OECD Environment, Health and Safety Publications

Series on Testing and Assessment No XX

and

Series on Pesticides No XX

Draft

GUIDANCE NOTES FOR THE ESTIMATION OF DERMAL ABSORPTION VALUES

Environmental Directorate
Organisation for Economic Co-Operation and Development
Paris 2008

PREFACE

The aim of this OECD project was to develop harmonised guidance on conducting independent evaluations of dermal absorption studies to estimate the dermal absorption factor of a pesticide. These *Guidance Notes* have been prepared for the purpose of assisting in interpretation of data on pesticides; they may have some use as guidance on evaluating studies for other groups of chemicals such as industrial chemicals thus enabling a consistent approach.

The objective of these *Guidance Notes* is to outline core concepts in order to obviate the need to make reference to large number of text books, but to refer the reader to other useful sources when more detailed or specific information is required.

These *Guidance Notes* do not address test methodology, recognising that there are numerous factors that can influence dermal penetration that include: species variation, application site, dosing regime, occlusion, sex and age. They are intended to complement OECD Test Guidelines and other publications by the OECD, especially OECD Test Guidelines (2004): 427 and 428 for the testing of chemicals and the OECD Guidance Document for the Conduct of Skin Absorption studies (2004). These notes are also designed to complement the IPCS/EHC document 235 *Dermal Absorption* (2006). All of these documents encourage harmonised approach to the conduct of dermal absorption studies and the EHC 235 monograph describes each study and its conduct.

These OECD Test Guidelines and the EHC 235 should be read in conjunction with the Guidance Notes. The EHC document serves to introduce dermal absorption at a broader level and the test Guidelines guide the conduct of the studies in contract with the Guidance Notes which assist with the assessment and interpretation of specific studies for the determination of dermal absorption factor to inform pesticide risk assessment.

The *Guidance Notes* aims to guide toxicology evaluators and risk assessors in the analysis and evaluation of data relevant to the dermal absorption (and therefore the extent of the systemic exposure). The primary focus of this document is on the use of such data for occupational health and public health risk assessment purposes.

While recognising that assessment of dermal absorption informs the exposure assessment and therefore the risk assessment, this document does not address the entire risk assessment process. Similarly, while it is recognised that different regions and countries of the world may have different policy approaches to the type of data required for the assessment of public health and occupational safety of compounds, this document does not attempt to reconcile such policies.

This document will consider the type of data that may be available to risk assessors in support of estimating or calculating the dermal absorption for the evaluation of public health or safety risks posed by a pesticide, and provides guidance on the interpretation of such a data to facilitate a harmonised approach.

The *Guidance Notes* were developed by the OECD Expert Group on Dermal Absorption, comprising experts from Australia (lead country), Canada, Germany, The Netherlands, the United Kingdom, the Unites States, industry/BIAC, the International Programme of Chemical Safety (IPCS) and the OECD Secretariat.

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1. INTRODUCTION

Chemicals present in workplace or other environment may come in contact with the skin and be absorbed. The skin absorption of pesticides needs to be minimised in occupational and home setting. Risk assessment is usually performed to determine the extent to which exposure to a pesticide is acceptable and therefore the extent to which the pesticide is safe to use.

The steps between the presence of a chemical in the environment and systemic exposure include a) the dermal exposure to the chemical, and b) the systemic exposure to the chemical following its c) absorption from the skin.

The purpose of this document is to provide:

1. an outline of data that may be available for estimating dermal absorption; and
2. practical guidance for using such data to estimate dermal absorption factor, including consideration of uncertainty associated with the estimate.

Risk assessment is a process which examines the potential exposure to a chemical in the context of the quantified hazard posed by that chemical. Dermal exposure is the assessment of the *extent* of absorption for a chemical to which people are exposed by the dermal route and is ultimately expressed as the estimate of the expected internal (systemic) level. Exposure assessment establishes the predicted exposure level for humans and is used in the risk assessment.

Dermal exposure assessment consists of:

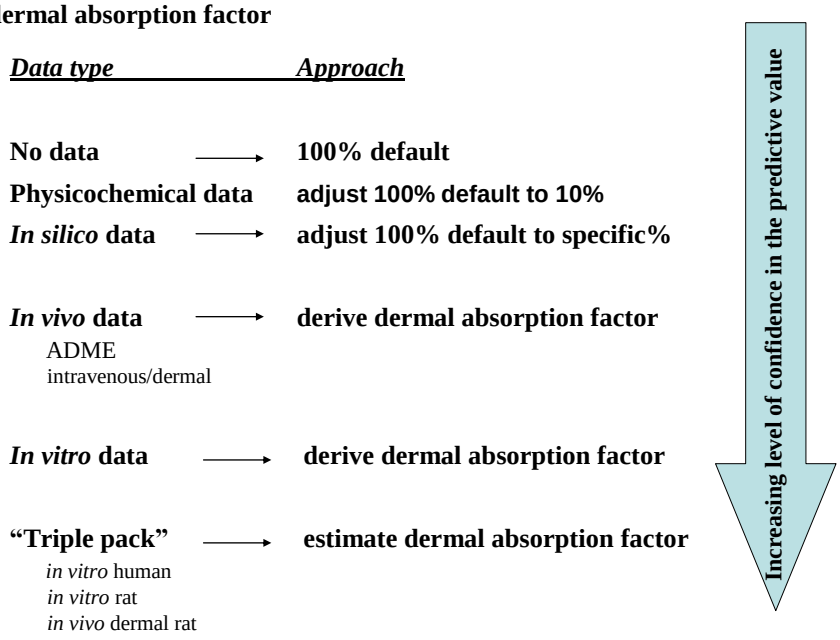
1) estimate of the amount of chemical people may come into contact with (how much gets on the skin);
2) estimate of how much of the chemical on the skin is absorbed (dermal absorption and dermal absorption factor);
3) estimate of the systemic dose utilising the dermal absorption factor where necessary .

These *Guidance Notes* focus **entirely** on the estimate of dermal absorption portion of this process.

A risk assessor considers experimental data *in vivo* and/or *in vitro* that allow for direct or indirect estimation of dermal absorption either of an active ingredient or a formulation through human skin. Estimation of dermal absorption or the dermal absorption factor (Figure 1) may be derived from the following data sources:

- Default values, including *in silico* considerations;
- *In silico* data;
- *In vivo* dermal penetration studies;
- *In vitro* dermal penetration studies; and
- Use of combined data in a so-called “triple pack” approach.

Figure 1
Decision tree – types of available data and approaches for estimating the dermal absorption factor



1.2 Definitions

Dermal (percutaneous, skin) absorption is a global term that describes the transport of chemical from the outer surface of the skin to the systemic circulation (OECD, 2004). This is often divided into:

- *Penetration*, which is the entry of a substance into a particular layer or structure, such as the entrance of a compound into the stratum corneum;
- *Permeation*, which is the penetration through one layer into a second layer that is both functionally and structurally different from the first layer; and
- *Resorption*, which is the uptake of a substance into the skin lymph and local vascular system and in most cases, will lead to entry into the systemic circulation (systemic absorption).

