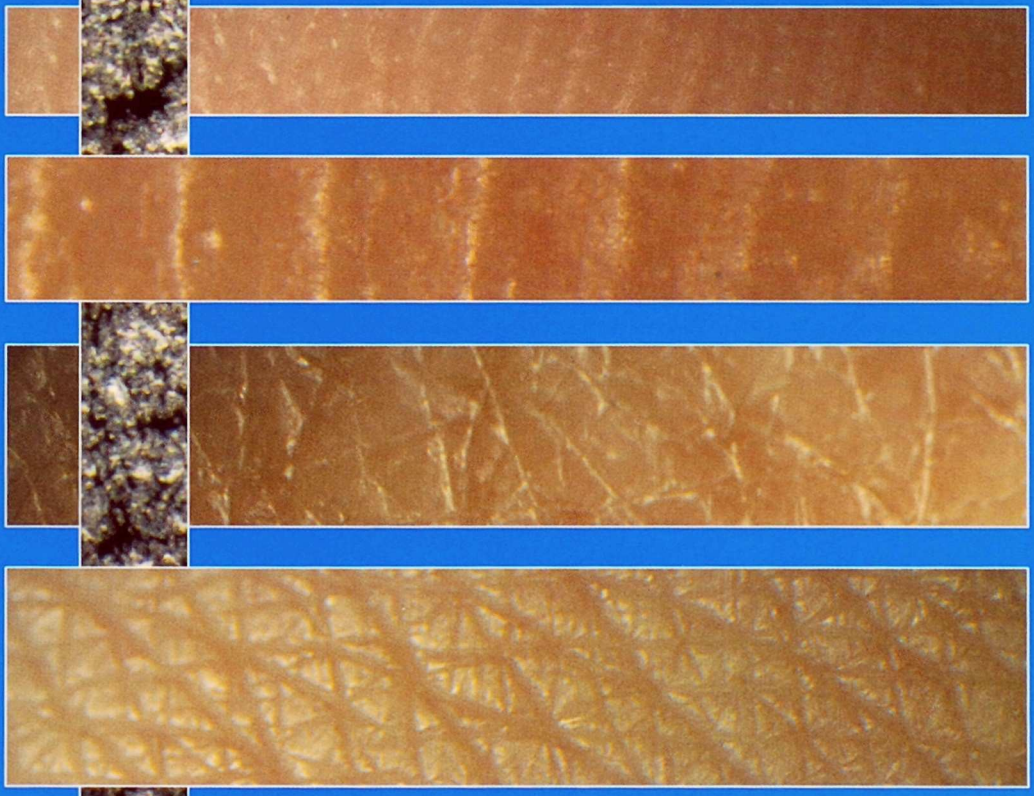
Dermal absorption of chemicals through normal and compromised skin

Ivone Jakaša



Dermal absorption of chemicals through normal and compromised skin

The studies presented in this thesis were carried out at the Coronel Institute of Occupational Health, University of Amsterdam, Amsterdam, the Netherlands. Part of this project was funded by European Union, Framework 5; Quality of Life, Environment and Health Key Action Funding, Project: Evaluations and predictions of dermal absorption of toxic chemicals, project number: QLKA-2000-00196 (project acronym: EDETOX).

Cover design: Niko Macura, Santpoort Noord
Printing: Ponsen & Looijen, b.v., Wageningnen
ISBN: 9064646414

Printing of this thesis was finantially supported by:
Astellas Pharma B.V., Huidstichting Chanfleury van IJsselsteijn, Stichting Nationaal Huidfonds and Novartis Pharma B.V.

Dermal absorption of chemicals through normal and compromised skin

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. mr. P.F. van der Heijden
ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Aula der Universiteit
op woensdag 31 mei 2006, te 10.00 uur

door Ivone Jakaša
geboren te Zagreb, Kroatië

Promotiecommissie

Promotores:	prof. dr. F.J.H. van Dijk prof. dr. J.D. Bos
Copromotor:	dr. M.M. Verberk
Overige leden:	dr. B.J. Blaauboer prof. dr. D.P. Bruynzeel prof. dr. M.L. Kapsenberg prof. dr. M.C. Michel dr. T.M. Pal

Faculteit der Geneeskunde

Contents

Glossary

Penetration	Entry of a substance into a particular layer of the organ
Diffusion	A velocity of a transport of a chemical through the skin layer
Permeation	The penetration through one layer into another, which is both functionally and structurally different from the first layer
Dermal absorption	The uptake of a substance into the vascular system (blood vessels) which acts as the central compartment
Steady-state	The part of an absorption profile where the absorption rate remains constant
Flux	Mass of test substance passing through a unit area of skin per unit of time
Absorption rate	Mass of test substance passing through a unit area of skin into the systemic circulation per unit of time
Lag time	The time needed to attain steady-state absorption

Chapter 1

Introduction

As the first organ in contact with the environment, the skin is frequently exposed to various chemicals by spills, splashes, immersion, or application of a consumer product. Absorption of a chemical into the skin may lead to local effects such as inflammation or sensitisation or to systemic effects after subsequent uptake in the circulation. At the work place the absorption of hazardous substances through the skin can contribute considerably to the total systemic uptake or can even be the main absorption route.[1] To protect individuals from the adverse health effects associated with exposure to chemicals, several exposure limit values have been developed by national and international regulatory or advisory agencies. While these exposure limits are set for inhalation and ingestion (e.g. maximum allowable concentration for occupational airborne exposure, and tolerable daily intake for food), at present there is only a qualitative indicator of hazard related to skin absorption known as the "skin notation".[2] The "skin notation" has only a warning function to identify substances that could contribute substantially to the total body burden by uptake via the skin.[3]

Different attempts were undertaken to develop quantitative dermal exposure limit values for the occupational practice; however, until now no consensus concerning establishment of these values has been reached.[3, 4] One of the main obstacles in development of an appropriate strategy for risk assessment of dermal exposure is that data on dermal absorption are often missing. This is partly due to the lack of reliable and feasible methods for the determination of dermal absorption. At present, laboratory animals are used to estimate dermal absorption for regulatory purposes. It is important, however, to realise that the human skin has specific properties and that dermal absorption data from animal studies have to be evaluated critically. As an alternative to animal models, *in vitro* assays with human skin are increasingly used. A number of guidelines have been established in an attempt to standardise these *in vitro* measurements.[5-8] However, to increase the applicability of these methods they have to be further validated, preferably by comparison with human *in vivo* studies.

One other point of concern in the evaluation of health risks associated with skin exposure is the occurrence of compromised skin which is not considered by risk assessors. The compromised skin barrier can be a consequence of skin disorders, physical damage (e.g. burned, shaved skin); chemical damage (caused by e.g. detergents, solvents); occluded skin (by wearing of gloves), increased hydration (caused by excessive hand washing), and even of psychological stress. Healthy skin is practically impermeable for molecules larger than 500 Da.[9] In contrast, it has been shown that in compromised skin penetration of larger molecules can result in cutaneous reactions.[10]

The unique barrier function of the skin originates from the particular structure of the skin consisting of several tissue layers (Fig 1).

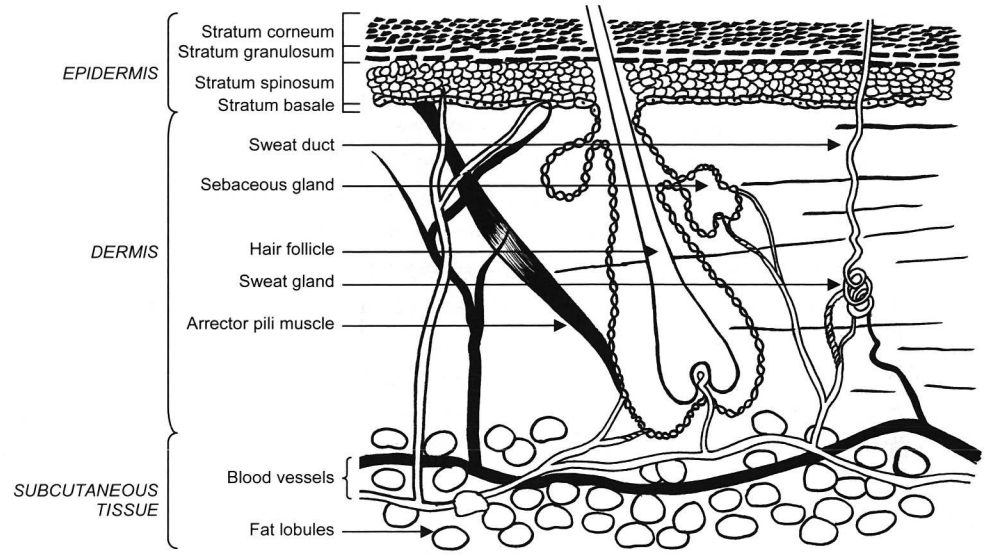


Fig 1: *Structure of the skin*

1.1. Structure of the skin

The outermost layer of the skin is the epidermis, which is separated from the dermis by a thin layer of basal membrane. The epidermis varies in thickness, depending on cell size and number of cell layers, ranging from about 0.8 mm on the palms and soles down to 0.06 mm on the eyelids. Keratinocytes are the primary cell type in the epidermis; they are metabolically active and able to divide.[11-12] Keratinocytes migrate from the lowest stratum (the stratum germinativum) up through different layers to the outermost stratum corneum (SC) and then are sloughed off through desquamation. As the keratinocytes migrate from the deepest layers of the epidermis to the SC they accumulate keratin and lipid granules. The intercellular lipids and the intercellular connections between these cells (desmosomes and tight junctions), provide the primary barrier to prevent fluid loss from the body and the absorption of foreign

chemicals into the body.[13] In addition to keratinocytes, the epidermis contains other specialised cell types e.g. melanocytes (responsible for melanin synthesis), Langerhans cells (major-antigen cells) and Merkel cells (associated with nerve endings).[12, 14] The dermis, at 3 to 5 mm thick, lies below the epidermis and is composed of a network of connective tissue, predominantly of collagen fibrils which provide support and elastic tissue which provide flexibility of the skin. It contains the sensory nerves and has the vascular network.[15] The blood supply reaches to within 0.2 mm of the skin surface, so that it readily absorbs most chemicals which penetrate through the stratum corneum and the viable epidermis. Due to high blood flow, dermis usually functions as a sink for the diffusing molecules which reach it during the process of dermal absorption. This sink condition ensures that the penetrate concentration in the dermis remains near zero and therefore the concentration gradient across the epidermis is maximal. The dermis contains several types of cells, namely fibroblasts which are the predominant cells, fat cells, dendritic cells, mast cells, and cells associated with the blood vessels and nerves of the skin. Three main appendages reaching the surface of the skin originate in the dermis: hair follicles, sebaceous and sweat glands. The subcutaneous fat layer acts as a bridge between the overlying dermis and the underlying body. This layer principally serves as insulation and provides mechanical protection against physical damage.

In dermal absorption research, the SC is often regarded as a separate membrane. It is only 10-50 μm thick over most of the body but provides a primary barrier for absorption of the chemicals as well as prevention of insensible loss or gain of water.[15-16] Typically, the SC consists of about 10-20 layers of flattened anucleated dead cells filled with keratin, known as corneocytes.[17] Each corneocyte is enclosed within a protein-rich cornified cell envelope embedded in a lipid-enriched intercellular matrix; this structure is often referred to as a 'bricks and mortar' model (Fig. 2).[18-21]

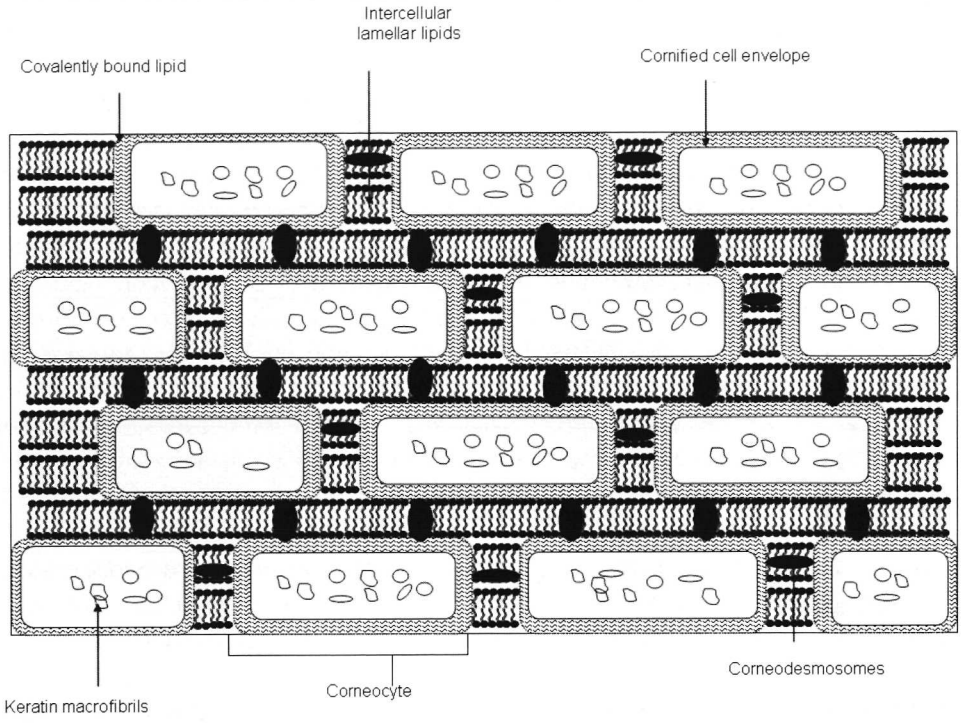


Fig 2: *Structure of the human Stratum Corneum*

Although the intercellular lipids account for only about 15% of the SC weight (the remainder being 70-80% proteins and 5-15% water) they are essential components in the barrier function.[22] The intercellular lipids, which form laminar bilayers, consist of about 40-50% of ceramides, 25% cholesterol, 15% of long chained fatty acids and 5% of other lipids such as cholesterol sulphate, cholesterol esters and glycosylceramides.[13, 22-23] The ceramides, structurally heterogenous and complex groups of sphinglolipids, are thought to play an important role in the barrier function of the SC. There are at least 8 major classes of ceramides present in the human SC matrix differing from each other by the structure of the head group and by the fatty acids chain length.[11, 22-23] The importance of these compounds in the preservation of the lipid bilayer structure has become clear in the studies of various skin disorders which are accompanied by altered ceramide composition (e.g. atopic dermatitis, psoriasis).[21-32] There are still many unanswered questions about the exact way in which the SC lipids are organized. In an attempt to explain the barrier properties of

the SC, several models of intercellular lipid structure have been proposed such as a "domain mosaic" model by Forslind, a "sandwich" model by Bouwstra and a "single gel phase" model proposed by Norlén.[33-35] The models describe the existence of different interconnected crystalline, semi- crystalline, gel and liquid crystal domains. The disruption of this rigid organisation of lipids is believed to be responsible for the damaging effect of solvents, soaps etc.[36]

1.2. Transport routes of chemicals through the skin

Chemicals have three potential routes from the skin surface to viable tissues (Fig 2). Dermal absorption of a chemical via the ***transcellular pathway*** implies that it has to cross the highly impermeable cell envelope to enter the cell, travel through the keratin rich cell and one more time cross the cell envelope on its way out. Additionally, it would have to cross the intercellular spaces as well. This thermodynamically and kinetically unfavourable route is not very likely and it remains controversial whether this route has any significance in the dermal absorption of chemicals.[37] The ***intercellular pathway*** involves diffusion through the lipid bilayers between the corneocytes and it has been widely accepted to be the principal route for permeation.[38-41] The exact mechanism of chemical diffusion through lipid bilayers is not clear. Recent studies indicate that hydrophilic and hydrophobic chemicals diffuse via different routes.[42-43] Hydrophilic chemicals seem to diffuse through the SC within the polar head groups while the lipophilic chemicals diffuse within nonpolar tail groups of the intercellular lipids. The transport between lipid bilayer occurs in the places where the bilayers show structural disorganization.[44] In the ***appendageal pathway*** the chemical is transported along the hair follicles, sweat glands and sebaceous glands, thereby by passing the corneocytes and lipid bilayers and entering directly into the epidermis.[45-47] The appendages occupy a relatively small area of the skin, generally less than 1% which is dependent upon the anatomical location.[15] For most chemicals penetration through appendages does not contribute significantly to the total dermal absorption.[44] However, these shunts become significant for large hydrophilic chemicals which are poorly absorbed through lipid bilayers.[48] Furthermore, this route is supposed to be the only route for macromolecules such as proteins and nanoparticles. [49-50]

1.3. Theoretical aspects of dermal absorption

The transport of chemicals through the skin is a complex process and occurs by passive diffusion.[51] Active transport and facilitated transport processes are absent from the SC because the corneocytes are anucleated and keratinized and cannot produce the specialised protein structures needed for active or facilitated transport.[11] For most chemicals the lipophilic SC is the rate-limiting barrier, and only in the case of very lipophilic chemicals and/or when the SC is damaged, the viable epidermis and dermis become the rate limiting barrier. Although the skin is a heterogeneous membrane, experimental results show that Fick's first law of diffusion offers a reasonable approximation of the processes of dermal absorption.[52]

$$J = D\,(C_{out}-C_{in})\,/\,L \qquad \textit{Eq. 1}$$

J is the steady-state flux or rate of mass transfer per unit area, L is the thickness of the SC, D is the diffusion coefficient and $(C_{out}-C_{in})$ is the concentration difference between two sides of the SC (C_{out} is the concentration of the chemical in the membrane at the outer side, and C_{in} is the concentration of the chemical at the inner side of the SC). Usually the concentration at the inner side of the SC is effectively zero and the equation can then be rewritten as:

$$J = D^{*}C_{out}\,/\,L \qquad \textit{Eq. 2}$$

The concentration C_{out} is related to the concentration of the chemical in the vehicle in which a chemical is applied (C_{veh}) by

$$C_{out} = C_{veh}{}^{*}K \qquad \textit{Eq. 3}$$

where K is the SC/vehicle partition coefficient. Often a permeability coefficient (K_p) is used which is defined as the steady-state flux divided by the concentration of the chemical in the vehicle (C_{veh}).

$$K_p = D\,K\,/\,L \qquad \textit{Eq. 4}$$

During non-steady state absorption, as e.g. by short exposures or during the initial period at longer exposures, there will be a non-linear change in concentration across the SC, the shape of which is described by Fick's second law of diffusion:

$$C(x) = KC_{veh}\left(1 - \frac{x}{L}\right) - \sum_{n=1}^{\infty} \frac{2}{n\pi} KC_{veh} \sin\left(\frac{n\pi x}{L}\right) \exp\left(\frac{-Dn^2\pi^2 t}{L^2}\right) \qquad \textit{Eq. 5}$$

where *C(x)* is the concentration profile of a chemical in the SC and *t* is exposure duration. The skin stripping technique, which was used in the studies presented to determine dermal absorption parameters, is based on the solution to the Fick's second law of diffusion (*Eq. 5*).

1.4. Factors affecting the transport of the chemicals through the skin

The extent and rate at which absorption of a chemical through the skin occurs depends upon a large number of variables including physico-chemical properties of the chemical and the vehicle in which the chemical is applied, skin condition, environmental factors and the exposure pattern.[53-54]

There is a large range of rates of dermal absorption between chemicals. Physico-chemical properties of the penetrant such as lipophilicity, polarity, charge and molecular size govern the partitioning of the chemical between the SC and vehicle and the diffusion. Due to lipophilic nature of the SC, lipophilic chemicals more readily partition into the SC than hydrophilic chemicals. Although lipophilic chemicals pass more readily through the SC, passage into and through the epidermis, and clearance from the dermis, may become rate limiting for the very lipophilic chemicals. Molecular size is an important factor in the SC diffusion. For molecules of similar polarity, those having the smaller molecular size will permeate faster. Instead of molecular size (e.g. molecular volume) molecular weight is more often used because of its availability and unambiguousness (not dependent on estimation methods as molecular volume).[53, 55] Experimental results have shown that the molecular weight dependence is more prominent for smaller molecules while it is more gradual for larger molecules.[56-58] It is generally considered that there is a molecular weight cut-off for effective permeation of healthy skin at 500 Da.[9]

The partitioning of a chemical from the vehicle into the SC is dependent on the physico-chemical properties of not only the chemical but also of the vehicle.[59-60] The vehicle can furthermore interact with the SC and alter the barrier properties, which might lead to an altered partitioning and diffusion of the chemical. Almost all vehicles alter the SC barrier to some extent and even water is known to be a penetration enhancer by interacting with polar head groups of the lipid bilayers.[57]

Dermal absorption varies with the anatomical site of the body due to thickness and composition of the SC, density of dermal appendages such as follicles which act as shunts and with, although to a lesser extent, differences in cutaneous blood flow.[45-47, 61] The maintenance of an intact skin is a prerequisite for a proper barrier function. However, the skin barrier can be compromised by physical damage (e.g. burned and shaved skin), chemicals (e.g. solvents, detergents and acids) or skin diseases, which might lead to the enhanced absorption.[11, 20, 62-68]

It has been shown that even a slight damage of the skin caused by exposure to sodium lauryl sulphate (SLS), which is a common ingredient of cosmetic products, can substantially increase the dermal absorption of chemicals covering a wide range of lipophilicity.[11, 62, 64-66, 69] Higher absorption was also found in the skin damaged by acetone and tape stripping.[61, 65, 67] Skin damage has been shown not only to increase absorption but also to facilitate the entrance of larger molecules.[42, 66-67]

Data on skin absorption in diseased skin are scarce and are obtained mostly from clinical studies. Higher absorption reported for diseased skin is based mostly on the topical treatment efficiency rather than on quantitative data.[70] Topical application of tacrolimus showed to be effective in the treatment of atopic dermatitis (AD); however, the absorption declined as the skin healed. [70-71] Persons with a history of AD showed signs of impaired skin barrier even on the sites visibly unaffected by the disease.[72-76] The higher skin permeability in AD has been linked to the different intercellular lipid composition and structure of the SC. Reduced ceramide content and decreased percentage of certain ceramides has been found in subjects with AD in both lesional and nonlesional skin.[24-25, 29, 32]

Environmental humidity has been shown to influence dermal absorption.[54, 77] The SC contains around 5-15% of water but this content can increase up to three-fold increasing the absorption of lipophilic chemicals.[78-81] The effect of hydration on the dermal absorption may be explained by the influence on the partitioning of a chemical into the SC, or by structural changes in lipid organization influencing diffusion.[52, 82] Increased hydration seemed to enhance especially the absorption of lipophilic chemicals, probably caused by increasing the transport across the SC/epidermis junction. Another factor in the diffusion is related to the kinetic energy of the diffusing molecule and is temperature dependent. Higher temperature may also affect organization of the intercellular lipid bilayers; the gel crystalline phase changes to the more fluid liquid crystalline phase.[83-85] This process improves the chemical diffusion through the SC.[86] In addition, the temperature can also affect the

blood flow which will increase the clearance from the skin. This may be important in the case of fast penetrating chemicals for which the clearance is rate-limiting.[57]

1.5. In vivo methods for measuring dermal absorption in humans

Human volunteer studies are considered as the "golden standard" against which all alternative methods such as in vitro and predictive mathematical models should be judged.[5] Because of technical and ethical concerns use of human volunteer studies is limited and their conduct is closely regulated.[87-88] Dermal absorption *in vivo* can be assessed using different approaches. Common methods for determination of in vivo dermal absorption include the measurement of parent chemical or metabolite levels in biological materials (e.g. blood, exhaled air and urine), microdialysis technique, and tape stripping. Each of these methods has its advantages and limitations.

Plasma and excreta measurements (Biological monitoring methods)

The extent of dermal absorption of chemicals can be assessed by analysing the parent chemical or its metabolite in plasma, exhaled air or urine.[89-91] The amount of the chemical determined after dermal exposure is compared to that after a reference exposure with a known input rate or dose such as intra-venous administration or inhalation. If the total amount of the chemical (e.g. total urine excretion), area under the blood concentration-time profile, is compared, only average absorption throughout the exposure can be deduced. A preferable method would be to estimate the dermal absorption rate- time profile. For that purpose, (de)convolution methods, using the concentration time profiles obtained from a dermal and a reference exposure, can be used.[92] The advantage of this approach is that, in addition to the average absorption into the skin, the maximum flux can be deduced and in the case of steady state absorption the permeability coefficient (K_p) can also be calculated.[92-93] This method has been widely used for the determination of dermal absorption of solvents, drugs and other chemicals.[94]

The measurement of parent chemical and/or its metabolite in plasma and excreta has practical importance for risk assessment. Especially in the case when dermal absorption contributes substantially to the total absorption, measurement of the internal dose by means of biological monitoring has to be preferred to environmental monitoring. In addition to the occupational exposure levels for airborne exposure, their biological equivalents known as Biological Exposure Indices have been set.[95]

Since dermal kinetics differ from those after inhalatory exposure the appropriate sampling strategy is important.

Microdialysis

Microdialysis is a technique that measures a dermally applied chemical in the extracellular space beneath the exposed skin site.[96-97] The principle of the technique is based on the passive diffusion of chemicals across the semi-permeable membrane of a microdialysis probe that is introduced into the cutaneous tissue parallel to the skin surface. The probe is usually slowly perfused with a physiological solution. Molecules able to pass the probe membrane will diffuse over the membrane into the perfusate which is collected for analysis. Due to the fact that the chemical is measured before it enters the systemic circulation, microdialysis is a very suitable technique to study skin metabolism. However, this technique has also its limitations. Implantation of the probe will elicit a tissue reaction, which in turn can influence skin absorption. Furthermore, when microdialysis is used with continuous perfusion of the probe, a true equilibrium with the perfusion fluid will not occur, and only a fraction of the chemical in surrounding tissue is recovered in the dialysate. This recovery depends on several experimental factors such as position of the tubing, physico-chemical properties of the chemical and perfusate. The most appropriate way of determining the relative recovery is still a matter of debate. An additional limitation of microdialysis is that it utilizes an aqueous perfusate and therefore can only dialyse water-soluble substances. Attempts to apply microdialysis of lipophilic chemicals following topical application have so far been unsuccessful.

Tape stripping

The tape stripping method is based on the determination of the amount of chemical in the separate layers of the SC. Generally, a predetermined area of the skin is exposed to a chemical for a certain period of time. After the end of exposure, the SC of the exposed skin site is removed sequentially by adhesive tape. The amount of recovered substance in each tape strip is determined with an appropriate analytical technique. Regarding the exposure period, time of SC harvesting, and the part of SC which is taken for the analysis, different approaches are proposed.[99-101]

In some, particularly older studies, dermal absorption was assessed by measuring the amount of chemical in only the superficial layers of the SC using one to three tape strips. It has been reported that the amount of the chemical in these SC layers was a good estimate of the total amount of the chemical absorbed into the systemic

circulation.[99-102] The main problem in that approach is the variability in the recovered amount of the chemical removed by each tape strip. This is influenced by several factors; type of adhesive tapes, vehicle in which the chemical is applied and the applied pressure on the tape prior to removal from the skin site. To avoid this source of variation, the amount of SC could be determined by measuring the weight of the SC in the strips or be estimated indirectly by e.g. the protein content or by assessing trans-epidermal water loss.[103-112]

The US Food and Drug Administration (FDA, 1998) proposed the tape stripping technique for the determination of bioequivalence of topically applied drugs.[113] The profile of a drug in the SC was determined during uptake and elimination phases. For the determination of the uptake, the drug is applied at multiple sites, each for different exposure duration. Immediately after the end of the exposure the SC is totally removed by tape strips. For the determination of the elimination, the drug is applied on multiple sites, but this time for the same exposure duration. The SC is then removed at different time points after the end of exposure. The determined concentration-time profile of a drug in the SC was used to estimate the rate and extent of diffusion and penetration of several drugs.[114-120] Due to poor reliability and reproducibility of this method, in 2002 the FDA withdrew the guidelines and the method is still in the investigative phase.

Instead of using the amount of the chemical in the SC as an estimate of dermal absorption, in some studies the concentration profile of the chemical across the entire SC depth has been determined.[103-105, 111, 121-122] The non-steady state diffusion equation (Eq. 4) is fitted to the data (Fig 3). From the fitted function, the rate constant for diffusion across SC (D/L^2, h^{-1}) and partition coefficient of the chemical between vehicle and SC (K) are deduced allowing estimation of the permeability coefficient (K_p).

Although the tape stripping technique has certain advantages, there are critical points. Some authors use the number of consecutive tape strips instead to measure the real SC depth assuming that the amount of the removed SC by each strip is linearly proportional to the number of strips.[123-124] However, the amount of removed SC varies considerably for different individuals and with the depth.[106, 124] The homogeneity of subsequent SC layers is also a point of concern: due to the furrows in the SC the amount of the chemical measured in the strip can come from different layers of the skin.[125] Furthermore, the time needed to remove the entire SC can be critical for the determination of chemicals which rapidly penetrate the SC.[126]

1.6. Objectives of the thesis and outline of the contents

As stated at the beginning of this chapter, understanding and quantifying dermal absorption of chemicals, as well as the factors which govern this process, are necessary for assessment of health risks following skin exposure. Reliable and validated methods are needed to determine dermal absorption. To date, dermal absorption has been mostly determined *in vitro,* while *in vivo* studies, in particular those in humans, are scarce. Still, such data are indispensable for validation of the *in vitro* methods and mathematical models for prediction of skin absorption.
In 2001, a project was initiated aiming to develop methodology for determination of dermal absorption and to generate new data on dermal absorption of a number of selected chemicals (EDETOX). A consortium of 12 participants from seven EU member states participated in this project. The work presented in this thesis was a part of this EDETOX project.

This dissertation reports studies conducted to:

I. Generate data on dermal absorption of 2-butoxyethanol in volunteers by using biological monitoring method (*Chapter section 2.1*) and microdialysis (*Chapter section 2.2)*
   These data were needed for the evaluation of *in vitro* methods and mathematical models for prediction of dermal absorption
II. Determine the influence of application vehicle (water) on dermal absorption of 2-butoxyethanol (*Chapter sections 2.1 and 2.2)*
III. Explore the possibility of biological monitoring of exposure to 2-butoxyethanol (*Chapter section 2.3*)
IV. Develop the skin stripping method for measurement of dermal absorption of polyethylene glycols (*Chapter 3*)
V. Study differences in the absorption of sodium lauryl sulphate and polyethylene glycols in the *SC* of normal skin and skin compromised by sodium lauryl sulphate (*Chapter section 4.3*) or by atopic dermatitis (*Chapter sections 4.1 and 4.2*)
VI. Investigate the influence of molecular size on absorption of polyethylene glycols in the *SC* of normal skin and skin compromised by sodium lauryl sulphate (*Chapter section 4.3*) or by atopic dermatitis (*Chapter sections 4.1 and 4.2*)
VII. Study the relation between the extent of absorption of an irritating chemical (SLS) and barrier impairment and skin inflammation (*Chapter 5*)

References:

1. Deutsche Forschungsgemeinschaft (DFG): List of MAK and BAT values 1999, Press Release No. 34 (13 July 1999) Wiley-VCH, Weinheim, 1999
2. Poet TS, McDougal JN: Skin absorption and human risk assessment. Chem Biol Interact 140: 19-34, 2002
3. Nielsen JB, Grandjean P: Criteria for skin notation in different countries. Am J Ind Med 45: 275-280, 2004
4. Bos PM, Brouwer DH, Stevenson H, Boogaard PJ, de Kort WL, van Hemmen JJ: Proposal for the assessment of quantitative dermal exposure limits in occupational environments: Prt 1. Development of a concept to derive a quantitative dermal occupational exposure limit. Occup Environ Med 55: 795-804, 1998
5. Howes D, Guy R, Hadgraft J, Heylings J, Hoeck U, Kemper F, Maibach H, Marty J-P, Merk H, Parra J, Rekkas D, Rondelli I, Schaefer H, Täuber U, Verbiese N: Methods for Assessing Percutaneous Absorption. Altern Lab Anim 24: 81-106, 1996
6. Jones AD, Dick IP, Cherrie JW, Cronin MTD, van de Sandt JJM, Esdaile DJ, Iyengar S, ten Berge W, Wilkinson SC, Roper CS, Semple S, de Heer C, Williams FM: CEFIC workshop on methods to determine dermal permeation for human risk assessment. Institute of Occupational Medicine, Edinburgh, Research report TM/04/07, 2004
7. OECD: Guideline for testing of chemicals. Guideline 428: Skin absorption: In vitro method (original guideline, adopted 13th April, 2004)
8. Skelly JP, Shah VP, Maibach HI, Guy RH, Wester RC, Flynn G et al: FDA and AAPS report of the workshop on principles and practices of in vitro percutaneous penetration studies: Relevance to bioavailability and bioequivalence. Pharm. Res 4: 265–267, 1987
9. Bos JD, Meinardi MMHM: The 500 Dalton rule for the skin penetration of chemical compounds and drugs. Exp Dermatol 9: 165-169, 2000
10. Smith-Pease CK, White IR, Basketter DA: Skin as a route of exposure to protein allergens. Clin Exp Dermatol 27: 296-230, 2002
11. Bouwstra JA, Honeywell-Nguyen PL, Gooris GS, Ponec M: Structure of the skin barrier and its modulation by vesicular formulations. Prog Lipid Res 42(1): 1-36, 2003
12. Williams AC: Transdermal and Topical Drug Delivery: From theory to clinical practice, Pharmaceuticals Press, London, U.K., 2003
13. Madison KC: Barrier function of the skin: "la raison d'etre" of the epidermis. J Invest Dermatol 121(2):231-41, 2003
14. Bos JD: Skin Immune System (SIS), In: (*Bos JD, editor*) Skin Immune System (SIS): Cutaneous Immunology and Clinical Immunodermatology. CRC Press, Boca Raton, page: 3-17, 2005
15. Singh S, Singh J: Transdermal drug delivery by passive diffusion and iontophoresis: a review. Med Res Rev 13(5): 569-621, 1993
16. Blank IH: Cutaneous barriers. J Invest Dermatol 45(4): 249-56, 1965
17. Ya-Xian Z, Suetake T, Tagami H: Number of cell layers of the stratum corneum in normal skin – relationship to the anatomical location on the body, age, sex and physical parameters. Arch Dermatol Res 291: 555-559, 1999
18. Elias PM: Epidermal lipids, barrier function, and desquamation. J Invest Dermatol 80 Suppl: 44s-49s, 1983

19. Elias PM, Cooper ER, Korc A, Brown BE: Percutaneous transport in relation to stratum corneum structure and lipid composition. J Invest Dermatol 76: 297-301, 1981
20. Elias PM, Menon GK: Structural and lipid biochemical correlates of the epidermal permeability barrier. Adv Lipid Res 24: 1-26, 1991
21. Michaels AS, Chandrasekaran SK, Shaw JE: Drug permeation through human skin: Theory and in vitro experimental measurement. Am Inst Chem. Eng J. 21: 985–996, 1975
22. Choi MJ, Maibach HI: Role of ceramides in barrier function of healthy and diseased skin. Am J Clin Dermatol 6(4): 215-223, 2005
23. Coderch L, Lopez O, de la Maza A, Parra JL: Ceramides and skin function. Am J Clin Dermatol 4(2): 107-129, 2003
24. Bleck O, Abeck D, Ring J, Hoppe U, Vietzke JP, Wolber R, Brandt O, Schreiner V: Two ceramide subfractions detectable in Cer (AS) position by HPTLC in Skin Surface Lipids of non-lesional skin of atopic eczema. J Invest Dermatol 113: 894-900, 1999
25. Di Nardo A, Wertz P, Giannetti A, Seidenari S: Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. Acta Derm Venereol 78: 27-30, 1998
26. Farwanah H, Raith K, Neubert RHH, Wohlrab J: Ceramide profiles of the uninvolved skin in atopic dermatitis and psoriasis are comparable to those of healthy skin. Arch Dermatol Res 296: 514-521, 2005
27. Fartasch M: Epidermal barrier in disorders of the skin Microsc Res Tech 38(4): 361-372, 1997
28. Ghadially R, Reed JT, Elias PM: Stratum corneum structure and function correlates with phenotype in psoriasis. J Invest Dermatol 107(4): 558-564, 1996
29. Imokawa G, Abe A, Jin K, Higaki Y, Kawashima M, Hidano A: Decreased level of ceramides in stratum corneum of atopic dermatitis: An etiological factor in atopic dry skin? J Invest Dermatol 96: 523-526, 1991
30. Matsumoto M, Umemoto N, Sugiura H, Uehara M: Difference in Ceramide Composition between "Dry" and "Normal" Skin in Patients with Atopic Dermatitis Acta Derm Venereol 79: 246-24, 1999
31. Motta S, Monti M, Sesana S, Mellesi L, Ghidoni R, Caputo R: Abnormality of water barrier function in psoriasis. Role of ceramide fractions. Arch Dermatol 130(4): 452-6, 1994
32. Yamamoto A, Serizawa S, Ito M, Sato Y: Stratum corneum abnormalities in atopic dermatitis. Arch Dermatol Res 283: 219-223, 1991
33. Bouwstra JA, Dubbelaar FER, Gooris GS, Ponec M: The lipid organization in the skin barrier. Acta Derm Venereol Supp 208: 23-30, 2000
34. Forslind B: A domain mosaic model of the skin barrier. Acta Derm Venereol 74(1): 1-6, 1994
35. Norlen L: Skin barrier structure and function: the single gel phase model. J Invest Dermatol 117(4): 830-836, 2001
36. Barry BW: Breaching the skin's barrier to drugs. Nat Biotechnol 22: 165 – 167, 2004
37. Flynn GL: In: Skin as a ropute of exposure to protein allergens, Gerrity TR, Henry CJ, eds. *Principles of route-to-route extrapolation for risk assessment.* Elsevier Science Publishing Co., Amsterdam, 1990

38. Bodde HE, van den Brink I, Koerten HK, de Haan FHN: Visualization of in vitro percutaneous penetration of mercuric chloride: Transport through intercellular space versus cellular uptake through desmosomes. J Control Release 15: 227– 236, 1991
39. Bouwstra JA, Gooris GS, Dubbelaar FE, Ponec M: Phase behavior of lipid mixtures based on human ceramides: coexistence of crystalline and liquid phases. J Lipid Res 42(11): 1759-1770, 2001
40. Kitson N, Thewalt JL: Hypothesis: The epidermal permeability barrier is a porous medium. Acta Derm Venereol 208: 12-15, 2000
41. Sznitowska M, Janicki S, Williams AC: Intracellular or intercellular localization of the polar pathway of penetration across stratum corneum. J Pharm Sci 87(9): 1109-1114, 1998
42. Billich A, Vyplel H, Grassberger M, Schmook FP, Steck A, Stuetz A: Novel cyclosporine derivatives featuring enhanced skin penetration despite increased molecular weight. Bioorg Med Chem 13: 3157-3167, 2005
43. Mitragotri S: Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. J Control Release 86(1): 69-92, 2003
44. Hadgraft J: Skin, the final frontier. Int J Pharm 224(1-2): 1-18, 2001
45. Feldman RJ, Maibach HI: Regional variation in percutaneous penetration of 14C-cortisol in man. J Invest Dermatol 48: 181-183, 1967
46. Maibach HI, Feldmann RJ, Milby TH, Serat WF: Regional variation in percutaneous penetration in man. Arch Environ Health 23: 208-211, 1971
47. Marzulli FN: Barriers to skin penetration. J Invest Dermatol 39: 387-393, 1962
48. Barry BW, Bennett SL: Effect of penetration enhancers on the permeation of mannitol, hydrocortisone and progesterone through human skin. J Pharm Pharmacol 39(7): 535-46, 1987
49. Chou CC, Riviere JE, Monteiro-Riviere NA: Differential relationship between the carbon chain length of jet fuel aliphatic hydrocarbons and their ability to induce cytotoxicity vs. interleukin-8 release in human epidermal keratinocytes. Toxicol Sci 69: 226–233, 2002
50. Eedy DJ: Carbon-fibre-induced airborne irritant contact dermatitis. Contact Dermatitis 35: 362–363, 1996
51. Crank J: Mathematics of diffusion. Oxford, Oxford Univ. Press, pp. 50-51, 1975
52. Scheuplein RJ, Blank IH: Permeability of the skin. Physiol Rev 51(4): 702-47, 1971
53. Patel H, ten Berge W, Cronin MT: Quantitative structure-activity relationships (QSARs) for the prediction of skin permeation of exogenous chemicals. Chemosphere 48(6): 603-13, 2002
54. Wiechers JW: The barrier function of the skin in relation to percutaneous absorption of drugs. Pharm Weekbl [Sci] 11: 185-198, 1989
55. Magnusson BM, Anissimov YG, Cross SE, Roberts MS: Molecular size as the main determinant of solute maximum flux across the skin. J Invest Dermatol 122: 993-999, 2004
56. Johnson ME, Blankschtein D, Langer R: Evaluation of solute permeation through the stratum corneum: Lateral bilayer diffusion as the primary transport mechanism. J Pharm Sci 86: 1162-1172, 1997
57. Kasting GB, Smith RL, Cooper ER: Effect of lipid solubility and molecular size on percutaneous absorption. In: Shroot B & Schaefer H (ed.)Skin

Pharmacokinetics.[Pharmacology and the skin] Basel, Karger Volume 1: 138-153, 1987

