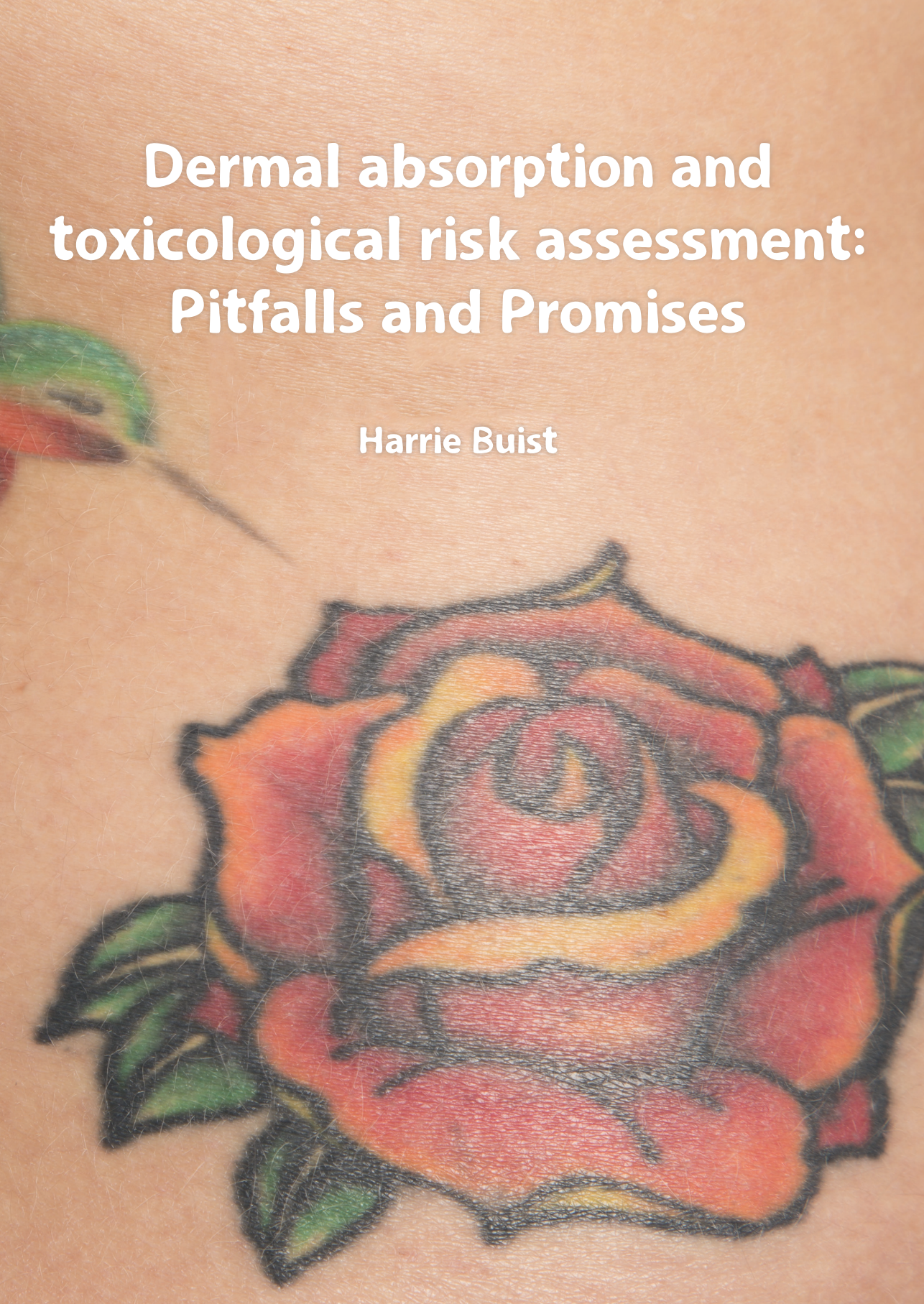
Dermal absorption and toxicological risk assessment: Pitfalls and Promises

Harrie Buist



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PITFALLS AND PROMISES

Harrie Buist

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Thesis

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1 Introduction

1.1 Background

1.1.1 Importance of the dermal route of exposure in risk assessment

Dermal contact is an important exposure route, as people are exposed to a variety of substances and products via the dermal route, either directly or indirectly while at work, at home or in public space (WHO, 2013).

Under occupational conditions, dermal exposure occurs mainly as a result of splashes, spills or drifts, during the application itself or from contact with contaminated surfaces. Pesticides, organic solvents and metalworking fluids are seen to be important contributors to adverse health effects due to occupational exposure via the dermal route (WHO, 2013).

In non-occupational settings, cosmetics, clothing and household products are the most relevant commodities with respect to dermal exposure, because of their conditions of use. For example, the use of cosmetics and clothing results in repeated direct skin contact, often of a large body surface area over a prolonged period of time (WHO, 2013).

In the context of dermal exposure, three different types of toxicological effects can be distinguished: local effects, sensitization and systemic effects (WHO, 2013).

Local effects are effects that occur at the portal of entry without the toxic compound becoming systemically available (ECHA, 2012a). Examples of local effects are skin irritation and skin tumours. By definition, absorption is of no relevance for these effects, although penetration of one or more skin layers may be relevant, e.g. in the case of tumours of the *dermis*, which lies immediately below the *epidermis* (see section 1.1.2 for more details on skin anatomy). In the latter case, comparative dermal penetration data (between tested animal and human), preferably including skin distribution, may be of importance in risk assessment in order to extrapolate animal dermal toxicity data to human exposure conditions.

Sensitizing effects are allergic responses in susceptible individuals, in which, after a first initialising (sensitising) dermal exposure, subsequent dermal exposures may cause allergic contact dermatitis or atopic dermatitis, characterised by symptoms such as erythema, oedema, and vesiculation (ECHA, 2012b). At first contact (the induction phase of skin sensitization), a chemical allergen enters the viable *epidermis*, after passing the *stratum corneum*, and forms a stable association with a protein ("haptenation"), which leads to the local secretion of proinflammatory cytokines and other signal proteins (Kimber *et al.*, 2011). The signal proteins activate Langerhans cells, which take up the haptenated allergen and move it from the *epidermis* to draining lymph nodes, where they initiate the cellular interactions leading to sensitization. Adverse health effects now may be initiated by renewed exposure of the sensitized person to this allergen, as allergen-specific T-cells gather at the place the chemical entered the viable *epidermis*, become activated and initiate

the immune reactions that lead to the symptoms of allergic contact dermatitis (elicitation phase). Both in the induction and the elicitation phase, the chemical allergen only needs to pass the *stratum corneum* and reach the viable *epidermis* to induce the adverse effect, without the need to become systemically available. In general, skin penetration data are not used in risk assessment with respect to skin sensitization, as in most cases the available data do not allow a quantitative assessment (ECHA, 2012). For sensitizers for which dose response data are available (mainly Local Lymph Node Assays aimed at establishing EC_3-values), the contribution of the degree of epidermal bioavailability to their potency is not clear (Jaworska *et al.*, 2013), which hampers the extrapolation of animal sensitization data to human exposure conditions or the distinction between one human exposure condition and another.

Systemic effects are effects that normally occur away from the portal of entry, after a substance has passed a physiological barrier like the skin, and has become systemically available (ECHA, 2012), i.e. has entered systemic circulation. Once a substance has passed from the portal of entry into the systemic circulation it is considered to be absorbed (WHO, 2006). This means that the degree of absorption of a substance is one of the determinants of its potency to cause systemic effects. Since the degree of absorption of a substance may vary with species, skin type and exposure conditions (section 1.1.5), dermal absorption data are of importance in toxicological risk assessment to extrapolate experimentally determined external dose levels to the expected levels of internal exposure. Furthermore, since in many cases mainly oral animal toxicity data are available, extrapolating oral animal toxicity data to human dermal exposure conditions is another issue that needs to be solved in dermal risk assessment. Also in this situation, data on absorption are needed. In some cases, when animal dermal toxicity data are available, risk assessment involves translating animal dermal exposure conditions to the expected human dermal exposure conditions, in which case comparative absorption data (for tested animal and exposed human) are needed. This thesis focuses on further developing, validating and improving methods to use dermal absorption data in the risk assessment of systemic toxic effects.

1.1.2 Skin structure

The outer most layer of the skin is the *epidermis* (Figure 1-1). It mainly consists of keratinocytes, cells that are generated by the innermost unicellular layer of basal cells (the *stratum germinativum*) by mitotic cell division and subsequent differentiation. From the thus formed *stratum spinosum*, keratinocytes move to the next layer of cells, the *stratum granulosum*. All these epidermal layers consist of living cells. The *epidermis* is topped by the non-living *stratum corneum*, which consists of cells called corneocytes. Corneocytes are mainly composed of keratin, elongated protein molecules interlinked by disulphide bridges. They possess a protein-rich cornified cell envelope and are linked to and surrounded by non-polar extracellular lipids. In humans, the *stratum corneum* is usually 15-20 cells thick (approximately 10-50 µm). Corneocytes are lost from the outer side of the *stratum corneum*

by a process called desquamation. The turnover time of keratinocytes is estimated to be 17 to 71 days, dependent on the region of the skin: e.g. 32-36 days for the human palm and 58 days for the anterior surface of the forearm (WHO, 2006).

The *epidermis* possesses two other types of cells, melanocytes and Langerhans cells. These are so called dendritic cells because of their branch-like extensions. The melanocytes are located directly next to the *stratum germinativum* and produce melanin, the main skin pigment. The Langerhans cells are situated in epidermal layers; they contain xenobiotic metabolising enzymes and have a function in the immune reactions of the skin (e.g. allergic reactions to sensitizers, see section 1.1.1). (WHO, 2006)

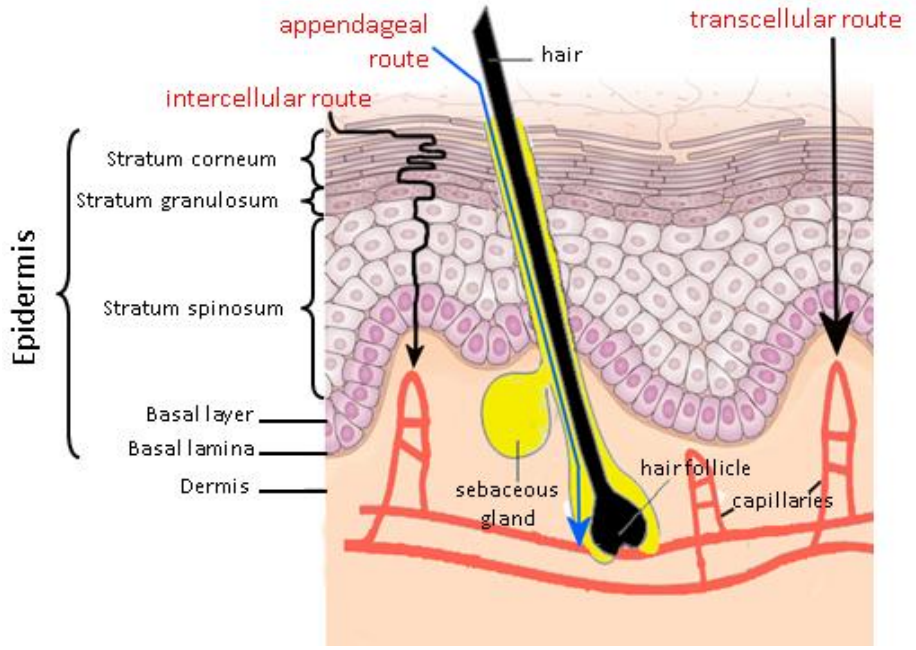

Figure 1-1 The skin and routes of absorption (based on figures published by Solanas & Aznar (2013) and Patient.co.uk (http://www.patient.co.uk/diagram/skin-cross-section-diagram).

Below the *epidermis*, the *dermis* is located which feeds the *epidermis*, which does not possess any vasculature. This skin layer is approximately 0.25 cm thick and consists mainly of a fibrous protein matrix (with a.o. collagen) embedded in an amorphous colloidal ground substance (WHO, 2006).

In the *dermis* also the sensory organs, blood vessels and lymph vessels of the skin are found. Two distinct layers can be observed in the *dermis*: the papillary *dermis*, directly underneath the *epidermis* and the reticular *dermis* (Young *et al.*, 2014). The papillary *dermis* contains the capillary network feeding the *epidermis*, while the reticular *dermis* contains coarse collagen

and elastic fibres and the larger vessels that transport blood in and out of the capillaries in the papillary *epidermis* (Young *et al.*, 2014).

The skin bears a number of appendages like sweat glands, sebaceous glands and hair follicles. The appendages all originate from the reticular *dermis* from where they cross the papillary *dermis* and the *epidermis* to terminate in an orifice at the outer skin surface (*stratum corneum*) enabling secretion of their products to the skin surface (or, in the case of hair follicles, to allow their hair filament to protrude from the skin) (WHO, 2006).

1.1.3 The process of dermal absorption

Dermal absorption is defined as the passage of chemicals from the outside of the skin into the systemic circulation (OECD, 2004a). It can be divided into three processes. The first is penetration, which is the entry of a substance into a particular skin layer or structure, the second is permeation, which is the passage of a substance through one layer of the skin into another and the third is resorption, which is the uptake of a substance into the dermal blood or lymph capillaries, thus entering directly or indirectly[1] systemic circulation (WHO, 2006).

In principle, there are three main pathways of skin absorption (Figure 1-1) (WHO, 2006):

1. The transcellular route
2. The intercellular route
3. The appendageal route

The surface area of the orifices of the appendages is relatively small (approximately 0.1 to 1% of the total skin surface). Therefore, the appendageal route is probably of little importance for most chemicals. Furthermore, the cornified envelope of the corneocytes is relatively impenetrable to most compounds hampering the transcellular route and rendering the intercellular route via the convoluted pathway of extracellular lipids between the corneocytes the major route of permeation of the *stratum corneum* (Figure 1-1) (WHO, 2006).

The crossing of the *stratum corneum* is mainly driven by passive diffusion, as active transport and facilitated diffusion by carrier proteins does not take place in this layer of dead cells (Jepps, *et al.* 2013). Since the matrix between the corneocytes consists mainly of apolar lipids, the *stratum corneum* is the main barrier for hydrophilic substances. On the other hand, the viable *epidermis* and the *dermis* are more likely to be the rate limiting barrier for lipophilic substances.

In the viable parts of the skin, active transport and facilitated diffusion may play a role in the absorption process. However, although the expression of many carrier proteins has been

[1] The lymph enters into the venous blood flow via large collecting lymph vessels (the thoracic duct and the right lymph duct), at the junction of the left or right internal jugular and subclavian veins (Guyton 1991a).

observed in the viable skin, so far no studies on the kinetics and thus of the importance of their contribution to skin absorption have been published (Jepps, *et al.* 2013). Until now the focus of research has been on passive diffusion as the most important driver of skin absorption.

Diffusion of substances across membranes is a chance process driven by the random thermal motion of molecules and a concentration gradient in the direction of their lower concentration (Eckert and Randall 1988). The rate of diffusion at steady state of a solute is described by Fick's first law, modified for membranes assumed to be pseudo-homogeneous (WHO, 2006):

$$-\frac{dM}{dt} = C \times k_p \times A$$ **Equation 1-1 Fick's first law**

in which

$-\frac{dM}{dt}$ = mass flux across the membrane in mg/h (expressed as decrease at the outer side),

C = the concentration of the substance in the vehicle in mg/cm^3 (assuming ideal sink conditions, at which the concentration underneath the membrane will be virtually zero).

k_p = permeation constant in cm/h and

A = the exposed skin area in cm^2.

It is assumed that the maximum flux of a permeant from saturated solutions across the skin is not dependent on the nature of the vehicle, unless the vehicle changes membrane permeability. Commonly, the *stratum corneum* is the rate limiting barrier and controls the rate of absorption. Therefore, when comparing permeant fluxes from different vehicles, the vehicle effect can be considered a change in the vehicle/*stratum corneum* partition coefficient $K_{SC/veh}$, which greatly influences the velocity with which a chemical penetrates the *stratum corneum*. The better soluble the penetrant in the vehicle, the more probable is its retention in the vehicle. This is described by Equation 1-2 in section 1.1.4.6.3 on page 30.

A more elaborate treatment of the mathematics of skin absorption can be found in section 1.1.4.6.3 (page 30 and further) and chapter 6.

1.1.4 Methods to estimate dermal absorption

The ultimate goal of measuring dermal absorption for toxicological risk assessment is to have a quantitative measure of human systemic exposure. Therefore, the studies most closely approaching real life human exposure are *in vivo* studies with humans, and the studies farthest away are *in vitro* studies with synthetic membranes. In this section the methods to measure dermal absorption of substances are discussed in decreasing order of resemblance with the human exposure situation.

1.1.4.1 In vivo human studies

In *in vivo* human studies the potential permeant is normally applied on the forearm or back skin, while whole-body exposure may be applied for vapours. There are basically two different parameters used to measure absorption in humans *in vivo*: systemic absorption or local penetration/permeation at the skin application site. For the former, the amounts of permeant and/or its metabolites present in plasma, excreta and exhaled air are determined (systemic recovery). Relative absorption can then be estimated as follows (WHO, 2006):

1. by comparing dermal systemic recovery with recovery after i.v. administration (100% systemic "absorption"),
2. by comparing recovery from the *stratum corneum*, excreta and exhaled air with the amount applied (mass balance method),
3. by comparing dermal systemic recovery with data from another administration route (e.g. oral).

Using these methods, systemic availability can be ascertained or at least approached, but k_p cannot be directly measured.

There are three experimental approaches to determine local penetration and/or permeation: cutaneous microdialysis, tape-stripping and dislodgeable dose (WHO, 2006).

Cutaneous microdialysis uses perfused dialysis to collect substances from the extracellular space beneath the skin for subsequent chemical analysis (WHO, 2006). One of the analytes can be a permeant that has been applied to the skin. The microdialysis appliance consists of microinjection pumps and microdialysis probes with semipermeable membranes. The probes can be inserted into blood vessels, the *dermis*, or the subcutaneous tissue. Receptor fluid passing through the microdialysis tubing represents the blood flow beneath the skin surface and collects the compounds diffusing from the surrounding tissue into the probes.

This method is, amongst others, applied to study dermal absorption kinetics. The amount of permeant recovered by microdialysis is only a small proportion of the amount present in the tissue, and results are usually expressed in terms of relative recovery. Therefore, microdialysis is principally of importance to compare the relative influence of different formulations and permeation enhancers on dermal absorption of specific compounds, e.g. drugs (Azeredo, *et al.* 2014). As long as recovery cannot be reliably established, it cannot be used to determine systemic bioavailability (Azeredo *et al.*, 2014) or a k_p.

Tape stripping of human *stratum corneum* is often applied to assess skin penetration and barrier function (WHO, 2006). The method is simple, cheap, and hardly invasive. The permeant is applied to a limited skin area for a certain period of time. Subsequently, the exposed *stratum corneum* is removed by repeated application and removal of strips of adhesive tape, and the mass of permeant on the tape strips is analysed.

One method to quantify k_p uses the concentration-depth profile of the permeant in the *stratum corneum* (WHO, 2006). By measuring the amount of *stratum corneum* on each tape

strip, the thickness of the skin layer removed can be assessed. The concentration-depth data are fitted into a solution of Fick's second law (Equation 1-5, page 31) to estimate the *stratum corneum*/vehicle partition coefficient ($K_{SC/veh}$) of the permeant and its diffusivity (D). Using the formula $k_p = K_{SC/veh} \cdot D_{SC}/L_{SC}$ (WHO, 2006), in which L_{SC} = thickness of the *stratum corneum,* k_p can then be calculated when the thickness is measured or assumed (e.g. on the basis of measurements as described by Xiao & Imhof (1997)).

An example is the study by Herkenne *et al.* (2008) on ibuprofen: for this drug, based on fitted concentration profile data, a k_p of 0.024 cm/h can be calculated[2] when dissolved in propylene glycol/water (25/75 v/v), while the EDETOX database lists a value of 0.036 cm/h for water as a vehicle and 0.088 cm/h for phosphate buffer of pH=3, values that are quite comparable. It should be noted that when using this method in human volunteers, usually no radiolabel can be applied and therefore sensitive analytical methods need to be available, certainly when the permeant hardly penetrates.

Another, simpler approach is one in which the permeant is applied and left on the skin for a certain period of time and then the amount washed off as well as the amount collected on tape strips after the washing procedure is determined (WHO, 2006). The amount absorbed is calculated by subtracting the sum of the amounts in the skin wash and the tape strips from the amount applied. This method is only reasonably precise when the amount absorbed is in the same order of magnitude as the amount applied.

1.1.4.2 In vitro studies with human skin

In *in vitro* studies with human skin, an excised piece of skin is used to measure permeation

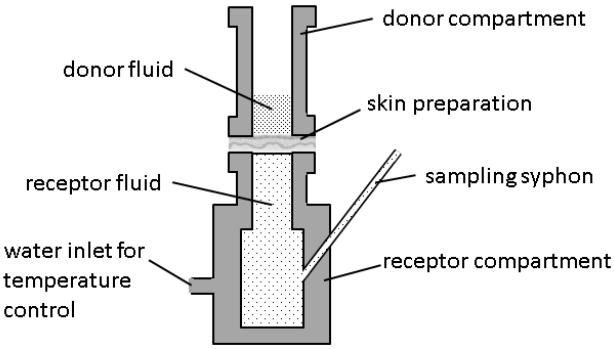

Figure 1-2 Franz cell for *in vitro* dermal absorption measurements (modified from Karpanen *et al.* (2008))

[2] The article lists a partition coefficient $K_{SC/veh}$ of 98 and a value of 0.12 h^{-1} for D/L_{SC}^2 (D= diffusivity of ibuprofen in the stratum corneum (SC) and L_{SC} is the thickness of the SC). These parameters were determined by fitting. The authors also determined L_{SC}^2 by transepidermal water loss measurements, but did not list their values. Therefore, I have used a default thickness of 20 µm based on Xiao & Imhof (1997). Since $k_p = K \cdot D/ L_{SC}$ (WHO, 2006), for ibuprofen, based on the tape strip data, it will be equal to 98 ($K_{SC/veh}$) × 0.12 h^{-1} (D/ L_{SC}^2) × 0.002 cm (L_{SC}) = 0.024 cm h^{-1}. It can be noted that nowadays confocal Raman spectroscopy is the gold standard method to measure SC thickness (Mateus, *et al.* 2013).

across the skin of chemicals applied to it (WHO, 2006). In 2004, the OECD has adopted and published a test guideline for *in vitro* dermal penetration (OECD, 2004b). The following descriptions are based on this guideline. The permeant is applied to the surface of a skin sample separating the two chambers of a diffusion cell, the donor compartment and the receptor compartment (Figure 1-2). These diffusion cells may vary from a simple "static" Franz cells to complex multi-jacketed flow-through cells.

The amount of permeant and/or its metabolites moving from the donor to the receptor compartment is measured over time. After a specified time, e.g. 8 or 24 hours, exposure is terminated by removing the permeant from the skin.

The static Franz diffusion cell is one of the most commonly used appliances to measure skin absorption *in vitro* (Figure 1-2). The receptor fluid beneath the skin is sampled at specific time intervals. The removed volume of receptor fluid is substituted by an equal volume of fresh receptor fluid. An important issue is the solubility of the permeant in the receptor fluid: A sufficient sink capacity has to be maintained to ensure that the concentration gradient across the skin is not significantly decreased by permeant accumulating in the receptor fluid. In flow-through cells receptor fluid is continuously replaced, which mimics *in vivo* blood flow. This method is particularly useful when a permeant hardly dissolves in the receptor fluid: The continuous replacement of receptor fluid maximises the concentration gradient. Skin permeability measurements using static and flow-through cells yield comparable results (Jakasa and Kezic, 2008).

By measuring, after exposure has been terminated, the amounts of permeant recovered from tape stripping and from the underlying viable skin also the penetration of the permeant into the *stratum corneum* and the underlying tissues can be determined. Often female abdominal and/or breast skin obtained from cosmetic surgery or autopsy is used, although cadaver skin from various body regions may be applied as well (WHO, 2006). Different skin preparations may be used (OECD, 2004b):

1. Full-thickness skin, including the *stratum corneum*, viable *epidermis*, and *dermis*.
   Systemic absorption may be underestimated as the *dermis* can act as a reservoir, especially for lipophilic substances (Jakasa and Kezic, 2008; Williams, 2006). Therefore, this skin preparation is less appropriate for regulatory use, unless the skin reservoir is included in the amount absorbed (the sum of the amounts in the receptor fluid and in the skin is often termed "potential absorption").
2. Dermatomed skin, from which the lower *dermis* has been removed, also called split-thickness skin.
   The excised skin is cut with a dermatome to obtain more uniform and reproducible preparations with respect to shape and thickness.
3. Epidermal membranes, consisting of the viable *epidermis* and the *stratum corneum*.
   The *epidermis* is separated from the *dermis* by applying heat, chemicals or enzyme treatment. The skin preparations used in chapter 6 are of this type. Epidermal membranes may overestimate human *in vivo* absorption (van de Sandt *et al.*, 2000).

4. Isolated *stratum corneum.*
   The *stratum corneum* is separated from epidermal membranes by treatment with trypsin. Isola-ted *stratum corneum* is principally applied in mechanistic studies and partition coefficient measurements.

The permeability coefficient k_p is determined (in of cm/h or cm/s) by dividing the steady-state flux (in µg/h per cm^2) by the permeant concentration (measured in µg/cm^3) in the vehicle present on the skin (WHO, 2006).

Steroids are well known for their skin reservoir effects. Another example of such a reservoir forming substance is didecyldimethylammoniumchloride (DDAC) (chapter 5). Depending on the physicochemical properties of the permeant and its interaction with skin components, e.g. proteins, it may form a reservoir in the *stratum corneum*, in the viable *epidermis*, or in the *dermis*. From the reservoir, the permeant may continue to diffuse further into the skin after exposure has terminated, in the end becoming systemically available via the lymphatic or sanguine capillary network in the *dermis* (WHO, 2006).

1.1.4.3 In vivo animal studies

The degree of dermal penetration and systemic absorption of the permeant can be assessed with greater precision in *in vivo* studies with animals than in studies with humans, since at the end of the study a full mass balance can be determined. *In vivo* dermal absorption studies are normally performed in rodents, mostly rats. Using rats has the advantage that other toxicity and toxicokinetic studies are mostly performed in this species. A major disadvantage is that animals have different skin permeability (Figure 1-4) and metabolism, distribution and excretion in comparison with humans. Also care must be taken to prevent the animals from directly or indirectly ingesting the permeant via grooming or the contamination of their surroundings.

In 2004, the OECD has adopted and published the guideline for determination of *in vivo* dermal absorption (OECD, 2004c). The following description is based on this guideline. The permeant is applied to 5-10% of the total skin surface in a suitable vehicle for a set period of time. The exposed area is often confined by a gauze covered ring to avoid spreading of the permeant and to ensure the animal does not tamper with the application site. Excreta are collected at set time intervals, and the amount of permeant and/or metabolite(s) in the samples is quantified. Expired air is also collected when excretion of permeant and/or its metabolites is to be expected via this route. As the degree of absorption is proportional to the exposure time, this time should be relevant to the scenario under consideration (usually 6 or 24 h). After exposure, the permeant is removed and excreta are continued to be collected until sacrifice of the animals.

Dermal absorption is normally expressed as a percentage of the dose applied, which is calculated by summing all amounts recovered from excreta, body fluids, expired air, and carcass, including the cleansed dose skin, and cage wash. Like in humans, occasionally tape

stripping is applied to study the process of penetration of the *stratum corneum*. Should the animals not be sacrificed, then an indirect measure of absorption can be used as is done in human volunteer studies (section 1.1.4.1).

In addition to OECD guideline 427 type of studies, also in animals cutaneous microdialysis may be performed to study dermal penetration (for a more elaborate description see section 1.1.4.1).

1.1.4.4 In vitro studies with animal skin

Instead of human skin, also animal skin can be used *in vitro*. The principles of the measurement are the same as described in section 1.1.4.2 for human skin. Rat skin is often chosen when the *in vivo* toxicity studies have been performed in rats, but also mouse, guinea pig and pig skin are frequently used. Furry animal skin is shaved before use to avoid non-uniform and badly reproducible dosing, and to better represent human skin (OECD, 2004b).

1.1.4.5 In vitro studies with artificial skin

Different types of synthetic skin equivalents have been developed. When used to simulate skin for instance for dermal absorption experiments in Franz cells, they are silicone based: e.g. polydimethylsiloxane (PDMS) or carbosil (Ng *et al.*, 2012). These membranes are hydrophobic, inert and do not show biological variation, which in principle makes them a good alternative for the *stratum corneum*. Karadzovska and Riviere (2013) compared absorption of five substances (cortisone, diclofenac, mannitol, salicylic acid and testosterone) by porcine skin mounted in diffusion cells with absorption values for three different artificial membranes (IPM, certramide and Strat-M™) determined using a high throughput technique with 96 wells plates. Correlation between porcine skin and artificial membrane absorption values was poor (R^2 = 0.38 to 0.56); the absorption values for artificial membranes tended to be lower.

Also living skin equivalent artificial models consisting of skin membranes grown in tissue culture are employed, these models may be monolayers (2D) or multi-layered, resembling more or less *in vivo* skin (3D) (Brohem *et al.*, 2011). However, percutaneous absorption cannot properly be assessed in 2D-models because they do not represent the functional physiology of the skin. 3D-models do represent this functional architecture, but are not able to form a well organised flat cornified epithelium (=*stratum corneum*) (Brohem *et al.*, 2011). Since the *stratum corneum* is the principal barrier of the skin (see a.o. WHO, 2006), also 3D-models are not a good option to measure skin absorption for regulatory purposes. This is confirmed by several researchers, e.g. Heylings *et al.* (2001) demonstrating that 3D reconstituted *epidermis* shows an abnormally high transepidermal water loss. Garcia *et al.* (2002) found that the permeability of reconstituted *epidermis* to caffeine is 20-25 times as high as that of normal human skin biopts, and Schmook *et al.* (2001) observed that the

permeability of a living skin equivalent and reconstituted *epidermis* for hydrocortisone, clotrimazole and terbinafine was several hundred times higher than for excised human skin.

1.1.4.6 In silico models

Besides actually measuring dermal absorption parameters for a specific permeant, they also may be calculated, or rather predicted, without doing experiments by applying *in silico* models. These models range from simple rules of thumb to complex models mimicking all toxicokinetic processes (absorption, distribution, metabolism and excretion) the permeant may be subjected to.

1.1.4.6.1 Rules of thumb

In Europe, simple rules of thumb to determine relative dermal absorption based on properties of the permeant are used as part of a tiered approach (see also section 7.7, Application in regulatory risk assessment).

In the REACH guidance (ECHA, 2012b), it is advised to use molecular weight and log K_{OW} of a chemical to estimate its dermal absorption in humans, when no acceptable experimental data on dermal absorption are available, based on a proposal published by de Heer *et al.* (1999): If its molecular weight is less than 500 and/or its log K_{OW} is between -1 and 4, dermal absorption is considered to be 100%, in all other cases the default is 10%. Although originally this approach was also used for pesticides within the legal framework for plant protection products (see e.g. EC, 2004), ECHA has redefined these rules of thumb, introducing the active substance concentration (= pesticide) as an additional criterion (ECHA, 2012b): If log K_{OW} < -1 or > 4 and MW > 500 a default dermal absorption value of 10% may be applied, else a value of 25% may be applied for products containing > 5% active substance and a value of 75% for products or in use dilutions containing ≤ 5% active substance.

Bos and Meinardi (2000) proposed the 500 Dalton rule for pharmaceuticals, stating that the molecular weight (MW) of a compound must be under 500 Dalton to permit skin absorption. This empirical rule was based on the observation that nearly all known contact allergens are smaller than 500 Dalton, that the most commonly used topical skin therapeutics are all under 500 Dalton, that all topical drugs used in transdermal drug-delivery systems are under 500 Dalton and that systemic cyclic immunosuppressants of ca. 800 Daltons and more are not effective when applied to the skin. However, this anecdotal evidence seems too weak a basis for such an absolute statement, principally because the dermal absorption of only very few drugs larger than 500 was assessed. Also the foremost *in vitro* dataset used in derivation of QSARs predicting dermal absorption, the Flynn database (section 1.1.4.6.2), does not contain many molecules greater than 500 Dalton (Figure 1-3). Moreover, the permeation constant data of the Flynn database do not show a clear trend towards an abrupt drop in absorption for molecules larger than 500 Dalton.

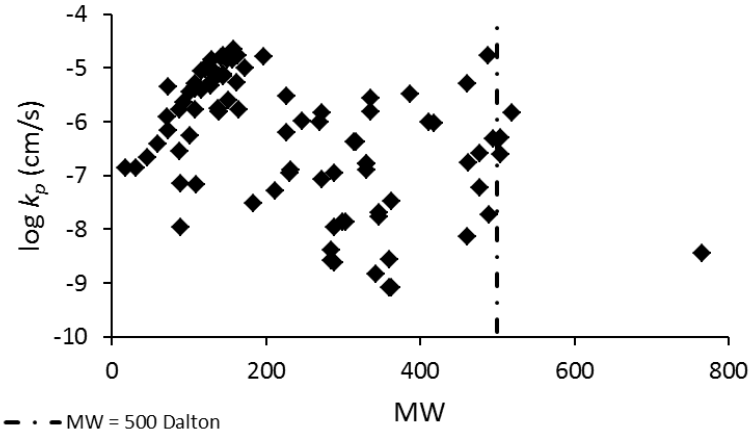

Figure 1-3 Flynn dataset as published by USEPA (2004)

1.1.4.6.2 QSARs

In general, Quantitative Structure Activity Relationships (QSARs) are quantitative predictions of some form of biological activity of chemicals based on one or more measures of their physicochemical and/or structural properties (Cronin and Schultz, 2003). Since strictly speaking, skin permeation is not a biological activity, but the result of a passive diffusion process (section 1.1.3), some authors use terms like Quantitative Structure Permeability Relationship (QSPR) or Quantitative Structure Permeation Relationship (QSPeR) (e.g. Geinoz *et al.*, 2004; Moss and Cronin, 2002). In this thesis the abbreviation QSPR will be used to indicate QSARs for dermal permeation parameters.

Most QSPRs developed to predict skin permeation of chemicals target the permeation constant (k_p), and a few maximum flux (J_{max}) (Mitragotri *et al.*, 2011). Occasionally, also QSPRs for percentage absorption have been developed (e.g. by Gute *et al.*, 1999), but although preferred from a regulatory point of view, they have limited value, since this parameter is highly influenced by exposure conditions like duration and dermal loading (see also section 1.1.5). Typically, QSPRs are based on training sets derived from *in vitro* dermal absorption experiments (Moss and Cronin, 2002).

A number of useful reviews of QSPRs have been published. Geinoz *et al.* (2004) provide an excellent historical overview of the different types of approaches used, combined with a statistical re-evaluation of landmark QSPRs and an appraisal of the most influential chemical properties determining the rate of dermal absorption. Also Moss *et al.* (2002) provide a good historical overview and parameter appraisal, and add a thorough discussion of the limitations the available database impose on QSPR development. Bouwman *et al.* (2008, 2005) evaluated over 30 QSPRs for their usefulness in regulatory risk assessment by assessing them against the OECD QSAR validation principles and tested the predictive quality of the QSPRs that fulfilled the OECD criteria against an external validation set of EDETOX and in-house TNO data. Mitragotri *et al.* (2011) presented a more succinct overview

of QSPRs, but also reported more recent developments and addressed the entire field of *in silico* models for skin permeation. External validation is the ultimate test of the quality of a QSAR (Cronin and Schultz, 2003), and besides Bouwman *et al.* (2008, 2005) also Lian *et al.* (2008) and Korinth *et al.* (2012) attempted such a validation, the former on five QSPRs using a dataset collected from public literature and the latter on 3 QSPRs using measured *in vitro* data of a varied set of 11 chemicals, generated in the same test system with the same protocol by one laboratory.

Table 1-1 lists the algorithms of the global QSPRs for aqueous solutions reviewed in the publications mentioned in the previous paragraph. The majority of these QSPRs relate permeation to the octanol-water partition coefficient (K_{OW}) and molecular weight (MW), indicating the importance of these physio-chemical properties in the permeation process. However, also hydrogen bonding, characterised by a number of different molecular descriptions, may play an important role (Geinoz *et al.*, 2004; Moss and Cronin, 2002). Also additional descriptors may be needed to fully predict skin permeation. As a matter of fact, when Degim *et al.* (2003) tried to develop a neural network predicting the permeation constants in the Wilschut database (Wilschut *et al.*, 1995), they could not find a significant solution based on MW and log K_{OW} alone, but when using the sum of the charges on the atoms in the molecule as an additional parameter, obtained excellent results. This is also illustrated by the bad performance of log K_{OW} and MW based QSPRs with external validation sets. All QSPRs predicting k_p tested by Bouwman *et al.* (2008, 2005) and Korinth *et al.* (2012) showed a correlation R^2 of less than 0.3 with the external validation set, although their datasets fell within the application domain of these QSPRs with respect to the input parameters (data not shown). The external validation by Lian *et al.* (2008) showed a much better performance of this type of QSPRs, but this was probably mainly due to the great overlap between the chemicals in their validation set and the training sets of the respective tested QSPRs: Seventy-five chemicals of the Flynn dataset published by USEPA (2004) are present among the 124 chemicals of the Lian *et al.* dataset. The Flynn database is most often used to derive the training set of QSPRs (Geinoz *et al.*, 2004). When the correlations of the predictions of the log K_{OW} and MW QSPRs evaluated by Lian *et al.* are calculated for the Bouwman *et al.* dataset, they all had a R^2<0.3 (unpublished results).

The QSPRs listed in using other molecular descriptors than log K_{OW} and MW, were dismissed for regulatory use by Bouwman *et al.* (2008), since they were considered not easily available to regulatory risk assessors. Chen *et al.* (2013) evaluated two QSPRs, one based on log K_{OW} and MW (Potts and Guy, 1992), one on solvatochromic parameters and three mechanistic brick-and-mortar models (see also section 1.1.4.6.3) against a dataset of hydrophilic molecules (defined as having a log K_{OW} <0.5). The QSPR models did not perform well (R^2<0.5), while the mechanistic models appeared to do reasonably well (highest R^2 = 0.60). However, for a number of molecules multiple values were present in the dataset (e.g. 16 values for water), giving more weight to these molecules in the analysis. When the correlations based on average log k_p values were calculated, also the performance of the

brick-and-mortar models was below par (highest R^2 = 0.48) (unpublished results). In view of the bad result of the QSPRs in external validation sets or the difficult access to the necessary molecular descriptors, their use in a regulatory setting is not feasible.

Another complicating factor for these QSPRs that are mainly derived by multiple linear regression, is that the relation between log k_p or J_{max} and at least some of the descriptors used is not always linear, e.g. Zhang *et al.* (2009) observed that J_{max} showed a more or less parabolic relationship with log K_{OW} for similar sized permeants, Magee (1998) (cited in Moss *et al.*, 2002) demonstrated that hydrogen bonding is more important in the most hydrophobic group of chemicals and Moss *et al.* (2011) showed that the relation between measured k_p-values and important properties determining permeation is non-linear.

New developments in QSPR development include neural network, nearest neighbour and Gaussian process models. These novel models are able to predict non-linear processes, and Gaussian process models have been shown to perform better than selected QSPRs (see Moss *et al.*, 2011) as have fuzzy models and artificial neural networks (Russell and Guy, 2009). However, they yet have to prove their practical value (Mitragotri *et al.*, 2011).

Little work has been done to include vehicle effects in QSPR predictions (Karadzovska *et al.*, 2013), most of it by Riviere and co-workers (see a.o. Ghafourian *et al.*, 2010a, 2010b; Guth *et al.*, 2014; Riviere and Brooks, 2005; Riviere *et al.*, 2014; Samaras *et al.*, 2012). Many of the QSPRs were based on Abraham descriptors with the addition of a mixture factor, a composite of descriptors like polarizability, refractive index and Henry's law constant. Each of these QSPRs was based on a small training set of less than 20 molecules, and all showed quite different mixture factors (Guth *et al.*, 2014; Riviere and Brooks, 2005; Riviere *et al.*, 2014). So it seems a global QSPR for vehicle effects is not within reach. Karadzovska *et al.* (2013) have published a recent overview on QSPR work on dermal absorption from different vehicles.

Table 1-1 List of global QSPRs for aqueous solutions.

Predicted parameter	Model	Input parameters	Reference
k_p	$\log k_p = -2.3 + 0.652\log K_{OW} - 0.00603MW - 0.623ABSQon - 0.313SsssCH$	ABSQon, K_{OW}, MW, SsssCH	Patel *et al.* (2002)
k_p	$\log k_p = -0.626\sum Ca - 23.8\sum(Q+)/\alpha - 0.289\ SsssCH - 0.0357SsssOH - 0.482I_B + 0.405B_R + 0.834$	$B_{R,}$ hydrogen bond descriptor ($\sum Ca$), I_B, $\sum(Q+)/\alpha$, SsssOH	Dearden *et al.* (2000)
k_p	$\log k_p = -2.724 - 0.00264MW \times charge + 0.59\log K_{OW}$	charge, K_{OW}, MW	Pugh *et al.* (2000)
k_p	$\log k_p = -0.07(\log K_{OW})^2 + 0.84\log K_{OW} - 0.27Hb - 1.84logMW + 0.8337$	Hb, K_{OW}, MW	Lien and Gao (1995)
k_p	If $-1.38 \le \log K_{OW} \le 1.96$: $\log k_p = 0.79 \log K_{OW} - 0.0371MR - 2.26$ If $1.53 \le \log K_{OW} \le 2.97$: $\log k_p = 1.05 \log K_{OW} - 0.0259MR - 3.24$ If $2.5 \le \log K_{OW} \le 5.49$: $\log k_p = 0.95 \log K_{OW} - 0.037MR - 0.107HBA - 2.92$	hydrogen bond descriptor (HBA), K_{OW}, MW, MR	Magee (1998) (cited by Gajewska *et al.*, 2014 in supplemental material)
k_p	$\log k_p = -1.36(3.54HBD - 0.37) - 3.39$	hydrogen bond descriptor (HBD)	Tayar *et al.* (1991) (cited by Geinoz *et al.*, 2004)
k_p	$\log k_p = 0.44R_2 - 0.49\pi_2^H - 1.48\Sigma\alpha_2^H - 3.44\Sigma\beta_2^H + 1.94V_x - 1.57$	Hydrogen bond descriptors ($\sum\alpha_2^H$, $\sum\beta_2^H$), R_2, V_x	Abraham and Martins (2004) (previous versions reported in Abraham *et al.* (1999, 1997, 1995))
k_p	$\log k_p = 0.0256MV - 1.72HD - 3.93HA - 1.29$	hydrogen bond descriptors (HA, HD), MV	Potts and Guy (1995)
k_p	$\log k_p = -0.6VEH - 0.0140MR - 0.157HBA - 0.29HBD - 0.140$	hydrogen bond descriptors (HBA, HBD), MR, VEH	Hostynek and Magee (1997) (cited by Geinoz *et al.*, 2004)
k_p	$k_p = 0.1\left[\frac{K_{OW}^{0.75}}{120 + K_{OW}^{0.75}}\right]$	K_{OW}	Brown and Rossi (1989)
k_p	$\log k_p = 0.820\log K_{OW} - 0.0093MV - 2.36 - 0.0039Mpt$	K_{OW}, Mpt, MV	Barratt (1995)
k_p	$\log k_p = 0.830\log K_{OW} - 0.0119MV - 2.31$	K_{OW}, MV	Geinoz *et al.* (2004)

Table 1-1 (continued)

Predicted parameter	Model	Input parameters	Reference
k_p	$k_p = \frac{k_{lip}}{1+\frac{k_{lip}\times\sqrt{MW}}{2.6}}$ $\log k_{lip} = -2.32 + 0.574\log K_{OW} - 0.005MW$	K_{OW}, MW	Cleek and Bunge (1993)
J_{max}	$J_{max} = \frac{C_{sat}}{15}\ (0.038 + 0.153K_{OW})\ e^{-0.016MW}$	K_{OW}, MW	Fiserova-Bergerova *et al.* (1990)
k_p	If MW < 150 and log K_{OW} < 0.5: log k_p = −3 If MW < 150 and log K_{OW} > 3: log k_p = −0.5 If MW < 150 and 0.5 ≤ log K_{OW} ≤ 3: log k_p = log K_{OW} − 3.5 If MW > 150 and log K_{OW} < 0.5: log k_p = −5 If MW > 150 and log K_{OW} > 3.5: log k_p = −1.5 If MW > 150 and 0.5 ≤ log K_{OW} ≤ 3: log k_p = log K_{OW} − 5.5	K_{OW}, MW	Flynn (1990) (cited in Geinoz *et al.*, 2004)
k_p	$\log k_p = 0.5356\log K_{OW} - 0.005227MW - 2.56$	K_{OW}, MW	Fujiwara *et al.* (2003)
k_p	$\log k_p = 0.93\log K_{OW} + 0.013MW - 2.11$	K_{OW}, MW	Geinoz *et al.* (2004)
k_p	$\log k_p = 0.8\log K_{OW} + 0.0108MW - 2.35$	K_{OW}, MW	Geinoz *et al.* (2004) (based on a subset (n=21) of the Flynn database, selected by El Tayar *et al.*, 1991)
k_p	$k_p = MW^{-2.404}\times\left[-5\cdot10^{-6}+\frac{0.0025}{-0.089+0.393\sqrt{K_{OW}}}\right]$	K_{OW}, MW	McKone and Howd (1992) (cited by Gajewska *et al.*, 2014 in supplemental material)
k_p	$\log k_p = 0.7\log K_{OW} - 0.0722MW^{3/4} - 1.6918$	K_{OW}, MW	Mitragotri (2002) (cited by Lian *et al.*, 2008)
k_p	$\log k_p = 0.766\log K_{OW} - 0.010MW - 2.281$ $\log k_p = 0.812\log K_{OW} - 0.04LMV - 0.230STW - 2.064$	K_{OW}, MW K_{OW}, LMV, STW	Moody and MacPherson (2003) The best fitting model is shown and the model with the easiest to obtain parameters
k_p	$\log k_p = 0.74\log K_{OW} - 0.0091MW - 2.39$	K_{OW}, MW	Moss and Cronin (2002)* (previous version published by Cronin *et al.*, 1999)

* The authors erroneously reported the unit of the k_p predicted by this algorithm to be cm/s instead of cm/h.