In addition, as summarised in EHC 235 (20080 and in Roberts & Walters (2008), the viable epidermis has various metabolic activities that may either deactivate or activate chemical toxicity following topical exposure.

Most of the barrier function of the skin resides in the outermost layer of the skin, the stratum corneum. Most compounds pass through this layer by diffusing through the intercellular lipids between the corneocytes. Any changes in the nature of this layer due to mechanical, chemical or disease processes may greatly facilitate the transport of chemicals through the skin.

2 NO DATA: USE OF DEFAULT VALUES BASED ON *IN SILICO* PREDICTIONS

In the absence of dermal absorption data, 100% conservative defaults have been adopted as a conservative approach for the dermal risk assessment However, wherever

possible, further refinement of this value is desirable to improve the predictive value of the dermal absorption factor in the subsequent risk assessment.

Consideration of molecular weight and log K_{ow} that distinguish between chemicals with high and low potential for dermal absorption (EC, 2003, 2004)

It has been traditionally accepted that 10% dermal absorption may be assumed where molecular weight is greater than 500 and log octanol-water (Pow) is smaller than -1 or higher than 4. Otherwise 100% dermal absorption is used. The reason for assuming 10% as the lower limit in under this rule is that the data presented in the literature indicates the occurrence of dermal absorption for tested compounds beyond the extremes of log K_{ow} and/or molecular weight values.

In principle, default values between 10% and 100% values can be made on a case-by-case expert judgement, taking into account other available and relevant data. This would include consideration of the water solubility, ionogenic state, molecular volume, oral absorption and dermal area dose in exposure situations in practice. Physico-chemical properties of the chemical that influence dermal absorption include:

- Molecular weight <500;
- Log Pow (octanol water partition coefficient) between -1 and +3.5 (peak absorption between 1 and 2); Note to the Expert drafting Group: do we need to specify pH?
- Solubility in water and non-polar solvents;
- Vapour pressure <5mmHg (1mmHg=0.13kPa)
- Boiling point (liquid/solid) >15^{o}C

2.1 Considerations in reducing the 100% default value

2.1.1 Quantitative estimate of dermal absorption below 100%

If an initial, very conservative risk assessment based on default value of 100% dermal absorption rate indicates that the exposure level is not acceptable/tolerable, a quantitative estimate of dermal absorption may be used. This assessment may include:

- Rule based on molecular weight and log K_{ow} that distinguish between chemicals with high and low potential for dermal absorption (EC, 2003, 2004), and applies a default value of 10% dermal absorption for those chemicals with a molecular weight >500 and log K_{ow} smaller than -1 or higher than 4. In all other cases 100% dermal absorption is assumed.

- The reason for assuming 10% as the lower limit in under this rule is that the data presented in the literature indicates the occurrence of dermal absorption for tested compounds beyond the extremes of log K_{ow} and/or molecular weight values.

Dressler and Walters (chapter in Dermatologic, Cosmeceutic and Cosmetic Development) commented that the Scientific Committee on Consumer Products (SCCP) used relationships between maximum flux (Jmax) of chemicals across skin

with molecular weight, log P, and the degree of saturation of the chemical in the formulation for 62 chemicals and proposed the following alternate default factors based on worst-case assumptions::

Jmax (μg/cm2/hr)	Default % dose absorbed per 24 hours
Non-reactive chemicals with MW 1000	Negligible
Jmax < 0.1	10
0.1 < Jmax < 10	40
Jmax >10	80

In addition, the SCCP panel included default retention factors of 0.01 or 0.1 (i.e., 1 or 10%) for certain rinse-off products and absorption be decreased 3-fold for ingredients used only once a week and 10-fold for products used less frequently.

2.1.2 Read-across

Dermal absorption default may be reduced by extrapolating knowledge of dermal absorption value from data for structurally-related chemicals.

2.1.3 Data from oral absorption studies

Generally, it is assumed that dermal absorption is not likely to exceed oral absorption. Therefore, adjusting the dermal value equivalent to oral absorption may be useful. If an appropriate oral absorption/ADME study is available, the results of the study have, at times been used to refine the default value for dermal absorption. Oral absorption may be determined at low dose levels in bile duct cannulated experimental animals to get an accurate estimate of the oral absorption. It is then claimed that based on theoretical grounds and supported by a comparison of oral and dermal absorption data available (for 12 pesticides) dermal absorption will not exceed oral absorption established by means of bile duct cannulation (unpublished data).

2.2 Other considerations

In silico predictions from maximum flux versus molecular weight data suggests that these defaults are only precise when small amounts of chemical are being applied. A preferable approach is to express predicted dermal absorption in terms of the amount absorbed over a given time period and for a given area, recognising that this can be predicted from maximum flux data and it is the amount that is most applicable for toxicity assessment.

2.3 An approach emphasising amount of chemical absorbed

The first general principle is that, in the absence of any other data, assume the entire applied dose is absorbed.

An important consideration is the exact amount of chemical dose absorbed over a time period of interest. In the absence of any enhancers in the formulations (see Table 2), Table 1 gives the safety limits for amount absorbed over 24 hr for differing amounts applied. Note that these estimations are based on molecular size (data based on upper 95% CI for maximum flux versus molecular weight for entire data base (n=278) of Magnusson et al (2004) – see Fig x below). Based on the upper 95% confidence interval for maximum flux versus molecular weight (Magnusson et al, 2004), approximate limits for human skin absorption (g/cm^2/24 hr) for a range of solutes (with molecular weights in brackets) are: 0.8 (100); 0.05 (200), 0.0001 (400) and 1.9-10 (800).

It is recognised that there usually is a lag time associated with skin penetration and it is also recognised that, in general, this time is difficult to estimate. As the time will lead to a relative reduction in flux relative to maximum flux and skin absorption may occur for an equivalent lag after a product is removed, lag effects have been ignored in these estimations.

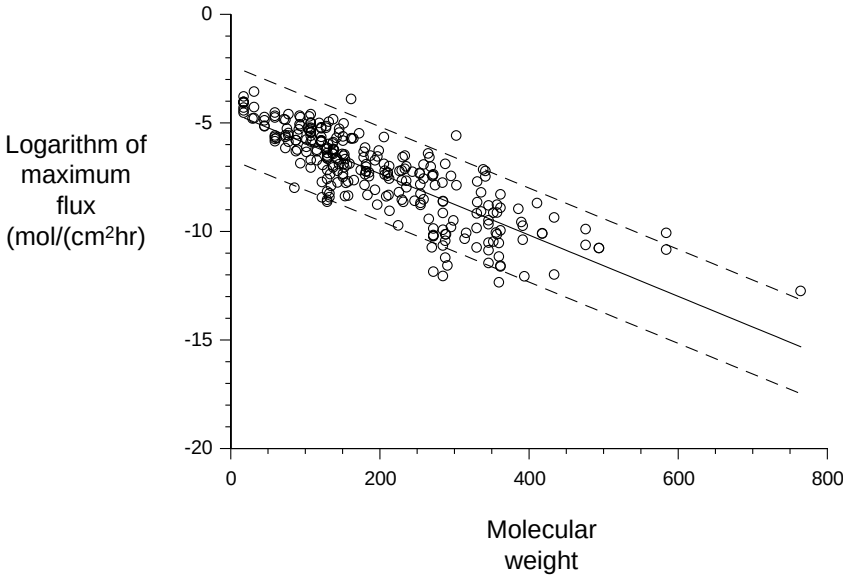


Fig. xx Max flux versus molecular weight for all available data (Magnusson et al, 2004)