58. Potts RO, Guy RH: Predicting skin permeability. Pharm Res 9: 663-669, 1992
59. Jacobi U, Tassopoulos T, Surber C, Lademann J: Cutaneous distribution and localization of dyes affected by vehicles all with different lipophilicity. Arch Dermatol Res 297(7): 303-310, 2006
60. Johanson G, Fernstrom P: Influence of water on the percutaneous absorption of 2-butoxyethanol in guinea pigs. Scand J Work Environ Health 14: 95-100, 1988
61. Benfeldt E: In vivo microdialysis for the investigation of drug levels in the dermis and the effect of barrier perturbation on cutaneous drug penetration. Studies in hairless rats and human subjects. Acta Derm Venereol Suppl 206: 1-59, 1999
62. Borras-Blasco J, Diez-Sales O, Lopez A, Herraez-Dominguez M: A mathematical approach to predicting the percutaneous absorption enhancing effect of sodium lauryl sulphate. Int J Pharm 269: 121-129, 2004
63. Fartasch M: Ultrastructure of the epidermal barrier after irritation. Microsc Res Tech 37(3): 193-199, 1997
64. Nielsen JB: Percutaneous penetration through slightly damaged skin. Arch Dermatol Res 296: 560-567, 2005
65. Tsai JC, Sheu HM, Hung PL, Cheng CL: Effect of barrier disruption by acetone treatment on the permeability of compounds with various lipophilicities: Implications for the permeability of compromised skin. J Pharm Sci 90 (9): 1242-1254, 2001
66. Tsai JC, Hung PL, Sheu HM: Molecular weight dependence of polyethylene glycol penetration across acetone-disrupted permeability barrier. Arch Dermatol Res 293: 302-307, 2001
67. Tsai JC, Shen LC, Sheu HM, Lu CC: Tape stripping and sodium dodecyl sulfate treatment increase the molecular weight cutoff of the polyethylene glycol penetration across murine skin. Arch Dermatol Res 295: 169-174, 2003
68. Yoshiike T, Aikawa Y, Sindhvananda J, Suto H, Nishimura K, Kawamoto T, Ogawa H: Skin barrier defect in atopic dermatitis: increased permeability of the stratum corneum using dimethyl sulfoxide and theophylline. J Dermatol Sci 5: 92-96, 1993
69. Wilhelm K P, Surber C, Maibach H I. Effect of sodium lauryl sulfate-Induced skin irritation on in vitro percutaneous penetration of four drugs. J Invest Dermatol 96: 963-967, 1991
70. Bos JD: Non-steroidal topical immunomodulators provide skin-selective, self-limiting treatment in atopic dermatitis. Eur J Dermatol 13: 455-461, 2003
71. Rubins A, Gutmane R, Valdmane N, Stevenson P, Foster C, Undre N: Pharmacokinetics of 0.1% tacrolimus ointment after first and repeated application to adults with moderate and severe atopic dermatitis. J Invest Dermatol 125: 68-71, 2005
72. Basketter DA, Miettinen, Lahti A: Acute irritant reactivity to sodium lauryl sulfate in atopics and non-atopics. Contact Dermatitis 38: 253-257, 1998
73. Berardesca E, Fideli D, Borroni G, Rabbiosi G, Maibach H: *In vivo* hydratation and water-retention capacity of stratum corneum in clinically uninvolved skin in atopic and psoriatic patients. Acta Derm Venereol 70: 400-404, 1990
74. Nicander I, Ollmar S: Clinically normal skin vs. non-atopic skin as seen through electrical impedance. Skin Res Technol 10: 178-183, 2004

75. Seidenari S, Giusti G: Objective assessment of the skin of children affected by atopic dermatitis: A study of pH, capacitance and TEWL in Eczematous and clinically uninvolved skin. Acta Derm Venereol 75: 429-433, 1995
76. Werner Y, Lindberg M: Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. Acta Derm Venereol 65: 102-105, 1985
77. Boman A, Maibach HI: Percutaneous absorption of organic solvents. Int J Occup Environ Health 6(2): 93-95, 2000
78. Barai ND: Effect of hydration on skin permeability.M.Sc. Thesis. College of Pharmacy, University of Cincinnati, Cincinnati, OH., 2002
79. Behl CR, Flynn GL, Kurihara T, Harper N, Smith W, Higuchi WI, Ho NFH, Pierson CL: Hydration and percutaneous absorption: I. Influence of hydratin on alkanol permeatin through hairless mouse skin. J Invest Dermatol 75: 346-352, 1980
80. Blank IH, Scheuplein RJ, MacFarlane DJ: Mechanism of percutaneous absorption. 3. The effect of temperature on the transport of non-electrolytes across the skin. J Invest Dermatol 49(6): 582-589, 1967
81. Bucks DA, McMaster JR, Maibach HI, Guy RH: Bioavailability of topically administered steroids: a "mass balance" technique. J Invest Dermatol 91(1): 29-33, 1988
82. Hadgraft J, Guy RH, editors: Transdermal drug delivery. Developmental issues and research initiatives. Marcel Dekker, New York, 1989
83. Jain AK, Panchagnula R: Effect of temperature on imipramine hydrochloride permeation: role of lipid bilayer arrangement and chemical compositin of rat skin. Int J Pharm 250: 287-293, 2003
84. Ogiso T, Hirota T, Iwaki M, HinoT, Tanito T: Effect of temperature on percutaneous absorption of terodiline, and relationship between penetration and fluidity of the stratum corneum lipids. Int J Pharm 176: 63-72, 1998
85. Pilgram GS, Engelsma-van Pelt AM, Bouwstra JA, Koerten HK: Electron diffraction provides new information on human stratum corneum lipid organization studied in relation to depth and temperature. J Invest Dermatol 113(3): 403-409, 1999
86. Murthy SN, Sen A, Li-Zhao Y, Hui SW: Temperature influences the postelectroporation permeability state of the skin. J Pharm Sci 93(4): 908-915, 2004
87. International Conference on Harmonisation: Guideline for Good Clinical Practice, 1996
88. World Medical Association Declaration of Helsinki, Ethical principles for medical research involving human subjects, adopted by the 18th World Medical Association general assembly, Helsinki, Finland, 1964 (as amended in 2004)
89. Akrill P, Cocker J, Dixon S: Dermal exposure to aqueous solutions of N-methyl pyrrolidone. Toxicol Lett 134(1-3): 265-269, 2002
90. Brooke I, Cocker J, Delic JI, Payne M, Jones K, Gregg NC, Dyne D: Dermal uptake of solvents from the vapour phase: an experimental study in humans. Ann Occup Hyg 42(8): 531-40, 1998
91. Cardona A, Marhuenda D, Marti J, Brugnone F, Roel J, Perbellini L: Biological monitoring of occupational exposure to n-hexane by measurement of urinary 2,5-hexanedione. Int Arch Occup Environ Health 65(1): 71-74, 1993
92. Opdam JJ: Linear systems dynamics in toxicokinetic studies. Ann Occup Hyg 35(6): 633-649, 1991

93. Kezic S, Janmaat A, Kruse J, Monster AC, Verberk MM: Percutaneous absorption of m-xylene vapour in volunteers during pre-steady and steady state. Toxicol Lett 153(2): 273-282, 2004
94. Kezic S, Monster AC, van de Gevel IA, Kruse J, Opdam JJ, Verberk MM: Dermal absorption of neat liquid solvents on brief exposures in volunteers. AIHAJ 62(1): 12-18, 2001
95. American Conference of Governmental Industrial Hygienists: Documentation of the Biological Exposure Indices, 7th Ed., Cincinnati USA, 2001
96. de Lange EC, de Boer AG, Breimer DD: Methodological issues in microdialysis sampling for pharmacokinetic studies. Adv Drug Deliv Rev 45(2-3):125-148, 2000
97. Kreilgaard M: Assessment of cutaneous drug delivery using microdialysis. Adv Drug Deliv Rev. 54 Suppl 1: S99-121, 2002
98. Schnetz E, Fartasch M: Microdialysis for the evaluation of penetration through the human skin barrier - a promising tool for future research? Eur J Pharm Sci 12(3):165-174, 2001
99. Chao YC, Gibson RL, Nylander-French LA: Dermal exposure to jet fuel (JP-8) in US Air Force personnel. Ann Occup Hyg. 49(7): 639-645, 2005
100. Dupuis D, Rougier A, Roguet R, Lotte C, Kalopissis G: In vivo relationship between layer reservoir effect and percutaneous absorption in human and rat. J Invest Dermatol 82: 353-356, 1984
101. Mattorano DA, Kupper LL, Nylander-French LA: Estimating dermal exposure to jet fuel (naphthalene) using adhesive tape strip samples. Ann Occup Hyg 48(2): 139-146, 2004
102. Rougier A, Dupuis D, Lotte C, Roguet R, Schaefer H: In vivo correlation between stratum corneum reservoir function and percutaneous absorption. J Invest Dermatol 81: 275-278, 1983
103. Alberti I, Kalia YN, Naik A, Guy RH: In vivo assessment of enhanced topical delivery of terbinafine to human stratum corneum.J Control Release 71(3): 319-327, 2001
104. Alberti I, Kalia YN, Naik A, Bonny JD, Guy RH: Effect of ethanol and isopropyl myristate on the availability of topical terbinafine in human stratum corneum, in vivo. Int J Pharm 219(1-2): 11-19, 2001
105. Alberti I, Kalia YN, Naik A, Bonny JD, Guy RH: Assessment and prediction of the cutaneous bioavailability of topical terbinafine, in vivo, in man. Pharm Res 18(10): 1472-1475, 2001
106. Bashir SJ, Chew AL, Angibogu A, Dreher F, Maibach HI: Physical and physiological effects of stratum corneum tape stripping. Skin Res Tehn 7: 40-48, 2001
107. Bommannan D, Potts RO, Guy RH: Examination of stratum corneum barrier function in vivo by infrared spectroscopy. J Invest Dermatol 95(4): 403-408, 1990
108. Chao YCE, Nylander-French LA: Determination of keratin protein in a tape-stripped skin sample from jet fuel exposed skin; Ann Occup Hyg 48: 65-73, 2004
109. Dreher F, Arens A, Hostynek JJ, Mudumba S, Ademola J, Maibach HI: Colorimetric method for quantifying human Stratum corneum removed by adhesive-tape stripping. Acta Derm Venereol. 78: 186-189, 1998
110. Marttin E, Neelissen-Subnel MT, De Haan FH, Bodde HE: A critical comparison of methods to quantify stratum corneum removed by tape stripping. Skin Pharmacol 9(1): 69-77, 1996

111. Pirot F, Kalia YN, Stinchcomb AL, Keating G, Bunge A, Guy RH: Characterization of the permeability barrier of human skin in vivo. Proc Nat Acad Sci USA 94: 1562-1567, 1997
112. Tsai JC, Lin CY, Sheu HM, Lo YL, Huang YH: Noninvasive characterization of regional variation in drug transport into human stratum corneum in vivo. Pharm Res 20(4): 632-638, 2003
113. US Food and drug administration (FDA): Guidance for Industry, Topical Dermatological Drug Product NDAs and ANDAs — In Vivo Bioavailability, Bioequivalence, In Vitro Release, and Associated Studies, 1998
114. Jiang SJ, Zhou XJ, Sun GQ, Zhang Y: Morphological alterations of the stratum corneum lipids induced by sodium lauryl sulfate treatment in hairless mice. J Dermatol Sci 32: 243-246, 2003
115. Loden M, Akerstrom U, Lindahl K, Berne B: Bioequivalence determination of topical ketoprofen using a dermatopharmacokinetic approach and excised skin penetration. Int J Pharm 284(1-2): 23-30, 2004
116. Pershing LK, Bakhtian S, Poncelet CE, Corlett JL, Shah VP: Comparison of skin stripping, in vitro release, and skin blanching response methods to measure dose response and similarity of triamcinolone acetonide cream strengths from two manufactured sources. J Pharm Sci 91(5): 1312-1323, 2002
117. Pershing LK, Corlett JL, Nelson JL: Comparison of dermatopharmacokinetic vs. clinicial efficacy methods for bioequivalence assessment of miconazole nitrate vaginal cream, 2% in humans. Pharm Res 19(3): 270-277, 2002
118. Pershing LK, Nelson JL, Corlett JL, Shrivastava SP, Hare DB, Shah VP: Assessment of dermatopharmacokinetic approach in the bioequivalence determination of topical tretinoin gel products. J Am Acad Dermatol 48(5): 740-751, 2003
119. Shah VP, Flynn GL, Yacobi A, Maibach HI, Bon C, Fleischer NM, Franz TJ, Kaplan SA, Kawamoto J, Lesko LJ, Marty JP, Pershing LK, Schaefer H, Sequeira JA, Shrivastava SP, Wilkin J, Williams RL: Bioequivalence of Topical Dermatological Dosage Forms - Methods of Evaluation of Bioequivalence 11(2): 117-124, 1998
120. Shah VP: Progress in methodologies for evaluating bioequivalence of topical formulations. Am J Clin Dermatol 2(5): 275-280, 2001
121. Kalia YN, Alberti I, Naik A, Guy RH: Assessment of topical bioavailability in vivo: the importance of stratum corneum thickness. Skin Pharmacol Appl Skin Physiol 14 Suppl 1: 82-86, 2001
122. Stinchcomb AL, Pirot F, Touraille GD, Bunge AL, Guy RH: Chemical uptake into human stratum corneum in vivo from volatile and non-volatile solvents. Pharm Res. 16(8): 1288-1293, 1999
123. Semple S: Dermal exposure to chemicals in the workplace: just how important is skin absorption? Occup Environ Med 61: 376-382, 2004
124. Tsai JC, Cappel MJ, Weiner ND, Flynn GL, Ferry J: Solvent effects on the harvesting of stratum corneum from hairless mouse skin through adhesive tape stripping in vitro. Int J Pharm 68: 127-133, 1991
125. van der Molen RG, Spies F, van 't Noordende JM , Boelsma E, Mommaas AM' Koerten HK : Tape stripping of human stratum corneum yields cell layers that originate from various depths because of furrows in the skin Arch Dermatol Res 289(9): 514-518, 1997

126. Reddy MB, Stinchcomb AL, Guy RH, Bunge AL: Determining dermal absorption parameters in vivo from tape strip data. Pharm Res 19(3): 292-298, 2002

Chapter 2: *Section 2.1*

Percutaneous absorption of neat and aqueous solutions of 2-butoxyethanol in volunteers

I. Jakasa, N. Mohammadi, J. Kruse, S. Kezic

Int Arch Occup Environ Health (2004) 77: 79-84

Abstract

Objectives: To study the influence of the presence of water on the dermal absorption of 2-butoxyethanol (BE) in volunteers.

Methods: Six male volunteers were dermally exposed to 50%, 90% or neat w/w BE for 4 h on the volar forearm over an area of 40 cm^2. An inhalation exposure with a known input rate and duration served as a reference dosage. The dermal absorption parameters were calculated from 24-h excretion of total (free + conjugated) butoxyacetic acid (BAA) in urine and BE in blood, measured after both inhalation and dermal exposure.

Results: The dermal absorption of BE from aqueous solutions was markedly higher than that of neat BE. The time-weighted average dermal fluxes were calculated from the urine and blood data and expressed in milligrammes per square centimetre per hour. The dermal fluxes obtained from cumulative 24-h excretion of BAA amounted to 1.34 ± 0.49, 0.92 ± 0.60 and 0.26 ± 0.17 mg cm^{-2} h^{-1} for 50%, 90% and neat BE, respectively. The dermal fluxes calculated from the BE blood data amounted to 0.92 ± 0.34 and 0.74 ± 0.25 mg cm^{-2} h^{-1} for 50% and 90% BE, respectively. The permeation rates into the blood reached a plateau between 60 and 120 min after the start of exposure, indicating achievement of steadystate permeation. The apparent permeability coefficient Kp, was $1.75 \pm 0.53 \cdot 10^{-3}$ and $0.88 \pm 0.42 \cdot 10^{-3}$ cm h^{-1} for 50% and 90% BE, respectively.

Conclusion: The percutaneous absorption of BE from aqueous solution increased markedly when compared with neat BE. Even water content as low as 10% led to an approximate fourfold increase in the permeation rates. These findings are important for the health risk assessment of occupational exposure to BE, since BE is commonly used in mixtures that contain water. Exposure to aqueous solutions of 50% and 90% of BE may result in substantial skin absorption: if a 60-min skin contact of 1000 cm^2 is assumed, dermal uptake would be fourtimes higher than the pulmonary uptake of an 8-h occupational exposure at a TLV of 100 mg m^{-3}. This clearly justifies the skin notation for BE. For the purpose of biological monitoring, both BE in blood and BAA in urine were shown to be reliable indicators of exposure.

Introduction

Ethylene glycol ethers are frequently used in industry and households as solvents, emulsifiers and detergents. The use of 2 ethoxyethanol and 2-butoxyethanol (BE) has increased after the removal of 2-methoxyethanol from nearly all formulations because of its toxicity [19]. They are used in great quantities because of their excellent hydrophilic and lipophilic properties. Because of the low vapour pressure and high rate of dermal absorption, significant systemic exposure can occur through contact with the skin [5, 6]. It has been shown that BE readily penetrates the skin in guinea pigs and rats in vivo an d in human, guinea pig and rat skin in vitro [1, 4, 5, 6]. The presence of water has been shown to enhance the percutaneous absorption of BE in vivo in guinea pig skin and in vitro in human skin [6, 20]. Percutaneous absorption of neat BE in humans has been demonstrated [7, 9]; however, dermal absorption from aqueous solutions of BE has not been studied. Since BE is commonly used in water mixtures, it is relevant to compare the absorption rate of neat BE and that of aqueous solutions in humans.

Material and methods

Subjects

Six male volunteers, aged 22–55 years and with no history of dermatological disease, participated in this study. They were in good health, had no visible skin damage and used no medication. The Ethical Committee of the Academic Medical Center, University of Amsterdam, approved the experiment protocol. Written informed consent was obtained from all subjects prior to experiments.

Reference inhalatory exposure

Each volunteer inhaled the solvent vapour for 30 min through a mouthpiece with a two-way valve connected to a Tedlar (DuPont, Delaware, USA) bag. The concentration of the vapour in the bag was approximately 93 ± 6.8 mg m^{-3} (mean value of six exposures), which is below the present occupational exposure limit in the Netherlands (100 mg m^{-3}) [11]. In order to determine the respiratory input rate we measured the total exhaled volume.

Dermal exposure

A bottomless glass chamber (area 40 cm^2) was placed on the volar forearm and filled with 8 ml of dosing BE solution. To prevent leakage, we glued the glass chamber onto the skin using UHU-Hart glue (UHU, Bu¨ hl, Germany). The concentration of BE

in the solution was measured before and after exposure. In order to avoid inhaling solvent vapour during the application of the solvent, the volunteer sat in a ventilated clean-air cabin with overpressure, and put his arm through an opening in the wall of the cabin. The exposure lasted for 4 h. Blood samples were collected for 8 h (16 samples per experiment). Urine samples were collected every 4 h during the 24-hour period. Each volunteer was exposed twice to a 50% BE solution (exposure on two different arms), once to 90% and once to neat BE. The period between two dermal exposures of the same skin site was at least 4 weeks.

Analytical methods

Chemicals

Acetone (p. a.), dichloromethane (p. a.), n-hexane (Lichrosolv), hydrochloric acid (conc., 37%), methanol (Lichrosolv), potassium carbonate (p. a.) and pyridine were purchased from Merck (the Netherlands). Phenoxyethanol (98%) and ethoxyacetic acid (98%) were purchased from Aldrich (the Netherlands). Pentafluorobenzoyl chloride (99%) and pentafluorobenzyl bromide (≥ 99%) were purchased from Fluka (the Netherlands). Butoxyethanol (99%) was purchased from Sigma (the Netherlands) and butoxyacetic acid from TCI (Japan).

Analysis of BE in plasma

Immediately after blood collection in Li-heparin tubes, the plasma samples were prepared and stored in safe-lock tubes at)18 °C until required for analysis. BE in plasma was determined with a slightly modified method of Johanson and Fernstrom [5]; and Johanson et al. [8], which is based on extraction with dichloromethane and derivatization with pentafluorobenzoyl chloride and electron capture detection (ECD). The limit of quantitation (LOQ) of the method was 0.014 mg L^{-1} and the coefficient of variation was 7%. Gas chromatographic (GC) analysis was carried out with a Hewlett-Packard 5890 GC (Hewlett-Packard, USA) equipped with a63Ni ECD. Two AT-1701 capillary columns (30 m·0.25 mm, 0.25-µm film thickness; Alltech, The Netherlands) were connected by glass connector. The initial column temperature was 50 °C, and the temperature was increased to 240 °C at 35 °C min^{-1} and held for 16 min. The injector temperature was 250 °C, the detector temperature was 260 °C and the column head pressure was 150 kPa. The sample (1 µL) was injected by means of the splitless injection technique.

Analysis of BAA in urine

After collection, 1.5 ml aliquots of urine were stored in safe-lock tubes at -18 °C until required for analysis. For 50% BE, the concentration of BAA was determined in all collected samples. Since the excretion of BAA was shown to be completed within a 24-h period, the concentration of BAA after exposure to 90% and neat BE was determined only in pooled 24-h urine. The analysis of BAA in urine samples was based on acid hydrolysis of conjugated BAA, subsequent derivatization with pentafluorobenzyl bromide (PFBBr) and GC-ECD analysis. For that purpose 50 µL of concentrated HCl was added to 50 µL of urine and heated for 60 min at 95 ± 5 °C. After the solution had cooled to room temperature, 2 ml of acetone, ± 0.15 g of potassium carbonate, 20 µL of ethoxyacetic acid solution (100 mg L^{-1}) as an internal standard and 20 µL of PFBBr were added and heated for 60 min at 95 °C. After being cooled to room temperature, a 100- µL aliquot of the acetone layer was transferred to a safe-lock tube containing 250 µL of 90% methanol in water and 750 µL of n-hexane. Samples were vortexed for 5 min and centrifuged for 30 s (11,860 g). GC analysis was carried out with a Carlo Erba HRGC 5300 GC (Interscience, The Netherlands) equipped with a 63Ni ECD. The column was HP-1 (25 m·0.32 mm, 0.52- µm film thickness, Alltech, The Netherlands). The initial column temperature was 100 °C, and the temperature was increased to 170 °C at 5 °C min^{-1} and subsequently to 200 °C at 45 °C min^{-1} and held for 1 min. The injector and detector temperatures were 250 °C and the column head pressure was 100 kPa. The sample (3 µL) was injected by the split injection technique (split ratio 1:50). The LOQ of the method was 3.3 mg L^{-1} and the coefficient of variation was 14%.

Calculations

Inhalation exposure

The respiratory input rate (IR) was calculated as follows [12]:

$$IR\ (\mu g\ min^{-1}) = C_{inh} \times (V_{inh} / t_{exp} - f \times V_d)$$

where V_{inh} (L) is total inhaled volume, C_{inh} (µg L^{-1}) is the concentration in inhaled air, t_{exp} (min) is duration of exposure, V_d (L) is dead-space volume taken as sum of the anatomical dead space (0.15 L) and the dead space of the mouthpiece (0.04 L) and f (vent min^{-1}) is individual ventilation frequency. We calculated the amount absorbed after inhalation exposure (INH_{abs}) by multiplying IR by exposure duration (30 min).

Dermal exposure

The amount of BE absorbed into the skin (DER_{abs}) was calculated from the excreted BAA measured after both inhalation (BAA_{inh}) and dermal (BAA_{der}) exposure as follows:

$$DER_{abs}\ (mg) = BAA_{der} / BAA_{inh} \times INH_{abs}$$

We calculated the average dermal flux throughout the exposure by dividing the amount of BE absorbed into the skin by exposure area and exposure time and expressed it in milligrammes per square centimetre per hour.

For the calculation of permeation rates and dermal fluxes we used the linear system dynamics method that is extensively described elsewhere [12, 13]. Briefly, we determined individual systemic kinetics from the reference inhalation experiment using the blood BE concentration-time data. Using a convolution method we fitted the data to a mathematical expression combining the kinetic response after bolus dose with exposure duration and concentration. The parameters obtained from a fitted function and the concentration-time data after dermal exposure were used to determine the permeation rates as function of time by deconvolution. The total amount of BE absorbed into the blood was determined from the area under the permeation rate-time curve. The amount of BE absorbed into the skin during exposure was considered to be equal to the amount absorbed into the blood. We calculated the average dermal flux from blood data in the same manner as from urine data, by dividing the amount absorbed into the skin by exposure area and exposure time, and expressed it in milligrammes per square centimetre per hour. The maximum permeation rates were determined from the slope of the cumulative absorbed mass vs time. When steady-state permeation is achieved the maximum permeation rate represents the apparent permeability coefficient Kp (cm h^{-1}). The principle of the method is illustrated in Fig 1, which shows the concentration-time course in blood following (a) inhalation, (b) dermal exposure and (c) corresponding permeation rate-time courses of BE.

Using the results of the (two) replicated dermal exposures, we calculated the intra-subject variability as well as the inter-subject variability in a restricted sense, i.e. after eliminating the intra-subject variability. For the latter we used the coefficient of variation = {([between subject variance)within subject variance] / 2)$^{1/2}$}/mean.

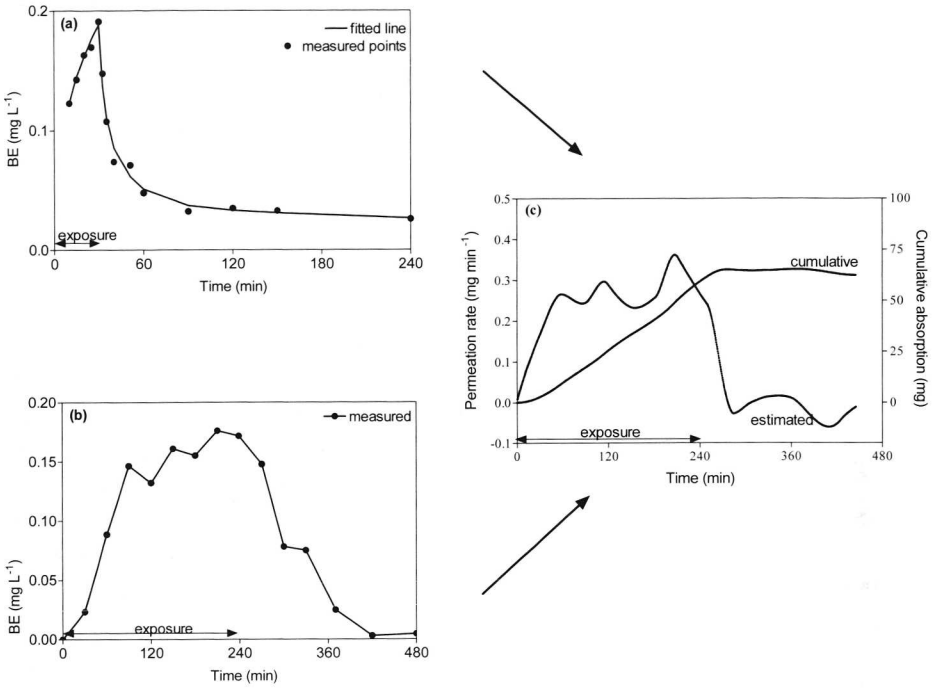


Fig 1a-c: *The linear system dynamics method used for the estimation of permeation rates and dermal fluxes in one subject exposed to 50% BE. (a) Concentration-time course of BE after inhalation exposure and the fitted function; (b) concentration-time course after dermal exposure; (c) estimated permeation rate-time course. Inhalation exposure concentration was 93 mg m^{-3} for 30 min; dermal exposure area was 40 cm^2 and dermal exposure duration was 4 h*

Results

The amount of BE in dosing solutions measured before and after dermal exposure to 50 % BE were 49.5 % and 49.0 ± 1.4 % (mean of 12 individual exposures), respectively. This indicates that the concentration throughout the exposure was constant (i.e. the dose was infinite). The concentration of BE in blood after exposure to neat BE was, in most of the samples, below the detection limit of the method. After exposures to 50 % and 90 % BE, the BE concentrations could be measured in all subjects at all time points. In none of the volunteers did skin irritation occur; however, after exposure the skin had a wrinkled appearance.

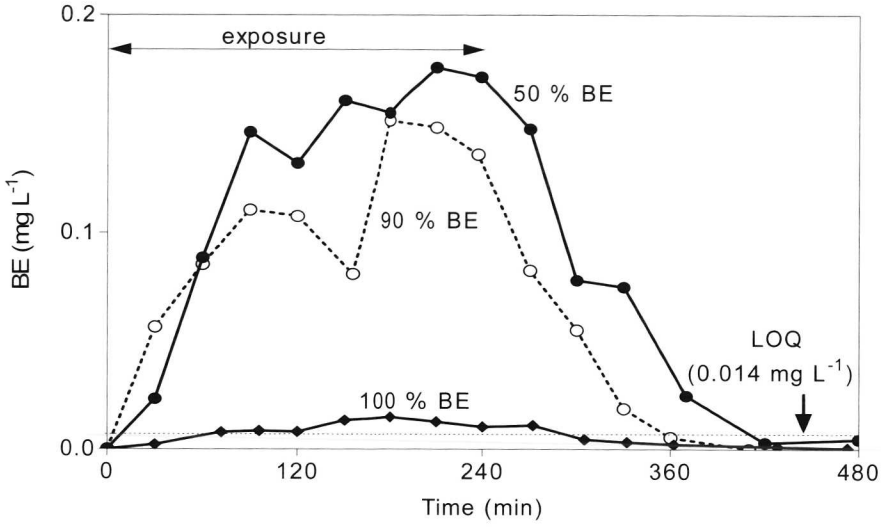


Fig 2: *Concentration–time profile of BE in blood in a subject dermally exposed to 50%, 90% and neat BE*

Figure 2 shows the typical concentration–time courses ofBE in blood, measured after exposure to 50%, 90%and neat BE in one volunteer. In all subjects, exposure to 50% and 90% BE resulted in higher blood concentrations than those after exposure to neat BE.

This is consistent with the higher 24-h cumulative excretion of BAA after exposure to aqueous solutions of BE than that after exposure to neat BE (Fig 3). As calculated from the individual BAA concentration-time curves determined after inhalation exposure, the average half-life of BAA amounted to 3.4 h (range 1.3 to 3.8 h). This implies that BAA is almost completely excreted in urine within 24 h of the start of the exposure. Using the amount of BE absorbed after inhalation exposure (the average value for six subjects was 20.9 ± 5.0 mg) and the cumulative excretion of BAA after inhalation, we calculated that, on average, 57% (range 42–70%) of absorbed BE was excreted as BAA.

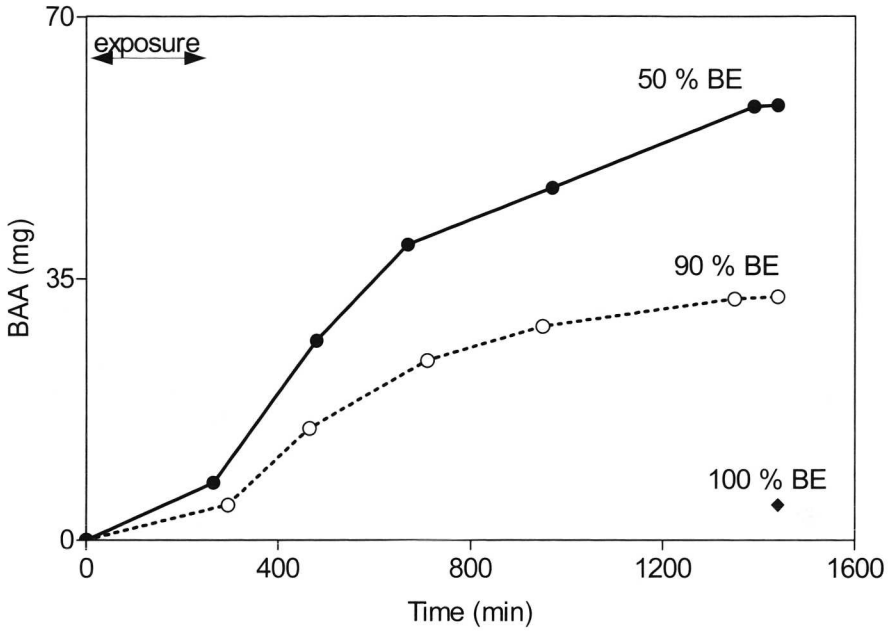


Fig 3: *Cumulative excretion of BAA in urine in a subject dermally exposed to 50%, 90% and neat BE. For neat BE only cumulative amount was measured*

Table 1. Average dermal fluxes of BE after exposure to 50%, 90% and neat BE obtained from blood and urine data. Values are means ± SD. *ND* not determined: blood concentrations were below limit of quantitation

Average dermal flux (mg cm^{-2} h^{-1})	50 % BE	90 % BE	Neat BE
Based on blood data	0.92 ± 0.34 [1](*n* =6)	0.74 ± 0.25 (*n* =3)	[2] ND (*n* =3)
Based on urine data	1.34 ± 0.49 [1](*n*=6)	0.92 ± 0.60 (*n* =5)	0.26 ± 0.17 (*n* =6)

[1]Each subject was exposed twice to 50 % BE
[2]Not determined: blood concentrations below limit of quantitation

The average dermal fluxes calculated from the BAA urine data as well as from the BE blood data are presented in Table 1. For the exposures to 50% and 90% BE the time courses of permeation rates were also determined. The permeation rates reached a plateau between 60 and 120 min after the start of exposure, indicating steady-state permeation. The maximum permeation rates, which are in fact the apparent permeability coefficients, are presented in Table 2.

Table 2. Apparent permeability coefficients of 50%, 90% and neat BE obtained from blood data. Values are means ± SD. *ND* not determined: blood concentrations were below limit of quantitation

Apparent permeability coefficients (x 10^{-3} cm h^{-1})		
50 % BE	90 % BE	Neat BE
1.75 ± 0.53 [1](*n* =6)	0.88 ± 0.42 (*n* =3)	[2]ND (*n* =3)

[1]Each subject was exposed twice to 50 % BE
[2]Not determined: blood concentrations below limit of quantitation

In order to obtain insight into intra-individual and inter-individual variation of dermal fluxes, we performed dermal exposure to 50% BE twice for each volunteer. The intra-individual and inter-individual variance of the dermal fluxes obtained from the blood and urine data are shown in Table 3. Table 3 shows that the inter-individual variation was approximately twice as high as the intra-individual variation, regardless of whether urine or blood data were taken.

Table 3. Intra-individual and inter-individual variations of dermal fluxes of BE after exposure to 50% BE

	Mean ± SD (mg cm^{-2} h^{-1})	90 % Confidence interval	Intraindividual CV (%)	Interindividual CV (%)
Based on blood data	0.92 ± 0.34	0.63 – 1.19	16	35
Based on urine data	1.34 ± 0.49	0.94 – 1.74	20	34

In order to assess the relevance of our results for occupational exposure to BE, we estimated the contribution of dermal uptake to the total uptake. The uptake after dermal exposure of 1000 cm^2 for 1 h was compared with an 8-h inhalatory uptake at TLV in the Netherlands [11]. Table 4 shows that such dermal exposure to aqueous solutions under the above-described exposure conditions leads to a substantial uptake that even exceeds the pulmonary uptake.

Table 4. Estimated dermal uptake of BE in relation to the pulmonary uptake during an 8-h exposure at the TLV (100 mg m^{-3})

[1]Pulmonary uptake (mg)	[2]Dermal uptake (mg)		
	50 % BE	90 % BE	100 % BE
346	1340	926	263

[1]Assumed minute volume of 10 L/min and alveolar retention of 72 % [9]
[2]Assumed dermal exposure of 1000 cm^2 skin for 1 hour

Discussion and conclusions

The presented results show that the presence of water in the applied solution markedly increases the percutaneous absorption of BE in humans in vivo. The time-weighted average fluxes calculated from the 24-h cumulative urine BAA excretion amounted to 0.26, 0.92 and 1.34 mg cm^{-2} h^{-1}, for neat, 90% and 50% BE, respectively. The presence of water has previously been shown to enhance the percutaneous penetration of BE. In an in vivo study [6], the permeability coefficients (Kp) of BE in the guinea pig were consistent with those reported in our study and were 0.30 x 10^{-3}, 0.84 x 10^{-3} and 1.82 x 10^{-3} cm h^{-1} for neat, 80% and 40% BE, respectively. The values of Kp that we obtained from blood results were 1.75 ± 0.53 x 10^{-3} and 0.88 ± 0.42 x 10^{-3} cm h^{-1} for 50% and 90% BE, respectively. Wilkinson and Williams reported in an in vitro study with human skin considerably higher apparent Kp values with aqueous solutions than with undiluted doses [20]. They suggested that those findings could be explained by better partitioning of BE between the stratum corneum (SC) and vehicle (aqueous solution), resulting in a proportionately higher concentration gradient across the skin, which hence promoted steady-state flux. It has been shown for a number of hydrophilic compounds, e.g. for BE, propoxyacetic acid and alcohols, that hydration of the SC leads to enhanced absorption [6, 10, 16]. It seems to us unlikely that the changed composition of either vehicle or SC would influence the partition of BE between those two phases to such

an extent (almost a fourfold increase of dermal flux in the presence of 10% water). A possible explanation could be that water affects the structure of the SC, in particular its lamellar organization, leading to a higher porosity and improved permeation of BE [18]. Recently Tang *at al.* [17] have found that hydration leads to induction of new pores/reduction of the tortuosity of existing pores in the SC, improving the percutaneous penetration of water-soluble compounds. Another explanation of the promoting effect of water on the permeation of BE was argued by Corley *et al.*, who suggested that BE may have "fixed" the skin at the higher concentrations [2]. However, the mechanism and the meaning of the "fixing" of the skin remained unclear in that study. It may also be that the use of neat BE will dehydrate the skin to a certain extent and that it will block the polar pathways through the skin.

Initially, we intended to use only blood BE concentrations to determine the dermal absorption kinetics. However, the absorption of neat BE was too low, and the blood BE concentrations were under the LOQ of the method used. In order to enable comparison of dermal fluxes of different BE concentrations, we also measured the urinary excretion of the main metabolite, BAA. Therefore, the comparison of dermal fluxes for all BE concentrations mainly relies on the BAA measured in urine, though dermal fluxes based on blood data of 50% and 90% BE were also calculated. The excretion of BAA in urine is of particular significance for occupational exposure and is used as a biological indicator of exposure [14, 15]. In most of the studies, only the free urinary fraction of BAA was measured. However, recent studies by Corley *et al.* [3], Rettenmeier et al. [14] and Sakai *et al.* [15] have shown that a substantial amount of BAA is excreted in human urine as a glutamate conjugate (on average 48, 71 and 66%, respectively), and that the conjugated BAA fraction is highly variable. This was the reason why, in the present study, we measured the excretion of the total (i.e. free and conjugated) BAAusing the acid hydrolysis procedure proposed by Rettenmeier *et al.* [14]. The average half-life of BAA in the present study was 3.4 h (range 1.3 to 3.8 h), which was in good agreement with values reported by Corley *et al.* (mean value 3.3 h, range 2.4 - 4.4 h) [3]. This half-life implies that the excretion of BAA was almost completed by 24 h.

The 24-h cumulative amount of BAA excreted in urine measured after inhalation accounted for 57% of the respiratory uptake of BE (range 42 to 70%). This was higher than the recovery of 17–55% reported in an inhalatory volunteer study by Johanson et al.; however, in that study only excretion of free BAA was measured [8].

The measurement of only non-conjugated BAA might also explain high intra-individual and inter-individual variation in cumulative excretion of BAA after dermal exposure of volunteers to neat BE [9]. The absorption rates reported in that study varied highly, from 0.05 to 0.74 cm h)1, suggesting that analysis of BAA might not be a reliable method for the assessment of the dermal absorption of BE. With regard to the variability of the absorption rates, the biological variability plus analytical variability in the flux through the skin of an individual was 16% (blood) and 20% (urine), assuming the systemic kinetics constant. If the inhalation exposure had also been replicated, a somewhat higher value would have been found (and a lower value for the inter-individual variation). These data are relevant in studies where subjects are compared on the basis of their skin permeability.

A comparison of the dermal uptake of aqueous solutions of BE with the respiratory uptake at the current occupational exposure limit for BE revealed substantial skin absorption. If one assumes a 60-min skin contact of 1000 cm^2 with a 50% or 90% aqueous solution of BE, the uptake would be three to four times higher than the pulmonary uptake of the 8-h occupational exposure.

In conclusion, dermal uptake of BE increases markedly in the presence of water. This has already been reported in vivo in guinea pigs and in in vitro studies with human skin; however, this is the first time that it has been shown in humans in vivo. These findings should therefore be considered in the health risk assessment of occupational dermal exposure to BE where water-based products containing glycol ethers are used.

BE showed substantial dermal absorption: if we assume 60-min skin contacts of approximate areas of 1000 cm^2 with 50% or 90% BE, dermal uptake would exceed the pulmonary uptake of the 8-h TLV exposure. This clearly justifies the skin notation for BE, and indicates biological monitoring (BM) as the preferred approach to exposure assessment. For the purpose of BM, both BE in blood or BAA in urine can be used. When BAA is chosen, total BAA instead of only free BAA in urine should be determined.

Acknowledgments

We are grateful to M.M. Verberk, PhD, and A.C. Monster, PhD, for their valuable discussion and criticism of the manuscript, and to Ms F. Calkoen, MSc, for technical assistance. The authors would like to thank the European Community for the financial support of this study, which was performed within the Fifth Framework Programme (project acronym: EDETOX).

