialysate was started 30 min after insertion of the microdialysis probe. Six dialysate samples of 90 µl were collected in 300 µl glass test tubes. After the experiment the probe depth was assessed by ultrasound imaging.

10.7.1 Placebo studies
In untreated rats two microdialysis probes were inserted in the skin and samples were collected using a 2 µl/min flow . The samples were investigated for compounds or background noise which could interfere with the analysis of betamethasone 17-valerate. Samples of 80 µl were collected. 125 µl of acetonitrile: buffer (50:50) was added to the samples, mixed and stored at 5 °C until analysis by HPLC.

10.7.2 Topical application of 4% betamethasone 17-valerate in ethanol
A solution of 4% betamethasone 17-valerate in ethanol (400 µl) was applied on 3 layers of filter paper (3.0 X 2.5 cm) placed on the skin. The papers were kept in place by a frame of Comfeel® (Coloplast A/S, Kokkedal, Denmark). An impermeable tape (Blenderm®, Coloplast A/S, Kokkedal) was applied to form an occlusive system. A bandage of Brend-a-Rete® (no.2, Artsana, Como, Italy) covered the application system and prevent the rat from tearing it of. The solution was applied on both sides of the rats twice a day for two days and before microdialysis was started in the morning of the third day. Two microdialysis probes were inserted in each test area, a total of four probes per rat. PTFE inlet tubings (Bolinder Laborgeräete, Lauder, Köningshofen, Germany) were glued to the membrane (2 cm), and at the efferent side three cm Peek tubings (MF5366, CMA/microdialysis, Stockholm, Sweden) were connected. The perfusion rate was 2 µl/min. Samples of 80 µl were collected. 125 µl of acetonitrile: buffer (pH 4.5) (50:50) was added to the samples and mixed to prevent betamethasone 17-valerate from conversion to betamethasone 21-valerate (Kubota et al., 1994a). The samples were stored at 5 °C before analysis for betamethasone 17-valerate. Four rats were used in the study.

10.7.3 Topical application of 4% betamethasone 17-valerate in 5% ethanolic IPM
Topical application of betamethasone 17-valerate in an ethanol solution containing 5% isopropyl myristate was performed in four hairless rats. The investigation was carried out similarly to the study described above with 4% betamethasone 17-valerate in ethanol.

10.7.4 Topical application of 4% betamethasone 17-valerate after SLS provocation
Sodium lauryl sulphate (SLS, Sigma Chemical, St. Louis, MO, USA) in 600 µl water (10%) was applied on the skin on each side of the back for 2 hours. The same dressing as described above was used for the SLS provocation study. TEWL was measured before and after SLS application to verify the damage to the skin barrier caused by the SLS (Agner and Serup, 1993). After 16 hours TEWL was measured and 4% betamethasone 17-valerate in 100 % ethanol was applied to the skin. The remaining part of the investigation followed the procedure described above. Four rats were studied.

10.7.5 Systemic administration of fusidic acid, betamethasone 17-valerate and calcipotriol.
Oral administration of fusidic acid, betamethasone 17-valerate and calcipotriol in a suspension vehicle was given to rats using a feeding tube. Two rats received 312.5 mg/kg fusidic acid for three days. One rat was given 188 mg/kg calcipotriol and two rats 158.0 mg/kg betamethasone 17-valerate. Microdialysis sampling was started 90 minutes after administration of drugs. Two microdialysis probes, 3 cm in length, were inserted on each side of the rat. Flow rate was 3 µl/min, 80 µl samples were collected. The fusidic acid and betamethasone 17-valerate samples were treated as described above. Calcipotriol samples were untreated prior to analysis.

When the *in vivo* results are plotted as dialysate concentrations as a function of time, the points are placed in the middle of the sampling interval. The dialysate concentration represents the average concentration of the sampling interval.

11.0 Results and discussion

11.1 Development of an HPLC method for fusidic acid

11.1.1 Derivatization in an aqueous micellar system

When the derivatization was performed in the aqueous micellar system numerous peaks appeared in the chromatograms from blank samples without fusidic acid. One of the peaks was located just besides the peak of the derivative of fusidic acid and interfered with the analysis of fusidic acid.

This impurity peak could be referred to Arkopal N-130 as it only appeared when Arkopal was incubated with the reagent BrMMC. Different mobil phases and different columns were tested in order to obtain a better separation between the derivative and the impurity which, however, did not result in a significantly better separation. Purification of Arkopal N-130 by extraction with organic solvents (isopropanol, cyclohexane, heptane, methanol, ethanol, benzene, toluene and acetonitrile) was investigated, but the impurity could not be removed. Finally Arkopal N-130 was substituted with Tween 20 and Tween 80, but without success.

This problem forced us to investigate the possibility of performing the derivatization in acetone. This requires removal of the water phase prior to derivatization.

11.1.2 Derivatization procedure in acetone

Also when the derivatization was performed in acetone a disturbing impurity was present at the position of the fusidic acid derivative. A validation with respect to linearity, precision and blank values was performed in order to judge the usefulness of the method.

Linearity

The linearity was determined in the range 0.0-1.5 µg/ml using three standard curves on three different days. A linear relationship was found between the standard concentrations and peak heights, range of r^2 = 0.9705 - 0.9984 (n= 9) (Table 4). The use of an internal standard might result in an even better correlation. The intercept with the y-axis and the lower correlation when the curve was forced through zero suggested that a peak is measured when the 0.0 µg/ml standard is injected.

Precision:

Standard	Day 1	Day 2	Day 3
0.25 µg/ml	12.60	9.66	10.40
1.00 µg/ml	6.80	5.00	3.51

Table 5 *The relative standard deviation of peak heights of 6 injections of 0.25 and 1.00 µg/ml fusidic acid on 3 different days.*

The relative standard deviation was higher for the low standard 0.25 µg/ml than for standard 1.00 µg/ml (Table 5). It was not investigated which parameter that contributed to the variation in peak heights. It can be the derivatization itself, the volume of standard or the chromatographic system.

Blank value

12 standards of 0.00 µg/ml were injected and the peak heights were calculated (Table 6). The variation in the peak heights of the blank was very large. The range was 26010 - 773674, which corresponds to 45 µl of a standard range 0.03 - 0.89 µg/ml added to the reaction vial (calculated from the curve forced through zero).

144668	773674	48900	26010
34112	42283	33695	50913
28635	34059	195837	44255

Table 6 *Peak heights of 12 injections of blank.*

The heights of the blanks were considerable and had a large variation. For this reason it was impossible to make corrections by subtracting the blank from the sample values in the calculations.

Some experiments were performed to investigate the origin of the blind peak. Emission and excitation spectra were obtained using a fluorescence detector. No useful differences between the spectra of the blind peak and the fusidic acid derivative peak was found.

All the components of the reaction mixture were tested by injecting acetone solutions into the HPLC. None of the substances caused a peak. Then the substances were combined in different ways and treated at 60 °C, again to test for appearance of the blind peak. The presence of BrMMC and potassium carbonate in the solution with or without acetic acid and freeze drying of the blank vial resulted in a blind peak. A mixture of 18-crown-6 ether and potassium carbonate did not show any blind peak. The basic milieu caused by potassium carbonate might influence BrMMC during incubation resulting in a new molecule, which resembles BrMMC in the chromatographic behaviour. This possibility was investigated in a series of experiments. Potassium carbonate was substituted by sodium sulphate, but this totally stopped the reaction. The presence and height of the blind peak might be dependent on the temperature during the reaction and the time of incubation. Different temperatures were investigated (40 °C, 50 °C, 60 °C and 80 °C). 40 °C lead to a decrease in peak heights as compare with 60 °C. 50 and 60 °C gave similar peak heights. 80 °C resulted in the highest peak of the 0.00 µg/ml standard. When the incubation time was increased from 30 min to 120 min a small increase of the blind peak was seen. A small and insignificant increase of the blind peak was also found when 10 mg potassium carbonate was added instead of 5 mg.

These experiments suggested that interactions between BrMMc and potassium carbonate are causing the observed peak from the blank solution or that one of the two substances might be contaminated. A spectrum of BrMMC by Nuclear Magnetic Resonance was run, but this did not give any conclusive results concerning the purity of BrMMC.

	Intercept with y-axis	Slope	r^2	Forced through zero	Slope	r^2
Day 1	38799.44	4599.82	0.9944	(0,0)	4830.92	0.9905
	10435.03	5724.10	0.9979	(0,0)	5665.72	0.9977
	64618.54	5120.33	0.9841	(0,0)	5482.46	0.9776
Day 2	149851.13	4253.51	0.9927	(0,0)	5224.66	0.9471
	285815.88	3951.00	0.9833	(0,0)	4587.16	0.8925
	185308.85	3338.90	0.9839	(0,0)	4587.16	0.8925
Day 3	59044.69	5307.86	0.9984	(0,0)	5652.91	0.9936
	97520.15	6199.62	0.9705	(0,0)	6775.07	0.9616
	96207.47	5577.25	0.9901	(0,0)	6146.28	0.9794
Mean ± SD	109733.46 ± 85260.43	4896.92 ± 927.84	0.9884 ± 0.0089		5439.15 ± 728.98	0.9592 ± 0.0410

Table 4 *The linearity of the derivatization in acetone for fusidic acid concentration from 0.0 to 1.5 μg/ml*

No further efforts were made to solve the problem with the blind peak, which is likely to be the reason for the variable precision of the method. It was judged that the limit of quantitation would be higher than expected, estimated to 50 ng/ml. At the same time in our laboratory an HPLC method using UV-detection and a narrowbore column had been developed for analysis of the active drugs in Fucicort® (Leo Pharmaceutical Products Ltd) i.e. fusidic acid and betamethasone 17-valerate. This method had acceptable limits of quantitation namely 50 ng/ml and 5 ng/ml for sodium fusidate and betamethasone 17-valerate, respectively. This method was therefore used in the *in vivo* microdialysis studies with fusidic acid and betamethasone 17-valerate.

11.2 In vitro microdialysis (Paper I)

The aim of the *in vitro* microdialysis studies was to determine parameters that influence recovery *in vitro*. These parameters will also influence recovery *in vivo*. Such basic knowledge is needed for subsequent *in vivo* microdialysis studies. The *in vitro* studies included microdialysis experiments with different kinds of drugs which allowed us to investigate the influence of the physico-chemical characteristics of the drug itself on recovery and loss. Furthermore, a method for estimation of the true concentration in the medium surrounding the probe was validated.

11.2.1 Determination of recovery and loss

The four tested drugs have different recoveries (Table 7). The more lipophilic the compound is the lower is the recovery (Table 3 and table 7). A similar pattern was not seen for the loss. The reproducibility of recovery and loss were found good in the *in vitro* microdialysis experiments.