Table 1
Estimations of amounts and % absorbed over 24 hr, applying the upper limit for maximal flux versus molecular weight (Magnusson et al, 2004)

Molecular weight	Maximum flux upper limit (mol/(cm^2hr)	Amount absorbed over 24 hr g/cm^2	% absorbed if 1 g applied	% absorbed if 1mg applied	%absorbed in 1μg applied
100	$10^{-3.5}$	0.76	76	100	100
200	10^{-5}	0.048	4.8	100	100
300	$10^{-6.5}$	0.0023	0.23	100	100
400	10^{-8}	0.00010	0.01	10	100
500	$10^{-9.5}$	3.8E-06	0.0004	0.38	100
600	10^{-11}	1.4E-07	1.44E-05	0.014	14.4
800	10^{-14}	1.9E-10	1.92E-08	1.92E-05	0.019

The above calculations for the amount and the percentage absorbed over 24 hours depend on the chemical being in a saturated solution.

2.3.1 Suspension

The above percentage absorbed will be reduced if chemical is in suspension and with the amount or percentage absorbed being given by either M_{24} X Fraction of dose existing in solution or $\%_{24}$ X Fraction of dose existing in solution.

2.3.2 Unsaturated solution

The above percentage absorbed will also be reduced if chemical is all in solution but only a fraction F_s of the saturation solubility. Here, the amount or percentage absorbed being given by either M_{24} X Fs or $\%_{24}$ X Fs.

2.3.3 Lipophilicity as a refinement in estimations of amount absorbed

In general, any algorithm based on permeability coefficients eg Potts-Guy should be limited to application of chemicals applied in pure aqueous solutions as almost all of these algorithms are based on these sets of data. The current limited evidence is that polar (solutes with an octanol water partition coefficient (log P) <-1) and lipophilic solutes (solutes with an octanol water partition coefficient (log P) >5) have a lower maximum flux than solutes with a log P between -1 and 5. However, the data is limited and Magnusson et al (2004) found consideration of liophilicity had only a marginal effect on prediction. Consequently, until better data is available, lipophilicity is not seen a key predictor, recognising that polar solutes are less likely to be absorbed.

2.3.4 Presence of penetration enhancers in formulations

If any of the penetration enhancers shown in Appendix 3 are present, 100% absorption should be assumed unless there is experimental skin penetration data to the contrary.

2.3.5 Relationship to existing rules

The current general rule used by regulators is to assume 100% absorbed for solutes with molecular weights less than 500 and 10% for molecular weights greater than 500. This rule is strictly only correct if the dose applied is 1μg and that the dose exists as a saturated solution as discussed below.

2.3.6 Experimental data

An important consideration is to ensure that the data used is equivalent in formulation, volume applied, area applied and method of application as would be used, or is as near to that in practice.

3. IN SILICO DATA

The theoretical advantages of predicting dermal absorption using *in silico* models include predictions with greater precision than given by the defaults with an

avoidance of costly and/or ethically challenging *in vitro* and *in vivo* testing involving human and laboratory animal experiments.

3.1 QSARs

Quantitative structure-activity relationships (QSARs), also known as quantitative structure-permeability relationships (QSPeRs) are frequently used to relate skin permeability to various physicochemical descriptors and/or structural properties of the molecule - but tend to commonly explain only 70% of the available data (Potts & Guy, 1992

Whilst maximum flux QSPeRs have the advantage that they apply across different solvents providing the solvent does not affect the membrane (Magnusson et al, 2004), experimentally measured dermal permeability coefficient K_p, which define the steady-state permeation rate for chemicals in infinitely dilute solutions, can also be used. The product of K_p and measured (or estimated) solubility in the same vehicle (usually water) provides an estimate of the maximum flux through the skin (Jones et al., 2004; Magnusson et al, 2004) to directly measured maximum flux values. The steady permeability coefficient or maximum flux has been used together with the lag time for absorption to describe non-steady-state or finite dose absorption (Roberts & Anissimov, 2005).

In principle, the observed or predicted absorption through human skin can be combined with an *in vivo* pharmacokinetic/pharmacodynamic model *in silico* to provide estimates of internal exposure, response and toxicity (Dancik et al, 2008).

3.2 Percentage absorption, "flux" or amount absorbed over a time period

Dermal absorption of chemicals is most often expressed as a percentage of the dose that is in contact with the skin. Fluxes (and the derived permeability coefficient values) have not been used to any great extent, except in cases to estimate the degree of acute exposure from a large amount or volume of chemical, for example in such cases as an incidental splash or contaminated swimming pool water. As mentioned earlier, a preferred approach being advocated here is the use of the amount absorbed from a given area over a specific time period for an in use topical product.

4 *IN VITRO* EXPERIMENTAL STUDIES USING HUMAN AND LABORATORY ANIMAL SKIN

A dermal absorption value can be generated from *in vitro* studies with human and/or rat skin (or other skin). *In vitro* methods are designed to measure the penetration of chemicals into and subsequent permeation across the skin into a fluid reservoir and can utilise:

- non-viable skin to measure penetration and permeation only or
- fresh, metabolically active skin to simultaneously measure permeation and skin metabolism. When integrity of skin can be proven, it can reasonably be assumed that its barrier function has been maintained *in vitro*. Then, in principle, the mechanism of skin penetration may be regarded the same as *in vivo*.

The basis of OECD Test Guideline 428 is that *in vitro* studies can predict *in vivo* absorption when the correct methodology for both tests is used.

4.1 Physical and chemical properties

Physical and chemical properties that define the penetration of molecules through the skin consist of:

- liposolubility (usually maximal when log Pow is between +1 and + 2);
- molecular weight (molecules with low MW pass more easily);
- electronic structure and dissociation constant (pKa) (highly ionised products and highly hydrogen bonded products do not penetrate well); and
- water and certain solvents favour penetration.

4.2 Predictive value of in vitro studies using animal skin

Many studies have compared *in vitro* and *in vivo* percutaneous absorption. In general, they verify the premise that properly conducted *in vitro* measurements can be used to predict *in vivo* absorption.

Permeation across the non-living outer layer of skin, the stratum corneum, is believed to depend upon permeant-specific factors, such as molecular weight, water and lipid solubility, polarity and state of ionisation. The permeability properties of the stratum corneum are unchanged after excision from the body, and a good agreement between *in vivo* and *in vitro* experiments with the same chemicals has been observed in this part of the skin.