References

1. Bartnik FG, Reddy AK, Klecak G, Zimmermann V, Hostynek JJ, Kunstler K (1987) Percutaneous absorption, metabolism and hemolytic activity of n-butoxyethanol. Fundam Appl Toxicol 8:59–70
2. Corley RA, Bormett GA, Ghanayem BI (1994) Physiologically based pharmacokinetics of 2-butoxyethanol and its major metabolite, 2-butoxyacetic acid, in rats and humans. Toxicol Appl Pharmacol 129:61–79
3. Corley RA, Markham DA, Banks C, Delorme P, Masterman A, Houle JM (1997) Physiologically based pharmacokinetics and the dermal absorption of 2-butoxyethanol vapor by humans. Fundam Appl Toxicol 39:120–130
4. Dugard PH, Walker M, Mawdsley SJ, Scott RC (1984) Absorption of some glycol ethers through human skin in vitro. Environ Health Perspect 57:193–197
5. Johanson G, Fernstrom P (1986) Percutaneous uptake rate of 2-butoxyethanol in the guinea pig. Scand J Work Environ Health 12:499–503
6. Johanson G, Fernstrom P (1988) Influence of water on the percutaneous absorption of 2-butoxyethanol in guinea pigs. Scand J Work Environ Health 14:95–100
7. Johanson G, Boman A (1991) Percutaneous absorption of 2-butoxyethanol vapor in human subjects. Br J Ind Med 48:788–792
8. Johanson G, Kronborg H, Naslund PH, Byfalt NM (1986) Toxicokinetics of inhaled 2-butoxyethanol (ethylene glycol monobutyl ether) in man. Scand J Work Environ Health 12:594–602
9. Johanson G, Boman A, Dynesius B (1988) Percutaneous absorption of 2-butoxyethanol in man. Scand J Work Environ Health 14:101–109
10. Meuling WJ, Franssen AC, Brouwer DH, van Hemmen JJ (1997) The influence of skin moisture on the dermal absorption of propoxur in human volunteers: a consideration for biological monitoring practices. Sci Total Environ 199:165–172
11. Ministerie van sociale zaken en werkgelegenheid (2001) Nationale MAC-lijst 2001. SDU, Den Haag, p 19
12. Opdam JJG (1989) The respiratory input in inhalation experiments. Br J Ind Med 46:145–156
13. Opdam JJ (1991) Linear systems dynamics in toxicokinetics studies. Ann Occup Hyg 35:633–649
14. Rettenmeier AW, Hennigs R, Wodarz R (1993) Determination of butoxyacetic acid and N-butoxyacetyl-glutamine in urine of lacquerers exposed to 2-butoxyethanol. Int Arch Occup Environ Health 65:S151–153

15. Sakai T, Araki T, Morita Y, Masuyama Y (1994) Gas chromatographic determination of butoxyacetic acid after hydrolysis of conjugated metabolites in urine from workers exposed to 2-butoxyethanol. Int Arch Occup Environ Health 66:249–254
16. Scheuplein RJ, Blank IH (1973) Mechanism of percutaneous absorption. IV. Penetration of nonelectrolytes (alcohols) from aqueous solutions and from pure liquids. J Invest Dermatol 60:286–296
17. Tang H, Blankschtein D, Langer R (2002) Prediction of steadystate skin permeabilities of polar and nonpolar permeants across excised pig skin based on measurements of transient diffusion: characterization of hydration effects on the skin porous pathway. J Pharm Sci 91:1891–1907
18. Tezel A, Sens A, Mitragotri S (2003) Description of transdermal transport of hydrophilic solutes during low-frequency sonophoresis based on a modified porous pathway model.J Pharm Sci 92:381–393
19. Vincent R, Rieger B, Subra I, Poirot P (1996) Exposure assessment to glycol ethers by atmosphere and biological monitoring. Occup Hyg 2:79–90
20. Wilkinson SC, Williams FM (2002) Effects of experimental conditions on absorption of glycol ethers through human skin in vitro. Int Arch Occup Environ Health 75:519–527

Chapter 2: *Section 2.2*

Percutaneous absorption and metabolism of 2-butoxyethanol in human volunteers: a microdialysis study

G. Korinth, I. Jakasa, T. Wellner, S. Kezic, J. Kruse, K.H. Schaller

(submitted to Arch Toxicol)

Abstract

Human in vivo studies on percutaneous absorption are very scarce although they are needed for the human risk assessment and evaluation of in vitro studies and mathematical predictive models.

In the present study we determined percutaneous absorption kinetics of 2-butoxyethanol (BE) in volunteers using microdialysis technique. This study was performed within a collaborative EU project (EDETOX) and had as the main objective generation of in vivo data for in vitro - in vivo comparison, and assessment of the feasibility of different in vivo methods for determination of percutaneous absorption.

Four male volunteers were dermally exposed on the left frearm to 90% and 50% aqueous solution (v/v) of BE for 4.5 hours. To determine percutaneous absorption kinetics the concentration of BE was measured in the dialysate samples collected at 30 min-intervals throughout exposure. The systemic absorption which is needed to determine recovery of the BE in the dialysate, was estimated from the concentration of the main metabolite of BE, free butoxyacetic acid (BAA) in urine.

A pseudo steady state percutaneous absorption of BE was reached approximately at 2 hours of exposure for both applied concentrations. The maximum dermal flux of 50% BE was higher than that of 90% BE (2.8 ± 1.0 and 1.9 ± 1.7 mg cm^{-2} h^{-1}), respectively, and the respective apparent permeability coefficient amounted to 6.1×10^{-3} and 2.5×10^{-3} mg cm^{-2} h^{-1}. The more diluted solution showed the shorter lag time; 25 *vs* 39 minutes. The average absorption rate into the skin amounted to 3.6 ± 1.3 and 2.3 ± 2.3 mg cm^{-2} h^{-1}. In one person we determined the amount of BAA in two dialysates collected at 4 and 4.5 hours in the exposure to 50 % BE and 90 % BE. The dermal metabolism seemed to be low, the amount of BAA was approximately 1% of the amount of BE in the same dialysate.

The presented study demonstrates applicability of microdialysis technique for assessment of percutaneous absorption kinetics and dermal metabolism without interference from the systemic compartment.

Introduction

For assessment of percutaneous absorption of chemicals mostly in vitro experiments using diffusion cells are performed, and in vivo human data are scarce. The lack of in vivo data makes it difficult to judge the validity of the use of in vitro data for the human risk assessment. In vivo studies are considered to be the gold standard for the evaluation of in vitro systems and predictive mathematical models (Howes et al. 1996), however, their wider use is limited due to ethical and practical considerations.

Although microdialysis is not frequently used in percutaneous absorption studies, it has been proposed as a useful method for the determination of in vivo percutaneous absorption of exogenous chemicals (Anderson et al. 1998, Surber et al. 1999). The microdialysis technique can be used in both in vivo and in vitro experiments (Anderson et al. 1991, Boutsiouki et al. 2001, Wellner and Korinth 2004, Klede et al. 2005).

The percutaneous absorption of the glycol ether 2-butoxyethanol (BE) has been studied in vitro (Wilkinson and Williams 2002, Korinth et al. 2005, Wilkinson et al. 2006), in vivo in experimental animals (Lockley et al. 2004, Lockley et al. 2005) and recently in human volunteers (Jakasa et al. 2004, Kezic 2004). The percutaneous absorption of BE was extensively studied within the EDETOX project (Williams 2004) as a model compound for inter-laboratory and in vivo-in vitro comparison.

The aim of the present study was to determine the percutaneous absorption kinetics of BE in volunteers using the microdialysis technique. Additonally, in a limited number of samples dermal metabolism of BE was investigated.

Material and Methods

Chemicals, subjects and experimental design

Four male Caucasian volunteers aged 27-37 and with no history of dermatological disease, participated in the study. They were in good health, had no visible skin damage and used no medication.

Percutaneous absorption of 90% and 50% aqueous solutions (v/v) of BE (≥ 99.8% purity, Fluka® Buchs, Switzerland) was assessed. The period between two dermal experiments exposures was at least two weeks. The volunteers were exposed on the left forearm to both, 90% and 50% BE in different exposure chambers (exposed skin

area of each chamber: 0.64 cm^2) for 4.5 hours. Single plasmapheresis hollow fibres (Plasmaflo OP–05(L) separator, Asahi medical, Tokyo, Japan) were used as capillaries for microdialysis. Two capillaries (material: polycarbonate; inner diameter (ID): 340 µm; wall thickness: 50 µm; pore size: 0.3 µm; cut-off: 3000 kDa) per exposure chamber were intradermally inserted without anaesthesia in parallel by linear technique at a length of ~ 2.8 cm in the skin and a distance of approximately 2.2 mm between both capillaries. Two rectangular (1 x 0.64 cm) exposure chambers (one for each concentration) of stainless steel were centred above microdialysis capillaries at a distance of about 3 cm and glued onto the skin surface using UHU®-hart (UHU, Bühl/Baden, Germany). The capillaries were connected by Tygon tubing (ID: 0.381 mm) (Cole-Parmer, Strongsville, OH) to the pulsation-free syringe pump PHD 2000 (Harvard apparatus, Holliston, MA) and perfused with saline as receptor fluid at a flow rate of 8 µl/min. The applied volume of BE solution in the exposure chambers was enough to ensure an infinite dose (200 µl/cm^2) and was covered with a foil to prevent evaporation. Receptor fluid samples were collected at 0.5 h intervals up to 4 hours. For assessment of dermal metabolism, free butoxyacetic acid (BAA) was determined in the last two collected dialysates of one subject (at 4 and 4.5 h), after exposure to 90 % BE and 50 % BE. Urine samples were collected at 4.5 h immediately after end of exposure.

Analysis of BE and BAA

BE was analysed in dialysates by gas-chromatography using flame ionisation detection. The concentration of BAA in dialysates and in urine samples was analysed by gas-chromatography followed usin electron capture detection as described in detail by Kezic et al. (2004).

Calculation of percutaneous absorption

To find out the proportion of BE that was recovered in the dialysates relative to the amount absorbed systemically, we measured the concentration of BAA in urine as a biological indicator of exposure. The systemic absorption was estimated using the results from another EDETOX study on percutaneous absorption of BE in volunteers (Kezic et al. 2004) that was based on biological monitoring (BM) method. In that study, the systemic absorption was assessed from the urine excretion of BAA measured after dermal and a reference inhalative exposure of a known dose. The average concentration of BAA in urine sample collected shortly after cesation of 4-h dermal exposure was 121.7 mmol/mol creatinine, and the average systemic uptake was 568 mg.

The total amount of systemically absorbed BE in the microdialysis experiment was derived from the equation:

$$BE_{m\ (systemic\ absorption)} = \frac{BAA_{m\ (mmol/mol\ creatinine)}}{BAA_{bm\ (mmol/mol\ creatinine)}} \times BE_{bm\ (mg)}$$

where BAA_m is the concentrations of BAA measured in our microdialysis study. BAA_{bm} is the concentration of BAA obtained in the BM study, BE_{bm} is dermal systemic absorption in the BM study. Recovery of BE in the dialysate was calculated for each exposure experiment from the ratio between the total amount of BE measured in the dialysates, and the estimated systemic absorption. Dermal fluxes were calculated for each exposure from the absorbed amount of BE in the dialysate adjusted for recovery divided by sampling period (30 min) and skin area (0.64 cm^2) as follows:

$$Dermal\ Flux\ BE\ (mg\,cm^{-2}h^{-1}) = \frac{BE_{dialysate\ (mg)}}{0.5\,(h) \times 0.64\ (cm^2) \times REC\,(\%)} \times 100\,(\%)$$

The maximum fluxes and lag times were determined from the slope of the cumulative amount of BE that was systemically absorbed vs. time. The average absorption rate into the skin (mg cm^{-2} h^{-1}) during the exposure period was calculated by dividing the amount of systemically absorbed BE (mg) by the exposed skin area (0.64 cm^2) and exposure duration (4 h).

From the results of both replicated dermal exposures, we calculated the intra- and inter-individual variability.

Results

Figure 1 shows the amount of BE in the dialysates collected over 30 min during exposure period, averaged for all volunteers. Dermal fluxes of BE reached plateau between 1.5 and 2 hours after the start of exposure indicating attainment of pseudo steady state absorption (Fig 2), although one volunteer showed in all four experiments a declining absorption rate after a maximum at 3 h exposure.

The average concentration of the free BAA excreted in the period 0 – 4.5 h after the start of exposure was 3.5 ± 2.0 mmol/mol creatinine. From the concentration of BAA in urine, the systemically absorbed amount of BE was estimated and shown in Table 1. Recovery of the absorbed amount in the dialysate was low and amounted less than 1% of the amount systemically absorbed. The percutaneous absorption parameters i.e. maximum flux, average absorption rate into the skin, permeability coefficient, and lag times are shown in Table 1. In general, 50 % BE showed higher and faster absorption. The lag time was shorter for 50 % BE (25.4 min) than that for 90 % BE (38.9 min). The respective intra-individual and inter-individual variation of the maximum dermal flux of BE amounted to 37 and 49 % for 50 % BE. For 90 % BE, the variation was considerable and amounted to 97 % and 91 %, respectively. This high variation after exposure to 90 % BE was primarily caused by one subject who showed significantly higher absorption.

The ratio of the amounts of BAA and BE in two microdialysis samples was 0.011. When corrected for the different molecular weight of BAA and BE dermal metabolism amounted to approximately 1.0%.

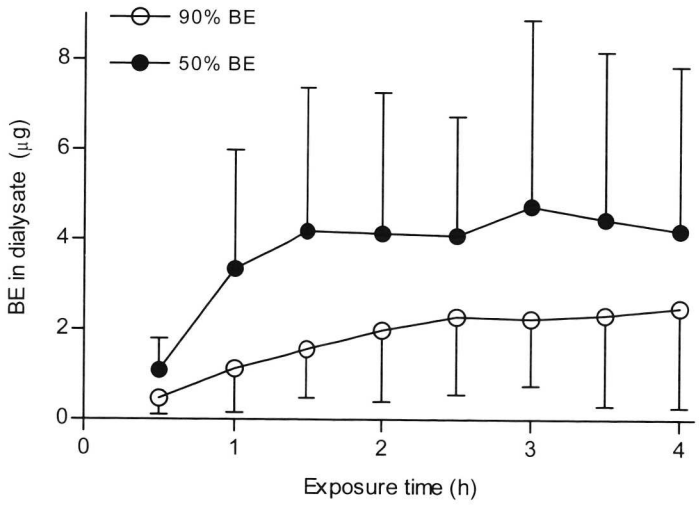


Fig 1: *The amount of 2-butoxyethanol (BE) in the dialysates during 4 hours exposure to 90% and 50% aqueous solution BE (mean ± SD)*

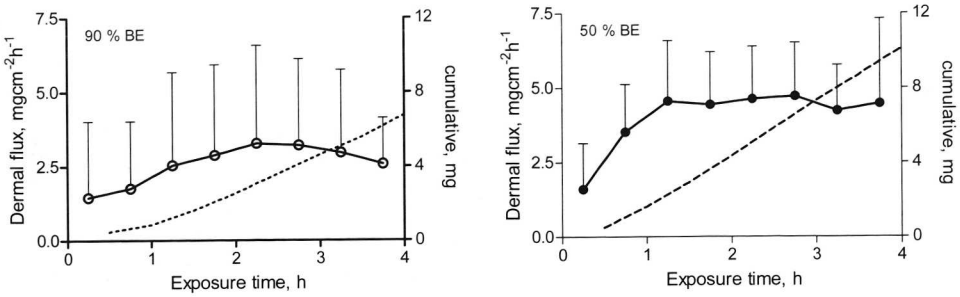


Fig 2: *Dermal flux (mean ± SD) and cumulative absorption of 90% and 50% aqueous solution 2-butoxyethanol (BE)*

Table 1. The amount of 2-butoxyethanol (BE) recovered in the dialysate, estimated systemic absorption, and the percutaneous absorption parameters for 90% and 50% BE (means ± SD)

	90% BE	50% BE
Cumulative amount of BE recovered in dialysate during 4 h (µg)	14.5 ± 11.7	30.2 ± 24.6
Systemic absorption (mg)	6.8 ± 6.6	10.1 ± 3.9
BAA in urine (mmol/mol creatinine)	3.5 ± 2.0	
Average absorption rate (mg cm^{-2} h^{-1})	2.4 ± 2.3	3.5 ± 1.3
Maximum dermal flux (mg cm^{-2} h^{-1})	1.9 ± 1.7	2.8 ± 1.0
Permeability coefficient (cm h^{-1})	2.5 ± 2.3 x 10^{-3}	6.1 ± 2.2 x 10^{-3}
Lag time (min)	38.9 ± 16.1	25.4 ± 10.6

Discussion

Application of the microdialysis technique has not yet attained wide acceptance in the research of percutaneous absorption, and only a few studies investigating occupationally relevant substances by this technique are available (Anderson et al. 1991, Boutsiouki et al. 2001, Klede et al. 2005). An important advantage of the microdialysis technique is that it measures the percutaneously penetrated chemical before entering systemic circulation (Schnetz and Fartasch 2001).

In the present study we have determined percutaneous absorption kinetics of 50% and 90% aqueous BE in volunteers. In addition, we were able, although in a very limited number of dialysates, to measure dermal metabolism.

Penetration of BE through the skin showed to be fast; lag times for 50% and 90% BEwere 25 and 39 mins, respectively. Since lag time and diffusion coefficient are interrelated ($t_{lag} = L^2/ 6 D$, where L is thickness of the stratum corneum, D is diffusion coefficient), this implies faster diffusion of 50% BE through the skin than that of 90% BE. Also average absorption rate into the skin and maximum flux were higher for more diluted BE. Average absorption rates amounted to 3.5 and 2.4 mg cm^{-2} h^{-1} for 50% and 90% BE, respectively, and the respective maximum fluxes 2.8 and 1.9 mg cm^{-2} h^{-1}. The apparent permeability coefficients of 50 % and 90 % BE amounted to 6.1 and 2.5 cm h^{-1}, respectively. The average absorption rates of 50 % and 90 % BE, in the volunteer study of Jakasa et al. (Jakasa *et al.* 2004) amounted to 1.3 mg cm^{-2} h^{-1} and 0.9 mg cm^{-2} h^{-1}. In that study also absorption of neat BE was determined, showing five times lower absorption rate than 50% BE. In another volunteer study, an average absorption rate of 3.5 mg cm^{-2} h^{-1} for 50% was reported (Kezic et al. 2004), which is very close to the value we obtained in the present study. The maximum flux of 50% BE in vitro in human skin was determined by eight laboratories participating in the EDETOX project, reporting the average value of 1.5 mg cm^{-2} h^{-1} (EDETOX, 2004). Also in vitro a marked influence of water on the percutaneous absorption of BE was found. In an in vitro study with human skin, Wilkinson and Williams (2002) reported considerably higher apparent permeability coefficients with aqueous solutions than with neat BE. This effect was assigned to a better partitioning of BE in hydrated stratum corneum. Our results show, that higher content of water increases diffusion of BE which might be caused by the altered structure of lipid bilayers of the stratum corneum. The enhancing effect of water has significant implications. BE is a solvent used on large scale in water based paints, cleaning agents or cutting oils and comes on the market normally as a water formulation. This stresses once again, the

necessity of measurement of percutaneous absorption of a chemical as applied in a formulation rather than that of a neat compound.

It is well known that skin has the capacity for local metabolism of chemicals. For instance, for benzyl alcohol dermal metabolism can reach, even in excised human skin, about 50% of the percutaneously absorbed amount (Bronaugh et al. 1999). Glycol ethers are oxidised by cytostolic alcohol dehydrogenase and aldehyde dehydrogenase (Lockley et al. 2005). Lockley et al. (2004) were not able to detect metabolism of BE in vitro or in vivo in rat skin. The authors attributed this to the rapid penetration of BE through the skin (Lockley et al. 2004), which would reduce the contact of metabolising enzymes of the skin with BE. Our results, although determined only in four experiments, showed that the extent of metabolism in the skin of BE was rather low in relation to the amount which penetrated through the skin. We found that the ratio of BAA and BE in the dialysate was approximately 0.01. Assuming the same recovery of BE and BAA across microdialysis capillaries, BE was dermally metabolised at a percentage of about 1%. Considering the findings of Lockley et al. (2004, 2005) the dermal metabolism of BE seems to be negligible.

One of the main problems of the microdialysis technique is the calibration i.e., the estimation of the penetrated amount recovered in the dialysate. We estimated recovery of BE in the microdialysis capillaries using biological monitoring. From the concentration of BAA measured in the urine sample at the end of exposure we estimated the amount of BE systemically absorbed. For that purpose we used the results of a research group that also participated in the EDETOX project and investigated extensively the excretion kinetics of BAA after dermal and reference inhalative exposure (Jakasa et al. 2004, Kezic et al. 2004). The results of the present study demonstrate that only a small part (less than 1%) of the amount that penetrated was recovered in microdialysis capillaries. In an in vitro microdialysis study with the 50% aqueous BE solution a recovery of approximately 40% (Maas et al. 2004) was reported. However, as pointed out by several authors, (Groth 1996, Stenken 1999), results from in vitro methods are often not reliable indicators for in vivo recovery.

The intraindividual variation in the average absorption rate into the skin of 37 % and 49 % for 50 % BE and 91 % BE, respectively and the respective interindividual variations of 97 % an 91 % was relatively high compared with studies based on biological monitoring. In the study by Jakasa et al. (2004), the coefficient of variation for the intra-individual and inter-individual variation was 20 % and 34 % when total BAA was used as a biological indicator. The higher variation in the present

microdialysis study was primarily caused by the high values in one person for exposure to 90 % BE. Often, relative large variability is found in percutaneous penetration studies by microdialysis (about 50-100%). Factors which are mostly argued to be responsible for this are insertion depth of microdialysis capillaries, the variations in the blood flow, and flow rate of the perfusate. It has been shown that insertion of the microdialysis capillaries is associated with a temporary increase in cutaneous blood flow, which subsides during exposure (Kreilgaard 2002). In one volunteer, we found that the maximum penetration was achieved fast, however, after 3 hours the penetration rate declined. A possible reason for this might be higher cutaneous flow in the beginning of exposure due to insertion of the microdialysis capillaries, but also to different percutaneous penetration behaviour compared to other volunteers since the kinetics in both duplicate experiments were similar.

The presented study shows some important advantages of the microdialysis technique. The skin penetration kinetics can be studied as a function of time enabling determination of maximum dermal fluxes and in the case of steady state the permeability coefficient. Methods which determine total systemic absorption, yield only average absorption rates into the skin. In addition, by using microdialysis, as shown in our study, the lag time and diffusion coefficient can be easily determined. Besides relevant information on the percutaneous absorption kinetics, the derived parameters enable direct comparison with in vitro experiments.

However, as already discussed, the question of recovery remains important issue. In this study, we determined the recovery using biological monitoring. We collected only one urine sample, directly after end of exposure. It would have been much better if we had measured the cumulative amount excreted; however, this was not possible in this study for practical reasons. However, the obtained percutaneous absorption parameters are consistent with those from volunteer studies (Jakasa et al. 2004, Kezic et al. 2004) and in vitro studies (EDETOX, 2004), so we may conclude that microdialysis is a reliable and useful technique for investigation of percutaneous absorption and dermal metabolism. Microdialysis is particularly suitable when comparative research is needed e.g. for studying influence of vehicle, exposure conditions, damage of the skin etc. In that case the main drawback of the technique – determination of recovery becomes less relevant.

Acknowledgement
This research was part of the project 'Evaluation and predictions of dermal absorption of toxic chemicals' (QLRT-2000-00196), which was supported by the Fifth Framework Programme of the European Commission (Coordinator: Prof. Faith Williams).

The experiments comply with the current laws of the countries in which the experiments were performed.

References

- Anderson C, Andersson T, Boman A (1998) Cutaneous microdialysis for human in vivo dermal absorption studies. In Dermal absorption and toxicity assessment (eds. Roberts, M.S. & Walters, K.A.) 231–244 (Marcel Dekker Inc., New York-Basel)
- Anderson C, Andersson T, Molander M (1991) Ethanol absorption across human skin measured by in vivo microdialysis technique. Acta Derm Venereol 71:389–393
- Boutsiouki P, Thompson JP, Clough GF (2001) Effects of local blood flow on the percutaneous absorption of the organophosphorus compound malathion: a microdialysis study in man. Arch Toxicol 75:321–328
- Bronaugh RL, Kraeling MEK, Yourick JJ, Hood HL (1999) Cutaneous metabolism during in vitro percutaneous absorption. In Percutaneous absorption. Drugs – cosmetics – mechanisms – methodology (eds. Bronaugh, R.L. & Maibach, H.I.) 347–374 (Marcel Dekker Inc., New York-Basel).
- Deutsche Forschungsgemeinschaft (DFG) (2005) List of MAK and BAT values 2005, report 41. Wiley-VCH, Weinheim
- EDETOX. (2004) Evaluations and predictions of dermal absorption of toxic chemicals. Final report for dissemination. Contract No QLK4-2000-00196 (http://www.ncl.ac.uk/edetox/index.html)
- Groth L (1996) Cutaneous microdialysis. Methodology and validation. Acta Derm Venereol Suppl (Stockh) 197:1–61
- Howes D, Guy R, Hadgraft J, Heylings J, Hoeck U, Kemper F, Maibach H, Marty J-P, Merk H, Parra J, Rekkas D, Rondelli I, Schaefer H, Täuber U, Verbiese N (1996) Methods for assessing percutaneous absorption. The report and recommendations of ECVAM workshop. ATLA (Alternatives to laboratory animals) 24: 81–106
- Jakasa I, Mohammadi N, Krüse J, Kezic S (2004) Percutaneous absorption of neat and aqueous solutions of 2-butoxyethanol in volunteers. Int Arch Occup Environ Health 77:79–84
- Kezic S, Meuling WJ, Jakasa I (2004) Free and total urinary 2-butoxyacetic acid following dermal and inhalation exposure to 2-butoxyethanol in human volunteers. Int Arch Occup Environ Health 77:580–586
- Klede M, Schmitz H, Göen T, Fartasch M, Drexler H, Schmelz M (2005) Transcutaneous penetration of toluene in rat skin a microdialysis study. Exp Dermatol 14:103–108

- Korinth G, Schaller KH, Drexler H (2005) Is the permeability coefficient Kp a reliable tool in percutaneous absorption studies? Arch Toxicol 79:155–159
- Kreilgaard M (2002) Assessment of cutaneous drug delivery using microdialysis. Advanced Drug Delivery Reviews 54 Suppl. 1 S99 –S121
- Lockley DJ, Howes D, Williams FM (2004) Percutaneous penetration and metabolism of 2-butoxyethanol. Arch Toxicol 78:617–628
- Lockley DJ, Howes D, Williams FM (2005) Cutaneous metabolism of glycol ethers. Arch Toxicol 79:160–168
- Maas WJM, van de Sandt JJM, Cnubben NHP, Korinth G (2004, Suppl. 1) Efficiency of microdialysis for the assessment of percutaneous absorption varies between test compounds. Drug Metab Rev 36:81 (Abstract)
- Schmelz M, Zeck S, Raithel M, Rukwied R (1999) Mast cell tryptase in dermal neurogenic inflammation. Clin Exp Allergy 29:695–702
- Schnetz E, Fartasch M (2001) Microdialysis for the evaluation of penetration through the human skin barrier - a promising tool for future research? Eur J Pharm Sci 12:165–174
- Stenken JA (1999) Methods and issues in microdialysis calibration. Analytica Chimica Acta 379:337–358
- Surber C, Smith EW, Schwarb FP, Maibach HI (1999) Drug concentration in the skin. in Percutaneous absorption. Drugs – cosmetics – mechanisms – methodology (eds. Bronaugh, R.L. & Maibach, H.I.) 347–374 (Marcel Dekker Inc., New York-Basel).
- Wellner T, Korinth G (2004) Percutaneous absorption of pyrene using in vitro microdialysis. In: Brain KR, Walters KA (Eds.) Perspectives in percutaneous penetration, STS Publishing, Cardiff, vol 9A (Abstract)
- Wilkinson SC, Williams FM (2002) Effects of experimental conditions on absorption of glycol ethers through human skin in vitro. Int Arch Occup Environ Health 75:519–527
- Wilkinson SC, Maas WJ, Nielsen JB, Greaves LC, van de Sandt JJ, Williams FM (2006) Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. Int Arch Occup Environ Health 25:1–9
- Williams FM (2004) EDETOX. Evaluations and predictions of dermal absorption of toxic chemicals. Int Arch Occup Environ Health 77:150–151

Chapter 2: *Section 2.3*

Free and total urinary 2-butoxyacetic acid following dermal and inhalation exposure to 2-butoxyethanol in human volunteers

S. Kezic, W.J.A. Meuling, I. Jakasa

Int Arch Occup Environ Health (2004) 77: 580-586

Abstract

Objectives: To assess excretion kinetics of free and total (free + conjugated) 2-butoxyacetic acid (BAA) following dermal and inhalation exposure to butoxyethanol (BE).

Methods: Six male volunteers were dermally exposed for 4 h to a 50% aqueous solution of BE on an area of 40 cm^2 of the volar forearm. Six other male volunteers were exposed by inhalation (mouth only) to 93 mg m^{-3} BE for 30 min. As biological indices of exposure, BE in blood and total and free BAA in urine, were measured.

Results: Following inhalation exposure, the 24-h cumulative excretion of free and total BAA in urine amounted to 5.5 ± 2.7 and 12.8 ± 4.0 mg, respectively. After dermal exposure, 147.1 ± 61.0 and 346 ± 52 mg, respectively, of free and total BAA were excreted in urine up to 48 h after the onset of exposure. The proportion of conjugated BAA in single urine samples increased after dermal exposure in time from 45 ± 30% in the first collection period to 92 ± 2% after 48 h. The elimination half-life of total BAA following dermal exposure was longer than that of free BAA (5.1 ± 0.6 and 3.8 ± 0.4 h, respectively). The interindividual variation in the cumulative excreted amount after inhalatory exposure was higher (49%) for free BAA than for total BAA (31%). The average dermal flux amounted to 3.5 mg cm^{-2} h^{-1} independently of whether free or total BAA was used for the calculation, and, again, the interindividual variation in the estimated fluxes was higher for free BAA than for total BAA (41% and 15%, respectively).

Conclusion: The interindividual variation in the extent of conjugation is large, and the degree of conjugation increases with time. Due to lower interindividual variability, total BAA is superior to free BAA as a biomarker of exposure.

Introduction

2-Butoxyethanol (BE) is a glycol ether frequently used in industry and households as a solvent, emulsifier and detergent. Due to the low vapour pressure and high rate of dermal absorption, a significant systemic exposure can occur through contact with skin (Jakasa *et al.* 2004; Johanson *et al.* 1986; Johanson and Boman 1991). BE is primarily metabolized to 2-butoxyacetic acid (BAA) in the liver (Ghanayem and Sullivan 1993). In humans, BAA can be conjugated with glutamine or glycine to form N-butoxyacetyl glutamine or N-butoxyacetyl glycine conjugates (Rettenmeier *et al.* 1993). The substantial skin uptake of BE indicates that in assessing the health risk, biological monitoring and the use of biological exposure indices are preferable to environmental monitoring. As a biological marker (BM) of exposure, free BAA in urine has been used (Angerer *et al.* 1990; Goen *et al.* 2002; Haufroid *et al.* 1997; Laitinen 1998). In Germany, the biological tolerance value (BAT) for BAA has been set to 100 mg/L (DFG 2003). In the United Kingdom, the guidance value for BAA is 240 mmol/mol creatinine (HSE 2002). However, as first shown by Rettenmeier *et al.* (1993), and supported by Sakai *et al.* (1994), Corley *et al.* (1997), and Jones and Cocker (2003), BAA is not excreted exclusively as free acid but also as acid-labile conjugates. Concerns raised in the literature about the large intraindividual and interindividual variation in the extent of the conjugation might be an important argument to question the validity of the present BM of BE exposure (Goen *et al.* 2002; Johanson *et al.* 1986; Johanson and Boman 1991).

In the context of an extensive study supported by the European Union (EU) on percutaneous absorption of chemicals (EDETOX) (European Commission 2000), we recently investigated dermal absorption of neat and aqueous solutions of BE in volunteers (Jakasa *et al.* 2004). Looking for the best biological indicators of exposure, we determined the concentrations of BE in plasma and of total BAA (the sum of conjugated and nonconjugated BAA after acid hydrolysis) in the urine of volunteers exposed dermally and by inhalation (reference exposure). To investigate the interlaboratory variation in the determination of dermal fluxes, another research group carried out a separate dermal exposure study under similar exposure conditions. In these volunteers, we determined the concentration of both free and total BAA. As the knowledge about the excretion pattern of BAA is of particular importance for occupational practice, the goal of this paper is to report the pattern and variability of the excretion of free and conjugated BAA after inhalatory and dermal exposure to BE.

Material and methods

Subjects

Two groups of six male volunteers, aged 22–55 years, with no history of dermatological disease, participated in the study. They were in good health, had no visible skin damage and used no medications. The Ethical Committee of the Academic Medical Center, University of Amsterdam (inhalatory exposure), and of the TNO Nutrition and Food Research, The Netherlands (dermal exposure), approved the experimental protocol. Written informed consent was obtained from all subjects prior to experiments.

Inhalatory exposure

The inhalatory experiment and the way the inspiratory input was calculated were described in detail previously (Jakasa *et al.* 2004). Briefly, each volunteer inhaled the solvent vapour for 30 min through a mouthpiece with a two-way valve connected to a Tedlar (DuPont, Delaware, USA) bag. The concentration of the vapour in the bag was 93 ± 6.8 mg m^{-3} (mean value of six exposures), which is below the present occupational exposure limit in The Netherlands (100 mg m^{-3}; see Ministerie van sociale zaken en werkgelegenheid 2001). The average respiratory uptake calculated from the inspiratory rate amounted to 20.9 ± 5.0 mg. Urine samples were collected before and after exposure at the following predetermined intervals: 0, 0-4, 4-8, 8-12, 12-16 and 16-24 h.

Dermal exposure

The second group of six male volunteers were exposed dermally. A bottomless glass chamber (area of 40 cm^2) was placed on the volar forearm and filled with 8 ml of dosing BE solution. To prevent leakage, the glass chamber was pressed onto the skin using elastic bands. The concentration of BE in the solution was measured before and after exposure. To avoid any inhalation of solvent vapour during the application of the solvent, the volunteer was sitting in a ventilated clean-air cabin, and put his arm through an opening in the wall of the cabin. The exposure lasted for 4 h. Blood samples were collected over a period of 8 h (16 samples per experiment). Urine samples were collected fractionated before and after exposure at the following predetermined intervals: 0, 0-4, 4-8, 8-12, 12-16, 16-24, 24-36 and 36-48 h.

Analytical methods

Chemicals

Acetone (p.a.), dichloromethane (p.a.), n-hexane (Lichrosolv), hydrochloric acid (conc., 37%), methanol (Lichrosolv), potassium carbonate (p.a.) and pyridine were purchased from Merck (The Netherlands). Phenoxyethanol (98%) and ethoxyacetic acid (98%) were purchased from Aldrich (The Netherlands). Pentafluorobenzoylchloride (99%) and pentafluorobenzylbromide (> 99%) were purchased from Fluka (The Netherlands). BE (99%) was purchased from Sigma (The Netherlands) and BAA from TCI (Japan).

Analysis of BE in plasma

The method for the measurement of BE in plasma is extensively described elsewhere (Jakasa *et al.* 2004). The method is based on extraction with dichloromethane and derivatization with pentafluorobenzoylchloride and electron capture detection (ECD). The limit of quantitation (LOQ) of the method was 0.014 mg L^{-1} and the coefficient of variation was 7%.

Analysis of BAA in urine

Total BAA The analytical method for the determination of total BAA was described in detail in our previous paper (Jakasa *et al.* 2004). The analysis was based on acid hydrolysis of conjugated BAA, subsequent derivatization with pentafluorobenzylbromide (PFBBr) and GC-ECD analysis. The limit ofdetection (LD; three times SD of the blank) of the method was 1.0 mg L^{-1}, and the coefficient of variation was 14%.

Free BAA The free BAA was analysed according to the procedure described in detail elsewhere (Kezic *et al.* 1997). Shortly, to 100 µl urine, 25 µl ethoxyacetic acid (internal standard) and 100 µl phosphate buffer (pH = 7) were added. The samples were left at 80 °C under a slightly reduced pressure (20 mm Hg) to evaporate to dryness. After cooling down, 500 µl 5% PFBBr in methanol was added and heated for 60 min at 95 °C. After cooling down, 500 µl water and 500 µl *n*-hexane were added. Samples were vortexed for 1 min and then centrifuged at 12,000 g. The GC analysis was identical to that used for the determination of total BAA. The LD of the method was 0.5 mg L^{-1}, and the coefficient of variation was 12%.

Calculations

The elimination half-life time of BAA was obtained from the slope of the curve of the log-linear excretion rate versus time data. The half-life was calculated if at least three

time points were available. Some of the urine samples were not collected exactly at the predetermined time. To calculate the average concentration at a point of time for all subjects, point-to-point curves were constructed for each set of individual excretion data (concentration versus time) (Graph-Pad Prism). We calculated the concentration of conjugated BAA as the difference between total and free concentration. The results are presented as the mean value ± standard deviation. The ratios between free and total BAA were calculated for each time point, and then averaged for six persons. The half-lives of free and conjugated BAA were compared using the paired Student's t test.

Results

As can be seen from Fig 1, the concentration of BE in plasma during inhalation exposure increased rapidly. The elimination of absorbed BE from blood after termination of exposure was similarly rapid with a mean half-life of 57 ± 20 min. Two hours after exposure, the concentration of BE declined under the limit of the quantitation of the method (0.014 mg L^{-1}). The area under the curve for the six volunteers was 8.0 ± 2.4 mg min L^{-1}. As calculated from the inhalation concentration and the total inspired volume, the inhalation dose amounted to 20.9 ± 5.0 mg.

After dermal exposure, the BE in the plasma was detected in all subjects already in the first sample, which was collected at 30 min (Fig 1). An apparent steady state was observed in four volunteers and was reached between 2 and 4 h. After termination of exposure, BE was rapidly cleared from the blood with an apparent elimination half-life of 73 ± 10 min. The area under the curve averaged for the six volunteers was 140.0 ± 63.2 mg min L^{-1}. Free and total BAA were detectable in all subjects already in the first urine samples, which were collected 4 h after the beginning of exposures. After inhalation exposure, the maximal excretion of both free and total BAA occurred in five volunteers in the first collection period (0-4 h), and in one volunteer during the second collection period (4-8 h). The maximal excretion after dermal exposure was in the second (4-8 h) and third (8-12 h) collection intervals. In Fig 2, the average excretion of free and total BAA for six volunteers is shown for the creatinine-corrected concentrations. A similar excretion pattern was obtained for uncorrected concentrations expressed as milligrams per litre (Fig 3), and excretion rates (mg/h) (figure not shown).

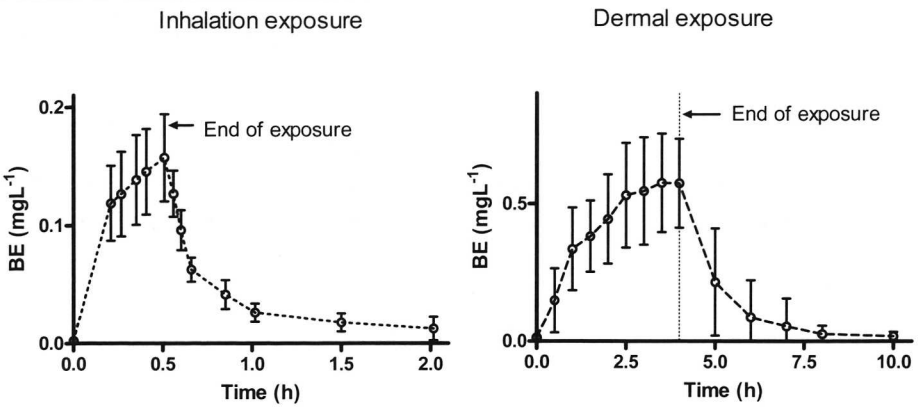


Fig 1: *The concentrations of BE in plasma during and after inhalation exposure to 93 mg m^{-3} for 30 min, and after dermal exposure to BE for 4 h (mean ± SD).*

Following inhalatory exposure, the concentration of free BAA in three volunteers decreased after 12 h under the detection limit of the method (0.5 mg/L) and therefore no meaningful half-life could be calculated. The half-life of free BAA in the three other volunteers amounted to 3.1 ± 0.7 h. The excretion rate of total BAA decreased with a half-life of 3.4 ± 0.4 h. After dermal exposure, half-lives could be calculated for both the free and total BAA in all subjects; they amounted to 3.8 ± 0.4 and 5.1 ± 0.6 h, respectively. Since the excretion kinetics of total BAA are the result of the excretion kinetics of free and conjugated BAA, we calculated also the half-life of conjugated BAA. After dermal exposure, this half-life amounted to 5.8 ± 1.0 h, which is higher than that of free BAA ($p < 0.001$).

Following inhalatory exposure, the concentration of free BAA in three volunteers decreased after 12 h under the detection limit of the method (0.5 mg/L) and therefore no meaningful half-life could be calculated. The half-life of free BAA in the three other volunteers amounted to 3.1 ± 0.7 h. The excretion rate of total BAA decreased with a half-life of 3.4 ± 0.4 h. After dermal exposure, half-lives could be calculated for both the free and total BAA in all subjects; they amounted to 3.8 ± 0.4 and 5.1 ± 0.6 h, respectively. Since the excretion kinetics of total BAA are the result of the excretion kinetics of free and conjugated BAA, we calculated also the half-life of conjugated BAA. After dermal exposure, this half-life amounted to 5.8 ± 1.0 h, which is higher than that of free BAA ($p < 0.001$).