Compound	Recovery %	Loss %	Recovery/ loss Ratio
Glucose	74.65 ± 0.99 n=3	75.69 ± 1.88 n=3	0.99
Sodium fusidate	40.99 ± 5.57 n=32	57.38 ± 7.51 n=32	0.71
Betamethasone dipropionate	36.40 ± 4.05 n=15	85.90 ± 3.18 n=6	0.42
Calcipotriol	31.12 ± 4.60 n=11	84.45 ± 5.08 n=12	0.37

Table 8 *Recovery, loss and ratio of recovery to loss. Values are mean ± SD. n: number of microdialysis probes.*

Glucose showed equal recovery and loss. Sodium fusidate, betamethasone dipropionate and calcipotriol all showed significant difference between recovery and loss demonstrated by the recovery/loss ratio, which is substantially below one. The deviation from one becomes more pronounced as the lipophilicity of the compound increases. The difference between recovery and loss may be caused by interactions between the diffusing compound and the dialysis membrane (Amberg and Lindefors, 1989). In our study these interactions were dependent of the lipophilicity of the dialysed compound. Burgio and Mcnamara (1993) also found a difference between recovery

and loss *in vitro* for various lipophilic benzodiazepines and antipyrine.

11.2.2 Parameters affecting recovery and loss

The recovery was dependent on the stirring rate, temperature, perfusion flow rate and the dialysis membrane length. The recovery of glucose was lower without stirring as compare with stirring of the test solution. Increasing the temperature in the standard solution leads to a higher recovery of glucose. Recovery of glucose, sodium fusidate and betamethasone dipropionate increased as the flow rate decreased, since there was more time for transport across the dialysis membrane (Figure 16).

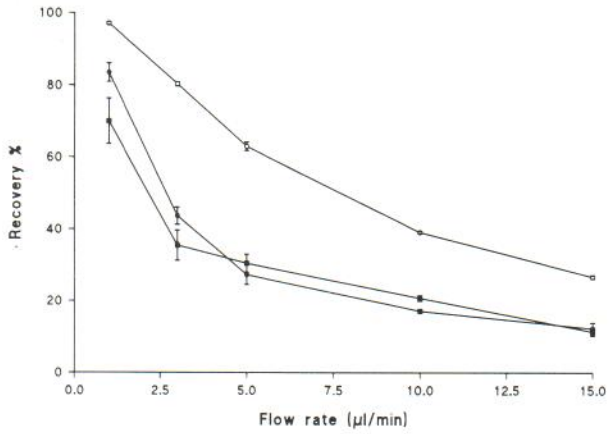

Figure 16 *The relationship between in vitro recovery and perfusion flow rate (n=3). Open: glucose 5.2 mM; Solid: sodium fusidate 50 µg/ml; Solid square: betamethasone 17,21-dipropionate 300 ng/ml*

The recovery of glucose and sodium fusidate increased as the membrane length increased until recovery approached a maximum. The maximum for sodium fusidate was only about 60%, which could not be explained on the basis of the present study (Figure 17).

A linear relationship between the concentrations in the surrounding medium and the concentrations in the dialysate was found for glucose, betamethasone dipropionate and calcipotriol with the slope illustrating recovery. This indicates that recovery is independent of the drug concentration in the medium surrounding the probe. When the concentration of the compound in the medium surrounding the probe increases or decreases, the concentration gradient across the membrane changes accordingly, thus keeping recovery constant. Thus, a one-way analysis of variance showed that recovery changed by changing the concentration of sodium fusidate in the outer medium. Recovery of sodium fusidate increased by increasing concentration, ranging from 34% to 44% in the interval 5-200 µg/ml. A possible reason may be a concentration dependent interaction between the dialysis membrane and sodium fusidate. The membrane becomes saturated as the concentration increases making the recovery higher. This sodium fusidate-membrane interaction may also be the reason for the concentration dependent loss of sodium fusidate. As the concentration in the perfusate increased, saturation of the membrane increased, loss became lower and approached the value of recovery. The difference between recovery and loss is dependent on concentration. Loss of betamethasone dipropionate was independent of the concentration in the perfusate. Landholt at al. (1991) found concentration dependent recovery of cysteine and glutathione *in vitro* due to compound interaction with the dialysis membrane.

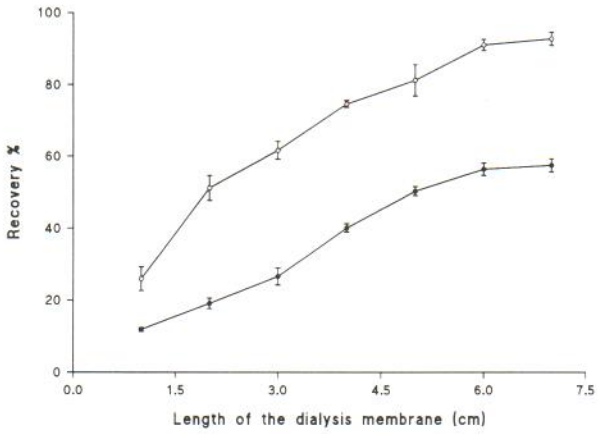

Figure 17 *The relationship between in vitro recovery and dialysis membrane length. Flow rate 3µl/min (n=3). Open: glucose 5.2 mM; Solid: sodium fusidate 50 µg/ml*

When albumin was added to the medium surrounding the probe, the unbound concentration became so low that dialysate concentration of sodium fusidate was undetectable. Rieutord et al. (1995) stated that fusidic acid is a drug sensitive to the albumin status of the media in which it is added.

The observed influence on recovery of flow rate and dialysis membrane length satisfied the diffusion theory of Fick.

Beside the above mentioned parameters the tubing which is glued to the membrane can influence the reproducibility of recovery. We had to use another tubing for betamethasone and calcipotriol because unstable recoveries were noted when the nylon tubing normally used for sodium fusidate and glucose was used. This indicates that the microdialysis technique must be adapted to the investigated compound and that lipophilic compounds may be more demanding as regards to methodological changes. Carneheim and Ståhle (1991) studied different perfusion media for lipophilic compounds and found a higher recovery of oleate when albumin was added to the perfusion fluid.

11.2.3 Point of no-net-flux

The true glucose concentration in the surrounding medium and the recovery *in vitro* was found using the point of no-net-flux method. A linear relationship between the perfusate concentration and the net increase in the dialysate of glucose was established (Figure 18). The estimated concentration (the intercept with the x-axis) deviated only by 1% from the true concentration. In contrast to glucose, two lines were obtained when the point of no-net-flux method was performed with sodium fusidate. One line below the concentration in the surrounding

medium, corresponding to recovery, and another line above the surrounding concentration, corresponding to loss, were found (Figure 19).

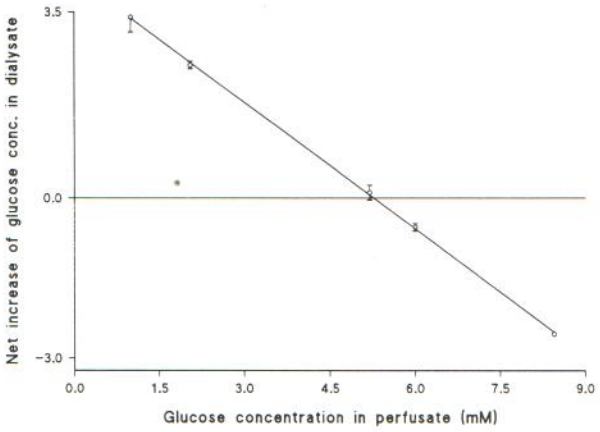

Figure 18 *In vitro calibration of glucose. The microdialysis probe was perfused with 1.0; 2.0; 5.2; 6.0 and 8.7 mM glucose. 5.2 mM glucose was added to the surrounding medium. The intercept with the perfusate concentration axis is the predicted concentration in the surrounding medium. The slope of the line is the recovery of glucose.*

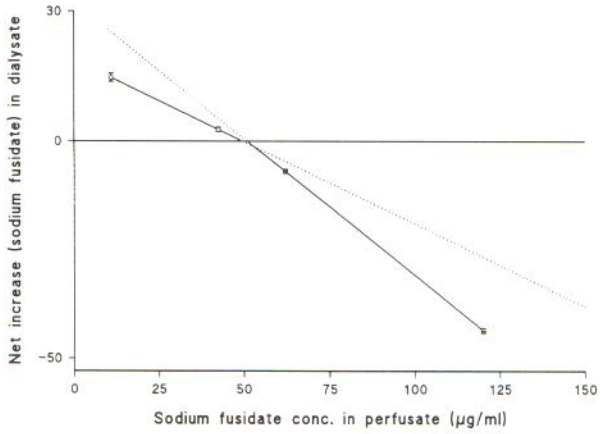

Figure 19 *In vitro calibration of sodium fusidate. The microdialysis probe was perfused with 10.0; 40.0; 60.0 and 100.0 µg/ml sodium fusidate. 50.0 µg/ml sodium fusidate was added to the surrounding medium. Dotted lines represent calculated lines.*

Theoretically the two lines are supposed to cross each other at the intercept with the x-axis, corresponding to the concentration in the medium surrounding the probe. The estimated concentration deviates by 10.58% ± 6.54 (n=20) from the true concentration. The difference between recovery and loss for sodium fusidate made it necessary to modify the point of no-net-flux method, i.e. using two linear relationships instead of one as for glucose. In addition, a large variation was found for the estimated concentration due to influence of sodium fusidate concentration on recovery and loss.

Lipophilic drugs tend to have different recovery and loss in contrast to more hydrophilic drugs. The differences between recovery and loss are matters of concern using the reference methods or the point of no-net-flux method to estimate the true extracellular concentration. In the reference methods recovery and loss need to be equal since *in vivo* loss is used to estimate *in vivo* recovery. This makes drugs like sodium fusidate and lipophilic drugs unsuitable for study by the reference methods. The difference in recovery and loss makes the point of no-net-flux more difficult and laborious since a larger number of perfusate concentrations have to be used.

11.2.4 Summary of in vitro microdialysis

Recovery depends on the perfusion rate, stirring rate, temperature and membrane length. Recovery and loss were independent of the concentration in the medium surrounding the microdialysis probes, except for sodium fusidate.

The more lipophilic substances have lower recoveries and lower ratio of recovery to loss.

The point of no-net-flux method is more laborious and the reference method are not suitable for substances having different recovery and loss, i.e. sodium fusidate, betamethasone dipropionate and calcipotriol.

Another concern is the low unbound concentration of sodium fusidate when protein was added to the test solution. These results indicates that *in vivo* microdialysis with sodium fusidate and other highly protein bound drugs would face problems and increase the demand for very sensitive analytical methods.

Microdialysis with lipophilic compounds and highly protein bound drugs cause lower recoveries and more problematic interpretations of the results. Furthermore the tubings had to be carefully selected to obtain stable and high recoveries. Microdialysis *in vivo* may therefore be limited to the study of hydrophilic compounds.

11.3 Trauma studies and effects of anaesthesia on rat skin.

11.3.1 Skin blood flow in awake and anaesthetized rats

The skin blood flow was compared in awake and anaesthetized rats, since skin perfusion can influence drug penetration in the skin. In the awake rats the recorded skin blood flow corresponds to their level of physical activity. When the rats moved around in the bowl the skin blood flow was higher and more unstable as compared with the tranquil rats. It was only possible to use the skin blood flow values for sleeping or tranquil rats.

Halothane anaesthesia

In five rats which were measured both in the awake and the anaesthetized conditions, the difference between the skin blood flow in the awake condition and in the anaesthetized condition of each rat was calculated (Table 8). There was found no significant difference in skin blood flow between the two conditions. A large variation in the difference between the two conditions was, however, observed.