Comment [l2]: Reference

4.2.1 Scientific evidence for correlation between in vitro and in vivo data

The IPCS/EHC 235 document on dermal absorption (2006) reports that there is, a generally good correlation between *in vivo* and *in vitro* dermal absorption data and that properly conducted *in vitro* measurements can be used to predict *in vivo* absorption. This conclusion is drawn from a small number of comparative studies. The best results in terms of a similar absorption rate were achieved when viable full-

thickness human skin membranes were used for the *in vitro* experiments and when the absorbed percentages of the applied total dose were compared instead of the flux rates (mainly based on Van de Sandt et al., 2000).

In general, it is agreed that there is a good correlation between in vivo and in vitro studies for homologous chemicals. However, for heterogeneous compound it is not well correlated. There are many literature studies in which good correlation have been demonstrated by manipulating the content of the receptor fluid to obtain results similar to in vivo study (IPCS 2008).

4.3 In vitro data as a "stand alone" to predict dermal absorption factor

The predictive value of the *in vitro* studies for dermal absorption *in vivo* is also agreed in the "Technical guidance document on risk assessment" of the European Chemical Bureau (ECB, 2003) to support EU regulation on new and existing industrial chemicals and biocides. In it is stated: "*At present, provided that skin levels are included in the overall percentage absorption figure, results from in vitro methods seem to adequately reflect those from in vivo experiments supporting their use as a replacement test to measure percutaneous absorption.*" However, reservations are expressed with regard to lipophilic compounds and furthermore, the use of reference compounds in *in vitro* studies is recommended.

In a frequently cited paper by van Ravenzwaay and Leibold (2004), the authors have shown that, at least in rats, *in vitro* studies tend to significantly overestimate dermal absorption measured *in vivo*. Even though this cannot be experimentally proven for humans, it seems reasonable to assume a similar ratio. This would give an additional margin of safety when human *in vitro* data is used as "stand alone" information.

Van de Sandt et al. (2000, also cited in the IPCS EHC 235, 2008) concludes that direct comparison was often difficult or even not possible because of the very different experimental conditions. They performed studies under standardized conditions with respect to dose, vehicle and exposure duration to overcome this lack of comparability. Their experiments with the pesticide propoxur demonstrated that the outcome of the *in vitro* study in human skin using viable full-thickness membranes (1.7 or 9.7% dermal absorption after 4 or 24 hr, respectively) correlated well with the human *in vivo* situation (mean values of 0.5 and 3.7% urinary excretion at 4 or 24 hr) when the amount in skin found *in vitro* was included. Slight overestimation of dermal absorption when only the *in vitro* study would be available would seem acceptable from a regulatory point of view and are thought to outweigh the uncertainties in the human *in vivo* study.

Using ortho-phenylphenol (Cnubben et al., 2002), after 48 hours after dosing (exposure period 4 hrs), about 33 % penetration through human viable skin, whereas in human volunteers about 15% of the dermally applied dose (4-hr exposure) was excreted in the urine within 48 hours, albeit there may be possible doubts about the integrity of skin *in vitro* when study duration exceeds 24 hours. Therefore, good comparatibility with, and some overestimation of, dermal absorption in the human skin *in vitro* experiments have been demonstrated, but only for the late and not for earlier time points of exposure.

4.4 Should chemical remaining in the skin be counted as absorbed?

There are differences of opinion on whether chemical remaining *in* the skin should be included in the value for the amount absorbed. Related to this is the issue of how skin residue is measured and what methods (such as tape-stripping) should be used to determine the amount of the chemical remaining in the outer layers of the dermis and therefore not likely to be available for absorption.

EHC 235 provides some guidance on this issue. Where a study is terminated immediately after the end of a 6 to 10-h administration period (and sometimes 24h) and all the animals are killed[1], the whole amount in skin is included in the estimate of absorption and total absorption is equal to the sum of radioactivity in the excreta and residues that were determined in all body compartments.

When sampling is continued by collecting excreta over a certain time period following commencement of treatment (at least up to 24 hours but often up to 7 days), the data obtained will enable greater confidence in the assessment of what part of the amount in skin should be actually included (absorbable dose). Guidance for how to do that is already given, in particular in the EU guidance document (i.e., the serial non-detects approach).

Comment [l3]: Note : last drafting group meeting indicated there are significant problems with the non-detects approach – should the Guidance Notes then indicate that while there is this approach, it is not recommended and give reasons why this would be so?

Subsequently, the amount that was systemically absorbed at 10 h (or, more generally, at the end of the exposure period), as given by the percentages found in excreta, blood, organs/tissues except skin and remaining carcass and the absorbable dose in skin, is summed to give the estimate for total dermal absorption.

The amount of penetrated substance found in the receptor fluid at the end of the experiment (generally 24 h) is considered to be systemically available. In addition, the amounts present in the epidermis and dermis at that time are considered to be systemically available.

The Guidance Notes recommend that in general, where the studies are carried out in accordance with the OECD methodology, the residue in the skin is counted in the amount absorbed.

4.5 Considerations in the assessment of in vitro skin absorption studies

4.5.1 Formulations

The experimental design and test conditions used may significantly affect the results obtained. Both *in vitro* and *in vivo* experiments have demonstrated there is an inverse relation between dose concentration and the percentage of absorption. The percentage

absorption is generally higher at low concentrations than percentage absorption at high concentrations. As a consequence, there is no standard absorption percentage for a given substance and dermal absorption studies should be done at different concentrations as a function of the planned agricultural practice.

These results emphasise the need to use skin absorption studies done with the actual formulation that is being assessed and mimicking its application method, area and duration using both an undiluted preparation and the preparation diluted to recommended concentrations for uses in the field. Since different solvents can modify chemical absorption percentages, skin absorption data should be requested for products which have a significantly different composition.

4.5.2 Type of skin used

Skin from human and laboratory animal can be used. Although the use of human skin samples gives data more appropriate to human *in vivo* conditions, these are not always available. Typical human *in vitro* experiments with viable skin involve the use of female abdominal or breast skin obtained at autopsy or from cosmetic surgery. Non-viable skin from several anatomical sites of male and female cadavers has also been used. The permeability of human skin differs depending on the site from which it is obtained. Whereas human skin is obviously the gold standard (EHC 235), other *in vitro* models involving pig skin as a surrogate for human skin, or *rat skin* for comparison of absorption with human skin, have been used.

4.5.3 Exposure period

Varying time periods have been proposed for *in vitro* studies on dermal absorption, with 6-8 hours being recommended in the case of agricultural chemicals to reflect the anticipated occupational exposure conditions. However it should be considered that this time period may be too short in some cases, for example in developing countries, or where agricultural circumstances require extended periods of operation.

The exposure period is terminated by washing of the skin surface. The procedure to remove the test preparation from the surface of the skin should mimic normal practice in use. During and after cessation of exposure, sampling should be frequent and long enough in order to get insight into the absorption kinetics, which is important for derivation of the maximum flux (European Commission, 2004).

Considerations of the skin viability and therefore the reliability of the experimental results suggest that 24 hour exposure for *in vitro* and longer if viability can continue to be demonstrated may provide a more predictive dermal absorption value.