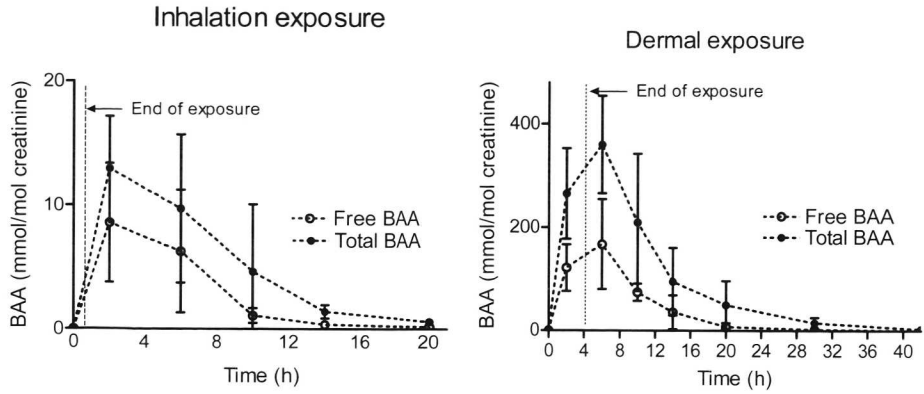


Fig 2: *Creatinine-corrected concentrations of free and total BAA after inhalation and dermal exposure to BE (mean ± SD).*

After inhalatory exposure, 56 ± 21% of the total 24-h excretion of BAA was in the form of conjugate. After dermal exposure, the percentage of conjugated BAA in the cumulative 48-h excretion was 58 ± 14%. The proportion of free and conjugated BAA in total excretion changed with time (Figs. 3 and 4). Following dermal exposure, the lowest proportion of conjugated BAA was found in the urine sample collected immediately after the exposure (45 ± 30%), increasing to 92 ± 2% at 48 h after start of exposure (Fig. 4).

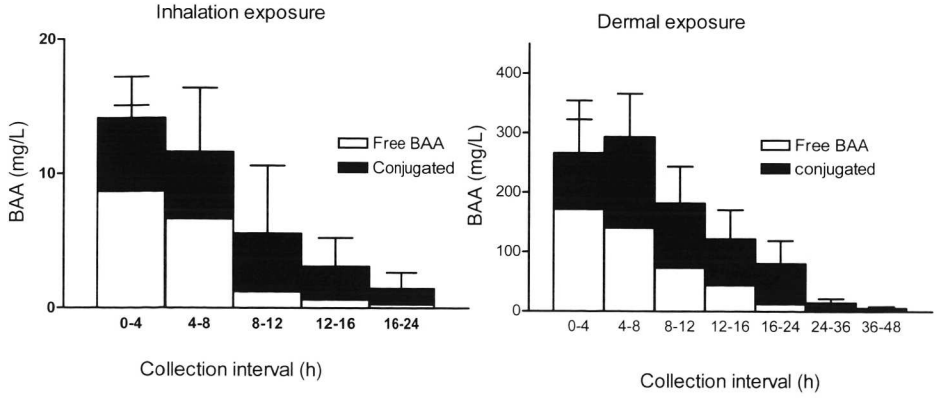


Fig 3: *The concentrations of free and conjugated BAA in urine following inhalation and dermal exposure to BE (mean ± SD).*

As presented in Fig 4, the interindividual variation in the extent of conjugation is substantial at any given time of collection. In one subject from the inhalatory exposed group, the relative excretion of the conjugated BAA was very high (92 - 100%) in all collected urine samples. On the other hand, in three urine samples-this time from different individuals-BAA was exclusively excreted in its free form.

As originally the purpose of this study was to estimate dermal absorption, the data on cumulative BAA excretion were used to calculate the average dermal flux. The 24-h cumulative urinary excretion of free and total BAA following a 30-min inhalation exposure amounted to 5.5 ± 2.7 and 12.8 ± 4.0 mg. After dermal exposure 147.1 ± 61.0 mg of free and 346 ± 52 mg of total BAA, respectively, were excreted in urine up to 48 h after the onset of exposure. The dermally absorbed amount was calculated from the ratio between the cumulative excretion of BAA after dermal and inhalation exposures, multiplied by the inhalation dose (20.9 ± 5.0 mg). The absorbed amount calculated using free and total BAA amounted to 568 and 566 mg, respectively. By dividing the absorbed amount by a skin exposure area of 40 cm^2 and exposure duration of 4 h, average fluxes of 3.5 ± 0.5 and of 3.5 ± 1.4 mg cm^{-2} h^{-1} were calculated using the excretion of total and free BAA, respectively. The skin flux that we calculated from the blood BE data (AUC) was somewhat lower (although not a statistically significant difference) and amounted to 2.4 ± 0.8 mg cm^{-2} h^{-1}.

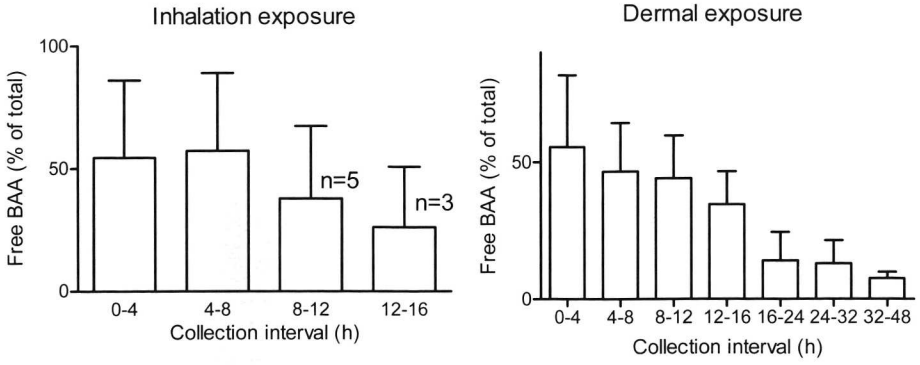


Fig 4: *The ratios of free and total BAA following inhalation and dermal exposure to BE (mean ± SD).*

For the purpose of biological monitoring, the measurement of free BAA in the post-shift urine has been proposed. The concentration of free and total BAA in the post-shift urine after inhalatory exposure were 8.6 ± 4.8 and 12.9 ± 4.2 mmol/mol creatinine, respectively. Following dermal exposure, the respective concentrations amounted to 122 ± 45 and 265 ± 88 mmol/mol creatinine.

Discussion

In the previous report on dermal absorption of BE we showed that the aqueous solution of BE is readily absorbed through the skin (Jakasa *et al.* 2004). Since BE is mainly used as a water-based mixture, even brief skin contact could result in substantial absorption. This clearly justifies the skin notation for BE and indicates BM as a preferable approach for exposure risk assessment. Urinary BAA was proposed as the most suitable marker for BM. In Germany (BAT; see DFG 2003) and the United Kingdom (Biological Guidance Values; see HSE 2002), the biological exposure limits were established at 100 mg/l urine (which corresponds to about 86 mmol/mol creatinine) and 240 mmol/mol creatinine, respectively (DFG 2003; HSE 2002). The guidelines propose the measurement of the free BAA in the postshift urine. However, due to big interindividual differences in excretion and the poor correlation between exposure levels and BAA values, the usefulness of BAA as a biological indicator of exposure has been questioned in earlier studies (Angerer *et al.* 1990; Goen *et al.* 2002; Johanson *et al.* 1988). As one of the possible reasons for the interindividual differences, the variation in the extent of the conjugation of BAA has been put forward (Corley *et al.* 1997; Jones and Cocker 2003; Rettenmeir *et al.* 1993; Sakai *et al.* 1994).

Our study confirms recent reports that urinary BAA is extensively conjugated, and that the extent of conjugation is highly variable between individuals. After inhalatory exposure with an absorbed amount of BE of 20.9 ± 5.0 mg, 55 ± 21% of the total excretion of BAA was in the form of conjugate. After dermal exposure with a much higher absorbed amount of BE (567 mg) nearly the same proportion (58 ± 14%) of conjugated BAA was found. The extent of conjugation is comparable to the results of Corley et al. (1997), who reported the proportion of conjugated BAA following a 2-h inhalation exposure to be 67 ± 9%. The interindividual variation in conjugation was higher in single urine samples. After inhalation, the proportion of conjugated BAA in the first postexposure sample amounted to 46 ± 33% (CV 72%). Following dermal exposure, that percentage of conjugated BAA in the first postexposure urine was 45 ± 26% (CV 58%). In the post-shift urines of workers

occupationally exposed to BE, various ratios of conjugated BAA were found by different authors. Jones and Cocker (2003) reported the value of 57% (95% CI, 44 - 70%), Rettenmeier et al. (1993) 48% (range 16 - 64%), and Sakai et al. (1994) 71% (range 44 - 92%). Our results show that the proportion of the conjugated fraction is dependent on the sampling time - that is the time that passed from the beginning of exposure. After dermal exposure, the lowest proportion of conjugated BAA (45 ± 30%) was found in the urines collected immediately after exposure, increasing in time to 92 ± 2% 48 h after the onset of exposure. This is consistent with the longer half-life of conjugated BAA in comparison to free BAA we found after dermal exposure (5.8 ± 1.0 and 3.8 ± 0.35 h, respectively). The half-life of total BAA, which is actually the result of respective half-lives of free and conjugated BAA, amounted to 5.1 ± 0.5 h. The slower elimination of conjugated BAA in comparison to free BAA we found is in agreement with the results of Sakai *et al.* (1994). In workers exposed to BE, they found that the accumulation of free BAA during the work week was relatively small in comparison with that of the conjugated form. On the other hand, Jones and Cocker (2003) reported similar half-lives for free and total BAA (5.9 ± 1.9 and 6.1 ± 2.4 h, respectively).

The maximal concentrations of BAA were measured in the first postexposure urines: for example, after a 30- min inhalation exposure in the urines collected 0-4 h, and after 4-h dermal exposure in the urines collected 4-8 h. The elimination kinetics of BAA in this study were consistent with two other studies (Johanson *et al.* 1986; Johanson and Boman (1991). In a 2-hr inhalation exposure, Johanson *et al.* (1986) found a maximal excretion of free BAA after between 2 and 10 h. In the second study, of Johanson and Boman, involving 2-h dermal exposure, the peak excretion occurred after 5 h (Johanson and Boman 1991). However, Jones and Cocker (2003) found the maximum excretion 6-12 h after the end of a 2-h inhalation exposure. Interestingly, they found a second maximum at 12 h postexposure time. In our study, a smooth regular decay in the concentrations and excretion rates was observed. As pointed out earlier, the use of BAA as an exposure indicator has been questioned due to high interindividual differences in excretion. In the present study, the interindividual coefficient of variation in the cumulative excretion after inhalation exposure was higher when free BAA was used (49%) than when calculated using total BAA (31%). Also, Jones and Cocker (2003) found in an experimental inhalation study a reduced variation when total excretion was used (fivefold and twofold variation for the excretion of free and total BAA, respectively). Also, after dermal exposure we found that the coefficient of variation of the cumulative excretion of total BAA was lower than that of free BAA (15% and 41%, respectively). This is in agreement with the respective values of 14% and 35% reported in a dermal study by

Corley *et al.* (1997). By contrast, Johanson *et al.* reported very high variations (excretion of BAA ranged from 8.7 to 313 µmol) after dermal exposure to neat BE; however, he determined only the free BAA (Johanson *et al.* 1988). The interindividual variation in the excretion of BAA in single urine samples was similar to that in cumulative urine samples. In the first postexposure urine after inhalatory exposure, the coefficients of variations were 56% and 33% for free and total BAA, respectively. Also, after dermal exposure the variation was higher for free BAA (37%) in comparison to total BAA (33%).

It is obvious that considerable variation in the conjugation may account in large part for the interindividual differences in the excretion of BAA reported in the earlier studies. In the present study, consistent with the findings of Jones and Cocker (2003), the degree of conjugation in the single urines varied nearly from 0 to 100%. In one subject, BAA was excreted almost exclusively (92 - 100%) in the conjugated form in all collected urines. On the other hand, we had no indication for the existence of two subgroups, that is fast versus slow conjugators or nonconjugators, in the sense that it is used in the literature for fast and slow acetylators. Although in three (out of 66) urine samples, BAA was excreted almost completely in the free form, these urine samples were from different individuals, and all of them were sampled immediately after the end of exposure. Jones and Cocker (2003) suggested that conjugation might be activated above a certain concentration level (above 50 mmol mol^{-1} creatinine). By contrast, Rettenmeier *et al.* (1993) suggested saturation of the conjugation at higher doses. Our data do not support either of these assumptions, we found the same extent of conjugation following inhalation and dermal exposure, although the systemic absorption resulting from these exposures differed more than 30 times.

The concentration of BAA in urine is of particular importance for biological monitoring of occupational exposure to BE. Firstly, BAA is believed to be responsible for toxic effects resulting from haemolysis of red blood cells (Ghanayen and Sullivan 1993). Secondly, being the main metabolite, BAA is a logical candidate for a biological indicator of exposure to BE. In this study, we used the excretion of BAA as a biological indicator of the internal dose to assess the dermal uptake. Using cumulative excretion of BAA after dermal and reference inhalation exposure, we estimated the average flux of BE through the skin. Nearly the same fluxes of 3.5 ±0.5 and of 3.5 ± 1.4 mg cm^{-2} h^{-1} were calculated by using the excretion of total and free BAA, respectively.

When using the BE blood data, a skin flux of 2.4 ± 0.8 mg cm^{-2} h^{-1} was found. These dermal fluxes were higher than the values of 1.34 and 0.92 mg cm^{-2} h^{-1} calculated from urine excretion of total BAA and blood BE values, respectively, in a study of Jakasa *et al.* (2004). The reason for this difference could be elastic bands that were used in the present study to fix the chamber onto the skin and prevent leakage. This might cause the stretching of the skin which might lead to higher penetration.

The results of the present study show that the cumulative excretion of BAA is a good indicator for the assessment of exposure to BE: the average skin fluxes were in agreement with those estimated from the BE blood data, with similar interindividual variability. As put forward by Jones and Cocker (2003), urinary BAA as a biomarker of exposure has different advantages over measurement of blood BE concentrations. Urine sampling is noninvasive, and the concentration of BAA in urine is higher than that of BE in blood, enabling more sensitive analyses. Furthermore, as shown by Corley *et al.* (1997), blood sampling can be confounded by locally high concentrations of BE.

In conclusion, this study reveals high intraindividual and interindividual variation in conjugation of BAA varying from nearly 0 to 100% of the total excretion. It is obvious that the use of free BAA will lead to an erroneous estimation of the internal absorption. Therefore, total BAA as a biomarker of exposure is superior to free BAA.

References

- Angerer J, Lichterbeck E, Begerow J, Jekel S, Lehnert G (1990) Occupational chronic exposure to organic solvents, XIII: glycol ether exposure during the production of varnishes. Int Arch Occup Environ Health 62:123–126
- Corley RA, Markham DA, Banks C, Delorme P, Masterman A, Houle JM (1997) Physiologically based pharmacokinetics and the dermal absorption of 2-butoxyethanol vapor by humans. Fundam Appl Toxicol 39:120–130
- European Commission (2000) Fifth Framework Programme of the European Commission. Contract QLRT-2000-00196. Brussels DFG (2003) List of MAK and BAT values. Wiley, VCH. ISBN 3-527-27512-6
- Ghanayem BI, Sullivan CA (1993) Assessment of the haemolytic activity of 2-butoxyethanol and its major metabolite, butoxyacetic acid, in various mammals including humans. Hum Exp Toxicol 12:305–311
- Goen T, Korinth G, Drexler H (2002) Butoxyethoxyacetic acid, a biomarker of exposure to water-based cleaning agents. Toxicol Lett 134:295–300

- Haufroid V, Thirion F, Mertens P, Buchet JP, Lison D (1997) Biological monitoring of workers exposed to low levels of 2-butoxyethanol. Int Arch Occup Environ Health 70:232–236
- HSE (2002) EH40/2002 occupational exposure limits 2002. Health and Safety Executive, London. ISBN 0 7176 2083 2
- Jakasa I, Mohammadi N, Kruse J, Kezic S (2004) Percutaneous absorption of neat and aqueous solutions of 2-butoxyethanol in volunteers. Int Arch Occup Environ Health 77:79–84
- Johanson G, Boman A (1991) Percutaneous absorption of 2-butoxyethanol vapor in human subjects. Br J Ind Med 48:788–792
- Johanson G, Kronborg H, Naslund PH, Byfalt NM (1986) Toxicokinetics of inhaled 2-butoxyethanol (ethylene glycol monobutyl ether) in man. Scand J Work Environ Health 12:594– 602585
- Johanson G, Boman A, Dynesius B (1988) Percutaneous absorption of 2-butoxyethanol in man. Scand J Work Environ Health 14:101–109
- Jones K, Cocker J (2003) A human exposure study to investigate biological monitoring methods for 2-butoxyethanol. Biomarkers 8:360–370
- Kezic S, Mahieu K, Monster AC, de Wolff FA (1997) Dermal absorption of vaporous and liquid 2-methoxyethanol and 2-ethoxyethanol in volunteers. Occup Environ Med 54:38–43
- Laitinen J (1998) Correspondence between occupational exposure limit and biological action level values for alkoxyethanols and their acetates. Int Arch Occup Environ Health 71:117–124
- Ministerie van sociale zaken en werkgelegenheid (2001) Nationale MAC-lijst 2001. SDU, The Hague, p 19
- Rettenmeier AW, Hennigs R, Wodarz R (1993) Determination of butoxyacetic acid and N-butoxyacetyl-glutamine in urine of lacquerers exposed to 2-butoxyethanol. Int Arch Occup Environ Health 65:S151–S153
- Sakai T, Araki T, Morita Y, Masuyama Y (1994) Gas chromatographic determination of butoxyacetic acid after hydrolysis of conjugated metabolites in urine from workers exposed to 2-butoxyethanol. Int Arch Occup Environ Health 66:249–254

Chapter 3

Determination of polyethylene glycol of different molecular weights in the stratum corneum

I. Jakasa, F. Calkoen, S. Kezic

J Chromatogr B Analyt Technol Biomed Life Sci (2004) 811: 177-182

Abstract

We developed a sensitive method for determination of polyethylene glycols (PEGs) of different molecular weight (MW) in the human stratum corneum (SC) obtained by tape stripping. The analysis is based on derivatization with pentafluoropropionic anhydride (PFPA) and gas chromatography–electron capture detection (GC–ECD). The identification and quantification of PEGs was done using individual oligomers. The method showed to be suitable for studying permeability in normal and impaired skin with respect to MW in the range of 150–600 Da.

1. Introduction

Penetration of chemicals into the body is primarily prevented by the outermost layer of the epidermis, the stratum corneum (SC). The molecular weight (MW) plays a significant roll in the percutaneous penetration of chemicals. The MW cut-off for normal skin has been reported to be approximately 500 Da [1]. However, it has been shown recently that in the compromised skin, this cut-off is shifted to higher MW [2] and [3]. As a result, the impaired skin barrier enables the entering of macromolecules, e.g. protein allergens leading to physiological effects. Since such compromised skin is common due to chemical or physical damage as well as in diseased skin, an evaluation of increased permeability is important. For studying the permeability of the healthy and affected skin, polyethylene glycols (PEGs) are suitable model compounds. PEGs have been extensively used in research of intestinal permeability since their introduction by Chadwick et al. [4-7]. They are available in a wide range of MW and their water/octanol partitioning ($P_{o/w}$) coefficient does not change greatly with molecular mass [8]. Due to low toxicity, they are used in many fields of cosmetic, chemical and pharmaceutical industries.

For the determination of PEGs in biological material, several high-performance liquid chromatographic (HPLC) and gas chromatographic (GC) methods have been described [4, 9-16]. The majority of the HPLC methods are based on refractive index detection. However, the HPLC methods were primarily developed for the analysis of PEGs in urine or blood and could not be applied in the present study due to their low sensitivity. Higher sensitivity was reported in the GC methods which were based on derivatization of hydroxy groups followed by gas chromatography–electron capture detection (GC–ECD) [9]. Derivatization with different reagents such as acetic acid anhydride, trifluoroacetic acid (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) was investigated. However, the major problem in these procedures was a poor stability of the derivatives [4-5, 9, 16].

In all reported methods, polydisperse mixtures containing PEGs of different MW were used and the identification and quantification of the PEG was performed only indirectly. Recently, PEGs are also available as individual monodisperse oligomers, which enabled us to validate the method. We developed a sensitive GC method for quantification of PEG oligomers of different MW in human SC. The method was applied for studying the differences in percutaneous penetration between normal and impaired skin.

2. Experimental

2.1. Chemicals and materials

PEG 150.17 Da (PEG150) was purchased from Sigma, The Netherlands and PEG 282.34 Da (PEG282) was purchased from Acros Organics, NY, USA. PEGs 326.4 Da (PEG326), 370.4 Da (PEG370), 502.6 Da (PEG502), 546.7 Da (PEG546) and 590.7 Da (PEG590) (all oligomers have chemical purity >99%, and oligomer purity >95%) were purchased from PolyPure, Norway. Polydisperse PEG600 (average MW) was purchased from Sigma, The Netherlands. Pentafluoropropionic anhydride and sodium lauryl sulfate (SLS) (>99% GC) were purchased from Fluka, The Netherlands and (1*S*,2*S*)-(−)-phenylpropylene oxide (PPO) (98%, GLC) was purchased from Aldrich, USA. Dichloromethane (p.a.), ethyl acetate (p.a.), *n*-hexane (Unisolv), hydrochloric acid (37%, p.a.), methanol (Lichrosolv), sodium hydroxide (p.a.) and sulphuric acid were purchased from Merck (The Netherlands). Pyridine was purchased from Alltech, The Netherlands. Bio-Rad protein assay (Cat. No. 500-0112) was purchased from Bio-Rad Laboratories (Germany). Diamond Ultra Clear tape (19 mm × 33 m) was purchased from Sellotape® Company, The Netherlands and Finn chambers® (18 mm in diameter) were purchased from Epitest Ltd., Finland.

2.2. Percutaneous penetration study

The volunteers were Caucasians of both genders aged 18–55 years (n = 20) and had no visible skin damage and no history of dermatological diseases. The subjects participating in the study were asked not to use lotion, cream or soap on the lower arm 3 days prior to and during the experiment. Written informed consent was obtained from all subjects prior to the experiment. The Ethical Committee of the Academic Medical Center approved the experiment protocol.

An application mixture of PEG was made by dissolving 47.5 mg PEG150, 50.1 mg PEG282, 102.9 mg PEG326, 199.1 mg PEG370, and 10 g of polydisperse PEG600 in 2 ml of water. One hundred and eighty microliters of the mixture was spread onto the filter paper in the Finn Patch test chamber®. After the whole amount of the mixture was absorbed, two chambers were applied for 6 h onto the lower volar forearm of the volunteers. Two other chambers containing the application mixture were applied onto the skin site pre-treated with SLS. The treatment with SLS was performed 24 h before the application of PEG. This was done by applying 200 µl of 5% (w/w) water solution of SLS for 4 h. Immediately after the removal of the chambers, the residue of the PEGs on the skin was gently wiped off with wet and, subsequently, dry paper tissue. Medical adhesive tape with a round marked area of

18 mm in diameter was fixed around the application site to ensure that the tapes were consistently applied to the same site. Fifteen minutes after the end of exposure, the SC was repeatedly stripped with precut Diamond tape pieces, i.e. strips. Prior to the experiment, the tape was mounted on a glossy side of a commercially available paper sheet and cut to the size of 1.9 cm × 2.0 cm. The tape was applied with tweezers to the test site and rolled over 20 times with a 1 kg stainless steel roller. The tapes were peeled off with one quick movement multidirectionally. Each skin site was striped 15–25 times until the SC was totally removed as observed by the shiny appearance of the skin and by measuring transepidermal water loss (TEWL) using portable VapoMeter (Delfin Technologies Ltd., Finland). Each subsequent tape was placed into a 20-ml glass vial and stored at −18 °C until analysis. For the calibration line and quality control (QC) samples, tape strips containing non-exposed SC of a volunteer were used (blank samples).

2.3. Analysis of PEGs

2.3.1. Calibration samples

The stock standard solution was prepared by dissolving 9.5 mg PEG150, 32.9 mg PEG282, 49.5 mg PEG326, 63.2 mg PEG370, 289.0 mg PEG502, 455.1 mg PEG546 and 871.6 mg PEG590 in 10 ml of distilled water. A working standard solution of PEGs was prepared by diluting 40 µl of stock solution with 20 ml of methanol. The calibration standards were prepared by adding 10–160 µl of the working standard to the 500 µl methanol solution of the blank samples. For internal standardization, 10 µl of the working standard of (1*S*,2*S*)-(−)-phenylpropylene glycol (PPG) in ethyl acetate was added. The concentration of PEGs in the SC was calculated by internal standardization using peak height measurements. All samples were analyzed in duplicates. Since PPG was not commercially available, it was synthesized by acid-catalyzed hydrolysis of PPO as described previously [17].

2.3.2. Extraction and derivatization

Two milliliters of methanol was added to the glass vials containing tape strips and they were mechanically shaken for 1 h. Five hundred microliters of aliquot of the methanol phase was transferred to 4 ml vials. Ten microliters of PPG as internal standard (IS) was added. The methanol aliquots were evaporated to dryness. To each sample, 500 µl of dichloromethane, 20 µl of pyridine and 20 µl of PFPA were added. The reaction mixtures were heated at 70 ± 5 °C for 30 min. After cooling down to room temperature the samples were evaporated and the residue was dissolved in 1 ml of *n*-hexane to which 15 µl of pyridine was added. Samples were vortexed for 1 min and transferred to safe-lock tubes, which were centrifuged for

15-30 s (11 860 × *g*) prior to the GC–ECD analysis. The samples were analyzed during a time period of 14 h.

2.3.3. GC–ECD analysis

The GC–ECD analysis was carried out with a Hewlett-Packard 5890 GC (Hewlett-Packard, USA) equipped with a ^{63}Ni ECD. The column was HP-1 (25 m × 0.32 mm, 0.17 µm film thickness, Agilent technologies, USA). The initial column temperature was 60 °C and the temperature was increased after 6 min to 150 °C at 15 °C min^{-1} and then programmed at 30 °C min^{-1} to 340 °C where it was held for 4 min. The injector and detector temperature was 350 °C and the column head pressure was 110 kPa. The sample (1 µl) was injected by the split injection technique (split ratio 1:40). The deactivated cup inlet liner suitable for split injection of high MW compounds (Cat. No. 20510) was purchased from Supelco, USA.

2.3.4. Determination of the stability of PFPA derivatives

To determine the stability of PEG derivatives, a standard SC sample was prepared by adding 80 µl of working standard (see Section 2.3.1) to 500 µl methanol solution of the blank samples. After extraction and derivatization (see Section 2.3.2), the hexane layer was distributed into the auto sampler vials and repeatedly injected into the GC–ECD during 14 h.

2.3.5. Recovery and precision

To determine the recovery, seven blank tape strips containing SC were placed into 20-ml glass vials and spiked with a working standard solution (see Section 2.3.1). The SC tape strips were dry within 1 h and thereafter analyzed according to the procedure (see Section 2.3.2). The same amount of the working standard solution was directly added to seven glass vials containing 2 ml methanol (100% recovery). The samples were then analyzed according to the standard procedure (see Section 2.3.2). The extraction recovery from the tape strips was determined by comparing the peak height ratios of standards not extracted with those of standards extracted from the tape strips. The repeatability (within-run precision) and reproducibility (between-run precision) of the method were determined by analysis of seven SC strips spiked at two and one concentration levels, respectively.

2.4. Protein analysis in the tape strip

The protein analysis was used to measure the amount of the SC removed by each tape strip, and to assess the depth of the consecutive SC strip [18] and [19].
The methanol residue containing tape strip with precipitated proteins on it after PEG analysis was evaporated. One milliliter of 1 M NaOH was added to the strip and the vials were shaken for 2 h. The samples were left at room temperature overnight and the next day they were shaken for 2 h one more time. One milliliter of 1 M HCl was added to the vials to neutralize the basic solution. The protein assay was based on the modified method of Dreher et al. and performed according to Bio-Rad DC protein microassay using commercially available bovine serum albumin (BSA) for standardization [20] and [21]. Absorbance at 655 nm was measured using the Bio-Rad 680 microplate reader (Bio-Rad, USA).

3. Results and discussion

3.1. Derivatization of the hydroxy groups with PFPA

To enable sensitive ECD detection and improve chromatographic performance, hydroxy groups were acylated with PFPA. In a pilot study, we investigated the optimal temperature and time duration for the derivatization. As concluded from the peak heights of formed derivatives, the derivatization performed at 70 °C was completed within 30 min. The addition of pyridine as an acid scavenger improved the derivatization; the absolute peak heights of the derivatives were higher for a factor of 10 when pyridine was added. Higher amounts of pyridine did not lead to a further increase in peak heights. Also, the stability of the derivatives was improved by addition of pyridine. The deterioration of the derivatives for all oligomers during a period of 14 h was limited and within the error of the method (Fig 1), except for oligomer with MW of 590 Da, for which the loss was somewhat higher (up to 15% within 14 h). The stability of the derivatives was better than that reported in the study of Fakt and Ervik [9]. They also used PFPA for acylation of polydisperse PEG400 reporting gradual deterioration of derivatives after only 1 h. After 24 h, the percentage of initial concentration in that study was about 60%. It was inconclusive from the article if all oligomers in PEG400 were investigated or just the highest peak in the mixture, which corresponded to MW of 326.4 Da. In their procedure, they used toluene as a solvent with no addition of pyridine.

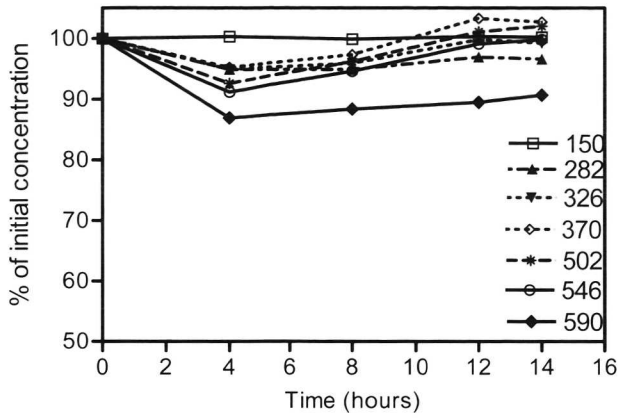


Fig 1: *Stability of PEG derivatives expressed as percentage of initial concentration.*

3.2. Chromatography

Fig 2 shows chromatograms of blank SC sample, blank SC sample spiked with mixture of individual oligomers and SC sample of a volunteer exposed to PEGs (fifth strip). As can be seen, good separation of all oligomers was achieved. In a pilot study, we investigated chromatographic performance using an identical column with a thicker stationary phase film (0.52 µm). However, the peaks of PEGs, especially those of higher MW, showed more peak tailing on that column. As concluded from the peak heights of the oligomers, we did not observe any deterioration of the polymers at high injector and column temperature (350 and 340 °C, respectively). This is consistent with the findings of Onigbinde et al., who also reported stability of the PEGs at the temperature of 330 °C [22]. The blank sample showed no major interfering peaks except for PEG282 and PEG590, although for these oligomers reproducible results were obtained (Table 1).

3.3. Identification and quantification of oligomers

Identification of oligomers in the application mixture and in the SC samples was achieved by direct comparison with individual oligomer standards which were analyzed under the same chromatographic conditions. This is in contrast with the previous studies, where the identification of the oligomers in the polydisperse mixtures was performed only indirectly [2-5, 9, 11, 14-15]. In most of these studies, the identification of the PEG oligomers in the chromatogram was done by the

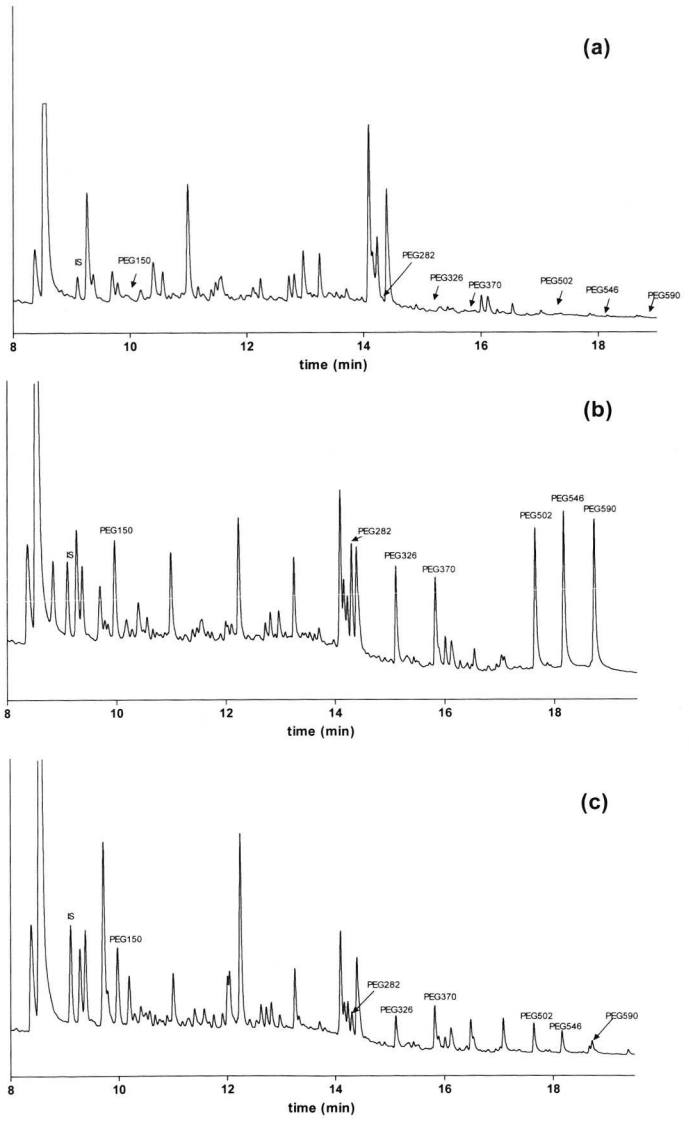


Fig 2: *Representative GC–ECD chromatograms of: (a) blank SC sample; (b) blank SC sample spiked with standard solution of individual PEG oligomers; and (c) SC sample obtained from a volunteer exposed to PEGs for 4 h at a skin site pre-treated with SLS (fifth strip).*

comparison of the retention time with that of a smaller individual oligomer (di-, tri- or tetraethylene glycol) which was available as a pure chemical [11, 14-15]. From this, the other oligomers were identified by sequential counting of the peaks. Another approach was to assign a MW closest to the average MW of the polymer to the highest peak in the chromatogram [2-3]. The standardization of these methods was also performed indirectly. The amount of each oligomer in the mixture was assessed from the relative peak areas, assuming equal detector response for oligomers of different MW. Under the assumption that the polydisperse mixture contained no impurities and that all oligomers in the mixture could be quantified from the chromatogram, this approach is less critical by HPLC methods. However, by GC methods, due to possible discrimination in the injector that might occur for higher MW compounds, the peak area might not be proportional to the mass fraction in the polydisperse mixture. In the study of Fakt and Ervik, the highest peak in a GC chromatogram of polydisperse PEG400 was reported to correspond to MW of 326 Da [9].

However, as confirmed by gel permeation chromatography, mass spectrometry and HPLC, in the polydisperse PEG400, PEG370 and PEG414 have the highest mass fraction [11, 13]. This clearly illustrates the advantage of using individual oligomers for the standardization of the method.

3.4. Extraction recovery

The results of extraction recovery of PEG oligomers from the tape are summarized in Table 1. Recoveries ranged from 88 to 97% and seemed not to be dependent on MW of oligomers. This is in contrast with the study of Ruddy and Hadzija, who reported recovery dependence on MW [11]. The recoveries in that study were found to increase with increasing MW ranging from 18 to 86% for PEG282 and PEG590, respectively. In that study, PEGs were isolated and purified by solid-phase extraction using large pore kieselguhr (Extrelut QE) cartridges.

3.5. Linearity

To determine the linearity of the assay the standard samples prepared as described in Section 2.3.1 (concentration ranges are reported in Table 1) were analyzed on seven different days. The regression analysis was performed using the least square method. The ratios of the peak heights of individual oligomers of PEGs versus IS were linearly related to the oligomer concentrations within the range of concentrations studied. The correlation coefficients (R) of the calibration curves ranged from 0.9909 to 0.9942.

Table 1. Analytical parameters of GC-ECD assay of PEG oligomers in human *SC* (CV=coefficient of variation, LOD=limit of detection, LOQ=limit of quantitation)

	N	PEG 150	PEG 282	PEG 326	PEG 370	PEG 502	PEG 546	PEG 590
Linear range (μg cm^{-2})	7	0.026-0.42	0.09-1.5	0.14-2.2	0.2-2.8	0.8-13	1.3-20	2.4-38
LOD (μg cm^{-2})	7	0.007	0.013	0.014	0.003	0.051	0.031	0.082
LOQ (μg cm^{-2})	7	0.024	0.042	0.046	0.011	0.169	0.104	0.272
Repeatability (CV %)	7	6.8	6.9	7.3	8.7	6.5	7.2	9.6
Concentration level (μg cm^{-2})		0.21	0.72	1.2	1.6	6.4	10.4	19.2
Repeatability (CV %)	7	5.2	9.9	9.3	7.8	6.2	7.0	8.5
Concentration level (μg cm^{-2})		0.05	0.18	0.3	0.4	1.6	2.6	4.8
Reproducibility (CV %)	5	3.2	9.3	10.7	10.8	9.5	10.1	12.8
Concentration level (μg cm^{-2})		0.21	0.72	1.09	1.39	6.35	10.01	19.16
Recovery (%)	7	94	88	93	97	96	96	93
Concentration level (μg cm^{-2})		0.21	0.72	1.2	1.6	6.4	10.4	19.2

3.6. Precision and limit of detection and quantitation

The limit of detection (LOD) was defined as three times the standard deviation (S.D.) obtained by repeated analysis of seven blank samples. The lower limit of quantitation (LLOQ) defined as the lowest standard on the calibration curve ranged from 0.026 μg cm^{-2} for PEG150 to 2.4 μg cm^{-2} for PEG590 (Table 1). As estimated from the injected amount of PEG that corresponds to the LLOQ, our method was much more sensitive than the existing GC method of Fakt and Ervik (0.4 and 16 ng, respectively) [9].

The coefficient of variation (CV) for all oligomers was less than 10% for repeatability and 13% for reproducibility. The results are summarized in Table 1.

3.7. Skin penetration study

The concentration of PEG oligomers was determined in the SC tape strips of the normal skin and in the skin after acute disruption by SLS. The amount of PEG oligomers in a subsequent strip normalized for the applied concentration was plotted as a function of the SC depth. This relationship was used for the estimation of the penetration parameters using the approach based on Fick's second law of diffusion described in details elsewhere [19]. Fig 3a–c illustrates the effect of SLS on the penetration of PEG150, PEG370 and PEG590 in a volunteer. The penetration of all PEG oligomers into the SC was enhanced in the skin impaired by SLS. Furthermore, the penetration enhancement was more prominent for PEGs with higher MW, which is in agreement with in vitro study in murine skin of Tsai et al. [3]. The results of this study will be published elsewhere.

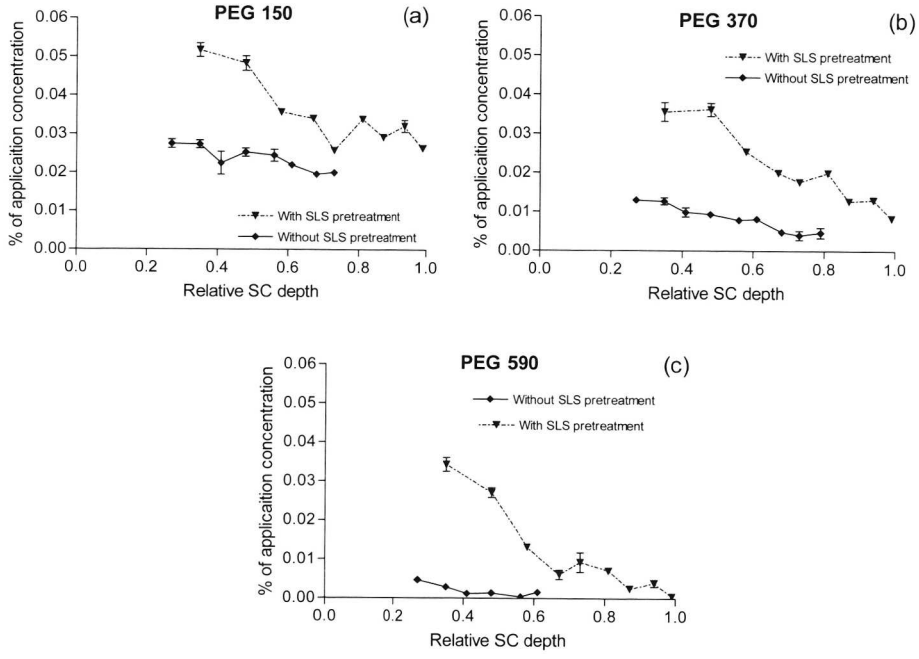


Fig 3: *The concentration of PEG150 (a), PEG370 (b) and PEG590 (c) in the SC normalized for the applied concentration (%) in a volunteer exposed to PEGs for 6 h. The relative SC depth was calculated from the ratio of the SC mass removed till i-th tape strip and the total SC mass removed by all tape strips.*

4. Conclusions

Our method, using individual oligomers, has proper identification, quantification and high sensitivity. The described method of extraction of the PEG oligomers with different MW from tape strips was fast and efficient showing no recovery dependence on MW. The derivatization procedure produced sufficiently stable derivatives for the analytical purpose. The method proved to be suitable for studying percutaneous penetration of PEGs with MW in the range from 150 to 600 Da in normal and impairedskin.