Rat	Awake	Anaesthetized	Difference in skin blood flow
1	8.33	7.00	1.33
2	11.33	10.00	1.33
3	12.68	8.00	4.68
4	11.56	13.90	-2.34
5	5.00	9.00	-4.00
Mean ± SD	9.78 ± 3.12	9.58 ± 2.66	0.2 ± 3.42

Table 9 *Difference between skin blood flow (arbitrary units) in the awake state and under halothane anaesthesia measured by laser Doppler flowmetry.*

Pentobarbital sodium anaesthesia

In six rats anaesthetized with pentobarbital sodium given in the peritoneal cavity, the difference in skin blood flow between the awake condition and the anaesthetized condition was calculated (Table 9). For pentobarbital sodium a significant difference in skin blood flow was found. The skin blood flow was in every rat lower in the anaesthetized condition as compared with the awake condition.

Rat	Awake	Anaesthetized	Difference in skin blood flow
1	13.33	9.50	3.83
2	12.67	6.50	6.17
3	7.70	5.75	1.42
4	11.67	5.75	5.92
5	12.50	5.83	6.67
6	10.00	7.00	3.00
Mean ± SD	11.31 ± 2.11	6.72 ± 1.45	4.50 ± 2.08

Table 10 *Difference between skin blood flow (arbitrary units) in the awake and under pentobarbital sodium anaesthesia measured by laser Doppler flowmetry.*

The mean skin blood flow of rats under pentobarbital sodium anaesthesia was lower than in rats under halothane anaesthesia and showed less variation, although not statistic significant, i.e. 6. 72 ± 1.45 and 9.58 ± 2.66 for pentobarbital sodium and halothane, respectively. This study suggests that pentobarbital sodium anaesthesia decreases the mean skin blood flow. In contrast halothane anaesthesia does not influence the mean skin blood flow. However, the larger variation in the halothane experiment may reflect some vascular effect of the halothane anaesthesia. Hoffman et al. (1982) found that skin blood flow was decreased as a function of halothane concentration in young and aged rats using radio active microspheres. No significant difference between young and aged rats was found.

11.3.2 Effect of anaesthesia on basal skin blood flow (Paper II)

Basal skin blood flow was measured by LDPI in two groups of rats, i.e. rats anaesthetized either by halothane or by pentobarbital sodium. This is an important parameter since blood flow changes in the skin may have profound effects on dermal pharmacokinetics (Singh and Roberts, 1994).

Halothane anaesthesia

The skin blood flow in rats anaesthetized with halothane was not constant. A significant drop was observed 15 min after induction (Figure 20) (mean decrease 0.49 ± 0.52, $p<0.05$, paired t-test). In eight rats a mean of 3.90 ± 0.41, range 2.65 - 5.57 was found during five hours anaesthesia. This shows a significant inter-individual variation between the rats regarding the mean blood flow in five hours ($p<0.05$).

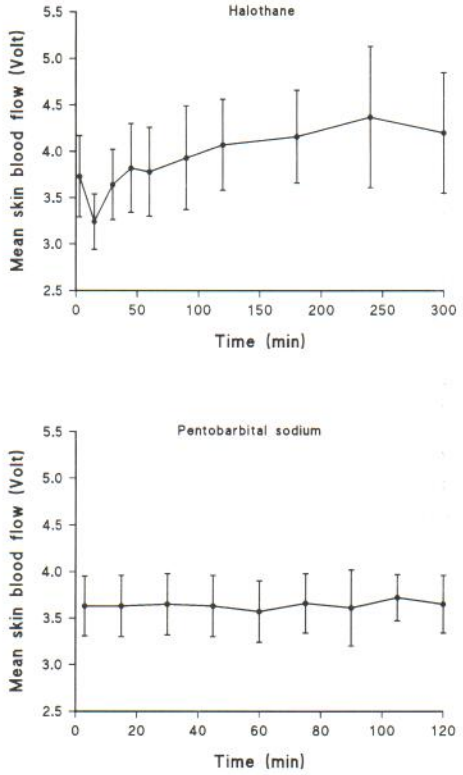

Figure 20 *Mean basal skin flow in rats anaesthetized with halothane (mean ± SD, n=8) and with pentobarbital sodium (mean ± SD, n=4) as a function of time.*

Pentobarbital sodium anaesthesia

The skin blood flow remained constant during a two hours period under pentobarbital sodium anaesthesia (Figure 20). The mean skin blood flow was 3.64 ± 0.29, range 3.04 - 4.01. The mean skin blood flow varied among the rats ($p<0.05$).

The skin blood flow was found more stable in rats anaesthetized with pentobarbital sodium than with halothane. The con-

stant blood flow in rats under pentobarbital anaesthesia is illustrated by the minor standard deviation in mean blood flow of each rat ranging from 0.05 to 0.14 as compared with rats under halothane anaesthesia, 0.27 to 0.68. The total mean blood flow in rats anaesthetized with pentobarbital sodium is lower than with halothane. A lower total range of mean blood flow after pentobarbital sodium anaesthesia than after halothane is also found. This is in accordance with the results of laser Doppler flowmetry. The initial drop in skin blood flow in rats anaesthetized with halothane is probably due to the high dose needed at the induction of anaesthesia. Lindgren et al. (1965) showed that more than 2% halothane causes variable perfusion of the skin as the blood flow was dependent on the depth of anaesthesia. In the present study the halothane dose is adjusted according to a subjective judgment of the depth of anaesthesia. This might lead to varying anaesthesia and varying skin perfusion. The study by the laser Doppler flowmeter suggested that halothane did not influence the mean skin blood flow. It is as mentioned above possible that effects of halothane on skin blood flow may be overlooked due to the large variation in the laser Doppler flowmetry study.

We have shown, however, that the skin blood flow is unstable in rats anaesthetized with halothane, but stable in rats anaesthetized with pentobarbital sodium. Pentobarbital is therefore preferable as anaesthetic in future microdialysis studies. In this way we can control the influence of changes in skin blood flow on skin penetration.

11.3.3 Effect of insertion trauma on skin blood flow ***(Paper II)***

The effect of insertion of a microdialysis probe on skin blood flow was measured in rats by LDPI. The rats were anaesthetized either with halothane or pentobarbital sodium.

Halothane anaesthesia

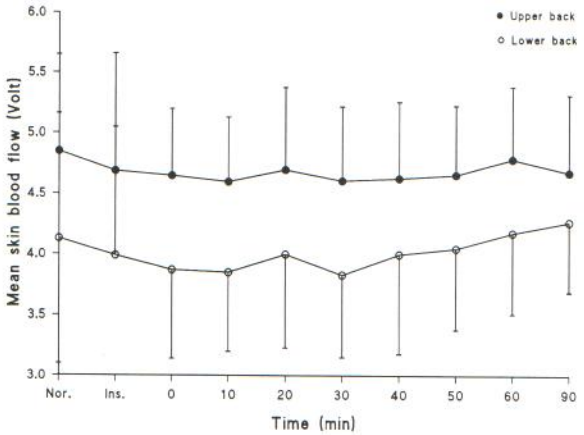

Figure 21 *Mean skin blood flow in rats as a function of time before and after insertion of a microdialysis probe (mean ± SD, n=16 insertions). Rats anaesthetized with halothane. Nor.: before insertion. Ins.: immediately after insertion.*

Figure 21 shows the mean blood flow as a function of time (n=16). Upper part and lower part of the back of one side of the rats are plotted. The mean skin blood flow in the upper part (insertion point of cannula) is higher than in the lower part (outlet point), i.e. 4.73 ± 0.56 and 4.00 ± 0.67 for upper and lower part respectively. In some rats a decrease in skin blood flow is observed in the beginning of the experiment. There was no significant difference between the blood flow before needle insertion and the blood flow immediately after insertion.

Pentobarbital sodium anaesthesia

As seen with halothane anaesthesia the mean skin blood flow was higher in the upper back versus the lower back i.e. 4.68 ± 0.35 and 3.87 ± 0.39 for upper and lower part, respectively (Figure 22).

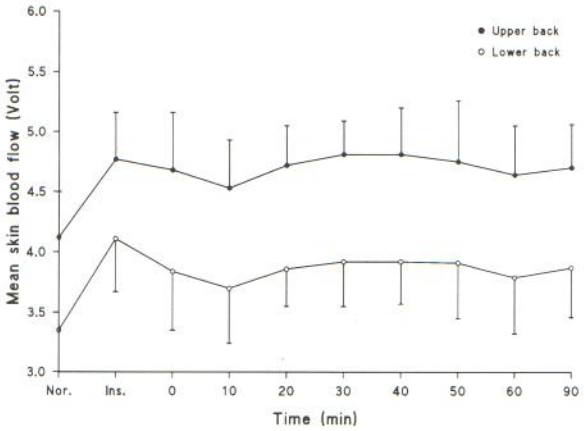

Figure 22 *Mean skin blood flow in rats as a function of time before and after insertion of a microdialysis probe (n=4 insertions). Rats anaesthetized with pentobarbital sodium. Nor.: before insertion. Ins.: immediately after insertion.*

There was a significant increase in blood flow following cannula insertion. The mean increase was 0.92 ± 0.19, range 0.73 - 1.17 ($p < 0.05$, paired t-test, mean of upper and lower scans of each insertion). Approximately 30 minutes after the insertion the blood flow diminished and became stable, at a level above baseline.

The needle insertion influenced skin blood flow in rats anaesthetized with pentobarbital sodium. In contrast the insertion of the probe did not cause skin blood flow changes in rats anaesthetized with halothane. The large variation in basal skin blood flow under halothane anaesthesia might dim a minor increase in blood flow following needle insertion. Especially the drop in skin blood flow 15 minutes after induction with halothane may influence the effect of the trauma. The baseline value in skin blood flow using pentobarbital sodium was, however, stable. Another explanation could be a difference in inhibition of the events of the trauma by the two anaesthetics. Pipkorn and Andersson (1987) stated that flare but not wheal response to allergen and histamine skin-prick test was inhibited by topical dermal anaesthesia. However, in the rats no flare or wheal were visually apparent after needle insertion although some minor components of factitial urticarial reaction cannot be excluded.

The difference in skin blood flow between the upper and lower part of the back of the rats is due to an anatomical difference in the rat and not to an effect of insertion since the difference was obvious before insertion.

11.3.4 Effect of insertion trauma on histamine release (Paper II)

The effect of insertion of a microdialysis probe on histamine release was studied. Furthermore, codeine phosphate induced histamine release was studied. The rats were anaesthetized either with halothane or pentobarbital sodium.

Trauma induced histamine release

The histamine release peaks momentarily at needle insertion, followed by an exponential decline to the baseline level at 20 ng/ml (Figure 23).