Table 1-1 (continued)

Predicted parameter	Model	Input parameters	Reference
k_p	$\log k_p = 0.71\log K_{OW} - 0.0061MW - 2.7$ $\log k_p = 0.74\log K_{OW} - 0.006MW - 2.8$ $\log k_p = 0.72\log K_{OW} - 0.0059MW - 2.8$	K_{OW}, MW	Potts and Guy (1992) Guy and Potts (1992) Geinoz *et al.* (2004)
k_p	$\log k_{pSC\text{-}intercellular} = 0.7318\log K_{OW} - 0.006832MW - 2.59$ $\log k_{pSC\text{-}transcellular} = -1.361\log MW - 1.367$ $k_p = k_{pSC\text{-}intercellular} + k_{pSC\text{-}transcellular}$	K_{OW}, MW	ten Berge (2009)
k_p	$\log k_p = 0.66\log K_{OW} - 0.0056MW - 2.80$	K_{OW}, MW	USEPA (USEPA, 2004, currently used in the DERMWIN skin absorption estimation software)
k_p	$\log k_p = 0.514\log K_{OW} - 0.0050MW - 2.44$ $\log k_p = 0.52\log K_{OW} - 1.58MW/T - 2.41$	K_{OW}, MW K_{OW}, MW, T	Vecchia and Bunge (2002b)
k_p	$\log k_p = 0.688\log K_{OW} + 0.181\sqrt{MW} - 1.526$	K_{OW}, MW	Wilschut *et al.* (1995) (cited by Gajewska *et al.*, 2014 in supplemental material)
k_p	$k_p = \left[\frac{1}{k_{lip} + k_{pol}} + \frac{1}{k_{aq}}\right]^{-1}$ $\log k_{lip} = 0.620\log K_{OW} - 0.159\sqrt{MW} - 1.286$ $k_{pol} = \frac{1 \cdot 10^{-10}}{\sqrt{MW}} k_{aq} = \frac{2.5}{\sqrt{MW}}$	K_{OW}, MW	Wilschut *et al.* (1995)
J_{max}	$\log J_{max} = -0.0141MW - 4.52$	MW	Magnusson *et al.* (2004)
k_p	$\log k_p = 0.0208\log V_e - 0.723N - 2.69$	N, V_e	Buchwald and Bodor (2001)

The unit for all models for k_p is cm/h. In case the original models had cm/s as unit, log 3600 (=3.56) has been added to the equation(s) for log k_p models and the equations for k_p have been multiplied with 3600. The unit for J_{max} of Fiserova-Bergerova is mg/(cm^2·h) and for Magnusson mol/(cm^2·h).

Table 1-1 Legend

ABSQon = is the sum of absolute charges on oxygen and nitrogen atoms
B_R = Number of rotable bonds
cb = the number of carbons not involved in a C= O bond
charge = the sum of the absolute values of the partial charges (calculation method described in Pugh *et al.* (2000))
C_{sat} = Concentration in water at saturation (expressed in mg/cm^3)
HA = Hydrogen bond acceptor activity
Hb = number of hydrogens
HBA = hydrogen bond acceptors
HBD = hydrogen bond donors
HD = Hydrogen bond donor activity
I_B = Balaban index
LMV = Liquid Molar Volume
log K_{OW} = octanol-water partition coefficient
Mpt = melting point
MR = molecular refractivity
MV = molar volume
MW = molecular weight
N = Number of affected hydrogen bonds, calculated by summing the number of N and O atoms (aliphatic twice, aromatic once) (Bodor and Buchwald 1997)
π_2^H = Solute dipolarity/polarizability
R_2 = Excess molar refraction
$\sum\alpha_2^H$ = Effective/overall hydrogen bond acidity
$\sum\beta_2^H$ = Effective/overall hydrogen bond basicity
∑Ca = HYBOT-PLUS H-bond acceptor free energy factor
∑(Q+)/α = HYBOT-PLUS positive charge per unit volume
SsssCH = the sum of E-state indices for all methyl groups/Electrotopological atom-type index for singly bonded CH
SsssOH = Electrotopological atom-type index for singly bonded OH
STW = Surface Tension in Water
V_e = Van der Waals effective molecular volumes, calculated according to Buchwald and Bodor (1998)
VEH = vehicle type (for acetone VEH=1 and for ethanol VEH=2)
Vx = McGowan characteristic volume

1.1.4.6.3 Mathematical skin models

The QSPRs described in the previous section merely predict a constant (k_p or J_{max}) of the skin permeation process. In order to calculate actual amounts of permeant crossing the skin under relevant exposure conditions, a mathematical model is needed. The following overview is mainly based on two recent reviews of mathematical modelling of dermal absorption by Anissimov *et al.* (2013) and Mitragotri *et al.* (2011).

The most simple model is to only consider the *stratum corneum*, being the major barrier for dermal absorption in most cases (section 1.1.3), and assume it is a (pseudo-)homogeneous compartment. In that case, the fundamental mathematical relationship is, based on Fick's first law (see a.o. Anissimov *et al.*, 2013; Mitragotri *et al.*, 2011; Russell and Guy, 2009):

$$k_{p,SC} = K_{SC/veh} \times D_{SC}/L_{SC}$$ **Equation 1-2 Permeation constant fraction of partitioning, diffusivity and diffusion path**

in which

$k_{p,SC}$ = permeation constant in cm/h

$K_{SC/veh}$ = vehicle/*stratum corneum* partition coefficient (unitless)

D_{SC} = diffusivity of the penetrant in the *stratum corneum* in cm^2/h

L_{SC} = length of the diffusion pathway through the *stratum corneum* in cm

At infinite dose and under ideal sink conditions (meaning that permeant concentration at the other side of the skin can be considered 0, because any permeant molecule that has crossed is rapidly removed), transport across the skin can be calculated using a non-differential version of Equation 1-1 from section 1.1.3 (Anissimov *et al.*, 2013):

$$M(t) = C \times k_p \times A \times (t - t_{lag})$$ **Equation 1-3 Infinite dose dermal permeation**

in which

M(t) = mass that has crossed the membrane at time *t*,

C = the concentration of the substance in the vehicle in mg/cm^3,

k_p = permeation constant of the *stratum corneum* in cm/h (which can be considered identical to the overall k_p of the skin when the *stratum corneum* is the principal barrier for the permeant, which is the case for comparatively hydrophilic molecules),

A = is the exposed in skin area in cm^2,

t = exposure time (expressed in hours) , and

t_{lag} = lag time (expressed in hours) = time needed to establish the steady state

When finite dose conditions need to be considered, steady state will not be reached, and the differential Equation 1-1 needs to be solved to calculate skin permeation, yielding:

$$M(t) = M(0) \times (1 - e^{-k_p/V_{veh} \times A \times (t - t_{lag})})$$ **Equation 1-4 Finite dose dermal permeation**

in which

M(0) = mass load on the membrane at time *t*=0, and

V_{veh} = volume of the vehicle the permeant is dissolved in (expressed in cm^3)

A more elaborate description of the use of this equation to estimate dermal absorption is given in chapter 6.

The chemical-specific parameters k_p and t_{lag} needed to use these equations can be either measured or predicted based on properties of the chemical. Since $t_{lag} = L_{SC}^2/6D_{SC}$ and k_p is given by Equation 1-2, L_{SC} needs to be known to be able to use Equation 1-3 or Equation 1-4 to predict dermal permeation based on the properties of the permeant: k_p can be predicted using an adequate QSPR, for water $K_{SC/veh}$ can be approximated by K_{OW}, and D_{SC} can be calculated once L_{SC} is known. For hydrophilic molecules that mainly follow the transcellular pathway, L_{SC} is approximately equal to the thickness of the *stratum corneum* (Mitragotri *et al.*, 2011), which can be measured. However, for lipophilic molecules, which mainly follow the tortuous intercellular pathway, it is not. Of course, when lag time is negligible compared to exposure time, one only has to rely on the predicted k_p, as lag time can assumed to be 0.

Also when the *stratum corneum* is not the rate limiting barrier, Equation 1-3 or Equation 1-4 may be used. Instead of representing the *stratum corneum*, the parameters can be considered values averaged over the different skin compartments (Anissimov *et al.*, 2013).

When the concentration of permeant at various depths in the *stratum corneum* needs to be taken into account, e.g. when using *in vivo* tape stripping to calculate diffusivity and the permeation constant (section 1.1.4.1), solutions to the second order differential equation constituting Fick's second law need to be found (see a.o Anissimov *et al.*, 2013):

$$\frac{\partial C(x,t)}{\partial t} = D_{SC}\frac{\partial^2 C(x,t)}{\partial^2 x}$$ **Equation 1-5 Fick's second law**

Various mathematically complex solutions to this differential equation have been elaborated, the description of which is outside the scope of this overview. For this, the reader may consult the excellent reviews of Mitragotri *et al.* (2011) and Anissimov *et al.* (2013).

More complex models of the skin mainly concern the *stratum corneum*, being often the rate-limiting barrier. Most of these models try to describe the structure of the *stratum corneum* more accurately as a so called "brick-and-mortar" model, in which the mortar represents the lipids and the bricks the corneocytes (Anissimov *et al.*, 2013; Mitragotri *et al.*, 2011). The simplest one only describes permeation through the lipid phase of the *stratum corneum* (intercellular pathway), others also consider the transcellular pathway through the corneocytes and the most complex ones subdivide the corneocytes (intercellular pathway) in protein fraction and a water fraction, allowing to take *stratum corneum* hydration into account (Mitragotri *et al.*, 2011). Although receiving less attention, also viable *epidermis* and avascular and vascular *dermis* have been modelled (Anissimov *et al.*, 2013). Every compartment added to the skin model implies an increase in parameters to describe partitioning between the different compartments and diffusion across them, and increases the mathematical complexity of the models and thereby the data needs of the models in

order determine the various chemical-specific and skin specific parameters (Anissimov *et al.*, 2013; Mitragotri *et al.*, 2011). This means that, in general, the usability of the more complex models in regulatory risk assessment will be limited, due to lack of data to populate the models with the necessary parameters. Furthermore, specialist numerical skills are required to use complex models (Anissimov *et al.*, 2013), which will hamper acceptance by regulatory risk assessors, who are by nature generalists. The latter objection is mitigated by the versatile and complex skin model called the Finite Dose Skin Permeation Model (FDSP) made available on the internet[3] by the US Centers for Disease Control and Prevention (CDC). It includes the *stratum corneum* (a brick-and-mortar model including hydration as a variable parameter), the viable *epidermis* and the *dermis*, and also takes into account the volatility of the permeant. The most recent description is provided by Dancik *et al.* (2013), and in chapter 7 its use as alternative for our model described in chapter 6 is assessed.

1.1.4.6.4 Physiology Based Pharmacokinetic (PBPK) models with a skin compartment

When evaluating systemic effects of permeants, it may be of importance to establish not only how much will pass the skin into the systemic circulation, but also what the fate of the permeant will be when it is distributed by circulation to other organs. This can be modelled with so called PBPK models, *in silico* models consisting of compartments representing different organs or groups of organs of an organism (e.g. rat, human), linked by circulating blood. Depending on the aim of a specific model, compartments are added, separated, subdivided or lumped. Transport of chemicals between the compartments and blood are modelled as perfusion or diffusion limited processes, dependent on chemical-specific blood-tissue partitioning coefficients and the physiological characteristics of the compartment. For some compartments (e.g. liver, kidney) metabolism may be modelled as well. By adding a skin compartment to this type of models, absorption, distribution, metabolism and excretion of chemicals entering the body via the skin can be modelled as well (Anissimov *et al.*, 2013). As these models are even more complex than the complex mathematical skin models and often require a great number of chemical specific parameters, they are even more difficult to use in regulatory risk assessment. Therefore, they are not further discussed here.

1.1.4.7 Selection of the most appropriate method for the purpose of risk assessment.

When using *in vivo* methods, the degree of dermal penetration and of systemic absorption of the permeant can be determined. The principal asset of *in vivo* studies in relation to *in vitro* studies is that they use skin that possesses its full metabolic and physiological competence. *In vivo* studies in humans are the gold standard for regulatory risk assessment. The principal set-back of using laboratory animals is that their skin permeability, metabolism, distribution and excretion may differ from those of humans.

[3] http://www.cdc.gov/niosh/topics/skin/finiteSkinPermCalc.html

The barrier characteristics of the *stratum corneum* are mostly unaltered after excision of a piece of skin from the body. Therefore, data from *in vitro* skin absorption tests correlate well with *in vivo* studies performed with the identical permeants (certainly for hydrophilic compounds) (Godin and Touitou, 2007; Jakasa and Kezic, 2008). *In vitro* tests are a suitable alternative for *in vivo* studies and also present some advantages over *in vivo* experiments, amongst others a saving in animals, costs and time, better reproducibility of results, and the possibility to test a wider range of different exposure parameters. Therefore, for regulatory risk assessment the use of *in vitro* dermal absorption studies with human skin is to be preferred, a conclusion also drawn by the WHO task group on environmental health criteria for dermal absorption (WHO, 2006). When metabolism is expected to be of importance, viable skin should be used. In that case, since the logistics of obtaining viable human skin are rather complicated, animal skin, e.g. rat skin, may be the best alternative.

Ideally, permeant application conditions in the *in vitro* test would be identical to those of the human exposure scenario's to be evaluated. Alternatively, especially when many different exposure conditions need to be evaluated, one could decide to only test an infinite dose to determine k_p and lag time and calculate absorption for different exposure conditions using a mathematical model with these parameters and the specific exposure parameters as input (chapter 6).

1.1.5 Factors influencing absorption

The many factors that may influence the rate and extent of dermal absorption can be divided in three main categories:

1. Physicochemical properties of the permeant and its vehicle
2. Properties and condition of the exposed skin
3. Exposure conditions

1.1.5.1 Physicochemical properties of the permeant and its vehicle

Since, in general, dermal absorption is a passive process involving diffusion down a concentration gradient and partitioning over lipophilic (e.g. *stratum corneum*) and hydrophilic (e.g. viable *epidermis*) media the octanol/water partition coefficient (log K_{OW}) and molecular size are among the most important chemical properties governing the rate and extent of dermal absorption (EFSA, 2011; WHO, 2006) (see also section 1.1.4.6.2). Molecular weight can be a proxy for molecular size, but only with confidence within a homologous series (EFSA, 2011).

The process described in section 1.1.3 refers to permeants diffusing across the skin from a solvent. However, chemicals may also come in contact with the skin in different physical states, e.g. solid, vapour, neat liquid.

Since diffusion is driven by random thermal movement, direct absorption of solids will be restricted, since in solid state the movement of molecules is restricted to oscillations.

Diffusion of entire particles across the skin is unlikely as well, since Watkinson *et al.* (2013) calculated, using two QSPRs for dermal absorption, that even nanoparticles are too big to diffuse in measurable amounts across the skin. Consequently, solid permeants first need to dissolve in a vehicle before they are able to penetrate into the *stratum corneum* (Heylings and Esdaile, 2008). The finer the solid particles, the more readily they may dissolve in e.g. moisture on the skin, to subsequently penetrate into the *stratum corneum* (Heylings and Esdaile, 2008). Still one would expect absorption to be less when permeants are in the solid state. Indeed, pesticides from solid formulations tend to be absorbed to a lower degree than pesticides from liquid formulations (Aggarwal *et al.*, 2015, 2014), although it cannot be excluded this is due to the properties of the pesticides since the authors did not control for that confounder. Romonchuk *et al.* (2006) found that the flux from pure powder (grains of approx. 50 µm) was over ten times slower than that from saturated solutions of 4-cyanophenol and methylparaben. However, the authors did not expect this as according to them the thermodynamic activity of the pure powder and the saturated solutions are the same and they did observe equal fluxes for both physical states when using an artificial membrane. Concluding, although the theoretical explanation is not clear, absorption from powders as opposed to from solutions seems to be slower.

On the other hand, permeants in the vapour phase have the maximum freedom of movement, but the amount of molecules near the skin surface will be much lower than will be the case for a solute in solvent. Therefore it may be expected that absorption from the vapour phase will be slow and negligible compared to absorption via the respiratory route, which occurs at an epithelial surface designed to take up molecules instead of being designed as a barrier. Still for some substances, dermal absorption from vapours may be an important contributor to the total uptake. For example, Weschler and Nazaroff (2014) collected measured values for the contribution of dermal uptake to the total uptake (via the dermal and respiratory routes) in humans for 19 volatile organic substances. For ten of them, dermal uptake contributed 20% or more to the total body burden, for some (2-butoxyethanol, aniline and 2-methoxyethanol) the contribution of dermal uptake was even higher than that of respiratory uptake. For the others, the contribution of dermal uptake was 7% or less.

A special case are the permeants that are present on the skin as neat liquids. One could expect that skin permeation of such a liquid (if miscible with the solvent, e.g. water), would simply increase with concentration from diluted to neat permeant, following Equation 1-1. More strictly formulated, neat k_p is expected to be equal to the aqueous k_p multiplied with the proportion of water solubility and density in the neat state, based on thermodynamic considerations (Frasch *et al.*, 2007). Indeed, a mixture of butoxyethanol and water does show this behaviour at higher dilutions of butoxyethanol, but in the mid region of dilutions the flux becomes constant and it decreases when the mixture approaches neat butoxyethanol (Bunge *et al.*, 2012). Based on the comparison between the permeation of butoxyethanol through rat skin *in vitro* and an artificial membrane (silicone), decreasing skin

hydration at higher concentrations of butoxyethanol is likely the cause of this phenomenon as skin hydration is an important factor in its permeability for hydrophilic compounds (Bunge *et al.*, 2012). The same behaviour has been observed for other glycol ethers, and ethanol (Bunge *et al.*, 2012). Also for a number of liquid compounds not miscible with water, neat application did not reflect the results obtained with saturated aqueous solutions. For example diethylphthalate's flux across hairless guinea pig skin *in vitro* was twice as high from a saturated solution compared to the neat form, while for dichloroethylene it was approx. 5 times lower (Frasch *et al.*, 2007). This is probably related to vehicle effects like (de)hydration (ethanol/water) or delipidisation (organic solvents) (Frasch *et al.*, 2007).

Of course, solubility is also an important factor for chemicals that are lodged on the skin dispersed or dissolved in a vehicle (solvent(s) and possibly other ingredients). When dissolved, the ionisation state of a molecule is of influence, since ionised molecules have lower absorption than the corresponding non-ionised forms, the permeability coefficient being lower by 1-2 orders of magnitude (EFSA, 2011). Consequently, also the pH of the vehicle, as well as the pK_a of the permeant will influence the rate of absorptions, since these values will largely determine the ionisation state of acids and bases. For ionisable substances log K_{OW} should be substituted by log D_{ow}, which is the distribution coefficient or apparent partition coefficient, and is given by the following formula (Mälkiä *et al.*, 2004):

$$D_{OW} = \frac{[HA]_{oct} + [A^-]_{oct}}{[HA]_{water} + [A^-]_{water}}$$

Equation 1-6 Partitioning coefficient ionogenic molecules

Also volatility of the permeant will be an important factor in dermal absorption as substances rapidly evaporating from the skin will be absorbed to a lesser extent than substances that do not readily evaporate. The relative importance of evaporation will be determined by the ratio between the rates of evaporation and penetration (see e.g. Kasting and Saiyasombati, 2001).

Protein binding properties of permeants will also influence their absorption rate, as binding to structural proteins in the skin may retard absorption and binding with carrier proteins may enhance it (Holmgaard *et al.*, 2014). However, the importance of the latter has never been demonstrated (section 1.1.3).

Other vehicle related factors that influence dermal absorption are (EFSA, 2011; WHO, 2006):

- Solvent drag, an absorbable solvent takes a dissolved lipophilic substance with it.
- Lipophilicity/hydrophilicity: Partitioning of the permeant between the *stratum corneum* and vehicle depends on its lipophilicity/hydrophilicity and that of the vehicle.
- Presence of irritants and/or sensitizers may cause significant skin irritation and therefore increased skin permeability.

- Presence of surfactants like quaternary ammonium compounds may alter the barrier properties of the *stratum corneum* and/or produce an irritant reaction that could increase dermal absorption (chapters 3 to 5).
- Volatility, when a solvent rapidly evaporates, the permeant, when not very volatile as well, will be deposited as a solid on the skin, a form in which it will less readily cross the skin.
- Presence of compounds that directly interact with *stratum corneum* lipids or proteins, which may enhance or retard permeation. E.g., a protein cross-linker like glutaraldehyde may decrease the permeability of the *stratum corneum* (chapter 5).

1.1.5.2 Properties and condition of the exposed skin

The main barrier to absorption of chemicals is the *stratum corneum* (EFSA, 2011; WHO, 2006). Hair follicle and sweat and sebaceous gland density may influence dermal absorption, as these appendages provide a short-cut through the *epidermis* (section 1.1.2). Consequently, there are differences in dermal absorption between anatomical sites and species. In humans, the hierarchy of absorption at different anatomical sites is: scrotum > forehead > torso and arms > palms and soles of feet (EFSA, 2011). The permeability of pig and monkey skin is comparable to human skin, while the skin of rats, guinea-pigs, and rabbits is more permeable (WHO, 2006). In a comparative study with different chemicals, dermal absorption decreased in this sequence: rabbit > rat > pig > human (Bartek *et al.* (1972, cited in WHO, 2006)). Also the EDETOX *in vitro* dermal absorption data show as a general tendency higher k_p values for animal skins (Figure 1-4). Remarkably, the values for snake skin, based on a limited data set, seem to be quite close to the human values, as are those of pig and minipig.

There are no systematic data on age-related differences in dermal absorption, but, based on incidental data, there are minor differences in skin absorption due to age, limited to certain skin areas (e.g. facial and back skin) (EFSA, 2011).

In principle, blood and lymph flow may influence absorption as the magnitude of the flow rate will determine how fast the permeated molecule is removed from the skin into the systemic circulation keeping the concentration gradient over the skin as large as possible. However, this is principally of interest for small, moderately lipophilic chemicals, as their removal from the skin by the blood flow is rate limiting for their systemic absorption (WHO, 2006).

Sweating and skin hydration may increase dermal absorption, but less than 2 fold (EFSA, 2011). So sweating and skin hydration appear to be of minor importance in skin absorption. Diffusion rates increase with temperature and therefore skin temperature will influence absorption (WHO, 2006). Temperature may also change the crystalline structure of the lipids in the *stratum corneum*, which can provoke higher permeability (WHO, 2006). Vecchia and Bunge (2002a) found a positive correlation between temperature and k_p in the Flynn dermal

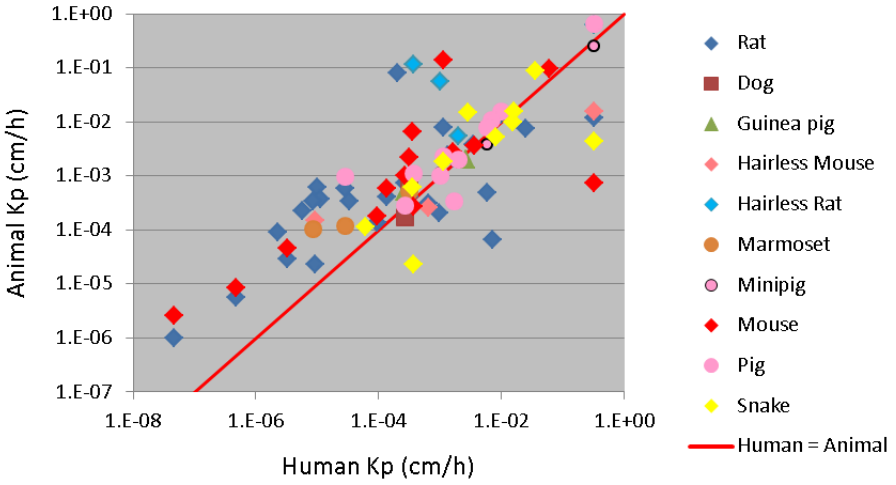

Figure 1-4 Comparison of human and animal k_p values, measured *in vitro* (data from the EDETOX project (http://edetox.ncl.ac.uk/))

absorption database. Further QSPR analysis, however, indicated the influence of absolute temperature on the absorption process was weak: adding absolute temperature to a QSPR, based on an amplified Flynn database and containing just MW and log K_{OW}, only increased the explained variability in log k_p from 55.1 to 56.4% (Vecchia and Bunge, 2002b). Furthermore, under *in vivo* conditions, ambient temperature influences the blood flow to the skin, which starts to increase from a temperature of approx. 24 ^{o}C (Guyton, 1991), thus potentially modifying absorption (see above).

Skin lesions caused by mechanical damage or disease may reduce the barrier function of the skin and increase its permeability. Mechanical lesions by either tape stripping or abrasion cause up to over a 100-fold increase of absorption, but this occurs only when the lesions reach the *dermis* (EFSA, 2011). In people suffering from atopic dermatitis, psoriasis or eczema skin permeability increased, sometimes even in not affected regions of the skin (Kezic and Nielsen, 2009). Based on an evaluation of the few *in vitro* studies available in public literature, the increase in absorption through damaged or diseased skin is modest when compared to intact skin and affects mainly the absorption of hydrophilic molecules (Gattu and Maibach, 2010).

The skin possesses xenobiotic metabolising enzymes. Both phase I and phase II enzymes are present in the viable *epidermis* and in the *dermis*, be it, in general, in lower activities than in the metabolising organ par excellence, the liver (see e.g. Oesch *et al.*, 2014, who give an overview of skin metabolizing capacity). For example, cultured skin cells and viable skin preparations in diffusion experiments are able to hydrolyse butoxyethanol to butoxyacetic acid and parabens to p-hydroxybenzoic acid (Williams, 2008). There is anecdotal evidence for the influence of skin metabolism on dermal absorption. In some experiments with

mouse skin induction of cutaneous xenobiotic metabolizing enzymes increased the permeation of benzo[*a*]pyrene 2- to 3-fold (Kao *et al.*, 1985, cited in WHO, 2006). However, metabolism may not be relevant if the *stratum corneum* is the rate limiting factor in percutaneous absorption. On the other hand, if the viable tissue is rate limiting, which may well be the case for lipophilic compounds, turnover to a more polar compound may increase permeation, e.g. *in vitro* absorption experiments with human and rat skin and esters of the pesticide fluroxypyr showed that the parent compound could only be detected in the *stratum corneum*, while only the metabolite could be detected in the viable skin and in the receptor fluid (equivalent to systemic absorption *in vivo*) (Hewitt *et al.*, 2000). This suggests that the esterase activity present in the viable skin may enhance absorption of these esters, although this should be confirmed by comparing it with a condition in which the esterase activity is inhibited (e.g. by using non-viable skin preparations). Also Beydon *et al.* (2014) when studying the absorption of phenoxyacid ester herbicides in viable rat and human skin *in vitro* only found the free esters in the receptor fluid. Still, the PPR Panel of EFSA considers that skin metabolism will not alter the calculated absorption significantly as the metabolically active cells are situated below the *stratum corneum* and therefore the main barrier to absorption has to be passed before any metabolism can occur (EFSA, 2011).

The outer layers of the *stratum corneum* are continuously removed by a process called desquamation (section 1.1.2). Therefore, chemicals present in the *stratum corneum* may be removed, and will no longer be available for systemic absorption. This will mostly be of importance for very lipophilic substances or substances that in another way remain associated with the *stratum corneum* (reservoir formation, see section 1.1.4.2). This is, amongst others, the case for DDAC (chapter 5)

1.1.5.3 Exposure conditions

Two distinct dosing regimens can be discerned: infinite and finite dose (Figure 1-5). At infinite dose, the amount of substance applied per unit skin area (dermal load) is so high that no appreciable dose depletion takes place, and the absorption rate is practically constant. How high the dermal load should be to reach infinite dose conditions will, amongst others, depend on the permeability of the chemical (the lower the permeability, the lower the dermal load necessary to reach infinite dose). Under these conditions, relative absorption is a meaningless number as increasing the load will not increase the absorption rate.

Under finite conditions, the applied dose will markedly change resulting in a decreasing absorption rate over time. In this case, absorption is usually expressed as a percentage of the dermal loading. Since this relative absorption will increase with time (Figure 1-5), it is important that the exposure conditions reflect the in-use situation with respect to these parameters. So if many different exposure conditions are to be evaluated for a certain compound, many different *in vitro* tests are needed or an *in silico* model capable of predicting absorption under these different exposure conditions (chapter 6).

When exposed, skin may be bare or covered with (protective) clothing. When skin is covered in such a way that evaporation from the skin is completely prevented, it is said to be occluded. An example of an occluding piece of clothing are latex surgical gloves. When the skin is covered by a fabric merely limiting evaporation from the skin, without completely preventing it, e.g. a cotton shirt, it is said to be semi-occluded. The effects of occlusion of the exposed skin, may be related to temperature and humidity, as occlusion may increase the hydration of the *stratum corneum* from 5-15% to as much as 50%, and may increase its temperature from 32 to as much as 37 ˚C (Bucks and Maibach, 2002). Bucks & Maibach (2002) have reviewed occlusion and *in vivo* penetration and demonstrated that there is an apparent trend of increased absorption due to occlusion with increasing permeant lipophilicity. Occlusion may cause up to 9-fold increases in absorption (EFSA, 2011).

As increased skin temperature and hydration may increase absorption (section 1.1.5.2), also raised ambient temperature and humidity conditions can be expected to augment absorption rates. However, the limited data available, which concern dermal absorption of vapours, show only a ca. 1.3-fold increase when ambient temperature was raised from 20 to 30 ˚C, while an increase in ambient relative humidity from 59 to 65% caused a somewhat higher increase, which however was not statistically significant (Jones *et al.*, 2003). For the moment, the impact of these two parameters seems limited, but more data are needed to draw more definitive conclusions.

1.2 Objectives and outline of this thesis

1.2.1 Objective

Given the possible importance of the dermal exposure route, the aim of this thesis is to characterise the role of dermal loading (the amount of chemical applied per cm^2 skin), of

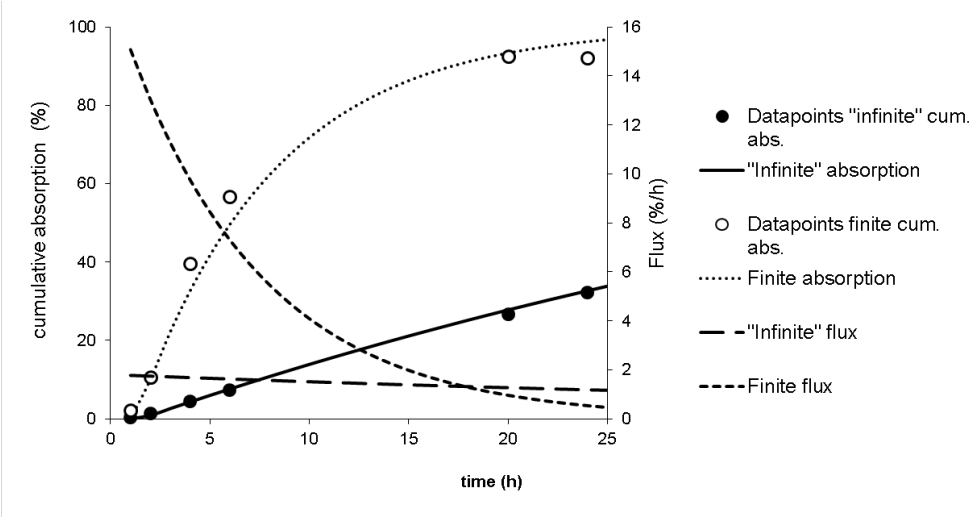

Figure 1-5 Relative cumulative absorption and flux under finite and (nearly) infinite dose conditions

irritative/corrosive potential, of frequency of exposure and of the vehicle used in dermal absorption and subsequent risk assessment in more detail, using *in vitro* models for dermal absorption and literature data whenever possible.

1.2.2 Outline

Chapter 1 presents an overview of the importance of dermal absorption in toxicological risk assessment, the factors influencing its rate and the *in silico*, *in vitro* as well as *in vivo* models currently available to measure and predict it. Many factors influence the rate of dermal absorption of a substance. In this thesis, four of these, dermal loading (chapters 3 and 6), irritative/corrosive potential (chapters 3 and 4), frequency of exposure (chapters 3, 4 and 5) and the vehicle used (chapter 6), were investigated in more detail.

Chapter 2 aims to investigate the relationship between relative dermal absorption and dermal loading (Buist *et al.*, 2009). It discusses the fallacy of the present common practice in regulatory toxicological risk assessment to ignore the impact of dermal loading on dermal absorption. Dermal loading is defined as the amount of substance per unit area of exposed skin. To illustrate the importance of dermal loading for dermal risk assessment, in chapter 2 an inventory is made of all dermal absorption data publicly available at the time of investigation including also a number of (anonymised) TNO data. The data are analysed with respect to the relation between relative dermal absorption and dermal loading. Furthermore the relevance of the results of this analysis for the estimation of dermal absorption under realistic exposure conditions in the framework of regulatory risk assessment is discussed.

In order to investigate the possible influence of the frequency of exposure, *chapter 3* aims to investigate the influence of single and repeated dermal exposure to biocidal active substances on *in vitro* skin permeability, based on public literature. This investigation has been performed on the basis of data available in public literature on dermal absorption of biocides. Also the potential influence of irritative and/or corrosive properties of biocides is discussed. Taking into account the results of this literature based evaluation, a proposal for addressing this issue via experimental research is formulated.

Chapter 4 (Buist *et al.*, 2005) aims to investigate the influence of single and repeated dermal exposure to biocidal active substances on *in vitro* skin permeability, using an experimental approach, based on the proposal formulated in chapter 3. The biocidal active substances were selected based on an inventory of biocidal products admitted on the market in the Netherlands, including their composition with respect to active substances (biocidal active substances) and their use categories (e.g. rodenticides, preservatives, disinfectants).

The biocidal active substances selected for this study were alkyldimethylbenzylammonium chloride (ADBAC), boric acid, deltamethrin, dimethyldidecylammonium chloride (DDAC), formaldehyde, permethrin, piperonyl butoxide, sodium bromide, and tebuconazole. Using

these model compounds the influence of single and repeated exposure on skin permeability using *in vitro* dermal absorption experiments was investigated. Also the potential role of irritative and/or corrosive properties of biocides is analysed.

Chapter 5 aims to determine the permeability coefficient (k_p) of the biocidal active substance DDAC, to investigate its skin permeation at a finite dose more representative of normal worker and consumer exposure and to probe the influence of various commercial biocidal formulations on its absorption (Buist *et al.*, 2007). The chapter zooms in on the consequences of skin barrier effects of single and repeated exposure for dermal absorption of a corrosive quaternary ammonium compound and biocidal active substance, DDAC. The influence of the use of different vehicles (dissolution media) on the dermatokinetics of DDAC was characterised using *in vitro* dermal absorption experiments.

Chapter 6 aims to develop and test a simple model to predict finite dose dermal absorption from infinite dose data (k_p and lag time) and the *stratum corneum*/water partition coefficient ($K_{SC,W}$) (Buist *et al.*, 2010). A simple mathematical model to predict finite dose dermal absorption from infinite dose data, which takes into account different exposure conditions with respect to dermal loading, concentration, exposed skin surface and exposure duration is developed. To test the model, a series of *in vitro* dermal absorption experiments was performed under both infinite and finite dose conditions using acetic acid, benzoic acid, bis(2-ethylhexyl)phthalate, butoxyethanol, cortisone, decanol, diazinone, 2,4-dichlorophenol, ethacrynic acid, linolenic acid, octylparaben, oleic acid, propylparaben, salicylic acid and testosterone.

Chapter 7 summarises the results obtained in this thesis and discusses how the parameters investigated affect dermal absorption. It also indicates how to best estimate dermal absorption under realistic exposure conditions for use in regulatory risk assessment, using a limited amount of experimental data. The chapter also presents future perspectives for the role and implementation of dermal absorption in regulatory risk assessment and the possibility to use alternative *in vitro* and/or *in silico* testing strategies.

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2 Relative absorption and dermal loading of chemical substances: Consequences for risk assessment

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2.1 Abstract

Quantification of skin absorption is an essential step in reducing the uncertainty of dermal risk assessment. Data from literature indicate that the relative dermal absorption of substances is dependent on dermal loading. Therefore, an internal exposure calculated with absorption data determined at a dermal loading not comparable to the actual loading may lead to a wrong assessment of the actual health risk. To investigate the relationship between dermal loading and relative absorption in a quantitative manner, 138 dermal publicly available absorption experiments with 98 substances were evaluated (87 *in vitro*, 51 *in vivo*; molecular weight between 40 and 950, log K_{OW} between -5 and 13), with dermal loading ranging mostly between 0.001 and 10 mg/cm^2. In 87 experiments (63%) an inverse relationship was observed between relative dermal absorption and dermal loading, with an average decrease of factor 33 ± 69. Known skin irritating and volatile substances less frequently showed an inverse relationship between dermal loading and relative absorption.

2.2 Introduction

The dermal route is the primary route of occupational exposure for most pesticides (Benford *et al.*, 1999; Wolfe, 1976) and biocides (EU, 2002). Despite the relatively high dermal exposure in occupational settings, regulation for pesticides has evolved from concern regarding consumers and the oral route of exposure, both in the United States and in Europe (EU, 1991, 1994; USEPA, 2008). As a consequence, studies requested for

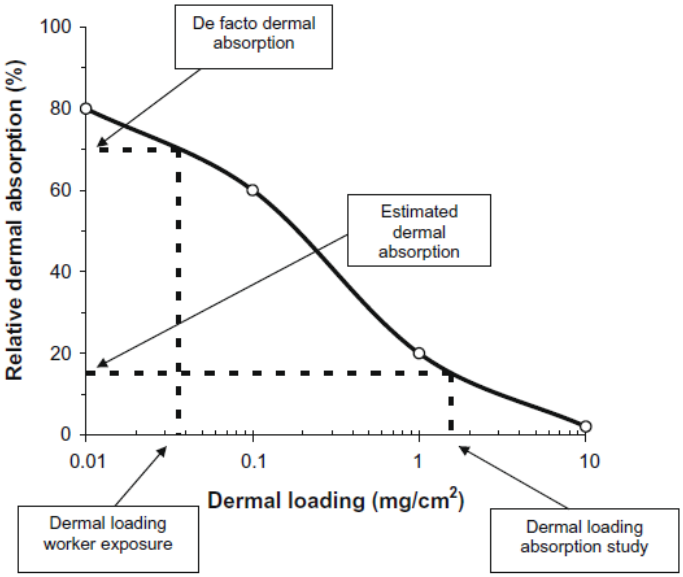

Figure 2-1 Effect on risk assessment of choosing unrealistic dermal loading, when there is an inverse relationship between loading and relative absorption.
In this case the consequence of choosing an excessively high dermal loading from a dermal absorption study is depicted.

registration purposes emphasise the oral route and low-level daily exposure (Krieger and Ross, 1993). Until recently the importance of dermal exposure has not been so clear for industrial chemicals. However, the results of the EU RISKOFDERM-project indicate that also for this category of chemicals dermal exposure may be substantial (see a.o. Marquart *et al.*, 2006), while toxicity studies available for risk assessment in the EU are mainly conducted via the oral route. Since systemic toxicity is dependent on the internal dose (or better, the internal concentration profile) some means must be provided to relate the bioavailability upon occupational dermal exposure to the (orally) absorbed dose in the toxicity study. This explains why dermal absorption studies may be requested for registration of pesticides (EU, 1991, 1994; USEPA, 2008) and biocides (EU, 1998). The risk assessment approach for pesticides and biocides in the EU differs from that for industrial chemicals. However, as in both regulatory frameworks oral toxicity data are often used to estimate dermal risks, oral-to-dermal route extrapolation is needed and an estimate of dermal absorption may be useful.

Dermal absorption estimates are generally derived from *in vivo* or *in vitro* dermal absorption studies, in which one or more concentrations are tested for relative dermal absorption. In rare cases, human internal exposure is estimated via biomonitoring techniques, eliminating the need for dermal absorption estimates to transform external exposure values into internal ones (van de Sandt *et al.*, 2007). In absence of (reliable) experimental data, default values for dermal absorption may be applied (EU, 2003) or sometimes QSPRs (van de Sandt *et al.*, 2007).

Some data in public literature indicate that relative dermal absorption of specific substances decreases with increasing dermal loading (i.e., amount of substance per unit area of skin) (e.g., Brewster *et al.*, 1989; Bunge, 2005; Wester and Maibach, 1976). An internal dose, therefore, when calculated without taking into account differences in relative absorption between the dermal loading applied in the animal study and the one workers are exposed to, may underestimate or overestimate the actual worker risk (Figure 2-1).

So far no substantiation has been presented with respect to the relative incidence of this inverse relationship nor has the practical relevance for worker risk assessment been probed in detail. In the present paper, we investigate the relevance of this phenomenon, by making an inventory and data analysis of existing dermal absorption studies reporting the relation between relative dermal absorption and dermal loading of specific substances.

2.3 Materials and methods

Public literature was screened for dermal absorption studies in which different dermal loadings or concentrations had been tested. Online databases, such as Toxline and Medline were used. In addition, in order to increase the potential number of suitable data, information provided in (drafts of) Risk Assessment Reports, prepared under Council Regulation EEC No. 793/93 on Existing Substances, including data from direct and indirect

references therein, was used. The searches were concluded in 2005. Most studies retrieved presented systemically absorbed doses or both systemically and potentially absorbed doses, a few studies presented only potentially absorbed doses or did not specify how absorption was defined. In contrast to the systemically absorbed dose, the potentially absorbed dose also includes that part of the dose which is present in the dosed skin site (after washing) at the end of the experiment. For *in vivo* studies the systemically absorbed dose equals the dose or dose equivalents recovered from excreta and all tissues (except the treated skin) over the total experimental period (usually between 1 and 7 days), while for *in vitro* studies it equals the recovery from the receptor fluid (usually over a period of 24 h). In order to obtain a homogeneous database, we selected only those studies which presented at least information on systemic absorption.

The relationships between relative absorption and dermal loading for all substances and experiments encountered were classified into two categories: inverse relationship between relative absorption and dermal loading and no inverse relationship between relative absorption and dermal loading, based on the following statistical analysis: before testing for differences between levels of dermal loading, missing standard deviations (SD) were imputed. Coefficients of variation (CV) were determined for each experiment for which an SD was available. Missing SDs were imputed as mean times the median CV value. A two-sided Dunnett's test (Dunnett, 1955) was used to test between levels of dermal loading, using the lowest dermal loading as reference. Per experiment, SDs were used to calculate a pooled SD. A *t*-statistic was calculated as (dermal loading *i* – dermal loading 1)/ $SD_{pooled} \times \sqrt{1/n_{dermal\ loading\ 1} + 1/n_{dermal\ loading\ i}}$ .The degrees of freedom for the test was equal to *N* - *k,* where *N* is the total number of available observations and *k* is the number of dermal loading groups including the reference dermal loading.

A continuously decreasing p-value with increasing dermal loading indicates an inverse relation between dermal loading and absorption. In case of significant p-values compared to the reference dermal loading, but a non-continuous decrease in p-values, consecutive dermal loadings were also tested. If in that case no statistically significant differences between these consecutive dermal loadings were found, the relation between dermal loading and absorption was also indicated as inverse.

For the analysis of the data also an "absorption decrease factor" was defined, i.e., the relative dermal absorption at low dermal loading divided by the relative dermal absorption at high dermal loading.

2.4 Results

The literature search produced more than 40 publications and other documents with a total of 138 dermal absorption experiments from which for a specific substance the relationship between relative dermal absorption and dermal loading could be established. Most experiments were performed *in vitro* (87), but also many *in vivo* studies are present in the

assembled database (51). The methods used to compute *in vivo* systemic dermal absorption were diverse, e.g., based on excreta only, on excreta and tissues or by comparing urinary excretion after intravenous and dermal application (see Annex describing the database, published on the internet site of this journal). *In vitro* ''systemic" dermal absorption figures were always based on the dose recovered from the receptor fluid at the end of the experiment. In 130 cases methods comparable to those described by OECD guidelines 427 and 428 (OECD, 2004a, b) were used and in eight cases they were not comparable or not described in sufficient detail. Whether or not OECD comparable methods were used, did not influence the relation found between dermal loading and relative absorption (analysis not shown). In approximately half of the cases the rat was the investigated species, and in one third of the cases human skin was exposed, while in the remainder of the studies pig, guinea pig, mouse or monkey skin was used. The species investigated did not significantly influence the nature of the relationship observed (data not shown). The average exposure time in the experiments was 22 h, while, on average, absorption was measured over a period of 41 h. The most frequent combinations of exposure time and sampling time were (in hours): 8/8 (n = 25), 8/24 (n = 12) and 24/24 (n = 37). In 65 of the 138 experiments, the exposure time was between 4 and 10 h, which constitutes a realistic worker exposure period, 60 had exposures times ≥24 h, and for 13 experiments exposure duration was not (clearly) indicated. The experiments with shorter (4-10 h) and longer (24 h and more) exposures did not differ significantly in the nature of the relationship between relative absorption and dermal loading (data not shown). Therefore, the results of all experiments with different exposure times were analysed together. Some experiments were performed under non-occlusive conditions (n = 54), some under semi-occlusive conditions (n = 46) and some under occlusive conditions (n = 27). For some experiments it was not clear under which condition of occlusion they were performed (n = 11). As the state of occlusion did not influence the relation between relative absorption and dermal loading (data not shown), these results

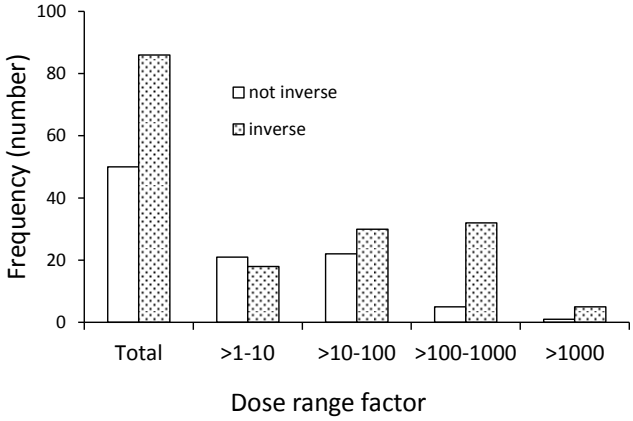

Figure 2-2 Relation between dermal loading range factor and change in relative absorption with dermal loading.