References

1. J.D. Bos and M.M.H.M. Meinardi, *Exp. Dermatol.* **9** (2000), p. 165
2. J.C. Tsai, P.L. Hung and H.M. Sheu, *Arch. Dermatol. Res.* **293** (2001), p. 302. [3] J.C. Tsai, L.C. Shen and C.C. Lu, *Arch. Dermatol. Res.* **295** (2003), p. 169.
3. V.S. Chadwick, S.F. Phillips and A.F. Hofmann, *Gastroenterology* **73** (1977) (2), p. 241.
4. V.S. Chadwick, S.F. Phillips and A.F. Hofmann, *Gastroenterology* **73** (1977) (2), p. 247.
5. T.H. Iqbal, M.A. Cox, K.O. Lewis and B.T. Cooper, *Clin. Sci.* **89** (1995) (3), p. 299.
6. J.B. Lloyd, *Clin. Sci.* **95** (1998) (1), p. 107. [8] P.P. Bijlsma, Ph.D. thesis, University of Amsterdam, 1999.
7. C. Fakt and M. Ervik, *J. Chromatogr. B* **700** (1997), p. 93.
8. E. Kwong, L. Baert and S. Bechard, *J. Pharm. Biomed. Anal.* **13** (1995) (1), p. 77.
9. S.B. Ruddy and B.W. Hadzija, *J. Chromatogr. B: Biomed. Appl.* **657** (1994) (1), p. 83.
10. H.A. Schwertner, W.R. Patterson, J.H. Cissik and K.W. Wilson, *J. Chromatogr.* **578** (1992), p. 297.
11. S.T. Lai, *J. Chromatogr.* **363** (1986), p. 444.
12. T. Meyer, D. Harms and J. Gmehling, *J. Chromatogr.* **645** (1993), p. 135. [15] R. Murphy, A.C. Selden, M. Fisher, E.A. Fagan and V.S. Chadwick, *J. Chromatogr.* **211** (1981), p. 160.
13. J.B. Bouska and S.F. Phillips, *J. Chromatogr.* **183** (1980) (1), p. 72[17] M.A. Wenker, S. Kezic, A.C. Monster and F.A. de Wolff, *Toxicol. Appl. Pharmacol.* **169** (2000) (1), p. 52.
14. F. Pirot, Y.N. Kalia, A.L. Stinchcomb, G. Keating and A. Bunge, *Pharmacology* **94** (1997), p. 1562.
15. M.B. Reddy, A.L. Stinchcomb, R.H. Guy and A.L. Bunge, *Pharm. Res.* **19** (2002) (3), p. 292.
16. F. Dreher, A. Arens, J.J. Hostynek, S. Mudumba, J. Ademola and H.I. Maibach, *Acta Derm. Venereol.* **78** (1998) (3), p. 186. [21] Bio-Rad Laboratories, DC Protein Assay: Microassay Procedure.
17. A. Onigbinde, G. Nicol and B. Munson, *Eur. J. Mass Spectrom.* **7** (2001), p. 279.

Chapter 4: *Section 4.1*

Percutaneous penetration of sodium lauryl sulphate is increased in uninvolved skin of atopic dermatitis patients compared to control subjects

I. Jakasa, C.M. de Jongh, M.M. Verberk, J.D. Bos, S. Kezic

Br J Dermatol (in press)

Abstract

Background: Involved regions of the skin in atopic dermatitis (AD) patients have been shown to have higher trans-epidermal water loss (TEWL) indicating compromised skin barrier. Whether also uninvolved skin has diminished barrier is controversial.

Objectives: To study the penetration of sodium lauryl sulphate (SLS) into uninvolved skin of AD patients compared to the skin of control subjects.

Methods: The percutaneous penetration was assessed using the tape stripping technique of the stratum corneum (SC). Twenty AD patients and 20 healthy subjects were exposed to 1% SLS for four hours on mid volar forearm. After the end of exposure the SC was removed by adhesive tape. In each consecutive strip, the amount of SLS was determined. Using Fick's second law of diffusion, diffusivity and partition coefficient of SLS between water and SC were deduced.

Results: The SC thickness was similar in both groups; however the TEWL was higher in AD patients compared to that of the control group (8.4 ± 4.3 and 6.3 ± 2.1 g m^2 h^{-1}, respectively). There was a correlation between SC thickness and TEWL in control subjects but no correlation was found in AD patients. The diffusivity of SLS through uninvolved AD skin was higher compared to normal skin (12.7 ± 5.8 x 10^{-9} and 6.1 ± 3.1 x 10^{-9} cm^2 h^{-1}, respectively) while the partition coefficient between SC and water was lower (137 ± 64 and 193 ± 101, respectively).

Conclusion: The results show different penetration profile of SLS into the SC of AD patients compared to control subjects. This indicates that even non-involved skin in AD patients has altered barrier emphasizing importance of skin protection and prevention of skin contact with chemicals.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyperactivity to environmental agents and is characterized by pruritic lesions with dryness and typical distribution and morphology. The affected regions of the skin in AD patients show higher trans-epidermal water loss (TEWL) in comparison to the normal skin, in other words, lower capacity to hold water[1]. Higher permeability of affected skin was shown also for theophylline[2] and polyethylene glycols (Jakasa *et al.*, manuscript in preparation). The literature data concerning permeability of uninvolved AD skin is contradictory. Some authors reported higher TEWL in patients with AD history[1;3-8] while others found no difference in comparison to normal skin[5;9-12]. In several studies it was suggested that the higher susceptibility to irritation in AD patients[6;10-11;13] might partly be explained by higher skin permeability[14]. Increased susceptibility of AD patients to irritation induced by sodium lauryl sulphate (SLS) was shown both in involved and uninvolved AD skin[6]. Impaired skin barrier in AD has often connected to the different lipid composition and structure of atopic skin. Previous studies have demonstrated that barrier impairment coincides with alterations in the amount and composition of stratum corneum (SC) ceramides in AD skin[5;15-16]. In involved as well as in uninvolved skin of AD patients reduced ceramide content and decreased percentages of Cer1 and Cer3 were reported.[5;15-17] In contrast to this, Matsumoto *et al.*[18] found that the reduction of Cer 1 is restricted to the involved AD skin and is not extended to uninvolved area, which is consistent with data of Farwanah *et al.*[19]

The question whether the barrier function in uninvolved skin in AD patients is compromised as compared to control skin is still open. In the present study we investigated the penetration of SLS into the SC of uninvolved skin of AD patients and into the SC of the skin of control subjects. SLS is a common ingredient of soaps and cosmetic products and atopic persons are known to be sensitive to these. In addition, in a previous study we have shown that atopic persons, although with no history of AD, appeared to have higher skin diffusibility and to be more susceptible to SLS irritation than non-atopic subjects.[14]

Subjects and methods

Study population

Twenty AD patients, 12 male and 8 female, mean age 29 years (range 18-54 years) and 20 healthy subjects, 11 male and 9 female, mean age 32 years (range 18-55

years), all Caucasians participated in the study. The study was carried out in June and July 2004.

AD patients were recruited from the outpatient clinic of the Academic Medical Center and diagnosed according to Hanifin & Rajka criteria.[20] We excluded patients that had received systemic therapy, such as corticosteroids and immunosuppressants, or photo-therapy in the past two years. Subjects with concomitant ichthyosis vulgaris were excluded. The test sites, both mid volar arms were free of dermatitis for at least 3 months prior to the experiment. The total eczema area and severity index[21] (EASI, the maximum is 72 points) was assessed in AD patients. The severity of the disease was mild in all patients and the median EASI score was 1.7 (ranging from 0.2 to 22.8). Twelve patients had active AD (pruritic lesions) and eight had inactive AD (free of dermatitis for at least 3 months, at the time showing only mild signs: scars, scaling, lichenification or dry skin) on body parts other than test sites.

Control subjects had no visible skin damage and no history of past or present AD and other dermatological diseases.

All subjects completed the Erlangen questionnaire from which an Atopy Score[22] (the maximum is 34 points and having a score ≥ 10 is considered as atopy) was derived. AD patients and control subjects had a score of 17.4 ± 6.6 and 3.0 ± 2.4 (mean ± SD), respectively.

Participants were not allowed to use soap, moisturizers or any other cosmetics and creams on the lower mid volar arms 48 hours prior to and during the experiments. Written informed consent was obtained from all subjects prior to the experiment.
The Medical Ethical Committee of the Academic Medical Center, University of Amsterdam approved the experimental protocol.

Penetration experiment

The subjects were exposed for four hours on both volar arms to 1% SLS in water (200 µL, ≥ 99% purity, Fluka, Buchs, Switzerland) using patch test chambers(Finn chambers®, 18 mm in diameter, Epitest Ltd., Finland). Before application and after patch removal, TEWL was measured on application sites. TEWL was measured with an Evaporimeter (VapoMeter SWL2g, Delfin Technologies, Ltd., Kuopio, Finland). The measurement was described in detail elsewhere[23]. Twenty minutes prior to the measurements, the subjects rested with their sleeves rolled-up in the examination room, where temperature was 20-22 °C and relative humidity ranged between 50 and 60%. Fifteen minutes after the end of exposure the SC layers were sequentially

removed with pre-cut Diamond tape pieces, 19 x 25 mm (Diamond Ultra Clear tape, The Sellotape® Company, the Netherlands). Templates of Scanpor® tape were fixed on the skin around application spot to limit the tape stripping area (18 mm in diameter). The tape pieces were consecutively applied to the test site and uniformly pressed with 1 kg stainless steel roller that was moved 20 times in two directions. The sites were stripped multidirectionally with one quick movement until the SC was totally removed as observed by shiny and reddish appearance of the skin, feeling of burning sensation by subjects when last tape strips were taken off and by measuring TEWL > 100 g m^{-2} h^{-1}. Each subsequent strip was placed into a glass vial and stored at -20 °C until analysis. The stripping of each site was completed within 40 minutes. The SC from non-exposed site was stripped off and served as negative control.

Analytical procedure

The concentration of SLS on each strip was determined spectrophotometrically using the adjusted method of Rusconi *et al.*[24] In brief, 2 ml of methanol (J. T.Baker, Deventer, The Netherlands) was added to the vials and shaken for one hour (TPM-2 shaker-Sarstedt, Numbreht, Germany) to extract the SLS from the tapes. Standards of SLS for the calibration curve were prepared in methanol (2.3 – 50 µg cm^{-3}) and 20 µl from each standard and sample was pipetted into a 96-wells plate. After evaporation of the methanol, 200 µl Stains-All® working solution was added to each well and absorbance was read at 450 nm (Model 680 Microplate reader, Bio-Rad Laboratories, Hercules, CA, USA).

A protein analysis was used to measure the amount of *SC* removed by each tape strip and to assess the depth of the consecutive *SC* strip. The methanol residue, containing tape strip with precipitated proteins on it after SLS analysis, was evaporated. 1 ml of 1 M NaOH was added to the strip and the vials were shaken for two hours. The samples were left at room temperature overnight and the next day they were once more shaken for two hours. 1 ml of 1M HCl was added to the vials to neutralize the basic solution. The protein assay was based on the modified method of Dreher *et al.*[25] and performed according to Bio-Rad DC protein microassay[26] using commercially available bovine serum albumin (BSA) for standardization. Absorbance at 655 nm was measured using the Bio Rad 680 microplate reader.

The concentration of SLS on each strip was normalized for the amount of proteins and expressed as µg SLS/µg protein. Assuming the SC density[27] of 1 g cm^{-3} and a uniform distribution of SC on the tapes the protein mass removed was converted to a volume enabling estimation of the depth of each strip in the SC and total thickness of the removed SC.

Data analysis

The concentration of SLS on each strip was plotted as a function of the relative SC depth. For the estimation of the penetration parameters we used the approach based on Fick's second law of diffusion described in detail elsewhere[28-29] (Fig 1) where C_{veh} is the applied SLS concentration (μg cm^{-3}), C(x) is the SLS concentration (μg cm^{-3}) at depth x, K is the SC/water partition coefficient, L is the total thickness of the SC (μm), D is the diffusivity of SLS through the skin (cm^2 h^{-1}) and t is the exposure duration (h). The non-steady state diffusion equation (Fig 1) was fitted to the data where the rate constant for diffusion across SC (D/L^2, h^{-1}) was obtained from the decay of C(x) as a function of x and K was obtained from the intercept at x = 0. The penetration parameters were derived from individual experiments and were averaged. The first strip was not included in the regression analysis, as it contained some residue of SLS on the surface of the skin after the end of exposure. For curve fitting and statistical calculations, Prism 4 software was used (Graph Pad, San Diego, CA, USA).

For statistical calculations Student's t-test and one-way ANOVA with Bonferroni post test were used. P value < 0.05 was considered significant.

$$C(x) = KC_{veh}\left\{1-\frac{x}{L}\right\} - \sum_{n-1}^{\infty}\frac{2}{n\pi}KC_{veh}\sin\left(\frac{n\pi x}{L}\right)\exp\left(\frac{-Dn^2\pi^2 t}{L^2}\right)$$

Fig 1: *Fick's second law of diffusion.*

Using the results of duplicate dermal exposures on two volar forearms we calculated the intra-subject variability as well as the inter-subject variability in a restricted sense, i.e. after eliminating the intra-subject variability. For the latter we used the coefficient of variation = {([between subject variance–within subject variance]/2)$^{1/2}$}/mean. We assume that the intra-individual variation predominantly consists of the measurement variation and in a small part of the difference in permeability between both measured sites.

Results

To remove the SC completely, on average 22 ± 9 strips for control subjects and 28 ± 9 for the AD patients were needed. The average amount of proteins removed from the exposure sites of the control subjects was 2206 ± 644 µg corresponding to SC thickness of 8.7 ± 2.5 µm that of AD patients was 2394 ± 491 µg corresponding to SC thickness of 9.4 ± 1.9 µm. Statistically acceptable ($r^2 \geq 0.95$) curve fitting was obtained for all control subjects and AD patients. For two control subjects and two AD patients fitting was obtained only for one of the duplicate measurement and for four control subjects curve fitting could only be performed using the pooled duplicate data. Figure 2 shows the SLS concentration profile across SC for one typical control subject and one AD patient as well as fitted curve obtained by non-linear regression analysis (dashed lines).

The penetration parameters are summarized in Table 1. We have found no substantial difference between the two groups for SC thickness. TEWL was higher in AD patients when compared to control subjects. A significant correlation between SC thickness and TEWL was found in control subjects ($r = -0.59$, $p = 0.003$): the thinner the SC, the higher the TEWL but no significant correlation was found in AD patients ($r = -0.14$, $p = 0.55$) (Figure 3).

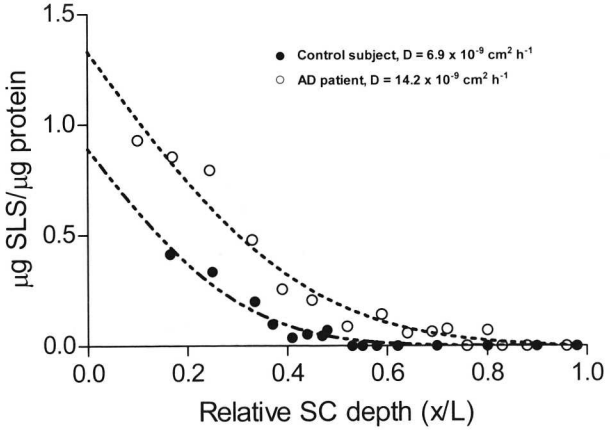


Fig 2: *SLS concentration decay as a function of SC depth (x/L) in one control subject and one AD patients after 4 hour exposure to 1% SLS. Non-linear regression analysis was used to fit the equation given in Fig 1 to the data (dashed lines).*

Table 1. SLS penetration parameters (*mean* ± *SD*) and corresponding inter- and intra-individual variations for AD patients and control subjects.

	Unit	Control subjects	AD patients	p-value*
Number of subjects		*20*	*20*	
SC thickness	µm	8.7 ± 2.5	9.4 ± 1.9	ns[2]
Baseline TEWL	g m^{-2} h^{-1}	6.3 ± 2.0	8.4 ± 4.3	0.015[1]
Diffusivity	10^{-9} cm^2 h^{-1}	6.2 ± 3.0	12.7 ± 5.8	< 0.0001[1]
SC/water partition coefficient		196 ± 107	137 ± 64	< 0.05[2]
Coefficient of variation (CV)		*n = 14*	*n = 18*	
Diffusivity				
Inter-individual	%	48	55	
Intra-individual	%	29	43	
SC/w partition coefficient				
Inter-individual	%	46	46	
Intra-individual	%	37	27	

*Independent sample T-test : [1](one-sided), [2](two-sided), ns = not significant

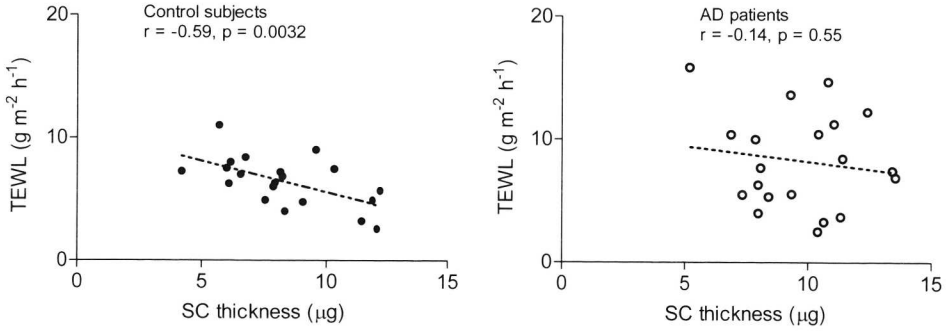


Fig 3: *Correlation between basal TEWL and SC thickness determined in control subjects and AD patients.*

The diffusivity was twice as high in AD patients ($12.7 \pm 5.8 \times 10^{-9}$ cm^2 h^{-1}) when compared to control subjects ($6.1 \pm 3.1 \times 10^{-9}$ cm^2 h^{-1}, $p < 0.001$). We also compared, using one-way ANOVA test, control subjects and AD patients according to state of disease; patients with active and inactive AD, and the mean values of diffusivity in three groups were significantly different ($p < 0.001$) (Figure 4). The mean value of diffusivity in patients with active AD was significantly higher compared to AD patients with inactive AD ($p = 0.017$) and control subjects ($p < 0.001$), but no significant difference was found between patients with inactive AD and control subjects ($p > 0.05$).

The partition coefficient was somewhat lower in AD patients ($p < 0.05$) than in control subjects. The mean values for the partition coefficient were not significantly different when we compared all three groups ($P > 0.05$) using ANOVA. Nevertheless, there was a trend of decreasing partition coefficient with state of disease (Figure 4).

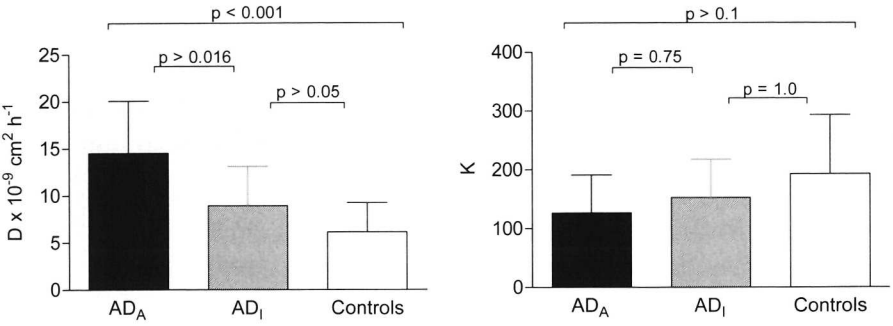


Fig 4: *Diffusivity (D) and SC/water partition coefficient (K) of SLS in patients with active AD (AD_A, n=12), inactive AD (AD_I, n=8), and control subjects (n=20).*

To obtain insight into intra-individual and inter-individual variation in diffusivity and partition coefficient we have used available duplicate data for 18 AD patients and 14 control subjects as shown in Table 1. The inter-individual variation, expressed as the coefficient of variation (CV), in diffusivity amounted to 48% and 55% in control subjects and AD patients, respectively, while CV for partition coefficient amounted to 46% in both groups. Overall, the inter-individual variation was higher than intra-individual variation in both parameters in both groups. The intra-individual variation in diffusivity was higher in AD patients, while for partition coefficient it was lower then in control subjects.

Discussion

In the present study we have assessed the penetration of SLS into the SC of AD patients and control subjects using the non-invasive tape stripping technique. We have shown an increased diffusion in uninvolved AD skin when compared to normal skin.

The thickness of SC as calculated from the amount of proteins removed by tape strips was nearly the same in both groups. The skin of AD patients, however, showed increased TEWL when compared to the skin of normal subjects (Table 1) indicating less effective skin barrier for water. This is in agreement with the results of Laudanska *et al.*[30], who also found higher TEWL in AD patients in the state of remission of the skin lesions and of Seidenari *et al.*[1] reporting higher TEWL in involved and uninvolved skin of children affected by AD. The TEWL in control subjects showed to be inversely dependent on the SC thickness, which is in agreement with other studies.[31,32] However this relationship was not found in AD patients suggesting that in atopic skin other factors besides skin thickness play a role in skin permeability for water. Altered composition and structure of the SC in atopic skin might at least partly be responsible for this. A reduction of Cer 3 was previously found to correlate with an increased TEWL in both involved as well as uninvolved skin.[5] Since epidermal lipids are essential for the proper barrier function and prevention of excessive water loss, the decreased amount of lipids would be responsible for the loss of barrier function and likely also for higher permeability of foreign substances.

The applied method of skin stripping enabled us to estimate two parameters which determined the permeability, diffusivity and partition coefficient of SLS. According to the Fick's law of diffusion, these two parameters determine the skin flux of a penetrant and its concentration in the SC. Both parameters, the diffusivity, which reflects the resistance of SC toward movement of SLS, and the partition coefficient, are dependent on the composition and structure of the SC. We found the average diffusivity across SC to be two times higher in the skin of AD patients when compared to control subjects. We have also looked into the differences in diffusivity of AD patients according to state of disease (Figure 4). The diffusivity was higher in patients with active AD when compared to those with inactive AD and control subjects but there was no significant difference between patients with inactive AD and control subjects. However, there is a clear trend of increasing diffusion of SLS with state of disease. This indicates that state of disease influences the permeability of the skin visibly not affected by AD.

These findings are in the line with a study of de Jongh *et al.*[14], where atopic persons (although with no history of AD) showed approximately 1.5 times higher diffusivity for SLS than non-atopics. In our accompanying study consisting of the same subjects we found higher diffusivity also for polyethylene glycols of different molecular sizes in the skin of AD patients compared to that of control subjects (Jakasa *et al.*, manuscript in preparation). Yosiike *et al.*[2] reported increased penetration of theophylline not only in involved but also in uninvolved AD skin compared to control subjects. All these findings indicate that uninvolved atopic skin is more permeable for different compounds concerning their hydrophilicity and molecular size.

The skin of AD patients showed a 30% lower solubility of SLS compared to normal skin, although the difference was not as high as by diffusion. The partition coefficient was not significantly different when two groups of AD patients and control subjects were compared; but there was a trend of decreasing partition of SLS into the SC with state of disease. Estimation of the partition coefficient, using the method applied in the present study, is associated with higher uncertainty compared to diffusivity as the quality of the first data points largely influences the estimation outcome. To overcome this problem, a second prolonged experiment was recommended[33] where the curve becomes a straight line at steady-state and estimation of partition coefficient is less dependent on the error from the superficial strips. However, in a present study we chose a relatively short exposure duration since longer exposure to SLS would more likely lead to the alteration of the skin barrier which might change the SC permeability.

In the present study we observed substantial inter- and intra-individual variation in both penetration parameters. The intra-individual variation in diffusivity was higher in AD patients compared to control subjects, which may be attributed to the more pronounced difference in composition and structure of the skin in different skin areas. At the same time the intra-individual variability in the partition coefficient was lower in AD patients. As mentioned earlier, the determination of partition coefficient in a non-steady state is largely influenced by the quality of the first data points. As the SLS concentration/SC depth curve (Fig 2) approaches linearity, as is the case with AD patients, the intercept value from which partition coefficient is derived is less dependent on first data points and, therefore, the intra-individual variation decreases.

To summarize, the skin of AD patients showed increased percutaneous penetration of SLS when compared to control subjects supporting the hypothesis of impaired skin barrier even in the non-involved skin. As a consequence, we expect that the defect skin barrier of AD patients will facilitate absorption of other chemicals, which could

lead to the higher susceptibility for local skin effects. This emphasizes the importance of continuous skin protection and maintenance of the skin barrier.

Acknowledgement

We are grateful to K. V. Sewnarain Sukul, C. E. T. Withagen and F. Calkoen-Kwa for technical assistance. The authors would like to thank the European Community for the financial support of this study, which was performed within the Fifth Framework Programme (project acronym: EDETOX).

References:

1. Seidenari S, Giusti G. Objective assessment of the skin of children affected by atopic dermatitis: A study of pH, capacitance and TEWL in Eczematous and clinically uninvolved skin. *Acta Derm Venereol* 1995; **75:** 429-433
2. YosiikeT, Aikawa Y, Sindhvananda J *et al.* Skin barrier defect in atopic dermatitis: increased permeability of the stratum corneum using dimethyl sulfoxide and theophylline. *J Dermatol Sci* 1993; **5:** 92-96
3. Agner T. Susceptibility of atopic dermatitis patients to irritant dermatitis caused by sodium lauryl sulphate. *Acta Derm Venereol* 1990; **71:** 296-300
4. Berardesca E, Fideli D, Borroni G *et al.* In vivo hydratation and water-retention capacity of stratum corneum in clinically uninvolved skin in atopic and psoriatic patients. *Acta Derm Venereol* 1990; **70:** 400-404
5. Di Nardo A, Wertz P, Giannetti A *et al.* Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm Venereol* 1998; **78:** 27-30
6. Tabata N, Tagami H, Kligman AM. A twenty-four-hour occlusive exposure to 1% sodium lauryl sulfate induces a unique histopathologic inflammatory response in the xerotic skin of atopic dermatitis patients. *Acta Derm Venereol* 19985; **78:** 244-247
7. Watanabe M, Tagami H, Horii I *et al.* Functional analyses of the superficial stratum corneum in atopic xerosis. *Arch Dermatol* 1991; **127:** 1689-1692
8. Werner Y, Lindberg M. Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Derm Venereol* 1985; **65:** 102-105
9. Basketter DA, Miettinen, Lahti A. Acute irritant reactivity to sodium lauryl sulfate in atopics and non-atopics. *Contact Dermatitis*; 1998; **38:** 253-257
10. Nicander I, Ollmar S. Clinically normal skin vs. non-atopic skin as seen through electrical impedance. *Skin Res Technol* 2004; **10:** 178-183

11. Seidenari S. Reactivity to nickel sulfate at sodium lauryl sulfate pretreated skin sites is higher in atopics: an echographic evaluation by means of image analysis performed on 20 MHz B-scan recordings. *Acta Derm Venereol* 1994; **74:** 245-249
12. Tanaka M, Zhen YX, Tagami H. Normal recovery of the stratum corneum barrier function following damage induced by tape stripping in patients with atopic dermatitis. *Br J Dermatol* 1997; **136:** 966-967
13. Goffin V, Pierard GE. Corneosurfametry and the compromised atopic stratum corneum. *Arch Dermatol Res* 1996; **288:** 489-491
14. De Jongh CM, Jakasa I, Verberk MM, Kezic S, Variation in barrier impairment and inflammation of human skin as determined by sodium lauryl sulphate penetration rate. *Br J Dermatol* 2005; (in press)
15. Bleck O, Abeck D, Ring J *et al.* Two ceramide subfractions detectable in Cer (AS) position by HPTLC in Skin Surface Lipids of non-lesional skin of atopic eczema. *J Invest Dermatol* 1999; **113:** 894-900
16. Imokawa G, Akihito A, Kumi J *et al.* Decreased level of ceramides in stratum corneum of atopic dermatitis: An etiological factor in atopic dry skin? *J Invest Dermatol* 1991; **96:** 523-526
17. Yamamoto A, Serizawa S, Ito M, Sato Y. Stratum corneum abnormalities in atopic dermatitis. *Arch Dermatol Res* 1991; **283:** 219-223
18. Matsumoto M, Umemoto N, SugiuraH, Uehara M. Difference in Ceramide Composition between "Dry" and "Normal" Skin in Patients with Atopic Dermatitis Acta Derm Venereol 1999; **79:** 246-247
19. Farwanah H, Raith K, Neubert RHH, Wohlrab J. Ceramide profiles of the uninvolved skin in atopic dermatitis and psoriasis are comparable to those of healthy skin. *Arch Dermatol Res* 2005; **296:** 514-521
20. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl* 1980; **92**:44-47
21. Hanifin JM, Thurston M, Omoto M et al. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. *Exp Dermatol* 2001; **10:** 11-18
22. Diepgen TL. Die atopische hautdiathese: Epidemiologie, Klinik und berufsdermatologische bedeutung. Stuttgart: Gentner Verlag, 1991; 1-128
23. Nuutinen J, Alanen E, Autio P *et al.* A closed unventilated chamber for het measurement of transepidermal water loss. Skin Res Technol 2003; **9:** 85-89
24. Rusconi F, Valton E, Nguyen R *et al.* Quantification of sodium dodecyl sulfate in microliter-volume biochemical samples by visible light spectroscopy. *Anal Bichem* 2001; **295:** 493-499
25. Dreher F, Arens A, Hostynek JJ *et al.* Colorimetric method for quantifying human stratum corneum removed by adhesive-tape stripping. *Acta Derm Venereol* 1998; **78**:186-189
26. Bio-Rad Laboratories, DC Protein Assay: Microassay Procedure
27. Anderson RL, Cassidy JM. Variation in physical dimensions and chemical composition of human stratum corneum. *J Invest Dermatol* 1973; **61**: 30-32
28. Crank J. Mathematics of diffusion. Oxford, Oxford Univ. Press, 1975; 50-51
29. Potts RO, Guy RH. Predicting skin permeability. *Pharm Res* 1992; **9:** 663-669

30. Laudanska H, Reduta T, Szmitkowska D. Evaluation of skin barrier function in allergic contact dermatitis and atopic dermatitis using method of the continuous TEWL measurement. 2003; **48:** 123-127
31. Ya-Xian Z, Suetake T, Tagami H. Number of cell layers of the stratum corneum in normal skin – relationship to the anatomical location on the body, age, sex and physical parameters. *Arch Dermatol Res* 1999; **291:** 555-559
32. Schwindt DA, Wilhelm KP, Maibach HI. Water diffusion characteristics of human stratum corneum at different anatomical sites in vivo. *J Invest Dermatol* 1998; **111**: 385-389
33. Reddy MB, Stinchcomb AL, Guy RH *et al.* Determining dermal absorption parameters in vivo from tape stripping data. *Pharm Res* 2002; **19:** 292-298

Chapter 4: *Section 4.2*

Altered penetration of polyethylene glycols into uninvolved skin of atopic dermatitis patients

I. Jakasa, M.M. Verberk, M. Esposito, J.D. Bos, S. Kezic

(submitted to *J Invest Dermatol*)

Abstract

Involved regions of the skin in atopic dermatitis patients have an altered barrier function. Whether uninvolved skin also has a diminished barrier is controversial.
To asses the barrier function of uninvolved skin in atopic dermatitis patients, the percutaneous penetration of polyethylene glycols of various molecular sizes was determined in atopic dermatitis patients and control subjects.
The percutaneous penetration was assessed using tape stripping of the stratum corneum. The diffusion coefficient and stratum corneum/vehicle partition coefficient were determined using Fick's second law of diffusion.
The stratum corneum thickness was similar in both groups, however, the trans-epidermal water loss was higher in atopic skin. The diffusion coefficient of polyethylene glycols through atopic skin was twice as high as through normal skin, and decreased with increasing molecular weight in both groups. The partition coefficient in the skin of atopic dermatitis patients was half of that for normal skin but as for normal skin there was no molecular weight dependency. Although atopic skin exhibited altered barrier with respect to diffusion coefficient and partitioning, the permeability coefficient, were nearly the same for atopic and normal skin.
The results support the hypothesis of altered skin barrier of AD patients even in the skin that is visibly unaffected by disease.

Introduction

The affected skin of patients with atopic dermatitis (AD) is known to have a defective barrier function. The alteration in the skin barrier is shown by the increase in trans-epidermal water loss (TEWL), in other words a poorer barrier to water transport (Seidenari and Giusti, 1995). On the other hand, the data on the permeability of uninvolved AD skin is contradictory. Some authors have reported higher TEWL in patients with AD history (Agner, 1990, Berardesca *et al*, 1990, Di Nardo *et al*, 1998, Seidenari and Giusti, 1995, Tabata *et al*, 1998, Watanabe *et al*, 1991, Werner and Lindberg, 1985), while others found no difference in comparison to normal skin (Basketter *et al*, 1998, Di Nardo *et al*, 1998, Nicander and Ollmar, 2004, Seidenari, 1994, Tanaka *et al*, 1997). From the results of several studies showing higher susceptibility to irritation in AD patients (Goffin and Pierard, 1996, Nicander and Ollmar, 2004, Seidenari, 1994, Tabata *et al*, 1998) it was suggested that this might be partly explained by higher skin permeability (de Jongh *et al*, in press). Impaired skin barrier in AD has often been linked to the different lipid composition and structure of atopic stratum corneum (SC). In this respect, reduced ceramide content and decreased percentages of Cer 1 and Cer 3 have been reported in both involved as well as in uninvolved SC of AD patients (Bleck *et al*, 1999, Di Nardo *et al*, 1998, Imokava *et al*, 1991, Yamamoto *et al*, 1991). In contrast to this, Matsumoto (Matsumoto *et al*, 1999) found that the reduction in Cer 1 is restricted to the involved AD skin and does not extend to uninvolved areas: this is consistent with Farwanah's data (Farwanah *et al*, 2005).

There are rather few data on the permeability of the compromised skin for chemicals. Yosiike (Yosiike *et al*, 1993) reported increased penetration of theophylline in both involved and uninvolved AD skin. In our parallel study, using the same subjects, the percutaneous penetration of sodium lauryl sulphate increased in uninvolved skin of AD patients (Jakasa *et al*, in press). Tsai *et al*. (Tsai *et al*, 2001a) investigated the effects of chemical and mechanical barrier disruption on skin permeability in an *in vitro* study using rat skin. They found enhanced skin permeability to both hydrophilic and amphipathic compounds in damaged skin. In other two studies by Tsai (Tsai *et al*, 2001b and 2003) not only was the penetration of polyethylene glycols (PEGs) of different sizes enhanced but larger molecules were also able to penetrate the skin when the barrier was compromised. Based on clinical experience, Bos and Meinardi proposed the 500 Da rule which states that the absorption of molecules through normal human skin declines rapidly as MW increases over 500 Da (Bos and Meinardi, 2000). On the other hand, topical tacrolimus (822 Da) and pimecrolimus (810 Da) showed to be effective in the treatment of AD (Bos, 2003) suggesting that

larger molecules can penetrate the diseased skin; interestingly, the absorption of tacrolimus declined as the skin healed (Rubins *et al*, 2005). As regards the role of molecular weight (MW) in penetration through compromised skin *in vivo* direct, evidence is lacking.

The present study investigated the skin penetration of PEGs of different MWs (150 – 590 Da) into uninvolved skin of AD patients compared to control subjects. PEG is a hydrophilic polymer widely used in corneal and intestinal permeability research. The octanol/water partition coefficient ($\log K_{o/w}$ ~ -1.6) does not change greatly with molecular size which makes PEGs, suitable model compounds that are not confounded by change in lipophilicity with molecular size (Hollander *et al*, 1989).

Subjects and methods

Study population

Twenty AD patients, 12 males and 8 females, with a mean age of 29 years (range 18-54 years) and 20 healthy subjects, 11 males and 9 females, with a mean age of 32 years (range 18-55 years), all Caucasians, participated in the study which was carried out in June and July 2004.

The AD patients were recruited from the outpatients clinic of the Academic Medical Center and diagnosed according to the Hanifin & Rajka criteria (Hanifin and Rajka, 1980). We excluded patients who had received systemic therapy, such as corticosteroids and immunosuppressants, or photo-therapy in the past two years. Subjects with concomitant ichthyosis vulgaris were also excluded. The test sites, both mid-volar arms had been free of dermatitis for at least three months prior to the experiment. The total eczema area and severity index (EASI, maximum 72 points) was assessed in the AD patients (Hanifin *et al*, 2001). The severity of the disease was mild in all the patients and the median EASI was 1.7 (ranging from 0.2 to 22.8). Twelve patients had active AD (pruritic lesions) and eight had inactive AD (they had been free of dermatitis for at least three months, showing only mild signs at the time - scars, scaling, lichenification or dry skin) on body parts other than the test sites.

The control subjects had no visible skin damage and no history of past or present AD or other dermatological diseases.

All the subjects completed the Erlangen questionnaire, from which an atopy score was calculated (maximum 34 points; a score ≥ 10 is considered as atopy) (Diepgen,

1991). AD patients and control subjects had a score of 17.4 ± 6.6 and 3.0 ± 2.4 (mean ± SD), respectively.

The participants were not allowed to use soap, moisturizers or any other cosmetics or creams on the lower mid-volar arms for 48 hours prior to and during the experiments. Written informed consent was obtained from all the subjects prior to the experiment. The Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, approved the experimental protocol. The study was conducted according to the Declaration of Helsinki Principles.

Penetration experiment

An application mixture of PEGs was made by dissolving 47.5 mg of monodispersed PEG150 (MW = 150.17 Da, Sigma, the Netherlands), 50.1 mg of monodispersed PEG282 (MW = 282.34 Da, Acros Organics, NY, USA), 102.9 mg of monodispersed PEG326 (MW = 326.4 Da, PolyPure, Norway), 199.1 mg of monodispersed PEG370 (MW = 370.4 Da, PolyPure, Norway), and 10 g of polydispersed PEG600 (average MW = 600 Da, Sigma, the Netherlands) in 2 ml of water. Subjects were exposed for six hours to the PEG application mixture (180 µL) on the mid-volar arms using patch test chambers (Finn Chambers®, 18 mm in diameter, Epitest Ltd., Finland). These prevented evaporation of water from the test site and this combined with excess PEG insured that the exposure concentration remained constant during the exposure. The TEWL was measured on application sites, before application and after patch removal, using an evaporimeter (VapoMeter SWL2g, Delfin Technologies, Ltd., Kuopio, Finland). Twenty minutes prior the application, the subjects rested with their sleeves rolled up in the examination room, where the temperature was 20-22 °C and the relative humidity ranged between 50 and 60%. Ten minutes after the end of exposure the SC layers were sequentially removed with pre-cut pieces of Diamond tape, 19 x 25 mm (Diamond Ultra Clear tape, The Sellotape® Company, the Netherlands). Templates of Scanpor® tape were fixed to the skin around the application spot to limit the tape stripping to exposed area (18 mm in diameter). The tape pieces consecutively applied to the test site and uniformly pressed with a 1 kg stainless steel roller which was moved 10 times in two directions. The total removal of the SC was evidenced by the shiny appearance of the skin and a TEWL > 100 g m^{-2} h^{-1}. Each individual strip was placed into a glass vial and stored at -20 °C until analysis. The SC from a non-exposed site was stripped off and served as negative control.

Analytical procedure

The gas chromatographic method for the determination of PEGs and the spectrophotometrical method for the analysing of proteins in tape strips have been described in detail elsewhere (Jakasa *et al*, 2004, Jakasa *et al*, in press).

The concentration of PEGs on each strip was normalized for the amount of proteins and expressed as μg of PEG/μg of protein. Assuming an SC density of 1 g cm^{-3} (Andersen and Cassidy, 1973) and uniform distributions of SC on the tapes and proteins within the SC (μg), the protein mass removed was converted to a volume, enabling the depth of each strip in the SC (x). In our calculation of the SC solute concentration it was assumed that the protein concentration in the SC was 1mg / μL SC.

Data analysis

To estimate the penetration parameters we used an approach based on Fick's second law of diffusion (Crank, 1975) as described by Pirot *et al*. (Pirot *et al*, 1997). In this method, two parameters, K and D/L^2, are determined by best-fit regression of the concentration pf PEGs as a function of relative SC depth (x/L) to the following equation

$$C(x) = KC_{veh}\left(1-\frac{x}{L}\right) - \sum_{n=1}^{\infty}\frac{2}{n\pi} KC_{veh}\sin\left(\frac{n\pi x}{L}\right)\exp\left(\frac{-Dn^2\pi^2 t}{L^2}\right) \qquad \textit{Eq. 1}$$

where C_{veh} is the applied PEGs concentration (μg cm^{-3}), C is the PEG concentration (μg cm^{-3}) at depth x (cm), K is the SC/water partition coefficient, L is the total thickness of the SC (cm), D is the effective diffusion coefficient of PEGs through the pseudo-homogeneous SC (cm^2 h^{-1}) and t is the exposure duration (h). The permeability coefficient (K_p, cm h^{-1}) for each PEG oligomer was calculated from the relationship $K_p = K*D/L$. The steady-state flux through the SC should equal the product of C_{veh} and the permeability coefficient (K_p, cm h^{-1}) and is defined as $K_p = K*D/L$. The first strip was not included in the regression analysis, as it contained some PEG residues on the surface of the skin after the end of exposure. All concentration data were weighted equally in the regression analysis. Prism 4 (Graph Pad Software Inc., San Diego, CA, USA) and SPSS software were used (SPSS Inc., Chicago, IL, USA) for curve fitting and statistical calculations. Student's t-test and one-way ANOVA with Bonferroni post-test were used for statistical calculations and p value < 0.05 was considered significant.

Results

To remove the SC completely, 28 ± 5 strips were needed on average for control subjects and 28 ± 7 for AD patients. The TEWL was higher in the AD patients (8.4 ± 4.3 g m^2 h^{-1}) than the control subjects (6.3 ± 2.1 g m^2 h^{-1}, p = 0.015). We found no substantial difference in SC thickness between the two groups (8.7 ± 2.5 µm and 9.4 ± 1.9 µm for control subjects and AD patients, respectively).

Fig 1 shows a typical concentration profile for PEG282 and PEG590 across SC in a control subject and an AD patient together with the fitted curve obtained by non-linear regression analysis (dashed lines). Statistically acceptable curve fitting ($r^2 \geq 0.95$) was obtained for all the control subjects and AD patients except in the case of PEG150. In four AD patients out of twenty a linear relationship was found between the PEG150 concentration and SC depth, indicating steady-state absorption, to which Fick's second law cannot be used to derive diffusion coefficient. In two control subjects out of twenty fitting could not be performed for PEG150 because of scattered data points.