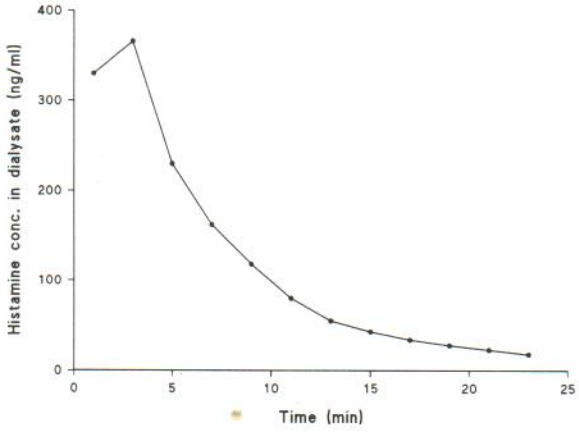

Figure 23 *Trauma induced histamine release. Histamine concentration in dialysate as a function of time after insertion of a dialysis probe. The rat was anaesthetized with halothane.*

The decline to normal level lasted 20 minutes after needle insertion. Mean C_{MAX} ± SD. for trauma induced histamine release were 274.0 ng/ml ± 63.76, range 221 ; 366 and 148.25 ng/ml ± 90.79, range 66 ; 278 for rats under halothane and pentobarbital sodium anaesthesia, respectively. A half-life of 4.59 ± 1.63 min^{-1} was found (correlation coefficient r^2 =0.9863 ± 0.0076, n = 8).

Codeine phosphate induced histamine release

In figure 24 the histamine release after injection of 50 µl 1 mg/ml codeine phosphate intradermally in a halothane anaesthetized rat is shown. The increase in histamine developed more slowly as compared to the increase after the insertion trauma. The curve reflects initially the local absorption and distribution of codeine phosphate and subsequently elimination of histamine. The mean C_{MAX} ± SD was 104.25 ng/ml ± 24.38, range 79 - 133, for halothane and pentobarbital sodium anaesthetized rats. The elimination half-life of histamine was 7.53 ± 0.88 min^{-1} (correlation coefficient r^2 = 0.9717 ± 0.0083, n = 8).

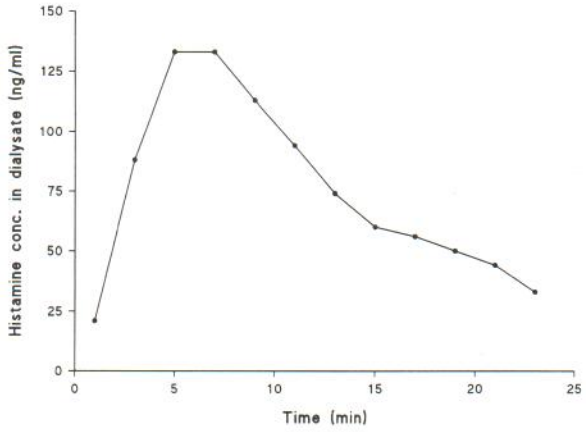

Figure 24 *Codeine phosphate induced histamine release. Histamine concentration in dialysate as a function of time after codeine provocation. The rat was anaesthetized with halothane.*

Trauma induced and codeine induced histamine release in rat skin was demonstrated in this study. This would be expected to vary as cutaneous blood flow responses to histamine and histamine plays an essential role in the triple-response (histamine secretion, wheal and flare) (Tur et al., 1994). A large variation was seen between rats both in trauma induced histamine release and codeine phosphate induced histamine release. Large variations in interindividual histamine release, distribution of mast cells and histamine content in human skin has been reported by others (Petersen et al., 1994; Eady et al., 1979), and rat skin may be equally variable. The variation observed in the rat may also be related to different probe depths, since the deeper dermis of the rat has a much higher density of mast cells than the superficial layer (Coleman and Salvatore, 1969; Riley and West, 1969).

No major difference was found between the trauma induced histamine release under halothane and pentobarbital sodium anaesthesia. However, it seemed that the trauma under halothane induced a stronger histamine release than trauma under pentobarbital sodium anaesthesia. Local anaesthetics such as lidocaine, procaine and tetracaine were shown to inhibit compound 48/80-induced histamine release from isolated rat mast cells (Kazimierczak et al., 1976).

After the intradermal injection of codeine a wheal was evident. The mean C_{MAX} for the codeine induced histamine release was lower than for the trauma induced histamine release. The codeine experiment was performed just after the trauma induced histamine release study, however, on intact skin of the same body region, some histamine in the mast cells might already have been depleted. This might explain the lowering of the histamine peak concentration of the codeine induced release.

The histamine release following needle insertion appears much larger in rat skin as compared to human skin and guinea pigs. The baseline level is also higher in rat skin than in skin of man and guinea pig (Petersen et al., 1994; Andersson et al., 1996; Okahara et al., 1995). Petersen et al. (1994) found a

monoexponential decline in the histamine elimination with a half-life of 3.81 ± 0.28 min^{-1} in humans after substance P provocation, which is in accordance with our finding of 4.59 ± 1.63 min^{-1} in the rat.

11.3.5 Effect of insertion trauma on skin thickness (Paper III)
The effect of insertion of the microdialysis probe on skin thickness was investigated by ultrasound scanning. The rats were anaesthetized either with halothane (n =14 insertions) or pentobarbital sodium (n= 6 insertions). In addition the probe depth was measured.

Halothane anaesthesia
Before needle insertion the skin thickness on the back of the rats was 1.12 ± 0.08 mm, range 1.00 - 1.26. Increase in skin thickness after cannula insertion was demonstrated. The skin thickness peaked 15-30 minutes after insertion. The increase 15 minutes after needle insertion was 0.42 ± 0.09 mm, range 0.30 - 0.63 ($p< 0.05$). This represents an increase of 37.5 % above basal skin thickness. 120 minutes after needle insertion the skin thickness had not declined to normal skin thickness as measured before insertion.
The microdialysis membrane has a outer diameter of 0.216 mm. If the probe thickness was subtracted from the skin thickness measurements, the skin thickening at 15 minutes was still significant, i.e. 0.20 mm ± 0.09, range 0.08 - 0.40.

Pentobarbital sodium anaesthesia
In this group of rats the initial skin thickness was 1.00 mm ± 0.10, range 0.85 - 1.13. Fifteen minutes after the insertion the mean increase was 0.38 mm ± 0.09, range 0.27 - 0.50 ($p<0.05$), which is a relative increase of 38.0 %. The increase was still significant even if the outer diameter of the probe was subtracted (0.10 mm ± 0.07). After 120 minutes the skin thickness had not reached the basal level.

Probe depth
The dialysis membrane was detected very easily with 20 MHz ultrasound scanning (Figure 25, colour insert, rear inside cover). The probe depth (measured from epidermal surface to echostructure of the probe within the dermis) depended of skin thickness. As the skin thickness increases, the probe depth becomes larger. The mean probe depth of the 14 insertions in the halothane anaesthetized rats was 0.92 mm ± 0.12, showing little variation. The mean probe depth of the six insertions in pentobarbital sodium anaesthetized rats was 0.78 mm ± 0.07. The difference between probe depths in the two groups is probably caused by the difference in basal skin thickness between the two groups of rats.
In both groups the variation of probe depth of any single probe measured at three different sites along the probe was occasionally high, illustrating that the probe cannot be inserted in exactly the same depth along the full four centimetres of length. The microdialysis probe was generally inserted in the lower part of the dermis.
We found a significant increase in skin thickness after needle insertion in rats anaesthetized with halothane and pentobarbital sodium (Figure 26).

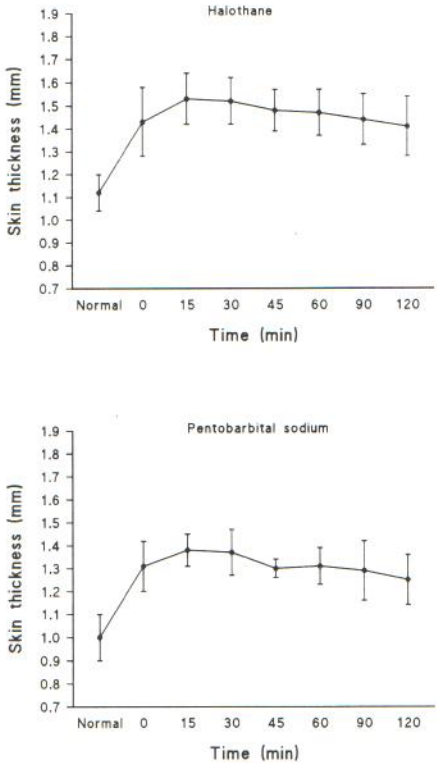

Figure 26 *Mean skin thickness as a function of time before and after insertion of a microdialysis probe (mean ± SD). Rats anaesthetized with halothane (n=14 insertions) and pentobarbital sodium (n=6 insertions).*
Nor.: before insertion. 0: immediately after insertion.

The increase in skin thickness is expected to be due to oedema formation in the skin. During the experiments low echogenic picture elements were obvious in the ultrasound measurements, especially fifteen to thirty minutes after needle insertion. Black and blue areas, which correspond to low echogenicity on the ultrasound image appeared around the probe (Figure 27, colour insert, rear inside cover). It has previously been demonstrated that the number of low echogenic pixels is proportional to the degree of dermal oedema (Seidenari and di Nardo, 1992). The oedema and resultant thickening appeared rapidly and was fully developed 15-30 minutes after insertion. Oedema was still prominent 120 minutes after needle insertion. No significant difference in skin thickening between rats anaesthetized with halothane and pentobarbital sodium was found.
The microdialysis probe was inserted reproducibly in the lower dermis. None of the probes were placed in the subcutaneous tissues. The reproducibility of the insertion procedure is most important, since the microdialysis process of a skin penetrating drug maybe dependent on probe depth (Andersson et al., 1995a; Müller et al., 1995c). Even though it is possible to place the probe reproducible, it is, however, still difficult to control the exact position of the probe along the full insertion length.
The formation of oedema around the microdialysis membrane

may effect recovery. Oedema increases the volume fraction of the extracellular space surrounding the dialysis membrane thereby increasing the area available for diffusion of drugs towards the membrane and the recovery. Recovery of glucose was increased during the implantation trauma phase and after challenging the skin with histamine, which induced a wheal (Petersen et al., 1992a). However, it is uncertain to which degree the changed volume fraction influences recovery. The skin thickening remains constant in the entire period of the experiment. This might suggest that the recovery also remains constant, provided that other biological factors influencing recovery remain constant.

11.3.6 Summary of trauma studies in rat skin.
The trauma studies in hairless rats showed that insertion of a microdialysis probe caused histamine release and oedema formation and an increase in skin blood flow. Approximately 30 minutes after cannula insertion the skin blood flow had stabilized and skin histamine levels had returned to normal. In contrast the skin thickness was still at a significantly higher level than normal (about 30%). For practical reasons it was decided to start the microdialysis experiment no earlier than 30 minutes after insertion. We have no knowledge of how long time the oedema remains after needle insertion. Furthermore, the experimental period needs to be acceptable in relation to keeping the animals anaesthetized and in a constant state. An equilibration period after needle insertion (the time required for levels to return to normal) of 30 minutes might not be enough for some endogenous compounds, which then may influence the experiment.