Dermal loading range factor = high dermal loading divided by low dermal loading.

were lumped together as well. The MW of the 98 substances investigated ranged between 40 and 950, while their log K_{OW} ranged between -5 and 13.

Of the 138 cases, 87 (63%) show an inverse relationship between relative dermal absorption and dermal loading (see Table 2-2). Within a 100-fold increase in dermal loading, relative absorption may decrease up to 100-fold (e.g., for nonylphenol ethoxylates in humans (Monteiro Riviere *et al.*, 2000)). For 10-fold or higher increases in dermal loading, the average decrease was a factor 42 ± 76 when an inverse relationship was present. The relative number of inverse relationships and the decrease factor tended to increase with increasing width of the dermal loading ranges investigated (see Table 2-2 (page 64) and Figure 2-2). Differences between dermal loading range classes were statistically significant (see Table 2-3A). Most dermal loading ranges tested were between 0.001 and 10 mg/cm^2 (see Annex describing the database, published on the internet site of this journal). Worker exposure for a diverse number of typical cases of hand exposure to industrial chemicals ranged from 0.0014 to 4.8 mg/cm^2 (Marquart *et al.*, 2006), while for non-agricultural pesticides a typical case value of 4.8 mg/cm^2 and a reasonable worst case value of 14 mg/cm^2 are mentioned in the Technical Guidance Document on Risk Assessment (EU, 2003). Therefore, the cases reported here seem to cover a range of exposures relevant for workers. The relative number of inverse relationships for substances with log K_{OW} > 1 was 73%, while for substances with log K_{OW} ≤1 it was 30% (see Table 2-3B, page 64).

The 29 volatile substances (V_P > 0.01 Pa)[4] tested showed significantly less cases of inverse proportionality than non-volatiles (see Table 2-3D), irrespective of state of occlusion. Skin irritants less often showed inverse proportionality between dermal loading and relative absorption (see Table 2-3C).

Particularly for pesticides vehicle effects may influence relative absorption at different skin loadings as field concentrations are often obtained by diluting a concentrate with water, changing the composition of the vehicle. Comparison between substances dissolved in water (mainly non-pesticides) and substances dissolved in aqueous dilutions of formulants (pesticides) seems to confirm this: pesticides showed a significantly larger proportion of inverse relationships compared to substances dissolved in water (see Table 2-3E). However, the width of the dermal loading ranges investigated may be a confounding factor, as the pesticides were studied over much larger dermal loading ranges and the proportion of inverse relationships increases with the width of the dermal loading range (see Table 2-3B).

[4] As defined in the EU Technical Guidance Document on Risk Assessment (EU, 2003).

Table 2-1 Dermal absorption data

Substance	Phase or vehicle	Species	Study	Dermal loadings ($\mu g/cm^2$)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
2,6-Di-tert-butyl-4-nitrophenol	Ethanol (100%)	Pig	*In vitro*	4-40	0.39-0.25 (0.78-0.2)	Not inverse	Inman *et al.* (2003)
2,6-Di-tert-butyl-4-nitrophenol	Ethanol (85%)	Pig	*In vitro*	4-40	1.1-0.14 (0.7-0.08)	Inverse	Inman *et al.* (2003)
2-Butoxyethanol	Water	Human	*In vitro*	940-1900	27-23 (6.6-8.7)	Not inverse	Wilkinson and Williams (2002)
2-Ethoxyethanol	Water	Human	*In vitro*	940-1900	5.1-18 (1.7-4.8)	Not inverse	Wilkinson and Williams (2002)
4,4'-Methylenedianiline (MDA)	Ethanol (95%)	Guinea pig	*In vitro*	200-2000	30-7.5 (10-n/a)	Not inverse	El-Hawari *et al.* (1986)
4,4'-Methylenedianiline (MDA)	Ethanol (95%)	Rat	*In vitro*	200-2000	54-6.6 (19-1)	Inverse	El-Hawari *et al.* (1986)
5-Aminolevulinic acid	Water	Mouse	*In vitro*	23,000-70,000-140,000	0.21-0.14-0.075 (0.11-0.13-0.044)	Inverse	van den Akker *et al.* (2003)
5-Aminolevulinic acid hexyl ester	Water	Mouse	*In vitro*	39,000-120,000-230,000	0.068-0.027-0.07 (0.062-0.014-0.026)	Not inverse	van den Akker *et al.* (2003)
5-Aminolevulinic acid methyl ester	Water	Mouse	*In vitro*	26,000-79,000-160,000	0.095-0.068-0.058 (0.043-0.058-0.042)	Not inverse	van den Akker *et al.* (2003)
7-(2H-naphtho[1,2-d]triazol-2- yl)-3-phenylcoumarin	Chloroform	Human	*In vitro*	10-50	1.5-0.3 (1.7-0.19)	Inverse	Yourick *et al.* (2004)
Atrazine	Acetone	Human	*In vitro*	9-91	11-8 (2.1-1.9)	Not inverse	Moody (2000)
Azinphos-methyl	No data	Rat	*In vitro*	0.95-9.2-93	42-22-18 (nd)	Inverse	Zendzian (2000)
BAS 220 F	No data	Rat	*In vitro*	0.3-3-30-300	13-13-9.9-4.5 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 352 F	No data	Rat	*In vitro*	2-20-200-2000	13-4.3-0.5-<0.22 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 421 F	No data	Rat	*In vitro*	7.5-3000	13-0.84 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 480 F	No data	Rat	*In vitro*	50-500	9.4-19 (nd)	Not inverse	van Ravenzwaay and Leibold (2004)

nd, no data; n/a, not applicable; Huntingdon, contribution of Huntingdon Life Sciences to work package 4 of the EDETOX project, unpublished data; TNO, confidential TNO-report

Table 2-1 continued

Substance	Phase or vehicle	Species	Study	Dermal loadings ($\mu g/cm^2$)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
BAS 490 F	No data	Rat	*In vitro*	7-50-350	12-5.8-2.3 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 500 F	No data	Rat	*In vitro*	15-75-380	0.64-0.85-0.51 (nd)	Not inverse	van Ravenzwaay and Leibold (2004)
BAS 505 F	No data	Rat	*In vitro*	12-60-300	3.3-1.4-0.75 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 510 H	No data	Rat	*In vitro*	10-100-1000	11-2.6-0.41 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 615 H	No data	Rat	*In vitro*	10-50-250	1.6-1.1-0.9 (nd)	Not inverse	van Ravenzwaay and Leibold (2004)
BAS 625 H	No data	Rat	*In vitro*	10-30-100	1.7-1.2-1.1 (nd)	Not inverse	van Ravenzwaay and Leibold (2004)
BAS 635 H	No data	Rat	*In vitro*	2-20-200	1.9-0.35-0.23 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 656 H	No data	Rat	*In vitro*	4-40-400	11-2.5-4.9 (nd)	Inverse	van Ravenzwaay and Leibold (2004)
BAS 9048 H	No data	Rat	*In vitro*	0.76-7.6-76-760	6.6-1.3-4-0.053 (nd)	Not inverse	van Ravenzwaay and Leibold (2004)
Bentazone	No data	Rat	*In vitro*	2-20-200-2000	1.2-1.9-1.5-0.79 (nd)	Not inverse	Clegg (1991)
Benzo[a]pyrene	Acetone	Guinea pig	*In vitro*	8.1-74	37-7.2 (0.9-1)	Inverse	Ng *et al.* (1992)
Benzoic acid	Solid	Human	*In vitro*	3-400-2000	37-26-14 (16-9.9-3.8)	Inverse	Wester and Maibach (1976, 1975)
Benzoic acid	Solid	Monkey	*In vitro*	40-2000	34-17 (5.1-1.2)	Inverse	Wester and Maibach (1976, 1975)
Butoxyethanol	Acetone	Rat	*In vitro*	1500-4600-8200	25-26-21 (6.6-9.4-4.8)	Not inverse	Sabourin *et al.* (1992)
Catechol	Ethanol	Rat	*In vitro*	120-780	49-81 (6.4-5.9)	Not inverse	Jung *et al.* (2003)
Decabromodiphenyl oxide	Tetrahydrofuran	Mouse	*In vitro*	8.8-44-88	0.34-0.09-0.07 (0.04-0.03-0.01)	Inverse	Hughes *et al.* (2001)
Di(2-ethylhexyl)phthalate (DEHP)	Acetone	Guinea pig	*In vitro*	14-60-120	6.1-2.4-2.5 (1.1-0.41-0.43)	Inverse	Ng *et al.* (1992)

nd, no data; n/a, not applicable; Huntingdon, contribution of Huntingdon Life Sciences to work package 4 of the EDETOX project, unpublished data; TNO, confidential TNO-report

Table 2-1 continued

Substance	Phase or vehicle	Species	Study	Dermal loadings (µg/cm^2)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
Ethylene glycol mono iso-propyl ether (EGiPE)	Water	Human	*In vitro*	90,000-180,000	1.6-0.91 (nd)	Inverse	Venier *et al.* (2004)
Diethylene glycol butyl ether (DEGBE, 2-(2-butoxyethoxy)ethanol)	Liquid	Rat (f)	*In vitro*	10,000-100,000	65-23 (17-11)	Inverse	van de Sandt and Rutten (1995)
Diethylene glycol butyl ether acetate (DEGBA)	Liquid	Rat (f)	*In vitro*	10,000-100,000	53-18 (9.9-5.5)	Inverse	van de Sandt and Rutten (1995)
Diethylene glycol mono butyl ether acetate (DEGBEA)	Water	Human	*In vitro*	13,000-200,000	42-3.4 (nd)	Inverse	Venier et al. (2004)
Dihydroxyacetone	Water	Human	*In vitro*	330-750	0.4-0.5 (0.42-0.39)	Not inverse	Yourick et al. (2004)
Diisononyl phthalate (DINP)	Liquid	Rat	*In vitro*	0.6-1.2 mg/kg bw	3.1-3.7 (nd)	Not inverse	Midwest Research Institute (1983)
Dinoconazole	No data	Rat	*In vitro*	4.9-49-490	8-10-0.6 (nd)	Inverse	Zendzian (2000)
Dipropylene glycol mono methyl ether (DPGME)	Water	Human	*In vitro*	95,000-190,000	0.27-0.32 (nd)	Not inverse	Venier et al. (2004)
Disulfoton	No data	Rat	*In vitro*	0.85-8.5-85	42-36-40 (nd)	Not inverse	Zendzian (2000)
EPTC	No data	Rat	*In vitro*	94-190-900-8800	5.6-3.1-5.8-8.6 (nd)	Not inverse	Zendzian (2000)
Ethoxyethanol	Acetone	Rat	*In vitro*	1200-3700-8400	27-17-20(7.8-5-9.4)	Not inverse	Sabourin et al. (1992)
Ethylene glycol mono methyl ether acetate (EGMEA)	Water	Human	*In vitro*	100,000-200,000	5.4-3.3 (nd)	Inverse	Venier *et al.* (2004)
Ethylene glycol mono n-propyl ether (EGnPE)	Water	Human	*In vitro*	91,000-180,000	3.3-1.6 (nd)	Inverse	Venier *et al.* (2004)
Fluazifop-butyl	Water + formulant	Human	*In vitro*	2.5-25-250	8-3.4-1.6 (1.6-0.77-0.36)	inverse	Ramsey *et al.* (1994, 1992)
Fluazifop-butyl	Water + formulant	Rat	*In vitro*	2.5-25-250	74-45-40 (15-22-13)	Not inverse	Ramsey *et al.* (1994)
Flurbiprofen	Acetone	Human	*In vitro*	44-310-2200	39-8.1-3.4 (14-2.3-1.9)	Inverse	Akhter and Barry (1985)
Furathiocarb	Acetone	Rat	*In vitro*	640-4800-9600-48,000	4-0.6-0.33-0.1 (nd)	Inverse	Liu and Kim (2003)
Glufosinate-ammonium	No data	Rat	*In vitro*	19-180-1800	41-43-33 (nd)	Not inverse	Zendzian (2000)

nd, no data; n/a, not applicable; Huntingdon, contribution of Huntingdon Life Sciences to work package 4 of the EDETOX project, unpublished data; TNO, confidential TNO-report

Table 2-1 continued

Substance	Phase or vehicle	Species	*Study*	Dermal loadings (μg/cm^2)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
Hexachlorobenzene	No data	Rat	*In vitro*	0.48-0.94-1.4	26-18-24 (nd)	Not inverse	Zendzian (2000)
Hydrocortisone	Solid	Human	*In vitro*	4-40	1.6-0.6 (1.4-0.3)	Not inverse	Wester and Maibach (1976, 1975)
Hydrogen cyanamide	No data	Rat	*In vitro*	8-80-800	1.7-1.8-10 (nd)	Not inverse	Zendzian (2000)
Ibuprofen	Acetone	Human	*In vitro*	44-310-2200	43-36-27 (16-13-10)	Not inverse	Akhter and Barry (1985)
Iprodione	No data	Rat	*In vitro*	31-310-31,000	7.4-3.2-0.19 (nd)	Inverse	Zendzian (2000)
Lauramide diethanolamine	Ethanol	Mouse	*In vitro*	780-1600-3100-12,000	49-67-69-50 (9.5-8.2-148.8)	Not inverse	Mathews *et al.* (1996)
Lauramide diethanolamine	Ethanol	Rat	*In vitro*	970-16,000	26-29 (6.4-9.6)	Not inverse	Mathews *et al.* (1996)
Malathion	Water	Human	*In vitro*	3.2-130	42-50 (43-19)	Not inverse	Wilkinson *et al.* (2005)
Methoxyethanol	Acetone	Rat	*In vitro*	900-2800-8300	19-27-21 (6.6-7.6-4.8)	Not inverse	Sabourin *et al.* (1992)
Mevinphos	No data	Rat	*In vitro*	0.45-2.5-13	14-14-20 (nd)	Not inverse	Zendzian (2000)
N,N-diethyl-m-toluamide	Ethanol	Human	*In vitro*	0.021-0.057-120-370-680-1500-2300-4800-8300	12-13-27-32-34-29-2521-17 (9.6-8.7-28-8.7-1225-34-39-30)	Not inverse	Santhanam *et al.* (2005)
Naloxone	Gel	Pig	*In vitro*	5100-10,000-15,000	10-6.8-4.9 (nd)	Inverse	Panchagnula *et al.* (2005)
N-methyl-2-pyrrolidone	Water	Rat	*In vitro*	25,000-50,000-100,000-200,000-400,000	61-64-51-42-35 (13-7.3-5.8-12-15)	Inverse	Payan *et al.* (2003)
NPE-4	PEG-400	Human	*In vitro*	3.1-31-310	0.97-0.08-0.011 (0.72-0.04-0.004)	Inverse	Monteiro Riviere *et al.* (2000)
NPE-4	PEG-400	Pig	*In vitro*	3.1-31-310	0.69-0.14-0.007 (0.46-0.1-0.002)	Inverse	Monteiro Riviere *et al.* (2000)
NPE-4	PEG-400	Rat	*In vitro*	3.1-31-310	0.45-0.06-0.005 (0.22-0.02-0.004)	Inverse	Monteiro Riviere *et al.* (2000)
NPE-9	PEG-400	Human	*In vitro*	3.1-31-310	0.86-0.08-0.009 (0.18-0.02-0.004)	Inverse	Monteiro Riviere *et al.* (2000)
NPE-9	PEG-400	Pig	*In vitro*	3.1-31-310	0.58-0.08-0.01 (0.14-0.06-0.006)	Inverse	Monteiro Riviere *et al.* (2000)

nd, no data; n/a, not applicable; Huntingdon, contribution of Huntingdon Life Sciences to work package 4 of the EDETOX project, unpublished data; TNO, confidential TNO-report

Table 2-1 continued

Substance	Phase or vehicle	Species	*Study*	Dermal loadings (µg/cm^2)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
NPE-9	PEG-400	Rat	*In vitro*	3.1-31-310	0.34-0.08-0.006 (0.14-0.04-0.004)	Inverse	Monteiro Riviere *et al.* (2000)
Parathion	Water	Human	*In vitro*	0.25-9.9	24-23 (nd)	Not inverse	Wilkinson *et al.* (2005)
Pentachlorphenol	Ethanol	Pig	*In vitro*	4-40	0.83-0.62 (0.67-0.47)	Not inverse	Baynes *et al.* (2002)
Pentachlorphenol	Ethanol (40%)	Pig	*In vitro*	4-40	3.2-4.4 (1.4-2.5)	Not inverse	Baynes *et al.* (2002)
Pesticide 1	Water + formulant	Human	*In vitro*	5-50-500	1.4-0.7-2.3 (nd)	Not inverse	Huntingdon
Pesticide 1	Water + formulant	Rat	*In vitro*	5-50-500	14-21-4.1 (nd)	Inverse	Huntingdon
Pesticide 2	Water + formulant	Human	*In vitro*	260-5200	34-80 (nd)	Not inverse	Huntingdon
Pesticide 2	Water + formulant	Rat	*In vitro*	260-5200	26-75 (nd)	Not inverse	Huntingdon
Pesticide 3	Water + formulant	Human	*In vitro*	36-470-4400	7.4-3.1-0.42 (nd)	Inverse	Huntingdon
Pesticide 3	Water + formulant	Rat	*In vitro*	36-470-4400	21-6.8-2.4 (nd)	Inverse	Huntingdon
Pesticide 4	Water + formulant	Human	*In vitro*	130-6200	1.2-0.06 (nd)	Inverse	Huntingdon
Pesticide 4	Water + formulant	Rat	*In vitro*	130-6200	4.3-0.25 (nd)	Inverse	Huntingdon
Pesticide 5	Water + formulant	Human	*In vitro*	60-540	1.4-0.2 (nd)	Inverse	Huntingdon
Pesticide 5	Water + formulant	Rat	*In vitro*	60-540	5.1-0.96 (nd)	Inverse	Huntingdon
Pesticide 5	Water + formulant	Rat	*In vitro*	6-550	16-1.6 (nd)	Inverse	Huntingdon
Pesticide 6	Water + formulant	Human	*In vitro*	21-530-2700	1.3-0.21-0.04 (nd)	Inverse	Huntingdon
Pesticide 6	Water + formulant	Rat	*In vitro*	21-530-2700	13-0.53-0.17 (nd)	Inverse	Huntingdon
Pesticide 6	Water + formulant	Rat	*In vitro*	21-530-2600	14-2.3-0.77 (nd)	Inverse	Huntingdon
Pesticide 7	Water + formulant	Human	*In vitro*	12-4800	2.5-0.12 (nd)	Inverse	Huntingdon
Pesticide 7	Water + formulant	Rat	*In vitro*	12-4800	19-1 (nd)	Inverse	Huntingdon
Pesticide 8	Water + formulant	Human	*In vitro*	9-4200	7.7-0.37 (nd)	Inverse	Huntingdon
Pesticide 8	Water + formulant	Rat	*In vitro*	9-4200	69-1 (nd)	Inverse	Huntingdon
Pesticide 8	Water + formulant	Rat	*In vitro*	18-5200	10-0.46 (nd)	Inverse	Huntingdon
Pesticide 9	Water + formulant	Human	*In vitro*	50-500	18-1.4 (nd)	Inverse	Huntingdon
Pesticide 9	Water + formulant	Rat	*In vitro*	50-500	28-22 (nd)	Not inverse	Huntingdon
Pesticide 10	Water + formulant	Human	*In vitro*	3-40	0.06-0.07 (nd)	Not inverse	Huntingdon
Pesticide 10	Water + formulant	Rat	*In vitro*	3-40	0.15-0.11 (nd)	Not inverse	Huntingdon
Pesticide 11	Water + formulant	Human	*In vitro*	4-700	0.45-0.07 (nd)	Inverse	Huntingdon
Pesticide 11	Water + formulant	Rat	*In vitro*	4-700	1.8-0.17 (nd)	Inverse	Huntingdon

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Table 2-1 continued

Substance	Phase or vehicle	Species	Study	Dermal loadings ($\mu g/cm^2$)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
Pesticide 11	Water + formulant	Rat	*In vitro*	3.8-680	4.7-0.92 (nd)	Inverse	Huntingdon
Pesticide 12	Water + formulant	Human	*In vitro*	1.5-1500	10-0.39 (nd)	Inverse	Huntingdon
Pesticide 12	Water + formulant	Rat	*In vitro*	1.5-1500	26-1.4 (nd)	Inverse	Huntingdon
Pesticide 12	Water + formulant	Rat	*In vitro*	1-1000	3.2-0.29 (nd)	Inverse	Huntingdon
Pesticide 13	Water + formulant	Human	*In vitro*	6.2-72-3800	0.33-0.27-0.035 (0.12-0.17-0.08)	Inverse	TNO
Pesticide 13	Water + formulant	Rat	*In vitro*	6.3-73-3800	3.5-1.6-0.06 (3.4-1.1-0.11)	Inverse	TNO
Pesticide 14	Water + formulant	Human	*In vitro*	82-720-9000	14-5.9-0.5 (2.8-0.9-0.1)	Inverse	TNO
Pesticide 14	Water + formulant	Rat	*In vitro*	82-720-9000	38-14-1.4 (8.8-3.4-0.1)	Inverse	TNO
Pesticide 15	Water + formulant	Human	*In vitro*	5.5-2300-4700	71-0.8-0.22 (13-0.37-0.05)	Inverse	TNO
Pesticide 15	Water + formulant	Rat	*In vitro*	5.5-2300-4700	90-0.99-0.44 (5.6-0.19-0.08)	Inverse	TNO
Pesticide 16	Water + formulant	Human	*In vitro*	39-420-1900	76-18-5.3 (19-4-1.5)	Inverse	TNO
Pesticide 16	Water + formulant	Rat	*In vitro*	39-420-1900	96-37-9.5 (6-12-1.7)	Inverse	TNO
Pesticide 17	Water + formulant	Human	*In vitro*	6.6-65-4000	1.2-1.5-19 (0.48-0.49-3.9)	Not inverse	TNO
Pesticide 17	Water + formulant	Rat	*In vitro*	6.6-65-4000	13-16-94 (13-7-4.8)	Not inverse	TNO
Pesticide 18	Water + formulant	Human	*In vitro*	130-710-2700	0.33-0.062-0.034 (0.1-0.02-0.005)	Inverse	TNO
Pesticide 18	Water + formulant	Rat	*In vitro*	130-710-2700	0.86-0.094-0.064 (0.15-0.03-0.04)	Inverse	TNO
Pesticide 19	Water + formulant	Human	*In vitro*	1-7.4-4200	8.5-9.9-0.019 (2.1-0.3)	Inverse	TNO
Pesticide 19	Water + formulant	Rat	*In vitro*	1-7.4-4200	29-28-0.1 (9.3-3.3-0.058)	Inverse	TNO
Pesticide 20	Water + formulant	Human	*In vitro*	7.3-82-1700	8.5-2.2-0.23 (nd)	Inverse	TNO
Pesticide 20	Water + formulant	Rat	*In vitro*	7.3-82-1700	31-3.8-0.35 (nd)	Inverse	TNO
Pesticide 21	Water + formulant	Human	*In vitro*	6.3-2500	13-1.6 (3.3-0.37)	Inverse	TNO
Pesticide 21	Water + formulant	Rat	*In vitro*	6.3-2600	13-2 (1.4-0.58)	Inverse	TNO
Pesticide 22	Water + formulant	Human	*In vitro*	0-130	0-0.22 (nd)	Inverse	TNO
Pesticide 23	Water + formulant	Human	*In vitro*	2.5-34-4300	0.036-0.026-0.17 (0.0750.015-0.028)	Inverse	TNO

nd, no data; n/a, not applicable; Huntingdon, contribution of Huntingdon Life Sciences to work package 4 of the EDETOX project, unpublished data; TNO, confidential TNO-report

Table 2-1 continued

Substance	Phase or vehicle	Species	Study	Dermal loadings ($\mu g/cm^2$)	Absorption (SD) (%)	% Absorption vs. dermal loading	Reference
Pesticide 23	Water + formulant	Rat	*In vitro*	3.3-35-4700	0.086-0.036-0.02 (0.0490.036-0.012)	Inverse	TNO
Pesticide 24	Water and acetone/ cremophore	Rat	*In vitro*	3.0-30 mg/kg bw	2.3-2.8 (nd)	Not inverse	TNO
Pesticide 25	Blank formulant	Rat	*In vitro*	10-100-1000	57-41-16 (nd)	Inverse	TNO
Phosmet	No data	Rat	*In vitro*	58-520-2700	7.8-1.7-0.23 (nd)	Inverse	Zendzian (2000)
Pyrene	Acetone	Guinea pig	*In vitro*	6.8-37	57-27 (2.8-0.9)	Inverse	Ng *et al.* (1992)
TCMTB	No data	Rat	*In vitro*	0.95-9.3-97	20-26-35 (nd)	Not inverse	Zendzian (2000)
Testosterone	Solid	Human	*In vitro*	3-30-400	12-8.8-2.8 (4.1-2-0.9)	Inverse	Wester and Maibach (1976, 1975)
Testosterone	Solid	Monkey	*In vitro*	4-40-250-400-1600-4000	18-6.7-2.9-2.2-2.9-1.4 (10-4.2-1.4-1-1.7-0.8)	Inverse	Wester and Maibach (1976, 1975)
Testosterone	WATER	Human	*In vitro*	0.53-20	42-27 (nd)	Inverse	Wilkinson *et al.* (2005)
Thiobencarb	No data	Rat	*In vitro*	5.2-50-490	72-73-42 (nd)	Not inverse	Zendzian (2000)
Triclosan	Water	Human	*In vitro*	0.23-9	15-25 (nd)	Not inverse	Wilkinson *et al.* (2005)
Trimethylamine	Water	Human	*In vitro*	310-3100-31,000	4.7-5.6-6 (3.8-2.1-1.6)	Not inverse	Kenyon *et al.* (2004)
Trimethylamine	Water	Rat	*In vitro*	310-3100-31,000	21-50-35 (7.5-21-18)	Not inverse	Kenyon *et al.* (2004)
Tris-(1,3-dichloro-2-propyl)phosphate	Acetone	Mouse	*In vitro*	0.013-0.067-0.13	57-45-39 (7.3-11-13)	Inverse	Hughes *et al.* (2001)
Vanillylnonanamide	Propylene glycol	Human	*In vitro*	1.4-4.4-15-44-110-340-1200-4200	46-39-37-36-23-20-8.33 (nd)	Inverse	Kasting (2001)
Zinc chloride	Water	Rat	*In vitro*	0.0092-1	6.1-3.6 (3.7-2.4)	Not inverse	Hallmans and Liden (1979)

nd, no data; n/a, not applicable; Huntingdon, contribution of Huntingdon Life Sciences to work package 4 of the EDETOX project, unpublished data; TNO, confidential TNO-report

Table 2-2 Relation between relative absorption and dermal loading.

Relation		Total	Dermal loading range factor examined				Dermal loading range factor (median)
			>1-10	>10-100	>100-1000	>1000	
Not inverse	Number	51	21	23	6	1	16
	Decrease factor ± SD	3.5 ± 17	1.3 ±0.8	1.0± 0.5	21 ± 50	0.7	
Inverse	Number	87	18	31	33	5	100
	Decrease factor ± SD	33 ± 69	4.4 ± 3.0	25 ± 31	40 ± 64	149 ± 203	
Total cases	Number	138	39	54	39	6	83

Decrease factor = relative absorption at low dermal loading divided by relative absorption at high dermal loading. Dermal loading range factor = high dermal loading divided by low dermal loading.

Table 2-3 Correlation between some parameters and the nature of the relationship relative absorption/dermal loading.

A. Influence extent of dermal loading range on nature of relationship relative absorption/dermal loading.

Relation	Dermal loading range factor (=high dermal loading/low dermal loading)				Total
	≤10	>10-100	>100-1000	>1000	
Not inverse	21	23	6	1	51
Inverse	18	31	33	5	87
Total	39	54	39	6	138

p-value χ^2: 0. 002 (null hypothesis = no difference between dermal loading range factor classes).

B. Influence log K_{OW} on nature of relationship relative absorption/dermal loading.

Relation	Log P				Total
	≤1	>1-3	>3-5	>5	
Not inverse	19	8	17	4	48
Inverse	8	23	49	7	87
Total	27	31	66	11	135

p-Value χ^2: <0. 0001 (null hypothesis = no difference between log K_{OW} classes).

C. Influence skin irritancy class on nature of relationship relative absorption/dermal loading.

Relation	Skin irritant		Total
	No	Yes	
Not inverse	3	25	28
Inverse	15	21	36
Total	18	46	64

p-Value χ^2: 0.001 (null hypothesis = no difference between skin irritancy classes). *Note:* Skin irritation class of the investigated substances was looked up in on-line databases (CTB, NTP, EPA, ATSDR) and in RTECS on CD-ROM).

D. Influence volatility of test substance on nature relationship relative absorption/ dermal loading.

Relation	Vapour pressure		Total
	>0.01 Pa	<0.01 Pa	
Not inverse	18	16	34
Inverse	11	33	44
Total	29	49	78

p-Value χ^2: 0.01 (null hypothesis = no difference between volatility classes).

E Influence pesticide formulant on nature relationship relative absorption/dermal loading.

Relation	Number			Dermal loading factor (mean ± SD (median))	
	Water	Water + formulant	Total	Water	Water + formulant
Not inverse	12	9	21	38 ±43 (23)	165 ± 251 (20)
Inverse	7	43	50	12 ±13 (6)	525 ± 896 (175)
Total	19	52	71	28 ± 37 (6)	463 ± 831 (129)

p-Value χ^2: <0.001 (null hypothesis = no difference between the two vehicle classes).
Dermal loading factor = high loading divide by low loading.

2.5 Discussion and conclusions

2.5.1 Relation between dermal loading and relative absorption

The experiments reviewed here provide a strong indication that in a majority of cases an inverse relationship exists between relative dermal absorption and dermal loading. Relative absorption may vary by more than a factor 100 within a dermal loading range that is relevant for occupational risk assessment. For the cases of inverse relationships reported here, relative absorption decreased on average with a factor 33 ± 69, when comparing low and high dermal loading (ranging from 0.009 μg/cm^2 to 400 mg/cm^2). A likely explanation of this relationship is saturation of the absorption capacity of the skin by the increasing dermal loading. When this capacity is saturated, the absorption rate will no longer proportionally increase with dermal loading, and thus the relative amount absorbed will decrease. Especially for poorly water-soluble substances, the very slow rate of dissolution in the water film on the skin or its rapid saturation may be the cause of levelling off of relative absorption in higher dermal loading ranges.

Although the majority of substances show an inversely proportional relationship between dermal loading and relative absorption, many do not. One explanation for this observation may be the dermal loading range, which in most experiments only covered one or two orders of magnitude: it may have been chosen in such a way that the penetration process was not yet saturated or not yet limited by the rate of dissolution into the dermal water film. Our results indeed show an increasing proportion of inverse relationships with wider dermal loading ranges (see Table 2-3A). Also skin irritating or corrosive properties of a substance may mask an inverse relationship, as rising concentrations of such a substance may decreasing the barrier function of the skin, increasing the permeability of the skin. Our results support this notion, as more than 80% of the proven non-irritants in our analysis show an inverse relationship while only 56% of the irritants exhibit this relationship (see Table 2-3C). Furthermore, volatile substances may not exhibit a clear inverse relationship between dermal loading and relative dermal absorption, because evaporation may rapidly reduce applied doses, thus preventing, e.g., saturation of the absorption process. This is corroborated by our observation that volatile substances showed relatively fewer cases of inverse relations between dermal loading and relative absorption than non-volatiles (see Table 2-3D).

2.5.2 Consequences for risk assessment

Underestimating the relative dermal absorption for the worker will lead to an underestimate of the internal exposure, which will in turn lead to an underestimation of the risk involved. Conversely, overestimating the dermal absorption for the worker will lead to an overestimation of the risk. Within a 100-fold range of exposure relative dermal absorption may vary up to a factor 100, therefore, as a consequence of the wrong choice of dermal loading, the risk index may be under- or over-estimated by the same factor.

In dermal exposure assessment often models are used which report their results in mg/person/day, without specifying the exposed area. If the underarms, hands and legs were exposed, the contaminated skin area would be ca. 8500 cm^2, while exposure of the hands only involves an area of 720 cm^2 (Paustenbach, 2000). Dermal exposure of 30 mg/day amounts to a dermal loading of 3.5 $\mu g/cm^2$ for arm, hand and leg exposure and to 42 $\mu g/cm^2$ for hands only. For e.g., 2,6-di-tert-butyl-4-nitrophenol these dermal loadings would mean a ca. eight times lower internal exposure for hands only (see Table 2-1) and therefore a lower risk than for the same absolute exposure to arms, hands and legs combined.

In European Risk Assessment Reports or pesticide monographs frequently only one value for relative dermal absorption is presented, often without specifying the dermal loading at which it was determined. For an appropriate evaluation, as illustrated by the example above, it is recommended to include the relevant data on dermal absorption studies in European Risk Assessment Reports and pesticide monographs, i.e., tested dermal loadings (expressed as $\mu g/cm^2$) with the corresponding relative absorption percentages. Furthermore, the tested dermal loadings should be similar to the actual human dermal loadings. In case of a (forcibly unnoticed) discrepancy in dermal loading between dermal absorption study and human exposure, relying on a single value may lead to a wrong estimate of internal exposure and therefore of risk.

2.6 Conclusions

Based on the data obtained, an inverse relationship between relative absorption and dermal loading exists for many chemical substances. Therefore, an internal exposure calculated with absorption data determined at a dermal loading not comparable to the actual loading may lead to a wrong assessment of the actual health risk. Some substances may exhibit deviating dermal absorption behaviour, likely caused by local toxicodynamic effects such as irritation or because of their volatility.

In order to prevent unsafe or overly conservative outcomes of risk assessments, the dermal loading dependency of relative dermal absorption should be taken into account. When using experimentally derived dermal absorption data, estimates determined at a dermal loading closest to that of the exposure of interest have to be used. Moreover, the inverse relation does stress the importance of examining, in dermal absorption studies, dermal loadings relevant for both human exposure and dermal toxicity studies.

Acknowledgments

We thank our colleague Carina Rubingh for her excellent statistical analysis of our data. The financial support the Dutch Ministry of Social Affairs and Employment for the research reported in this manuscript is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this chapter can be found, in the online version of the paper, at doi:10.1016/ j.yrtph.2009.04.002.

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3 The influence of repeated exposure to biocides on the skin barrier: A literature study

This chapter is largely based on TNO rapport V 5491 “Dermal absorption after repeated exposure to biocides. A discussion paper and research proposal” by H.E. Buist, J.G.M. Bessems, J.J. van de Sandt, F. Schurz, C. de Heer, published on November 19th, 2003.

3.1 Abstract

Differences between absorption after single and repeated dermal exposure were investigated based on a literature scan, which demonstrated that the effect of repeated exposure does not show a unique trend. However, an increase in daily absorption was frequently observed upon repeated daily exposure. The little information available on possible differences between single and repeated exposure was mostly related to pharmaceuticals. However, consumers and workers may be repeatedly exposed to other type of chemicals, like disinfectants and cleaning products, which often contain biocidal active substances that may decrease the barrier function of the skin, especially after repeated exposure. These biocidal products, therefore, may present a safety risk that is not covered by the present risk assessment practice. Consequently, it was decided to investigate the importance of this issue for biocide safety evaluation. As the present literature search revealed that hardly any data on absorption upon repeated dermal exposure to biocides are available, it was concluded that data need to be generated by testing.

To cover the entire range of biocidal products in such future testing, a representative series of biocidal substances needs to be tested, making *in vitro* testing of dermal absorption the preferred choice over *in vivo* testing. Based on an inventory made it appeared that the 16 product types represented among the biocidal products authorised in the Netherlands could be clustered into 6 more or less homogeneous categories based on similarity in active substances. Thus it can be concluded that the required *in vitro* testing can be limited to testing a representative congener from each of these 6 categories.

3.2 Introduction

The skin is a relevant route of internal exposure for workers and consumers (see 1.1.1). However, toxicity studies submitted within the European regulatory frameworks mostly concern the oral route of administration. This often implies that oral-to-dermal route extrapolation needs to be performed in order to compare dermal exposure to limit values derived from oral toxicity studies (ECB, 2003): this route-to-route extrapolation should ideally be based on internal exposure, which is calculated by correcting the measured or modelled external exposure values for the percentage absorption of the evaluated substance via the respective routes. Dermal absorption of the evaluated substances, commonly expressed as percentage of the applied dose, is usually determined through *in vivo* or *in vitro* dermal absorption studies executed according to globally accepted guidelines (OECD, 2004a and b; ECB, 2003). In the EU, default dermal absorption values of 100% or 10% (depending on the MW and log K_{OW} of the permeant) are used in case of oral-to-dermal route extrapolation, as a worst-case approach when data are not available (see section 1.1.4.6.1).

The OECD guidelines on *in vivo* or *in vitro* dermal absorption studies are not explicit on whether single or repeated exposure should be investigated (OECD, 2004a and b). Common

practice is that dermal absorption studies submitted within the EU regulatory frameworks are restricted to single exposure (ECB, 2003). Occupational dermal exposure is generally repeated in nature: e.g. disinfectant exposure in workers performing the daily disinfection of slaughterhouses (Preller & Schipper, 1999), metal working fluid exposure in metal workers (van Wendel de Joode, 2005), polycyclic aromatic compound exposure in asphalt pavement workers (McClean, 2012), exposure to floor and bathroom cleaning products in hospital cleaners (Bello, 2009). Also consumer dermal exposure, e.g. to cosmetics or household cleaning products, may be repeated in nature (WHO, 2013). Therefore, differences in absorption between single and repeated dermal exposure could be an issue in toxicological risk assessment.

In the present study the issue of repeated dermal exposure was investigated by scanning public literature (section 3.3.1). Based on this literature study, the possible differences between absorption after single and repeated dermal exposure and their potential causes are discussed. As few relevant data were found, an experimental approach to determine the importance of these differences in toxicological risk assessment was considered necessary. Chemically reactive substances are most likely to influence the barrier function of the skin (see section 1.1.5.1) and thus dermal absorption. Repeated exposure may aggravate these effects. In view of their function, many biocides are such reactive substances (see e.g. SCENIHR, 2009) and biocides are often repeatedly applied (e.g. disinfectants). Therefore, it was decided to use biocides as the model compounds to investigate the effects of repeated exposure on dermal absorption, since they represent worst case compounds. To select relevant and representative active substances used in biocides, an inventory was made of the biocides authorised in the Netherlands, their composition, molecular weights, log K_{OW}'s and skin irritating properties of their active substances (section 3.4.2). Hierarchic clustering provided insight into the subcategories that could be defined within the group of biocides providing a basis for definition of priorities for future experimental testing.

3.3 Methods

3.3.1 Literature scan on dermal absorption after repeated exposure

A search of the Medline database on CD-ROM was performed, covering the period 1966 to July 2003. The following key words were used: “skin” AND “absorption” AND (“repeated dose” OR “repeated exposure” OR “repeated”). Relevant references were collected and analysed. In 2015, the search performed in 2003 was updated to include references up to August 2015, in order to check whether additional references providing new insights could be gained from recent literature.

3.3.2 Inventory of biocides admitted in the Netherlands

An inventory of biocidal products authorised on the market in the Netherlands in 2003, their composition with respect to active substances and their area(s) of application was provided

by the Dutch Board for the Authorisation of Plant Protection Products and Biocides (CTGB), and also obtained from their web-site (http://www.ctgb.org/). Both datasets were combined to yield a complete inventory. The different biocide product types were grouped on the basis of the degree of overlap between the active substances used in the biocidal products of these product types, using the hierarchical clustering algorithm with city block as distant measure from the MATLAB software release 13 of The Mathworks Inc.

The CAS numbers of the active substances used in biocides were obtained using the on-line ChemFinder search engine (http://www.chemfinder.com/). The molar weights and log K_{OW}'s were picked from the on-line SRC Interactive PhysProp Database (http://esc.syrres.com/interK_{OW}/physdemo.htm). When no experimental log K_{OW} was found in this database, the SRC's on-line Interactive Log K_{OW} (KowWin) estimation software was used to calculate log K_{OW} (http://esc.syrres.com/interKow/Kowdemo.htm). The skin irritating properties of the active substances were collected from European chemical Substances Information System (ESIS) at that time provided on-line by the European Chemicals Bureau (http://ecb.jrc.it/)[5] and the on-line HSDB and Toxline databases accessed through TOXNET, portal provided by the US National Library of Medicine (http://toxnet.nlm. nih.gov/).

3.4 Results

The literature search performed in 2003 produced approximately 180 references, of which 57 seemed relevant for the issue at hand based on title and, if available, abstract. For fifty-two of these 57 references the full papers could be obtained for detailed evaluation, of which 27 indeed concerned the issue of absorption after repeated dermal exposure. Most substances reported in these references were dermally applied pharmaceuticals. One pesticide (malathion) was addressed, and no biocide. Nearly all references described *in vivo* studies (rats, mice, man). Most exposures investigated lasted up to 24 hours per day, and the substances were mostly applied daily, with a varying number of total applications. The vehicles in which the permeants were applied ranged from simple solvents, such as ethanol and DMSO, to creams and gels (see Table 3-1 to Table 3-3). Also the state of occlusion varied between studies.

The retrieved studies revealed some differences between the results of dermal absorption after single and repeated exposure. The clearest effect was the increase in daily absorption often observed when exposure was repeated each day (see Table 3-1 and Figure 3-1). This observation is based on experiments with 12-13 different chemicals. Blood concentrations showed a similar tendency, although, in general, the fold-change was lower and not always statistically significant (see Table 3-2 and Figure 3-2). Only 6-7 different substances with data on blood concentrations after single and repeated dermal exposure were retrieved from public literature. Very few studies on skin permeability were located: Roberts and

[5] Nowadays provided on-line by ECHA at http://echa.europa.eu/information-on-chemicals/cl-inventory-database.

Harlock (1978) found that salicylic acid may increase or decrease skin permeability, depending on the pattern of repeated exposure, while Campbell *et al.* (2002) did not find any influence of repeated exposure to lidocaine on skin permeability. A remarkable result was obtained by Vänttinen and Moravcova (2001): when repeating dermal exposure to two phytoestrogens after one and subsequently after five months, excretion of these chemicals and their final metabolites was lower after each repetition (a factor 10 or more). This big decrease in absorption is probably not related to changes in skin permeability, but rather to induction of metabolic changes, since baseline excretion of these compounds (measured one day before repetition of the exposure) had declined to the same degree.

An updated literature search performed in 2015 yielded 34 additional potentially relevant papers, of which only 14 contained information relevant to the issue at hand. Repeated exposure to benzophenone-3 (Gonzalez, *et al.* 2006), to butyl paraben (Janjua, *et al.* 2008) and N,N-dimethylformamide (Chang, *et al.* 2005) led to increased daily absorption compared to single exposure, as measured by urinary excretion. In four different studies on tacrolimus absorption during treatment of atopic dermatitis with a skin ointment containing this medicine, two also showed increases in daily absorption upon repeated exposure: Reitamo *et al.* (2009) found an increase in the plasma $AUC_{0\text{-}24}$ after 14 days of repeated exposure and Undre *et al.* (2009) observed that blood concentrations of tacrolimus were twice as high on day 14 of repeated exposure than on day 1. Plasma concentrations of tacrolimus were low: Undre *et al.* (2009) demonstrated that tacrolimus concentrations in the skin were more than 700 times higher than in blood. Furthermore, absorption tended to reduce with increased skin healing (Rubins, *et al.* 2005). These observations may explain

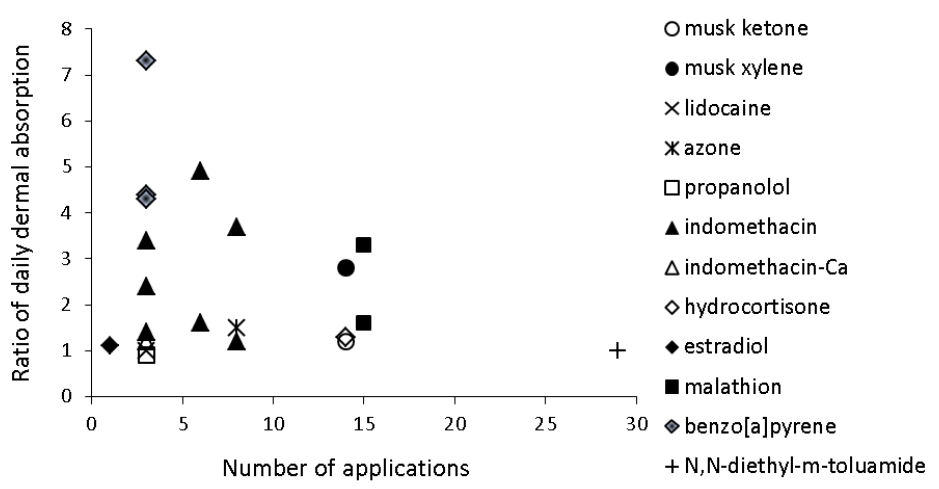

Figure 3-1 Dermal absorption after single and repeated daily dermal dosing
Data from studies with daily exposures, listed in Table 3-1. Daily dermal absorption after repeated exposure was divided by that after a single dose to obtain the ratio. Note that nearly all values are > 1 indicating increased absorption after repeated exposure as compared to single dose exposure.

why the other two studies (Harper, *et al.* 2005, Rubins, *et al.* 2005) did not find any increase in tacrolimus absorption upon repetition of exposure. Repeated dermal exposure of rats to progesterone led to a decrease in trough plasma concentrations[6] with one formulation ("F10") and no change with another (Progestelle®) (Matsui, *et al.* 2015).