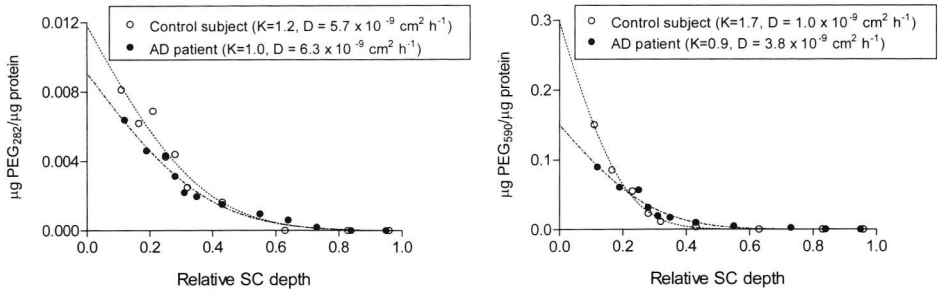


Fig 1: *Concentration decay of PEG282 and PEG590 as a function of normalized position (x/L) in the SC in one control subject and one AD patients after 6 hours exposure to PEG mixture. Non-linear regression analysis was used to fit the equation (Eq. 1) to the experimentally obtained data (dashed lines). The effective diffusion coefficient is calculated from D/L^2 value determined from the slope of the curve, while the partition coefficient is determined from K^*C_{veh} at the intercept at x = 0.*

The results are summarized in Table I. The diffusion coefficient decreases with increasing MW of the penetrant. It was approximately twice as high for all PEGs in the AD patients as in the control subjects, except for PEG150, where it was only 60 % higher in the AD patients ($p < 0.0001$ for all PEGs). We also compared the control subjects and the AD patients according to state of disease using one-way ANOVA test. The patients were divided into two subgroups, patients with active and inactive AD. The mean values of diffusion coefficient in three groups were significantly different ($p < 0.05$ for all PEGs). The mean value of diffusion coefficient in patients with active AD was significantly higher than in the control subjects for all PEG molecules ($p < 0.05$ for all PEGs). No significant difference was found between patients with inactive AD and the control subjects, or between AD patients with active and inactive AD (Fig 2).

Table I: Penetration parameters of polyethylene glycols (*mean* ± *SD*) for AD patients and control subjects.

MW Da	D x 10^{-9} (cm^2 h^{-1})		K (unitless)		K_p x 10^{-5} (cm h^{-1})	
	AD	Ctrl	AD	Ctrl	AD	Ctrl
150	13.3 ± 6.7	8.4 ± 5.2	1.02 ± 0.29	1.66 ± 0.70	1.34 ± 0.42	1.25 ± 0.39
282	9.2 ± 5.1	4.5 ± 2.9	0.93 ± 0.38	1.76 ± 1.00	0.84 ± 0.55	0.68 ± 0.24
326	8.2 ± 4.2	4.5 ± 2.8	0.86 ± 0.35	1.72 ± 0.99	0.69 ± 0.30	0.66 ± 0.25
370	7.6 ± 3.8	4.1 ± 2.7	0.86 ± 0.37	1.74 ± 0.96	0.65 ± 0.40	0.60 ± 0.22
414	6.7 ± 3.6	3.5 ± 2.2	0.85 ± 0.37	1.78 ± 0.94	0.54 ± 0.19	0.55 ± 0.21
458	6.2 ± 3.2	3.2 ± 1.9	0.82 ± 0.38	1.78 ± 0.92	0.48 ± 0.22	0.51 ± 0.19
502	6.1 ± 3.0	2.9 ± 1.7	0.81 ± 0.38	1.78 ± 0.85	0.49 ± 0.27	0.48 ± 0.19
546	5.0 ± 3.0	2.4 ± 1.4	0.80 ± 0.43	1.87 ± 0.78	0.37 ± 0.23	0.44 ± 0.19
590	3.8 ± 2.8	1.9 ± 1.1	0.96 ± 0.53	1.85 ± 0.82	0.34 ± 0.27	0.35 ± 0.19

D = diffusion coefficient, K = stratum corneum/water partition coefficient, K_p = permeability coefficient , AD = AD patients (*n=20*), Ctrl = control subjects (*n=20*)

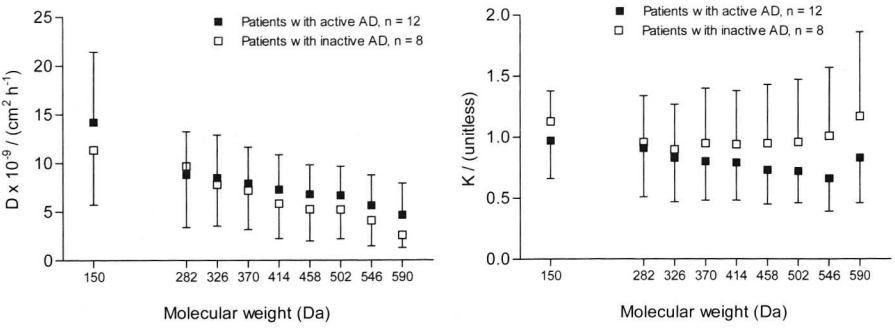


Fig 2: *Average diffusion coefficient (D) and partition coefficient (K) of PEGs (150-590 Da) in patients with active and inactive AD after 6 hours dermal exposure. The results are shown as mean ± SD.*

The partition coefficient for AD patients was approximately half as large as in the control subjects as for all PEG molecules, except for PEG150, where it was about 60 % of that in the control subjects ($p < 0.0001$ for all PEGs). We compared all three groups of subjects using ANOVA; again a significant difference was found for all PEGs ($p < 0.01$ for all PEGs). The partition coefficient was also significantly lower in patients with active and inactive AD than in the control subjects for all PEGs ($p < 0.05$). There was no significant difference between patients with active and inactive AD, however, the partition coefficient did tend to decrease with state of disease for all PEGs (Fig 2).

Calculated permeability coefficients were nearly the same for both the AD patients and control subjects (Table I).

The inter-individual differences in diffusion coefficient, partition coefficient and permeability coefficient were considerable in both the AD patients and the control subjects. The coefficient of variation in AD patients was 49 % to 73 % for the diffusion coefficient and 29 % to 55 % for the partition coefficient and 32 % to 80 %for permeability coefficient. The coefficient of variation in control subjects was 58 % to 66 % for the diffusion coefficient and 42 % to 58 % for the partition coefficient and 31 % to 56 % for permeability coefficient.

Discussion

The present study assessed the penetration of PEGs ranging in MW from 150 to 590 Da into the SC of AD patients and control subjects. The skin stripping method used enabled us to estimate two penetration parameters, the effective diffusion coefficient in the SC and the partition coefficient between SC and vehicle from which the permeability coefficient could be calculated. According to Fick's law of diffusion, when the SC control mass transfer through the skin, the skin flux of a penetrant is the product of permeability coefficient and the concentration in the SC.

The average diffusion coefficient across SC was about twice as high in AD patients as in the control subjects for all PEG oligomers. The diffusion coefficient also tended to increase with state of disease (Fig 2). These findings are in line with our parallel study using the same subjects, where we found significantly higher diffusion coefficient of sodium lauryl sulphate in uninvolved skin of AD patients (Jakasa *et al*, in press). The increase in the diffusion coefficient of PEGs was more pronounced in the case of larger PEG oligomers in patients with active AD, which might support suggestion that substantially impaired skin is more prone to high MW compounds entering [Bos, 2003, Rubins *et al*, 2005]. The higher diffusion of PEG we found in AD skin is consistent with the study by Tsai (Tsai *et al*, 2001b, 2003), who investigated the penetration of PEGs *in vitro* in hairless mice. That study assessed the penetration of polydispersed PEG (PEG300, PEG600 and PEG1000) through normal skin and skin damaged by acetone, sodium lauryl sulphate or tape stripping. The penetration of PEGs, expressed as a percentage of the applied dose, increased with the degree of barrier disruption as measured by TEWL in all three disruption models. Shifting of the MW cut-off value for PEG in the damaged skin was also reported. A cut-off value of 414 Da was found in normal skin, but in the skin damaged by acetone, sodium lauryl sulphate or tape stripping, MW cut-off values of 590, 766 and 986 Da respectively were found for the same range of TEWL. However, the amount of larger PEGs that penetrated the skin and reached the receptor fluid was very low and the determined cut-off might reflect the detection limit rather than the real cut-off value [Tsai *et al*, 2001b, 2003]. This is supported by the finding that the cut-off in that study also shifted to a higher MW after partial tape stripping; this effect was even more pronounced than after skin was damaged by sodium lauryl sulphate or acetone. Tape stripping removes part of the SC which is the rate-limiting barrier for penetration, however, it does not change the composition or structure of the remaining SC, only the thickness of the membrane. A higher penetration can therefore be expected after tape stripping, but the increase will be relatively the same for all MWs.

The diffusion coefficient of a solute decreases as the solute size increases. With respect to diffusion coefficient in the stratum corneum, different authors have proposed different functional forms to describe the effect of solute size (Potts and Guy, 1992, Kasting *et al*, 1987). In these functions, the diffusion coefficient decreases exponentially with increasing molecular size and predicted a stronger effect of molecular size than observed in this study. Our results showed a gradual decrease of diffusion coefficient of PEGs with increasing MW; in the range of MW from 280 to 590, this relation seemed to be linear (Fig 3).

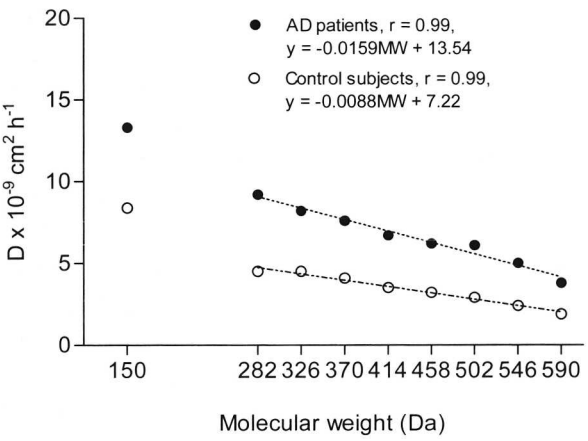


Fig 3: *A linear regression analysis for the average diffusion coefficients (D) versus corresponding molecular weights of PEG 282-590 Da of AD patients and control subjects after 6 hours dermal exposure to PEG mixture.*

This is in good agreement with experimental *in vitro* data on skin permeability of hydrophilic compounds presented in the paper of Mitragotri (Mitragotri, 2003, Billich *et al*, 2005). Recent studies revealed that the impact of molecular size on the skin permeability depends on the penetrant hydrophilicity (Mitragotri, 2003). Billich(Billich *et al*, 2005) showed that *in vitro* penetration of cyclosporins of MW > 1000 could be substantially increased by introduction of polar side chains. All together, these studies support the existence of different penetration pathways in the stratum corneum, dependently on the physico-chemical properties of the solute. Lipophilic solutes which penetrate predominantly through lipid bilayers exhibit strong size-selectivity: when they approach the MW of the lipid molecules (~ 400 Da),

the diffusion coefficient decreases rapidly. The skin permeation of lipophilic solutes, which represents a large majority of active agents for therapeutic application, would thus be in line with the 500 Da rule. There is increasing evidence however, that the penetration of hydrophilic compounds such as PEGs, occurs via hydrophilic pores in the SC, referred to as porous pathways (Mitragotri, 2003). The existence of such pores in the SC has been hypothesized to be a result of the imperfections in the lipid bilayers. Structure defects are commonly observed in lipid lamellar systems (Cotigan *et al*, 2000). The permeation rate will depend on the radius of these pores and the radius of the penetrant, while the radius and number of these pores will be determined on the structure and composition of the SC. Higher diffusion coefficient of PEGs in AD skin could be therefore explained by the different lipid composition observed in AD patients in both involved and uninvolved skin (Bleck *et al*, 1999, Di Nardo *et al*, 1998, Imokava *et al*, 1991, Yamamoto *et al*, 1991), which might result in the presence of more and/or larger pores. Not only the diffusion coefficient but also the partitioning of PEGs into AD skin was different from that in the control skin: the partition coefficient was twice as high in the control subjects as in the AD patients. Although there was no significant difference between patients with active and inactive AD, the tendency for partition to decrease with state of disease was present for all PEG oligomers. Decreased partitioning of PEGs into diseased skin might be explained by the dryness of the AD skin: a smaller amount of water will decrease the partitioning of hydrophilic PEGs into the SC. In contrast to the diffusion coefficient, the partition coefficient was similar for all PEG oligomers. This was consistent with a similar octanol-water partition coefficient over a broad range of MWs (Hollander *et al*, 1988).

Although the diffusion coefficient was significantly higher in AD patients, the permeability coefficient was nearly the same in both groups due to a compensating decrease in partition coefficient. However, for less hydrophilic compounds, which would more favorabpartition into the SC of atopic patients; the influence of diffusion coefficient would result in an increased permeability coefficient. The permeability coefficient, we calculate from the experimentally determined diffusion and a partition coefficient was for the PEGs of MW > 500 Da lower than 5×10^{-6} cm h^{-1}.

The compounds with such a low permeability will probably have a low relevance for pharmaceutical purposes. However, from a toxicological point of view, one has to keep in mind that also larger molecules are able to penetrate the skin, and as shown in this study this will be even more pronounced when the skin is impaired. Since skin damaged mechanically, chemically or physiologically is not uncommon, when

evaluating the health risk associated with skin exposure, penetration of higher MW compounds should be considered.

To summarize, the skin of AD patients showed increased diffusion coefficients for all PEGs when compared to control subjects, and the effect seems to be more pronounced in the case of larger oligomers in patients with active AD. This supports the hypothesis that skin barrier function is altered even in skin that is visibly unaffected and that diffusion coefficient depends on the overall severity of disease. As a consequence, we would expect the defective skin barrier of AD patients to facilitate the absorption of other chemicals, which might result in higher susceptibility and local skin effects.

Acknowledgements

We are grateful to prof. A. L. Bunge and dr. J. Kruse for valuable comments on the manuscript. We are also grateful to N. Mohammadi for the help during development of tape stripping method, and C. E. T. Withagen and F. Calkoen-Kwa for technical assistance. The authors would like to thank the European Community for providing financial support for part of this the study, which was carried out under the Fifth Framework Programme (project acronym: EDETOX).

References:

- Agner T(1990). Susceptibility of atopic dermatitis patients to irritant dermatitis caused by sodium lauryl sulphate. *Acta Derm Venereol* **71**: 296-300.
- Anderson RL, Cassidy JM(1973). Variation in physical dimensions and chemical composition of human stratum corneum. *J Invest Dermatol* **61**: 30-32.
- Basketter DA, Miettinen, Lahti A (1998). Acute irritant reactivity to sodium lauryl sulfate in atopics and non-atopics. *Contact Dermatitis* **38**: 253-257.
- Berardesca E, Fideli D, Borroni G, Rabbiosi G, Maibach H (1990). *In vivo* hydratation and water-retention capacity of stratum corneum in clinically uninvolved skin in atopic and psoriatic patients. *Acta Derm Venereol* **70**: 400-404.
- Billich A, Vyplel H, Grassberger M, Schmook FP, Steck A, Stuetz A (2005).Novel cyclosporine derivatives featuring enhanced skin penetration despite increased molecular weight. *Bioorg Med Chem* **13**: 3157-3167.
- Bleck O, Abeck D, Ring J, Hoppe U, Vietzke JP, Wolber R, Brandt O, Schreiner V (1999). Two ceramide subfractions detectable in Cer (AS) position by HPTLC in Skin Surface Lipids of non-lesional skin of atopic eczema. *J Invest Dermatol* **113**: 894-900.

- Bos JD (2003). Non-steroidal topical immunomodulators provide skin-selective, self-limiting treatment in atopic dermatitis. *Eur J Dermatol* **13**: 455-461.
- Bos JD, Meinardi MMHM (2000). The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol* **9**: 165-169.
- Cotigan SC, Booth PJ, Templer RH (2000). Estimations of lipid bilayer geometry in fluid lamellar phases. *Biochim Biophys Acta* **1468**: 41-54.
- Crank J (1975) *Mathematics of diffusion*. Oxford Univ. Press: Oxford, p: 50-51.
- De Jongh CM, Jakasa I, Verberk MM, Kezic S: Variation in barrier impairment and inflammation of human skin as determined by sodium lauryl sulphate penetration rate. *Br J Dermatol* advance online publication 27/07/2005
- Diepgen TL (1991). *Die atopische hautdiathese: Epidemiologie, Klinik und berufsdermatologische bedeutung*. Gentner Verlag: Stuttgart, p: 1-128.
- Di Nardo A, Wertz P, Giannetti A, Seidenari S (1998). Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm Venereol* **78**: 27-30.
- Farwanah H, Raith K, Neubert RHH, Wohlrab J (2005). Ceramide profiles of the uninvolved skin in atopic dermatitis and psoriasis are comparable to those of healthy skin. *Arch Dermatol Res* **296**: 514-521.
- Goffin V, Pierard GE (1996). Corneosurfametry and the compromised atopic stratum corneum. *Arch Dermatol Res* **288**: 489-491.
- Hanifin JM, Rajka G (1980). Diagnostic features of atopic dermatitis. *Acta Derm Venereol* **92** (Suppl): 44-47.
- Hanifin JM, Thurston M, Omoto M, Cherill M, Tofte SJ, Graeber M, the EASI evaluator group (2001). The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. *Exp Dermatol* **10**: 11-18.
- Hollander D, Koyama S, Dadufalza V, Tran DQ, Krugliak P, Ma T, Ling KY (1989). Polyethylene glycol 900 permeability of rat intestinal and colonic segments *in vivo* and brush border membrane vesicles *in vitro*. *J Lab Clin Med* **113**: 505-515.
- Imokawa G, Abe A, Jin K, Higaki Y, Kawashima M, Hidano A (1991). Decreased level of ceramides in stratum corneum of atopic dermatitis: An etiological factor in atopic dry skin? *J Invest Dermatol* **96**: 523-526.
- Jakasa I, Calkoen F, Kezic S (2004). Determination of polyethylene glycols of different molecular weight in the stratum corneum. *J Chromatogr B* **811**(2): 177-182.
- Jakasa I, de Jongh CM, Verberk MM, Bos JD, Kezic S. Percutaneous penetration of sodium lauryl sulphate is increased in uninvolved skin of atopic dermatitis patients compared to control subjects. *Br J Dermatol* (in press)
- Kasting GB, Smith RL, Cooper ER (1987). Effect of lipid solubility and molecular size on percutaneous absorption. In: Skin Pharmacokinetics (Shroot B, Schaefer H, eds) Karger: Basel 138-153.
- Matsumoto M, Umemoto N, Sugiura H, Uehara M (1999). Difference in Ceramide Composition between "Dry" and "Normal" Skin in Patients with Atopic Dermatitis *Acta Derm Venereol* **79**: 246-24.
- Mitragotri S: Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. *J Contrl* Rel **86**: 69-92. 2003

- Nicander I, Ollmar S (2004). Clinically normal skin vs. non-atopic skin as seen through electrical impedance. *Skin Res Technol* **10**: 178-183.
- Pirot F, Kalia YN, Stinchcomb AL, Keating G, Bunge A, Guy RH (1997). Characterization of the permeability barrier of human skin in vivo. *Proc Nat Acad Sci USA* **94**: 1562-1567.
- Potts RO, Guy RH (1992). Predicting skin permeability. *Pharm Res* **9**: 663-669.
- Rubins A, Gutmane R, Valdmane N, Stevenson P, Foster C, Undre N (2005). Pharmacokinetics of 0.1% tacrolimus ointment after first and repeated application to adults with moderate and severe atopic dermatitis. *J Invest Dermatol* **125**: 68-71.
- Seidenari S (1994). Reactivity to nickel sulfate at sodium lauryl sulfate pretreated skin sites is higher in atopics: an echographic evaluation by means of image analysis performed on 20 MHz B-scan recordings. *Acta Derm Venereol* **74**: 245-249.
- Seidenari S, Giusti G (1995). Objective assessment of the skin of children affected by atopic dermatitis: A study of pH, capacitance and TEWL in Eczematous and clinically uninvolved skin. *Acta Derm Venereol* **75**: 429-433.
- Tabata N, Tagami H, Kligman AM (1998). A twenty-four-hour occlusive exposure to 1% sodium lauryl sulfate induces a unique histopathologic inflammatory response in the xerotic skin of atopic dermatitis patients. *Acta Derm Venereol* **78**: 244-247.
- Tanaka M, Zhen YX, Tagami H (1997). Normal recovery of the stratum corneum barrier function following damage induced by tape stripping in patients with atopic dermatitis. *Br J Dermatol* **136**: 966-967.
- Tsai JC, Sheu HM, Hung PL, Cheng CL (2001a). Effect of barrier disruption by acetone treatment on the permeability of compounds with various lipophilicities: Implications for the permeability of compromised skin. *J Pharm Sci* **90** (9): 1242-1254.
- Tsai JC, Hung PL, Sheu HM (2001b). Molecular weight dependence of polyethylene glycol penetration across acetone-disrupted permeability barrier. *Arch Dermatol Res* **293**: 302-307.
- Tsai JC, Shen LC, Sheu HM, Lu CC (2003). Tape stripping and sodium dodecyl sulfate treatment increase the molecular weight cutoff of the polyethylene glycol penetration across murine skin. *Arch Dermatol Res* **295**: 169-174.
- Watanabe M, Tagami H, Horii I, Takahashi M, Kligman AM (1991). Functional analyses of the superficial stratum corneum in atopic xerosis. *Arch Dermatol* **127**: 1689-1692.
- Werner Y, Lindberg M (1985). Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Derm Venereol* **65**: 102-105.
- Yamamoto A, Serizawa S, Ito M, Sato Y (1991). Stratum corneum abnormalities in atopic dermatitis. *Arch Dermatol Res* **283**: 219-223.
- Yoshiike T, Aikawa Y, Sindhvananda J, Suto H, Nishimura K, Kawamoto T, Ogawa H (1993). Skin barrier defect in atopic dermatitis: increased permeability of the stratum corneum using dimethyl sulfoxide and theophylline. *J Dermatol Sci* **5**: 92-96.

Chapter 4: *Section 4.3*

Increased permeability for polyethylene glycols through skin compromised by sodium lauryl sulphate

I. Jakasa, M.M. Verberk, A.L. Bunge, J. Kruse, S. Kezic

(submitted to *Exp Dermatol*)

Abstract

In this in vivo human study we assessed the influence of skin damage by sodium lauryl sulphate (SLS) on percutaneous penetration of polyethylene glycols (PEGs) of different molecular weights (MW).

Percutaneous penetration of PEGs of various molecular sizes was determined using tape stripping of the stratum corneum (SC). The forearm skin of volunteers was pre-treated with 5 % w/w SLS for 4 hours, and 24 hours later patches with PEGs were applied for 6 hours. After the end of application, the layers of the SC were removed consecutively using adhesive tape. The diffusion coefficient, stratum corneum/vehicle partition coefficient and the permeability coefficient were deduced by data regression to Fick's law written for unsteady-state diffusion across membrane from a constant source to a sink.

The trans-epidermal water loss (TEWL) was increased as a result of SLS treatment from 6.3 ± 2.1 g m^2 h^{-1} (mean ± SD) to 17.9 ± 8.7 g m^2 h^{-1}. The diffusion coefficient for all PEGs was increased in the SLS damaged skin. The magnitude of the increase was smaller for higher MW. Also the partition coefficient of PEGs between SC and water was larger in the SLS compromised skin when compared to the normal skin and showed a tendency to increase with MW. The permeability coefficient decreased gradually with increasing MW of PEGs in both normal and SLS-compromised skin. SLS caused a three-fold increase of the permeability coefficient for all MWs ranging in normal skin from 0.34 to 0.70 x 10^{-5} cm h^{-1} and in the SLS compromised skin from 1.20 to 2.09 x 10^{-5} cm h^{-1} for MW of 590 to 282 Da.

Results of this study show the deleterious effect of SLS on the skin barrier for hydrophilic PEGs. A defective skin barrier will facilitate absorption of other chemicals that could lead to higher susceptibility and local skin effects.

Introduction

Most of the available skin permeability data are from normal intact human and animal skin. Data on permeability of chemicals in the compromised skin, in particular in humans in vivo, are scarce. Experimentally induced damage of the skin barrier has been used to study the influence of common disrupting chemicals (e.g. detergents) and also to model diseased skin characterized by defective skin barrier. As a result, various models based on chemical or mechanical barrier disruption have been developed. Tsai *et al.* investigated in an in vitro study using rat skin the influence of chemical barrier disruption on skin permeability [1-3]. In that study, it was shown that skin barrier disruption by acetone significantly enhanced permeability to both hydroplilic and amphiphilic compounds [1]. The influence of the pre-treatment of the skin with sodium lauryl sulphate (SLS) has shown to increase permeability of hydrophilic to moderately lipophilic compounds while influence of SLS was absent for highly lipophilic compounds [4-6].

Many surfactants, from pharmaceutical preparations to food products, are widely used as emulsifying, suspending, solubilizing and stabilizing agents. Anionic surfactants can penetrate and interact strongly with skin producing alterations in the barrier properties [7]. SLS in particular is able to cause variations in structural organization of lipids in the stratum corneum (SC) which are thought to be the most important pathway for chemical penetration and diffusion through SC [8-10].

In the present study we investigated the influence of SLS on the SC penetration of PEGs of different MWs (282 – 590 Da) in the human skin in vivo. PEG is a hydrophilic polymer widely used in corneal and intestinal permeability research. The octanol/water partition coefficient ($K_{o/w}$) does not change considerably with molecular size, which makes PEGs suitable model compounds that are not confounded by changes in lipophilicity with molecular size [11]. In the study of Tsai *et al.* using in vitro rat skin, the penetration of polyethylene glycols (PEGs) of different sizes was shown to be enhanced by SLS [2-3]. Furthermore, in our parallel study we have found increased diffusion of PEGs in the skin of atopic dermatitis (AD) patients who are known for their defective barrier function [12). Regarding the effect/role of molecular weight in penetration through skin compromised by SLS in vivo direct information/evidence is lacking.

Subjects and methods

Study population

Twenty healthy subjects, 11 males and 9 females, mean age 32 years (range 18-55 years), all Caucasians, participated in the study. Participants had no visible skin damage and no history of past or present AD and other dermatological diseases.
All subjects completed the Erlangen questionnaire from which the Atopy Score (the maximum is 34 points and a score ≥ 10 is considered as atopy) was derived [13]. The atopy score, expressed as mean ± SD, was 3.0 ± 2.4. Participants were not allowed to use soap, moisturizers or any other cosmetics or creams on the lower mid volar arms 48 hours prior to and during the experiments. Written informed consent was obtained from all subjects prior to the experiment. The Medical Ethical Committee of
the Academic Medical Center, University of Amsterdam approved the experimental protocol. The study was conducted according to the Declaration of Helsinki Principles.

Penetration experiment

An application mixture of PEGs was made by dissolving 47.5 mg of monodispersed PEG150 (MW = 150.17 Da, Sigma, the Netherlands), 50.1 mg of monodispersed PEG282 (MW = 282.34 Da, Acros Organics, NY, USA)), 102.9 mg of monodispersed PEG326 (MW = 326.4 Da, PolyPure, Norway), 199.1 mg of monodispersed PEG370 (MW = 370.4 Da, PolyPure, Norway), and 10 g of polydispersed PEG600 (average MW = 600 Da, Sigma, the Netherlands) in 2 ml of water. Subjects were exposed to the PEG application mixture (180 µL) using patch test chambers (Finn chambers®, 18 mm in diameter, Epitest Ltd., Finland) on volar arm for six hours. These prevented evaporation of water from the test site and this combined with excess PEG insured that the exposure concentration remained constant during the exposure. Another chamber containing the application mixture was applied onto the skin site pre-treated with SLS (≥ 99% purity; Fluka, Buchs, Switzerland). The treatment with SLS was performed 24 hrs before the application of PEG. This was done by applying 200 µL of a 5% (w/w) water solution of SLS for 4 h. Before application of the patch containing SLS or PEG and during tape striping the TEWL was measured using an Evaporimeter (VapoMeter SWL2g, Delfin Technologies, Ltd., Kuopio, Finland). Twenty minutes prior to application the subjects rested with their sleeves rolled-up in the examination room, where the temperature was 20-22 °C and relative humidity ranged between 50 and 60%. After the removal of the patch containing PEG, a piece of dry cell tissue was gently attached to the skin site to remove the residue of PEG mixture. Ten minutes after the end of exposure to PEGs, the SC layers were

sequentially removed with pre-cut Diamond tape pieces, 19 x 25 mm^2 (Diamond Ultra Clear tape, The Sellotape® Company, the Netherlands). Templates of Scanpor® tape were fixed on the skin around the application spot to limit the tape stripping of the exposed area (18 mm in diameter). To limit the tape stripping to the exposed area (18 mm in diameter) templates of Scanpor® tape were fixed on the skin around the application spot. The tape pieces were consecutively applied to the test site and uniformly pressed with 1 kg stainless steel roller that was moved 20 times in two directions. Total removal of the SC was evidenced by shiny appearance of the skin and by the TEWL > 100 g m^{-2} h^{-1}. Each individual strip was placed into a glass vial and stored at -20 °C until analysis. The SC from a non-exposed site was stripped off and served as negative control.

Analytical procedure

The gas chromatographic method for determination of PEGs and the spectrophotometrical method for analysis of proteins in tape strips have been described extensively elsewhere [14, 15].

Concentration of PEGs on each strip was normalized for the amount of proteins and expressed as µg PEG/µg protein. Assuming an SC density of 1 g cm^{-3} (Andersen and Cassidy, 1973) and uniform distributions of SC on the tapes and proteins within the SC (µg), the protein mass removed was converted to volume, enabling the depth of each strip in the SC (x). [16]. In our calculation of the SC solute concentration it was assumed that the protein concentration in the SC was (on average) 1g / mL SC.

Data analysis

The concentration of PEGs on each strip was plotted as a function of relative SC depth. To estimate the penetration parameters we used an approach based on Fick's second law of diffusion as described by Pirot *et al.* and a recent series of papers by Alberti *et al.* [17-21]. In this method, two parameters, K and D/L^2, are determined by best-fit regression of the concentration of PEGs as a function of relative SC depth (x/L) to the following equation

$$C(x) = KC_{veh}\left(1-\frac{x}{L}\right) - \sum_{n=1}^{\infty}\frac{2}{n\pi} KC_{veh}\sin\left(\frac{n\pi x}{L}\right)\exp\left(\frac{-Dn^2\pi^2 t}{L^2}\right) \qquad \textit{Eq. 1}$$

where C_{veh} is the applied PEGs concentration (µg cm^{-3}), C is the PEG concentration (µg cm^{-3}) at depth x (cm), K is the SC/water partition coefficient, L is the total thickness of the SC (cm), D is the effective diffusion coefficient of PEGs through the pseudo-homogeneous SC (cm^2 h^{-1}) and t is the exposure duration (h). The non-

steady state diffusion equation (Eq. 1) was fitted to the data and the rate constant for diffusion across SC (D/L^2, h^{-1}) was obtained from the decay of C(x) as a function of x, and K was obtained from the intercept at x = 0. The permeability coefficient (K_p, cm h^{-1}) for each PEG oligomer was calculated from the relationship $K_p = K^*D/L$. All concentration data were weighted equally in the regression analysis. The first strip was not included in the regression analysis, as it contained some residue of PEGs on the surface of the skin after end of exposure. For curve fitting and statistical calculations Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) was used. For statistical calculations Student's paired two-tailed t-test was used and the p value < 0.05 was considered significant.

Results

To remove the SC completely, on average 28 ± 5 and 23 ± 6 strips were needed for normal and SLS pre-treated sites, respectively. The SC thickness, expressed as mean ± SD was 8.7 ± 2.5 µm for the normal site, and 6.2 ± 3.0 µm, for the SLS pre-treated site. The amount of proteins in the SLS treated skin was in some subjects up to three times lower compared to the normal site. To enable comparison of the SC penetration between SLS treated and normal skin, only data obtained from the subjects with comparable (difference less then 15 %) protein amount were included in the calculations. In these subjects (*n=10*), the SC thickness, expressed as mean ± SD, was 9.1 ± 2.5 µm for the normal site and 8.4 ± 2.6 µm for the SLS treated site. Basal TEWL, expressed as mean ± SD (*n=20*), was 6.3 ± 2.1 g m^2 h^{-1} and increased to 17.9 ± 8.7 g m^2 h^{-1} 24 hours after SLS pretreatment (time point of the PEG application).

Figure 1 shows a typical concentration profile for PEG282 and PEG590 across SC of normal skin and skin compromised by SLS in one subject together with the fitted curve obtained by non-linear regression analysis (dashed lines). Statistically acceptable curve fitting ($r^2 \geq 0.95$) was obtained for all subjects without SLS pre-treatment. However, after SLS pre-treatment curve fitting could not be performed for all MWs in two subjects due to scattered data points (Table 1). To enable paired analysis the calculations did take into consideration only the data obtained for both normal and SLS-compromised skin.

The results showed that the diffusion coefficient decreased as MW of the PEGs increased in both normal and SLS pretreated skin (Fig 2). The diffusion coefficient was approximately two times higher for PEG282 in SLS compromised skin and it

gradually decreased to be approximately 1.5 times higher for PEG590 when compared to normal skin ($p < 0.05$ for PEG282-370 while it was not significant for PEG414-590) (Fig 2).

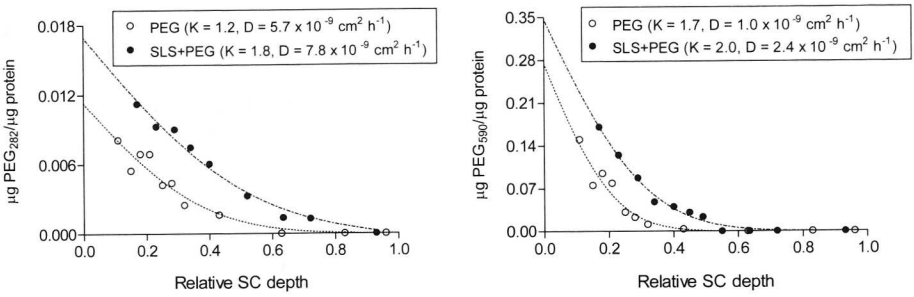


Fig 1: *Concentration decay of PEG282 and PEG590 as a function of normalized position (x/L) in the SC in normal and SLS compromised skin of one control subject after 6 hours exposure to PEG mixture. Non-linear regression analysis was used to fit the equation (Eq. 1) to the experimentally obtained data (dashed lines). The effective diffusion coefficient is calculated from D/L^2 value determined from the slope of the curve, while the partition coefficient is determined from $K*C_{veh}$ at the intercept at x= 0.*

Table 1: The ratios of penetration parameters for PEGs of different molecular weights before and after SLS treatment (geometric mean and 90%confidence interval)

MW (Da)	D x 10^{-9} (cm^2 h^{-1})	90% CI (D)	K (unitless)	90% CI (K)	K_p x 10^{-5} (cm h^{-1})	90% CI (K_p)
282	2.08	1.03 - 4.18	1.20	0.82 - 1.77	2.94	1.87 - 4.62
326	1.79	0.88 – 3.62	1.28	0.89 - 1.86	2.68	1.67 - 4.31
370	1.79	0.95 – 3.37	1.36	0.94 - 1.98	2.87	1.88 - 4.37
414	1.76	1.02 - 3.03	1.51	1.09 - 2.09	3.06	2.10 - 4.48
458	1.66	0.99 - 2.77	1.60	1.15 - 2.23	3.08	2.10 - 4.52
502	1.45	0.87 - 2.41	1.85	1.32 - 2.58	3.09	2.07 - 4.61
546	1.44	0.89 - 2.33	1.78	1.22 - 2.58	2.96	1.86 - 4.72
590	1.48	0.97 - 2.25	2.04	1.45 - 2.89	3.48	2.35 - 5.16

*MW = Molecular weight, D = Diffusion coefficient, K = Partition coefficient,
K_p = Permeability coefficient, CI = Confidence interval

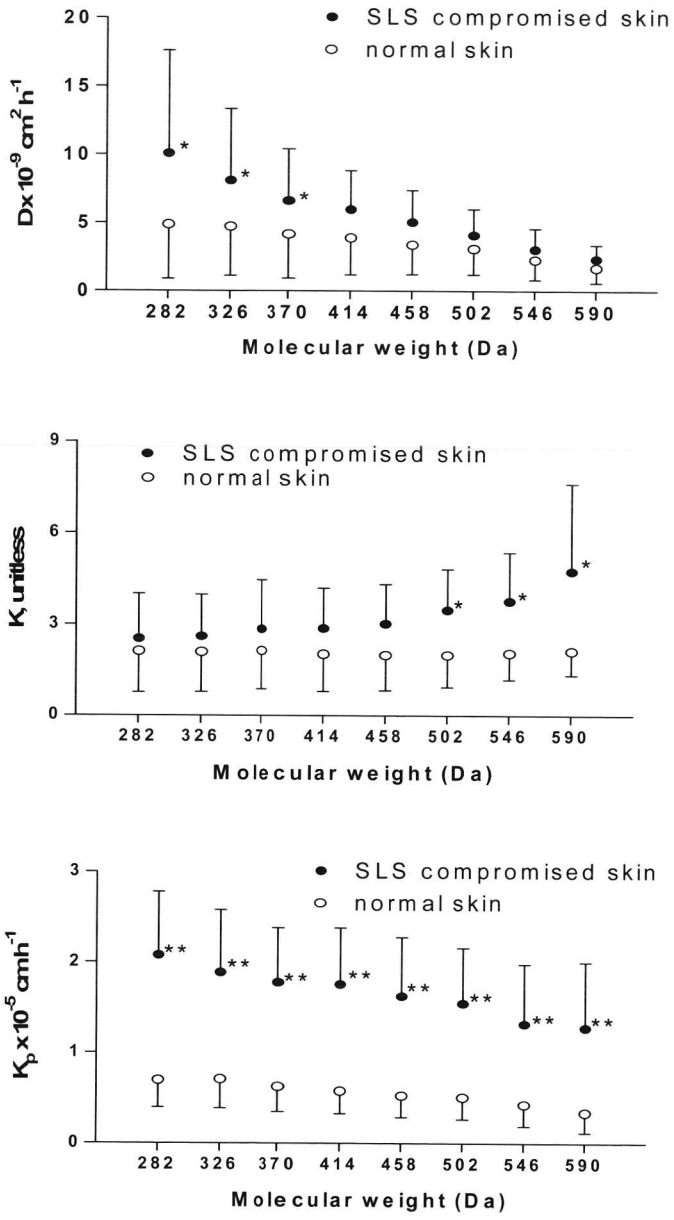


Fig 2: *Diffusion coefficient (D), partition coefficient (K) and permeability coefficient (K_p) of PEGs (282-590 Da) in normal and SLS compromised skin after 6 hours dermal exposure. The results are shown as mean ± SD, (* $p < 0.05$, ** $p < 0.01$).*

The partition coefficient for normal skin was similar for all PEGs while in SLS compromised skin it had a tendency to increase with increased MW being more prominent for PEG 502-590 ($p < 0.05$) (Fig 2).

The permeability coefficient of PEGs of different MW was approximately three times higher in SLS compromised skin ($p < 0.01$ for all PEGs), and it gradually decreased with increasing MW in both normal and SLS-compromised skin. We found no correlation between basal TEWL or TEWL measured 24 hours after SLS pre-treatment and any of the estimated penetration parameters.

Additionally, we calculated the ratios of all penetration parameters between normal skin and skin pre-treated with SLS. The geometric mean values of the ratios and corresponding 90 % confidence interval are shown in Table 1.

The inter-individual differences in all measured penetration parameters were considerable for both normal and SLS compromised skin. The coefficient of variation for normal skin amounted from 64% to 82% for the diffusion coefficient, from 37% to 64 % for the partition coefficient, and from 43% to 64% for the permeability coefficient. The coefficient of variation for SLS compromised skin amounted from 44% to 75% for the diffusion coefficient, from 39% to 61% for the partition coefficient, and from 34% to 56% for the permeability coefficient.

Discussion

In the present study we assessed the penetration of PEGs ranging in the MW from 282 to 590 Da into the normal skin and skin compromised by SLS. From the amount of PEGs determined in the SC layers we estimated the effective diffusion coefficient and partition coefficient between SC and vehicle from which the permeability coefficient could be calculated. According to Fick's law of diffusion, when the SC controls mass transfer through the skin, the skin flux of a penetrant is the product of the permeability coefficient and the concentration in the SC.

The SLS treatment caused moderate barrier impairment, the TEWL increased from 6.3 ± 2.1 g m^2 h^{-1} to 17.9 ± 8.7 g m^2 h^{-1}. The average number of strips needed to remove the whole SC and the protein amount were significantly lower after SLS pretreatment, in some persons up to 3-fold. A possible explanation for the smaller amount of proteins could be partial loss of the SC after SLS pre-treatment. SLS is known to change the cohesiveness of the SC. The loss of proteins might have

occurred during the period between the removal of the SLS patch and the application of PEG leading to a thinner SC. Since SC is reported not to be homogenous across entire length, the comparison of the penetration between SLS treated and normal skin was not justified. Another reason for the lower protein amount in some subjects might be loss of SC during the removal of the PEG exposure chamber followed by gentle attaching of dry tissue paper. In this case, the upper layers of SC containing PEG will be lost. Of course, both possibilities might contribute to the loss of proteins making the outcomes unreliable. Therefore, for data analysis we have included only the paired data of subjects who had similar protein amount before and after SLS application ($n = 10$). The percutaneous penetration parameters and the TEWL values assessed before application of SLS did not statistically differ in these two groups of subjects (data not shown).