11.4 Effects of insertion trauma and anaesthesia on human skin (Paper IV)

11.4.1 Effect of insertion trauma on skin blood flow and erythema with prior anaesthesia, ***part 1***
Seven male and five female subjects participated. Six had the probe inserted in the left arm and 6 in the right arm. The subjects were given a local anaesthetic in both arms. Afterwards a microdialysis probe was inserted in one of the arms. Significant increases in skin blood flow ($p<0.01$), skin colour (a*, erythema, Minolta chromameter) ($p<0.01$) and erythema (Dermaspectrometer) ($p<0.01$) were measured after the insertion of the microdialysis probe (Figure 28,29,30). The skin blood flow had not normalized until 90-120 minutes after the insertion. At least 60-80 minutes was needed for the erythema to normalize as measured by the Minolta chromameter. The time-to-normalization for erythema measured by the Dermaspectrometer was longer, i.e. 100 minutes.
After insertion of a commercial microdialysis probe in the skin Anderson et al. (1994) also found increased skin perfusion measured by LDPI. After 60 min the skin perfusion values were close to resting levels. In the present study 90-120 min elapsed until the skin perfusion had reached baseline level. Petersen et al. (1992a) suggested 90-135 min should elapse after insertion of a microdialysis probe similar to the one we used, according to assessment by laser Doppler flowmetry. The longer period of equilibration might be explained by double insertions, i.e. entry and outlet for the probe we use, as compared with only one insertion needed for the commercial probe.

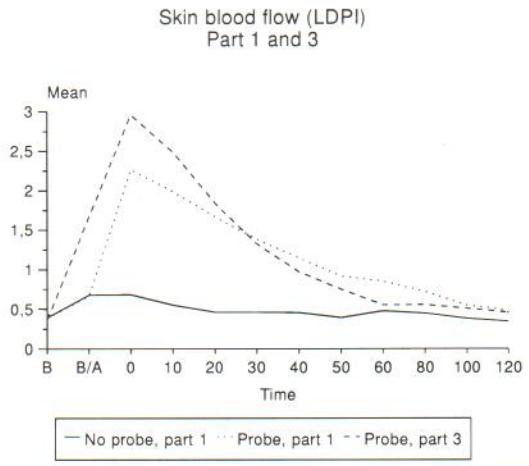

Figure 28 *Mean skin blood flow (Volt) in human skin as a function of time before and after insertion of a microdialysis probe. n=12 insertions for* ***part 1*** *and n=4 for* ***part 3****.*
B: baseline before insertion B/A: baseline after anaesthesia
O: immediately after insertion.

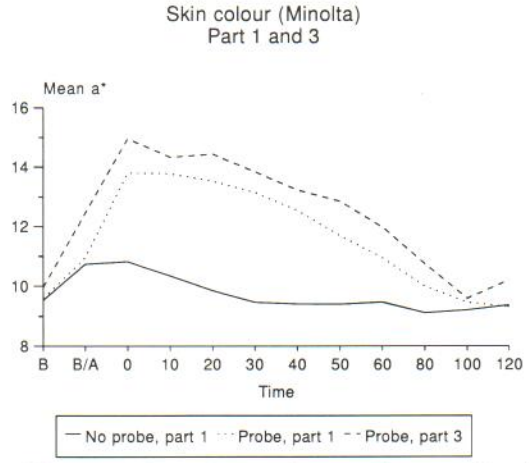

Figure 29 *Mean skin colour in human skin as a function of time before and after insertion of a microdialysis probe.*
n=12 insertions for ***part 1*** *and n=4 for* ***part 3****.*
B: baseline before insertion B/A: baseline after anaesthesia
O: immediately after insertion.

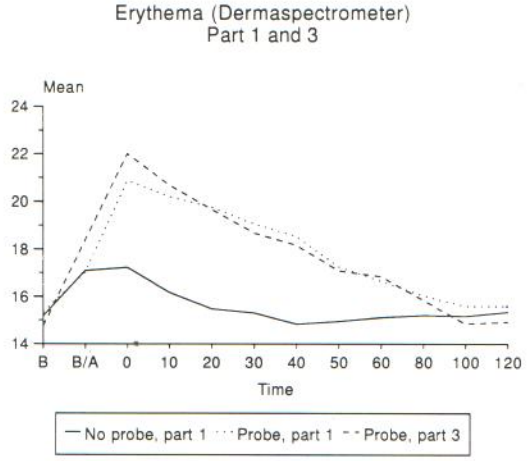

Figure 30 *Mean erythema in human skin as a function of time before and after insertion of a microdialysis probe. n=12 insertions for* ***part 1*** *and n=4 for* ***part 3****. B: baseline before insertion B/A: baseline after anaesthesia O: immediately after insertion.*

11.4.2 Effect of insertion trauma on skin thickness, ***part 2***

Increase in skin thickness was obvious after insertion of the microdialysis probe (Figure 31).

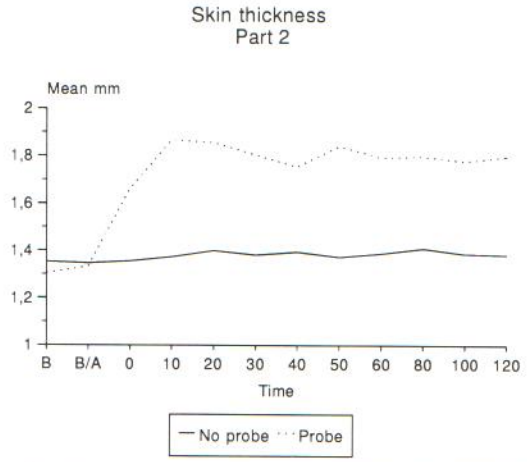

Figure 31 *Mean skin thickness in human skin as a function of time before and after insertion of a microdialysis probe. n=12 insertions for* ***part 1*** *and n=4 for* ***part 3****. B: baseline before insertion B/A: baseline after anaesthesia O: immediately after insertion.*

The difference between the arm with and without insertion of a needle was highly significant in the entire experimental period. The outer diameter of the membrane (0.216 mm) may contribute to skin thickening. However, even if this value was subtracted from all the differences, the significance of the difference remained at any time. Thickening was largest 10 min after needle insertion and decreased a little to a constant level which corresponded to a relative mean skin thickening of 38%. The investigation period was not long enough to estimate when the skin thickness ultimately became normal. The skin thickness might first be normal after the microdialysis probe is removed.

As observed in the hairless rat, the skin thickening in human skin also represents traumatic oedema formation, confirmed by the findings of a low echogenic area around the probe in the ultrasound image known to correspond to oedema.

The relative skin thickening and the relative stability of the oedema after needle insertion are similar in rat and human skin.

11.4.3 Effect of insertion trauma on skin blood flow and erythema without prior anaesthesia, ***Part 3***

Three males and one female subjects were included. Two had the probe inserted in the right arm and two in the left arm without prior anaesthesia. Increases in skin blood flow ($p<0.01$), skin colour ($p<0.01$) and erythema ($p<0.01$) were found after needle insertion (Figure 28,29,30). Baseline blood flow was retained after 90-120 min. No significant difference in erythema and skin colour were found 40 min after needle insertion, indicating that the skin had reached the pre-insertion level. However, care must be taken not to base the normalization time entirely on the obtained p-values, since mean values were larger and with a greater variation in this study with 4 subjects than with the study with local anaesthesia. The figure (29 and 30) suggest that skin colour and erythema first were retained after 100 min.

11.4.4 Comparison of the effects with or without prior anaesthesia

The mean differences from baseline for skin blood flow, skin colour and erythema as a function of time are shown for the trauma studies with and without prior anaesthesia (Figure 32,33,34).

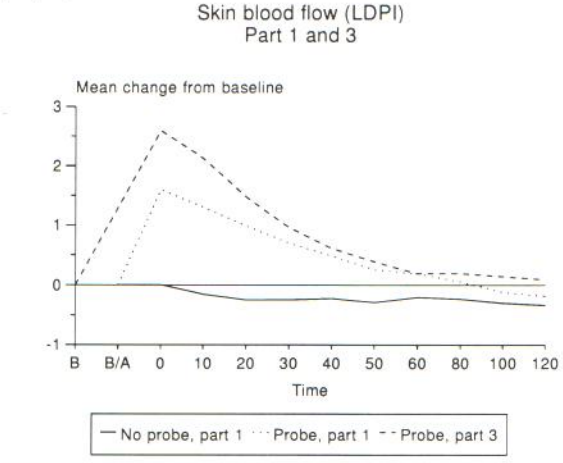

Figure 32 *Mean change of skin blood flow (Volt) from baseline as a function of time after insertion of a microdialysis probe. n=12 insertions for* ***part 1*** *and n=4 for* ***part 3****. B: baseline before insertion B/A: baseline after anaesthesia O: immediately after insertion.*

The mean difference from baseline was always larger in the study without prior anaesthesia as compared with anaesthesia. For erythema the differences were significant in 6 out of ten measurements (independent t-test at each time between part 1 and part 3, $p<0.05$). Skin colour and blood flow both showed significant differences three times. This shows that the vascular

effects of trauma after insertion of a microdialysis probe were reduced by the injection of local anaesthetic prior to insertion as compared with no local anaesthesia. Anderson et al. (1994) found that anaesthesia at the site of insertion abolished trauma reactions. We found that anaesthesia prior to insertion only reduced the effects of trauma. Anderson et al. (1994) suggested that local anaesthesia abolishes the axon reflex flare and that no flare was shown in the anaesthetized skin. Accordingly, in the present study we found less pronounced erythema, which is part of the flare in the anaesthetized skin as compared with the unanesthetized skin.

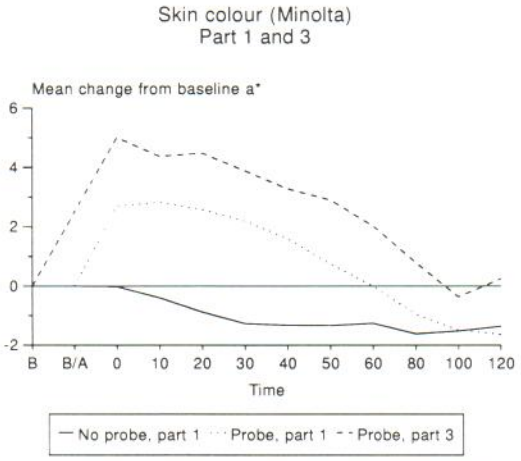

Figure 33 *Mean change of skin colour from baseline as a function of time after insertion of a microdialysis probe. n=12 insertions for* ***part 1*** *and n=4 for* ***part 3****.*
B: baseline before insertion B/A: baseline after anaesthesia O: immediately after insertion.

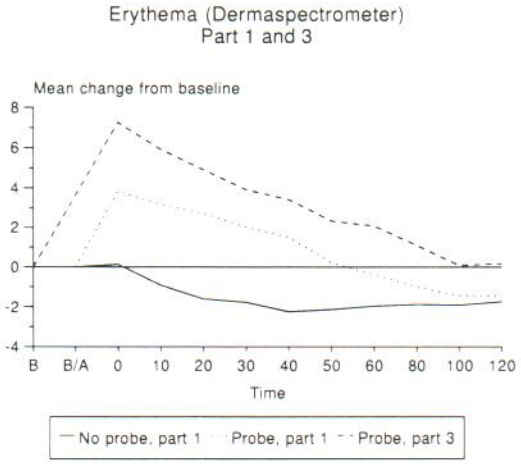

Figure 34 *Mean change of erythema from baseline as a function of time after insertion of a microdialysis probe. n=12 insertions for* ***part 1*** *and n=4 for* ***part 3****.*
B: baseline before insertion B/A: baseline after anaesthesia O: immediately after insertion.