Three studies concerned dermal drug delivery systems (a gel, a patch and a transdermal delivery system called Buprederm™) designed to maintain a steady state plasma concentration. The drugs investigated were oxybutynin (Dmochowski, *et al.* 2011), scopolamine (Nachum, *et al.* 2006) and buprenorphine (Park, *et al.* 2008). Repeated exposure (or continuous exposure with slow release) indeed produced a more or less stable plasma concentration of the drug or its active metabolite.

In short, these more recent papers confirmed the results of the previous investigation that there exists a tendency of increased daily absorption after repeated dermal exposure. New data on skin permeability after repeated dermal exposure were not found. Furthermore, relevant and consistent data remained scarce, making it difficult to draw general conclusions and to gain real insight in the importance of including the effects of repeated dermal exposure in toxicological risk assessment. In a next step it was therefore decided to investigate this issue via an experimental approach focused on biocides. In order to prepare a well-balanced research plan, an inventory of biocides authorised in the Netherlands was made and their active substances characterised with respect to a number of properties influencing dermal absorption.

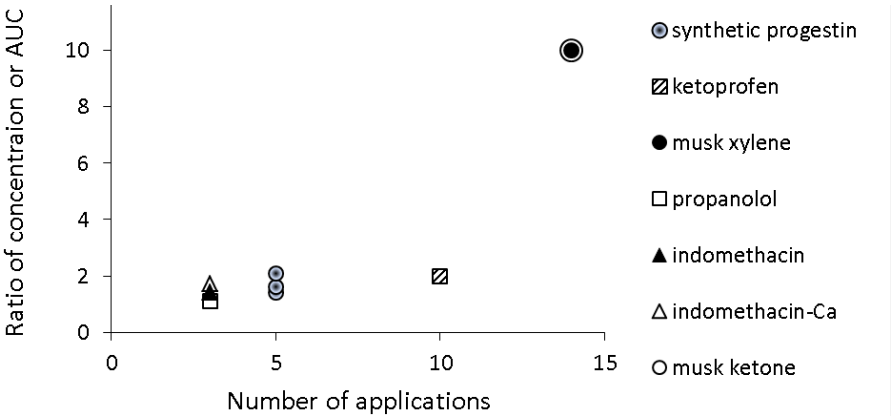

Figure 3-2 Blood (whole blood, plasma or serum) concentrations or AUC after single and repeated daily dermal dosing

Data from studies with daily exposures, listed in Table 3-2. AUC or blood concentration after repeated exposure was divided by the same parameter after single dose to obtain the ratio. Note that nearly all values are > 1 indicating increased absorption after repeated exposure as compared to single dose exposure.

[6] Through plasma concentrations are the dips in the plasma concentration time curves.

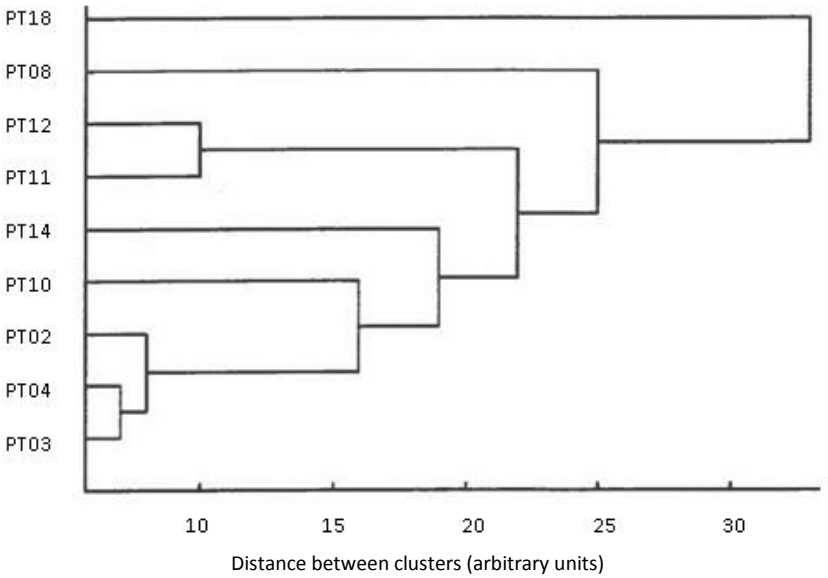

Figure 3-3 Clustering of areas of application of biocidal products

PT02 = Private area and public health area disinfectants and other biocidal products; PT03 = Veterinary hygiene biocidal products; PT04 = Food and feed area disinfectants; PT08 = Wood preservatives; PT10 = Masonry preservatives; PT11 = Preservatives for liquid-cooling and processing systems; PT12 = Slimicides; PT14 = Rodenticides; PT18 = Arthropodicides

In October 2003, 714 biocides were authorised in the Netherlands, covering 16 of the 23 biocidal product types defined by the EU Biocidal Products Directive (BPD) 98/8/EC (EU, 1998) (see Table 4-1). Quite a number of biocidal products were used in more than one area of application, on average the 714 different biocidal products were authorised under more than two biocidal product types. There is also a certain measure of overlap between biocides with regard to the active substances present, even when used in different areas of application. Thus the product types can be clustered on basis of the degree of overlap in their active substances. Figure 3-3 depicts the results of clustering of biocidal product types, using the hierarchical clustering algorithm with city block as distant measure from the MATLAB. This clustering revealed that biocides used as disinfectants across various product types (PT02-04) are quite alike, while also slimicides and preservatives for liquid cooling and processing systems (PT11-12) are chemically closely related. The arthropodicides (PT18) are unlike all other biocidal products.

Table 3-1 Repeated dermal exposure studies: Absorption data

Substance		Vehicle	Study type/ Species	dose /conc.	skin load (mg/ cm^2)	occlusion	h /day - n - frequency (day^{-1})	Abs. measurement method	Absorption				Reference
Category	Name								single	repea-ted	unit	repea-ted/ single	
cosmetic	musk ketone	ethanol/ phenyl ethyl alcohol (99/1)	*in vivo*/ rat	1 mg/L	0.011	occluded	6 - 14 - 1	Radiolabel in urine and faeces over 24 h	11.3[a,k,n]	13.9[a,k,n]	%	1.2	Hawkins & Ford, 1999
	musk xylene								7.4[a,k,n]	20.5[a,k,n]	%	2.8	
industrial chemical	dimethyl tere-phthalate	water, 1% Triton-X100	*in vivo*/rat	80 mg	nd	semi-occluded	48 - 5 - 0.5	urinary and faecal excretion, and organs	18.3[m,o]	16[m,o]	%	0.9	Moffitt *et al.*, 1975
	terephthalic acid								5.1[m,o]	9.4[m,o]	%	1.8	
insect repellent	N,N-diethyl-m-toluamide	absolute ethanol	*in vivo*/ rabbit	50 mg/kg bw	nd	non-occlusive	24 - 29 - 1	urinary excretion of radiolabel after 29 days	43[k,l,n]	43[k,l,n]	%	1	Snodgrass *et al.*, 1982
				100 mg/kg bw					47[k,l,n]	47[k,l,n]	%	1	
				500 mg/kg bw					48[k,l,n]	48[k,l,n]	%	1	
permeation enhancer	azone	cream	*in vivo*/ human	100 mg	2	semi-occluded	24 - 8 - 1	24-h urinary excretion of tracer radiolabel	1.84[k,n]	2.76[k,n]	%	1.5	Wester *et al.*, 1994
pesticide	malathion	toluene	*in vivo*/ Guinea pig		5	unoccluded	24 - 15 - 1	urinary excretion of tracer radiolabel over 7 days after application	1.6[k,o]	5.3[n]	%	3.3	Bucks, Marty, Maibach, 1985

Table 3-1 continued

Substance		Vehicle	Study type/ Species	dose /conc.	skin load (mg/ cm^2)	occlu-sion	h /day - n – frequency (day^{-1})	Abs. measurement method	Absorption				Reference
Category	Name								single	repea-ted	unit	repea-ted/ single	
pharma-ceutical	aescin	water	*in vivo*/ *pig*	0.7 mg	nd	occluded	24 - 3 - 3	urinary excretion 24 h after last application, normalised to 70 µg/kg	0.63[m,n]	0.87[m,n]	%	1.4	Lang, 1977
pharma-ceutical	daidzein	olive oil	*in vivo*/ human	9-10 mg	0.18-0.20	nd	nd - 3 - 0.017	Radiolabel in urine, period not specified, probably 24 h	16[k,n]	0.16[k,n]	%	0.01	Vänttinen & Moravcova, 2002
pharma-ceutical	diclofenac diethyl-ammonium salt	Voltarene emulgel	*in vivo*/ human	50 mg[f]	0.063	semi-occluded	2-24[g] - 14 - 1.8	Plasma AUC 0-24 h	237[n]	308[n]	nmol·h/ L	1.3	Sioufi *et al.*, 1994
pharma-ceutical	estradiol	acetone	*in vivo*/ human		0.004	un-occluded	<23 - 14 - 1	urinary excretion of tracer radiolabel over 7 days after application	9.9[k,o]	11[n]	%	1.1	Bucks, Maibach, Guy, 1985
pharma-ceutical	flurbiprofen	patch	*in vivo*/ human	40 mg	0.29	occluded	12 - 13 - 1.9	relative bioavailability based on plasma AUC(0-12h) dermal/AUC(0-∞) oral	5.2[e,k,n]	3.5[e,k,n]	%	0.67	Taburet *et al.*, 1995

Table 3-1 continued

Substance		Vehicle	Study type/ Species	dose /conc.	skin load (mg/ cm^2)	occlu-sion	h /day - n - frequency (day^{-1})	Abs. measurement method	Absorption				Reference
Category	Name								single	repea-ted	unit	repea-ted/ single	
pharma-ceutical	genistein	olive oil	*in vivo*/ human	9-10 mg	0.18-0.20	nd	nd - 3 - 0.017	Radiolabel in urine, period not specified, probably 24 h	$7.7^{k,n}$	$0.02^{k,n}$	%	0.0026	Vänttinen & Moravcova, 2001
pharma-ceutical	hydro-cortisone	acetone	*in vivo*/ human		0.004	un-occlu-ded	<23 - 14 - 1	urinary excretion of tracer radiolabel over 7 days after application	$2.6^{k,o}$	3.4^{n}	%	1.3	Bucks, Maibach, Guy, 1985
pharma-ceutical	indo-methacin	gel ointment (+azone)	*in vivo*/ rat	1 %	140	occlu-ded	24 - 3 - 1	AUC(24 h) in plasma relative to i.v. administration	$16^{j,k,n}$	$23^{j,k,n}$	%	1.4	Ogiso *et al.*, 1987
pharma-ceutical	indo-methacin	ointment	*in vivo*/ rabbit	3.3 %	3.3	occlu-ded	10 - 3 - 1	plasma AUC (0-∞ single, 0-10 h repeated)	9.56^{o}	32.9^{n}	µg·h/mL	3.4	Tsai *et al.*, 1985
							10 - 6 - 1		9.56^{o}	47.1^{n}	µg·h/mL	4.9	
							10 - 8 - 1		9.56^{o}	35.3^{n}	µg·h/mL	3.7	
							10 - 3 - 1		14.9^{o}	35.1^{n}	µg·h/mL	2.4	
							10 - 6 - 1		14.9^{o}	24.0^{n}	µg·h/mL	1.6	
							10 - 8 - 1		14.9^{o}	17.7^{n}	µg·h/mL	1.2	
pharma-ceutical	indo-methacin-Ca	gel ointment (+azone)	*in vivo*/ rat	1 %	140	occlu-ded	24 - 3 - 1	AUC(24 h) in plasma relative to i.v. administration	$53^{j,k,n}$	$65^{j,k,n}$	%	1.2	Ogiso *et al.*, 1987
pharma-ceutical	lidocaine	patch	*in vivo*/ *human*	2100 mg	5	occlude d	12 - 3 - 1	Plasma 24h-AUC parent compound	$3^{m,k,n}$	$3^{m,k,n}$	%	1	Campbell et al., 2002

Table 3-1 continued

Substance		Vehicle	Study type/ Species	dose /conc.	skin load (mg/ cm^2)	occlu-sion	h /day - n - frequency (day^{-1})	Abs. measurement method	Absorption				Reference
Category	Name								single	repea-ted	unit	repea-ted/ single	
pharma-ceutical	Methyl-predni-solone aceponate	ointment	*in vivo*/ human	nd	0.005	semi-occluded	24 - 8 - 1	7-day urinary excretion of tracer radiolabel	0.56[k,o]	0.36[k,o]	%	0.64	Täuber & Matthes, 1992
pharma-ceutical	oxatomide	gel	*in vivo*/ human	85 mg	nd	nd	24 - 13 - 1.9	plasma AUC parent compound, probably 0-12 h	15[n]	25[n]	ng·h/mL	1.7	Benvenuti *et al.*, 1992
pharma-ceutical	propanolol	ointment (including azone!)	*in vivo*/rabbit	150 mg	9.4 or 6	occluded	24 - 3 - 1	AUC(24 h) in plasma relative to i.v. administration	15[j,k,n]	14[j,k,n]	%	0.9	Ogiso *et al.*, 1988
pharma-ceutical	testosterone	acetone	*in vivo*/ human		0.004	un-occluded	<23 - 14 - 1	urinary excretion of tracer radiolabel over 7 days after application	21[k,o]	17[k,o]	%	0.81	Bucks, Maibach, Guy, 1985
polycyclic aromatic hydro-carbon	benzo[a]pyrene	olive oil/ acetone	*in vivo/rat*	10 µmol/kg bw	nd	occluded	24 - 3 - 1	urinary excretion of 3-OH-benzo[a] pyrene over 24 h after appli-cation	0.98[n]	4.32[n]	µg	4.4	Jongeneelen et al., 1984
				20 µmol/kg bw					0.59[n]	4.32[n]	µg	7.3	
				50 µmol/kg bw					1.77[n]	7.59[n]	µg	4.3	

Table 3-1 Legend

[a] Calculated by dividing the sum of µeq. excreted by the amount applied (99 µg)	[i] values for one subject
[b] concentration unit provided by authors, probably AUC is meant	[j] calculated by author of this thesis
[c] peak concentration read from graph	[k] relative to daily dose
[d] C_{max}	[l] relative to daily dose, steady state, reached after 24 h according to Snodgrass *et al.* (data not shown), percentages calculated from bar graph recovery after 29 days of exposure
[e] Bioavailability relative to orally dosed	[m] relative to total dose applied
[f] Expressed as diclofenac sodium	[n] daily absorption
[g] Subjects were not allowed to shower or bathe for 2 h after application	[o] absorption over more than a one day period
[h] 24 h after last application	

AUC = area under the curve; i.v. = intravenous, n = number of exposures; nd = no data

Table 3-2 Repeated dermal exposure studies: Blood values (whole blood, plasma or serum)

Substance		Vehicle	Study type/ Species	dose / conc.	skin load (mg/cm^2)	occlusion	h /day - n - frequency (day^{-1})	Blood/plasma/serum concentration				Reference
Category	Name							single	repeated	unit	repeated/ single	
cosmetic	musk ketone	ethanol/phenyl ethyl alcohol (99/1)	*in vivo*/ rat	1 mg/L	0.011	occluded	6 - 14 - 1	0.094	0.24	µg eq./g tissue	2.6	Hawkins & Ford, 1999
	musk xylene							0.012	0.12	µg eq./g tissue	10	
organic solvent	n-butanol	none	*in vivo*/ guinea pig	0.5 ml	nd	un-occluded	0.017 - 8 - 8	2.5	13	µM[a]	5.2	Boman & Maibach, 1996
	n-toluene							2.5	2	µM[a]	0.8	
pharma-ceutical	daidzein	olive oil	*in vivo*/ human	9-10 mg	0.18-0.20	nd	nd - 3 - 0.017	45	20	nmol.h^{-1} [b]	0.4	Vänttinen & Moravcova, 2001
pharma-ceutical	diclofenac diethyl-ammonium salt	Voltarene emulgel	*in vivo*/ human	50 mg[c]	0.063	semi-occluded	2-24[d] - 14 - 1.8	15	33	nmol/L[e]	2.2	Sioufi *et al.*, 1994
pharma-ceutical	flurbiprofen	patch	*in vivo*/ human	40 mg	0.29	occluded	12 - 13 - 1.9	43	103	ng/ml[b]	2.4	Taburet *et al.*, 1995

Table 3-2 continued

Substance		Vehicle	Study type/ Species	dose / conc.	skin load (mg/cm^2)	occlusion	h /day - n - frequency (day^{-1})	Blood/plasma/serum concentration				Reference
Category	Name							single	repeated	unit	repeated/ single	
pharmaceutical	genistein	olive oil	*in vivo*/ human	9-10 mg	0.18-0.20	nd	nd - 3 - 0.017	120	44	$nmol.h^{-1}$ [b]	0.4	Vänttinen & Moravcova, 2001
pharmaceutical	indomethacin	gel ointment (+azone)	*in vivo*/ rat	1 %	140	occluded	24 - 3 - 1	4.1	5.9	µg/ml [b]	1.4	Ogiso *et al.*, 1987
pharmaceutical	indomethacin-Ca							10	17	µg/ml [b]	1.7	
pharmaceutical	ketoprofen	gel	*in vivo*/ human	375 mg	0.5	semi-occluded	24 - 10 - 1	144	294	ng/ml [b]	2	Flouvat *et al.*, 1989
pharmaceutical	oxatomide	gel	*in vivo*/ human	85 mg	nd	nd	24 - 13 - 1.9	2.9	5	ng/ml [b]	1.7	Benvenuti *et al.*, 1992
pharmaceutical	propanolol	ointment (including azone!)	*in vivo*/ rabbit	150 mg	9.4 or 6	occluded	24 - 3 - 1	64	70	ng/ml [b]	1.1	Ogiso *et al.*, 1988
pharmaceutical	synthetic progestin ST-435	Progestogel/progesterone	*in vivo*/ human	2.3 mg	nd	semi-occluded	24 - 5 - 1	80	112	pmol/L [f]	1.4	Haukkamaa *et al.*, 1991
							24 - 5 - 1	184	296	pmol/L [f]	1.6	
							24 - 5 - 1	224	477	pmol/L [f]	2.1	

[a] peak concentration read from graph
[b] C_{max}
[c] Expressed as diclofenac sodium
n = number of exposures; nd = no data
[d] Subjects were not allowed to shower or bathe for 2 h after application
[e] 24 h after last application
[f] values for one subject

Table 3-3 Repeated dermal exposure studies with pharmaceuticals in which flux was measured

Sub-stance	Vehicle	Study type/Species	dose/ conc.	skin load (mg/cm^2)	occlusion	h/day - n - frequency (day^{-1})	Flux ($\mu g/cm^2/h$)			Remark	Reference
							single	repeated	repeated/ single		
lidocaine	patch	*in vivo*/human	2100 mg	5	occluded	12 - 3 - 1	12.6	12.4	1.0	--	Campbell *et al.*, 2002
salicylic acid	oint-ment	*in vivo*/*in vitro*/rat	1 %	nd	occluded	7.5 - 4 - 0.14	14	14	1.0	repeated exposure was executed *in vivo*, permeability of exposed skin was measured *in vitro*	Roberts & Horlock, 1978
				nd		7.5 - 3 - 1	16	22	1.4		
				nd		7.5 - 5 - 1	16	27	1.7		
			5 %	nd		7.5 - 4 - 0.14	61	27	0.4		
				nd		7.5 - 3 - 1	62	127	2.0		
				nd		7.5 - 5 - 1	62	88	1.4		
			10 %	nd		7.5 - 4 - 0.14	78	42	0.5		
				nd		7.5 - 3 - 1	80	173	2.2		
				nd		7.5 - 5 - 1	80	149	1.9		

n = number of exposures; nd = no data

Table 3-4 Top twenty biocidal active substances and some of their characteristics

Rank	Name	CAS No.	MW	Log K_{OW}	Skin irritant?	Number of products
1	didecyldimethylammoniumchloride	7173-51-5	362	4.7	Yes	166
2	sodium hypochlorite	10022-70-5	74	?	Yes	64
3	hydrogen peroxide	7722-84-1	34	-1.6	Yes	46
4	potassium hydroxide	1310-58-3	56	?	Yes	39
5	glutaraldehyde	111-30-8	100	-0.2	Yes	41
6	alkyldimethylbenzylammoniumchloride	63449-41-2	372	?	Yes	42
7	peracetic acid	79-21-0	76	-1.1	Yes	33
8	sodium dichloro-isocyanurate	2893-78-9	220	-0.06	Doubtful	30
9	sodium hydroxide	1310-73-2	40	-3.9	Yes	25
10	2-methyl-4-isothiazolin-3-one	2682-20-4	115	?	Yes	23
11	5-chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	150	-0.3	Yes	23
12	formaldehyde	50-00-0	30	0.4	Yes	23
13	l-bromo-3-chloro-5,5-dimethyl-hydantoine	16079-88-2	241	-0.9	?	20
14	deltamethrin	52918-63-5	505	6.2	No	18
15	piperonyl butoxide	51-03-6	338	4.8	Yes	17
16	diethyl-m-toluamide	134-62-3	191	2.2	Yes	17
17	pyrethrins	121-29-9	372	4.3	No	15
18	2,2 dibromo-3-nitrilopropionamide	10222-01-2	242	0.8	Yes	15
19	difenacoum	56073-07-5	445	7.6	?	15
20	copper(I)oxide	1317-39-1	143	?	?	15

A total of 100 active substances were represented in the 714 authorised biocidal products. The top 20 compounds are listed in Table 3-4, along with some of their characteristics. Of the 714 authorised biocidal products 452 contain only one active substance, 152 contain two, 43 three and 6 four active substances. The 20 most frequent pairs of active substances are listed in Table 3-5. The top 3 contains lye, an acid and oxidising agents. The fourth ranked pair consists of the known sensitizers 2-methyl-4-isothiazolin-3-one and 5-chloro-2-methyl-4-isothiazolin-3-one (see http://echa.europa.eu/information-on-chemicals), while ranks 5 to 7 contain aldehydes and surfactants. Approximately 60% of the biocidal substances with skin irritation data are classified as skin irritants (see Table 3-6).

MW and log K_{OW}

Molecular weight and log K_{OW} are important determinants for the ability of chemicals to penetrate the skin barrier (De Heer *et al.,* 1999; ECB, 2003). Therefore, these properties are important to consider when selecting representative biocidal active substances for the experiments to be planned. Most biocidal active substances are expected to exhibit high dermal absorption since their MW is lower than 500, while only few are expected to exhibit low dermal absorption, having a MW >500 and a log K_{OW} >4 or <-1 (see section 1.1.4.6.1). Figure 3-4 depicts the log K_{OW} and MW distribution for biocidal active substances.

In general, preservatives and disinfectants tend to be small, hydrophilic molecules, while rodenticides and arthropodicides tend to be larger and more lipophilic (see Figure 3-2). Wood preservatives are an exception to this rule, having an intermediate position between these two groups.

Table 3-5 The twenty most frequent pairs of active substances in registered biocidal products containing two or more active substances

Rank	Pair	Number of products
1	potassium hydroxide and sodium hypochlorite	42
2	peracetic acid and hydrogen peroxide	34
3	sodium hypochlorite and sodium hydroxide	25
4	2-methyl-4-isothiazolin-3-one and 5-chloro-2-methyl-4-isothiazolin-3-one	23
5	didecyldimethylammoniumchloride and formaldehyde	22
6	didecyldimethylammoniumchloride and glutaraldehyde	22
7	formaldehyde and glutaraldehyde	20
8	piperonyl butoxide and pyrethrins	14
9	alkyldimethylbenzylammoniumchloride and didecyldimethylammoniumchloride	10
10	1-bromo-3-chloro-5,5-dimethylhydantoine and l,3-dichloro-5-ethyl-5-methyl hydantoine	7
11	alkyldimethylbenzylammoniumchloride and glutaraldehyde	7
12	diurone and copper(I)oxide	6
13	carbendazim and thiram	5
14	carbendazim and ziram	5
15	copper(I)oxide and zinc oxide	5
16	potassium hydroxide and sodium hydroxide	5
17	ziram and thiram	5
18	1,3-dichloro-5-ethyl-5-methylhydantoine and l-3-dichloro-5,5-dimethylhydantoine	4
19	fenoxycarb and piperonyl butoxide	4
20	permethrin and fenoxycarb	4

Table 3-6 Classification of biocidal active substances with respect to skin irritation

Classification	Number	% of biocidal active substances with skin irritation data
Skin irritant	43	59
Doubtful	7	9.6
Non skin irritant	23	32
No skin irritation data found	30	n/a
Total investigated	**103**	

3.5 Discussion and conclusions

The scan of public literature demonstrated no clear trend in the effects of repeated dermal exposure on absorption. This is probably caused by differences in the chemical nature of the permeants, as well as the variety of exposure conditions and vehicles used. However, one effect was frequently observed: an increase in daily absorption when exposure was repeated daily. This implies that single exposure dermal absorption experiments often used

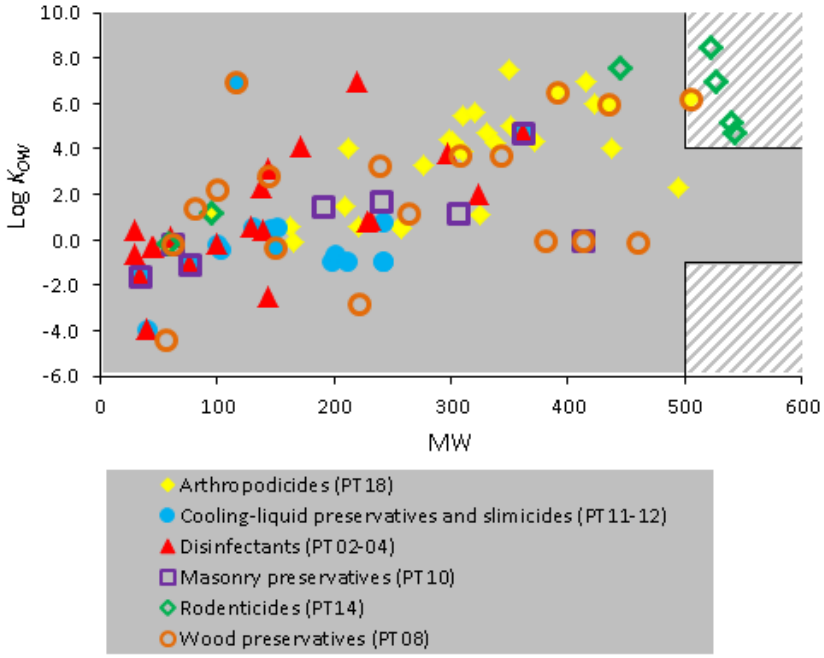

Figure 3-4 Distribution of log K_{OW} and MW of biocidal active substances
The hashed areas contain the chemicals with a low (default) dermal absorption and the uniformly grey area the ones with a high (default) absorption, based on the rule of thumb that dermal absorption of substances with a log K_{OW} <-1 or >4 and a MW >500 is 10% and that of all other substances 100%.

in regulatory settings may underestimate daily absorption of chemicals when repeatedly applied, e.g. daily.

The results of the inventory of the biocides authorised in the Netherlands confirmed their possible impact on the skin barrier, since many of them were reactive chemicals like lye, acids, aldehydes, peroxides and/or have corrosive, irritant or skins sensitising properties. This warrants experimental research into the effect of repeated exposure to biocides on the skin barrier, since the usual single exposure dermal absorption experiments may not suffice to adequately record the possible impact and literature data on this issue are lacking.

To cover the entire range of biocidal products, a representative series of biocidal substances needs to be investigated. Aspects to be included are the different functions biocides may have, their corrosive and/or irritating properties and their physicochemical properties like log K_{OW} and MW. In order to achieve a representative overview, many biocides would need to be tested, making *in vitro* testing of dermal absorption the preferred choice over *in vivo* testing, as the former costs less time and money. Results of the present inventory revealed that the 16 product types represented among the biocidal products authorised in the Netherlands could be clustered into 6 more or less homogeneous categories based on

similarity in active substances. Thus it is concluded that the required *in vitro* testing can be limited to testing a representative congener from each category.

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4 Effects of single and repeated exposure to biocidal active substances on the barrier function of the skin *in vitro*

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Effects of single and repeated exposure to biocidal active substances on the barrier function of the skin *in vitro.*
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4.1 Abstract

The dermal route of exposure is important in worker exposure to biocidal products. Many biocidal active substances which are used on a daily basis may decrease the barrier function of the skin to a larger extent than current risk assessment practice addresses, due to possible skin effects of repeated exposure. The influence of repeated and single exposure to representative biocidal active substances on the skin barrier was investigated *in vitro*. The biocidal active substances selected were alkyldimethylbenzylammonium chloride (ADBAC), boric acid, deltamethrin, dimethyldidecylammonium chloride (DDAC), formaldehyde, permethrin, piperonyl butoxide, sodium bromide, and tebuconazole. Of these nine compounds, only the quaternary ammonium chlorides ADBAC and DDAC had a clear and consistent influence on skin permeability of the marker compounds tritiated water and [^{14}C]propoxur. For these compounds, repeated exposure increased skin permeability more than single exposure. At high concentrations the difference between single and repeated exposure was quantitatively significant: repeated exposure to 300 mg/L ADBAC increased skin permeability two to threefold in comparison to single exposure. Therefore, single and repeated exposure to specific biocidal products may significantly increase skin permeability, especially when used undiluted.

4.2 Introduction

The dermal route of exposure is important in worker exposure to biocidal products (TNsG, 2002). For a number of biocidal product types, professional operators are likely to be exposed five days a week for extended periods of time (TNsG, 2002), e.g., in slaughterhouses which are disinfected daily (HeRy *et al.*, 1999). Many biocidal active substances are classified as “corrosive” or “skin irritants” by the EU or, if EU classification is lacking, are listed in public literature as “skin irritants”. In October 2003, 714 biocidal products were admitted in the Netherlands, covering 16 of the 23 biocidal product types defined by the EU Biocidal Products Directive (BPD) 98/8/EC (EU, 1998) (see Table 4-1). The total number of active substances represented in the 714 admitted biocidal products is 100. More than one third of these products contain two or more biocidal active substances. Of the biocidal active substances used in disinfectants registered in the Netherlands (EU Product Types (PT) 02-05) 50% are classified as “corrosive” by the EU and 7% as “skin irritant,” while another 25% is listed in public literature as “skin irritant” (data not shown). Disinfectants represent by far the highest number of registered biocidal products in the Netherlands.

Corrosive or skin irritating substances are able to damage the barrier function of the skin, thereby potentially increasing the systemic exposure to these substances. Repeated exposure may increase the inflicted skin damage, thereby further augmenting systemic exposure.

Table 4-1 Distribution of the biocidal products registered in The Netherlands over the different product types

Product type	EU-code	Number of products	Product type	EU-code	Number of products
Private area and public health area disinfectants and other biocidal products	PT02	297	Masonry preservatives	PT10	100
Veterinary hygiene biocidal products	PT03	208	Preservatives for liquid-cooling and processing systems	PT11	184
Food and feed area disinfectants	PT04	433	Slimicides	PT12	124
Drinking water disinfectants	PT05	5	Metalworking-fluid preservatives	PT13	10
In-can preservatives	PT06	4	Rodenticides	PT14	37
Film preservatives	PT07	1	Repellents and attractants	PT19	18
Wood preservatives	PT08	58	Insecticides, acaricides, and products to control other arthropods (“arthropodicides”)	PT18	162
Fibre, leather, rubber, and polymerised materials preservatives	PT09	1	Antifouling products	PT21	52

A considerable number of biocidal products are registered under two or more product types

In toxicological risk assessment, dermal absorption of the evaluated substance (e.g., a biocide) is usually determined through *in vivo* or *in vitro* dermal absorption studies executed according to OECD or EPA guidelines (ECB, 2003; EPA, 1998; OECD, 2004a; OECD, 2004b). Common practice is that dermal absorption studies submitted within the EU regulatory frameworks are restricted to single exposures (ECB, 2003). Therefore, possible differences in absorption between single and repeated dermal exposure are not taken into account, which may lead to underestimation of toxicological risks.

Since many biocidal active substances are used on a daily basis, they have the potential to affect the barrier function of the skin to a larger extent than current risk assessment practice addresses. A survey of public literature revealed that the public literature contains limited information on the influence of biocidal active substances on skin permeability (see chapter 3). Furthermore, information on repeated exposure appears to be restricted to chemicals which are not representative of biocidal products. In this paper, experimental research on the influence of single and repeated dermal exposure to biocidal active substances on *in vitro* skin permeability is reported. Nine biocidal substances were selected for the experiments, based on pre-set criteria.

Table 4-2 Selected biocidal active substances and their characteristics

Parameter	Selected biocidal active substances								
	ADBAC[a]	DDAC	Formaldehyde	Piperonyl butoxide	Permethrin	Boric acid	Tebu-conazole	Deltamethrin	Sodium bromide
MW	354[b]	362	30	338	391	62	308	505	103
log K_{OW}	3.4[c]	4.7	0.4	4.8	6.5	-0.2	3.7	6.2	-0.4
Skin classification[d]	Corrosive	Corrosive	Corrosive	Not classified	Not classified	Not classified	Not classified	Not classified	Not classified
Skin irritant? (public literature)	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Unknown
Number of products	42	166	23	17	15	6	1	18	10
Number of product types	7	7	3	1	2	2	1	2	2
Combinations with other active substances	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Dermal absorption	Not reported	Not reported	10% in rat and guinea pig after 72 h (dose: 10 mg/mL, 0.1 mg/animal) (Jeffcoat *et al.*, 1983)	ca. 2% in humans after 8 h (dose: ca 125 µg/cm^2) (Selim *et al.*, 1999)	ca. 2% in rat, mouse and pig *in vitro* after 8 or 24 h (dose 500 µg/cm^2) (Baynes *et al.*, 1997)	Poorly absorbed (Beyer *et al.*, 1983)	Not reported	Incompletely absorbed in mammals (Perger and Szadkowski, 1994); poorly absorbed in rats (Kavlock *et al.*, 1979)	Not reported

[a] Mixture of various alkyl chain lengths, e.g., 60% dodecyl- and 40% tetradecyldimethylbenzylammoniumchloride (marketed by Aldrich).
[b] Average of molecular weights of dodecyl- and tetradecyldimethylbenzylammoniumchloride.
[c] Average of log K_{OW} calculated with KowWin for dodecyl- and tetradecyldimethylbenzylammonium chloride.
[d] Classification for skin sensitisation not included.

4.3 Materials and methods

4.3.1 Selection of biocidal active substances

The biocidal active substances were selected based on an inventory of biocidal products admitted on the market in the Netherlands, including their composition with respect to active substances (biocidal active substances) and their area(s) of application. The molar weights and octanol water partition coefficients (log K_{OW}) were retrieved from the on-line SRC Interactive PhysProp Database (http://esc.syrres.com/interK_{OW}/physdemo.htm). If no experimental log K_{OW} was available in this database, the SRC's on-line Interactive log K_{OW} (KowWin) estimation software was used to calculate log K_{OW}[7]. The present EU-classification (EU, 2004) of the biocidal active substances with respect to skin irritating properties was retrieved from the European Chemical Substances Information System provided on-line by the European Chemicals Bureau[8]. Non-classification by the EU does not necessarily imply a biocidal active substance has been tested negatively: it may simply mean it was not tested for that particular characteristic. Therefore, if the biocidal active substance was not classified as "corrosive" or "skin irritating" by the EU or was not listed, data from the on-line HSDB and Toxline databases, accessed through TOXNET[9], were used to judge the skin irritation potential.

The following criteria have been applied in order to select biocidal active substances representative of the biocidal products admitted in the Netherlands for testing:

1. The selection should reflect the variety in molecular weights and log K_{OW} values among biocidal active substances.
2. The selected active substances should be quantitatively important, as measured by the number of products and product types they are part of.
3. Inorganic and organic molecules should be represented.
4. Both skin irritants and non-irritants should be represented.
5. The selected substances should occur in combination with other active substances (since one active substance may influence the skin absorption of another active substance in the product).

Based on these criteria, initially eight substances were selected for the *in vitro* assays. Table 4-2 lists the selected substances and their selection parameter values, based on which they were considered fairly representative of the biocidal active substances admitted on the Dutch market. As the preliminary results indicated that alkyldimethylbenzylammonium chloride (ADBAC) was the only substance showing a clear difference in skin permeability between single and repeated exposure, its structural analogue didecyldimethylammonium chloride (DDAC) was later added. Its characteristics are quite similar to those of ADBAC, and

[7] http://esc.syrres.com/interK_{OW}/K_{OW}demo.htm)
[8] http://ecb.jrc.it/
[9] http://toxnet.nlm.nih.gov/

it is represented in even more biocidal products (166). Ten products consist of a combination of ADBAC and DDAC.

4.3.2 *In vitro* skin permeability assay

The assay was performed in static diffusion cells using cryopreserved human abdominal skin, as previously described by van de Sandt *et al.* (1993, 2000). The skin originated from seven female donors, aged 31-57 years (average 39). The *dermis* was partly removed using forceps and scissors, and the average skin thickness was 0.702 ± 0.062 mm. The receptor fluid (total volume 1.2 mL) consisted of a physiological salt solution (0.9% NaCl w/v) containing 0.01% sodium azide. The skin preparations were exposed to 10 µL/cm^2 of a solution of the selected biocidal active substance for 4h (single exposure, starting 48 h after onset of the experiment) or for 3 times 4h (repeated exposure, starting at 0, 24, and 48 h, respectively). If applicable, the ethanol solvent was evaporated under N_2, immediately after application. Each biocidal active substance was tested at 3-4 concentrations, representing the range of concentrations in which the biocide can be present in the products and in the prescribed application dilutions. The specifications, concentrations, and solvents used are listed in Table 4-3. The experimental design is illustrated in Figure 4-1

Each exposure period was finished by washing off the test substance using 4 cotton swabs humidified with a 3% Teepol solution and subsequently drying the skin preparations using 2 dry cotton swabs. The same washing procedure was applied to the control skins. In the repeated as well as in the single exposure experiments all skins were washed three times, in order to control for a possible effect of the washing procedure on skin permeability. For each biocide/concentration combination, the single and multiple dosing experiments were

Table 4-3 Test substance concentrations applied

Test substance (purity, %w/w)	*Supplier*	Concentrations applied (mg/ml)	Vehicle
ADBAC (not specified)[a]	*Sigma*	300, 50, 1	Water
Boric acid (>99%)	*Aldrich*	50, 25, 10	Water
DDAC[b] (ca. 50% 2-propanol-water solution)	*Merck*	100, 25, 2.5, 0.005	7.4% 2-propanol in water
Deltamethrin (99%)	*Supelco*	3, 1, 0.2	Ethanol
Formaldehyde (36.5-38% aqueous solution)	*Sigma*	30, 5, 0.2	Water
Permethrin (94.4%)	*Riedel de Haan*	10, 1, 0.1	Ethanol
Piperonyl butoxide (tech. 90%)	*Aldrich*	30, 10, 1	Ethanol
Sodium bromide (minimum 99.5%)	*Sigma*	500, 1, 0.003	Water
Tebuconazole (99.4%)	*Riedel de Haan*	10, 5, 1	1% carboxymethyl-cellulose

[a] Alkyldimethylbenzylammonium chloride; the alkyl group is predominantly *n*-$C_{12}H_{25}$, but the product also contained C_{14} and C_{16} homologues. It passed the Sigma quality control with respect to impurities.
[b] Didecyldimethylammonium chloride.

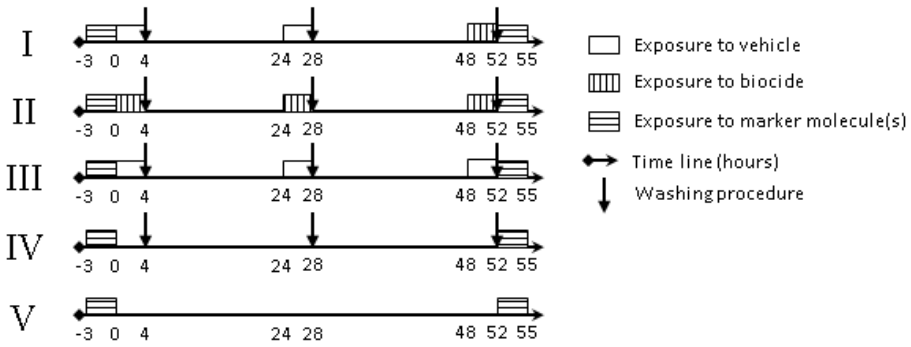

Figure 4-1 Experimental design.
I, single exposure; II, multiple exposure; III, vehicle control (ethanol, 1% carboxymethylcellulose, 7.4% 2-propanol in water); IV, vehicle control (water); and V, control without washing.

always performed with skin from the same donor. The proper control experiments were performed in parallel with every individual experiment.

Prior to the start of the experiment, integrity of the skin preparations was assessed by determining the permeability coefficient (k_p) of tritiated water, as described by van de Sandt *et al.* (1993, 2000), using a cut-off value of 4.0 x 10^{-3} cm/h. Alongside with tritiated water, [^{14}C]propoxur, a pesticide with MW 209, log K_{OW} 1.5, was used as marker of skin permeability. At the end of the last exposure period, 500 µL of a mix of [^{3}H]H_2O (Perkin Elmer Life Sciences, purity not indicated, ca. 37 kBq/mL) and ca. 31 mM [^{14}C]propoxur (TNO-Prins Maurits Laboratory, Rijswijk, the Netherlands, purity >99%,40.7 MBq/mL) was applied to the skin preparations. During the following 3h, 200 µL samples of receptor fluid were collected every hour and assayed for ^{3}H- and ^{14}C-radioactivity by liquid scintillation counting in a Wallac Pharmacia scintillation counter. Directly after each sampling the original volume of the receptor fluid was restored by adding 200 µL fresh receptor fluid to each well. The amount of radiolabelled marker molecules that had penetrated the skin was plotted against time. For [^{3}H]H_2O the penetration rate was calculated by linear regression analysis over 3h. Since for [^{14}C]propoxur no steady state was reached, the maximum penetration rate was calculated over the last hour of marker exposure. The permeability constant (k_p, expressed in cm/h) for [^{3}H]H_2O and the apparent k_p for [^{14}C]propoxur of the treated and control skins were calculated by dividing the maximum penetration rate (µg/cm^2/h) by the applied concentration (µg/cm^3).

Skin permeability may vary considerably between donors or due to differences in solvents. In order to control for these variables, relative (apparent) k_p values were calculated by dividing the (apparent) k_p of an exposed skin by the (apparent) k_p of its control skin. These relative (apparent) k_p values were used to measure the effects of biocidal active substances on the skin barrier function. Each exposure condition was performed in quadruplicate. Occasionally, outlying relative (apparent) k_p values which differed more than 3 standard

deviations from the mean of the other three repeat values, were excluded from the results. To detect treatment effects as such, comparisons were made for absolute k_p values for each substance and for each treatment level and the pertaining control. The assumption was made that the samples were from a normal distribution and the following procedure was applied:

1. First it was tested whether a substance had a statistically significant effect on the cell membrane per se by means of *t* tests between treatments and the pertaining control. The homogeneity of variances was tested and a pooled or a separate variance *t* test was applied.
2. Relative k_p values were analysed separately for tritiated water and propoxur, using a two-way ANOVA with factors Group (different concentrations) and Application (single/repeated). If either one or two factors or the interaction yielded a significant effect ($p < 0.05$), post hoc *t* tests were applied between single-repeated application for each concentration level.
3. If variances in relative k_p values were not homogeneous (according to Levine's test for equality of variances), Brown-Forsythe two-way ANOVA was applied instead, followed by, if the ANOVA was significant ($p < 0.05$), separate variance post hoc *t* tests.
4. Linear trend tests (orthogonal contrasts) were applied to relative k_p values across increasing concentrations for every substance, separately for the single and repeated factor.

Power analysis showed that a two-fold increase or decrease in tritiated water permeability, on average, can be detected with sufficient confidence (with a power of 91 ± 17% for differences between control and exposed groups, and of 91 ± 13% for differences between single and repeated exposure). Changes in propoxur permeability could only be detected with sufficient power from threefold increases or decreases upwards (power 92 ± 13% for differences between control and exposed groups and of 89 ± 18% for differences between single and repeated exposure). Therefore, the test procedure is sensitive enough to detect small, but relevant differences in permeability for tritiated water.