As expected, the diffusion coefficient was inversely dependent on MW in both normal and in SLS-compromised skin. The diffusion coefficient after pre-treatment with SLS increased for all PEG oligomers and this increase was more prominent for smaller oligomers. Enhanced diffusion coefficient in SLS pre-treated skin could be explained by the influence of SLS on the lipid bilayers of the SC. SLS has been shown to cause distinct abnormalities of the SC in its intercellular lipid domain leading to fluidization of the intercellular lipid domain [8, 9]. Recent work showed that SLS removed hydrophobic lipids (8-15%) while no removal of ceramides occurred [10, 22-23]. A similar effect of enhanced diffusion coefficient was found for PEGs, SLS and theophylline in patients with atopic dermatitis, whose skin is characterized by impaired intercellular lipid domain [12, 15, 24].

The partition coefficient between SC and vehicle was similar for all PEG oligomers in normal skin, which is consistent with similar octanol-water partition coefficients of PEGs over a broad range of MWs [11]. The partitioning of PEGs in the SLS-compromised skin was higher than in normal skin. This better solubility of PEGs in the SC due to SLS pre-treatment might be explained by removal of hydrophobic lipids and induced hydration leading to more hydrophilic SC, as already suggested by others [18, 25-26]. In contrast to normal skin, partition coefficient was increasing with the MW of PEGs. The reason for this is not clear to us. In our parallel study with AD patients known for skin dryness, we found lower partition of PEGs in the SC compared to control subjects; however, this decrease was similar for all MWs [12].

The permeability coefficient, calculated from diffusion coefficient and partition coefficient values, was three-fold larger in the SLS compromised skin compared with the normal skin for all PEG oligomers, ranging 0.34 to 0.70 x 10^{-5} cm h^{-1} in normal

skin from and from 1.20 to 2.09 x 10^{-5} cm h^{-1} in the SLS compromised skin (MW 590 to 282 Da). This was consistent with the values of 5 x 10^{-5} cm h^{-1} to 8.3 x 10^{-5} cm h^{-1} for PEG414 - PEG282 obtained in hairless mouse model [27]. The higher permeability coefficient of PEG found in the SLS compromised skin is consistent with the study of Tsai *et al.*, who investigated the penetration of polydispersed PEGs (PEG300, PEG600 and PEG1000) in vitro in hairless mice after SLS pre-treatment [3]. In that study, the penetration of PEGs expressed as percentage of the applied dose increased with the degree of barrier disruption as measured by TEWL. Furthermore, they reported the shifting of the MW cut-off value for PEG in the damaged skin. In the normal skin a cut-off value of 414 Da was found; however, in the skin compromised by SLS the MW cut-off value was shifted to 766 Da. But, as discussed earlier, the reported cut-off value was probably more the result of the detection limit of the analytical method than the real molecular cut-off [3, Jakasa *et al*, submitted for publication]. In our study we see a gradual decrease of PEG penetration with increasing MW in both normal and SLS compromised skin. This is in accordance with Johnson *et al.*, who reported that the diffusion coefficient of larger solutes (~350-500 Da) appear not to decrease at the same dramatic rate but instead remain relatively constant [28].

The effect of SLS on the percutaneous penetration has been shown to be dependent on the lipophilicity of the penetrant. Nielsen *et al.* investigated in vitro percutaneous penetration of a number of pesticides varying in lipophilicity through skin that was pre-treated with 0.1 and 0.3 % SLS for 3 hours [5]. He found that percutaneous penetration of more hydrophilic compounds was affected more. Penetration of lipophilic compounds ($\log K_{o/w} > 3$) through the SLS treated skin increased little, whereas for less lipophilic compounds ($\log K_{o/w}$ of 0.7 and 1.7) the penetration increased two-fold. This is in agreement with the study of Borras-Blasco *et al* which investigated the effect of SLS on in vitro percutaneous absorption through rat skin of a number of compounds with a wide range of lipophilicity values ($\log K_{o/w}$ from -0.95 to 4.42) [4]. They showed that permeability increased only for compounds with $\log K_{o/w} < 3$. For a hydrophilic compound with a $\log K_{o/w}$ of -0.95 which was similar to that of PEGs ($\log K_{o/w} = -1.6$) the permeability coefficient increased ten-fold. In our study the permeability coefficient was increased three- fold for all PEG oligomers. It has to be pointed out that the effect of SLS measured in various studies was influenced by the experimental design, in particular with reference to the time difference between SLS pre-treatment and penetrant application. In some studies, the penetrant was applied simultaneously with SLS, while in others immediately after removal of SLS or 24-48 hours after the SLS pre-treatment, when the irritation effect is highest. The nature and the magnitude of the effect will be therefore different.

In the present in vivo human study we have shown that SLS damages the skin leading to increased skin permeability of hydrophilic PEGs. The increase in permeability was due to enhancement in both diffusion and partitioning into the SC. In the skin that has been damaged by SLS exposure, the penetrated amount will be higher and larger molecules could penetrate in amounts that might be sufficient to exceed toxic levels. Therefore, when performing the risk assessment of dermal exposure, damaged skin, which occurs commonly, should be taken into consideration.

Acknowledgement

We are grateful to N. Mohammadi for the help during development of tape stripping method, and C. E. T. Withagen and F. Calkoen-Kwa for technical assistance. The authors would like to thank the European Community for providing financial support for part of this the study, which was carried out under the Fifth Framework Programme (project acronym: EDETOX).

References:

1. Tsai J C, Sheu H M, Hung P L, Cheng C L. Effect of barrier disruption by acetone treatment on the permeability of compounds with various lipophilicities: Implications for the permeability of compromised skin. J Pharm Sci 2001: 90(9): 1242-1254.
2. Tsai J C, Hung P L, Sheu H M. Molecular weight dependence of polyethylene glycol penetration across acetone-disrupted permeability barrier. Arch Dermatol Res 2001: 293: 302-307.
3. Tsai J C, Shen L C, Sheu H M, Lu C C. Tape stripping and sodium dodecyl sulfate treatment increase the molecular weight cutoff of the polyethylene glycol penetration across murine skin. Arch Dermatol Res 2003: 295: 169-174.
4. Borras-Blasco J, Diez-Sales O, Lopez A, Herraez-Dominguez M. A mathematical approach to predicting the percutaneous absorption enhancing effect of sodium lauryl sulphate. Int J Pharm 2004: 269: 121-129.
5. Nielsen J B. Percutaneous penetration through slightly damaged skin. Arch Dermatol Res 2005: 296: 560-567.
6. Wilhelm K P, Surber C, Maibach H I. Effect of sodium lauryl sulfate-Induced skin irritation on in vitro percutaneous penetration of four drugs. J Invest Dermatol 1991: 96: 963-967.
7. Walters K A. Surfactants and percutaneous absorption. In: Scott R C, Guy R H, Hadgraft J. Prediction of percutaneous penetration: Methods, measurements, modeling. Ibc Technical Services, London 1989: 148-162.

8. Jiang S J, Zhou X J, Sun G Q, Zhang Y. Morphological alterations of the stratum corneum lipids induced by sodium lauryl sulfate treatment in hairless mice. J Dermatol Sci 2003: 32: 243-246.
9. Ribaud C, Garson J C, Doucet J, Leveque J L. Organization of stratum corneum lipids in relation to permeability: Influence of sodium lauryl sulfate and preheating. Pharm Res 1994: 11: 1414-1418.
10. Fartasch M. Ultrastructure of the Epidermal Barrier after irritation. Microsc Res Tech 1997: 37: 193-199.
11. Hollander D, Koyama S, Dadufalza V *et al.* Polyethylene glycol 900 permeability of rat intestinal and colonic segments in vivo and brush border membrane vesicles in vitro. J Lab Clin Med 1989: 113: 505-515.
12. Jakasa I, Verberk M M, Esposito M, Bos J D, Kezic S. Increased diffusion of polyethylene glycols in uninvolved skin of atopic dermatitis patients. J Invest Dermatol (to be submitted, 2006)
13. Diepgen T L. Die atopische hautdiathese: Epidemiologie, Klinik und berufsdermatologische bedeutung. Stuttgart: Gentner Verlag 1991: 1-128.
14. Jakasa I, Calkoen F, Kezic S. Determination of polyethylene glycols of different molecular weight in the stratum corneum. J Chromatogr B 2004: 811(2): 177-182.
15. Jakasa I, de Jongh CM, Verberk MM, Bos JD, Kezic S: Percutaneous penetration of sodium lauryl sulphate is increased in uninvolved skin of atopic dermatitis patients compared to control subjects. Br J Dermatol (in press)
16. Anderson R L, Cassidy J M: Variation in physical dimensions and chemical composition of human stratum corneum. J Invest Dermatol 1973: 61: 30-32.
17. Crank J: Mathematics of diffusion. Oxford: Oxford Univ. Press, 1975: 50-51.
18. Pirot F, Kalia YN, Stinchcomb AL, Keating G, Bunge A, Guy RH: Characterization of the permeability barrier of human skin in vivo. Proc Nat Acad Sci USA 94: 156201567, 1997
19. Alberti I, Kalia Y, Naik A, Bonny J D, Guy R H. In vivo assessment of enhanced topical delivery of terbinafine to human stratum corneum. J Controlled Release 2001a: 71:319-327.
20. Alberti I, Kalia Y N, Naik A, Bonny J D, Guy R H. Effect of ethanol and isopropyl myristate on the availability of topical terbinafine in human stratum corneum, in vivo. Int J Pharm 2001b: 219:11-19.
21. Alberti I, Kalia Y N, Naik A, Bonny J D, Guy R H. Assessment and prediction of the cutaneous bioavailability of topical terbinafine in vivo. Pharm Res 2001c: 18:1472-1475.
22. Froebe C L, Simion F A, Rhein L D, Cagan R H, Kligman A. Stratum corneum lipid removal by surfactants: Relation to in vivo irritation. Dermatologica 1990: 181: 277-283.
23. Lévêque J L, de Rigal J, Saint-Léger D, Billy D. How does sodium lauryl sulfate alter the skin barrier function in man? A multiparametric approach. Skin Pharmacol 1993: 6: 111-115.
24. Yoshiike T, Aikawa Y, Sindhvananda J *et al.* Skin barrier defect in atopic dermatitis: increased permeability of the stratum corneum using dimethyl sulfoxide and theophylline. J Dermatol Sci 1993: 5: 92-96.

25. Gloor M, Senger B, Langenauer M, Fluhr J W. On the course of the irritant reaction after irritation with sodium lauryl sulphate. Skin Res Technol 2004: 10: 144-148.
26. Rhein L D, Robbins C R, Fernee K and Cantore R. Surfactant structure effects on swelling of isolated human stratum corneum. J Soc Cosmet Chem 1986: 37: 125–139.
27. Mitragotri S: Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. *J Contrl* Rel **86**: 69-92. 2003
28. Johnson M E, Blankschtein D, Langer R. Evaluation of solute permeation through the stratum corneum: Lateral bilayer diffusion as the primary transport mechanism. J Pharm Sci 1997: 86: 1162-1172

Chapter 5

Variation in barrier impairment and inflammation of human skin as determined by sodium lauryl sulphate penetration rate

C.M. de Jongh, I. Jakasa, M.M. Verberk, S. Kezic

Br J Dermatol (2006)154: 651-657

Abstract

Background Skin irritability after a brief exposure to the model skin irritant, sodium lauryl sulphate (SLS), is known to vary considerably between individuals. A difference in the skin barrier to SLS may contribute to this variation. To date, no human *in vivo* data have been available on SLS penetration into the skin.

Objectives We studied whether the SLS penetration rate into the stratum corneum (SC) is related to impairment of the water barrier function and inflammation of the skin.

Methods The penetration of SLS into the SC was assessed using a noninvasive tape-stripping procedure in 20 volunteers after a 4-h exposure to 1% SLS. Additionally, the effect of a 24-h exposure to 1% SLS on the skin water barrier function was assessed by measuring the transepidermal water loss (TEWL). The accompanying inflammation was quantified by measuring erythema.

Results The mean ± SD diffusivity of SLS (D) and the SLS permeability coefficient (K_p) were $1{\cdot}4 \pm 0{\cdot}6 \times 10^{-8}$ cm^2 h^{-1} and $1{\cdot}5 \pm 0{\cdot}7 \times 10^{-3}$ cm h^{-1}, respectively. A multiple regression analysis showed that the baseline TEWL, SC thickness and SLS penetration parameters K (SC/water partition coefficient) and D clearly influenced the increase in TEWL after the 24-h irritation test (explained variance: $r^2 = 0{\cdot}80$). Change in erythema was mainly influenced by SC thickness.

Conclusions We found that variation in the barrier impairment and inflammation of human skin depends on the SLS penetration rate, which was mainly determined by SC thickness.

Repeated cutaneous exposure to mild irritants is the leading cause of occupational contact dermatitis.[1] High-risk occupations for developing chronic irritant contact dermatitis (ICD) include hairdressing,[2,3] healthcare[3] and metalworking.[4] For example, in a study of hairdressing apprentices, the incidence rate of ICD during the first year was 31·7 per 100 person-years.[2] The mechanism of development of chronic ICD and the factors that determine an individual's susceptibility are not completely understood. The 'irritation threshold' to the model irritant sodium lauryl sulphate (SLS) has been suggested as a possible indicator of individual susceptibility to chronic ICD.[5-7] The irritation threshold has been defined as the lowest SLS concentration that leads to visual skin inflammation, assessed in an acute 4-h patch test. The investigators used visual grading of the erythema reaction as a parameter of irritation. These studies revealed substantial interindividual differences in acute skin inflammation susceptibility: the irritation threshold ranged from < 0·1% to > 20% SLS.

We assumed that the irritant effect of SLS on the stratum corneum (SC)[8] and the underlying viable tissues[9] is related to the ability of SLS to penetrate into the SC. Thus, the differences in the skin barrier to SLS may be one of the factors contributing to the interindividual differences in skin irritability. In studies with healthy volunteers, interindividual differences of up to a factor of 4 were found in the skin penetration rate of several chemicals.[10] In this regard, one important factor is the SC thickness, which varies between individuals by a factor of 2–3.[11] As the penetration of chemicals into the skin is a diffusion process, the amount absorbed will be inversely proportional to the thickness of the SC membrane.

Several studies have been performed to obtain insight into SLS penetration into the skin. Most of these studies were *in vitro*, using mammalian[9,12-14] or human skin.[9,15-17] In two studies, the ability of SLS to penetrate into the skin was investigated *in vivo*, using rat models.[9,18] None of these studies addressed the relationship between the SLS penetration and the skin inflammation reaction.

Penetration of topically applied substances into the SC *in vivo* can be studied noninvasively, using a tape stripping procedure.[19,20] In the present study, we investigated the relationship between SLS penetration into the SC and the impairment of the skin water barrier function and skin inflammation. To this end, we performed two exposures in volunteers. One exposure was a 24-h exposure to 1% SLS intended to evoke skin irritation: 24 h after patch removal, the effect of SLS on the skin water barrier function was measured by transepidermal water loss (TEWL), and the inflammation was assessed by measurement of erythema (skin redness). The second exposure was a 4-h application of 1% SLS to assess the rate of

penetration of SLS into the SC. The results presented here were part of a larger study designed to investigate individual susceptibility factors for developing chronic ICD (de Jongh *et al.*, in preparation).

Subjects and methods

Study population

Twenty healthy volunteers with no visible skin abnormalities participated in the study (13 women, mean ± SD age 24 ± 3 years and seven men, mean ± SD age 25 ± 8 years). The study was approved by the ethics committee of the Academic Medical Centre, and all subjects gave their written, informed consent. The subjects were not allowed to use soap or moisturizers on the lower arms for 24 h prior to, and during the days of, the experiments. All participants filled in the Erlangen Atopy Questionnaire,[21] which was used to derive an atopy score.

Single 24-h sodium lauryl sulphate irritation test

To evoke skin irritation, the dominant midvolar forearm was exposed over a 24-h period to a 1% w/v SLS solution (200 µL, ≥ 99% purity; Fluka, Buchs, Switzerland), using a patch test chamber (Finn chambers® of 18 mm diameter and filter paper discs; Epitest, Tuusula, Finland). Before application and 24 h after patch removal, TEWL and erythema were measured on the exposed site and on a control site on the dominant forearm. TEWL was measured with an Evaporimeter (VapoMeter SWL2g; Delfin Technologies Ltd, Kuopio, Finland). Nuutinen *et al.* describe this measurement device in detail.[22] For at least 20 min prior to the measurements, the volunteers rested with their sleeves rolled up in the examination room, where the temperature was 20–22 °C and the relative humidity ranged between 50% and 60%. The erythema index was measured using an erythema meter (DermaSpectrometer; Cortex Technology, Hadsund, Denmark), as described by Clarys *et al.*[23]

Sodium lauryl sulphate penetration test

In addition to the single 24-h irritation test site, two other sites on the dominant distal volar forearm were exposed for 4 h to 200 µL of a 1% SLS solution using patch test chambers to determine the SLS penetration rate. The chambers were separated by 15 mm and attached with adhesive tape (Scanpor® tape; Norgeplaster, Vennesla, Norway). A third, nonexposed site on this forearm served as a control.

Fifteen minutes after the chamber removal, the SC was sequentially removed using pieces of 19 × 25 mm adhesive tape (Diamond Ultra Clear Tape, 19 mm × 33 m;

The Sellotape® Company, Eindhoven, the Netherlands). Templates of Scanpor® tape were fixed on the skin around each application spot to limit the tape stripping area (18 mm diameter). Pieces of tape were consecutively applied to the sites and homogeneously pressed on to the skin by moving a 1·0-kg stainless steel roller[24] 10 times in two directions. The tapes were then slowly removed at an angle of 170° with the skin. The sites were multidirectionally stripped until it appeared that the SC was totally removed, as observed by the shininess and redness of the surface and a TEWL > 100 g m^{-2} h^{-1}. Each tape strip was collected in a glass vial and stored at − 20 °C. The tape stripping of the three sites (two SLS-exposed and one control, simultaneously) was completed after 20 min.

The amount of SLS on each strip was analysed using an adjusted method based on that of Rusconi *et al.*[25] The total amount of protein on each strip was analysed following a slightly modified procedure based on that of Dreher *et al.*[26] Blank tapes were processed and assayed as a negative control. To extract the SLS from the tape, 1 mL methanol (J.T. Baker, Deventer, the Netherlands) was added to each vial and the vials were shaken for 1 h (TPM-2; Sarstedt, Numbrecht, Germany). The methanol fraction was removed and stored at − 20 °C for SLS analysis.

The concentration of SLS was determined spectrophotometrically, using a dye (Stains-All®; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). Standards of SLS for the calibration curve were prepared in methanol (2·3–50 μg mL^{-1}) and 20 μL from each standard and sample was pipetted into a 96-well plate. After evaporation of the methanol, 200 μL of Stains-All® working solution was added to each well and absorbance was read at 450 nm (Model 680 Microplate reader; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

The remaining methanol in the vials containing tape strips was evaporated, using nitrogen. Subsequently, 1 mL of 1 mol L^{-1} NaOH was added. The vials were then shaken for 2 h and left at room temperature overnight. After addition of 1 mL of 1 mol L^{-1} HCl, the sample was ready for the protein assay. The total protein on each strip was determined with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories), using the supplied bovine serum albumin as the standard. Standards and samples were pipetted into a 96-well plate and assayed following the kit instructions. The concentration of SLS on each strip was expressed as μg SLS μg^{-1} protein. Assuming an SC density[11] of 1 g cm^{-3} and a uniform distribution of SC on the tape strips (stripped skin area: 2·54 cm^2), the protein mass removed was converted to a volume enabling estimation of the thickness of SC on each strip and the cumulative SC thickness.

Data analysis

After the 4-h SLS penetration test, analysis of the sequentially tape-stripped SC yielded values of the SLS concentration as a function of the relative depth (x/L) into the SC. The SLS concentration profile is given by a solution to Fick's Second Law of Diffusion (Fig 1),[19,20] where $C(x)$ is the SLS concentration (μg cm^{-3}) at depth x in the SC, K is the SC/water partition coefficient of SLS, C_{veh} is the applied SLS concentration (μg mL^{-1}) in the vehicle (water), L is the total thickness of the SC (cm), D is the diffusivity of SLS in the membrane (cm^2 h^{-1}), and t is the exposure duration (h).

The rate constant for diffusion across the SC (D/L^2) is obtained by fitting this nonsteady-state diffusion equation to the data of the SLS concentration profile. KC_{veh} equals the intercept at $x = 0$. The SLS permeability coefficient (K_p) across the SC layer is calculated by $K_p = KD/L$. The calculation of penetration parameters from the concentration vs. depth curve has been described in detail elsewhere.[19,20]

Data of duplicate SLS penetration tests were pooled to obtain one regression curve for each subject. The first strip was not included in the regression analysis, as it contained some SLS that remained on the surface of the skin. Prism 4 (GraphPad, San Diego, CA, U.S.A.) was used for curve fitting.

Statistics

For our statistical analysis, we used Student's two-sample t-test to compare subgroups, and the Pearson correlation coefficient. For the effect of SLS penetration rate on skin water barrier function, we used a multiple linear regression analysis with the change in TEWL as the dependent parameter, and the baseline TEWL, K, L and D as the independent parameters. For the effect on skin inflammation, we used the change in erythema as the dependent parameter, and K, L and D as the independent parameters. $P \leq 0{\cdot}05$ was considered significant.

Results

Atopy score

The subjects' Erlangen Atopy Scores ranged from 3 to 16 (the maximum for the questionnaire is 34), and the mean ± SD score was 7·9 ± 3·9. Six subjects had a score ≥ 10 points, which is considered as having atopy.[21]

$$C(x) = KC_{veh}\left(1-\frac{x}{L}\right) - \sum_{n=1}^{\infty}\frac{2}{n\pi} KC_{veh}\sin\left(\frac{n\pi x}{L}\right)\exp\left(\frac{-Dn^2\pi^2 t}{L^2}\right)$$

Fig 1: *Fick's Second Law of Diffusion.*

Single 24-h sodium lauryl sulphate irritation test

One day after the 24-h irritation test with 1% SLS, the mean ± SD TEWL had increased from 9.0 ± 3.0 g m^{-2} h^{-1} (baseline) to 81 ± 42 g m^{-2} h^{-1}, and the degree of erythema from 7.8 ± 2.1 arbitrary units (AU) to 12.2 ± 3.2 AU.

Sodium lauryl sulphate penetration test

On average, 13 ± 4 tape strips were removed from each SLS test site and 20 ± 4 strips from the control site. The mean ± SD total amount of protein removed from one site was 2140 ± 570 µg, corresponding to a SC thickness of 8.6 ± 2.4 µm.

Acceptable curve fitting was obtained for 19 volunteers. The data on two volunteers had to be excluded from the analysis. This was because a linear relationship was found between the SLS concentration and SC depth, indicating a steady-state absorption to which Fick's Second Law of Diffusion is not applicable. Figure 2 shows the SLS concentration profiles across the SC for two selected subjects. Nonlinear regression was used to obtain the best fit of the equation given in Figure 1 (dashed lines).

The SLS penetration results are summarized in Table 1. We observed fairly high interindividual variation in all penetration parameters: the coefficients of variation (CV) ranged from 24% to 51%. The mean values were also calculated separately for the subjects with (n = 6) and without (n = 11) atopy. No difference in K was found between these two groups. In the atopy group, the SC tended to be thinner, and D/L^2 and K_p were almost doubled (P = 0.002 and P = 0.001, respectively).

Table 1 shows that changes in TEWL (ΔTEWL) and erythema (Δerythema) were higher in atopics than in nonatopics (P = 0.04 and P = 0.06, respectively) after the 24-h irritation test. Baseline TEWL did not clearly differ between subjects with and without atopy (Table 1).

Table 2 shows the relationship between ΔTEWL and Δerythema after the 24-h irritation test, and the obtained penetration parameters K, L, D and D/L^2. A good relationship was found between the effect parameters, ΔTEWL and Δerythema, and D/L^2 (r = 0.74, Fig 3, and r = 0.58, respectively). Moreover, L had a clear influence on ΔTEWL (r = − 0·64) and Δerythema (r = − 0.63), as well as an influence on the baseline TEWL (r = − 0.43). No clear relationship was found between K and D and the skin irritation parameters. As regards the baseline TEWL, subjects with a higher baseline value also had a higher ΔTEWL (r = 0.62). As the lag time to reach steady state is defined as $L^2/6D$, it appeared that the lag time in our subjects would be 10.5 ± 6.0 h.

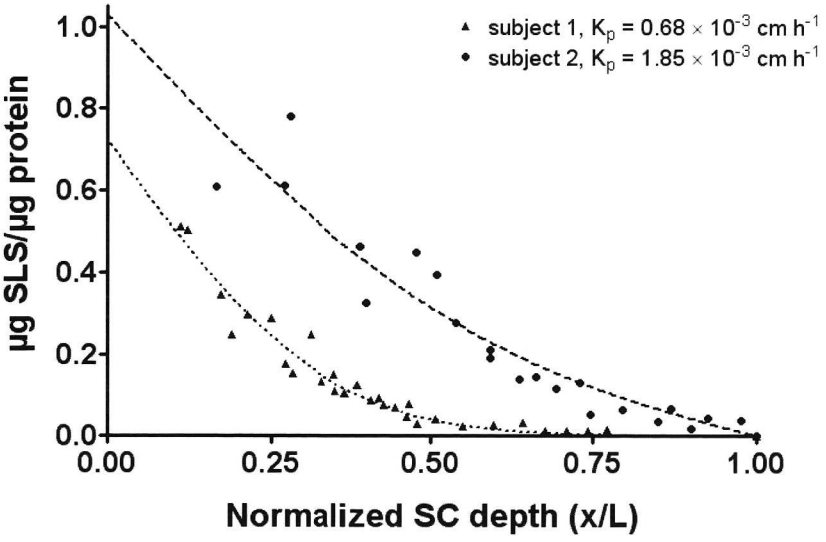


Fig 2: *Sodium lauryl sulphate (SLS) concentration obtained by tape stripping in two subjects as a function of stratum corneum (SC) depth (x/L) after a 4-h exposure to 1% SLS. Experimental data were fitted to the equation given in Figure 1, using a nonlinear regression analysis (dashed lines; r = 0.95–0.97)* Kp = SLS permeability coefficient.*

Table I. SLS penetration parameters (mean ± SD) and skin irritation parameters for subjects with and without atopy.

Parameter	Unit	All subjects	Atopy	No atopy	P-value*
		n = 17	*n = 6*	*n = 11*	*+/- atopy*
SC/water partition coefficient (K)		93 ± 22	95 ± 12	91 ± 27	n.s.
SC thickness (L)	µm	8.6 ± 2.4	7.6 ± 1.3	9.2 ± 2.7	n.s.
Diffusion coefficient (D)	$\times 10^{-8}$ cm^2 h^{-1}	1.4 ± 0.6	1.8 ± 0.5	1.2 ± 0.5	0.05
Rate constant for SLS diffusion (D/L^2)	$\times 10^{-2}$ h^{-1}	2.1 ± 1.1	3.1 ± 0.8	1.6 ± 0.7	0.002
Permeability coefficient (K_p)	$\times 10^{-3}$ cm h^{-1}	1.5 ± 0.7	2.2 ± 0.5	1.2 ± 0.5	0.001
Baseline TEWL	g m^{-2} h^{-1}	8.6 ± 3.0	9.2 ± 3.9	8.3 ± 2.5	n.s.
ΔTEWL	g m^{-2} h^{-1}	66 ± 40	93 ± 45	52 ± 30	0.04
Δerythema	AU	4.1 ± 2.9	5.9 ± 2.5	3.2 ± 2.7	0.06

SC, stratum corneum; TEWL, transepidermal water loss; NS, not significant; AU, arbitrary units. [a]Independent sample t-test (two-sided)

To estimate the contribution of individual skin properties on the skin irritation parameters, we performed a multiple linear regression analysis. The following relationship was obtained [estimated coefficients (SE)]: ΔTEWL = 23 (38) + 5.3 (1.9) baseline TEWL + 0.63 (0.24)K − 1·1 (0.25) × $10^5 L$ + 2.8 (0.91) × $10^9 D$ with an explained variance of r^2 = 0·80 (P = 0·001). In a model with only the baseline TEWL and L as the independent parameters, the explained variance was 0.55. For Δerythema, we obtained: Δerythema = 11 (3.0) − 1.2 (2.6) × $10^{-2} K$ − 8.8 (2.6) ×$10^3 L$ + 1.6 (1.0) × $10^8 D$ with an explained variance of r^2 =0.50 (P = 0.024). Regarding ΔTEWL, all independent factors showed a significant contribution. By contrast, only L contributed significantly for Δerythema.

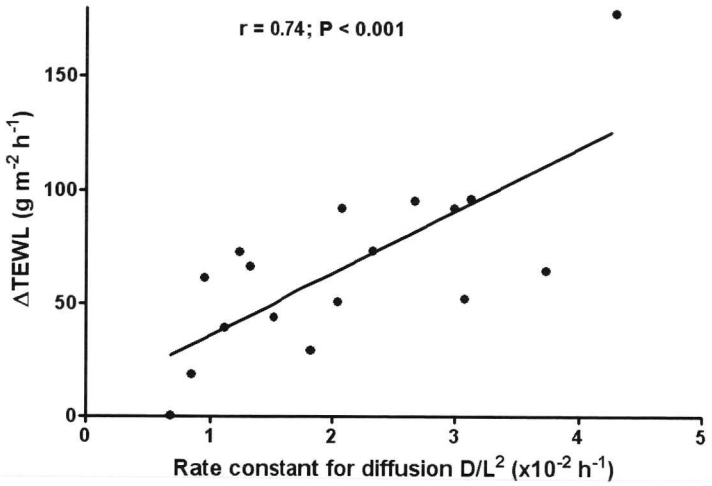


Fig 3: *Increase in transepidermal water loss (DTEWL) after a 24-h irritation test as a function of the rate constant (D/L^2) for diffusion of sodium lauryl sulphate across the stratum corneum.*

Table II. Relationships between SLS penetration parameters and skin irritation parameters in volunteers.

Parameter	SC/water partition coefficient (K)	SC thickness (L)	Diffusivity of SLS (D)	Rate constant for SLS diffusion (D/L^2)
	r	r	r	r
baseline TEWL	-0.26	-0.43*	0.02	0.43*
ΔTEWL	0.10	-0.64**	0.22	0.74***
Δerythema	-0.25	-0.63**	0.09	0.58**

r = Pearson correlation coefficient, n = 17, * P < 0.05, ** P < 0.01, *** P < 0.001 (one-sided)

Discussion

Exposure to 1% SLS over 24 h led to a decrease in the skin water barrier function and to skin inflammation. The mean increase in TEWL and erythema was 72 g m^{-2} h^{-1} and 4·4 AU, respectively, with considerable interindividual variation. These findings are in agreement with those of other studies.[27-29]
In the present study, we aimed to investigate the influence of the skin penetration rate of SLS on skin irritation effects. The differences in the SLS penetration rate have been suggested as one of the possible sources of high interindividual differences in SLS irritability.[5]

In this study, we determined the penetration of SLS into the SC by means of an SC tape-stripping method. This noninvasive method is increasingly being used to determine the penetration parameters of compounds with various physicochemical properties, e.g. naphthalene,[30] 4-cyanophenol[19,31] and cimetidine.[31] The advantage to this method is that it enables measurement of the local SLS concentration, close to the effect site. The individual penetration parameters, K and D, were deduced from the SLS concentration in subsequent tape strips based on Fick's Second Law of Diffusion. The SLS permeability coefficient (K_p) calculated from these penetration parameters was $1.5 \pm 0.7 \times 10^{-3}$ cm h^{-1}.

To date, no human *in vivo* data have been available on SLS penetration. However, some data has been published on *in vitro* experiments.[9,12,15-18] *In vitro* assays showed that after a 24-h dermal application, only 2-3% of the applied SLS penetrated through the skin into the receptor fluid.[16,17] Due to this low permeability, the blood concentrations of SLS will be too low to assess *in vivo* permeation of SLS. The experimental conditions in the mentioned *in vitro* studies differed from those used in our study. For example, the *in vitro* SLS exposure was longer (> 24 h), which makes changes in the barrier due to SLS effects more likely.[8,32,33] Furthermore, the types of skin and methods of sampling in those studies were different. It is difficult therefore to compare the results obtained in our study with the *in vitro* values reported in the literature. As an alternative to experimentally determined data, for practical purposes the K_p of a compound can be predicted by skin permeation models. The Environmental Protection Agency has proposed an empirical model, which is based on the molecular weight and the octanol/water partition coefficient.[34] This model estimates a K_p for SLS of 4.5×10^{-4} cm h^{-1}. Considering the SC as the major rate-limiting barrier to SLS penetration, this K_p is only a factor of 3 lower than our result: 1.5×10^{-3} cm h^{-1}.

In general, K_p describes the diffusion of a compound through a membrane under steady-state conditions. For our 24-h irritation test, the lag time to reach steady state ($L^2/6D$) can be estimated roughly at 10.5 ± 6.0 h. So, for the purpose of predicting the increase of TEWL and erythema, K_p has limited value, because it reflects only the steady-state flux. The sooner the steady state is reached, the higher the time-weighted concentration of SLS in the SC of a subject will be, and the more intense the effect on TEWL and erythema. So the finding that the rate constant for diffusion (D/L^2) appeared to be a good predictor of the irritation effects was according to expectations.

With respect to K, the estimation of this parameter from the curve of the SLS concentration vs. SC depth depends very much on the quality of the first data points. To improve the reliability of this estimation Reddy *et al.*[35] suggested conducting two exposure experiments: one experiment in the nonsteady-state condition to determine D and another experiment with a longer exposure to determine K. As the curve of the SLS concentration vs. SC depth becomes a straight line at the steady state, the estimation of K depends less on the error in the superficial strips. However, longer exposure could lead to alteration of the skin barrier to SLS.

It is possible that the SLS penetration rate into the skin may have been altered during the 4-h exposure, leading to a higher SLS diffusion. To minimize this risk, we opted for a relatively short exposure time and a low concentration. Moreover, in a pilot study, we observed no differences in penetration between a 1% SLS solution and a 0·1% solution. In the light of that, we assume that during a 4-h exposure SLS has a minimal effect on alterations in the barrier to SLS.

In the present study, we obtained substantial interindividual variation in all the measured penetration parameters (CV ranged from 24% to 51%). Fullerton *et al.* also found considerable interindividual variation (CV = 44%) in the *in vitro* penetrated amount of SLS in the epidermis of five different donors.[16] We found higher penetration of SLS in atopics than in nonatopics.

The main objective of this study was to investigate the relationship between SLS penetration rate and the impairment of the skin water barrier function and skin inflammation. Several mechanisms are involved in skin irritation after exposure to SLS. In the SC, SLS interacts with the protein components of the SC[8,36,37] and causes disorganization of the lipid bilayers.[8,32,33] Both of these processes lead to barrier perturbation, resulting in an increased TEWL. In the epidermis, SLS has a direct toxic effect on the keratinocytes.[38] As a consequence of epidermal cell

damage, primary cytokines such as interleukin-1α are released from the keratinocytes, triggering the production of secondary cytokines and other inflammatory mediators by epidermal cells. Subsequently, these mediators induce an inflammatory reaction with cellular infiltrate in the epidermis and vasodilation in the dermis.[39–41]

The effect of SLS on the skin water barrier function is known to vary considerably between subjects.[24,42] The permeability of the SC to SLS will contribute to those interindividual differences, as it determines the concentration of SLS in the SC and, by consequence, the extent of barrier perturbation and increase in TEWL. The permeability of the SC depends on the composition and structure of the SC, reflected in the parameters K and D. The partition coefficient of SLS between water and SC, K, will determine the amount of SLS that enters the SC. The diffusion coefficient, D, will dictate the rate by which the SLS moves through the SC. Both parameters and the SC thickness will determine the actual concentration in the SC. Taken alone, the baseline TEWL and SC thickness had a fair predictive value ($r^2 = 0.55$) in predicting the impairment of the skin water barrier function after a 24-h SLS irritation test. This predictive value increased to $r^2 = 0.80$ when the SLS penetration parameters were added. The positive relationship between the baseline TEWL and the increase in TEWL after SLS exposure fell in line with the findings of other SLS susceptibility studies.[7,43,44]

In skin inflammation, as assessed by erythema, considerable interindividual differences were found after SLS exposure.[5–7,24] In our study, the SC thickness seemed to be the most important factor influencing the extent of erythema after a 24-h SLS irritation test.

The observed interindividual variation in SLS penetration only partly explains the considerable variation in the SLS irritation effects. In our study, the range (P_5–P_{95}, data not shown) of SLS penetration between subjects was about a factor of 4 (3.6 for K_p and 4.4 for D/L^2). However, the SLS irritation threshold in acute exposure was found to differ by a factor of 200 between individuals (< 0.1% to > 20%).[5–7] It is likely therefore that factors other than permeability play a role in individual susceptibility to SLS irritation. Aside from differences in skin barrier properties, one important factor may be the cytokine profile after exposure to SLS, which will affect the inflammatory response. Allen *et al.* showed an association between the individual irritant threshold for SLS and the presence of the polymorphism at position −308 in the *TNFA* gene.[45]

We expect that individuals who have a higher SLS permeability also have a higher permeability for other substances, which makes them more susceptible to skin damage, e.g. in occupational exposure. This emphasizes that such individuals at risk have a greater need for skin protection in order to achieve a sufficient barrier to environmental chemicals. In conclusion, we have shown that the variation in barrier impairment and inflammation of human skin depends on the SLS penetration rate.

Acknowledgments

The authors thank Dr J. Krüse for his advice and assistance with the data analysis, and C.E.T. Withagen for her technical assistance.

References

1. Chew AL, Maibach HI. Occupational issues of irritant contact dermatitis. *Int Arch Occup Environ Health* 2003; 76: 339–46
2. John SM, Uter W, Schwanitz HJ. Relevance of multiparametric skin bioengineering in a prospectively-followed cohort of junior hairdressers. *Contact Dermatitis* 2000; 43: 161–8.
3. Smit HA, van Rijssen A, Vandenbroucke JP *et al.* Susceptibility to and incidence of hand dermatitis in a cohort of apprentice hairdressers and nurses. *Scand J Work Environ Health* 1994; 20: 113–21
4. Berndt U, Hinnen U, Iliev D *et al.* Is occupational irritant contact dermatitis predictable by cutaneous bioengineering methods? Results of the Swiss Metalworkers' Eczema Study (PROMETES). *Dermatology* 1999; 198: 351–4.
5. Judge MR, Griffiths HA, Basketter DA *et al.* Variation in response of human skin to irritant challenge. *Contact Dermatitis* 1996; 34: 115–7.
6. McFadden JP, Wakelin SH, Basketter DA. Acute irritation thresholds in subjects with type I–type VI skin. *Contact Dermatitis* 1998; 38: 147–9.
7. Smith HR, Rowson M, Basketter DA *et al.* Intra-individual variation of irritant threshold and relationship to transepidermal water loss measurement of skin irritation. *Contact Dermatitis* 2004; 51: 26–9.
8. Fartasch M. Ultrastructure of the epidermal barrier after irritation. *Microsc Res Tech* 1997; 37: 193–9.
9. Howes D. The percutaneous absorption of some anionic surfactants. *J Soc Cosmet Chem* 1975; 26: 47–63.
10. Williams FM. EDETOX. Evaluations and predictions of dermal absorption of toxic chemicals. *Int Arch Occup Environ Health* 2004; 77: 150–1.
11. Anderson RL, Cassidy JM. Variation in physical dimensions and chemical composition of human stratum corneum. *J Invest Dermatol* 1973; 61: 30–2.

12. Faucher JA, Goddard ED. Interaction of keratinous substrates with sodium lauryl sulfate. II. Permeation through stratum corneum. *J Soc Cosmet Chem* 1978; 29: 339–52.
13. Moore PN, Puvvada S, Blankschtein D. Challenging the surfactant monomer skin penetration model: penetration of sodium dodecyl sulfate micelles into the epidermis. J Cosmet Sci 2003; 54:29–46.
14. Patil S, Singh P, Maibach H. Cumulative irritancy in man to sodium lauryl sulfate: the overlap phenomenon. Int J Pharm 1994; 110:147–54.
15. Agner T, Fullerton A, Broby-Johansen U et al. Irritant patch testing: penetration of sodium lauryl sulphate into human skin. Skin Pharmacol 1990; 3:213–7.
16. Fullerton A, Broby-Johansen U, Agner T. Sodium lauryl sulphate penetration in an in vitro model using human skin. Contact Dermatitis 1994; 30:222–5.
17. Loden M. The simultaneous penetration of water and sodium lauryl sulfate through isolated human skin. J Soc Cosmet Chem 1990; 41:227–33.
18. Patil S, Singh P, Sarasour K et al. Quantification of sodium lauryl sulfate penetration into the skin and underlying tissue after topical application—pharmacological and toxicological implications. J Pharm Sci 1995; 84:1240–4.
19. Pirot F, Kalia YN, Stinchcomb AL et al. Characterization of the permeability barrier of human skin in vivo. Proc Natl Acad Sci USA 1997; 94:1562–7.
20. Stinchcomb AL, Pirot F, Touraille GD et al. Chemical uptake into human stratum corneum in vivo from volatile and non-volatile solvents. Pharm Res 1999; 16:1288–93.
21. Diepgen TL. Die Atopische Hautdiathese: Epidemiologie, Klinik und Berufsdermatologische Bedeutung. Stuttgart: Gentner Verlag 1991; 1–128.
22. Nuutinen J, Alanen E, Autio P et al. A closed unventilated chamber for the measurement of transepidermal water loss. Skin Res Technol 2003; 9:85–9.
23. Clarys P, Alewaeters K, Lambrecht R et al. Skin color measurements: comparison between three instruments: the Chromameter (R), the DermaSpectrometer (R) and the Mexameter (R). Skin Res Technol 2000; 6:230–8.
24. Koopman DG, Kezic S, Verberk MM. Skin reaction and recovery: a repeated sodium lauryl sulphate patch test vs. a 24-h patch test and tape stripping. Br J Dermatol 2004; 150:493–9.
25. Rusconi F, Valton E, Nguyen R et al. Quantification of sodium dodecyl sulfate in microliter-volume biochemical samples by visible light spectroscopy. Anal Biochem 2001; 295:31–7.
26. Dreher F, Arens A, Hostynek JJ et al. Colorimetric method for quantifying human stratum corneum removed by adhesive-tape stripping. Acta Derm Venereol (Stockh) 1998; 78:186–9.
27. Aramaki J, Loffler C, Kawana S et al. Irritant patch testing with sodium lauryl sulphate: interrelation between concentration and exposure time. Br J Dermatol 2001; 145:704–8.
28. Lee JY, Effendy I, Maibach HI. Acute irritant contact dermatitis: recovery time in man. Contact Dermatitis 1997; 36:285–90.
29. Leveque JL, de Rigal J, Saint-Leger D et al. How does sodium lauryl sulfate alter the skin barrier function in man? A multiparametric approach. Skin Pharmacol 1993; 6:111–5.