11.4.5 Effect of local anaesthesia

Paired t-test was performed to analyze the changes in skin blood flow, skin colour, erythema and skin thickness before and after local anaesthetic injection in the control arm, where no probe was inserted. Skin blood flow, skin colour and erythema increased significantly following anaesthesia. This effect already declined 20 min after local anaesthesia. No effect on skin thickness of local anaesthesia was found.

11.4.6 Probe depth in dermis

Probe depth was measured in all of the three trauma studies. The mean probe depth was 0.98 ± 0.22 mm, 0.79 ± 0.15 mm and 1.31 ± 0.13 mm for part 1, 2 and 3, respectively. The low standard deviation indicates that the insertion of the microdialysis probes was performed reproducibly. The reason for the larger probe depth in the study without prior anaesthesia was not obvious. The skin was stretched a little by the injected volume of anaesthetic which may ease the insertion. The probes were mostly placed in the lower part of the dermis. However, B-mode scanning showed that the probe was located within the reticular dermis in every case.
Thus, we could not demonstrate a relationship between the traumatic reactions and the probe depths, whereas Anderson et al. (1994) observed a more pronounced increase in skin perfusion with more superficial probe levels, though only based on two individuals.

11.4.7 Summary of trauma studies in human skin

Anaesthetized and unanesthetized human skin was affected by insertion of a microdialysis probe with respect to skin blood flow, skin colour, erythema and skin thickness. Except for increase in skin thickness, the reactions to the insertion appeared immediately. The precise time until the skin was totally normalized concerning skin blood flow, skin colour and erythema is not well defined. It would be erroneous to estimate the period of equilibration only judged by the p-values as large variations were found. However, the studies indicate that skin blood flow requires 90-120 min to stabilize after needle insertion. Erythema, measured by the Minolta chromameter and the Dermaspectrometer, reached baseline level approximately 90 min after cannula insertion in the anaesthetized skin. A period of 90-120 min is required in human skin, allowing the vascular effects of trauma to normalize. Comparable to the study of rats the skin thickness did not normalize during the investigation. Local anaesthesia prior to insertion reduced the vascular effects of trauma. The local anaesthesia injection itself induced a minor trauma, which faded out fast. Probe depth did not show any influence on the effects of trauma.

11.5 *In vivo* microdialysis

11.5.1 Placebo studies

The dialysate samples from two untreated rats did not contain any compounds which interfered with the HPLC analysis of betamethasone 17-valerate.

11.5.2 Topical application of 4% betamethasone 17-valerate

High-dose topical application of betamethasone 17-valerate, 4% in different vehicles was studied. The concentrations in the initial and sometimes later dialysate samples were surprisingly high. This might be due to contamination with betamethasone

17-valerate during insertion of the cannula. Cleaning of the skin surface with ethanol and water at the insertion point and use of a stilette in the cannula during insertion was attempted. For this reason the calculation of mean dialysate concentration only includes observations after 100 min.

A one-way analysis of variance (5% significance level) was used to analyze the effect of the treatment. The mean dialysate concentrations from the 3-4 microdialysis probes were used as the observation in each rat. The variation between the probes of each rat was small as compared with the variation among the rats. At first a test was performed of the interaction between time and treatment. No significant interaction was found. Then effect of time on the dialysate was tested and found insignificant. This indicates that at 100 min after the sampling is started, a steady state of the dialysate is established (Figure 35).

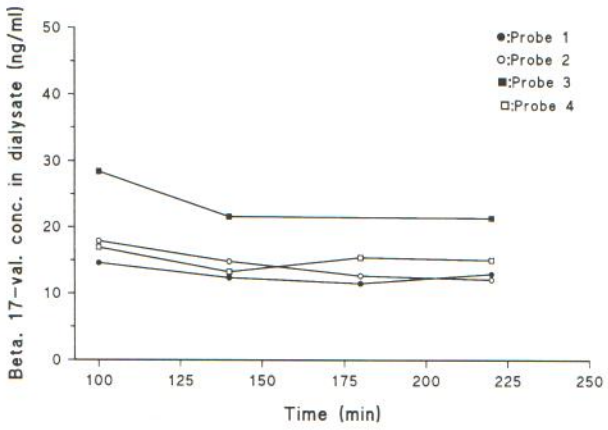

Figure 35 *Mean dialysate concentrations after topical application of betamethasone 17-valerate in 5% ethanolic IMP solution in a single rat.*

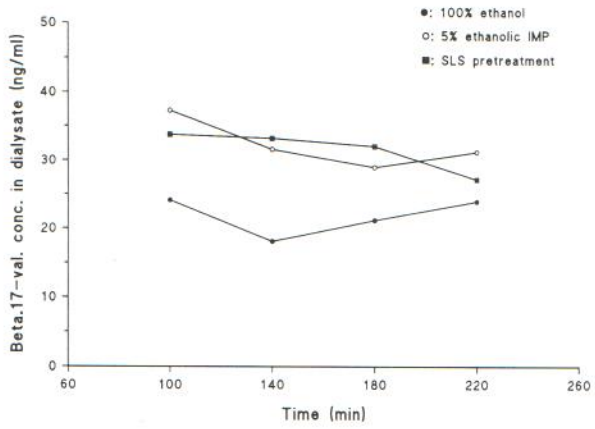

Figure 36 *Mean dialysate concentrations after topical application of betamethasone 17-valerate. 100% ethanol: n=13 microdialysis probes. 5% ethanolic IPM: n=15 microdialysis probes. SLS: n=10 microdialysis probes.*

No significant effects of the treatments were found (p=0.09). Standard deviations up to 50% of the mean were found in some of the rats, which weakens the analysis. Thus, the figure 36 only indicates a difference between the SLS and IPM groups and the plain ethanol group, i.e. the concentration in the dermis is higher after topical application of 4% betamethasone 17-valerate with 5% IPM, and after SLS corrosive pretreatment of the skin than compared to 4% betamethasone 17-valerate in pure ethanol applied on normal skin. The SLS provocation prior to the betamethasone 17-valerate application did, however, not significantly increase the dialysate concentration. TEWL, indicating water barrier disruption, was measured before drug administration and found increased following SLS provocation in all of the three experiments. Simultaneous administration of hydrocortisone and SLS did not show a dose-effect relation between SLS concentration and *in vitro* penetration of hydrocortisone (Frankild et al., 1995). Pretreatment of the skin with 4% SLS for 0.5 to 8 hours did not result in time-effect relationship for *in vitro* penetration of hydrocortisone, whereas pretreatment with 10% for 2 hours increased the penetration of hydrocortisone 4.3 times. These authors explained the minor effect of SLS on hydrocortisone penetration as compared with nickel and tritiated water by an association of hydrocortisone molecules with SLS micelles, formed at SLS concentration above the critical micelle concentration. As micelles do not penetrate the skin, only unassociated hydrocortisone molecules will be able to penetrate. However, Frankild et al. (1995) observed increased penetration of hydrocortisone following the same treatment of the skin as in the present study, where 10% SLS may have enhanced the penetration of betamethasone 17-valerate a little. Wilhelm et al. found increased penetration of hydrocortisone after pretreatment for 24 h with SLS in guinea pig skin *in vitro* and *in vivo* (Wilhelm et al., 1991a; 1991b). However, the repair mechanism after SLS provocation is negligible in *in vitro* studies, whereas in our *in vivo* study in rats this mechanism clearly functions, accompanied by skin metabolism.

The present study also indicated a higher penetration when 4% betamethasone 17-valerate in 5% isopropyl myristate was compared with a 100% ethanolic solvent. Minor intradermal haemorrhage was observed in the rats treated with 5% IPM twice daily for 3 days. Four skin biopsies, two from each rat, were taken from the area with bleeding. Histology studies showed a severe, irritative, acute dermatitis with erythrocyte extravasation. This severe damage to the skin barrier was together with occlusion expected to result in high penetration rates of betamethasone 17-valerate in the present study, but this was not the case. Wester and Maibach (1992) studied percutaneous absorption in diseased/ damaged skin. The data suggested that diseased skin can retain barrier properties, and that characteristic differences will exist for different drugs and different disease conditions. However, 5% IPM enhanced the flux of indomethacin *in vitro* by increasing the fluidity of the stratum corneum lipids (Ogiso et al., 1995). Fang et al. (1996) found increased flux of sodium nonivamide acetate through pig skin *in vitro* and in humans *in vivo* when the substances were formulated with 3.8% IPM as compared with the original vehicle without isopropyl myristate.

A relationship between the mean dialysate concentration and the probe depth in the treatment groups was not found. This relationship may be unclear because of variations in the study due to different skin thickness of the rats and different effect of SLS and IPM on the skin, among others. The measurement of probe depth by ultrasound was also more difficult than experienced earlier. The microdialysis probes were perfused for at least four hours. When the membrane becomes wet, it is more difficult to detect by ultrasound. This problem influences the otherwise high precision of probe depth measurements, leading to a poorer correlation between dialysate concentrations and probe depth in the present study. The probe depth measurements in *in vivo* microdialysis studies need further validation.
The study suggest that more animals in each treatment group is required to obtain significant results.

Betamethasone 17-valerate ointment 0.1% (Betnovat® ointment, Glaxo, UK) was in an additional experiment applied on two rats once daily under occlusion for three days. A dose of 60 mg was given on a 12 cm^2 test area on each side of the rat. Before the ointment was applied a 10% SLS exposure was made to the test area for two hours, and the damage to the barrier was demonstrated by an increase in TEWL. A microdialysis probe was inserted in each area. No betamethasone 17-valerate was detected in the dialysate samples.
Thus, unrealistic and extreme topical treatments such as organic solvents and enhancers must be used to measure betamethasone 17-valerate in the skin by cutaneous microdialysis. The affinity of betamethasone 17-valerate to the aqueous perfusate is low because of the lipophilic nature of the drug, as compared with the affinity to the tissues. A higher affinity and thereby a higher *in vivo* recovery might be obtained if the perfusate contained for instance a lipophilic adjuvant or an emulgator.

11.5.3 Systemic administration of betamethasone 17-valerate
After oral administration 158.0 mg/kg of betamethasone 17-valerate the mean dialysate concentration of the two probes in each rat was determined (Table 10).

Rat	Right side ng/ml	Left side ng/ml
A	43.55 ± 10.09	39.39 ± 19.76
B	25.65 ± 6.63	24.52 ± 4.33

Table 11 *Mean betamethasone 17-valerate in dialysates after oral administration of betamethasone 17-valerate.*

Steady state of the dialysate concentrations was found. The drug concentrations from the left and right probe were the same in each rat, but rat A and rat B differed in concentration levels. This reflects variations between the rats in for instance absorption fraction. The drug concentrations in the dialysate following oral treatment was comparable to those found in the topically treated rats. However, in rats treated orally, high concentrations at the start of sampling were not observed. In humans the plasma half-life of betamethasone 17-valerate was 8.1 h^{-1} and 16.6 h^{-1} a mean AUC of 75.4 and 7.74 ng $ml^{-1}h$ was found after oral and topical administration, respectively (Kubota et al., 1994b). The skin levels were higher in humans treated orally than those treated topically in contrast to the rats in our study. This may be due to different pharmacokinetics of betamethasone 17-valerate in rats and humans.
Oral doses of 15 mg/kg and 60 mg/kg were administered to rats, but this did not lead to detectable levels of betamethasone 17-valerate in the dialysate.