4.4 Results

Nine biocidal active substances were tested for their effect on skin barrier function. Only the quaternary ammonium chlorides ADBAC and DDAC had a clear and consistent influence on skin permeability as measured with propoxur and tritiated water. After single exposure to ADBAC, skin permeability increased most at a concentration of 50 mg/L: approximately twofold (Table 4-4 and Figure 4-2). Single exposure to DDAC caused the highest increase in permeability at 100 mg/L, being ca. three to fourfold (Table 4-4 and Figure 4-3). After repeated exposure, the maximum increase in skin permeability after ADBAC exposure was observed at a concentration of 300 mg/L and was ca. three to fourfold, while for DDAC this was ca. four to fivefold at a concentration of 100 mg/L. Except for tritiated water

Table 4-4 Skin permeability for $^{3}H_2O$ and [^{14}C]propoxur after single and repeated exposure to a number of biocidal active substances

Biocide	Concen-tration (mg/L)	Relative (apparent) k_p (% of control ± SD)				Ratio repeated/single (±SD)[a]	
		$^{3}H_2O$		[^{14}C]propoxur			
		Single	Repeated	Single	Repeated	$^{3}H_2O$	[^{14}C]Propoxur
Boric acid	10	116 ± 28	87 ± 31	82 ± 40	77 ± 17	0.8 ± 0.3	0.9 ± 0.5
	25	96 ± 26	112 ± 31	94 ± 27	91 ± 19	1.2 ± 0.5	1.0 ± 0.3
	50	101 ± 30	108 ± 22	81 ± 43	105 ± 40	1.1 ± 0.4	1.3 ± 0.8
Sodium bromide	0.003	123 ± 50	94 ± 36	117 ± 25	102 ± 44	0.8 ± 0.4	0.9 ± 0.4
	1	83 ± 26	133 ± 51	96 ± 32	101 ± 55	1.6 ± 0.8	1.1 ± 0.7
	500	106 ± 35	113 ± 48	107 ± 38	156 ± 95	1.1 ± 0.6	1.5 ± 1.0
ADBAC	1	91 ± 16	89 ± 13	43 ± 12	60 ± 17	1.0 ± 0.2	1.4 ± 0.6
	50	*188 ± 36*	*228 ± 23*	*242 ± 68*	*360 ± 46*	1.2 ± 0.3	**1.5 ± 0.5**
	300	123 ± 19	*410 ± 151*	155 ± 42	*364 ± 32*	**3.3 ± 1.3**	**2.3 ± 0.7**
DDAC	0.005	108 ± 13	113 ± 40	*165 ± 6*	115 ± 20	1.0 ± 0.4	**0.7 ± 0.1**
	2.5	141 ± 25	161 ± 30	*278 ± 66*	*279 ± 94*	1.1 ± 0.3	1.0 ± 0.4
	25	*274 ± 49*	*254 ± 14*	*238 ± 15*	*300 ± 23*	0.9 ± 0.2	**1.3 ± 0.1**
	100	*312 ± 28*	*438 ± 39*	*435 ± 24*	*512 ± 21*	**1.4 ± 0.2**	**1.2 ± 0.1**
Formaldehyde	0.2	82 ± 13	*67 ± 5*	63 ± 44	58 ± 14	0.8 ± 0.1	0.9 ± 0.7
	5	88 ± 10	108 ± 24	82 ± 35	88 ± 55	1.2 ± 0.3	1.1 ± 0.8
	30	84 ± 19	*76 ± 14*	87 ± 29	172 ± 131	0.9 ± 0.3	2.0 ± 1.7
Piperonyl butoxide	1	101 ± 30	88 ± 14	70 ± 23	64 ± 18	0.9 ± 0.3	0.9 ± 0.4
	10	99 ± 19	*84 ± 6*	103 ± 6	91 ± 11	0.8 ± 0.2	0.9 ± 0.1
	30	84 ± 16	97 ± 12	54 ± 46	88 ± 18	1.2 ± 0.3	1.6 ± 1.4
Deltamethrin	0.2	100 ± 48	72 ± 13	108 ± 50	85 ± 17	0.7 ± 0.4	0.8 ± 0.4
	1	98 ± 17	107 ± 14	*154 ± 12*	99 ± 5	1.1 ± 0.2	**0.6 ± 0.1**
	3	122 ± 37	98 ± 18	72 ± 7	*61 ± 11*	0.8 ± 0.3	0.9 ± 0.2
Permethrin	0.1	74 ± 20	84 ± 45	81 ± 50	93 ± 31	1.1 ± 0.7	1.1 ± 0.8
	1	*73 ± 6*	92 ± 13	66 ± 5	*155 ± 18*	1.3 ± 0.2	**2.3 ± 0.3**
	10	*150 ± 32*	107 ± 17	82 ± 32	94 ± 35	**0.7 ± 0.2**	1.1 ± 0.6
Tebuconazole	1	106 ± 29	89 ± 10	76 ± 5	72 ± 9	0.8 ± 0.2	0.9 ± 0.1
	5	91 ± 16	129 ± 53	90 ± 20	149 ± 94	1.4 ± 0.6	1.7 ± 1.1
	10	98 ± 8	94 ± 16	114 ± 36	93 ± 17	1.0 ± 0.2	0.8 ± 0.3

SD = standard deviation.
Italicised values indicate statistically significant difference (p ≤0.05) between experimental and control group.
Bold printed values indicate a statistically significant difference (p ≤0.05) between single and multiple exposure.
[a] Relative (apparent) k_p after repeated exposure divided by relative (apparent) k_p after single exposure.

permeability after single exposure to ADBAC (p = 0.10 in linear trend test), permeability increased linearly with increasing concentration (p < 0.05). The observation that repeated

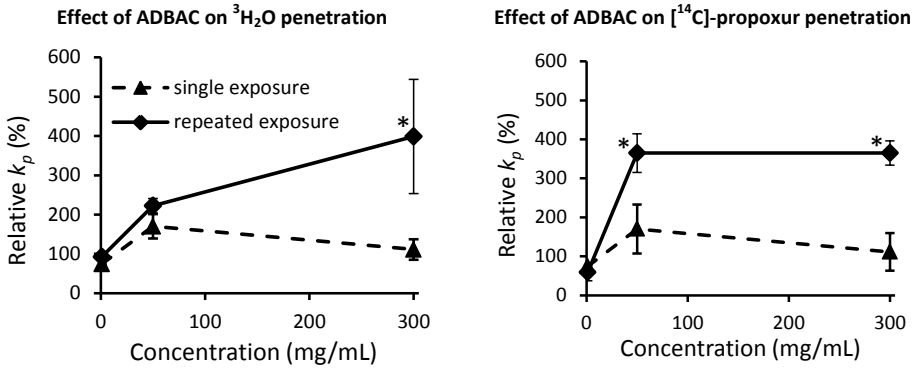

Figure 4-2 Influence of ADBAC on skin permeability.
* Statistically different from single exposure at corresponding concentration (p- value ≤0.05).

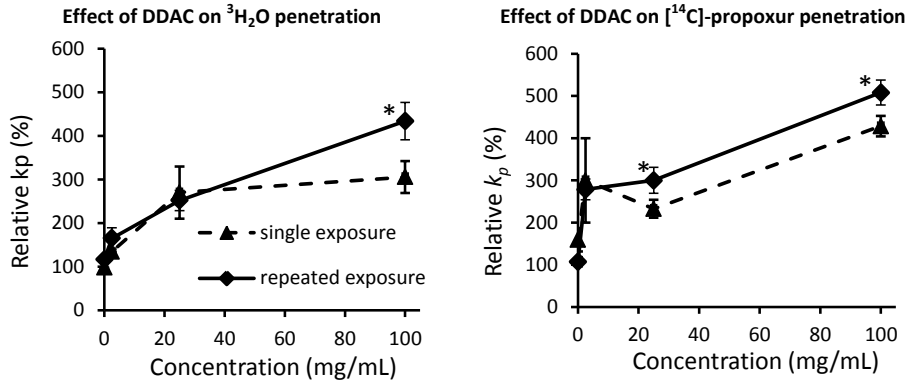

Figure 4-3 Influence of DDAC on skin permeability.
* Statistically different from single exposure at corresponding concentration (p- value ≤0.05).

exposure to quaternary ammonium chlorides increased skin permeability more than single exposure was especially clear at high concentrations: repeated exposure to 300 mg/L ADBAC increased skin permeability two to threefold more than after single exposure.

Formaldehyde had a tendency for reducing skin permeability, especially after repeated exposure. However, the effect was only in very few cases statistically significant, and did not exhibit a clear dose relationship (Table 4-4): it did not show a statistically significant linear trend. Statistically significant effects on permeability of piperonyl butoxide, deltamethrin, and permethrin were few and often contradictory. Most likely these effects are chance findings.

In the control groups (groups III and IV in Figure 4-1), the k_p for tritiated water increased from a mean of 1.6 ± 0.8 cm/h x 10^{-3} before onset of the experiments to a mean of 2.6 ± 1.0 cm/h x 10^{-3} at the end of the experiment (55 h later). On average the k_p increased twofold (see Table 4-5). The control skin preparations that were not subjected to the washing procedure (group V in Figure 4-1) had virtually the same k_p for tritiated water as the corresponding control group that was subjected to the washing procedure (Figure 4-4). The

apparent k_p for propoxur for the non-washed group was approximately two times lower as for the washed control group. However, the difference was not statistically significant (p-value 0.11).

4.5 Discussion and conclusions

4.5.1 Characteristics of the test system

Over prolonged periods of time, the barrier function of the skin preparations may deteriorate in a diffusion cell when using a simple physiologic saline receptor fluid. In order to determine whether the quality of the skin preparations remained intact during the experimental period (55 h), we measured the permeation of tritiated water in non-exposed control groups (groups III and IV). We observed a twofold increased permeability, which indicates that the barrier was slightly reduced. Apart from deterioration, the increased hydration of the skin due to the humid incubation conditions may have contributed to this increase. In addition, most outliers removed from the calculations (see Section 4.2) were only deviant for one of the markers (tritiated water or [^{14}C]propoxur), not for both markers. Therefore, we conclude that the quality of the skin preparations was sufficient to obtain reliable data.

The washing procedure is another factor that may influence skin permeability in our test system (Bucks *et al.*, 1985). However, the k_p values for tritiated water were very similar between skin preparations which were not subjected to the washing procedure (group V) and those which were washed 3 times (group IV). Although propoxur permeability was in some cases slightly higher in the washed group, we conclude that the washing procedure had only a very limited effect on the skin permeability in our experiments.

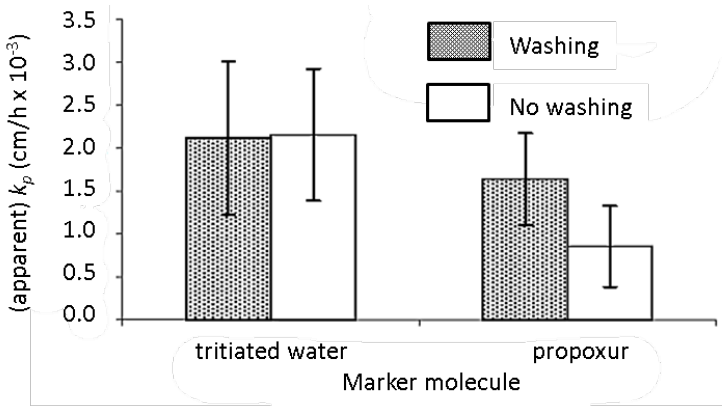

Figure 4-4 Influence of washing procedure on skin permeability.

4.5.2 Influence of biocide exposure on skin permeability

Of the nine biocidal active substances tested, only the quaternary ammonium chlorides ADBAC and DDAC clearly and consistently changed skin permeability for the two marker molecules, both after single and repeated exposure. These quaternary ammonium chlorides increased skin permeability up to six fold, probably due to structural changes induced by these compounds. ADBAC was proven to cause an increase of eosinophilic staining, nuclear condensation, cellular swelling, and separation between *dermis* and *epidermis* in *in vitro* human skin organ cultures, at concentrations of 10 and 50 mg/mL (van de Sandt and Rutten, 1995). All these changes were dose-related. Likewise in a dose-related fashion, epidermal cell proliferation was significantly inhibited, MTT conversion was decreased and pro-inflammatory hydroxyl fatty acids were released. These changes indicate that ADBAC causes structural and functional damage to cultured human skin at a concentration (50 mg/mL) provoking increased permeability, both to tritiated water and propoxur, in the experiment reported in the present manuscript. This property is probably linked to the amphipathic, soap-like character of these molecules. Soap is reported to increase permeability of guinea pig skin (Bucks *et al.*, 1985). Furthermore, it has been suggested that high water and lipid solubility enhances the corrosive character of quaternary ammonium compounds (Lin and Hemming, 1996). Another explanation may be the skin irritant properties; both investigated quaternary ammonium chlorides are classified as “corrosive” by the EU (EU, 2004). However, also formaldehyde is classified “corrosive”, but did not increase permeability in our experiments. This may be a reflection of a different underlying molecular mechanism. Quaternary ammonium chlorides are surfactants and therefore solubilisers, while formaldehyde is a chemical cross-linker (Heck and Casanova-Schmitz, 1984) which may very well have a different effect on skin structure and barrier function.

Both DDAC and ADBAC considerably increased *in vitro* skin permeability after single application of relatively low concentrations. This suggests that quaternary ammonium chlorides probably enhance their own dermal uptake and that of other biocidal active

Table 4-5 Control skin permeability for ^3H_2O at the start and the end of the experiments

Control group #	Vehicle	k_p (cm/h x 10^{-3}) at 0 h	k_p (cm/h x 10^{-3}) at 55 h	Ratio 55/0 h
1	None	0.8 ± 0.2	2.3 ± 0.2	2.8 ± 0.6
2	None	2.7 ± 0.8	4.5 ± 1.2	1.7 ± 0.5
3	Ethanol	1.8 ± 1.0	2.9 ± 0.2	2.0 ± 1.1
4	Ethanol	2.2 ± 0.5	3.2 ± 0.5	1.5 ± 0.3
5	None	0.9 ± 0.3	1.7 ± 0.04	2.1 ± 1.0
6	1% CMC	1.7 ± 0.5	2.7 ± 0.5	1.7 ± 0.5
7	Ethanol	1.6 ± 1.0	2.4 ± 1.0	1.6 ± 0.3
8	7.4% 2-propanol in water	1.1 ± 0.6	1.6 ± 0.3	1.9 ± 1.1
9	None	0.9 ± 0.4	2.1 ± 0.9	2.3 ± 0.3
Mean ± SD		1.6 ± 0.8	2.6 ± 1.0	2.0 ± 0.7

SD = standard deviation.

substances, when products with multiple active substances or a combination of different products are used. At these low concentrations, no difference in permeability effects was observed between single and repeated exposure. In contrast, a relatively high concentration of ADBAC (300 mg/L) and DDAC (100 mg/L) induced a further increase of skin permeability after repeated exposure. Therefore, repeated exposure can be a quantitatively important issue, especially during the task of mixing and/or loading of undiluted products containing quaternary ammonium chlorides. It should be noted, however, that in our *in vitro* experimental setting, daily exposure can be simulated for a maximum of 3 days, while operator exposure to biocidal active substances may take place 5 times a week for periods covering several months on a row. Therefore, we should not exclude that also at low concentrations of quaternary ammonium chlorides repeated exposure may be a significant factor.

Based on the results, we conclude that repeated exposure may be a quantitatively important issue for some specific biocidal products. The risks of the use of quaternary ammonium chlorides may be underestimated when the skin permeability enhancing properties are not taken into account, especially during repeated mixing and/or loading of undiluted products.

Acknowledgments

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5 Dermatokinetics of didecyldimethylammonium chloride and the influence of some commercial biocidal formulations on its dermal absorption *in vitro*

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Dermatokinetics of didecyldimethylammonium chloride and the influence of some commercial biocidal formulations on its dermal absorption *in vitro*
Regulatory Toxicology and Pharmacology 48 (2007): 87-92

5.1 Abstract

The *in vitro* dermal absorption kinetics of didecyldimethylammonium chloride (DDAC) was studied after single and multiple exposure. In addition, the influence of biocidal formulations on the absorption of DDAC was investigated. Following dermal exposure to DDAC in aqueous solution, less than 0.5% of the applied dose reached the receptor fluid after 48 h. The apparent permeability coefficient (k_p) was 5 ± 1 cm/h x 10^{-6} for concentrations <12.5 mg/mL, and 12 ± 3 cm/h x 10^{-6} for concentrations ≥12.5 mg/mL, suggesting that DDAC decreases the skin barrier function. DDAC distributed readily into the *stratum corneum,* but the *dermis* appeared to be the main barrier for DDAC penetration. Multiple dosing of DDAC increased its flux across the skin, when applied in high concentrations (>11 mg/mL). However, the amount of DDAC reaching the receptor fluid remained low (<1% over a 48 h period). Selected biocidal formulations tended to reduce DDAC skin absorption. The degree of reduction appeared to be correlated to the amount of aldehydes present. Based on the comparison of the distribution of DDAC in full-thickness skin and epidermal membranes, we conclude that approximately one- third of the DDAC measured in the full-thickness membranes resides in the *dermis.* As a reasonable worst case assumption, this fraction could be considered systemically available when estimating the daily systemic body burden of DDAC.

5.2 Introduction

The quaternary ammonium chlorides alkyldimethylbenzylammonium chloride (ADBAC) and didecyldimethylammonium chloride (DDAC) have been shown to reduce the skin barrier function *in vitro* (Buist *et al.*, 2005). Both compounds clearly and consistently increased the skin permeability of the marker compounds [^{3}H-]water and [^{14}C-]propoxur after single application of relatively low concentrations. These results suggest that quaternary ammonium chlorides may also enhance their own dermal uptake. A survey of the biocidal products on the market in The Netherlands revealed that of the 714 products admitted on the market at that time, 42 contained ADBAC and 166 contained DDAC. The biocidal products that contain these quaternary ammonium chlorides are disinfectants and preservatives. Especially when used in disinfectants, the probability of workers and consumers being exposed via the dermal route is high. Relatively little is known about the systemic toxicity of quaternary ammonium chlorides, but the acute oral LD_{50} of DDAC in rats (84 mg/kg bw/d, BIBRA, 1990), would lead to the classification “toxic” according to EU criteria. Systemic effects (reduced urinary excretion of potassium and chloride) were observed at single oral doses of 10-50 mg/kg bw. The LD_{50} of ADBAC in rats is higher than that of DDAC: 234-525 mg/kg bw, and an oral administration of 250 mg/kg bw led to severe congestion of liver and kidneys (Xue *et al.*, 2004). According to EU criteria ADBAC would be classified as ‘harmful’. In view of its frequent use as a disinfectant, its potential systemic toxicity and because of its effects on skin permeability, the absorption kinetics of DDAC was studied after multiple and single exposure. We used an *in vitro* model to conduct our

research, as it allowed us to conduct many more experiments than would have been the case with an *in vivo* model or, even more so, with human volunteers. Properly conducted *in vitro* measurements can be used to predict *in vivo* absorption (see a.o. the recent WHO monograph on dermal absorption (WHO, 2006) and the evaluation by Hakkert *et al.* (2005)). In order to determine the permeability coefficient (k_p) of DDAC, the initial experiments were performed using a high volume of exposure (313 µL/cm^2) in order to approach the infinite dose conditions. Further experiments were executed using a low volume of exposure (10 µL/cm^2), which is more representative of normal worker and consumer exposure (finite dose). As absorption may be considerably influenced by the nature of the vehicle (Hakkert *et al.*, 2005), the influence of various commercial biocidal formulations on DDAC absorption was investigated as well.

5.3 Materials and methods

5.3.1 *In vitro* experiments

The *in vitro* skin penetration assays were performed in static diffusion cells using cryopreserved human abdominal skin, as previously described by van de Sandt *et al.* (1993, 2000). The skin originated from four female donors, aged 28-60 years (average 43). The *dermis* was partly removed using forceps and scissors, attaining an average skin thickness of 0.565 ± 0.064 mm. Most experiments were executed with these full-thickness skin preparations. The receptor fluid (total volume 1.2 mL) consisted of a physiological salt solution (0.9% NaCl w/v) containing 0.01% sodium azide and 3% BSA. Some experiments were executed using epidermal membranes instead of full-thickness skin. They were prepared by incubating skin overnight in a 2 M NaBr solution in saline, after which the *epidermis* was peeled off the *dermis* using forceps.

In the high volume experiments, the skin preparations were exposed to 313 µL/cm^2 of DDAC in 7.4% 2-propanol in water for 48 h at concentrations of 0.5, 2.5, 12.5 and 50 mg DDAC/mL. In the low volume experiments, the skin preparations were exposed to 10 µL/cm^2 of DDAC in 7.4% 2-propanol in water or formulation for 4 h (single exposure) or for three times 4 h (repeated exposure, starting at 0, 24 and 48 h).The DDAC concentrations applied were 50 mg/ mL (Roloxid 50 formulation), 27.5 mg/ mL (Bakta Steril) and 11 mg/ mL (MS Macrodes), respectively. These formulations were compared to equivalent solutions of DDAC in 7.4% 2-propanol in water. The experimental designs are illustrated in Figure 5-1.

Each exposure period was finished by washing off the test substance using four cotton swabs humidified with a 3% Teepol solution and subsequently drying the skin preparations using two dry cotton swabs. In the repeated as well as in the single low volume experiments all skin preparations were washed three times, in order to control for a possible effect of the washing procedure on skin permeability. Each experiment was performed with skin from one donor. Most experiments were performed each with skin from a different donor.

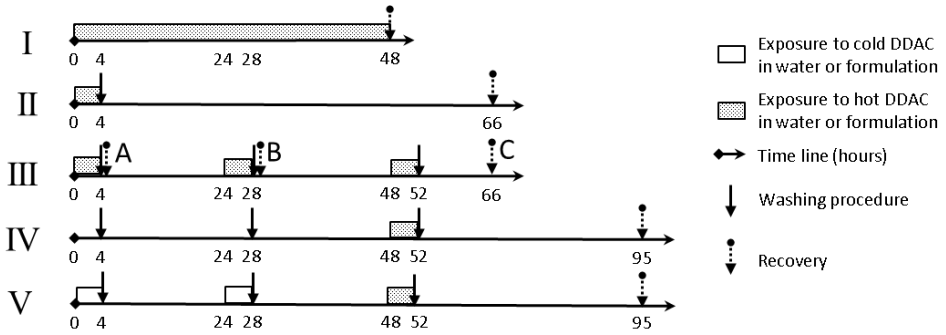

Figure 5-1 Experimental design.
(I) High volume exposure full-thickness skin. (II) Single low volume exposure full-thickness skin and epidermal membrane. (III) Repeated low volume exposure full-thickness skin. (IV) Single low volume exposure DDAC in water or formulation (full-thickness skin). (V) Repeated low volume exposure DDAC in water or formulation (full-thickness skin).

Prior to the start of the experiment, integrity of the skin preparations was assessed by determining the permeability coefficient (k_p) of tritiated water, as described by van de Sandt *et al.* (1993, 2000). Only skin preparations with a k_p of less than 3.0 x 10^{-3} cm/h for tritiated water were used in the subsequent experiments.

After the start of (the first) exposure, 500 µL samples of receptor fluid were collected at regular intervals and assayed for ^{14}C-radioactivity by liquid scintillation counting in a Wallac Pharmacia scintillation counter. Directly after each sampling the original volume of the receptor fluid was restored by adding 500 µL fresh receptor fluid to each well. The amount of [^{14}C]-DDAC that had penetrated the skin was plotted against time and the penetration rate was calculated by linear regression analysis. At the end of each experiment, the recovery was determined by measurement of radioactivity in the cotton swabs fraction (cotton swabs were extracted with ethanol and a subfraction was counted for radioactivity) and the skin membrane fraction (skin membranes were dissolved in 1.5 M KOH in 20% ethanol and a subfraction was counted for radioactivity). In some experiments the skin preparations were tape-stripped seven times using D-squame (Monoderm, Monaco). Each first tape strip was collected and counted separately. Subsequent tape strips were pooled in groups of three. Radioactivity in the tape strip fractions was determined by direct addition of scintillation fluid.

[^{14}C]-DDAC (labelled at the methyl-groups, s.a. 2.15 GBq/mmol, radiochemical purity >99%) was purchased from Amersham, England. Unlabelled DDAC (50% solution in 2-propanol/water (2:3)) was purchased from Merck KgaA, Darmstadt, Germany. The commercially available biocides Bakta Steril, Roloxid 50 and MS Macrodes were obtained from Fisher Emergo B.V. Landsmeer, Netherlands.

5.3.2 Statistics

Statistical calculations were executed using MS Excel version 2003. First equality of variances was tested using the FTEST function to calculate the *F* (folded) statistic. If the two-tailed probability of a greater *F*-value was >0.05, equality of variances was assumed, and the null hypothesis of no differences between groups was tested using the unpaired homoscedastic TTEST function. If the probability was ≤0.05, inequality of variances was assumed, and the null hypothesis was tested using the heteroscedastic TTEST function. As the groups were relatively small (usually $n = 4$), normal distribution was assumed and not further tested.

Table 5-1 Statistical analysis of the differences between k_p-values determined at different concentrations of DDAC, using the χ^2-test

p-values of χ^2 -test →			
Concentration DDAC ↓	0.5	2.5	12.5
50	0.014[a]	0.005[a]	0.258
12.5	0.0009[a]	0.005[a]	
2.5	0.11		

[a] Statistically significant difference between k_p-values of the two corresponding concentrations ($p < 0.05$); number of replicates is 4.

5.4 Results

The high volume experiments (design I, Figure 5-1) with DDAC showed that the k_p-value increased with increasing concentration (see Figure 5-2). Cumulative penetration of DDAC showed almost linear kinetics between 28 and 48h after application for the two lower concentrations, while for the two higher concentrations tested the absorption rate increased with time (see Figure 5-3). Based on this observation and on the statistical analysis of the data (see Table 5-1), two categories of DDAC concentrations could be discerned with respect to k_p-values: for concentrations <12.5 mg/ mL the k_p was 5 ± 1 cm/h x 10^{-6}, while for concentrations ≥12.5 mg/ mL it was 12 ± 3 cm/h x 10^{-6}.

In the experiments in which the k_p-values were determined, the cumulative absorption in the receptor fluid remained very low and never exceeded 0.1% of the administered dose, 48 h after the start of exposure (see Table 5-2). A far greater fraction remained associated with the skin (tape strips and membrane). The relative size of this fraction increased with decreasing DDAC concentration, ranging from ca. 4% at 50 mg/ mL to ca. 40% at 0.5 mg/mL. The contribution of the *stratum corneum* (adhered to tape strips) relative to the rest of the skin increased with decreasing concentration, from ca. one ninth of the total radiolabel associated with skin at 50 mg/ mL to more than one fourth at 0.5 mg/mL.

In the low volume experiments (design II, Figure 5-1), mimicking realistic worker exposure conditions, DDAC was washed off the skin preparation after four hours of exposure, using cotton swabs. After exposure to 50 mg/ mL DDAC, a larger fraction of the applied dose was recovered from the receptor fluid when epidermal membranes were used (ca. 5%) instead

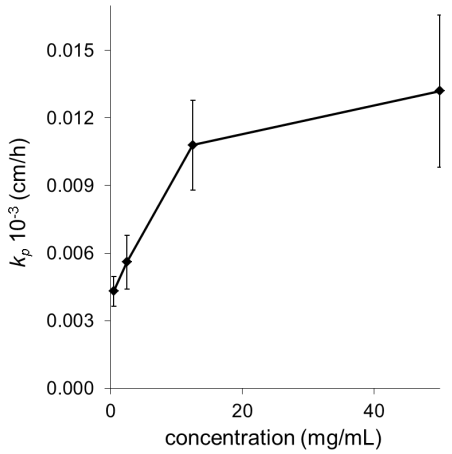

Figure 5-2 Permeability of human skin for DDAC in relation to its concentration (high volume application).

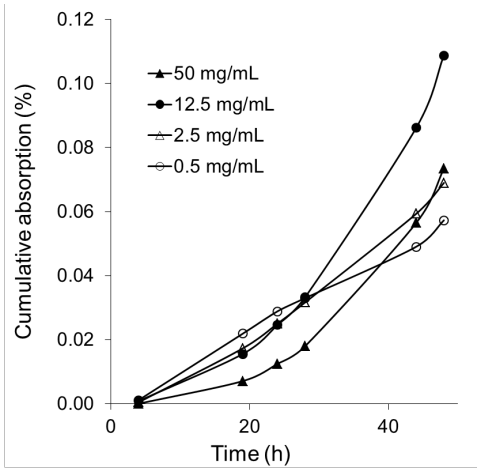

Figure 5-3 Cumulative relative absorption of DDAC after continuous exposure to various concentrations dissolved in water/propanol (313 µL/cm^2).

of full-thickness skin (ca. 0.5%), 66 h after start of exposure (Table 5-4). Interestingly, the amount of the applied dose recovered from the skin was lower for epidermal membranes (ca. 14% for epidermal membranes and ca. 18% for full-thickness skin), resulting in a total absorption of ca. 19% for both test systems.

Table 5-2 Distribution of radiolabel in skin preparations after 48 h of exposure to DDAC solution (313 µL/cm^2)

Amounts in percentage of administered dose (± SD)

	DDAC concentration (mg/mL)			
	50	12.5	2.5	0.5
Receptor	0.05 ± 0.01	0.08 ± 0.02[a]	0.05 ± 0.01	0.04 ± 0.00
Membrane	3.9 ± 0.6[a]	8.5 ± 1.1[a]	13.0 ± 3.2[a]	28.8 ± 5.7[a]
Tape strips	0.5 ± 0.2	1.3 ± 0.3	3.1 ± 0.6[a]	11.4 ± 3.1[a]
Swabs	88.8 ± 2.2	86.6 ± 1.3	80.4 ± 3.0[a]	47.5 ± 3.8[a]
Recovery	93.2 ± 2.6	96.5 ± 0.1	96.5 ± 0.4	87.7 ± 0.9[a]

[a] Statistically significantly different from all other concentrations ($p < 0.05$). Number of replicates is 4.

Repeated exposure to low volumes of 50 mg/ mL DDAC (design III, see Figure 5-1) did not tend to increase the absolute amount of DDAC initially present in the *stratum corneum*[10] after the first exposure (ca. 20 µg), while the amount of DDAC in the rest of the skin layers clearly increased with time from ca. 15 µg to ca. 70 µg (see Figure 5-4). However, the increase after the third exposure was considerably less (ca. 10 µg) than after the second exposure (ca. 35 µg). A similar saturation of the *stratum corneum* was observed when full-thickness skin preparations exposed to a low volume of 50 mg/mL DDAC in Roloxid 50 or

[10] Represented by the radiolabel recovered from tape strips 2-7; tape strip 1 is assumed to represent DDAC not removed from the skin by swabbing.

Table 5-4 Distribution of radiolabel in full-thickness skin preparations and epidermal membranes after 4-h exposure to DDAC solution (10 µL/cm^2, 50 mg/mL)

	Full-thickness skin	Epidermal membrane
Receptor	0.3 ± 0.0[a]	5.3 ± 0.7
Membranes	18.4 ± 2.1[a]	13.9 ± 1.5
Swabs	75.0 ± 3.9[a]	69.3 ± 2.3
Recovery	93.8 ± 4.3	88.5 ± 3.1

The data are expressed as % of the applied dose (means ± SD), 62 h after the exposure period of 4 h (n = 4).
[a] Statistically significant difference between epidermal membrane and full-thickness skin ($p < 0.05$).

water/propanol were compared: although the amount absorbed from water/propanol into the entire skin was three times higher, the amount present in the *stratum corneum* was virtually the same (data not shown).

When skin preparations were exposed to DDAC in commercial formulations (Roloxid 50, Bakta Steril, MS Macrodes: see Table 5-3; designs IV and V, Figure 5-1), the behaviour of DDAC was similar to the behaviour of unformulated DDAC: a skin reservoir was built up and relatively little DDAC eventually reached the receptor fluid. There were no statistically significant differences in skin reservoir built up between skin preparations that received multiple and single exposures (see Table 5-6), although penetration rates of DDAC tended to be higher when preceded by two daily 4h-exposures (see Table 5-5). However, the tested formulations clearly reduced the amount of DDAC built up in the skin as well as DDAC penetration rates, compared to when water/propanol was used as a vehicle (see Table 5-6 and Table 5-5). The more formaldehyde and glutaraldehyde the formulations contained, the greater the reduction of the skin reservoir and of the penetration rate. It was also remarkable that the skin preparations exposed to the formulation containing the highest concentration of formaldehyde and glutaraldehyde were more difficult to solubilise before being assayed for radio-activity than the other exposed skin preparations.

Table 5-3 Overview of the active ingredients of the tested formulations

Formulation	Concentration (mg/mL)				In-use dilution factors[a]
	DDAC	ADBAC	Formaldehyde	Glutaraldehyde	
Bakta Steril	27.5	—	—	—	50x, 100x
Roloxid 50	100	—	32	80	30x, 60x, 100x, 200x, 300x
MS Macrodes	11	30	196	106	50x, 67x

[a] According to the official Instructions for Use published by the Dutch Board for the Authorisation of Pesticides (http://www.ctb-wageningen.nl/).

5.5 Discussion and conclusions

We studied the dermatokinetics of DDAC in order to determine to which extent this frequently used biocide may pass the barrier of the skin into systemic circulation. Our results show that DDAC poorly penetrates human skin *in vitro.* Following exposure conditions relevant for workers and consumers (10µL/cm^2, 0.5-50 mg/mL), only less than

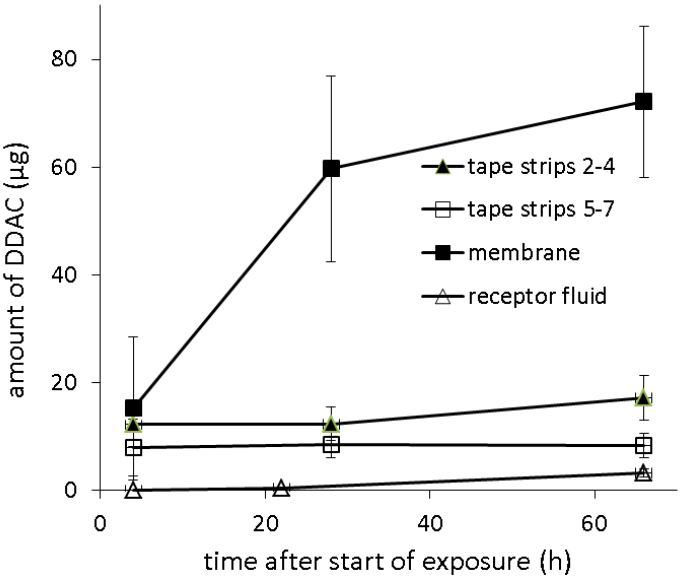

Figure 5-4 Distribution of radiolabel in full-thickness skin preparations during multiple exposure to DDAC solution (10 µL/cm^2, 50 mg/mL), at different times after the start of the experiment.

Skin preparations were three times exposed to [^{14}C]-DDAC for the following periods after the start of the experiment: *t* = 0-4, 24-28 and 48-52 h

0.5% of the applied dose passed the full-thickness skin preparation and reached the receptor fluid. In an experiment in which we applied a high volume of DDAC, the permeability constant k_p of DDAC appeared not to be independent of the concentration applied, as it was ca. two times higher at high concentrations (≥12.5 mg/mL) than at low concentrations. This finding is in line with our earlier study, in which we observed that DDAC increased the skin absorption of two marker molecules (Buist *et al.*, 2005). It can be hypothesized that DDAC damages the *stratum corneum* by solubilisation since its structural analogue ADBAC has been shown to effectively extract cholesterol from the *stratum corneum* (Aki and Kawasaki, 2004). ADBAC also affected the viability of human skin organ cultures, as was observed by a series of histopathological changes (van de Sandt and Rutten, 1995) using exposure conditions similar to those employed in the present experiments. Therefore, the higher skin permeability constant of DDAC measured after exposure to high concentrations is likely to be due to the damage inflicted by this surfactant to the barrier function of the skin thus violating one of the conditions for Fick's law to be applicable, that is that the substance applied should not interfere with the skin's permeability.

DDAC diffused readily into the *stratum corneum*, as at high concentrations of DDAC this skin layer was already saturated after the first of three exposure periods. The underlying part of the skin appeared to be more resistant to DDAC penetration than the *stratum corneum,* as it was only (nearly) saturated after the second exposure period. This is in line with the high log

Table 5-5 Influence of various formulations on the distribution of DDAC after single and multiple exposure

Formulation	DDAC conc. (mg/mL)	Aldehyde conc. (M)	Exposure (10 µL/cm^2)	DDAC in receptor fluid (% dose ± SD)	DDAC in receptor fluid Formulation/ water (fraction ± SD)[a]	DDAC in membrane (% dose ± SD)	DDAC in membrane Formulation/ water (fraction ± SD)[a]
Roloxid 50	50	0.9	Multiple	0.16 ± 0.10	0.16 ± 0.12	8.7 ± 2.8	0.53 ± 0.3
Roloxid 50	50	0.9	Single	0.10 ± 0.04	0.13 ± 0.06	7.1 ± 2.7[d]	0.32 ± 0.16[c]
Bakta Steril	27.5	0.0	Multiple	0.79 ± 0.22[b]	0.81 ± 0.48	15.3 ± 1.0[d]	0.76 ± 0.17
Bakta Steril	27.5	0.0	Single	0.47 ± 0.10[b]	1.28 ± 0.38	12.4 ± 2.4[e]	0.68 ± 0.26
MS Macrodes	11	7.6	Multiple	0.03 ± 0.00	0.03 ± 0.01[c]	4.2 ± 1.6	0.11 ± 0.05[c]
MS Macrodes	11	7.6	Single	0.05 ± 0.02	0.05 ± 0.03[c]	4.8 ± 1.9	0.11 ± 0.05[c]

Number of replicates was 4, unless indicated otherwise. Each exposure lasted 4h. Single exposures were executed with radiolabelled DDAC. The first two exposures of the multiple exposure regime were executed with unlabelled DDAC and the last with radiolabelled.

[a] Calculated by dividing % dose recovered with formulation by % dose recovered with the corresponding water control group.

[b] Statistically significant difference between multiple and single exposure ($p < 0.05$)

[c] Statistically significantly different from water control group ($p < 0.05$).

[d] Two replicates in control group.

[e] Three replicates in control group.

K_{OW} of DDAC of 4.7, indicating high lipophilicity. Furthermore, the structurally related alkyldimethylbenzylammonium chloride (ADBAC) has been demonstrated to possess high affinity for the *stratum corneum* lipids ceramide and cholesterol, while its affinity for defatted *stratum corneum* was much less (Aki and Kawasaki, 2004). In line with these properties of DDAC, the *dermis* appeared to be the main barrier for DDAC penetration, as removal of this skin compartment resulted in a ca. 10-fold increase of DDAC penetration into the receptor fluid after a single exposure to a low volume of 50 mg/ mL DDAC.

In previous experiments, DDAC exposure decreased the barrier function of the skin, as measured by the penetration of marker molecules (tritiated water and propoxur) (Buist *et al.*, 2005). Multiple dosing of relatively high concentrations increased this effect. In the present experiments, multiple dermal dosing of DDAC in commercial formulations increased its flux across the skin compared to single dosing, when applied in relatively high concentrations (>11 mg/mL). However, the flux differences between single and multiple dosing were relatively limited and not always statistically significant. It should be noted that these differences may in fact be somewhat greater than observed in this study, as radiolabelled DDAC was only applied after the second of the repeated exposures, and dilution of the radiolabelled DDAC with the previously applied non-labelled DDAC may have led to an underestimation of the amount penetrated after multiple exposure. In previous experiments tritiated water penetration was also slightly increased after triple exposure to 100 mg/ mL DDAC, as compared to a single exposure (Buist *et al.*, 2005). Taken together, it can be concluded that the total amount DDAC reaching the receptor fluid was low (less than 0.5% over 48 h) and repeated exposure did not affect this in a quantitatively significant fashion, relative to one single exposure.

Table 5-6 Influence of various formulations on the DDAC flux through skin preparations during single and multiple exposure

Vehicle	n	DDAC conc. (mg/mL)	Exposure (10 µL/cm^2)	Flux (µg/cm^2/h)	Relative flux formula/water (fraction ± SD)[a]
Roloxid 50	4	50	Multiple	0.083 ± 0.045	0.20 ± 0.13[c]
Roloxid 50	4	50	Single	0.043 ± 0.018	0.12 ± 0.07[d]
Bakta Steril	4	27.5	Multiple	0.13 ± 0.03[b]	0.49 ± 0.26[d]
Bakta Steril	4	27.5	Single	0.08 ± 0.02[b]	0.95 ± 0.35[e]
MS Macrodes	4	11	Multiple	0.002 ± 0.001	0.03 ± 0.01[c]
MS Macrodes	4	11	Single	0.003 ± 0.001	0.05 ± 0.02[c]

Number of replicates was 4, unless indicated otherwise. Each exposure lasted 4 h. Single exposures were executed with radiolabelled DDAC. The first two exposures of the multiple exposure regime were executed with unlabelled DDAC and the last with radiolabelled.

[a] Calculated by dividing the flux measured with formulation by the flux measured with the corresponding water control group containing the same concentration of DDAC.

[b] Statistically significant difference between multiple and single exposure ($p < 0.05$).

[c] Statistically significantly different from water control group ($p < 0.05$).

[d] Two replicates in control group.

[e] Three replicates in control group.

It is well known that the nature of the vehicle in which a substance is applied to the skin may alter the rate at which it is absorbed, either accelerating it or slowing it down. The magnitude of the vehicle effect needs to be taken into account in the risk assessment of substances. In our study, we showed that several selected commercial formulations tended to reduce skin penetration of DDAC. This was most pronounced with the formulation MS Macrodes, which contains the highest concentration of formaldehyde (196 mg/mL) and glutaraldehyde (106 mg/mL), and reduced the flux of DDAC across the skin by 95%. The reduction caused by Bakta Steril, the only tested formulation not containing any aldehydes, was smallest and did not reach statistical significance. In our previous experiments, formaldehyde exhibited a tendency to decrease skin permeability for tritiated water and propoxur, when tested up to concentrations of 30 mg/ mL (Buist *et al.*, 2005). The results obtained in the present study confirm this early finding. A possible explanation of this phenomenon is the ability of formaldehyde and glutaraldehyde to cross-link proteins (Usha and Ramasami, 2005). Cross-linking of proteins in the *epidermis*, such as keratins, could turn the skin less permeable. This is corroborated by our observation that skin preparations exposed to MS Macrodes were more resistant to solubilising than those exposed to one of the other formulations.

Although DDAC increased skin permeability, it is unlikely that this would lead to a considerable increase of its systemic availability. DDAC remains closely linked to the *stratum corneum* and even more than 40 h after termination of exposure to DDAC in commercial formulations, the cumulative dose present in the receptor fluid did not exceed 1% of the applied dose. Furthermore, repeated exposure did not lead to a quantitatively significant increase of DDAC in the receptor fluid. It is likely that DDAC ultimately will be lost from the

stratum corneum by desquamation. The DDAC fraction associated with the *dermis* may not be lost and could become systemically available via the local microcirculation. However, its release into systemic circulation is expected to be slow and, consequently, its contribution to the daily systemic body burden would be low. Based on the comparison of the distribution of DDAC over receptor fluid and skin preparation between full-thickness skin and epidermal membranes after 4 h of low volume exposure, we conclude that approximately one-third of the DDAC measured in the full-thickness membranes resides in the *dermis*. As a reasonable worst case assumption, this fraction could be considered systemically available when estimating the daily systemic body burden of DDAC. Consequently, when using the *in vitro* absorption data determined with epidermal membranes (not containing any *dermis*), the fraction of DDAC present in the membrane does not need to be taken into account when estimating the daily systemic body burden.

Acknowledgments

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6 New *in vitro* dermal absorption database and the prediction of dermal absorption under finite conditions for risk assessment purposes

Harrie E. Buist, Johan A. van Burgsteden, Andreas P. Freidig, Wilfred J.M. Maas, Johannes J.M. van de Sandt.
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Regulatory Toxicology and Pharmacology 57 (2010): 200-209.

6.1 Abstract

Most QSPRs for dermal absorption predict the permeability coefficient, k_p, of a molecule, which is valid for infinite dose conditions. In practice, dermal exposure mostly occurs under finite dose conditions. Therefore, a simple model to predict finite dose dermal absorption from infinite dose data (k_p and lag time) and the *stratum corneum*/water partition coefficient ($K_{SC,W}$) was developed. To test the model, a series of *in vitro* dermal absorption experiments was performed under both infinite and finite dose conditions using acetic acid, benzoic acid, bis(2-ethylhexyl)phthalate, butoxyethanol, cortisone, decanol, diazinone, 2,4-dichlorophenol, ethacrynic acid, linolenic acid, octylparaben, oleic acid, propylparaben, salicylic acid and testosterone. For six substances, the predicted relative dermal absorption was not statistically different from the measured value. For all other substances, measured absorption was overpredicted by the model, but most of the overpredictions were still below the European default absorption value. In conclusion, our finite dose prediction model provides a useful and cost-effective estimate of dermal absorption, to be used in risk assessment for non-volatile substances dissolved in water at non-irritating concentrations.

6.2 Introduction

Dermal absorption data are needed in toxicological risk assessment of chemical substances in order to estimate internal dose after dermal exposure to these chemicals. This internal dose can then be compared to an internal limit value for systemic effects to assess the safety of the dermal exposure (see a.o. the EU technical guidance on risk assessment: EU, 2004). Dermal absorption can be estimated using *in vivo* or *in vitro* studies with humans or animals, which need to be conducted under conditions mimicking those expected to occur

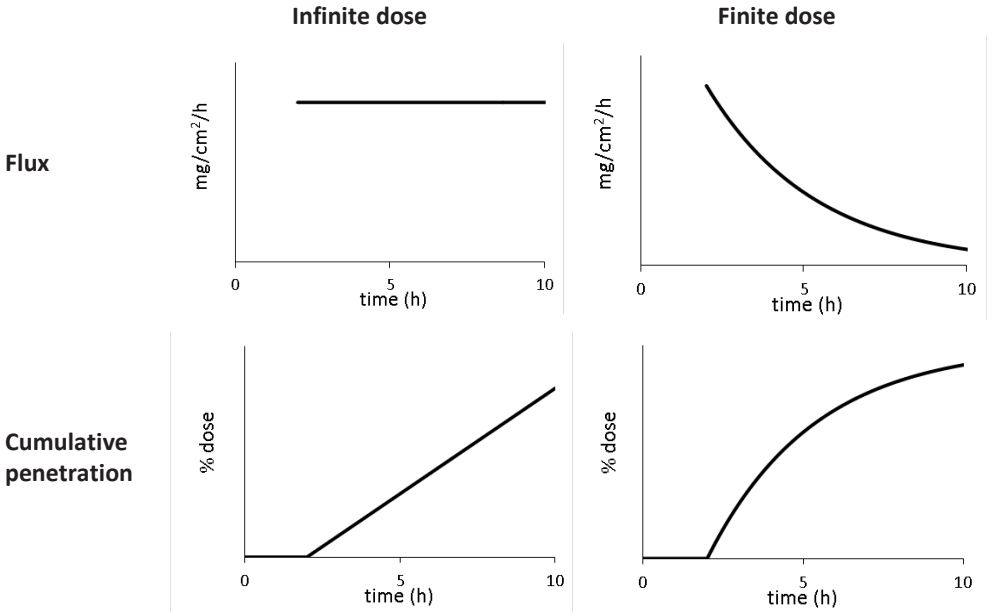

Figure 6-1 Comparison of infinite and finite dose dermal penetration

during the exposure(s) to be evaluated for toxicological risk (EU, 2004). When no experimental data are available, European regulatory authorities assume 100% dermal absorption unless the chemical possesses a molecular weight >500 and a log K_{OW} smaller than -1 or higher than 4, in which case 10% absorption is assumed (EU, 2004). In our experience, most molecules evaluated in regulatory risk assessment are assumed to be totally absorbed, when applying this rule.