4.5.4 Study design

Not all studies available to the assessor are fully compliant with the OECD Test Guideline 428. Therefore, study designs need to be closely examined to determine the value of the data to predict dermal absorption. A major point often looked at is the correlation between dermal absorption rates measured *in vivo* and *in vitro*. In one analysis of the available data (Roberts, xxx), regression analysis yielded the following correlation values (R^2):

	In vitro human – *in vivo* volunteers	*In vitro* rat – *in vivo* volunteers	*In vitro* rat – *in vivo* volunteers*
R^2	0.84	0.66	0.10

However, as mentioned above, neither of the methods has been formally validated to date for the range of pesticides and other compounds of interest. Limited validation between human skin *in vitro* and *in vivo* exists for topically applied pharmaceuticals (Dancik et al, 2008). A typical regression is shown in Fig. 1.

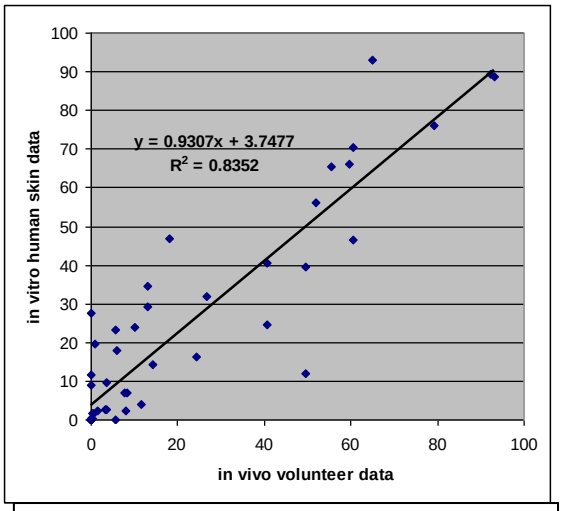


Fig. 2. In vitro – in vivo skin penetration correlations (see references for data source)

Even so, such comparisons are often flawed by methodical deficiencies and, therefore, cannot be always relied on. For instance, Ramsey et al 1994 showed percentages of applied dose absorbed *in vitro* varied from 33 to 80% for various doses, whereas the corresponding in *vivo* data for the same doses shows percentages of applied dose absorbed ranging from 1.6 to 8% only. It is even more inappropriate to compare data obtained in rats *in vivo* with penetration rates through human skin measured *in vitro* because of the morphological and perhaps also biochemical differences. If human *in vitro* and *in vivo* data for the same substance are considered, the amount retained in skin cannot be determined in humans and is sometimes not measured in skin samples. Thus, what is really compared is often not more than the urinary excretion vs. the absorbed portion found in the receptor fluid.

Perhaps the most important reason for concern about the actual predictive value of *in vitro* data is the large variability of results that has been proven in a recent inter-laboratory comparison (Van de Sandt et al., 2004; IPCS, 2006). It is anticipated that standardisation of experimental conditions will contribute to the reduction of this variability, but it may be some time before the results of these efforts will become apparent by a reduction in variability.

5 IN VIVO DATA

5.1 Laboratory animal data

The rat is the most commonly used species for animal *in vivo* studies, having the advantage that information from other toxicity and toxicokinetic studies is mostly obtained from this species and is therefore directly comparable. Data from other species may be used, providing that it is established that they are known to have skin absorption more similar to humans than the rat.

It is accepted that data from rat studies generally overestimate dermal absorption in humans. Monkey and pigs may show dermal absorption similar to that of humans, but these species are comparatively difficult and expensive to maintain as test species and there are ethical considerations for their uses.

There are three types of *in vivo* animal (rat) data that are useful for the estimation of the dermal absorption factor. Measurements following dermal application of the chemical are:

- blood or excreta,
- the skin by biopsy or other method, and
- all tissues (residue analysis). The test chemical is applied to an area of skin in a solvent or formulation for a defined period. Body fluids, tissue or excreta may be collected at predefined intervals, and the quantity of chemical and/or its metabolites in the sample measured.

In studies where the animals are not sacrificed, then an indirect measure of absorption (by monitoring urine, faeces of blood) is generated as a measure of the dermal absorption of the chemical (EHC 235).

However this measure does not allow for material which accumulates in the body (including in the skin) and is not excreted. Lipophilic, anionic compounds are particularly likely to accumulate in the skin. This reservoir effect commonly applies to the stratum corneum, but the epidermis, dermis and underlying tissues may also act as reservoirs. This effect must be taken into consideration when determining levels of dermal absorption based on these studies.

5.1.1 Dermal in vivo ADME/kinetic studies

Dermal *in vivo* ADME/kinetic studies (measuring absorption, distribution, metabolism, excretion and mass balance) estimate the extent of cutaneous uptake and/or the systemic absorption of the chemical. The methodology for these studies is detailed in an OECD test guideline for *in vivo* dermal absorption (OECD 2004). Determination of dermal absorption in these studies is generally based on mass balance considerations. An adequate mean recovery is in the range of 100 ± 10%. The test methodology requires that recoveries outside the given range need to be justified. As an alternative to the mass balance recovery method, absorption can also be assessed through a comparison with an intravenous dose with the % absorbed being given by:

$$\%Absorbed = \frac{AUC_{topical}}{AUC_{\text{int}\,ravenous}} \frac{Dose_{\text{int}\,raveous}}{Dose_{topical}} x100$$

$$\%Absorbed = \frac{Urinaryre\,\text{covery}_{topical}}{Urinaryre\,\text{covery}_{\text{int}\,ravenous}} \frac{Dose_{\text{int}\,raveous}}{Dose_{topical}} x100$$

Where the area under the curve (AUC) is the plasma concentration of unchanged chemical in the plasma, blood or serum and urinary recovery can refer to unchanged chemical, metabolite or unchanged chemical plus metabolite. The latter methodology

is the one of choice when recovery is outside the accepted mass balance recovery range.

The dermal absorption of a chemical is usually given as a percentage of the dose applied and is obtained by addition of amounts recovered from faeces, urine and cage washing, expired gas, blood and remaining carcass, and dose skin. However, such data should be treated with caution as it is the extent of absorption that is important in toxicity assessment. For instance, if a suspension of chemical was applied, the same amount of chemical would be absorbed if the volume of application was doubled whereas the percentage absorbed would be halved.

Tape stripping may be performed in order to obtain information on test chemical deposition in the stratum corneum. Considerations on the validity of tape stripping methodology to measure the extent of chemical absorption are discussed later in this section.

Where animals are not sacrificed, an indirect measure of absorption (by monitoring urine, faeces, or blood) is performed, including an adjustment to account for material that is absorbed but not measured in the fluid that is monitored. Pharmacokinetic analyses are usually undertaken to estimate the rate and the extent of absorption. The overall extent of absorption is defined in terms of absolute bioavailability, best determined by comparison with plasma/blood or urine levels of unchanged solute achieved with intravenous administration of the solute (EHC 235).

In cases where an intravenous study is available for comparison of availability, the percentage of dermal absorption is calculated from:

$$\text{Percentage dermally absorbed} = \frac{\text{total \% excreted after topical dose}}{\text{total \% excreted after intravenous dose}} \times 100$$

In the absence of intravenous studies, the percentage absorbed has to be estimated by mass balance which is given by the amount excreted divided by the amount applied (Roberts and Walters, 1998; EHC 235).