11.5.4 Systemic administration of fusidic acid
In two rats treated with oral fusidic acid suspension, sodium fusidate was found in the dialysate. Figure 37 shows the concentration of sodium fusidate in the dialysate plotted as a function of time for rat A and rat B.

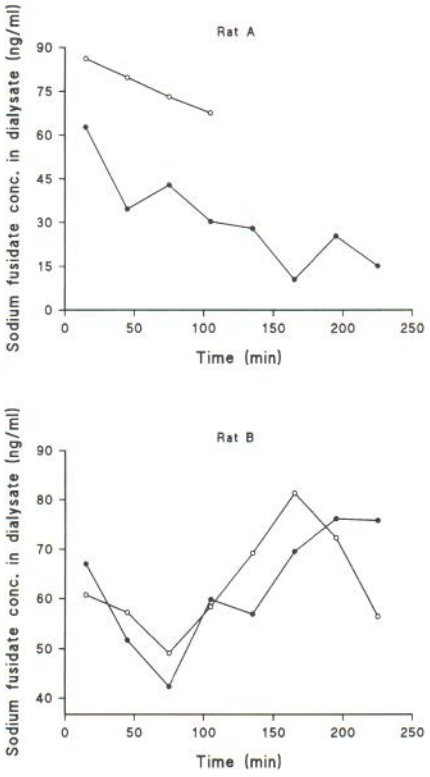

Figure 37 *Mean dialysate concentrations after oral administration of 312.5 mg/kg fusidic acid for two rats.*

In rat A the concentrations in the dialysate from two microdialysis probes declines exponentially with a half-life of fusidic acid of 4.22 h^{-1} and 1.93 h^{-1}, respectively. The plasma half-life of fusidic acid in humans is about 5 h^{-1} (Reeves, 1987). Reeves (1987) stated that fusidic acid is extensively metabolized to form a glucuronide of fusidic acid, a di-carboxylic metabolite and a 3-keto metabolite. The high metabolization rate and the high protein binding may explain why fusidic acid was not found in dialysates after oral administration of 80 mg/kg twice daily for three days. Thus, this study shows, even though only

two probes were included, that it is possible to study the elimination of fusidic acid in the skin by cutaneous microdialysis. In Rat B, also treated with 312,5 mg/kg a special pattern in the dialysate concentration was found, since this animal deteriorated and finally died before the last sample was collected. The dialysate concentration increased in both probes parallel to worsening of the condition ending with a circulating collapse. This suggests that decreased skin blood flow was associated with a decrease in exchange of drug between the tissue and the capillaries resulting in a higher recovery. In contrast, Ståhle et al. (1991b) found that theophylline dialysate levels fell as a consequence of death, i.e. to 1/4 of "alive" levels. They were not able to explain this result by the passive diffusion model and concluded that active transport processes are involved. Singh and Roberts (1993) showed that the concentrations of lidocaine and salicylic acid in the underlying tissue (dermis, subcutis and fascia) after dermal application are significantly increased in the presence of phenylephrine, a local vasoconstrictor. This is consistent with our findings of increased dialysate concentration due to decreased clearance of fusidic acid from the skin when the circulation collapsed.

Human skin blister fluid contained 21 ± 5 μg/ml and 79 ± 11 μg/ml after oral doses of 250 and 500 mg fusidic acid, respectively, administered twice daily for 6 days (Vaillant et al., 1992). These drug levels are about 1000 times higher than the levels found in the dialysate from rat skin. However, the skin blister fluid includes bound and unbound drug concentration in contrast to the samples obtained in microdialysis. The free concentration of fusidic acid present in the blister fluid would approximately be 630 ng/ml (97% binding) after a 250 mg oral tablet. This is about 10 times the dialysate level. The difference might be explained by a more extensive metabolism in the rat as compared with man and a low recovery of fusidic acid.

Some investigations with topically applied fusidic acid were performed. Rats (n = 3) were treated with Fucidin cream® (Leo Pharmaceutical Products, Ballerup, Denmark) twice daily for two days. In another group of rats the same cream was applied twice daily for two days following SLS pretreatment (10% for 2 hours) with and without occlusion (n = 4). Two rats were treated for 3 days with a 20 mg/ml sodium fusidate solution applied to the skin following SLS provocation (10% for 2 hours). Finally 10% fusidic acid in ethanol was applied topically under occlusion to two additional rats once daily for 3 days. None of the topical experiments with fusidic acid or sodium fusidate resulted in measurable concentrations in the dialysate from the probes inserted in the dermis of the rats. The free concentration of fusidic acid in the dermis after the different treatments was clearly too low to be measured by cutaneous microdialysis using our present HPLC method. The recovery of sodium fusidate *in vitro* is 41%. It is expected that the *in vivo* recovery is lower, as described earlier. Addition of albumin to the surrounding medium had a major impact on *in vitro* recovery due to the high protein binding of fusidic acid. The same influence of proteins is expected *in vivo*.

Brown and Percival (1978) found that fusidic acid penetrates well into isolated cells resulting in concentrations of between 40 and 100% of those present extracellularly.

The penetration of fusidic acid was studied by Vickers (1969) in whole-thickness excised human skin using glass permeation chambers. Approximately 2% of the applied dose formulated as alcoholic solutions of sodium fusidate and fusidic acid were absorbed through intact skin. Absorptions from cream and ointment were slightly lower. Stüttgen and Bauer (1988) studied the penetration and permeation of fusidic acid in different formulations using permeation chambers and they also analyzed drug levels in skin sections. They concluded that the normal, intact, horny layer offers marked resistance to the penetration of sodium fusidate/fusidic acid, but this resistance diminishes markedly when the horny layer is damaged (tape stripping) or shows pathological changes. These penetration studies did not take the metabolism of fusidic acid into account, as in our *in vivo* microdialysis studies.

In vitro studies may show unrealistic high drug levels in the skin. The extraction of fusidic acid from the skin sections, furthermore, influence the total amount of drug measured in skin *in vitro*.

It can be concluded from the study on topical application that at present we have found it impossible to determine fusidic acid in the skin by cutaneous microdialysis. The technique needs some methodological improvements. One interesting option is the addition of protein to the perfusate. In this way the protein in the perfusate will compete with the protein binding in the tissue. However, this solution requires a series of manipulations to extract the drug from the perfusate. It is not very attractive considering the very small perfusate volumes.

11.5.5 Systemic administration of calcipotriol

It was not possible to detect calcipotriol in the dialysate after oral administration of 188 mg/kg calcipotriol to two rats. This dose is extremely high, as compare with the dose applied with the commercial ointment which contains 50 μg/g. The oral absorption of calcipotriol is approximately 60% in rats with a half-life of 12 min (Calcipotriol monograph). During the 90 minutes period from administration of the drug to the start of sampling of dialysate approximately 8 half-lives have elapsed. Autoradiography studies show that calcipotriol is concentrated in the liver, kidney and intestines in rats (Calcipotriol monograph). Distribution of calcipotriol to the skin might thus be very low. The pharmacokinetics of calcipotriol may explain why we were not able to detect calcipotriol in the dialysate. The dialysate was not investigated for metabolites of calcipotriol.

11.5.6 Summary of in vivo microdialysis studies in animals

The summary table (Table 11) shows that only extreme treatments (SLS pretreatment, ethanol vehicle and high dose) either topically or orally with betamethasone 17-valerate and fusidic acid result in detectable drug levels in the dialysate. However, we found that to some degree it is possible to study effects of vehicles after topical application, drug levels in the skin after oral administration, elimination in the skin after oral administration and the influence of blood flow changes on skin penetration. The unsuccessful outcome of realistic clinical treatments is due to low doses, low free concentrations of fusidic acid in the skin due to protein binding and flux into cells and

low recovery of betamethasone 17-valerate due to the strongly lipophilic character of this substance. Müller et al. (1995c) also found that microdialysis with lipophilic drugs result in low recoveries. The sensitivity of the HPLC methods failed to meet the very high demands of the vivo microdialysis samples.
The *in vivo* recovery of substances is in high competition with the absorption to the blood vessels in the tissues, since the *in vivo* recovery increased as the skin blood flow decreased.

Substances and formulations	Measurable	Non-measurable
Fucidin® cream		X
Fucidin® cream SLS and ± occlusion		X
10% fusidic acid in ethanol, occlusion		X
Oral fusidic acid 80 mg/kg		X
Oral fusidic acid 312,5 mg/kg	X	
Betnovat® ointment SLS and occlusion		X
4% betamethasone 17-valerate in ethanol, occlusion	X	
4% betamethasone 17-valerate in 5% IPM ethanol solution, occlusion	X	
4% betamethasone 17-valerate in ethanol, SLS, occlusion	X	
Oral betamethasone 17-valerate 15 mg/kg and 60 mg/kg		X
Oral betamethasone 17-valerate 158 mg/kg	X	
Oral calcipotriol 188 mg/kg		X

Table 12 *Summary of in vivo microdialysis studies.*

12.0 Conclusions

In vitro microdialysis studies of model substances with different physico-chemical characteristics, i.e. glucose, sodium fusidate, betamethasone 17,21-dipropionate and calcipotriol were performed. The experimental setup for the *in vitro* studies resulted in stable and reproducible recoveries. Recovery depends on perfusion rate, stirring rate, temperature and membrane length. Recovery and loss were independent of the concentration in the medium surrounding the microdialysis probe, except for sodium fusidate.

For lipophilic substances recovery was found lower than loss. More lipophilic substances had lower recoveries and lower ratio of recovery to loss.

The recovery of sodium fusidate was significantly reduced when protein was added to the surrounding medium.

The point of no-net-flux method requires modifications in order to study substances having different recovery and loss, successfully.

Microdialysis of lipophilic or highly protein bound substances requires careful selection of tubings which, if not suited, may cause low recovery and problematic interpretations of the results.

Sodium pentobarbital anaesthesia decreases the mean skin blood flow in rats as compared with unanesthetized rats. However, skin blood flow was stable in rats anaesthetized with sodium pentobarbital, but unstable in rats anaesthetized with halothane. Thus, sodium pentobarbital is the preferred drug for anaesthesia.

Insertion of the microdialysis probe causes trauma with increase in skin blood flow and skin thickness and releases histamine in rat skin. An equilibration period after insertion of minimum 30 minutes is suitable for cutaneous microdialysis in the rat.

Insertion of the microdialysis probe in human skin similarly caused increase in skin blood flow, erythema and skin thickness. Local anaesthesia with lidocaine subcutaneously prior to insertion reduced the vascular effects of the trauma. At least 90 minutes is required after insertion of a microdialysis probe in human skin to allow effects of trauma to diminish and become negligible.

The insertion of the microdialysis probe was well tolerated, even in subjects without prior anaesthesia.

No correlation was found between probe depth and the trauma reactions.

The microdialysis probes were placed reproducibly in the lower dermis in both man and rat. The increase in human and rat skin thickness after needle insertion was due to oedema formation.

In vivo cutaneous microdialysis in rats treated topically or orally with fusidic acid, betamethasone 17-valerate and calcipotriol was studied.