At the end of the year 2006, the EU parliament accepted new legislation on the registration of chemicals, known under the acronym REACH[11]. REACH entered into force on 1st June 2007. Based upon it, over 50,000 existing chemicals will need to be evaluated for environmental and health safety under the conditions they are manufactured, used and

Table 6-1 Test compounds and concentrations.

Test compound	Final concentrations (radioactivity in MBq mL^{-1})	
	Experiments on concentration dependence of k_p	Finite dose prediction experiments
[^{14}C]Acetic acid	95.8; 21.0; 4.66; 0.82; 0.26 mg mL^{-1} (0.22; 0.25; 0.25; 0.25; 0.14)	100 mg mL^{-1} (0.45)
[^{14}C]Benzoic acid	--	33 mg mL^{-1} (0.19)
[^{14}C]Bis(2-ethylhexyl)phthalate		2.47 µg mL^{-1} (0.0027)
[^{14}C]Butoxyethanol	451.5; 90.3; 18.1; 3.61; 1.01 mg mL^{-1} (0.16; 0.17; 0.050; 0.010; 0.0028)	451 mg mL^{-1} (0.020)
[^{14}C]Cortisone	--	195 µg mL^{-1} (0.0035) (infinite dose) 22.2 µg mL^{-1} (0.13) (finite dose)
[^{14}C]Decanol	0.74; 0.142; 0.0265; 0.00416 mg mL^{-1} (0.0016; 0.021; 0.029 ; 0.014)	2.5 mg mL^{-1} (0.024)
[^{14}C]Diazinone	--	16.95 µg mL^{-1} (0.11)
1^{14}C]2,4-Dichlorophenol	383; 50.8; 2.67; 0.52 µg mL^{-1} (0.087 ; 0.027; 0.0047; 0.0025)	370 µg mL^{-1} (0.029)
[^{14}C]Ethacrynic acid	--	11.1 µg mL^{-1} (0.020)
[^{14}C]Linolenic acid	--	10.84 µg mL^{-1} (0.073)
[^{14}C]0ctylparaben	104; 19; 3.13; 0.101 µg mL^{-1} (0.029; 0.024 ; 0.024; 0.00076)	89 µg mL^{-1} (0.046)
[^{14}C]Oleic acid	--	10.81 µg mL^{-1} (0.072)
[^{14}C]Propylparaben	503; 104; 21.2; 4.92; 0.99 µg mL^{-1} (0.032; 0.031; 0.032; 0.031; 0.0075)	502 µg mL^{-1} (0.041)
[^{14}C]Salicylic acid	--	361 µg mL^{-1} (0.014)
[^{14}C]Testosterone	21.0; 8.03; 5.79; 1.74; 0.61 µg mL^{-1} (0.13; 0.052; 0.037 ; 0.011; 0.0039)	25.6 µg mL^{-1} (0.16)

[11] Registration, Evaluation Authorisation and restriction of CHemicals.

discarded. All substances should be evaluated by the end of 2017. For many chemicals, data on dermal absorption under many different exposure conditions will need to be generated. To save time and money, a simple *in silico* method, not automatically leading to a default of 100% absorption, would be advantageous. Extensive overviews of available *in silico* methods have been published recently (WHO, 2006; Bouwman *et al.*, 2008). Most QSPRs calculate the permeability coefficient, k_p, of a molecule, which is a measure of the skin permeability of a molecule under steady state conditions. Steady state is reached after a certain lag time, which amongst others depends on the nature of the molecule. The k_p is usually expressed in cm/h, and from it the flux of solute over the skin under steady state conditions can be calculated for so called infinite dose conditions, i.e., when the concentration of the solution applied to the skin does not change (appreciably) over time. In practice, however, dermal exposure will mostly occur under finite instead of infinite dose conditions, meaning the concentration of the solute in solution on the skin will clearly change over time. Under these conditions, the flux of the substance across the skin will decrease with time as the solution on the skin is depleted of its solute (see Figure 6-1). Using k_p values and initial concentrations to calculate absorption would thus lead to overestimation. Therefore, we developed a simple model to predict finite dose absorption from infinite dose data (k_p and lag time). In order to test this finite dose absorption prediction model, a series of *in vitro* dermal absorption experiments was performed with 15 different substances, both under infinite and finite dose conditions.

6.3 Materials and methods

6.3.1 *In vitro* dermal absorption experiments

The *in vitro* skin absorption assays were executed according to standard protocols used in our laboratory to perform *in vitro* dermal absorption studies according to OECD test guideline 428 (OECD, 2004). The assays were performed in static diffusion cells using cryopreserved human abdominal skin (exposed area 0.64 cm^2), as previously described by van de Sandt *et al.* (1993, 2000). The skin originated from nine female donors, aged 29-53 years (average 39 ± 7.4). Epidermal membranes were used, which were prepared by incubating skin overnight in 2 M NaBr solution in saline, after which the *epidermis* was peeled from the *dermis* using forceps. The receptor fluid (total volume 1.2 mL) consisted of a physiological salt solution (0.9% NaCl w/v) containing 0.01% sodium azide and 6% polyoxyethylene (20) oleyl ether, the latter having been added to ensure also lipophilic test substances would be readily soluble in it. Prior to the start of the experiment, integrity of the epidermal membranes was assessed by determining the permeability coefficient (k_p) of tritiated water, as described by van de Sandt *et al.* (1993, 2000). Epidermal membranes with a k_p for tritiated water of less than 3.0 x 10^{-3} cm/h were used in the subsequent experiments.

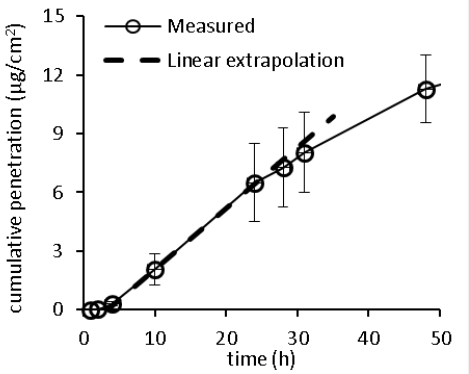

Figure 6-2 Exemplary plot of cumulative penetration versus time.

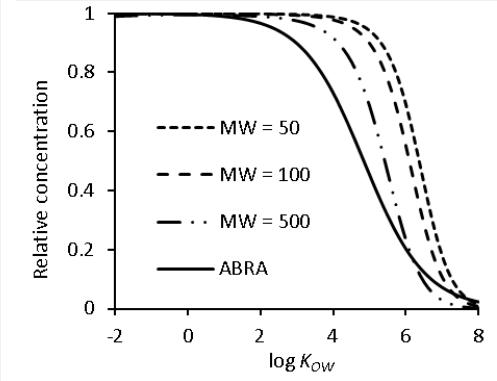

Figure 6-3 Concentration of substances in the donor cell relative to the total amount present the *stratum corneum* and the donor cell at steady state as a function of log K_{OW}.
The relative concentration was calculated using Eq. (3) and QSARs for $K_{SC,W}$: ABRA, the QSAR of Abraham *et al.* (1995); MW. the QSAR for $K_{SC,W}$ of Hui *et al.* (1995) for the MW indicated.

The epidermal membranes were exposed to 780 µL (the maximum volume the donor cells of our system can accommodate) or 16 µL aqueous dose solution/cm^2, representing infinite and finite dose experiments[12], respectively.

For the absorption experiments in which k_p was assessed over a broad range of test concentrations for seven substances, the aim was to test 5x a 1:5 dilution of the test compounds. For a number of substances, the highest test concentrations were selected based on maximum solubility in water, which was determined experimentally before preparation of the solutions (octylparaben, propylparaben, testosterone, 2,4-dichlorophenol and decanol). As the flux is determined by the concentration of the substance and its k_p, it is not useful to test beyond maximum water solubility. For the other compounds solubility was not a limiting factor. Butoxyethanol and ethylene glycol were tested at a maximum concentration of 50% (v/ v) (corresponding to, respectively, ca. 470 and ca. 530 mg mL^{-1}). Acetic acid was tested at a lower maximum concentrations of 100 mg mL^{-1}, as this compound is known to have corrosive potential. According to EU classification, 10% acetic acid constitutes the lower limit for classification as “irritating”. This means a series of concentrations was used up to the point where, according to EU classification, irritancy starts. In the absorption experiments it was shown that the k_p is relatively constant over a broad range of test concentrations. Therefore, test concentration selection for the other experiments was less critical. The test concentrations of the different test compounds are presented in Table 6-1. All concentrations chosen were below the maximum solubility of the test substance in water. All test solutions were prepared using a [^{14}C]- radiolabel. Depending

[12] For very slowly penetrating substances even 16 µl/cm^2 may constitute an infinite dose. However, as the results for our experiments show, this was not the case for our test substances.

on the specific activity of the radiolabel and the desired test concentration, the radiolabel was mixed with unlabelled test compound. The dose compartments were occluded using a glass coverslip fixed to the rim of the donor cell with Vaseline, in order to avoid evaporation.

In all experiments, the epidermal membranes (3-4 per test group) were exposed to the dose solutions for the whole experimental period. The duration of the finite dose experiments was set to 8 h to represent one working day. The infinite dose experiments were much longer in duration (48-51 h), in order to make sure linear steady state penetration would be observed (during which k_p can be measured, see Figure 6-2), even for substances with a long lag time. These long exposures usually did not affect the skin barrier, as steady state penetration was normally maintained throughout this period. If towards the end of this period penetration rates did change, only the linear portion of the time versus cumulative penetration curve was used to measure k_p.

After the start of exposure, 500 µL samples of receptor fluid were collected at regular intervals and assayed for ^{14}C-radioactivity by liquid scintillation counting (LSC) using a Wallac Pharmacia scintillation counter. Directly after each sampling the original volume of the receptor fluid was restored by adding 500 µL fresh receptor fluid to each well. The amount of test substance that had penetrated the skin was plotted against time and the penetration rate was calculated by linear regression analysis. At the end of each experiment, the test substance was removed from the skin surface by washing off the test substance using four cotton swabs humidified with a 3% Teepol solution and subsequently drying the epidermal membranes using a dry cotton swabs. For the

infinite dose experiments the remaining dose was first removed by means of a pipette. For the finite dose experiments, recovery was determined by measurement of radioactivity in the remaining dose, the cotton swabs fraction (cotton swabs were extracted with ethanol and a sub-fraction was counted for radioactivity) and the epidermal membrane fraction (epidermal membranes were dissolved in 1.5 M KOH in 20% ethanol and a sub-fraction was counted for radioactivity). Each series of experiments with a single substance was performed with skin from one single donor.

6.3.2 Selection of the chemicals

The radiolabelled chemicals were selected in such a way as to obtain a representative sample of small organic molecules with respect to the main chemical specific determinants of skin penetration used in most QSPRs: MW and log K_{OW} (see a.o. Bouwman *et al.*, 2008).

6.3.3 Calculation of k_p and lag time in *in vitro* infinite dose experiments

The calculations were performed using a standardised Excel spreadsheet. A cumulative amount penetrated per unit skin area versus time curve was constructed from the amount of test substance in the receptor fluid, and the maximum penetration rate was determined from the steepest, linear portion of this curve (see Figure 6-2). k_p values were calculated by

dividing the maximum penetration rate by the (initial) concentration of test substance applied to the epidermal membrane. The lag time was determined by extrapolating the steepest linear portion of the curve to a cumulative amount penetrated of 0 mg/cm^2.

Basic differential equation for flux (modified Fick's law)

$$-\frac{dM_{don}}{dt} = C \times k_p \times A \quad \cdots\cdots (1)$$

Stratum corneum/water partitioning coefficient ($K_{SC,W}$)

$$K_{SC,W} = \frac{C_{SC}}{C_{don}} = \frac{M_{SC}/V_{SC}}{M_{don}/V_{don}} \quad \cdots\cdots (2)$$

Correction factor (f) for steady state distribution into the *stratum corneum*

$$f = \frac{M_{don}}{M_{SC} + M_{don}} = \frac{V_{don}}{V_{SC} \times K_{SC,W} + V_{don}} \quad \cdots\cdots (3)$$

Calculation of $K_{SC,W}$

For alkanols (Abraham *et al.*, 1995):

$$\log K_{SC,W} = 0.514 \log K_{OW} + 0.104 \quad \cdots\cdots (4)$$

For all other chemicals (Hui *et al.*, 1995):

$$\log K_{SC,W} = 0.078 (\log K_{OW})^2 + 0.868 \log MW - 2.04 \quad \cdots\cdots (5)$$

Prediction of cumulative penetration ($M_{rec,t}$)

$$M_{rec,t} = M_{don,0}\left(1 - e^{-\frac{f \times k_p' \times A}{V_{don}} \times (t - t_{lag})}\right) \quad \cdots\cdots (6)$$

Prediction of the *in vitro* skin reservoir (M_{SC})

$$M_{SC} = (M_{don,0} - M_{rec,t}) \times (1 - f) \quad \cdots\cdots (7)$$

QSPRs for k_p

Potts and Guy, 1992:

$$\log k_p = -2.72 + 0.71 \log K_{OW} - 0.0061 MW \quad \cdots\cdots (8)$$

Patel *et al.*, 2002 :

$$\log k_p = -2.3 + 0.652 \log K_{OW} - 0.00603 MW - 0.623 ABSQon - 0.313 SsssCH \quad \cdots\cdots (9)$$

Cleek & Bunge (1993) correction:

$$k_{p,\,corrected} = \frac{k_p}{1 + \frac{k_p \times \sqrt{MW}}{2.6}} \quad \cdots\cdots (10)$$

Explanation of symbols used

A	= exposed skin area in cm^2
ABSQon	= the sum of absolute values of charges on nitrogen and oxygen atoms of the penetrant
f	= fraction of substance present in donor fluid (relative to the total amount present in donor fluid and stratum corneum)
k_p	= measured permeation constant in cm/h
$K_{SC,W}$	= stratum corneum/water partition coefficient
Log K_{OW}	= logarithm of octanol/water partition coefficient
L_{SC}	= thickness of stratum corneum = 0.002 cm (default, based on Xiao & Imhof (1997)
$M_{don,0}$	= initial mass of substance present in donor fluid in mg
$M_{rec,t}$	= amount of substance in receptor fluid in mg at time t
M_{SC}	= amount of substance present in the *stratum corneum* in mg
SsssCH	= the sum of E-state indices for all methyl groups of the penetrant
t	= exposure time in h
t_{lag}	= measured lag time in h
V_{don}	= volume of donor fluid in mL
V_{sc}	= volume of stratum corneum in mL = A (cm^2) × L_{SC} (cm) = 0.64 × 0.002 = 0.00128 mL

Figure 6-4 Equations

6.3.4 Calculation of experimentally determined relative absorption

In order to determine relative absorption, the amount of radio-label recovered from the epidermal membrane at the end of the experiment after washing was added to the amount of radiolabel accumulated in the receptor fluid. This sum was divided by the amount of radiolabel applied at the start of the experiment.

6.3.5 Prediction of absorption *in vitro* under finite dose conditions

The absorption in finite dose *in vitro* experiments was predicted as follows from the k_p and lag time, which were both measured in the infinite dose experiments:

1. Correction of the measured k_p for distribution into the *stratum corneum*:

$$k_p' = \frac{k_p \times (V_{SC} \times K_{SC,W} + V_{don})}{V_{don}}$$

2. Estimation of the cumulative amount penetrated after exposure:

$$M_{rec,t} = M_{don,0}\left(1 - e^{-\frac{f \times k_p' \times A}{V_{don}} \times (t - t_{lag})}\right)$$

3. Estimation of the amount still present in the *stratum corneum,* immediately after cessation of exposure:

$$M_{SC} = \left(M_{don,0} - M_{rec,t}\right) \times (1 - f)$$

4. Calculation of relative absorption in %:

$$\%A_{pot} = \frac{M_{rec,t} + M_{SC}}{M_0} \times 100$$

These values were calculated for each combination of k_p and lag time from the three to four replicates, and averaged. Also standard deviations were calculated based on these outcomes.

The equations are described in Figure 6-4, and the calculation of a number of parameters is explained in more detail below.

6.3.6 Correction factor (*f*) for steady state distribution into the *stratum corneum*

The flux of a substance across the skin can be described by a modification of Fick's law (Eq. (1) in Figure 6-4) (WHO, 2006). In this approximation, the amount of substance distributed into the *stratum corneum* is neglected. However, for finite dose conditions the amount of penetrant present in the *stratum corneum* may be significant compared to the amount still present in the donor cell. Under these circumstances, the initial distribution into the *stratum corneum* will significantly decrease the concentration of the penetrant in the donor cell. As this concentration is the force driving its flux across the skin, the flux will be lower than predicted by Eq. (1). Under steady state conditions, the distribution of the penetrant over donor fluid and *stratum corneum* is given by the *stratum corneum*/water partition coefficient ($K_{SC,W}$, Eq. (2) in Figure 6-4) (WHO, 2006). From this equation the proportion (*f*) of the substance still present in the donor fluid after distribution into the *stratum corneum* can

be calculated, when the volumes of the *stratum corneum*, and of the donor and receptor cell fluids are known (see Eq. (3) in Figure 6-4).

According to Xiao and Imhof (1997), the thickness of the *stratum corneum* of the volar side of the fore-arm is ca. 20 µm. As the exposed skin area in our *in vitro* set-up is 0.64 cm^2, its volume (V_{SC}) in our system would be 1.28 µL, assuming the abdominal skin we used possesses a *stratum corneum* of approximately the same thickness. In our calculations of *f* we used this value.

As there are no data available on the $K_{SC,W}$ of the substances we investigated, with the exception of testosterone, we depended on QSARs to estimate the partition coefficients necessary to calculate *f*. These QSARs are listed in Figure 6-4 (Eqs. (4) and (5)). For testosterone we used the measured $K_{SC,W}$ reported by Abraham *et al.* (1995).

6.3.6.1 Correction measured k_p

Usually, k_p is determined under infinite dose conditions and calculated by dividing the measured flux by the (initial) driving concentration of the substance in the donor fluid. It is assumed that under these conditions no significant amount of substance will distribute into the *stratum corneum* relative to the amount remaining in the donor compartment. However, taking into account the parameters used in our infinite dose experiments, a substantial amount of substance will be present in the *stratum corneum* during steady state if the substance is rather lipophilic (see Figure 6-3). This means that also under infinite dose conditions the driving concentration may be significantly decreased by the distribution of substance into the *stratum corneum,* at least for lipophilic substances. Therefore, the k_p measured in the infinite dose experiments was corrected by dividing it by the correction factor *f* for a donor cell volume of 500 µL.

6.3.6.2 Prediction of cumulative penetration ($M_{rec,t}$) under finite dose conditions

Cumulative penetration was calculated using a formula derived from the differential equation of the modified Fick's law as shown in Figure 6-5. The correction factor (*f* for partitioning between donor cell fluid (10 µL) and *stratum corneum* was inserted in it as well as the lag time, measured under infinite dose conditions. The resulting formula is presented as Eq. (6) in Figure 6-4.

6.3.6.3 Prediction of the in vitro skin reservoir (M_{SC}) under finite dose conditions

The amount of penetrant present in the *stratum corneum* under steady state conditions was used as an approximation for the total amount present in the epidermal membrane. It was calculated from the predicted amount in the receptor fluid, the initial amount applied and the partitioning between donor cell fluid (10 µL) and *stratum corneum*, using Eq. (7) (see Figure 6-4).

In equation (1) the concentration term can be substituted by M_{don}/V_{don}, in which M_{don} is the mass of substance present in the donor cell on the skin in mg and V_{don} is the volume in which it is dissolved in mL (cm^3)/cm^2. Subsequently, differential equation (1) can be solved in the following steps:

$$-\frac{dM_{don}}{dt} = k_p \times \frac{M_{don}}{V_{don}} \times A$$

$$\int \frac{dM_{don}}{M_{don}} = -\int \frac{k_p \times A}{V_{don}} \times dt$$

$$\ln M_{don} = -\frac{k_p \times A}{V_{don}} \times t + c$$

$$M_{don,\,t} = e^{-\frac{k_p \times A}{V_{don}} \times t + c}$$

$$M_{don,\,t} = M_{don,\,0}\, e^{-\frac{k_p \times A}{V_{don}} \times t}$$

in which $M_{don,\,0}$ = initial mass of substance present on the skin in the donor cell in mg/cm^2.
Also taking into account the lag time (t_{lag}), the cumulative penetration into the receptor cell at time t ($M_{rec,\,t}$) in mg/cm^2, assuming that the total mass present is equal to the sum of M_{don} and M_{rec} (= $M_{don,\,0}$), is thus given by:

$$M_{rec,t} = M_{don,\,0} - M_{don,\,0}\, e^{-\frac{k_p \times A}{V_{don}} \times (t - t_{lag})} = M_{don,0}\left(1 - e^{-\frac{k_p \times A}{V_{don}} \times (t - t_{lag})}\right)$$

When distribution into the stratum corneum is fast compared to the flux across the skin, the penetrant will accumulate in that skin compartment. In that case, the amount left in the donor cell at time t will be less than the difference between the applied amount ($M_{don,\,0}$) and the penetrated amount ($M_{rec,\,t}$). Equation (3) of figure 6-4 gives the fraction *f* of the substance initially present on the skin after distribution into the stratum corneum. To correct for the decreased concentration due to distribution into the stratum corneum, the driving concentration (C) is substituted by f × C in the derivation of the cumulative penetration equation above from equation (1). This yields equation (6) of figure 6-4.

Figure 6-5 Derivation of Eq. (6) for cumulative penetration.
The equation numbers refer to those used in Figure 6-4. The symbols used are explained in Figure 6-4.

6.3.7 Log K_{OW}

Log K_{OW} of the investigated substances were obtained using the KowWin software (Meylan and Howard, 1999). KowWin calculates log K_{OW} of molecules based on its molecular structure, and also provides experimental values when available in its database. Unless otherwise stated, experimental values of log K_{OW} were used.

6.3.8 QSPRs for k_p

The evaluated QSPRs for k_p are listed in Figure 6-4 (Eqs. (8-10)). E-state indices for the methyl groups in a particular chemical were calculated according to the method detailed by Hall and Kier (Hall and Kier, 1995; Kier and Hall, 1997), and summed to obtain SsssCH (i.e., the sum of the E-state indices) of that chemical. Optimised geometries for each of the chemicals within the dataset were obtained using the AM1 Hamiltonian described by Dewar *et al.* (1985) available within the software package Gaussian 03 (Frisch *et al.*, 2003). The ABSQon descriptor was then obtained by summing the Mulliken charges upon the nitrogen and oxygen atoms present in a particular molecule as calculated with Gaussian 03.

6.3.9 Statistical analysis

Correlations between concentration and k_p were investigated using Spearman correlation coefficients. An absolute correlation coefficient that is higher than 0.8 was considered to reveal a good correlation, an absolute correlation coefficient between 0.6 and 0.8 was considered to reveal a moderate correlation. An absolute correlation below 0.6 was considered to be non-relevant. Differences in k_p between levels of concentration were tested with ANOVA. A Tukey Kramer correction was performed to correct for multiple comparison. In all tests performed, the null hypothesis of no difference was rejected at the 0.05 level of probability.

Differences between measurements and predictions of absorption in the 8 h finite dose experiments were tested for statistical significance using SAS for Windows (SAS 9.1.3; SAS Institute Inc., Cary, USA). A two-sided exact non-parametric Kolomogov-Smirnov test for two samples was used to test whether the measurements and predictions had identical distributions. The null hypothesis was rejected at the 0.05 level of probability (α = 5%).

6.4 Results and discussion

6.4.1 Measured and predicted k_p values

k_p values determined under infinite dose conditions are listed in Table 6-2 and Table 6-3; the latter also includes a complete overview of the measured infinite dose data. For the dataset described in this article, predictions of the k_p values by two selected QSPRs (Patel *et al.*, 2002; Potts and Guy, 1992) were, in general, in the same order of magnitude as the measured values for hydrophilic substances, but not for very lipophilic substances (log K_{OW} ≥4). However, performance of these QSPRs improved considerably when the k_p values were adjusted for lipophilicity according to the formula of Cleek and Bunge (1993; see Eq. (10) in Figure 6-4), at least when compared to measured values corrected for distribution into the *stratum corneum*. The k_p values for decanol, ethacrynic acid, octylparaben and salicylic acid, which still were not well predicted in spite of the adjustment for lipophilicity, were overestimated by a factor 5-10 (see Table 6-3).

6.4.2 Prediction of absorption

In EU risk assessment procedures, dermal absorption based on *in vitro* experiments is calculated by adding the amount that penetrated the skin to the amount present in the skin (yielding absorption). This is a worst case approach, since especially lipophilic substances can be retained in the *stratum corneum* and therefore may not become systemically available (see a.o. Buist *et al.*, 2007). In analogy, predictions of dermal absorption for risk assessment purposes should take the skin reservoir into account. Calculating the amount present in the *stratum corneum* under steady state conditions can give an indication of the total amount present in the membrane at the end of the experiment. Therefore, we used

Table 6-2 Concentration dependence of k_p for seven substances.

Substance	Concentration (mg mL^{-1})	k_p (10^{-3} cm/h ± SD)	% of maximum	Spearman correlation
Acetic acid	9.6×10^{1}	4.0 ± 2.1	100 ± 43	Good positive correlation (ρ = 0.86)
	2.1×10^{1}	3.2 ± 1.3	79 ± 31	
	4.7×10^{0}	1.4 ± 0.4	34 ± 11	
	8.2×10^{-1}	0.9 ± 0.1	21 ± 2	
	2.6×10^{-1}	0.8 ± 0.1	21 ± 3	
Butoxyethanol	4.5×10^{2}	5.4 ± 1.5	65 ± 18	No correlation (ρ = 0.18)
	9.0×10^{1}	8.4 ± 2.5	100 ± 30	
	1.8×10^{1}	4.4 ± 0.2	53 ± 2	
	3.6×10^{0}	5.0 ± 1.5	60 ± 18	
	1.0×10^{0}	5.2 ± 1.0	62 ± 12	
Decanol	7.4×10^{-1}	12.5 ± 0.8	71 ± 5	No correlation (ρ = -0.27)
	1.4×10^{-1}	16.0 ± 4.1	91 ± 23	
	2.6×10^{-2}	13.2 ±0.2	75 ± 33	
	4.2×10^{-3}	17.6 ±0.03	100 ± 35	
2,4-Dichlorophenol	3.8×10^{-1}	95.2 ± 5.5	100 ± 5	No correlation (ρ = 0.47)
	5.1×10^{-2}	57.2 ± 9.7	60 ± 10	
	2.7×10^{-3}	70.6 ± 3.2	74 ± 3	
	5.2×10^{-4}	66.1 ± 4.5	69 ± 5	
Octylparaben	1.0×10^{-1}	12.3 ± 0.6	76 ± 4	No correlation (ρ = 0.25)
	1.9×10^{-2}	16.3 ± 4.8	100 ± 30	
	3.1×10^{-3}	15.1 ± 1.1	93 ± 7	
	1.0×10^{-4}	10.5 ± 2.0	64 ± 12	
Propylparaben	5.0×10^{-1}	27.8 ± 4.0	100 ± 14	Moderate positive correlation (ρ = 0.73)
	1.0×10^{-1}	23.8 ± 3.0	86 ± 11	
	2.1×10^{-2}	24.4 ± 3.2	88 ± 12	
	4.9×10^{-3}	19.7 ± 2.5	71 ± 9	
	9.9×10^{-4}	19.7 ± 2.0	71 ± 7	
Testosterone	2.1×10^{-2}	11.1 ± 3.4	100 ± 31	No correlation (ρ = 0.51)
	8.0×10^{-3}	9.7 ± 2.2	87 ± 19	
	5.8×10^{-3}	9.4 ± 2.0	85 ± 18	
	1.7×10^{-3}	8.6 ± 1.4	77 ± 12	
	6.1×10^{-4}	7.8 ± 1.2	70 ± 10	

ρ = Spearman correlation coefficient.

our finite dose absorption prediction model to calculate the amounts present in the receptor fluid and the *stratum corneum* and summed them in order to estimate absorption.

This approach was tested in a series of *in vitro* experiments, in which epidermal membranes were exposed for 8 h to finite dose conditions. The k_p and lag times to be used in the prediction of absorption were determined in parallel experiments under infinite conditions. Measured and predicted values of relative absorption are listed in Table 6-4 and depicted in Figure 6-6. A complete overview of all measured finite dose data is given in Table 6-5.

For six substances the predicted absorption was not statistically different from the measured values. For all substances for which the predictions were statistically different from the measured values, absorption was overpredicted. Even so, most of these overpredictions were still below the European default absorption value for these substances (see Table 6-4).

Table 6-3 Results of infinite dose experiments (used for finite dose predictions), including k_p values calculated with selected QSARs.

Substance	MW	log K_{ow}	EU classification for skin irritancy[3]	Concentration (mg mL^{-1})	*n*	k_p values (10^{-3} cm/h)						Lag time ± SD (h)
						Measured		Calculated with QSAR				
						Uncorrected	Corrected[f]	P&G	P&G$_a$	Pat	Pat$_a$	
Acetic acid	60	-0.17	Corrosive[b]	1.0×10^{2}	*4*	3.0 ±1.3	2.9 ±1.3	0.6	0.6	0.7	0.7	9.6 ± 6.1
Benzoic acid	122	1.87	Not classified	3.3×10^{1}	3	7.6 ±1.1	7.6 ±1.1	7.3	**7.1**	**6.6**	**6.4**	1.9 ± 1
Bis(2-ethylhexyl) phthalate	391	8.39	Not classified	2.5×10^{-3}	4	0.08 ± 0.03	101 ±35	7100	**129**	500	**104**	0 ± 0
Butoxyethanol	118	0.83	Irritating[c]	4.5×10^{2}	4	3.6 ±1.1	3.6 ±1.1	1.4	1.4	1.4	1.4	3.6 ± 2.9
Cortisone	361	1.47	Not classified	2.0×10^{-1}	4	0.15 ±0.03	0.15 ±0.03	**0.13**	**0.13**	0.019	0.019	0 ± 0
Decanol	158	4.57	Not classified	2.5×10^{0}	4	11 ±0.5	18.1 ±0.9	362	132	300	122	1 ± 0.3
Diazinone	304	3.86	Not classified	1.7×10^{-2}	4	8.7 ± 0.7	9.1 ± 0.7	15	14	**12**	**11**	2.4 ± 0.2
2,4-Dichlorophenol	163	3.06	Corrosive[d]	3.7×10^{-1}	4	31 ±18	32 ±18	**26**	**23**	**34**	**29**	1.5 ± 1.5
Ethacrynic acid	303	3.69	Not classified	1.1×10^{-2}	3	0.15 ±0.15	0.16 ±0.16	11	10	3.8	3.7	15.8 ± 7.6
Linolenic acid	278	7.3	Not classified	1.1×10^{-2}	4	3.7 ± 1.1	168 ±49	5900	**152**	2444	**146**	4.4 ± 0.3
Octylparaben	250	5.43[e]	Not classified	8.9×10^{-2}	4	9.3 ±1.6	14.5 ± 2.5	408	117	174	84	3.4 ± 0.5
Oleic acid	282	7.73	Not classified	1.1×10^{-2}	4	1.3 ±0.13	184 ± 18	11200	**153**	4400	**149**	15 ± 0.8
Propylparaben	180	3.04	Not classified	5.0×10^{-1}	4	14 ±1.2	14.3 ± 1.2	20	18	**12**	**11**	1.1 ± 0.3
Salicylic acid	138	2.24	Not classified	3.6×10^{-1}	4	0.93 ± 0.27	0.93 ± 0.27	11	10	5.0	4.9	18 ± 0.9
Testosterone	288	3.3	Not classified	2.6×10^{-2}	4	7.7 ± 1.6	7.9 ±1.6	**7.6**	**7.2**	0.89	0.89	3.3 ± 0.3

Donor cell solution volume = 500 µL; exposed area = 0.64 cm^2; exposure time = 48-51 h.

P&G = QSAR derived by Potts and Guy (1992); Pat = QSAR derived by Patel *et al.* (2002); P&G$_a$/Pat$_a$ = QSARs adjusted for lipophilicity according to Cleek and Bunge (1993).

Calculated k_p values printed in bold are within or close to the range of corrected measured values.

[a] Retrieved from the EU internet database ESIS (http://ecb.jrc.ec.europa.eu/esis/).

[b] Concentration limits: ≥25% = corrosive, between 10% and 25%: irritating.

[c] Tested with undiluted pure substance, 50% solution in polyethylene glycol was not irritating.

[d] Tested with undiluted pure substance.

[e] Calculated value.

[f] Correction for distribution into the *stratum corneum* by dividing it by ƒ (see Eq. (3), Figure 6-4, page 129).

Table 6-4 Prediction of potential absorption in finite dose experiments with 15 substances.

Substance	(%) Potential absorption		
	Measured	Predicted	EU default[b]
Acetic acid	43 ± 18	**19 ± 26**	100
Benzoic acid	48 ± 7	**93 ± 4**	100
Butoxyethanol	45 ± 10	**57 ± 25**	100
Cortisone	21 ± 7	**27 ± 1**	100
Decanol	60 ± 6	98 ± 0	100
Diazinone	73 ± 6	89 ± 1	100
2,4-Dichlorophenol	90 ± 5	**93 ± 14**	100
Ethacrynic acid	54 ± 17	**66 ± 0**	100
Linolenic acid	55 ± 14	100 ± 0	100
Octylparaben	76 ± 12	97 ± 0	100
Oleic acid	60 ± 9	100 ± 0	100
Bis(2-ethylhexyl) phthalate	82 ± 11	100 ± 0	100
Propylparaben	90 ± 5	99 ± 0	100
Salicylic acid	20 ± 1	17 ± 0	100
Testosterone	63 ± 5	84 ± 4[a]	100

Donor cell solution volume, 10 µL; exposed area, 0.64 cm^2; exposure time, 8 h.
Bold no statistical difference between measured and predicted value ($p > 0.05$ in two-tailed Kolomogov-Smirnov test for two samples ($\alpha = 5\%$).
[a] Predicted using measured $K_{SC,W}$ (Abraham *et al.*, 1995).
[b] 10% for substances with MW > 500 and log K_{OW} <-1 or >4, otherwise 100% (EU, 2004).

Table 6-5 Mass balance of finite dose experiments.

Substance	Concentration (mg mL^{-1})	n	% of applied dose ± SD				
			Donor cell	Membrane	Receptor cell	Potential absorption[a]	Recovery
Acetic acid	1.0×10^{2}	4	52 ± 18	15 ± 3.8	27 ± 15	43 ± 18	95 ± 2.2
Benzoic acid	3.3×10^{1}	4	49 ± 8.0	24 ± 1.8	24 ± 5.8	48 ± 7.5	98 ± 0.8
Bis(2-ethylhexyl) phthalate	2.5×10^{-3}	4	24 ± 4.4	77 ± 9.4	4.4 ± 2.5	82 ± 11	105 ± 11
Butoxyethanol	4.5×10^{2}	4	55 ± 13	10 ± 1.3	35 ± 10	45 ± 10	100 ± 3.9
Cortisone	2.2×10^{-2}	4	78 ± 6.3	21 ± 6.8	0.6 ± 0.2	21 ± 7.0	99 ± 1.5
Decanol	2.5×10^{0}	4	37 ± 7.9	29 ± 5.7	32 ± 1.6	60 ± 5.6	97 ± 7.4
Diazinone	1.7×10^{-2}	4	27 ± 3.2	53 ± 7.7	20 ± 4.0	73 ± 6.4	100 ± 5.2
2,4-Dichlorophenol	3.7×10^{-1}	3	15 ± 3.0	24 ± 2.4	66 ± 3.9	90 ± 5.0	104 ± 2.6
Ethacrynic acid	1.1×10^{-2}	3	33 ± 24	52 ± 17	1.1 ± 0.4	54 ± 17	89 ± 13
Linolenic acid	1.1×10^{-2}	4	41 ± 13	48 ± 15	7.4 ± 2.8	55 ± 14	96 ± 3.8
Octylparaben	8.9×10^{-2}	4	33 ± 8.3	58 ± 10	18 ± 3.9	76 ± 12	109 ± 12
Oleic acid	1.1×10^{-2}	4	36 ± 6.7	59 ± 9.1	1.2 ± 0.3	60 ± 9.3	96 ± 5.2
Propylparaben	5.0×10^{-1}	4	10 ± 3.8	26 ± 3.8	64 ± 6.9	90 ± 4.8	100 ± 1.2
Salicylic acid	3.6×10^{-1}	4	80 ± 3.3	16 ± 1.6	4.3 ± 0.8	20 ± 1.3	100 ± 3.7
Testosterone	2.6×10^{-2}	4	36 ± 7.4	22 ± 2.2	42 ± 4.0	63 ± 5.5	100 ± 2.2

Donor cell solution volume, 10 µL; exposed area, 0.64 cm^2; exposure time, 8 h.
[a] Sum of relative amounts recovered from the skin membrane and the receptor cell.

For acetic acid, no good predictions could be made for concentrations other than the one used to determine k_p. The reason for this was that its k_p value increased considerably with increasing concentration (ca. a factor 5 over the investigated concentration range; see Table 6-2). This concentration dependency was not observed to this extent with any of the other substances tested, and is probably related to the corrosive nature of acetic acid, causing a decrease in the barrier function of the skin.

The only other compounds classified by the EU as irritating (butoxyethanol) or corrosive (2,4-dichlorophenol) tested positive as pure undiluted substances (see Table 6-3). A 50% dilution of butoxyethanol in polyethylene glycol tested negative for skin irritancy, therefore the tested aqueous concentrations of this substance (up to 450 mg mL^{-1}) are probably not skin irritating.

In the skin irritation study used by the EU for 2,4-dichlorophenol, no dilutions were tested. The highest concentration of 2,4-dichlorophenol used in this paper (0.38 mg mL^{-1}) showed a statistically significantly higher permeability coefficient than the other three concentrations (using ANOVA with Tukey correction for multicomparison). However, the increase compared to the other concentrations was small: ca. a factor 1.5. Concluding, these classified substances were probably not tested at skin damaging concentrations.

For our predictions we used QSARs to estimate the $K_{SC,W}$, a parameter necessary to calculate the impact of distribution into the *stratum corneum*. These are based on limited datasets: Hui *et al.* (1995) used seven lipophilic substances (five pesticides, one steroid hormone and aminopyrine) and five hydrophilic substances (dopamine, glycine, urea, glyphosate and theophylline), whilst Abraham *et al.* (1995) used a dataset of eight aliphatic alcohols (C_1-C_8) and 14 steroid hormones. Judging by their log K_{OW} and MW, the substances tested in this study are inside or at least close to the applicability domain of these QSARs. However, for wider application of our method, the QSARs for $K_{SC,W}$ should be based on a larger and more varied dataset. Alternatively, $K_{SC,W}$ could be determined experimentally *in vitro*, as described by Hui *et al.* (1995) and Abraham *et al.* (1995). These QSARs do not take into account possible molecular interaction (e.g., binding, adsorption) between substances and the *stratum corneum*. For substances that have a great tendency to do so, this may lead to an underestimation of the amount associated with the *stratum corneum*. On the other hand, such substances will not easily migrate from the *stratum corneum* to the viable *epidermis* and from there, eventually, to the receptor fluid (which represents systemic circulation in the *in vivo* situation). As the reason for including the skin reservoir in the estimation of dermal absorption is the fact that it may migrate into the receptor fluid, neglecting these strong interactions may not lead to a serious underestimation of dermal absorption. This is illustrated by the example of DDAC, which shows a strong association with the *stratum corneum*, but hardly penetrates into the receptor fluid (Buist *et al.*, 2007).

Another critical parameter in our calculations is the thickness of the *stratum corneum*. We used a value of 0.002 cm, determined by Xiao and Imhof (1997) for the thickness of the *stratum corneum* of the volar side of the fore-arm. In order to test the influence of *stratum corneum* thickness on our prediction model, we executed the calculations with different thicknesses of the *stratum corneum.* Only in 3 out of 15 cases notable differences were observed between the predicted absorption values: in these cases, when assuming a 4x thicker *stratum corneum*, absorption was ca. twice as high (data not shown). Therefore, our model does not seem very sensitive to changes in *stratum corneum* thickness.

Our prediction method uses experimentally derived values for k_p and lag time. However, these values could in principle also be obtained by using QSARs for the determination of $K_{SC,W}$ and k_p. The lag time may be calculated using the fixed relationship between lag time (t_{lag}), $K_{SC,W}$, k_p for the *stratum corneum* (k_{pSC}) and thickness of the *stratum corneum* (L_{SC}), derived by Shah *et al.* (1994): $t_{lag} = K_{SC,W} \times L_{SC}/6k_{pSC}$. However, the predictive power of the tested QSPRs for k_p does not seem very high, at least not for our data set, although their performance improves when adjusted for lipophilicity according to the method of Cleek and Bunge (1993; see Table 6-3). Poor performance of QSPRs for k_p has also been observed for other data sets (Bouwman *et al.*, 2008). Furthermore, the relationship of Shah *et al.* (1994) did not hold very well under the assumptions and simplifications we used: *stratum corneum* is rate limiting and a homogeneous barrier, neglecting the concentration gradient inside *stratum corneum* (data not shown). Therefore, at present, our prediction is insufficiently valid when using QSPR data only, and needs experimentally derived values for k_p and lag time.

Often very diverse scenarios need to be evaluated for regulatory risk assessment purposes. Therefore, our method provides an advantage for risk assessment, as one only has to determine k_p and lag time under conditions of infinite dose to be able to calculate (relative) dermal absorption for a whole range of exposure scenarios, differing in exposure time, exposure concentration, dermal loading and exposed skin area. This eliminates the need to perform many dermal absorption experiments under different exposure conditions. Furthermore, the method can be used to more easily analyse which changes in, e.g., operational procedures may lead to a safe use scenario.

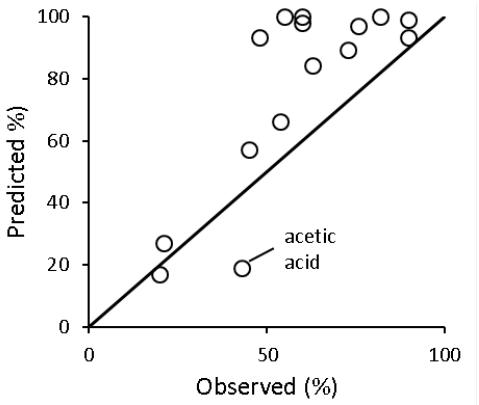

Figure 6-6 Prediction of finite dose absorption.
Predictions using k_p and lag time measured under infinite dose conditions, with correction for distribution into the *stratum corneum*. The solid line represents the perfect prediction.

6.5 Conclusions

Our finite dose prediction model provides a tool to estimate dermal absorption for many different (finite) exposure scenarios, using k_p and lag time measured under infinite dose

conditions and a QSAR for the *stratum corneum*/water partition coefficient ($K_{SC,W}$). The model predicted values which were either not statistically different from measured *in vitro* values or overestimated them. Overestimated values still were most often less conservative than the default European approach. Therefore, our model provides a useful and cost-effective estimate of dermal absorption, to be used in risk assessment for non-volatile substances dissolved in water at non-irritating[13] concentrations.

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6.7 Glossary (including abbreviations)

ABSQon	a molecular descriptor: the sum of absolute charges on the oxygen and nitrogen atoms in a molecule
Corrosive	to skin: capable of full-thickness destruction of skin tissue
DDAC	didecyldimethylammonium chloride
Default absorption values	absorption values used in absence of measured or predicted data
Dermal absorption value	in *in vitro* dermal absorption experiments: the amount of substance that has penetrated the skin plus the amount still present in the skin preparation
Descriptor	in this article: short for molecular descriptor = a specific characteristic of molecular structures, which can be expressed in a numeric value
Donor compartment	part of the diffusion cell positioned on the outside of the skin preparation to which the exposure solution is added
Epidermal membrane	Skin preparation consisting of *stratum corneum* and *epidermis*
f	amount of penetrant in the donor compartment divided by the sum of the amounts in the donor compartment and the *stratum corneum*
Finite dose	applied amount of donor solution for which of the concentration of the penetrant significantly decreases during dermal exposure
Flux	amount of penetrant that crosses the skin per unit of time and unit of exposed skin area, often expressed in $mg/cm^2/h$
In silico	calculated or predicted by computer (analogous to *in vitro* and *in vivo*)
Infinite dose	applied amount of donor solution for which of the concentration of the penetrant remains (more or less) constant during dermal exposure
Irritating	to skin: causing significant inflammation of the skin
K_{OW}	octanol/water partition coefficient
k_p	permeation or permeability constant, often expressed in cm/h
$K_{SC,W}$	*stratum corneum*/water partition coefficient

Lag time	the time intercept for penetration equal to zero of the linear portion of the cumulative penetration versus time graph (see Figure 6-2)
Limit value	regulatory exposure value which should not be exceeded
Log K_{OW}	10-base logarithm of the octanol/water partition coefficient, also known as log P
Mulliken charges	Mulliken charges arise from the Mulliken population analysis and provide a means of estimating partial atomic charges from calculations carried out by computational chemistry packages like Gaussian 03.
MW	molecular weight
Occluded	in *in vitro* dermal absorption experiments: donor compartment covered in such a way that no evaporation of solvent can take place
Penetrant	in *in vitro* dermal absorption experiments: the substance of which the absorption value or k_p is being determined
Penetration	in *in vitro* dermal absorption experiments: the crossing of the skin into the receptor compartment
Permeability coefficient	constant describing the permeability of a specific skin preparation for a specific molecule dissolved in water, usually expressed in cm/h
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation Authorisation and restriction of CHemicals
Receptor compartment	part of the diffusion cell positioned on the inside of the skin preparation, filled with liquid in which the investigated chemical penetrates if the skin is permeable to it (and a concentration gradient is maintained across the skin).
Skin reservoir	amount of penetrant still present in the skin after cessation of exposure
SsssCH	a molecular descriptor: the sum of E-state indices for all methyl groups in a molecule
Static diffusion cell	apparatus to determine *in vitro* dermal absorption from which the liquid in the receptor compartment is not continuously refreshed, but only after specific time intervals
Steady state	in *in vitro* dermal absorption experiments: conditions under which penetration into the receptor fluid occurs at a constant rate
Stratum corneum	non-viable upper layer of the skin
Systemic effects	effects that occur if and when substances enter systemic circulation

7 General discussion

7.1 Introduction

Given the possible importance of the dermal exposure route, the state-of-the-art of models to estimate dermal absorption and the many factors influencing the efficiency of this exposure route outlined in chapter 1, the objective of this thesis was to further develop, evaluate and improve methods for including dermal absorption data in toxicological risk assessment.