These approaches provide a measure of internal (plasma) exposure and are not necessarily applicable if the exposure of interest is in the viable epidermis. The viable epidermis does have a metabolic capacity which leads to a significant skin first pass effect for many compounds (EHC 235).

The main disadvantage of using laboratory animals is that they have different skin permeability and may also have different systemic disposition of chemicals when compared with humans.

The skin of rats, guinea pigs and rabbits is more permeable than that of humans, whereas skin permeability of pigs and monkeys is more similar to that of humans (OECD, 2004). There are numerous publications that show that the rat dermal absorption is equal too, or in excessive of human absorption by a factor of 2 to 10. i.e. use of the rat dermal values provide a conservative measure.

The main advantage of *in vivo* data is that they are generated from a physiologically and metabolically intact system (OECD 2004). Since for the vast majority of substances under study a higher permeability of rat (or rabbit) skin as compared to human skin has been demonstrated, this approach will mostly overestimate dermal absorption in humans and provide an additional margin of safety

5.2 Human in vivo data

Dermal in vivo studies in humans to estimate dermal absorption is the gold standard. The major limitations of this approach include the possible systemic and local toxicity of applied and/or administered chemicals and the need for human ethical approval prior to such studies. Whilst dilute chemicals may be used, it is uncertain whether the absorption found will mimic the use of the chemical in the actual dose form under real-life exposure situations. In addition, *in vivo* absorption depends on application method, skin type, location etc and these have the potential to confound any reported findings.

In vivo studies with human volunteers performed under relevant test conditions provide definitive data for the assessment of the absorption of chemicals through human skin. These mass balance or biomonitoring studies quantitate the amount of chemical that is recovered e.g. in body fluids after dermal application.

Human volunteer data from controlled studies allows the establishment of the relationship between levels in the biological fluids and the dermally applied dose (Jakasa et al 2004 – EHC 235). Knowledge of the chemical half-life, metabolic profile, clearance and the enzymes involved are required to interpret biological monitoring data, unless i.v.dosing is available to "normalise" the data. They include:

- *Plasma, excreta and breath analysis* - in this approach, the amount recovered (in the stratum corneum and in urine) is compared with the amounts applied, providing an estimate of average absorption rate (Roberts and Walters 1998 EHC 235). Biomonitoring gives an indication of the internal dose of a chemical by monitoring levels of chemicals and metabolites in the blood, urine or breath.

- *Tape stripping* – stratum corneum tape stripping is an approach that has been advocated in assessing the bioequivalence of topical dermatologicals but has been associated with considerable variability. Its methodology is described separately (OECD methodology).

- *Pharmacodynamic assessments* – specific pharmacodynamic tests are widely used to assess the absorption of topical dermatological substances and include the vasoconstrictor "blanching" test for topically applied corticosteroids, rubefacient or increase in blood flow caused by vasodilators and alterations in physiology as measured by trans-epidermal water loss (TEWL), skin pH, skin hydration and skin micro-relief after any treatment that modifies the stratum corneum (Walters & Roberts, 2008). The pharmacodynamic measurements most relevant to chemical exposure are corrosion, irritation and sensitisation

The advantage of these approaches are that human studies carry a greater regulatory value because they are performed under experimental conditions which may be

controlled and provide data on the relevant species, albeit that they often provide poor mass balance data. These studies are likely to become more important given the increasing consumer resistance to the use of animals in testing of chemicals.

The first (plasma, excreta and breath analysis) and last (pharmacodynamic) approach to dermal absorption estimate are considered to be of value in risk assessment, albeit that care needs to be taken in interpreting such data.

5.2.1 Tape stripping

Tape stripping provides information on test chemical deposition into the stratum corneum. Most recent studies with stratum corneum tape stripping have shown that reliable percutaneous absorption estimates can only be obtained when both the weight of each strip and the residual stratum corneum barrier function (by TEWL) are assessed (Guy et al). However, these studies appear, at this time, have only been undertaken in the academic laboratories proposing the refined methodology and hence the capacity to generalise this refined technique is unknown. In addition, tape striping methods generally do not take into account the effects of skin metabolism and chemical uptake by the dermal blood supply as determinants for skin absorption. Walters & Brain (date) concluded that "*the variability in experimental parameters that exist at present preclude the acceptance of the tape stripping method as a means to evaluate the bioequivalence of topically applied drugs, except for those compounds such as antifungal agents whose main site of activity is the stratum corneum itself*."

Tape stripping of human stratum corneum has frequently been used for investigation of skin penetration. In a tape-stripping experiment, an area of skin is exposed to a chemical for a fixed application period. After exposure, stratum corneum from the exposed skin site is removed sequentially by successive application of adhesive tape. The amount of the substance recovered by the tape is then determined. The number of tape strips needed to remove a given fraction of the stratum corneum may vary with the type of tape, the pressure applied and the peeling force, as well as the anatomical site and the age, sex and ethnicity of the subject.

The skin-stripping technique may be used to determine the concentration profile of a chemical in the stratum corneum in relation to depth within this skin layer. This allows the derivation of the partition coefficient of a chemical between the stratum corneum and the vehicle, and diffusivity, which enables deduction of the chemical permeability coefficient (Kp). However, this method is not very reliable and usually needs both an accurate estimate of the weight of the strip and the trans-epidermal water loss rate present following the removal of the strip. Tape-stripping has the obvious advantage that it permits *in vivo* observations on dermal absorption in humans.

The main value in tape stripping is to define the residue amount remaining in the skin after topical application.

5.2.2 Skin-bound residue

Skin-bound residue should be considered on a case-by-case basis. The OECD test guideline requires washing the skin after exposure and subsequent monitoring of

bioavailability of skin bound radioactivity. Depending on the bioavailability of skin bound radioactivity, materials remaining on the skin may or may not be added to the absorbed dose. If the study shows significant depletion of radioactivity from the application site following washing and corresponding increase in absorbed dose over time, radioactivity remaining at the application site is considered available for further skin absorption. In this case, the dermal absorption value at post wash (typically 24-72 hours) is selected and the radioactivity depleted from the application site is added to derive the dermal absorption factor.

Alternatively, if the study indicates that skin bound residues remain in/on the skin (i.e. no depletion of radioactivity) throughout the duration of the study, then radioactivity remaining at the application site is considered unavailable for further absorption and is not added to derive the total absorbed dose. Sample collection (urine and faeces) intervals after skin washing are essential in interpretation of bioavailability of bound skin residues. Bound skin residues are considered absorbed if bioavailability cannot be determined

However, in practice, the skin is often washed after removal and this could be extensive in some cases eg. in a warm water shower or bath that would promote skin hydration and absorption of the chemical. Such effects have been well documented for topical pharmaceuticals. In addition, issues exist for lipophilic solutes, as raised earlier.