When rats received extreme treatments (high oral doses or topical treatment following sodium lauryl sulphate induced barrier disruption) measurable drug concentrations in the dialysate were found. However, following realistic and clinical relevant topical treatments with the same drugs they could not be detected by microdialysis. This is probably due to the lipophilic and highly protein bound character of the drugs. Lipophilic and highly protein bound substances have very low recovery *in vivo* and demands an extremely low limit of quantitation of the used analytical method in order to measure the low free concentrations present in the dialysate.

With our present analytical technique, cutaneous microdialysis is limited to the study of hydrophilic substances of low protein affinity and of relatively low molecular weight and to endogenous substances such as histamine and mediators of inflammation and glucose.

Cutaneous microdialysis is a potent technique in experimental dermatological research. Cutaneous microdialysis is the only method which enables continuously sampling of drugs, metabolites and endogenous substances in animal and human skin with a negligible trauma. The technique represents a unique addition to traditional methods for the study of skin penetration. Its application in the dermatological research is just at the beginning.

13.0 Potentials and further aspects of cutaneous microdialysis

Application of cutaneous microdialysis to study lipophilic substances needs further investigations to aim at higher, stable and reproducible recoveries. These investigations should include studies of alternative perfusion media which may increase the partition of substances into the perfusate and decrease adsorption and interaction with the membrane and tubings. Other possibilities may be to lower the flow rate, to test new tubings and microdialysis membranes of different materials and larger cut-off values.

Estimation of the true extracellular concentration of substances which have different recovery and loss needs improvement. The reference methods and retrodialysis are not suitable for the study of these drugs and must be modified. The point of no-net-flux is relatively laborious for these substances and the modification presented in this study must be verified in *in vivo* studies.

Previous studies have found correlation between dialysate concentration and probe depth in the dermis. We found no such relation. Further investigations must be conducted to finally conclude this relationship.

The drug concentrations of dialysate samples obtained by cutaneous microdialysis are typically low and development of sensitive analytical methods is an essential prerequisite both for hydrophilic and lipophilic compounds.

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Figure 12:
Experimental setup for in vitro microdialysis studies.

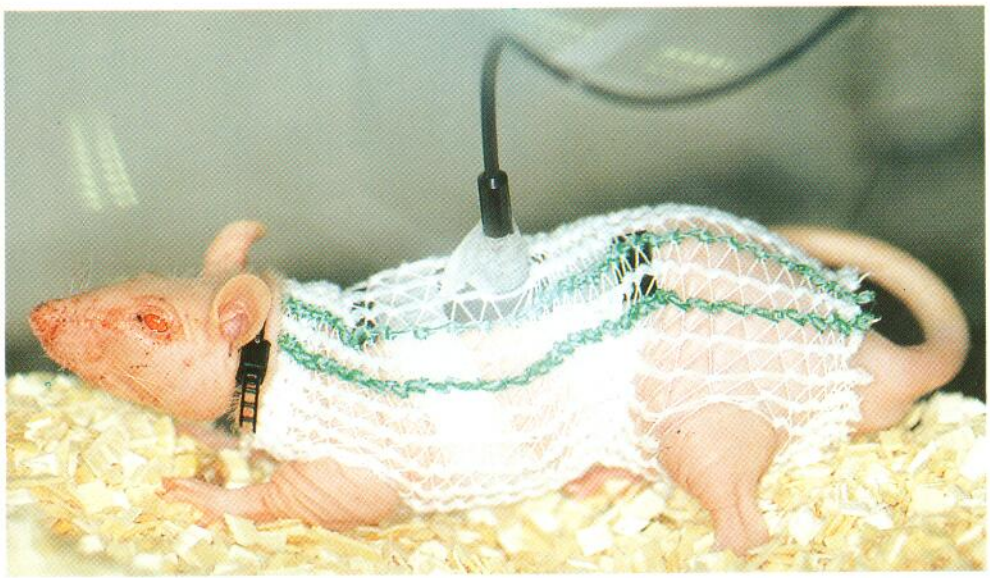

Figure 13:
Measurements of skin blood flow in a hairless rat by laser Doppler flowmetry.

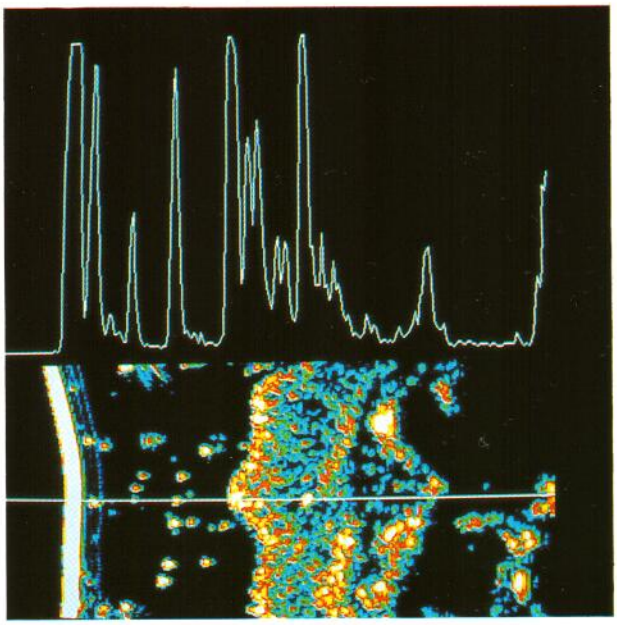

Figure 14:
A-mode scan (upper half) and B-mode scan (lower half) of rat skin. An entrance echo corresponding to the epidermal surface (left) and an echo corresponding to the inter-phase between the dermis and the subcutaneous tissue (right) are seen. Approximately in the middle an echo from the microdialysis membrane is seen.

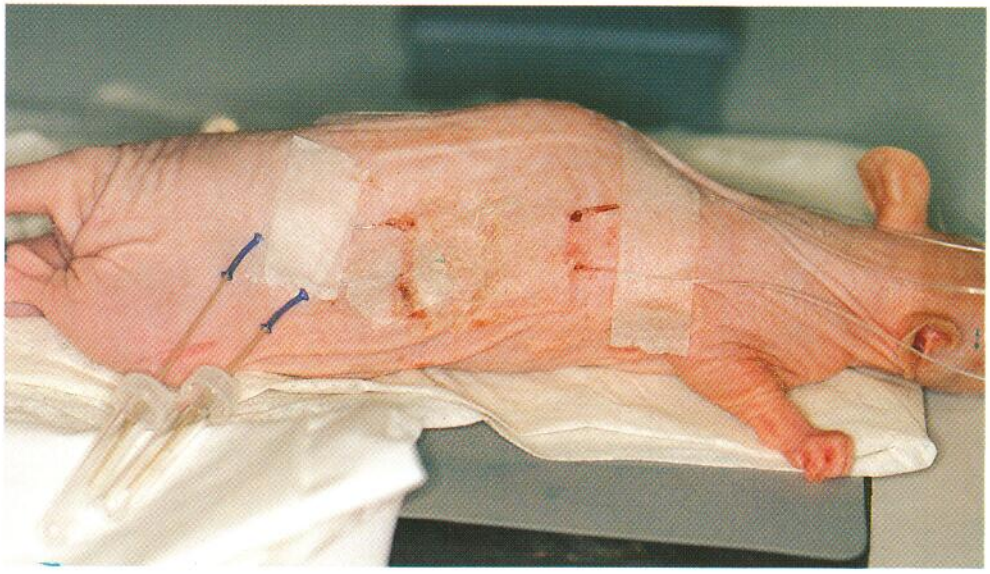

Figure 15:
Cutaneous microdialysis in a hairless rat.

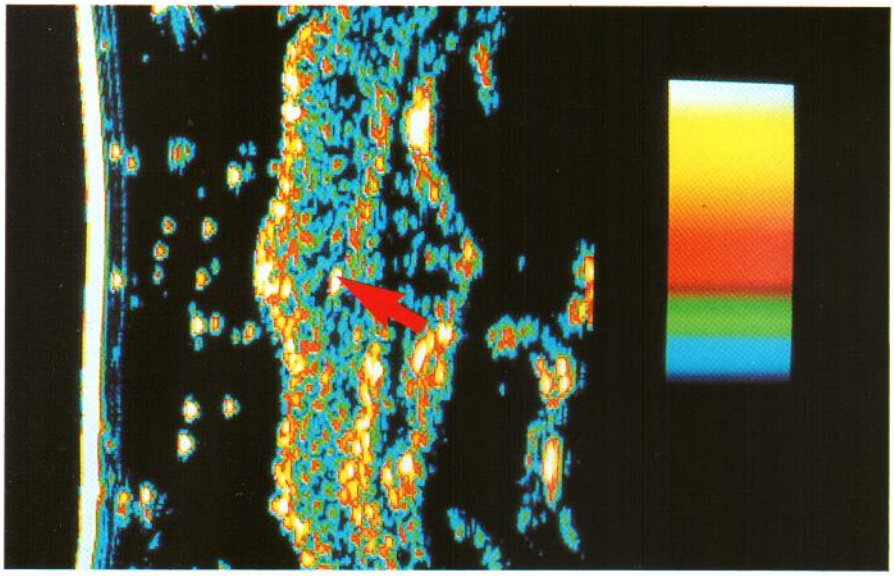

Figure 25:
A B-mode scan of rat skin. The marked white dot represents the microdialysis membrane within the dermis.

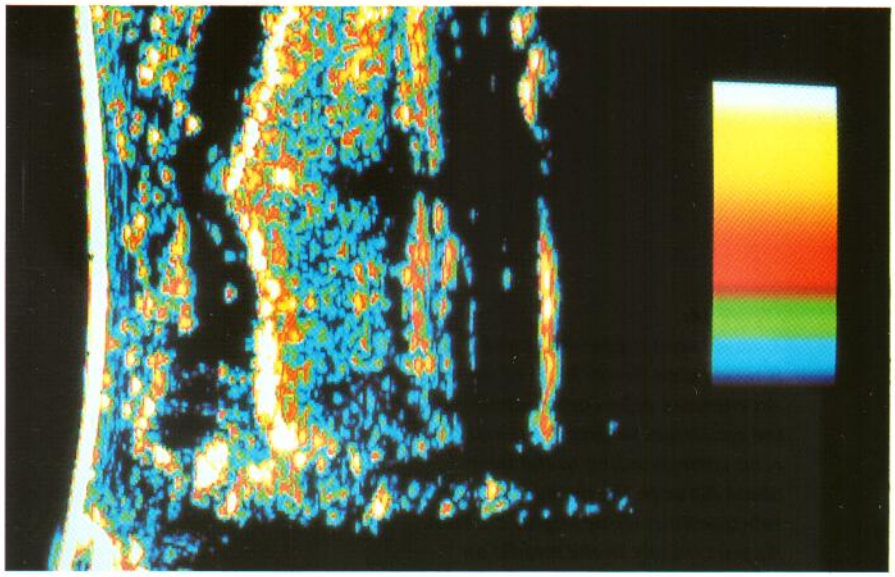

Figure 27:
Ultrasound image of rat skin 15 minutes after probe insertion. Note the low echogenic area around the probe.
Colour scale of echogenicity: White > yellow > red > green > blue > black.