Adequate quantification of dermal absorption is an essential step in reducing the uncertainty of dermal risk assessment and essential to avoid unrealistic worst case assumptions. Many factors influence the rate of dermal absorption of a substance. In this thesis, four of these factors were investigated in more detail, i.e. dermal loading (chapter 2), irritative/corrosive potential (chapters 3 and 4), frequency of exposure (chapters 3-5), and the formulation used (chapter 5). To conclude, in chapter 6 of this thesis a model to extrapolate infinite dose absorption data to finite dose conditions, tentatively called Dermal Absorption Model for Extrapolation (DAME), was developed and tested. In the following sections the investigations described and discussed in chapters 2 to 6 will be put into perspective, the pitfalls and promises for toxicological risk assessment emerging from them will be discussed, future perspectives will be presented and general conclusions will be drawn.

7.2 Dermal loading and absorption

In regulatory risk assessment it is common practice to use relative dermal absorption determined at a specific dermal loading (amount of chemical per unit skin surface) to calculate internal exposure. In chapter 2 of this thesis, the relationship between dermal loading and relative absorption (e.g. expressed as percentage of the applied dose) was investigated using published data. Nearly two-thirds of the evaluated *in vitro* and *in vivo* absorption experiments showed an inverse relationship between dermal loading and relative absorption. It should be noted that in absolute terms dermal absorption still may increase with dermal loading. However, the focus was on relative dermal absorption (often expressed as percentage of the applied dose) as this is a measure frequently reported and used in toxicological risk assessment.

A likely explanation for the inverse relationship between dermal loading and relative dermal absorption may be that in many cases the range of loadings tested represented (nearly) infinite dose conditions, meaning that the amount of chemical applied on the skin does not significantly decrease by the disappearance of chemical from the skin through the process of absorption. Under infinite conditions the amount of absorption is flux-limited, and under finite conditions the amount of absorption is delivery-limited, i.e. dependent on dermal loading. When absorption is flux-limited, the amount absorbed per unit time will be constant, irrespective of the dermal loading, meaning relative absorption will decrease with increasing dermal loading under these conditions.

Kissel *et al.* (2011) have introduced the fraction N_{derm} to assess whether dose conditions are *de facto* infinite. For experimental exposures, N_{derm} is defined as the applied dermal load divided by the product of the maximum flux (J_{max}) and the exposure time: $N_{derm} = \frac{\text{Dermal load (mg/cm}^2\text{)}}{J_{max}\ \text{(mg/cm}^2\text{/h)} \times \text{exposure time (h)}}$. Conditions of maximum flux are reached when the skin is exposed to saturated solutions of a chemical. N_{derm} was later redefined by Frasch *et al.* (2014) as dermal load divided by the product of *steady state* flux (J_{SS}) and exposure time: $N_{derm} = \frac{\text{Dermal load (mg/cm}^2\text{)}}{J_{SS}\ \text{(mg/cm}^2\text{/h)} \times \text{exposure time (h)}}$. Steady state flux is the constant *in vitro* flux of a chemical across the skin preparation that is reached under (nearly) infinite dose conditions. Using this definition, N_{derm} is also applicable to dermal exposure to dilute solutions, which is more common. Infinite dose conditions are by definition, conditions under which the applied concentration of the chemical on the skin does not change noticeably, and the flux of the chemical is constant, determining the total amount of chemical being absorbed during the time exposure continues, in other words, the absorption is limited by the rate (flux) with which the chemical crosses the skin barrier, and will not increase when more chemical is added to the skin. By consequence, N_{derm} will be high as the amount that disappears from the dermal load is negligible compared to the load present, and a high N_{derm} is an indication of flux-limited absorption. In these circumstances, relative absorption is a meaningless figure, as the rate of absorption is independent of the applied dose. By definition, under finite dose conditions the concentration of the chemical on the skin will decrease due to absorption and the rate of absorption will decrease over time: N_{derm} will be low. In this case, absorption is delivery-limited as increasing the amount of chemical on the skin will increase the rate of absorption.

Frasch *et al.* (2014) have reanalysed the data described in chapter 2, Table 2-1, by plotting N_{derm} calculated by them, against the measured percentage absorption reported in Table 2-1. They found a distinctive decrease of percentage absorption with increasing N_{derm}, although a clear cut-off value between delivery- and flux-limited absorption was not found. Unfortunately, N_{derm} can only be calculated when measured values like k_p, J_{SS} or J_{max} are available, since the predictive performance of Quantitative Structure-Permeability Relationships (QSPRs) for these variables is not good enough to be relied upon (section 1.1.4.6.2). Therefore, N_{derm} is above all useful for the evaluation of (*in vitro*) dermal absorption experiments (Frasch *et al.*, 2014).

When in the studies evaluated in this thesis an inverse relation between relative absorption and dermal loading was observed, relative dermal absorption at low dermal loading could be up to a factor 100 higher than at high dermal loading. Therefore, a pitfall in risk assessment is to calculate internal exposure with relative absorption data determined at a dermal loading not representative for the actual loading, which may lead to an incorrect assessment of human systemic exposure and its resulting health risk. This is illustrated in Figure 2-1 (page 52), which depicts a generalized example of an inverse relation between dermal loading and relative absorption. It demonstrates that using a relative absorption

value derived from a high dermal loading will lead to an underestimation of systemic absorption at low dermal loading. Therefore, dermal absorption data should be derived as much as possible under exposure conditions reflecting the scenario to be evaluated or be extrapolated to these conditions, e.g. by using a model like DAME (section 7.6).

7.3 Skin irritants/corrosives and absorption

Skin irritants have been subject of discussion in chapters 2 to 6 of this thesis, and are substances that may cause reversible damage of the skin following their application for up to 4 hours, while corrosives may cause irreversible damage to the skin, all the way down to the *dermis* (OECD, 2015). These local effects are concentration driven, and substances that are corrosive at high concentrations may be merely irritants at intermediate concentrations or may cause no local effects at all at low concentrations. Hence, concentration limits have been established for this type of local toxicants under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (UN, 2015). As an example, aqueous solutions of HCl with a concentration ≥25% (w/w) are classified as "skin corrosive", when the HCl-concentration is ≥10% but <25%, the solution is classified as "skin irritant" and solutions with a concentration <10% are not classified with respect to this local toxic property[14].

It is generally assumed that skin corrosives and irritants may decrease the barrier function of the skin and hence increase its permeability (EFSA, 2011; WHO, 2006). In various instances, this is supported by the results of our investigations. Classified skin irritants less often show an inverse effect between dermal loading and absorption: 46% against 83% of the positively identified non-irritants (Table 2-3C, page 64). This may be explained by the assumption that the decrease of relative absorption with increasing dermal loading is offset by an increase in absorption due to the rising skin permeability if dermal loading is increased by raising the concentration of the skin irritating chemical.

Also the experimental research presented in this thesis (described in chapters 4 to 6) showed results indicative of altered, mostly decreased, barrier function by skin irritants/corrosives. Table 7-1 shows an overview of the classification for skin irritation/corrosion of the chemicals used in the experiments described in this thesis. The classification has been updated in 2015, as, since the REACH-regulation entered into force in the EU on 1 June 2007 (EU, 2006), many additional data have become publicly available through the ECHA-website (http://echa.europa.eu).

[14] http://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/cl-inventory/view-notification-summary/105223

Table 7-1 ECHA classifications for skin corrosion/irritation of the substances tested in this thesis for influence on skin permeability

Name	CASRN	Classification ECHA[a]	Harmonised[b]	Remark	Experiments in thesis
ADBAC	8001-54-5	Skin Corr. 1B	No		$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Boric acid	10043-35-3	conclusive but not sufficient for classification	Yes	study in ECHA registration dossier	$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
DDAC	7173-51-5	Skin Corr. 1B	Yes		$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Deltamethrin	52918-63-5	No data	Yes	no ECHA registration dossier	$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Permethrin	52645-53-1	No data	Yes	no ECHA registration dossier	$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Piperonyl butoxide	51-03-6	conclusive but not sufficient for classification	No	study in registration dossier	$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Sodium bromide	7647-15-6	conclusive but not sufficient for classification	No	study in ECHA registration dossier	$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Tebuconazole	107534-96-3	No data	Yes	no ECHA registration dossier	$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4)
Formaldehyde	50-00-0	Skin Corr. 1B: C ≥ 25% Skin Irrit. 2: 5% ≤ C < 25%	Yes		$[^3H]H_2O$ and $[^{14}C]$propoxur permeability (chapter 4) influence on DDAC dermatokinetics (chapter 5)
Glutaraldehyde	111-30-8	Skin Corr. 1B: C ≥ 10% Skin Irrit. 2 : 0.5% ≤ C < 10%	Yes		influence on DDAC dermatokinetics (chapter 5)
2,4-Dichlorophenol	120-83-2	Skin Corr. 1B	Yes	tested with undiluted pure substance and as an 80% solution in water	concentration dependency of k_p (chapter 6)
Acetic acid	64-19-7	Skin Corr. 1A: C ≥ 90% Skin Corr. 1B: 25% ≤ C < 90% Skin Irrit. 2: 10% ≤ C < 25%	Yes		concentration dependency of k_p (chapter 6)
Butoxyethanol	111-76-2	Skin Irrit. 2	Yes	pure substance, 50% solution in PEG not irritating, not in ECHA registration dossier.	concentration dependency of k_p (chapter 6)

Table 7-1 continued

Name	CASRN	Classification ECHA[a]	Harmonised[b]	Remark	Experiments in thesis
Decanol	112-30-1 / 36729-58-5	Skin Irrit. 2 / conclusive but not sufficient for classification	No	pure substance, 5 adequate studies in REACH registration dossier: 4 negative, 1 positive (all borderline)	concentration dependency of kp (chapter 6)
Octylparaben	1219-38-1	Skin Irrit. 2	No	One other entry: no classification due to lack of data; no ECHA registration dossier	concentration dependency of k_p (chapter 6)
Propylparaben	94-13-3	conclusive but not sufficient for classification	No	ECHA registration dossier: propylparaben tested in hydrophilic ointment and read-across to pure ethyl- and methylparaben tested as powders.	concentration dependency of k_p (chapter 6)
Testosterone	58-22-0	conclusive but not sufficient for classification	No	ECHA registration dossier: based on read-across to androst-4-ene-3,17-dione	concentration dependency of k_p (chapter 6)

% = concentration in % (w/w); C = concentration; CASRN = Chemical Association Substance Registry Number; Corr. = corrosive; Irrit. = irritative

[a] 1A = Corrosive responses in at least one animal following exposure ≤ 3 min during an observation period ≤ 1 h; 1B = Corrosive responses in at least one animal following exposure > 3 min and ≤ 1 h and observations ≤14 days; 2 = Irritative responses in 2 out of 3 animals (or in 1 out of 3 if clearly positive and pronounced variability between animals exists); conclusive but not sufficient for classification = not corrosive/irritative to skin, based on adequate data.

[b] Substances have a harmonized classification when included in Part 3 of Annex VI to Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP). Part of the harmonization procedure is evaluation by experts of the EU member states.

In the skin permeability assays with biocidal active substances presented in chapter 4 of this thesis, two out of three substances classified for skin corrosion/irritancy, the quaternary ammonium chloride compounds didecyldimethylammonium chloride (DDAC) and alkyldimethylbenzylammonium chloride (ADBAC), caused a clear concentration-dependent increase in skin permeability for [^{14}C]propoxur and tritiated water, both after single and repeated application. The third corrosive compound tested, formaldehyde, did not show such an effect, but showed a tendency to decrease skin permeability for [^{14}C]propoxur and tritiated water, although not in a statistically significant manner. This effect observed for formaldehyde was confirmed by the experiments carried out with different biocidal

formulations of DDAC, some of which contained aldehydes (chapter 5). These formulations tended to reduce DDAC dermal absorption as compared to pure water as a vehicle (Figure 7-1). A possible explanation for this phenomenon is the ability of formaldehyde and glutaraldehyde to crosslink proteins (Usha and Ramasami 2005). Crosslinking of proteins, such as keratins, in the *stratum corneum* could turn the skin less permeable. This is corroborated by our observation that the digestion of skin preparations[15] exposed to MS Macrodes, the commercial biocidal formulation used with the highest concentration of aldehydes, took more time than the digestion of skin preparations exposed to one of the other formulations.

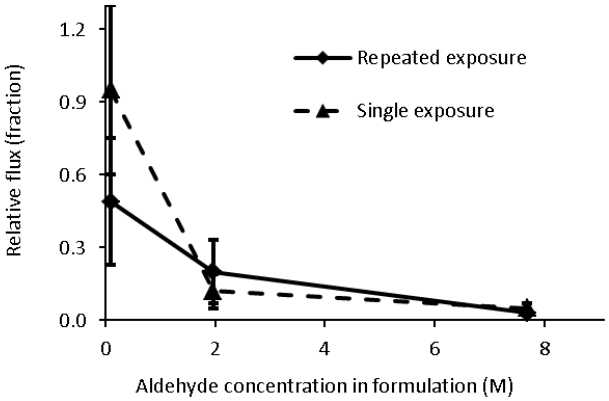


Figure 7-1 Relative flux of DDAC across the skin versus the aldehyde concentration in the formulations used

The flux is expressed relative to the flux of the same amount of DDAC dissolved in propanol/water instead of formulation. Data from Table 5-6 of chapter 5.

In principle, the permeation constant k_p is independent of the concentration of the chemical (Equation 1-2, page 30), provided the chemical does not influence the skin barrier. Therefore, for compounds that do change skin permeability in a concentration related manner, the k_p values should depend on their concentration. Indeed, the corrosive/irritative potency of the tested substances and the degree to which their k_p-value is influenced by their concentration, does show a positive correlation between the corrosion/irritation potency and skin permeability (R = 0.76, Table 7-2).

Concluding, by and large our results confirm the influence of skin corrosives/irritants on skin permeability. This influence does not necessarily imply an increase in permeability, as is illustrated by the permeability decreasing effect of two corrosive aldehydes, formaldehyde and glutaraldehyde. It should be noticed that skin corrosion or irritation is not only determined by the chemical nature of the substances the skin is exposed to, but also by their concentration, meaning that even a corrosive substance like acetic acid may not increase skin permeability, provided its concentration is sufficiently low. A distinct pitfall in

[15] This digestion was performed in order to analyse the DDAC concentration in the skin preparations.

the case of skin corrosives/irritants is to assume that their k_p is independent of concentration and extrapolate absorption values obtained at one concentration to another, as can be done with substances that do not influence skin permeability. Therefore, skin absorption data to be used in risk assessment of substances classified for skin corrosion/ irritation should be obtained at exposure concentrations representative of the exposure scenarios to be evaluated.

Table 7-2 Relation between corrosion/irritation potency and concentration dependence of k_p.

Substance	Corrosion/Irritation potency[a]	Degree of concentration dependence of k_p[b]
2,4-Dichlorophenol	2	1
Acetic acid	3	2
Butoxyethanol	0.5	0
Decanol	0.5	0
Octylparaben	1	0
Propylparaben	0	1
Testosterone	0	0
Correlation (Pearson)	**R = 0.76**	

[a] Based on the following grading of highest classification of a substance: corrosive 1A = 3; corrosive 1B = 2; irritant 2 = 1; borderline irritant 2 = 0.5. The classifications are listed in Table 7-1 (page 147).

[b] Based on the following grading of the concentration dependence: 2 = good correlation between k_p and concentration; 1 = moderate correlation or statistically significant difference between k_p at highest concentration and lower concentrations; 0 = no influence of concentration on k_p. The concentration dependencies are listed in Table 6-2 (page 134).

[c] The Pearson correlation between corrosion/irritation potency and degree of concentration dependence was calculated using the "CORREL" function of MS-Excel from MS-Office Professional Plus 2010.

7.4 Repeated dermal exposure and absorption

In chapters 3 to 5 of this thesis the effect of repeated dermal exposure on absorption was investigated. The OECD guidelines on *in vivo* and *in vitro* dermal absorption studies (OECD 2004a, OECD 2004b) are not explicit on whether single or repeated exposure should be investigated. However, common practice is that dermal absorption studies submitted within the EU regulatory frameworks are restricted to single exposure, while in practice dermal exposure of workers and consumers may be repeated in nature, e.g. in professional cleaning and in use of cosmetics.

A literature search presented in chapter 3, revealed that repeated dermal exposure often has a cumulative effect on daily systemic absorption, although less than would be expected based on addition of daily doses. The magnitude of this effect cannot be exactly predicted based on dermal absorption data alone since it will depend on the net effect of absorption, distribution, metabolism and excretion. However, it is a potential pitfall in dermal route toxicological risk assessment, since the use of single exposure dermal absorption data may lead to underestimation of systemic exposure, and consequently of the risk associated with it.

In various guidelines for dermal absorption the amount retained in the skin after cessation of exposure is considered to be (potentially) absorbed (EFSA, 2012; OECD, 2004a; USEPA, 2007). This may, at least partly, compensate for cumulative effects of repeated exposure. For instance, this is the case for repeated dermal exposure to DDAC : After single *in vitro* dermal exposure, the amount present in the skin (excluding tape strips) is approx. 15 µg while after three exposures only approximately 3 µg has fully penetrated into the receptor fluid (equivalent of systemic circulation, see Figure 5-4, page 118). Consequently, in this example, including the skin reservoir after single exposure in the dermal absorption value, as demanded by the cited guidelines, would prevent an underestimation of systemic exposure after repeated exposure, and ultimately an underestimation of the risk. It also shows that, certainly for a reservoir forming substance like DDAC, systemic absorption may be considerably overestimated by including the skin reservoir in the absorption estimation. Furthermore, the ratio of the amount of chemical encountered in the skin preparation and the amount of chemical encountered in the receptor fluid in finite dose experiments indicates that including the amount retained in the skin in the dermally absorbed dose would compensate for the cumulative effects of repeated dermal exposure for at least half of the chemicals (Figure 7-2), when assuming accumulation of doses by repeated exposure will increase daily systemic absorption with a factor 1 to 5 (based on the data depicted in Figure 3-1, page 77). Probably, the chemicals in Figure 7-2 showing a lower ratio are also the substances with a smaller cumulative effect, since the skin reservoir is the source of the cumulation. However, more consistent and complete data are needed to reach a definitive conclusion.

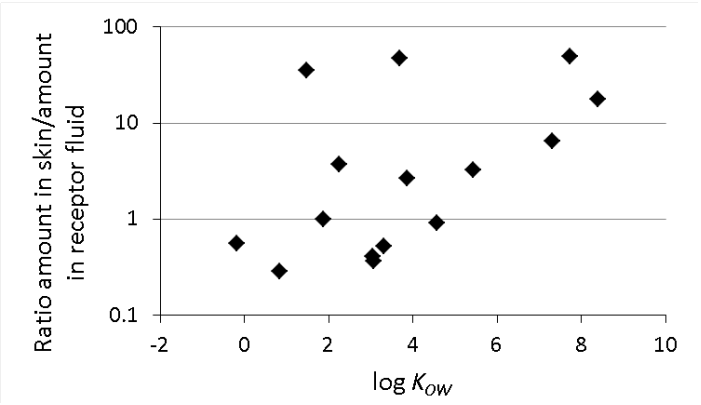


Figure 7-2 Ratio chemical retained in skin and receptor fluid in finite dose absorption experiments
Data taken from Table 6-5, page 136.

Furthermore, *in vitro* skin permeability experiments with a number of biocides indicated that repeated exposure to high concentrations of corrosive substances like quaternary ammonium chlorides may increase skin permeability more than single exposure (chapter 4). The degree of increase after three times repeated exposure compared to single exposure was approximately a factor 1.2 to 3 (Table 4-4, page 103). Non-corrosives/irritants do not

seem to influence skin permeability to a higher degree after repeated exposure, if at all. More data on the effects of other corrosive, irritant and non-irritant substances are needed to obtain a more general picture on the influence of repeated exposure to these categories of substances.

Since from these few results it appears that only high concentrations of corrosive substances may (further) increase skin permeability after repeated exposure, its practical relevance could be low, as only professional users may be expected to be repeatedly exposed to high concentrations of corrosives for prolonged times, and they will be likely to wear protective clothing.

7.5 Formulations and absorption

As already indicated in chapter 1, the vehicle in which the chemical is applied to the skin may influence its absorption. This was investigated in some more detail in chapter 5 of the thesis. In case a vehicle is a more or less complex mixture designed for a specific purpose (e.g. pest control, drug delivery), it is called a formulation. In principle, when a vehicle does not alter skin permeability or enhances the solubility of the chemical in the skin, the permeation constant, k_p, of a chemical in a specific vehicle can be derived from its saturation concentration in that vehicle and its maximum flux, J_{max}, in another vehicle, e.g. water. This follows from Equation 1-1 (Fick's first law) and Equation 1-2 (relation between permeation constant and partition coefficient, diffusivity and diffusion path) (page 16 and 30, respectively). Adapting Equation 1-1 to obtain J_{max} (expressed per unit skin area) yields:

$J_{max} = C_{veh,\,sat} \times k_{p,veh}$ **Equation 7-1 Calculating J_{max}**

in which

J_{max} = maximum mass flux across the membrane in mg/h,
$C_{veh,\,sat}$ = the saturation concentration of the substance in the vehicle in mg/cm^3, and
$k_{p,\,veh}$ = permeation constant for a specific vehicle in cm/h

Filling out the result of Equation 1-3 for k_p ($k_p = K_{SC/veh} \times D_{SC}/L_{SC}$) in Equation 7-1 gives:

$J_{max} = C_{veh,\,sat} \times K_{SC/veh} \times D_{SC}/L_{SC}$

in which

$K_{SC/veh}$ = vehicle/*stratum corneum* partition coefficient (unitless)
D_{SC} = diffusivity of the penetrant in the *stratum corneum* in cm^2/h
L_{SC} = length of the diffusion pathway through the *stratum corneum* in cm

As by definition $K_{SC/veh} = C_{SC,\,sat} / C_{veh,\,sat}$, J_{max} is given by:

$J_{max} = C_{SC,\,sat} \times D_{SC}/L_{SC}$ **Equation 7-2 J_{max} determined by constants**

in which

$C_{SC,\,sat}$ = the saturation concentration of the substance in the *stratum corneum* in mg/cm^3

Since all the independent variables in Equation 7-2 are constants for a given chemical and skin preparation, the J_{max} of a chemical is the same for every vehicle. This is illustrated by

Guy (2010), who used the constancy of J_{max} (determined in aqueous solutions) to predict the absorption under infinite dose conditions of 14 fragrances from formulations in which they were (assumed to be) dissolved to saturation. With two exceptions, his predictions were within one order of magnitude of the experimentally derived values; most of them overestimating the experimental values (Figure 7-3). Therefore, extrapolation of dermal absorption of a chemical from one formulation to another on the basis of measured J_{max} and saturation concentration of the chemical in the formulation would seem a viable approach.

The pitfall of this approach is that vehicles indeed may change skin permeability and the solubility of the chemical in the skin, as pointed out in chapter 1 (section 1.1.5.1, pages 33 to 36) and as illustrated by the skin permeability changing corrosive substances discussed in section 7.3. Instead of enhancing absorption, some formulations may also limit absorption, e.g. when they contain aldehydes (as observed in chapters 4 and 5 of this thesis).

In short, there are various ways in which a vehicle may influence dermal absorption of chemicals and thus violate the condition under which the k_p derived for one vehicle can be extrapolated to another using J_{max} and its saturation concentration in the vehicle of interest. This perhaps explains why QSPRs developed to predict dermal absorption from different mixtures have not been very successful (section 1.1.4.6.2, page 25). Therefore, measuring dermal absorption of chemicals using the vehicles/formulations in which they will be exposed to the skin, is at present the only viable option, unless one can be certain the vehicles will not alter the skin's properties.

7.6 Prediction of dermal absorption for risk assessment

In chapter 6 of this thesis a Dermal Absorption Model for Extrapolation (DAME) was

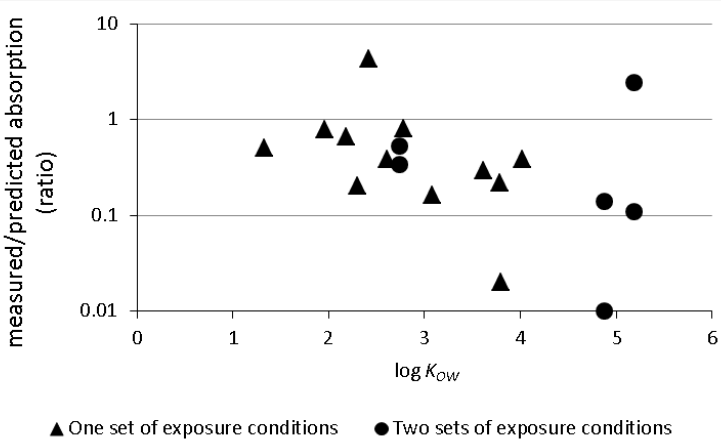


Figure 7-3 Ratios of measured and predicted absorption for 14 fragrances, plotted against log K_{OW}
Data derived from Guy (2010). Most fragrances were tested under one set of exposure conditions (that is a single combination of quantity, exposed area and exposure time) (triangles), while some were tested under two different sets of conditions (bullets). (Two bullets at the same log K_{OW} represent one fragrance)

introduced. DAME basically models the Franz diffusion cell (Figure 1-2, page 18) and predicts *in vitro* dermal absorption from aqueous solutions, defined as the sum of the amounts of a chemical encountered in the *epidermis* and the receptor fluid. The Franz cell dimensions represented in DAME are the donor cell volume, that is the amount of liquid applied to the skin, and the exposed skin area, set at 1 cm^2. Finite testing conditions typically concern volumes of up to 10 µL/cm^2 (OECD, 2004b), while a maximum capacity of the donor cell of 500-1000 µL/cm^2 usually represent infinite dose conditions. Chemical-specific inputs needed by DAME are the measured permeation constant (k_p), lag time and *stratum corneum*/water partition coefficient ($K_{SC,W}$). The permeation constant and lag time should be measured in an infinite dose *in vitro* absorption experiment. Usually, a measured $K_{SC,W}$ is not available, in which case the model uses a QSAR to calculate it. For this QSAR two additional chemical-specific parameters are needed: its MW and log K_{OW}.

In comparison with *in vivo* dermal absorption, *in vitro* dermal absorption experiments cost less time and money to perform, can be characterised by just a few parameters and may be executed with human skin, while in the EU human *in vivo* data, even if obtained in an ethically sound way, are not allowed to be used (EFSA, 2012). This means it is easier to construct and validate an *in silico* model for *in vitro* than for *in vivo* absorption via human skin. Furthermore, *in vitro* data correlate well with *in vivo* data (Godin and Touitou, 2007; Jakasa and Kezic, 2008), and the *in vitro* dermal absorption test with human skin is the experimental method preferred by the European Food Safety Authority (EFSA, 2012). Still, building *in silico* models on *in vitro* data introduces an additional uncertainty in the outcome, since in the end it is the *in vivo* situation one wants to assess. However, since in general, *in vitro* dermal absorption overestimates *in vivo* dermal absorption, one would err on the safe site, since a higher dermal absorption is assumed than will be reached *in vivo*.

Since the publication of our DAME model, presented in chapter 6 of the thesis, the US Centers for Disease Control and Prevention (CDC) have made the Finite Dose Skin Permeation (FDSP) model available on the internet[16]. This model was developed by Fedorowicz *et al.* (2011) and published in peer-reviewed literature (Dancik *et al.*, 2013; Kasting and Miller, 2006; Kasting *et al.*, 2008; Miller and Kasting, 2010; Wang *et al.*, 2007). Besides predicting skin permeation under finite conditions, this FDSP model also facilitates predictions based on measured permeation constants. According to calculations of Dancik *et al.* (2013), the k_p-predictions of the FDSP model highly correlate with those based on the Potts & Guy QSPR (Potts and Guy, 1992) with the Cleek & Bunge correction (Cleek and Bunge, 1993) (R^2 = 0.99). In comparison with DAME, the FDSP model has the advantage of taking evaporation into account and can therefore be applied to volatile compounds as well.

[16] http://www.cdc.gov/niosh/topics/skin/finiteSkinPermCalc.html

Table 7-3 Parameters used to calculate finite dose absorption for the database of Buist *et al.* (2010)

Name	Chemical formula	Number of rings	Number of double bonds	Water solubility (mg/L)	Water solubility at °C[1]	MW	Log K_{OW}	MP (°C)	BP (°C)	VP at 32 °C (mmHg)	pKa	k_p (cm/h) corrected[3])
Acetic acid	C2H4O2	0	1	1000000	25	60	0.17	16.6	117.9	2.52×10^{1}	4.76	0.0029
Benzoic acid	C7H6O2	1	4	3400	25	122	1.87	122.4	249.2	6.71×10^{-3}	4.2	0.0076
Bis(2-ethylhexyl) phthalate	C24H38O4	0	5	0.27	25	391	8.39[2]	-55	384	4.50×10^{-5}		0.101
Butoxyethanol	C6H14O2	0	0	100000	20	118	0.83	-74.8	168.4	8.33×10^{-1}	15.1	0.0036
Cortisone	C21H28O5	4	4	280	25	361	1.47	222	500.54	2.10×10^{-12}		0.00015
Decanol	C10H22O1	0	0	37	25	158	4.57	6.9	231.1	2.17×10^{-2}		0.0181
Diazinone	C12H21N2O3P1S1	1	4	40	25	304	3.86	87.58	125	1.20×10^{-4}		0.0091
2,4-Dichlorophenol	C6H4Cl2O1	1	3	4500	20	163	3.06	45	210	1.37×10^{-1}	7.85	0.032
Ethacrynic acid	C13H12Cl2O4	1	6	20.59	25	303	3.69	122.5	406.63	1.80×10^{-6}	2.5	0.00016
Linolenic acid	C18H30O2	0	4	0.124	25	278	7.3[2]	-16.5	231	2.80×10^{-5}	4.77	0.168
Octylparaben	C15H22O3	1	4	15	30	250	5.43	116.6	351.94	1.10×10^{-5}	8.3	0.0145
Oleic acid	C18H34O2	0	2	0.0115	25	282	7.73[2]	44	237.5	1.30×10^{-4}	5.02	0.184
Propylparaben	C10H12O3	1	4	500	25	180	3.04	97	285.14	7.60×10^{-4}	8.5	0.0143
Salicylic acid	C7H6O3	1	4	2240	25	138	2.24	158	211	8.40×10^{-5}	2.97	0.00093
Testosterone	C19H28O2	4	2	23.4	25	288	3.3	155	390.02	5.80×10^{-8}		0.0079

[1] Solubility at 32 °C is preferred (skin temperature), but was not easily obtainable.
[2] Log K_{OW} = 5.5 was used as the model does not accept higher values.
[3] See Buist *et al.* (2010)

To compare its performance with DAME, the FDSP model was applied to predict the results of the finite experiments reported in chapter 6, using the k_p's from our infinite dose experiments as input parameter instead of the k_p calculated by the FDSP model based on physicochemical parameters. Since these experiments were executed under occlusion, meaning the applied dose was coated with a cover preventing evaporation, the FDSP model was run with vapour pressure set to 0, to exclude evaporation as factor.

In general, the predictions by the FDSP model do not correlate well with the results of our experiments ($R^2 = 0.12$), and are outperformed by the DAME predictions ($R^2 = 0.64$) (Figure 7-4). The FDSP model does not use lag time, that is the time between the start of exposure and the first appearance of the permeating chemical in the receptor fluid, as an input parameter, while DAME uses measured lag time as input parameter. Dancik *et al.* (2013) noted the lag times predicted by FDSP, using Fick's second law and estimations of $K_{SC,W}$ (Wang *et al.*, 2007), may deviate substantially from the experimental values, and also our experimental lag times were not well predicted using a comparable method derived by Shah *et al.* (1994)(section 6.4.2). This may, at least in part, explain the better correlation obtained with DAME, which uses experimental lag times from infinite dose experiments. For a more definitive assessment of the merits of both models, more finite dose experiments should be evaluated applying both models. In addition, including the approach used by the FDSP model for volatile substances in DAME could be a next step forward.

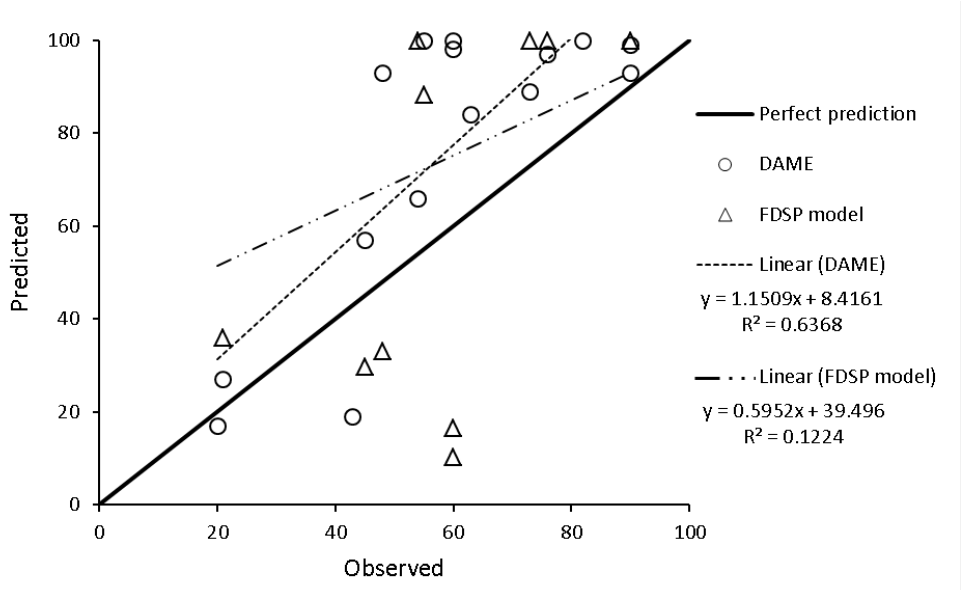


Figure 7-4 Prediction of finite dose results of Buist *et al.* (2010) using DAME and the FDSP model.
Potential absorption predicted by the FDSP model was obtained by summing the percentages predicted to be retained in the *stratum corneum* and the viable *epidermis* and the percentage predicted to be systemically absorbed. Line with short dashes: linear regression on the data calculated with DAME (taken from chapter 6 of this thesis). Line with long and short dashes: linear regression on the data computed with the FDSP model.

7.7 Application in regulatory risk assessment

Eventually the dermal absorption data discussed in this thesis are to be used in regulatory risk assessment of chemicals. In the final paragraphs of this discussion, it will be pointed out how the results obtained could be applied to refine the use of dermal absorption data in regulatory risk assessment. The EU has developed and published a tiered approach for such use (EC, 2004; Figure 7-5, page 158). This tiered approach was developed within the legal framework regarding the admittance of Plant Protection Products on the EU market, and focuses on dermal exposure of workers, comparing at each tier the internal exposure via the dermal route, calculated using an assumed or estimated relative absorption, with the systemic Acceptable Operator Exposure Level (AOEL). The AOEL is the maximum amount of active substance, expressed on a bodyweight basis, to which the operator may be exposed without any adverse health effects (CRD, 2013). When at a certain tier it is concluded that internal exposure via the dermal route is lower than the systemic AOEL, no further refinement of the evaluation is deemed necessary, since no adverse health effects are to be expected as a result of this dermal exposure. The higher the tier the more refined (and more costly) the assessment becomes. At each tier, the worst case (highest) dermal absorption is assumed given the available information.

At the first tier, it is simply assumed that 100% of the substance to be evaluated is absorbed. In principle, this may occur within the standard exposure time of six or twenty-four hours, under finite dose conditions. Some substances indeed approach this value (e.g. 2,4-dichlorophenol and propylparaben of which 90% is absorbed under finite dose conditions (Table 6-4, page 136)). In practice, e.g. in case of pesticides, this value will hardly be reached: Dewhurst *et al.* (2010) analysed all dermal absorption values reported for 64 pesticides evaluated in the EU, and found only one pesticide reaching a highest value of 70% (benthiavalicarb, when diluted); average relative absorption for in-use dilutions of pesticide formulations was 13 ± 14% and for the concentrated formulations it was even less (4.7 ± 6.1%). So there is sufficient room for refinement of this first crude tier.

In the second tier, a reduced relative absorption of 10% of the applied dose is assumed for chemicals with a MW >500 and a log K_{OW} <-1 or >4. All other molecules are still assumed to be 100% absorbed. This rule of thumb is rather coarse, and leads to classification of most pesticidal active substances in the 100% absorption category (62 out of 64 in the database published by Dewhurst *et al.* (2010)). Also absorption data obtained from the survey reported in chapter 2 clearly show this: for all substances considered in tier II to have a default dermal absorption of 100% the actual dermal absorption ranged from 1 to 78%, the average maximum absorption being 39 ± 24% (Table 7-4). Consequently, tier II constitutes only a small improvement over the first tier. Furthermore, the scientific basis for the selection of the cut off values is not clear (OECD, 2011).

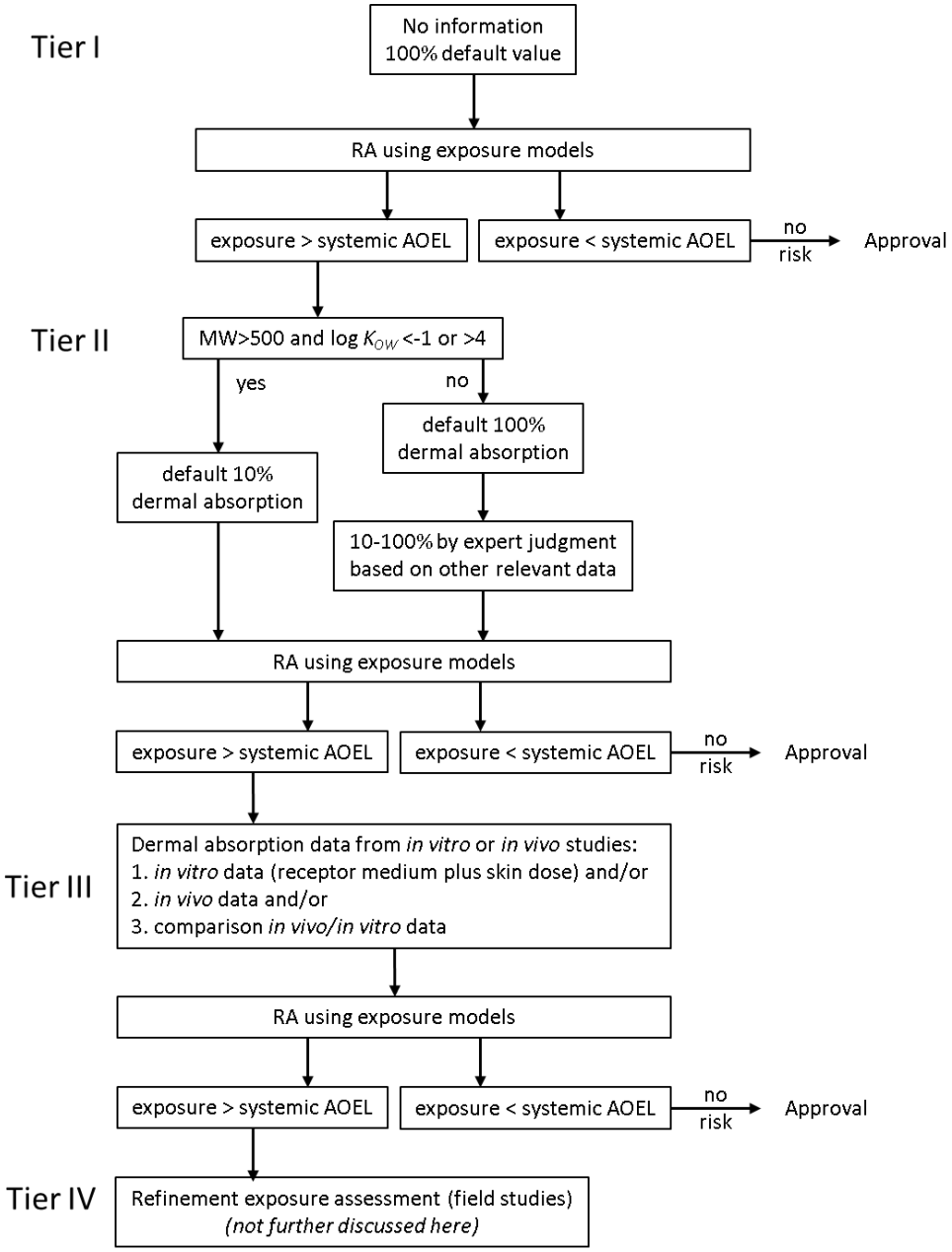


Figure 7-5 Tiered approach for dermal risk assessment (reproduced from EC, 2004)
AOEL = Acceptable Operator Exposure Level

Further refinement of tier II could be achieved by employing DAME, using the Potts & Guy QSPR predictions of k_p (Potts and Guy, 1992) as input, ignoring lag time and putting in

default parameters for *in vitro* finite dose dermal absorption conditions to generate different categories of default absorption values based on the log K_{OW} and MW of chemicals (Figure 7-6, page 160). The reason to ignore lag time is that it cannot be well predicted (section 7.6, page 153). The introduction of this worst case assumption (that absorption starts immediately at t = 0) is offset by also ignoring a potential skin reservoir. The output of this adapted DAME could then be used to classify the chemical in a default absorption category of, e.g., 10, 25, 50, 75 or 100% (Figure 7-6). Currently this model is being tested and fine-tuned using a number of different datasets (Buist *et al.*, in preparation).

For tier III of the approach depicted in Figure 7-5, it is advised in current regulatory practice to perform dermal absorption experiments reflecting actual exposure conditions (e.g. with respect to dermal loading, chemical concentration and exposure time) (EFSA, 2012; OECD, 2004a). When many different exposure conditions are to be tested, either substantially higher costs are incurred due to an increased number of experiments to be performed, or one has to restrict oneself to the (finite dose) conditions expected to lead to the highest percentage absorption, as suggested by EFSA (2012), thus accepting an overestimation of absorption for other exposure conditions. As has been demonstrated in chapter 2, different conditions of dermal loading may cause substantial differences in relative absorption. Also differences in exposure time will lead to differences in relative absorption (Figure 1-5, page

Table 7-4 Actual dermal absorption percentages for a number of substances with default dermal absorption of 100%

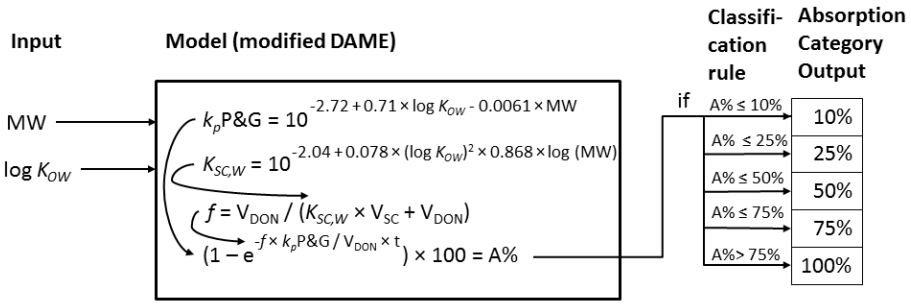
Substance	MW	Log K_{OW}	Dermal absorption range (%)	Species
4,4'-methylenedianiline (MDA)	198	1.59	14 - 54	rat
cyclohexane	84	3.44	4 - 60	rat
methyl-*t*-butyl ether	88	0.94	16 - 34	rat
di(2-ethylhexyl) phthalate (DEHP)	391	7.6	39 - 47	guinea pig
pyrene	202	4.88	41 - 70	guinea pig
benzo[*a*]pyrene	252	6.13	40 - 67	guinea pig
diethylene glycol butyl ether	162	0.56	6 - 65	rat
testosterone	288	3.32	1 - 18	monkey
benzoic acid	122	1.87	17 - 34	monkey
testosterone	288	3.32	3 - 12	man
hydrocortisone	362	1.61	1 - 2	man
benzoic acid	122	1.87	14 - 37	man
methoxyethanol	76	-0.77	19 - 27	rat
ethoxyethanol	90	-0.32	17 - 27	rat
butoxyethanol	118	0.83	21 - 26	rat
fluazifop-butyl	383	4.5	2 - 8	man
fluazifop-butyl	383	4.5	40 - 74	rat
propoxur	209	1.52	27 - 71	man
lindane	291	3.72	5 - 28	rat
cyromazine	166	-0.155	7 - 11	rat
bentazone	240	2.34	1 - 2	rat
trinexapac-ethyl	252	-0.38	48 - 78	rat

Data derived from Table 2-1 in chapter 2. Percentages rounded off to the nearest integer. Differences between minimum and maximum absorption are caused by differences in dermal loading.