The disadvantages of human *in vivo* data include the fact that studies in human volunteers have ethical and scientific limitations, certain disadvantages that cannot be readily overcome and are also flawed by experimental uncertainties. Ideally, the amount absorbed (systemic exposure) after topical can be assessed when the clearance from the body is known or when mass balance estimations are possible. Clearance is best assessed by comparison to an intravenous dose but such data is likely to be unavailable. Comparison is then best made to dosing given by another route. Mass balance estimations are chemical-dependent, as the routes of metabolism and excretion for a compound depend on their chemical nature and size. The entero-hepatic excretion of a polar conjugate of a chemical compound in the bile or sequestration of a chemical in lipid stores within the body can make a mass balance approach very difficult. The validity of a mass balance approach must them be made on a chemical by chemical case.

Each of the approaches to generating *in vivo* human dermal absorption has major limitations in determining dermal absorption for topically applied chemicals.

Cutaneous microdialysis probes have been employed, being inserted 200 μm or more below the skin surface. As they are below the dermal capillary blood supply to the viable epidermis, levels are often up to 100 fold less than entering the blood. The insertion of the probes can cause an inflammatory response (erythema) which generally subsides after ~30min. Variations in local blood flow can affect levels observed and poor recoveries are often observed for lipophilic compounds.

6 COMBINATION OF ANIMAL IN VITRO AND IN VIVO AND HUMAN IN VITRO DATA

When valid (guideline-compliant and GLP-like) *in vitro* studies on human skin are performed and subsequently accepted by regulatory authorities, a common approach is to estimate *in vivo* human equivalent dermal percentage absorption by using a combination of rat *in vitro* and *in vivo* data and human *in vitro* data (OECD 427 and 428), the so-called **"**triple pack" approach. Much discussion has centred on whether the "triple pack" should be preferably used for prediction of dermal absorption, rather than in vitro skin results alone.

The triple pack approach allows moderation of *in vitro* results using rat skin by correcting for any differences between *in vitro* and *in vivo* rates in that species as well as for species differences, which is important since absorption by human skin is usually lower than that by rat skin. As such, the combined use of data offers greater precision in estimating dermal absorption.

This approach has the capacity to provide the most predictive estimate of the dermal absorption factor for the subsequent exposure and risk assessment.
Comment [l4]: Note to the Drafting Group: do we need guidance about the advantages and disadvantages of this approach to guide the assessor?

The dermal absorption estimate using data from the "Triple Pack" is derived from the following approach:

In vivo human absorption = (*In vitro* human absorption) x (*In vivo* rat absorption) / (*In vitro* rat absorption).

The following examples are provided for illustration:

Example 1

The fungicide fluquinoconazole is currently subject to EU evaluation (*Note: is this finalised?*) The available dermal absorption studies were assessed with the following results:

> *in vitro* human skin, concentrate about 1.2%;
> *in vitro* rat skin, concentrate about 2.6%, revealing a (human vs. rat) ratio of 1:2.2;
>
> *in vitro* human skin, dilution about 6.1%;
> *in vitro* rat skin, dilution 6.8%, revealing a ratio of 1:1.1.

These ratios were used to correct the dermal absorption rates obtained *in vivo* in male rats of 1.6% for the concentrate and 15.8% for the dilution. Because of this correction, dermal absorption rates of 0.7% (conc.) and 14% (dilution) have been proposed.

When the human skin *in vitro* data would have been used instead, assumptions of a higher dermal absorption for the concentrate (1.2%) but a lower one for the dilution (6.1%) had to be made. Such differences might have regulatory consequences. To ensure that the regulatory approach is conservative, the *Guidance Notes* suggest the use of the worst-case assumption.

Note to the Drafting group:

In order for these examples to evaluated, it is necessary to confirm the exact definition of what is absorbed: does it include the skin residue with or without the stratum corneum. If the stratum corneum is included, then the example where the in vivo human equivalent value is lower than the measured in vitro human can be understood, because the human in vitro data may contain the assumption that the stratum corneum residue will be absorbed. The variation in approaches cited in these examples justifies the need for clear guidance which this document is mandated to provide.

7 OTHER CONSIDERATION

7.1 Other potential sources of information on dermal absorption

Other information may be relevant when estimating dermal absorption factor, albeit as an additional information which may be used to modify conservative estimates. Each of these have significant limitations. They include:

- Biomonitoring, which includes gathering data in the field from blood, urine or breath samples as an indication of exposure levels;
- Evidence from clinical toxicology, including data collected from poisoning cases; and
- Cutaneous microdialysis, which is an *in vivo* sampling technique for the extracellular space beneath the skin using perfused dialysis, used in human volunteers as well as laboratory animals.

7.2 Use of data from acute oral route to estimate dermal absorption

An estimate of dermal absorption cannot be deduced from the results of acute toxicity studies because differences in, for example, oral and dermal LD_{50} values are not necessarily a result of differences in absorption. Firstly, the result in a dermal LD_{50} study is dependent on the size of the exposed area and can be changed by altering the exposed area. Secondly, differences in toxicity after oral and dermal exposure could be the result of first-pass effects. Furthermore, the toxicity of a substance is also influenced by the rate of absorption. Generally, and especially in acute (gavage) studies, oral absorption will be relatively fast, resulting in a peak concentration in the body, whereas the absorption after dermal exposure is generally more gradual.

7.3 Comparison of data from repeat-dose oral and dermal toxicity study to estimate dermal absorption

It is desirable to generate experimental data *in vivo* and/or *in vitro* that allow for direct or indirect estimation of dermal penetration either of an active ingredient or a formulation through human skin. If such special dermal absorption studies are not available, not conclusive or not accepted for any reasons, a comparison between repeat dose *oral* and repeat dose *dermal* toxicity studies have been taken into consideration as a possible alternative to using default values. However, this approach has significant problems and is not an accepted.

If default values on the basis of physico-chemical properties are used but still in dispute, data obtained from this extrapolation approach may give additional information to facilitate the decision whether a value less than 100% was more appropriate. In cases where specific dermal absorption studies have been conducted, such a comparison should not be used to vary the expected absorption rate, but may increase the confidence in the study results.

A meaningful comparison of the LOAELs in oral (feeding or gavage) and dermal toxicity studies can be made only on condition that:

- Animal species and sex in the studies to be compared were the same. (It is preferred to have data on both male and female animals);

- Duration of treatment was the same or at least very similar. (Usually, 21- or 28-day dermal studies will be compared to subacute oral studies. However, if available, 90-day or even long-term studies with dermal and oral administration may be used);

- Acute toxicity studies must not be used for this purpose since they are usually conducted at the limit dose;

- The number of animals per sex and group was sufficient for reliable statistical analysis;

- The range of parameters investigated was the same or at least very similar in both types of studies and was large enough to detect target organ toxicity. (Sometimes, oral short-term studies performed for dose finding purposes will not be suitable for a comparison with a dermal experiment because of the limited data obtained.) Usually, clinical observations, monitoring of body weight (gain) and food consumption, haematological and (blood and urine) clinical chemistry, gross pathology, organ weights and (limited) histopathology should be included;

- Unusual kinetic, e.g. for carbamates may preclude this approach;

- Clear LOAELs were obtained in all studies to be compared. (If no effects were observed, the highest dose is considered the NOAEL);

- In cases where no adverse effects were seen in the dermal study, testing should have been done up to the limit dose (1000 mg/kg bw/d;

- Systemic effects must have occurred and a LOEL was identified at least in the oral study. (This requirement implies that studies of similar duration with no effects up to a limit dose after both oral and dermal exposure would not be suitable for a comparison of dermal absorption.); and

- In cases where a systemic effect was observed after dermal administration, too, it should be similar to the findings in the oral study in terms of the target organ and the parameters that were affected. (*Note: Rather quantitative differences such as a higher or lower percentage of organ weight changes or a different degree of*

histopathological lesions may occur. Isolated organ weight increases with no concomitant histopathological findings - mostly liver - are often regarded as toxicologically not relevant but are thought to indicate exposure to a xenobiotic. Therefore, they can be relevant for comparison of oral and dermal exposure and should be taken into consideration).