30. Chao YC, Nylander-French LA. Determination of keratin protein in a tape-stripped skin sample from jet fuel exposed skin. Ann Occup Hyg 2004; 48:65–73.
31. Tsai JC, Lin CY, Sheu HM et al. Noninvasive characterization of regional variation in drug transport into human stratum corneum in vivo. Pharm Res 2003; 20:632–8.
32. Jiang SJ, Zhou XJ, Sun GQ et al. Morphological alterations of the stratum corneum lipids induced by sodium lauryl sulfate treatment in hairless mice. J Dermatol Sci 2003; 32:243–6.
33. Ribaud C, Garson JC, Doucet J et al. Organization of stratum corneum lipids in relation to permeability: influence of sodium lauryl sulfate and preheating. Pharm Res 1994; 11:1414–8.
34. Potts RO, Guy RH. Predicting skin permeability. Pharm Res 1992; 9:663–9.
35. Reddy MB, Stinchcomb AL, Guy RH et al. Determining dermal absorption parameters in vivo from tape strip data. Pharm Res 2002; 19:292–8.
36. Faucher JA, Goddard ED. Interaction of keratinous substrates with sodium lauryl sulfate. I. Sorption. J Soc Cosmet Chem 1978; 29: 323–37.
37. Ananthapadmanabhan KP, Yu KK, Meyers CL et al. Binding of surfactants to stratum corneum. J Soc Cosmet Chem 1996; 47:185–200.
38. Willis CM, Stephens CJ, Wilkinson JD. Epidermal damage induced by irritants in man: a light and electron microscopic study. J Invest Dermatol 1989; 93:695–9.
39. 39 Corsini E, Galli CL. Epidermal cytokines in experimental contact dermatitis. Toxicology 2000; 142:203–11.
40. 40 Elias PM, Wood LC, Feingold KR. Epidermal pathogenesis of inflammatory dermatoses. Am J Contact Dermatitis 1999; 10:119–26.
41. Levin CY, Maibach HI. Irritant contact dermatitis: is there an immunologic component? Int Immunopharmacol 2002; 2:183–9.
42. Welzel J, Metker C, Wolff HH et al. SLS-irritated human skin shows no correlation between degree of proliferation and TEWL increase. Arch Dermatol Res 1998; 290:615–20.
43. Agner T. Noninvasive measuring methods for the investigation of irritant patch test reactions. A study of patients with hand eczema, atopic dermatitis and controls. Acta Derm Venereol (Stockh) 1992; 173 (Suppl.):1–26.
44. Pinnagoda J, Tupker RA, Coenraads PJ et al. Prediction of susceptibility to an irritant response by transepidermal water loss. Contact Dermatitis 1989; 20:341–6.
45. Allen MH, Wakelin SH, Holloway D et al. Association of TNFA gene polymorphism at position)308 with susceptibility to irritant contact dermatitis. Immunogenetics 2000; 51:201–5.

Summary

Understanding and quantifying dermal absorption of chemicals and identifying factors which govern this process is necessary for assessment of human health risk.
The project described in this thesis aimed at *(1)* generating *in vivo* human data by using different methodology and *(2)* investigating factors which govern absorption processes.

Dermal absorption was assessed for the following model chemicals: 2-butoxyethanol (BE), sodium lauryl sulphate (SLS) and polyethylene glycol oligomers (PEG) ranging in molecular weight (MW) from 150 to 590 Da. Due to its excellent lipophilic and hydrophilic properties, BE is a frequently used solvent in industry and household, and information on the percutaneous absorption of this chemical in humans *in vivo* is limited. SLS is an anionic surfactant which is a common constituent of detergents and soaps and dermal exposure to this chemical in everyday life is frequent. SLS is a potent skin irritant, and the extent of absorption might therefore contribute to the extent of irritation. PEG is a polymer frequently used in intestinal and corneal permeability research. The advantages of PEG as a model compound include its availability in a wide range of molecular weights and the fact that the solubility of individual oligomers is not confounded by the molecular weight.

Among factors which may affect the extent and rate of dermal absorption we investigated *(1)* the influence of water as a vehicle on dermal absorption of BE, *(2)* the influence of molecular size of PEGs on dermal absorption and *(3)* the role of skin condition in the absorption of SLS and PEGs in the skin of atopic dermatitis (AD) patients and in the absorption of PEGs in the skin compromised by SLS.

Chapter 2 describes the assessment of dermal absorption of BE by using two different methods: biological monitoring (BM) and microdialysis (***chapter section 2.1 and 2.2***).

Using BM method, dermal absorption of neat BE, 90 % and 50 % aqueous solution of BE was determined by measuring the concentration of BE in blood and of its major metabolite 2-butoxyacetic acid (BAA) in urine after dermal exposure and after inhalation exposure, the latter serving as a reference dosage. The average dermal absorption rates of neat, 90 % and 50 % aqueous solutions of BE as determined from the 24-hour excretion of BAA in urine amounted to 0.26 ± 0.17, 0.92 ± 0.60 and 1.34 ± 0.49 mg cm^{-2} h^{-1}. More detailed dermal kinetics could be deduced from the time course of BE concentration in blood. Using the linear system dynamics method based on mathematical (de)convolution, the dermal absorption rate as a function of

time was obtained. This enabled us to calculate the maximal absorption rate and the permeability coefficient. These two parameters are important because they are used for comparison with *in vitro* assays. In addition, mathematical predictive models are based on permeability coefficients. The permeability coefficients of 50% and 90% aqueous solutions of BE were $1.75 \pm 0.53 \times 10^{-3}$ cm h^{-1} and $0.88 \pm 0.42 \times 10^{-3}$ cm h^{-1}, respectively. The permeability coefficient of neat BE could not be determined because the concentrations of BE in blood were under the detection limit of the analytical method.

Microdialysis showed to be a useful technique for determination of dermal absorption kinetics. In this study, semi permeable microdialysis probe was inserted in the dermis under the exposed skin site, in parallel to the skin surface. This probe was continuously perfused with physiological solution and dialysate was collected at regular intervals for the analysis of BE. Although the respective permeability coefficients for 50% and 90% aqueous solutions of BE of $6.1 \pm 2.2 \times 10^{-3}$ cm h^{-1} and $2.5 \pm 2.3 \times 10^{-3}$ cm h^{-1} were higher than the values obtained by BM method, the enhancing effect of water was consistent in both studies. In addition to the permeability coefficient and maximum flux, which were also determined using the BM method, the microdialysis technique enabled determination of lag time and diffusion coefficient. The microdialysis study revealed a higher diffusion coefficient of more diluted solution BE, so the mechanism of action could at least be partly explained by the damaging effect of water on the lipid bilayers of the stratum corneum (SC).
Assessment of the recovery, i.e. part of the chemical that is sampled in the dialysate relative to the amount penetrated through the skin, is a specific problem of microdialysis. To overcome this problem we measured the excretion of BAA in urine from which the systemic absorption of BE was derived. In that way the amount of BE found in the dialysate could be adjusted for recovery. The microdialysis technique showed to be suitable for studying skin metabolism without interference from the systemic compartment. The dermal metabolism seemed to be low, the amount of BAA was approximately 1 % of the amount of BE in the same dialysate.

BE is readily absorbed through the skin and the results showed that dermal absorption of BE from water solution is increased markedly compared to neat BE. Even water addition as low as 10% led to an approximate four-fold increase in absorption rates. These findings are important for health risk assessment of occupational exposure to BE, since BE is commonly used in mixtures containing water. The dermal uptake of aqueous solutions of BE was substantial: assuming a 60- minute skin contact of an area of 1000 cm^2, the dermal uptake would be four

times higher than the pulmonary uptake of an 8-hour exposure at the occupational limit value for BE. The results clearly justify the introduction of a skin notation for BE. To explore the applicability of BAA as a biological indicator of exposure to BE we studied the excretion pattern of free and conjugated BAA after both inhalation and dermal exposure to BE (***chapter section 2.3***). The results revealed high intra- and inter-individual variation in conjugation of BAA varying from 2-100% of total excretion. The use of only free BAA as indicator of exposure in present BM programs will therefore lead to erroneous estimation of the internal dose. Since conjugation changes with time, the time and duration of sampling would influence the outcome. The result of our study indicated total BAA, due to lower inter-individual variability, as a superior biomarker of exposure over free BAA.

Measurement of dermal absorption *in vivo* by the BM method is difficult for certain classes of chemicals. The concentration of the chemical in body fluids and excreta can be too low to be determined, due to low systemic absorption or extensive metabolism. Since absorption through the SC is for most chemicals the rate limiting step, the concentration of the chemical absorbed in the SC can give an estimate of systemic absorption. This approach has been applied for the determination of dermal absorption of PEG. Existing methods for PEG determination, mostly high-performance liquid chromatography (HPLC) methods, were not sensitive enough to detect PEGs in the SC layers. For this purpose we developed a method for determination of PEG in the SC obtained from the volunteers' forearm by means of tape stripping (described in **Chapter 3**). The method was based on extraction of PEGs from the SC on the tape and subsequent derivatization of the hydroxyl groups, which enabled sensitive measurement by gas chromatography using electron capture detection (GC-ECD). Furthermore, previously reported methods used polydispersed mixtures where only indirect (relative) determination of individual PEGs was possible. In our method identification and quantification of PEGs were done for the first time directly, by using individual PEG standards. The developed method was used to study permeability of normal and compromised skin. To overcome poor reproducibility caused by non-uniform tape stripping we normalized the concentration of PEGs on each strip by the amount of protein. The protein content was also used to determine the SC thickness and depth of each tape strip sample. This is a more reliable approach than using only tape strip number.

In **Chapter 4** absorption of model chemicals was determined in normal and compromised skin by means of the tape stripping technique. After end of exposure the whole SC was removed subsequently by adhesive tapes on which the amount of the chemical was determined. The penetration parameters, i.e. diffusion coefficient and partition coefficient, were determined by best-fit regression of the concentration of SLS or PEGs as a function of relative SC depth using an approach based on Fick's second law of diffusion. In healthy subjects the diffusion coefficient for SLS was 6.2 ± 3.0 x 10^{-9} cm^2 h^{-1} and for PEGs it ranged from 1.9 ± x 10^{-9} (590 Da) to 8.4 ±x 10^{-9} cm^2 h^{-1} (150 Da). The partition coefficient was 196 for SLS and it ranged from 1.66 (150 Da) to 1.87 (590 Da) for PEGs.

AD patients showed increased trans-epidermal water loss (TEWL): 8.4 ± 4.3 g m^{-2} h^{-1} compared to 6.3 ± 2.0 g m^{-2} h^{-1} in controls. Given the similar SC thickness in both groups this implicates a less effective barrier for water. The diffusion of both SLS and PEGs through uninvolved AD skin was enhanced, being twice as high as through normal skin, while the partition coefficient between the SC and water was 30 % and 50 % lower, respectively. The observed enhanced diffusion and lower partitioning tended to be more pronounced in patients with active AD compared to those with inactive AD. This indicates that state of disease influences permeability of the skin which is visibly not affected by AD.

Absorption of PEGs into the SC was also investigated in the skin compromised by SLS (***chapter section 4.3***). The SLS pre-treatment caused moderate barrier impairment: the TEWL increased from 6.3 to 17.9 g m^{-2} h^{-1}. The skin compromised by SLS showed both an increased diffusion and partitioning of PEGs into the SC.

As expected, the diffusion of PEGs decreased with the MW in normal skin, skin of AD patients and in SLS-compromised skin. The gradual decrease of diffusion with increasing molecular weight is in agreement with recent findings that hydrophilic chemicals show less strong dependence of diffusion on the molecular weight than lipophilic chemicals. This might support existence of two different transport pathways through the SC for hydrophilic and lipophilic chemicals.
The partition coefficient showed no MW dependence in normal and AD skin; however, in the skin compromised by SLS the partitioning showed an unexplained increase with increasing MW.

These studies, are the first to have experimentally shown *in vivo* that the barrier for chemicals other than water is altered in the visibly not affected skin of AD patients.

Chapter 5 addressed the relation of the SLS penetration rate into the SC to both, the impairment of the water barrier and the inflammation of the skin. The penetration of 1% SLS into the SC was assessed in healthy volunteers by means of the tape stripping procedure (chapter 4). The volunteers were also exposed to 1% SLS for 24 hours to induce damage to the skin barrier and inflammation, which were assessed by measurement of TEWL and erythema, respectively.

The extent of barrier impairment and inflammation appeared to depend on individual penetration rate. Furthermore, TEWL, erythema and penetration rate of SLS were higher in atopics than non-atopics. The atopic status was assessed by Erlangen Atopy Questionnaire.

This study supports the assumption that the more permeable the skin is the more susceptible it is to local effects of chemicals.

Based on the findings in the studies described in this thesis several recommendations were formulated referring to methods of volunteer studies, effects of chemical mixtures and importance of skin condition for dermal absorption.

Samenvatting

Het begrijpen en kwantificeren van opname van chemische stoffen door de huid en het identificeren van de factoren die dit proces beïnvloeden is noodzakelijk voor het vaststellen van gezondheidsrisico's voor de mens.

Het doel van het onderzoek dat beschreven wordt in dit proefschrift was *(1)* het genereren van *in vivo* onderzoekgegevens bij mensen door gebruik van verschillende onderzoeksmethoden, en *(2)* onderzoek naar de factoren die absorptie door de huid beïnvloeden.

Als modelstoffen is gebruik gemaakt van 2-butoxyethanol (BE), van natriumlaurylsulfaat (SLS) en van oligomeren van polyethyleenglycol (PEG) die in molecuulgewicht variëren van 150 tot 590 Dalton (Da). Dankzij zijn uitstekende lipofiele en hydrofiele eigenschappen is BE een veel gebruikt oplosmiddel in zowel industriële als huishoudelijke toepassingen; tegelijk is informatie over de dermale absorptie *in vivo* van deze stof bij mensen schaars. SLS is een surfactans dat veel wordt gebruikt als bestanddeel van detergentia en zeep; in het dagelijks leven komt de huid veelvuldig met deze stof in aanraking. SLS is sterk irriterend voor de huid, en het is goed denkbaar dat de mate van absorptie bijdraagt aan de mate van irritatie. PEG is een polymeer, dat veel wordt gebruikt bij onderzoek naar de permeabiliteit van de darmen en van het hoornvlies. Het voordeel van PEG als modelstof is dat het verkrijgbaar is in een ruim bereik van molecuulgewichten en het feit dat de oplosbaarheid van de verschillende oligomeren niet door het molecuulgewicht wordt beïnvloed.

Van de factoren die een rol kunnen spelen bij de dermale absorptie is onderzocht: *(1)* de invloed van water als vehikel bij dermale absorptie van BE, *(2)* de invloed van de molecuulgrootte van PEG's op de dermale absorptie en *(3)* de rol die de conditie van de huid speelt bij de absorptie van modelstoffen: SLS en PEG's in de huid van patiënten met atopische dermatitis (AD) en PEG's bij gezonde personen na behandeling van de huid met SLS.

In **hoofdstuk 2** wordt een beschrijving gegeven van de bepaling van dermale absorptie van BE volgens twee onderling verschillende methoden: biologische monitoring (BM) en microdialyse (**hoofdstuk 2.1 en 2.2**).

Door middel van BM werd de dermale absorptie bepaald door het gehalte te meten van 2-butoxyazijnzuur (BAA) in urine en BE in bloed na blootstelling via de huid en na inademing. De laatste werd gebruikt als referentiedosis. Uit de 24-uurs excretie

van BAA in urine kon alleen de gemiddelde dermale absorptie gedurende de hele blootstellingsperiode worden berekend. Deze absorptie bedroeg voor onverdunde BE 0.26 ± 0.17 mg cm^{-2} h^{-1} en voor 90% en 50% waterige oplossingen van BE respectievelijk 0.92 ± 0.60 en 1.34 ± 0.49 mg cm^{-2} h^{-1}. Meer gedetailleerde gegevens van de dermale kinetiek konden worden afgeleid uit het tijdsverloop van de BE concentratie in bloed. Met behulp van lineaire systeemdynamica, gebaseerd op wiskundige (de)convolutie, werd de dermale absorptiesnelheid als functie van de tijd verkregen. Hierdoor konden de maximale absorptiesnelheid en de permeabiliteitscoëfficiënt berekend worden. Deze beide parameters zijn van belang omdat zij voor vergelijking met *in vitro*-onderzoeksgegevens worden gebruikt. Ook wiskundige predictiemodellen zijn op permeabiliteitscoëfficiënten gebaseerd. Bij gezonde personen werd een permeatiesnelheid gevonden voor waterige oplossingen van 50 % en 90 % BE van respectievelijk 1,75 ± 0,53 x 10^{-3} cm h^{-1} en 0,88 ± 0,42 x 10^{-3} cm h^{-1}.

Microdialyse lijkt een geschikte methode te zijn voor het bepalen van de dermale absorptiekinetiek. In deze studie werd de concentratie van BE en BAA gemeten in de halfdoorlaatbare microdialyse capillairen, welke intradermaal ingebracht waren parallel aan het huidoppervlak. De capillairen werden doorgespoeld met een fysiologische zoutoplossing die opgevangen werd voor het bepalen van de BE concentratie. De permeabiliteitscoëfficiënt bedroeg 6,1 ± 2,2 x 10^{-3} cm h^{-1} en 2,5 ± 2,3 x 10^{-3} cm h^{-1} voor waterige oplossingen van respectievelijk 50% en 90% BE. Hoewel deze waarden hoger zijn dan de waarden verkregen met de BM methode, was het versterkend effect van water consistent in beide studies. Naast de permeabiliteitscoëfficiënt en de maximale flux, welke ook zijn bepaald door middel van de BM methode, kon met microdialyse de lag time en de diffusiecoëfficiënt worden bepaald. De microdialyse studie liet een hogere diffusiecoëfficiënt zien voor de meer verdunde BE oplossing, waaruit blijkt dat het werkingsmechanisme gedeeltelijk verklaard kan worden door de beschadigende werking van water op de lipiden dubbellaag van het stratum corneum (SC).

Het bepalen van de fractie van de stof in het dialysaat in verhouding tot de hoeveelheid binnengedrongen door de huid (recovery), is een probleem dat eigen is aan deze techniek. Om dit probleem te ondervangen is de excretie van BAA in urine gemeten waaruit de systemische absorptie van BE werd afgeleid. Op deze wijze kon de hoeveelheid BE in het dialysaat worden geschaald. Deze methode blijkt ook geschikt voor het onderzoeken van metabolisme in de huid zonder verstoring door het systemische compartiment.

BE wordt gemakkelijk in de huid geabsorbeerd en de resultaten laten zien dat de dermale absorptie van BE vanuit een waterige oplossing sterk verhoogd is in vergelijking met pure BE. Zelfs bij toevoeging van slechts 10% water neemt de absorptiesnelheid met ongeveer een factor 4 toe. Deze bevindingen zijn van belang voor het bepalen van gezondheidsrisico's bij beroepsmatige blootstelling aan BE, aangezien BE veel wordt gebruikt in waterhoudende mengsels. De dermale opname van waterige BE-oplossingen is aanzienlijk: uitgaande van 60 minuten huidcontact met een oppervlak van 1000 cm^2 zou de dermale opname vier maal hoger zijn dan de opname via de luchtwegen bij een 8 uur durende blootstelling aan BE op het niveau van de grenswaarde voor de arbeid. Deze uitkomsten maken duidelijk dat het toekennen van een 'huidnotatie' voor BE nodig is.

Om de toepasbaarheid van BAA als biologische indicator van blootstelling aan BE te onderzoeken, is het excretiepatroon onderzocht van vrij en geconjugeerd BAA na zowel blootstelling via de inademing als dermale blootstelling aan BE (**hoofdstuk 2.3**). In beide blootstellingsscenario's neemt de mate van conjugatie toe in de tijd. De resultaten laten een grote intra- en interindividuele variatie zien van de conjugatie van BAA, uiteenlopend van 2 tot 100% van de totale excretie. Daarom zal het gebruik van uitsluitend vrij BAA als blootstellingsindicator in de huidige BM-programma's een onnauwkeurige schatting van de interne dosis tot gevolg hebben. Aangezien de mate van conjugatie in de tijd verandert, worden de uitkomsten bovendien beïnvloed door het tijdstip en de duur van de monstername. Er is aangetoond dat het totale BAA als gevolg van de lagere interindividuele variabiliteit, een betere biomarker van blootstelling is dan alleen vrij BAA.

Het *in vivo* meten van dermale absorptie met de methode van biologische beschikbaarheid is bij bepaalde categorieën van stoffen moeilijk. De concentratie van een stof in lichaamsvloeistoffen en uitscheidingsproducten kan te laag zijn om te worden gemeten, als gevolg van een lage systemische absorptie of intensieve stofwisseling. Omdat absorptie door het SC voor de meeste chemische stoffen de snelheidsbepalende stap is, kan de concentratie van de geabsorbeerde stof in het SC een schatting van de systemische absorptie opleveren. Deze aanpak is toegepast bij PEG. Echter, de bestaande methoden voor de bepaling van PEG – met name "hoge druk vloeistof chromatografie" methoden – blijken niet gevoelig genoeg voor de geringe hoeveelheid PEG in de dunne lagen van het SC. Daarom werd een methode ontwikkeld voor het bepalen van PEG's in het SC verkregen van de onderarm door middel van tapestripping (**hoofdstuk 3**). De methode is gebaseerd op extractie van PEG's uit het SC op de tape, gevolgd door derivatisering van de

hydroxylgroepen, die een gevoelige meting door middel van gaschromatografie met electron capture-detectie (GC-ECD) mogelijk maakt. Verder gebruikten eerder beschreven methoden polydisperse mengsels waarbij slechts indirecte (relatieve) bepaling van afzonderlijke PEG's mogelijk was. Bij onze methode werd de identificatie en kwantificatie van PEG's voor de eerste maal rechtstreeks uitgevoerd, door afzonderlijke oligomeren te gebruiken als standaarden. De ontwikkelde methode is toegepast in onderzoek naar de permeabiliteit van normale en beschadigde huid. Ter voorkoming van slechte reproduceerbaarheid ten gevolge van niet-uniforme tapestripping hebben we de concentratie van PEG's op elke strip genormaliseerd d.m.v. het eiwitgehalte. Het eiwitgehalte is eveneens gebruikt voor het bepalen van de dikte van het SC en de diepte van elk monster verkregen m.b.v. tapestrips. Deze aanpak is betrouwbaarder dan alleen gebruik te maken van de rangnummers van de strips.

Hoofdstuk 4 beschrijft het bepalen van de absorptie van modelstoffen in normale en aangetaste huid door middel van tapestripping. Na het einde van de blootstelling werd het SC met behulp van strookjes plakband geheel verwijderd en werd de hoeveelheid van een chemische stof op deze tapes bepaald. De penetratieparameters, d.w.z. de diffusiecoëfficiënt en de partitiecoëfficiënt, werden bepaald door middel van best-fitregressie van de concentratie van SLS of PEG's als functie van de relatieve diepte van het SC. Hierbij werd gebruik gemaakt van een aanpak die gebaseerd is op de tweede diffusiewet van Fick. De diffusiecoëfficiënt en de partitiecoëfficiënt voor SLS bedroegen bij gezonde personen respectievelijk $6{,}2 \times 10^{-9}$ cm^2 h^{-1} en 196. Voor PEG's varieerde de diffusiecoëfficiënt van $1{,}9 \times 10^{-9}$ cm^2 h^{-1} (590 Da) tot $8{,}4 \times 10^{-9}$ cm^2 h^{-1} (150 Da) en de partitiecoëfficiënt varieerde van 1,87 (590 Da) tot 1,66 (150 Da).

Bij patiënten met AD werd een toename van het transepidermaal waterverlies (TEWL) waargenomen: $8{,}4 \pm 4{,}3$ g m^{-2} h^{-1} vergeleken met $6{,}3 \pm 2{,}0$ g m^{-2} h^{-1} bij controlepersonen. Aangezien de dikte van het SC in beide groepen gelijk was, impliceert dit een minder effectieve waterbarrière. De diffusie van zowel SLS als PEG's door de symptoomvrije huid van AD patiënten neemt toe tot twee maal de diffusie via de normale huid, terwijl de partitiecoëfficiënt tussen het SC en water respectievelijk 30 en 50 procent lager blijkt. Een tendens is waargenomen dat de toename van de diffusiecoëfficiënt en de afname van de partitiecoëfficiënt meer uitgesproken zijn bij patiënten met actieve AD dan bij degenen met inactieve AD. Dit duidt erop dat het stadium van de ziekte van invloed is op de permeabiliteit van de huid die op het oog niet door AD is aangetast.

De absorptie van PEG's in het SC is ook onderzocht in door SLS aangetaste huid (**hoofdstuk 4.3**). De voorbehandeling met SLS veroorzaakte een matige verslechtering van de waterbarrière: de TEWL neemt toe van 6,3 tot 17,9 g m^{-2} h^{-1}. Bij de door SLS aangetaste huid is een toename van zowel de diffusie als de partitiecoëfficiënt voor PEG's waargenomen.

Zoals verwacht is de diffusie van PEG's afgenomen met het molecuulgewicht; dit geldt voor normale huid, huid van AD patiënten en door SLS aangetaste huid. De langzame afname van de diffusie bij toenemend molecuulgewicht is in overeenstemming met recente bevindingen dat hydrofiele verbindingen qua diffusie in mindere mate afhankelijk zijn van het molecuulgewicht dan lipofiele verbindingen. Dit impliceert dat er twee verschillende transportroutes door het SC voor hydrofiele en lipofiele stoffen zouden zijn.

In normale en door AD aangetaste huid blijkt de partitiecoëfficiënt niet afhankelijk van het molecuulgewicht. In de door SLS aangetaste huid vertoont de partitiecoëfficiënt een niet verklaarde toename met het molecuulgewicht.

Deze onderzoeken zijn de eerste waarbij experimenteel *in vivo* bij mensen is aangetoond dat de huidbarrière voor andere stoffen dan water in de symptoomvrije huid van de AD patiënten is veranderd.

In **hoofdstuk 5** wordt de relatie onderzocht tussen enerzijds de penetratiesnelheid van SLS in het SC en anderzijds de verslechtering van de waterbarrière en de ontsteking van de huid bij gezonde mensen. De penetratie van 1% SLS in het SC werd bepaald met behulp van tapestripping (hoofdstuk 4). Ook werden vrijwilligers 24 uur lang blootgesteld aan 1% SLS met het doel schade aan de huidbarrière en ontsteking te veroorzaken, die werden beoordeeld door bepaling van respectievelijk de TEWL en het optreden van erytheem.

De mate van barrièreverslechtering en ontsteking blijkt afhankelijk te zijn van de individuele penetratiesnelheid. Ook de waarden van TEWL, erytheem en SLS-penetratiesnelheid zijn hoger bij atopici dan bij niet-atopici. De atopische status werd bepaald met behulp van de Erlangen Atopie Vragenlijst.

Dit onderzoek ondersteunt de veronderstelling dat de huid vatbaarder is voor lokale effecten van stoffen naarmate de permeabiliteit toeneemt.

Op grond van de bevindingen van de onderzoeken die in dit proefschrift zijn beschreven, zijn enkele aanbevelingen geformuleerd met betrekking tot methoden van onderzoek met vrijwilligers, effecten van chemische mengsels en het belang van de conditie van de huid voor dermale absorptie.

Conclusions and recommendations

The results presented in this thesis, considered in the context of those achieved within the EDETOX project, help in focusing the scope of needed investigations and in defining our recommendations.

We strongly encourage performance of human volunteer studies

The present studies showed that well designed human *in vivo* experiments are a valuable tool for assessment of dermal absorption. They uses a physiologically and metabolically intact system, and no inter species extrapolation is needed. The preference for alternative methods, such as *in vitro* measurements, in vivo animal studies or predictive mathematical modelling, over human *in vivo* studies is usually argumented by ethical and practical considerations. However, recent methodology and sensitive measurement techniques allow for exposures at concentration levels which are far below current occupational exposure limits. Although we recognize that human volunteer studies are laborious, the value and necessity of in vivo human data more than compensates for it.

In vivo studies should be designed in such a way that absorption kinetics rather than systemic dose is derived

Most *in vivo* methods are based on measurement of total systemic absorption yielding only average dermal absorption rate into the skin. This might be sufficient for practical purposes, e.g. for biological monitoring (BM) of occupational exposure. Realising that skin absorption is a time dependent process, these data are poorly translatable to other exposure scenarios and are not suitable for evaluation of alternative methods, such as *in vitro* or mathematical predictive models. Determination of dermal absorption rate as a function of time by using, e.g. mathematical (de)convolution, should be preferred. As shown in the study on 2-butoxyethanol, this approach enables determination of detailed dermal kinetics including permeability coefficient, maximum skin flux and lag time. These absorption parameters are needed for human risk assessment and allow direct comparison with *in vitro* data and predictive mathematical models.

Additional work is needed to enable reliable determination of recovery before the microdialysis technique becomes suitable for wider application

Although microdialysis is a complex and relatively invasive technique, it showed its potential in studying influence of the vehicle on dermal absorption of a chemical. Additionally, as demonstrated in the presented study on 2-butoxyethanol, metabolism occurring in the skin can be measured by this technique without interference from the systemic compartment. However, assessment of recovery i.e. the proportion of the chemical that was recovered in the dialysate relative to the amount penetrated through the skin, remains one of the main problems of this technique.

Further research is needed in evaluation of the tape stripping technique with emphasis on standardization and development of a valid and feasible method for measurement of stratum corneum thickness

Tape stripping is a relatively non-invasive and simple technique for determination of dermal absorption, and it is not surprising that the interest in this technique is growing in the area of cosmetics, pharmaceuticals, and risk assessment. The value of this technique is in the fact that it measures diffusion coefficient and partition coefficient separately, which showed to be advantageous in studying absorption mechanism. However, at present there are no approved guidelines and the used procedures are poorly standardised. In addition, the measurement of the stratum corneum thickness is very error prone, which is a problem as it is essential for proper data analysis.

There is a need for further investigations of the effect of vehicle formulation and mixtures on absorption

Data on dermal absorption are usually obtained from studies with neat chemicals. As demonstrated in the study on 2-butoxyethanol, even a small addition of water to the application solution enhanced absorption of 2-butoxyethanol dramatically. Since in occupational settings and everyday life the skin is usually exposed to chemical mixtures, further research is necessary to understand the mechanism by which chemical mixtures affect dermal absorption.

There is a need for more in vivo data on dermal absorption of chemicals in damaged and diseased skin.

Data on dermal absorption through damaged skin in humans *in vivo* are missing. The studies on percutaneous penetration of two model chemicals demonstrated altered skin barrier in patients with atopic dermatitis even on the skin sites visibly not affected by disease. Higher dermal absorption was also demonstrated in the skin damaged by sodium lauryl sulphate. Compromised skin not only increases absorption, but would facilitate entrance of larger molecules such as proteins which normally would not be able to pass through the skin. This emphasizes the importance of continuous skin protection and maintenance of the skin barrier. Since a compromised skin barrier due to environmental damage or skin disease is not uncommon we would strongly encourage more studies on dermal absorption of compromised skin. The extent of dermal absorption is not only important for systemic effects, but as shown in this study, permeability of the skin plays an important role in the individual susceptibility to local skin effects of chemicals.

Due to lack of data on dermal absorption of chemicals and insufficient knowledge about the factors which influence this process, the setting up of quantitative exposure limits for dermal exposure is not possible yet.

For chemicals which are substantially absorbed through the skin, when possible, biological monitoring should be performed

The study on 2-butoxyethanol showed that systemic absorption due to dermal exposure can be substantial and may exceed the inhalation route which is traditionally seen as the most important entry route for chemicals in occupational setting. As long as there are no occupational exposure limits for skin exposure, when available, biological monitoring should be used. Sampling strategy by development and implementation of biological monitoring in occupational settings should take into account the differences between dermal and inhalation kinetics.

Publications

1. Kezic S, Jakasa I, Wenker M, *Determination of mandelic acid enantiomers in urine by gas chromatography and electron capture or flame ionisation detection,* J. Chromatogr. B. **738** (2000) 39-46

2. Kezic S, Jakasa I, Wenker M, Boogaard PJ, Monster AC, de Wolff FA, *Gas chromatography-electron capture determination of styrene-7,8-oxide enantiomers in human liver mocrosomes,* J. Chromatogr. B. **749** (2000) 265-74.

3. Jakasa I, Mohammadi N, Krüse J, Kezic S, *Percutaneous absorption of neat and aqueous solutions of 2-butoxyethanol in volunteers,* Int. Arch. Occup. Environ. Health. **77** (2) (2004) 79-84

4. Kezic S, Meuling MJW, Jakasa I, *Free and total urinary 2-butoxyacetic acid following dermal and inhalation exposure to 2-butoxyethanol in human volunteers,* Int. Arch. Occup. Environ. Health. **77** (2004) 580-586

5. Jakasa I, Calkoen F, Kezic S, *Determination of polyethylene glycol of different molecular weights in the stratum corneum*, J. Chromatogr. B.- Analyt Technol Biomed Life Sci **811** (2) (2004): 177-182

6. Jakasa I, Verberk MM, Esposito M, Bos JD, Kezic S, *Differences in percutaneous absorption in normal and atopic dermatitis skin in relation to molecular weight* J Invest Dermatol **125**-3 (2005): 612-612 (Meeting Abstract)

7. de Jongh CM, Jakasa I, Verberk MM, Kezic S, *Variation in barrier impairment and inflammation of human skin as determined by SLS penetration rate*, Br J Dermatol **154** (2006): 651-657

8. Jakasa I, de Jongh CM, Verberk MM, Bos JD, Kezic S. *Percutaneous penetration of sodium lauryl sulphate is increased in uninvolved skin of atopic dermatitis patients compared to control subjects* Br J Dermatol **(in press)**

9. Jakasa I, Verberk MM, Kezic S. *Increased permeability for polyethylene glycols of the skin compromised by sodium lauryl sulphate* (submitted to *Exp Dermatol*)

10. Jakasa I, Verberk MM, Esposito M, Bos JD, Kezic S, *Altered penetration of polyethylene glycols into uninvolved skin of atopic dermatitis patients* (submitted to *J Invest Dermatol*)

11. Korinth G, Jakasa I, Wellner T, Kezic S, Kruse J, Schaller KH, Percutaneous absorption and metabolism of 2-butoxyethanol in human volunteers: a microdialysis study *(submitted to Arch Toxicol)*

Acknowledgement

This thesis is the result of four years of work at Coronel Institute whereby I have been accompanied and supported by many people. It is with great pleasure that I now take the opportunity to express my gratitude to all of them.

First I would like to thank my direct supervisors dr. Sanja Kežić and dr. Maarten Verberk for their immense involvement and help in planning and executing all the work on time. Their encouragement and guidance helped in sorting my thoughts and changing the way of thinking in a totally new field. You always stepped in when needed. From shared experiences with other PhD students I realised that I was more then lucky to have such good supervisors and to be able to work in a relaxed atmosphere.

Dear Sanja, thank you for believing in me all the way. Without your help and support this thesis would not have been completed. You have pushed my limits to the very end. You taught me that there is always more that needs to be learned. Our talks, laughs, and time spent together outside of work gave me a sense of having a family not so far away. Hvala, najdraža teta.

Dear Maarten, thank you also for the help whenever something had to be written in Dutch, especially the Dutch version of the summary. Even when reading was such a painful task. Not talking to me in English after one year, helped to cross my boundaries and start speaking Dutch. Did I have a choice?! ("het" and "de" are still a mystery to me!)

I would like to thank my promoters professor Frank van Dijk and professor Jan D. Bos, who kept a critical eye on the progress of my work. Prof Bos, thank you for your advice and suggestions in the patient study. With no medical background, I found your help invaluable. Frank, you didn't strike me as a stereotypical department head when I first met you. But your informality, openness and interest in my work will always be remembered. I wish to thank you one more time for the critical reading of the last (final) text.

I would like to thank my colleague Cindy de Jongh. My roommate: all the laughs, talks and discussions helped in melting some of the stress we both had. You helped me a great deal in sorting out a "Nederlandse samenvatting" after so many changes. Dear Cindy, good luck with your dissertation. I am sure, knowing you as a

perfectionist, that your thesis will be a great one. But don't forget the text discreetly placed on our desk (by whom!?!) that we looked at every day (see Stelling).

I would like to thank Nadira Mohammadi, Florentine Calkoen and Carien Witthagen for the all the analyses of samples. I wouldn't have managed it all without your help. Dear Nadira, thank you for the joyful time during our working together,, especially for your involvement in the developing of the stripping technique and chromatographic method. Florentine, I realise that life with highly demanding AIOs is not the easiest task, so thanks for your patience. Dear Carien, your constant good mood helped in surviving all that "stripping" work. I wish you all the success in your new job.
Aart Monster, we really worked nicely together. We had not even a single exposure experiment fail. Your technical assistance was very valuable to me. Enjoy your retirement.

My thanks also go to Jaap Kruse for the valuable lectures and discussions on permeability and mathematical modelling. Jaap, we still have one article to go! I am looking forward to it.

I am grateful to Herman Pieters, who was always willing to help whenever my computer or the lab computer decided to think for themselves. It was so frustrating sometimes.....Somehow you always managed to persuade them to work properly.

Thank you, dear Jelka, for editing most of the text in this thesis on a short notice.
I think that the last part of my thesis was as stressful for you as it was for me.

And of course, I would like to thank all my "proefpersonen". Without full cooperation and sticking to the instructions this thesis would not have been possible.

I also want to say thank you to my mother, grandmother and aunt for their support during my pursuit of higher education. They never pressured me about school and yet they managed to instil in me the need to become a well educated person. Without them none of this would have been possible. Dear Saša, thank you for all the support and for melting most of the stress during our 6 years apart.

Curriculum Vitae

Ivone Jakaša, born in 1971 in Zagreb

2002-2006	PhD student at Coronel Institute of Occupational Health, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
2001	PhD student at Department of Chemistry, University of Waterloo, Waterloo, Canada
2000	M.Sc. in Chemistry, Faculty of Science, University of Zagreb, Zagreb, Croatia
1998-1999	Working on experimental part of MSc thesis entitled "Determination of of chiral metabolites of styrene in biological material", Coronel Institute, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
1997-1998	Working as a chemical engineer in the Research and Field Department of Chromos-Paints and Varnishes, Zagreb, Croatia
1996	Dipl. Eng. in Chemistry, Faculty of Science, University of Zagreb, Zagreb, Croatia
1995-1996	Working on experiemental part of diploma paper entitled 'Determination of chlorinated aliphatic hydrocarbons in drinking water', Institute for Occupational Health and Medical research, University of Zagreb, Zagreb, Croatia
1985-1989	School for Catering and Hotel management, Zagreb, Croatia
1977-1985	Primary school in Zagreb, Croatia

Uitnodiging

voor het bijwonen van de openbare verdediging van mijn proefschrift

op woensdag 31 mei 2006
om 10.00 uur
in de Aula van de Universiteit van Amsterdam,
Oude Lutherse Kerk
Singel 411 (hoek Spui)
in Amsterdam

Receptie na afloop van de promotie

Het Spui is goed bereikbaar met de tramlijnen 1, 2 en 5, of lopend vanaf het Centraal Station (ca. 15 minuten)

Parkeren nabij het Spui is nauwelijks mogelijk. Er zijn parkeergarages op 15 minuten loopafstand: Muziektheater/Bijenkorf/Europarking

Ivone Jakaša
Weesperzijde 80-E
1091 EJ Amsterdam
020-5663249
i.jakasa@amc.uva.nl

Stellingen

behorende bij het proefschrift

"Dermal absorption of chemicals through normal and compromised skin"

1. The value and necessity of *in vivo* studies outweigh their laboriousness and price *(this thesis)*

2. Use of dermal absorption data of neat chemicals to predict the risk of dermal exposure to mixtures, as usual in current risk assessment, will result in wrong conclusions *(this thesis)*

3. "Is there anything that the skin can't do? In short, the question is impossible to answer in any condensed form. Besides, we live in many skins, from head to foot" *(Albert M. Kligman. In: What is the true function of skin? Experimental Dermatology 2002:11:159–180)*

4. The measurement of dermal exposure has many uncertainties , therefore biological monitoring when available should be the method of choice for the assessment of health risk of workers *(this thesis)*

5. Proper barrier function of normal looking skin should not be taken for granted *(this thesis)*

6. The more permeable skin is, the more susceptible it is to local effects of chemicals *(this thesis)*

7. "The need for accuracy must be weighted against the need for finality" *(Comments by the Justices of the U.S. Supreme Court bringing to a close the uncertain outcome one month after U.S. Presidential elections in 2000 between G.W. Bush and A. Gore)*

8. The "Inburgeringcursus" should start with: How to keep your "fiets" from being stolen

9. In pursuing the scientific career, one has to be prepared to live with suitcases in his/her hand

Ivone Jakaša, May 31, 2006