39). As an alternative, one could perform an *in vitro* infinite dose dermal absorption experiment with human skin in order to determine k_p and lag time of the chemical. Subsequently, for each of the different exposure conditions, one may calculate potential absorption using DAME. The model can handle finite as well as infinite dose conditions: for the latter one simply has to increase the donor cell volume in accordance with the expected exposure volume.

When all possible refinements of the assessment of dermal absorption are exhausted and still the estimated internal level of the skin chemical is higher than the systemic AOEL, external exposure assessment can be refined (tier IV). A variety of measurement strategies and mathematical models can be used (see WHO, 2013 for a comprehensive review), but that discussion is outside the scope of this thesis, as the aim of external exposure assessment is purely to establish dermal loading, without considering the possibility of absorption.

In 2012, EFSA updated the tiered approach for dermal risk assessment of pesticides (Figure 7-7). Tier I was eliminated and the approach for molecules with a MW <500 and/or a log K_{OW} between -1 and 4 was changed. In this changed approach for concentrates (defined as formulations with a concentration of active substance >5%), dermal absorption is now assumed to be 25% while for dilutions (defined as (in-use) dilutions with a concentration of active substance ≤5%), a default of 75% was set. These modifications are based on the observation made by Dewhurst *et al.* (2010) that for the pesticides evaluated by the EU no concentrate and no in-use dilution measured exceeded their respective cut-off value for



A% = absorption relative to applied dose expressed in %
f = fraction of unpenetrated chemical still in donor cell volume
k_pP&G = permeation constant, calculated using the Potts & Guy QSPR (Potts and Guy 1992)
$K_{SC,W}$ = partition coefficient *stratum corneum*/water, calculated using the QSPR of Hui *et al.* (1995)
log K_{OW} = partition coefficient octanol/water of chemical
MW = molecular weight of chemical
t = exposure time = 8 h
V_{DON} = donor cell volume = 0.01 mL ("finite dose volume")
V_{SC} = volume of 1 cm^2 *stratum corneum* = 0.002 mL (Xiao and Imhof 1997)

Figure 7-6 Establishing different dermal absorption classes for finite dose, based on MW and Log K_{OW}

dermal absorption. For concentrated pesticides, this is a sizeable improvement compared to the old tier II default values as it considerably reduces the overestimation of their

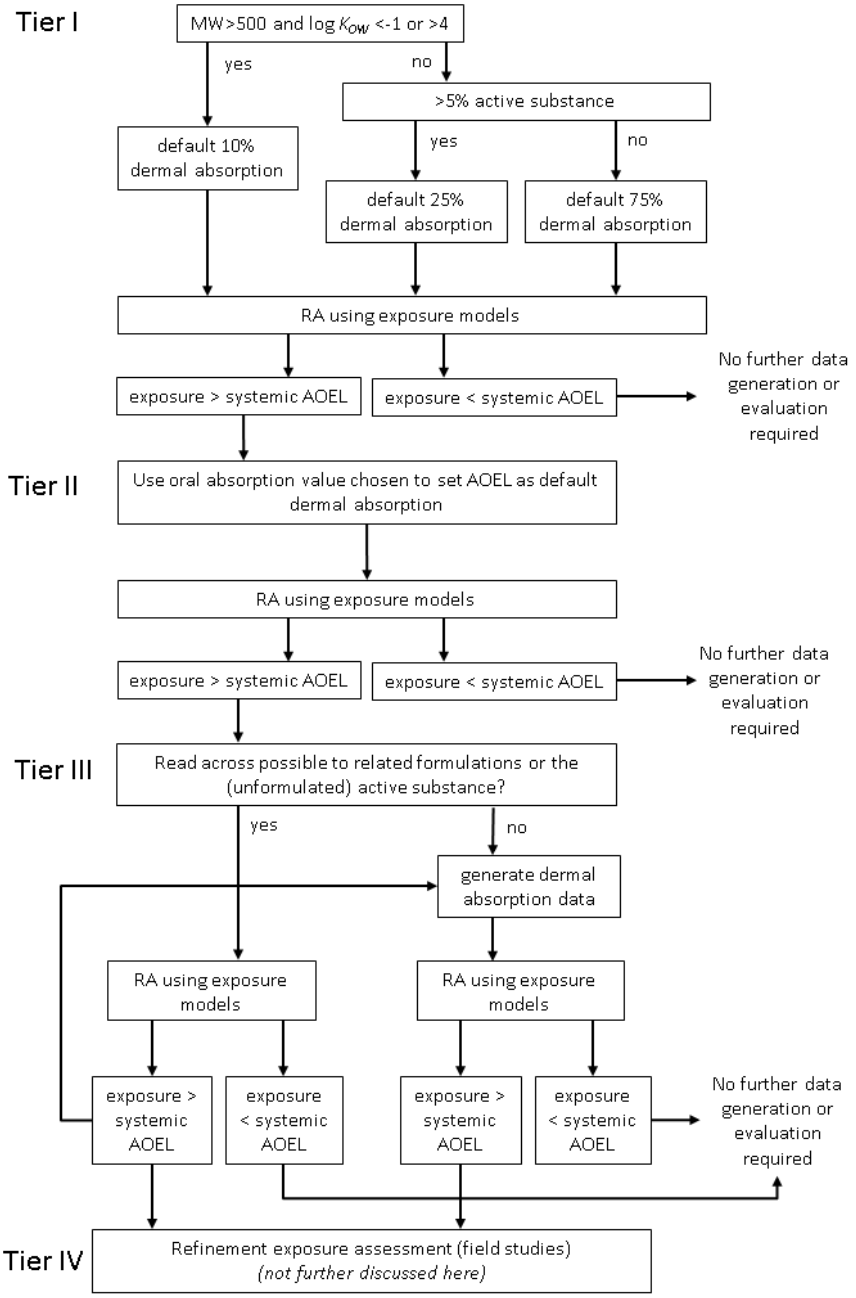


Figure 7-7 Current EFSA tiered approach for dermal risk assessment (adapted from EFSA 2012b)
AOEL = Acceptable Operator Exposure Level. Active substance is the pesticidal chemical present in the pesticide formulation.

absorption, which in the old tier II was set at 100%, but for in-use dilutions the improvement is marginal. Tier III is now explicitly expanded with the possibility to read across to similar formulations containing the active substance or even “unformulated” active substance (that is contained in a simple vehicle like water), instead of immediately having to generate dermal absorption data.

7.8 Future perspectives

In this section possible future developments on the main issues discussed in this thesis are described.

7.8.1 Dermal loading and relative absorption

The issue of the influence of dermal loading on relative absorption can be addressed by using the Dermal Absorption Model for Extrapolation (DAME) to extrapolate from the conditions under which absorption was measured to those under which consumers or workers are expected to be exposed, provided measured k_p and lag time are available. As pointed out in section 7.6 of this thesis, before employing DAME for such extrapolations, its predictive performance needs to be evaluated more elaborately (section 7.8.5).

7.8.2 Skin corrosive/irritant substances

Data on skin corrosion/irritation are included in the basic data requirements for chemicals falling under REACH (ECHA, 2012), as well as for pesticides (EU, 2013), which are evaluated by the European Food Safety Authority (EFSA). While under REACH toxicokinetic studies, like dermal absorption studies, are not mandatory at any production/importation level, for pesticides dermal absorption studies are mandatory. Since more data on the effects of skin corrosive/irritant substances are needed to obtain a more general picture on the influence of single and repeated exposure to this kind of substances (section 7.3 of this thesis), especially publicly available risk assessments of pesticides could be a data source. However, whether a substance will exert corrosive or irritant effects is highly dependent upon its concentration, while regulatory studies are not aimed at establishing a dose-response curve, but just at categorising, and liquids and solids are applied in their pure form in these studies (OECD, 2015). Therefore, additional applied research aimed at establishing the relation between the dose-response of skin corrosive/irritant effects and skin permeability after single and repeated exposure will probably be needed to complete the picture.

7.8.3 Repeated dermal exposure

The approach to include the amount retained in the skin (skin reservoir) in the dermally absorbed dose obtained from single exposure *in vitro* experiments in order to compensate for the cumulative effect of repeated dermal exposure, needs to be analysed for a more consistent and complete dataset before it can be applied with confidence in risk assessment (section 7.4 of this thesis). Special attention needs to be given to chemicals showing a low ratio of skin reservoir versus amount recovered from the receptor fluid (representing

systemic absorption). Starting point for such an evaluation could be the *in vivo* data on dermal absorption after repeated dosing listed in Table 3-1 (page 80). Subsequently single exposure *in vitro* absorption data for the listed substances could be collected from public literature or experimentally generated, so that it can be checked whether combining skin reservoir and amount recovered from the receptor fluid would provide an adequate prediction of *in vivo* dermal absorption in the context of regulatory risk assessment.

Since at the end of the day, the effect of repeated daily dermal exposure on daily absorption will depend upon the net result of absorption, distribution, metabolism and excretion, prediction of absorption using a generic Physiology Based Pharmacokinetic (PBPK) model that can be run with a few, easily obtainable physicochemical parameters would be an important next step. A recent example of a generic PBPK model is the IndusChemFate model developed by Jongeneelen and ten Berge (2011). The model addresses the three main routes of exposure: the oral, dermal and respiratory routes, is implemented in MS-Excel™ and is freely available from the website of the European Chemical Industry Council (CEFIC)[17]. Currently, its output is limited to the time courses of alveolar air, blood and urine concentrations of the parent compound and possible metabolites, but since the datasheets and Visual Basic source code are not password-protected, it can be easily adapted to provide e.g. blood AUC's as a measure of absorption. Furthermore, in IndusChemFate dermal absorption is calculated using a modified version of the QSPRs developed by ten Berge (2009) and Wilschut *et al.* (1995) (see also Table 1-1). In principle, these predictions can be substituted by measured k_p values by adapting the Visual Basic code, so that substance-specific data are used, reducing uncertainty.

7.8.4 Vehicles and formulations

A vehicle is the solvent in which the chemical to be absorbed is applied to the skin, while a formulation is a more complex vehicle consisting of a chemical mixture in which an active pharmacological, biocidal or pesticidal substance is applied. In the text below, formulations are included when the term "vehicle" is used.

Measuring dermal absorption of chemicals using the vehicles in which they will be exposed to the skin, is at present the only reliable option, unless one can be certain the vehicles will not alter the skin's barrier properties (section 7.5 of this thesis). As a first approach, one could assume that vehicles not classified for skin corrosion or irritancy will not alter the barrier properties of the skin, and hence dermal absorption of active substances from these vehicles can be extrapolated from e.g. experiments with water as a vehicle or another non-irritant vehicle. This approach could be initially evaluated using data on pesticides, since pesticide regulatory dossiers will contain both data on vehicle skin corrosion/irritation and

[17] http://cefic-lri.org/lri_toolbox/induschemfate/

dermal absorption data on undiluted and diluted vehicles. Pesticide risk assessment reports containing summaries of all submitted studies are available at the EFSA website[18].

Another approach could be to test the influence of the vehicle on skin permeability by using radiolabelled model substances like tritiated water, as has been reported in chapter 4 of this thesis. If these tests show that the vehicle does not influence skin permeability, extrapolation of dermal absorption values is a possibility. One could, of course, opt to use the radiolabelled active substance to begin with, but producing radiolabelled chemicals on demand is costly, while radiolabelled model substances will be available on the commercial market at lower costs.

As indicated in section 7.5, at present modelling approaches predicting dermal absorption of chemicals from different vehicles have not been very successful so far. In view of the great variability in formulations with respect to the nature and concentrations of ingredients and solvents and the many different ways formulations may influence permeability and absorption (e.g. solvent drag, damaging the barrier by delipidation or dehydration, protein crosslinking), only developing very local models (that are valid for a limited group of similar formulations) is likely to offer some perspective, but the development of more universally valid, global models for prediction of dermal absorption from different vehicles would require too many data to be feasible, at least in the short run.

To conclude, future development of efficient, animal-free, dermal toxicological risk assessment of vehicles and active substances can expected to be spearheaded by the cosmetics industry, since the EU has implemented a ban on marketing finished cosmetic products and ingredients that have been tested on animals[19].

7.8.5 Prediction of dermal absorption

For a more definitive assessment of the merits of DAME, more finite dose experiments should be evaluated applying the model (section 7.6 of this thesis). For such an evaluation, the on-line available EDETOX-database[20] could give access to relevant data. Unfortunately, one has to revert to the original papers from which the *in vitro* absorption data were extracted, since the database does not list some of the variables needed by DAME, e.g. concentration of the chemical to be tested and the volume applied in the donor cell.

In future, the present QSPRs for k_p could be replaced by models with better predictive qualities. When that is the case, DAME may use predicted instead of measured k_p values. Based on the overview presented in section 1.1.4.6.2 (pages 23-29), it can be concluded that besides log K_{OW} and MW, the physical parameters that are used in most of the presented QSPRs, an additional predictive independent variable is needed which describes the

[18] http://dar.efsa.europa.eu/dar-web/provision
[19] http://ec.europa.eu/growth/sectors/cosmetics/animal-testing/index_en.htm
[20] http://edetox.ncl.ac.uk

chemical reactivity of the substance, e.g. hydrogen bonding (Geinoz *et al.*, 2004; Moss and Cronin, 2002). Furthermore, the k_p model to be developed should be non-linear with respect to the relationships between k_p and the predictive independent variables, since non-linear models seem to outperform the traditionally developed linear models (Moss *et al.*, 2011; Russell and Guy, 2009). A disadvantage of non-linear models is that more data are needed to develop them than for linear models, and that they are more prone to overfitting. Therefore, existing dermal absorption datasets, both public and private, should be combined, curated and filtered to build a large homogeneous and consistent dataset that can be used to develop such non-linear models to predict k_p. The on-line available EDETOX-database[21] provides a good structure to gather these data and already a fair number of curated k_p data.

7.9 General conclusions

The influence of vehicles on absorption and the impact of irritative or corrosive vehicles or chemicals on the skin barrier have been demonstrated in this thesis. Until to date the significance and magnitude of these effects can only be experimentally established. The impact of vehicles and of corrosive of irritative vehicles or chemicals on absorption appears to be modest judging by the results presented in this thesis. However, more data on the effects of skin corrosive/irritant chemicals and formulations are needed to obtain a more general picture on their influence on dermal absorption after single or repeated exposure.

Expressing dermal absorption as a single percentage is a misleading practice as it suggests universal validity, although under different conditions relative absorption of the same chemical may be considerably higher or lower, as demonstrated in chapter 2 of this thesis.

An *in silico* model called DAME was developed that is able to account for a number of these different conditions, based on the measured k_p, the measured lag time, the MW and the log K_{OW} of the chemical. DAME enables the user to evaluate a variety of dermal exposure scenarios with limited experimental data and easy to obtain physico-chemical properties. At present, the applicability of DAME is restricted to non-volatile substances dissolved in aqueous solvents that do not affect the barrier function of the skin.

Altogether, it is concluded that dermal exposure can be an important factor in risks posed by chemicals and should be taken into account in risk assessment. The methods to actually do this are still open for further improvement to better account for the various factors influencing dermal absorption and to develop adequate combinations of *in vitro* and *in silico* models that can accurately predict human systemic exposure.

[21] http://edetox.ncl.ac.uk

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8 Summary

In *chapter 1* of this thesis, the background of the research reported in chapters 2 to 6 is presented and the research objectives are set out.

The role of dermal absorption in toxicological risk assessment is relevant because dermal contact can be an important exposure route, as people are exposed to a variety of substances and products via the skin, either directly or indirectly, while at work, at home or in public space. Under occupational conditions, dermal exposure occurs mainly as a result of splashes, spills or drifts, during the application itself or from contact with contaminated surfaces. Pesticides, organic solvents and metalworking fluids are seen to be important contributors to adverse health effects due to occupational exposure via the dermal route. In non-occupational settings, cosmetics, clothing and household products are the most relevant commodities with respect to dermal exposure, because of their conditions of use.

In the context of dermal exposure, three different types of toxicological effects can be distinguished: local skin effects, skin sensitization and systemic effects. This thesis is centred around the process of dermal absorption, which is principally relevant for the assessment of systemic toxicological effects, since local effects do not require absorption and the importance of absorption (or rather penetration into the viable *dermis*) for skin sensitization appears to be limited.

Given the possible importance of the dermal exposure route in toxicological risk assessment, the state-of-the-art of models to estimate dermal absorption and the many factors influencing the efficiency of this exposure route, the objective of this thesis was to further develop, evaluate and improve methods for including dermal absorption data in toxicological risk assessment.

In this thesis, four factors influencing dermal absorption, namely dermal loading (chapters 3 and 6), irritative/corrosive potential (chapters 3 and 4), frequency of exposure (chapters 3, 4 and 5) and the vehicle used (chapter 5), were investigated in more detail. Furthermore, a model to extrapolate infinite dose absorption data to finite dose conditions, baptized Dermal Absorption Model for Extrapolation (DAME), was developed and tested.

In *chapter 2* of this thesis, the relationship between relative dermal absorption and dermal loading was investigated. Hundred-and-thirty-eight dermal publicly available absorption experiments with 98 substances were evaluated. The results obtained revealed that dermal loading ranged mostly between 0.001 and 10 mg/cm^2. In 87 experiments (63%), an inverse relationship was observed between relative dermal absorption and dermal loading. On average, relative absorption at high dermal loading was 33 times lower than at low dermal loading. Known skin irritating and volatile substances less frequently showed an inverse relationship between dermal loading and relative absorption. It was concluded that when using relative dermal absorption in regulatory risk assessment, its value should be determined at or extrapolated to dermal loadings relevant for the exposure conditions being evaluated.

In *chapter 3* of this thesis, a literature search was presented with the aim to investigate whether neglecting the effects of repeated exposure may lead to an incorrect estimate of dermal absorption. The results demonstrated that the effect of repeated versus single exposure does not demonstrate a unique trend. Nevertheless, an increase in daily absorption was frequently observed upon repeated daily exposure. The little information available mostly concerned pharmaceuticals. However, consumers and workers may be repeatedly exposed to other types of chemicals, like disinfectants and cleaning products, which often contain biocidal active substances that may decrease the barrier function of the skin, especially after repeated exposure. These biocidal products, therefore, may present a safety risk that is not covered by the current risk assessment practice since absorption data are usually obtained by single exposure experiments. Consequently, it was decided to investigate the importance of this issue for biocide safety evaluation. As the literature search revealed that hardly any data on absorption upon repeated dermal exposure to biocides are available, it was concluded that data need to be generated by testing.

To cover the entire range of biocidal products in such testing, a representative series of biocidal substances should be tested, making *in vitro* testing of dermal absorption the preferred choice over *in vivo* testing. Based on an inventory made, it appeared that the 16 product types represented among the biocidal products authorised in the Netherlands could be clustered into 6 more or less homogeneous categories based on similarity in active substances. This result could facilitate experimental testing by providing a basis for selection of a limited number of representative compounds to be evaluated.

In *chapter 4* of this thesis, the importance of the effect of repeated dermal exposure on skin permeability for biocide safety evaluation was investigated, using a selection of nine representative biocides from the inventory made in chapter 3. The *in vitro* dermal penetration of tritiated water and [^{14}C]propoxur was chosen as a measure of the permeability and integrity of human abdominal skin after single and repeated exposure. The results indicated that single and repeated exposure to specific biocidal products (e.g. the quaternary ammonium chlorides DDAC and ADBAC) may significantly increase skin permeability, especially when the compounds are applied at high concentrations, while a substance like formaldehyde may reduce skin permeability under specific conditions.

In *chapter 5* of this thesis, the *in vitro* dermal absorption kinetics of the quaternary ammonium compound didecyldimethylammonium chloride (DDAC) during single and repeated exposure was studied in more detail. In addition, the influence of biocidal formulations on the absorption of DDAC was investigated, because it was expected that formulation characteristics may be another factor influencing its dermal absorption. The analysis of biocidal products on the Dutch market, reported in chapter 3, indicated that DDAC is often used in combination with other active ingredients. DDAC was most frequently combined with formaldehyde, glutaraldehyde and/or alkyldimethylbenzylammonium chloride (ADBAC). Consequently, commercial formulations containing one or more of these

additional active ingredients were selected, in addition to one formulation containing only DDAC as an active ingredient. The selected commercial formulations tended to reduce skin penetration of DDAC. This was most pronounced with the formulation containing the highest concentration of formaldehyde (196 mg/mL) and glutaraldehyde (106 mg/mL), which reduced the flux of DDAC across the skin by 95%. The reduction caused by the only tested formulation containing no other active ingredients than DDAC, and thus incorporating no aldehydes, was smallest, and did not reach statistical significance.

In *chapter 6* of this thesis, a simple *in silico* model to predict finite dose dermal absorption from infinite dose data (k_p and lag time) and the stratum corneum/water partition coefficient ($K_{SC,W}$) was developed. This model was tentatively called **D**ermal Absorption Model for Extrapolation (DAME). As dermal exposure may occur under a large variety of conditions leading to quite different rates of absorption, such a predictive model using simple experimental or physicochemical inputs provides a cost-effective means to estimate dermal absorption under different conditions.

To evaluate the DAME, a series of *in vitro* dermal absorption experiments was performed under both infinite and finite dose conditions using a variety of different substances. The k_p's and lag times determined in the infinite dose experiments were entered into DAME to predict relative dermal absorption value under finite dose conditions. For six substances, the predicted relative dermal absorption under finite dose conditions was not statistically different from the measured value. For all other substances, measured absorption was overpredicted by DAME, but most of the overpredicted values were still lower than 100%, the European default absorption value for the tested compounds.

In conclusion, our finite dose prediction model (DAME) provides a useful and cost-effective estimate of *in vitro* dermal absorption, to be used in risk assessment for non-volatile substances dissolved in water at non-irritating concentrations.

In *chapter 7* of this thesis, the results of the research reported in chapters 2 to 6 were put into perspective, the pitfalls and promises emanating from them discussed and general conclusions drawn. The possible influence of vehicles on absorption and the possible impact of irritative or corrosive vehicles or chemicals on the skin barrier have been demonstrated in this thesis. An *in silico* predictive model tentatively called DAME was developed, which enables the user to evaluate a variety of dermal exposure scenarios with limited experimental data (k_p and lag time) and easy to obtain physicochemical properties (MW and log K_{OW}). The predictions of our experiments reported in chapter 6 were compared to those of the Finite Dose Skin Permeation (FDSP) model published on the internet by the US Centers for Disease Control and Prevention (CDC). DAME outperformed FDSP (R^2 of the correlation predicted/measured potential absorption 0.64 and 0.12, respectively). At present, the applicability domain of DAME is limited to non-volatile substances dissolved in

aqueous solvents. However, in future the model will be adapted to include volatile substances as well.

Altogether, it is concluded that dermal exposure can be an important factor in risks posed by chemicals and should be taken into account in risk assessment. The methods to actually do this are still open for further improvement to better account for the various factors influencing skin penetration and to develop adequate combinations of *in vitro* and *in silico* models that can accurately predict human dermal absorption.

9 Glossary and abbreviations

In this thesis the definitions and abbreviations as applied by the OECD (2011, 2004a, 2004b, 2004c) and the WHO (2006) have been used as much as feasible.

9.1 Glossary

absorption decrease factor	the relative dermal absorption at low dermal loading divided by the relative dermal absorption at high dermal loading
allergen	a molecule that may elicit an allergic reaction, that is a disproportionate immune reaction
amphipathic	having a hydrophobic and a hydrophilic part (said of molecules)
apolar	not having separate positive and negative (partial) charges
appendageal route	dermal absorption route via skin appendages like hair follicles and sweat glands
applied dose	mass of permeant applied to the skin
arthropodicide	biocidal product designed to kill arthropods (animals with jointed appendages like insects and spiders)
Bakta Steril	a commercial biocidal product
biocidal product	product used to protect humans, animals, materials or articles against harmful organisms like pests or bacteria (definition of biocidal product according to EU Regulation 528/2012)
biocide	chemical used to kill organisms
charge	as a molecular descriptor: the sum of the absolute values of the partial charges in a molecule (calculation method described in Pugh *et al.* (2000)
corneocytes	cornified keratinocytes of the *stratum corneum*
corrosive	to skin: capable of full-thickness destruction of skin tissue
Dalton	unit of molecular weight
default absorption values	absorption values used in absence of measured or predicted data
dendritic cell	immune cell that presents antigens
dermal absorption	transport of chemicals from the outer surface of the skin into the systemic circulation

dermal absorption value	in *in vitro* dermal absorption experiments: the amount of substance that has penetrated the skin plus the amount still present in the skin preparation
dermal load(ing)	amount of applied dose per unit skin surface (e.g. expressed in mg/cm^2)
dermatokinetics	the processes by which permeants move through the skin
dermatomed skin	skin preparation from which the lower part of the *dermis* has been removed with a dermatome (an instrument to finely and precisely cut skin)
dermis	the lowest layer of the skin, containing a.o. the skin capillary network
descriptor	in this thesis: short for molecular descriptor = a specific characteristic of molecular structures, which can be expressed in a numeric value
diffusion	movement of molecules down a concentration gradient
donor compartment	compartment of a diffusion cell to which the permeant is added in an *in vitro* dermal absorption experiment
elicitation phase	in skin sensitisation experiments: the phase during which a possibly sensitised individual is challenged with the sensitizer in order to elicit an immune reaction of the skin
epidermal membrane	skin preparation consisting only of the *epidermis*
epidermis	upper layer of the skin, consisting of a viable part and the non-viable *stratum corneum*
esterase	an enzyme capable hydrolysing an ester bond (a bond originating from the condensation reaction between an alcoholic hydroxyl group and an acid hydroxylic group)
facilitated diffusion	movement of molecules down a concentration gradient aided by a transporter protein
finite dose	applied amount of donor solution for which the concentration of the penetrant significantly decreases during dermal exposure
flow-through cell	apparatus to measure dermal absorption in which the receptor fluid passes continuously below the skin preparation
flux	amount of penetrant that crosses the skin per unit of time and unit of exposed skin area, often expressed in $mg/cm^2/h$

formulation	chemical mixture in which an active pharmacological, biocidal or pesticidal substance is contained
Franz cell	apparatus to measure dermal absorption in which a donor cell is separated from a receptor cell by the skin preparation to be investigated
full-thickness skin	skin preparation consisting of the *epidermis* and the entire *dermis*
haptenation	the linking of (part of) an allergen to an immune protein, turning it capable of eliciting an immune response
Henry's law constant	physical constant of a molecule describing the relation between its partial gas pressure and its solubility in water
heteroscedastic	when applied to a statistical test: comparison of two groups of measurements with different variabilities
homologous series	series of chemically similar molecules which are obtained by repeatedly adding an identical chemical group to the molecular chain (e.g. alkanoic acids)
homoscedastic	when applied to a statistical test: comparison of two groups of measurements with similar variabilities
hydrophilic	preferably dissolving in water
in silico	calculated or predicted by computer (analogous to *in vitro* and *in vivo*)
in vitro	measured/executed in labware
in vivo	measured/executed in/with living organisms
induction	in skin sensitisation experiments: the phase during which a non-sensitised individual exposed to a possible sensitizer in order to try to make him immunoresponsive to this molecule
infinite dose	applied amount of donor solution for which the concentration of the penetrant remains (more or less) constant during dermal exposure
intercellular route	in dermal absorption: passage of the permeant between the cells of the skin (also called paracellular)
irritating	to skin: causing significant inflammation of the skin
keratin	structural protein produced by the keratinocytes
keratinocytes	cells that constitute the *epidermis*

kinetics	in physiology: the process in the body by which a molecule is absorbed, distributed, metabolised and excreted
lag time	the time intercept for penetration equal to zero of the linear portion of the cumulative penetration versus time graph (see Figure 6-2)
Langerhans cell	dendritic cell (=immune cell that presents antigens) of the skin
limit value	regulatory exposure value which should not be exceeded
lipophilic	preferably dissolving in lipids
log K_{OW}	10-base logarithm of the octanol/water partition coefficient, also known as log P or log K_{OW}
melanocytes	pigment producing cells of the skin
microdialysis	miniature dialysis instrument which is inserted below the skin surface on which the permeant is applied, to measure dermal absorption *in vivo*
mitotic cell division	cell division in which the daughter cells maintain the original (diploid) number of chromosomes
MS Macrodes	a commercial biocidal product
Mulliken charges	Mulliken charges arise from the Mulliken population analysis and provide a means of estimating partial atomic charges from calculations carried out by computational chemistry packages like Gaussian 03.
non-occluded	dermal exposure in which the skin is not covered, or covered in such a way that evaporation is not prevented
occluded	dermal exposure in which the skin is covered in such a way that evaporation is prevented
passive diffusion	unaided movement of molecules down a concentration gradient
penetration	the entry of a substance in a particular layer or structure
percutaneous absorption	transport of chemicals from the outer surface of the skin into the systemic circulation
permeability coefficient	constant describing the permeability of a specific skin preparation for a specific molecule dissolved in water, usually expressed in cm/ h

permeant	chemical that penetrates through one layer into a second, functionally and structurally different, layer
permeation	penetration through one layer into a second, functionally and structurally different, layer
pesticide	biocidal product used to protect plants against harmful organisms (pesticides are not encompassed by the definition of biocidal products under EU Regulation 528/2012)
phase I enzymes	enzymes of the first phase of detoxification, in which toxicants are made more polar
phase II enzymes	enzymes of the first phase of detoxification, in which toxicants are conjugated to endogenous molecules, facilitating their excretion
polarizability	a molecular descriptor: ratio of the induced dipole moment of a molecule and the electrical field that produces it (a measure of the degree to which electrical charges in a molecule may be separated)
portal of entry	the site where a molecule enters the body (e.g. lungs, intestines, skin)
potential absorption	in dermal absorption: the sum of the amount of permeant that has entered systemic circulation (*in vivo*) or the receptor fluid (*in vitro*) and the amount remaining in the skin after exposure has ceased.
receptor compartment	part of the diffusion cell positioned on the inside of the skin preparation, filled with liquid in which the investigated chemical penetrates if the skin is permeable to it (and a concentration gradient is maintained across the skin).
receptor compartment	compartment of a diffusion cell to which the permeant is transported in an *in vitro* dermal absorption experiment
refractive index	ratio between the speed of light in a pure substance and the speed of light in vacuum
rodenticide	biocidal product designed to kill rodents
Roloxid 50	a commercial biocidal product
semi-occluded	dermal exposure in which the skin is covered in such a way that evaporation is only partially prevented
semipermeable membrane	a membrane that is permeable to a limited number of smaller molecules (e.g. water)

skin absorption	transport of chemicals from the outer surface of the skin into the systemic circulation
skin reservoir	amount of penetrant that remains in the skin after exposure has finished and in principle still may be systemically absorbed
skin reservoir	the amount of permeant present in the skin after cessation of exposure
skin sensitization	the process by which the skin is rendered sensitive to a skin allergen
slimicide	biocidal product designed to kill micro-organisms that produce slime
solvatochromic properties	molecular properties linked to its changing colour in solvents of different polarity
split-thickness skin	skin preparation from which the lower part of the *dermis* has been removed with a dermatome (an instrument to finely and precisely cut skin)
static diffusion cell	apparatus to determine *in vitro* dermal absorption from which the liquid in the receptor compartment is not continuously refreshed, but only after specific time intervals
static Franz cell	diffusion cell to measure dermal absorption in which the receptor fluid remains immobile
steady state	in *in vitro* dermal absorption experiments: conditions under which penetration into the receptor fluid occurs at a constant rate
Strat-M™	a commercial synthetic membrane used to imitate human skin, consisting of two layers of polyethersulfone and one layer of polyolefin
stratum corneum	non-viable upper layer of the skin
stratum germinativum	the germinal layer of the *epidermis*, it is its innermost layer
stratum granulosum	epidermal layer with a granular appearance, located just below the *stratum corneum*
stratum spinosum	epidermal layer with a spiny appearance, located just above the *stratum germinativum*
surfactant	surface active substance, that is a substance that when dissolved in water will reduce its surface tension

systemic absorption	uptake of a chemical into systemic circulation after having been transported across an outer body surface (e.g. skin, lung epithelium)
systemic effects	effects that occur if and when substances enter systemic circulation
Teepol	a commercial detergent
transcellular route	in dermal absorption: passage of the permeant across the cells of the skin
trough plasma concentration	plasma concentrations at the dip in the plasma concentration time curve of a periodically administered drug
t-statistic	statistical measure of the deviation of a measured parameter value from an expected of predicted value, in relation to the standard error of the measured value
unoccluded	dermal exposure in which the skin is not covered or covered in such a way that evaporation is not prevented
vehicle	solvent in which the permeant is administered
xenobiotic	a chemical normally not present in a specific species in appreciable concentrations (literal meaning: “strange to life”)

9.2 Abbreviations

ABSQon	a molecular descriptor: the sum of absolute charges on the oxygen and nitrogen atoms in a molecule
ADBAC	AlkylDimethylBenzylAmmonium Chloride
ANOVA	ANalysis Of VAriance
AOEL	Acceptable Operator Exposure Level
AUC	Area Under the Curve
B_R	Number of rotable bonds
BSA	Bovine Serum Albumin
cb	the number of carbons not involved in a C= O bond
CD-ROM	Compact Disk - Read-Only Memory
C_{sat}	Concentration in water at saturation (expressed in mg/cm^3)
CTGB	Dutch Board for the Authorisation of Plant Protection Products and Biocides
CV	Coefficient of Variation
DAME	Dermal Absorption Model for Extrapolation

DDAC	DidecylDimethylAmmonium Chloride
DMSO	DiMethyl SulfOxide
D_{ow}	distribution coefficient or apparent partition coefficient of ionogenic molecules
D_{SC}	diffusivity of the penetrant in the *stratum corneum* in cm^2/h
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EU	European Union
f	amount of penetrant in the donor compartment divided by the sum of the amounts in the donor compartment and the *stratum corneum*
FDSP	Finite Dose Skin Permeation
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
HA	Hydrogen bond acceptor activity
Hb	number of hydrogen bonds
HBA	hydrogen bond acceptors
HBD	hydrogen bond donors
HD	Hydrogen bond donor activity
L_{SC}	length of the diffusion pathway through the *stratum corneum* in cm
HSDB	Hazardous Substances Data Bank
I_B	a molecular descriptor: Balaban index
IPM	IsoPropyl Myristate
J_{max}	maximum flux
J_{SS}	steady-state flux
kg bw/d	kg bodyweight/day
K_{OW}	octanol/water partition coefficient (in some publications also abbreviated as P_{oct}, in many publications log K_{OW} is designated as log P).
k_p	permeation or permeability constant, often expressed in cm/h
$k_{p,SC}$	*stratum corneum* permeation constant in cm/h
$K_{SC,W}$	*stratum corneum*/water partition coefficient
$K_{SC/veh}$	vehicle/*stratum corneum* partition coefficient
LD_{50}	lethal dose for 50% of the individuals in the exposed group

LMV	a molecular descriptor: Liquid Molar Volume
L_{SC}	thickness of the *stratum corneum*
Mpt	melting point
MR	a molecular descriptor: molecular refractivity
MV	a molecular descriptor: molar volume
MW	molecular weight
N	when used as a molecular descriptor: Number of affected hydrogen bonds, calculated by summing the number of N and O atoms (aliphatic twice, aromatic once) (Bodor and Buchwald, 1997)
N_{derm}	dermal load divided by the product of *steady state* flux (J_{SS}) and exposure time (Frasch *et al.*, 2014)
OECD	Organisation for Economic Co-operation and Development
PBPK	Physiology Based Pharmacokinetic (model)
PDMS	polydimethylsiloxane
π_2^H	a molecular descriptor: Solute dipolarity/polarizability
pKa	negative 10-base logarithm of the acid dissociation constant
PT	for biocidal products: Product Type
QSAR	Quantitative Structure-Activity Relationship
QSPeR	Quantitative Structure-Permeation Relationship
QSPR	Quantitative Structure-Permeability Relationship
R_2	a molecular descriptor: Excess molar refraction
REACH	Registration, Evaluation Authorisation and restriction of CHemicals
RISKOFDERM	Acronym of a model for Risk Assessment of Occupational Dermal Exposure to Chemicals
$\sum\alpha_2^H$	a molecular descriptor: Effective/overall hydrogen bond acidity
$\sum\beta_2^H$	a molecular descriptor: Effective/overall hydrogen bond basicity
∑Ca	a molecular descriptor: HYBOT-PLUS H-bond acceptor free energy factor
SD	Standard Deviation
∑(Q+)/α	a molecular descriptor: HYBOT-PLUS positive charge per unit volume
SRC	Syracuse Research Corporation
SsssCH	a molecular descriptor: the sum of E-state indices for all methyl groups/ Electrotopological atom-type index for singly bonded CH

SsssOH	a molecular descriptor: Electrotopological atom-type index for singly bonded OH
STW	Surface Tension in Water
t_{lag}	lag time (expressed in hours) = time needed to establish the steady state
US	United States
V_e	a molecular descriptor: Van der Waals effective molecular volumes, calculated according to Buchwald and Bodor (1998)
Vx	a molecular descriptor: McGowan characteristic volume

9.3 References

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It is a cliché, but only because it is true: I could not have written this thesis alone. Many people have contributed directly and indirectly towards this achievement, and I would like to thank some of them explicitly and many more implicitly.

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This brings me to another group of people for whose contribution I am very grateful: my co-authors. Cees de Heer introduced me to the art of toxicological risk assessment and the role played by dermal absorption. Han van de Sandt taught me about the theory and practice of dermal absorption experiments. Johan van Burgsteden performed the experiments that are the core of chapters 4, 5 and 6, a role Wilfred Maas took over when Johan left TNO. Their contribution was crucial, as I am not known for my dexterity in the laboratory. Jos Bessems introduced me to the intricacies of toxicokinetics and I enjoyed our many discussions on the fundamentals of toxicological risk assessment, as well as a broad range of other subjects. Andreas Freidig guided me on the path to DAME, the model described in chapter 6. Last but not least, I would like to thank Franziska Schurz and Gerwin Schaafsma who did a lot of work on chapters 3 and 2, respectively, and were as eager to learn from me as I was to learn from them.

Others also directly contributed to the papers included in this thesis. My TNO-colleague Carina de Jong-Rhubing designed and performed the statistical analyses for chapters 2, 3 and 6. My former TNO-colleague Jos Hagenaars completed the statistical work for chapter 4. Steve Enoch from the School of Pharmacy and Chemistry of Liverpool John Moores University calculated some of the chemical properties necessary for chapter 6. Finally, although I never met them in person, Matt Miller of the James L. Winkle College of Pharmacy of the University of Cincinnati and Adam Fedorowicz of the US National Institute for Occupational Safety and Health kindly guided me in the use of the Finite Dose Skin Permeation model, which I discussed in chapter 7.

For over 16 years I have been working now in the group of regulatory toxicologists at TNO in Zeist, in several departments under more names than I care to remember, but there was always one constant factor, the presence of enthusiastic, involved, smart-ass and caring colleagues without whom I would not have had the pleasure and motivation to work on my

thesis papers in addition to all of the other papers, statements and reports that desk jockeys like us produce.

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Curriculum vitae

Harrie Buist was born on January 12, 1955 in Delfzijl, and grew up in Utrecht and De Bilt. After graduating from grammar school, he started to study Biology at the University of Utrecht in 1973, with Chemistry as a second major. For his Master, he executed one major research project of 12 months in Chemical Animal Physiology on fumarate reductase in *Mytilus edulis L.* and two minor ones of six months each in Molecular Biology on the initiation of eukaryotic protein synthesis and Protozoology & Immunology on the transfer of protection against *Babesia rodhaini* by injection of spleen cells, and obtained his Teaching Certificate in Biology. He obtained his Master's degree in 1981.

After his studies, he started to work as a biology teacher, first at a secondary school in Barendrecht (1981-1982), then at two different secondary schools in Mozambique, one in Pemba (1983-1984) and one in Maputo (1985). During his stay in Mozambique he was asked to be a lecturer at the recently reopened Biology Faculty of the Eduardo Mondlane University in Maputo, where he taught Human Biology, Basic Biology, Animal Physiology and Molecular Cell Biology (1986-1990).

Upon return to the Netherlands, in 1990, he stared to attend the Postgraduate Education in Toxicology courses, which he successfully concluded with obtaining the Certificate, in 1992. In the meanwhile, he was employed by the Free University of Amsterdam as Project Officer for its Centre for Development Cooperation Services in which capacity he managed interuniversity projects in the area of sciences (biology, chemistry, physics and mathematics) and medicine and developed biology teaching materials for use in third-world countries (1992-1995, 1999). During his employment by the Free University Amsterdam he was stationed as a biology lecturer at the Department of Biology of the School of Sciences of the University of Lusaka, Zambia (1996-1998). At this university he developed and taught a biology bridging course, aimed at bridging the gap between Zambian secondary school O-level education and the A-level required by the University.

In 1999, he was hired by TNO as a regulatory toxicologist, based in Zeist. In that function, he wrote numerous toxicological risk assessment reports on pesticides, biocides, industrial chemicals and chemicals in food and feed. Besides working as a risk assessor, he also has been project leader of knowledge investment and business-to-business projects and performed research on specific topics (e.g. dermal absorption, blood:air partitioning, prediction of nitrosamine carcinogenicity, health effect factors of nanomaterials), which was published in peer-reviewed journals. Part of these published papers formed the basis of this thesis. He is a Board Certified Toxicologist with the Netherlands Society of Toxicology since June 17, 2003 as well as with EUROTOX since November, 20, 2003. After completing his PhD, he will continue to work at TNO as a toxicologist.

List of publications

Buist, H.E., Hischier, R., Westerhout, J., Brouwer, D. (2016). Derivation of health effect factors for nanoparticles to be used in LCIA. Toxicology and Applied Pharmacology (*accepted for publication*).

Fransman, W., Buist, H., Kuijpers, E., Walser, T., Meyer, D., Zondervan, E., Westerhout, J., Klein-Entink, R., Brouwer, D. (2016). Implementing Comparative Human Health Impact Assessment of Engineered Nanomaterials in the framework of Life Cycle Assessment. *Manuscript submitted for publication.*

Buist, H.E., Krul, L., Leeman, W.R. (2016). A TTC threshold for acute oral exposure to non-genotoxic substances, Regulatory Toxicology and Pharmacology, Available online 4 February 2016, DOI: 10.1016/j.yrtph.2016.01.022.

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Buist, H.E., Devito, S., Goldbohm, R.A., Stierum, R.H., Venhorst, J., Kroese, E.D. (2015). Hazard assessment of nitrosamine and nitramine by-products of amine-based CCS: alternative approaches. Regulatory toxicology and pharmacology, 71 (3), pp. 601-623. DOI: 10.1016/j.yrtph.2014.01.017

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http://www.cdc.gov/niosh/topics/skin/OEESC2/AbPost025Buist.html.

Overview of completed training activities

Discipline specific activities

Name of the course	Graduate school/institute	Year
Postgraduate training Toxicology / Clinical Toxicology	Utrecht University	1990
Postgraduate training Toxicology / Epidemiology	Utrecht University	1990
Postgraduate training Toxicology / Introduction into Toxicology	Wageningen University	1991
Postgraduate training Toxicology / Laboratory Animal Science	Utrecht University	1991
Postgraduate training Toxicology / Occupational Toxicology	Nijmegen University	1991
Postgraduate training Toxicology / Pathobiology	Utrecht University	1991
Postgraduate training Toxicology / Risk Assessment	Wageningen University	1991
Postgraduate training Toxicology / Cell Toxicology	Limburg University	1992
Postgraduate training Toxicology / Ecotoxicology	Free University Amsterdam	1992
Postgraduate training Toxicology / Genetic Toxicology	Leiden University	1992
Postgraduate training Toxicology / Internship Genetic Toxicology	Wageningen University/ Duphar B.V. Weesp	1992
Postgraduate training Toxicology / Molecular Toxicology	Free University Amsterdam	1992
Introduction Completeness check pesticide notification dossiers in the Netherlands	CTB	2000
Symposium New developments in Risk Assessment	NVT	2001
Workshop Reproduction toxicity	RIVM	2001
Conference Food safety & dietary risk	AgChem Forum	2002
Symposium New developments in Risk Assessment	NVT	2002
Forum Tox '03 Toxicology in the Pharma Industry	Vision in Business	2003
Conference on Occupational and Environmental Exposures of Skin to Chemicals (OEESC 2005)	NIOSH, Karolinska Institutet (Stockholm)	2005
Short course Assessment of dermal exposure (part of OEESC 2005)	TNO	2005
Short course In vivo studies for prediction of percutaneous penetration (part of OEESC 2005)	Dept. of Dermatology, University of Copenhagen	2005
Short course Modelling percutaneous penetration (part of OEESC 2005)	Colorado School of Mines, USA	2005
Symposium Combination Toxicology and Risk Assessment	NVT	2005
Workshop Consequences of pesticides for immunologic health	Europit (Maastricht University)	2005
REACH workshop	TNO	2006
Course Benchmark-Dose Modelling	IRAS/RIVM, University of Utrecht	2007
Course exposure modelling	TNO	2007
Integrated Probabilistic Risk Assessment	RIVM, Bilthoven, Netherlands	2008
Long range Research Initiative symposium	CEFIC, Brussels, Belgium	2008
Mode of Action workshop	John Moores University, Liverpool, UK	2008
Structural Alerts workshop	OECD, Utrecht, Netherlands	2008
Hearing Toxicological Threshold of Concern	EU, EU Health and Consumer Protection Directorate General, Brussels, Belgium	2009
Workshop PBPK modelling and risk assessment	WHO/IPCS, Berlin, Germany	2009
Conference on Nanotoxicology (2 days)	An international scientific committee of nanotoxicologists	2014

General courses

Name of the course	Graduate school/institute	Year
Course small fire extinguishers	TNO	2001
Course SAP for project leaders	TNO	2002
Spider training	TNO	2006
Course Communication styles	GITPL, Amsterdam, Netherlands	2008
Course Data mining (4 days)	School of Mathematics and Informatics, Technical University of Eindhoven, Netherlands	2011

Optionals

member PBPK working group WHO (2006-2010)

member dermal absorption working group EFSA (2015-now)

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