When all these criteria are met, the LOELs from the oral and dermal studies may be compared to estimate the dermal absorption. If there were effects in the oral study but not (up to a limit dose) in the dermal one (where investigations were performed and would have detected them if occurring), the ratio between the oral LOEL and the dermal limit dose (usually 1000 mg/kg bw/d) should be calculated, providing upper bound estimate of dermal absorption. Since for the vast majority of substances under study a higher permeability of rat (or rabbit) skin as compared to human skin has been demonstrated, this figure will mostly overestimate dermal absorption in humans and, thus, provide an additional margin of safety for subsequent exposure assessment.

While comparable oral and dermal studies are frequently available in rats, the situation is even more complex when a dermal study had been performed in rabbits. Usually, a developmental toxicity study is then the only other study in this species within the test regime that is available for a comparison. However, such a comparison between non-pregnant and pregnant animals is not recommended because of the following uncertainties:

- Metabolism in pregnant (certainly in liver and placenta but perhaps also in skin) is different from that in non-pregnant females. Changes such as a different distribution of water in the body compartments during pregnancy may influence skin absorption itself but in particular the kinetics and fate of the absorbed amount; and
- The range of parameters investigated in pregnant females at scheduled termination is in most cases smaller than in a sub-acute dermal study and, thus, comparability must be questioned.

In rare cases, developmental toxicity studies may be performed in one or the other species by the oral and the dermal route. In principle, these studies could be used for estimation of dermal absorption but their results can be misleading for the estimation of dermal absorption factor. If similar effects would occur at the same or not very different dose levels, this could point on one hand to a very high dermal absorption but, on the other hand, might result from a lack of first-pass effect after dermal administration, irrespective of the actual dermal absorption rate.

A criticism of comparing the results of oral and dermal toxicity studies following repeated dosing approach has been that, at dermal doses approaching the limit doses (*ca* 1000 mg/kg bw) the depth of test material could be such that much of it is not in contact with the skin and is unavailable for absorption. This would tend to compromise the reliability of the estimated systemic exposure, as opposed to applied dose, in the dermal toxicity study.

In the dermal absorption guidelines (OECD 427 & 428) the recommended application of test material is stated as 1 to 5 $mg.cm^{-2}$ for solids and $10\mu L.cm^{-2}$ for liquids. This is

intended to produce a thin film and optimise contact between the mass of test material and the skin.

In the repeat dose percutaneous toxicity guidelines (OECD 410 & 411), the recommendation is that the dose is applied to *ca* 10% of the surface area of the animal. The rats used in these studies will typically be 150 – 250g and have a total surface area of 200 to 400 cm^2. At the limit dose of 1000 mg/kg bw/day the application of test material will be *ca* 150 mg to 20 cm^2, equal to 7.5 $mg.cm^{-2}$. This value is not inconsistent with the applications rates used in the dermal absorption studies.

Comment [l6]: [these values are taken from some studies and odd references – are they OK or does anyone have a definitive / alternative set of numbers? Para 54 of the OECD guidance doc 28 on skin absorption indicates 10cm^2 is 5 – 10% of a rat surface].

For rabbits the equivalent values are 2 – 3 kg and a total surface area of 1000 – 2000 cm^2. At the limit dose of 1000 mg/kg bw/day the application of test material will be *ca* 2000 mg to 100 cm^2, equal to 20 $mg.cm^{-2}$. This value is significantly above the applications rates recommended in the dermal absorption studies for solids and above the upper recommendation for liquids.

Comment [l7]: [alternative numbers] ?

Subject to general caveats in estimating dermal absorption by comparing findings in dermal and oral toxicity studies (i.e. that they use same strain and sex, and the study is not a Cmax driven end-point, level of investigation) there is no reason to believe that the thickness of the test material 'layer' will be a significant confounding factor in rats exposed at up to 1000 mg/kg bw/day or in rabbits exposed at up to 300 mg/kg bw/day. Caution should be exercised in attempting to use data from dermal studies using dose levels above these, particularly for solids.

8 METHODOLOGICAL CONSIDERATIONS IN ASSESSING THE VALUE OF THE AVAILABLE INFORMATION IN ESTIMATING THE DERMAL ABSORPTION FACTOR

While the methodology for the conduct of dermal absorption studies is detailed in separate OECD methodology monographs, the assessor needs to take into consideration a number of issues either not covered in detail in the monographs, and/or to assist in the assessment of older studies that may not be compliant with OECD methodology. These issues are raised and discussed briefly below to provide the assessor with a perspective on the value of the study being assessed and potentially used to derive a dermal absorption factor.

8.1 Finite vs. infinite dose

Studies are conducted with dosing conditions reflecting in use application (finite doses) which reflect real life applications. Infinite doses (large donor volumes) are frequently used to yield steady state fluxes but their relationship to finite doses is

often vague due to diffusion and skin hydration differences (Cross et al 2001). Differences can also exist between in vivo and in vitro results due to the difference in application techniques and conditions. For instance, Wester et al 1998 showed 1000-fold greater boric acid k_p for infinite dose *in vitro* vs. *in vivo* penetration, whereas finite dose *in vitro* boric acid k_p is 10 times greater. In practice, it is technically demanding to apply finite dose to biological membrane such as skin. This may explain some variation in human skin *in vitro* studies (van de Sandt, 2004, Mavon et al, 2007).

8.2 Full- vs. split-thickness skin vs separated epidermal membranes.

Different forms of skin preparations are used in *in vitro* studies. Cross & Roberts (2007, 2008) point out that partial and full-thickness skin, in which a substantial amount of dermis is present, reflect infinite vasoconstriction whereas epidermal membranes reflect infinite vasodilatation. From the viewpoint of evaluating a chemical's safety from the perspective of potential maximum absorption, the use of epidermal membranes is preferred.

Caution should always be taken in assessing studies that use the whole skin (full thickness) data, as the dermis can act as both a reservoir and barrier to penetration, leading to underestimates in the extent of absorption. Beckley-Kartey et al (1997) and Cnubben et al (2002) suggest that a permeant reservoir may be especially formed for more lipophilic compounds in full-thickness skin due to presence of dermis, into which the compound must partition. Using human/rat split-thickness epidermis, Cnubben et al 2002 showed that, for the lipophilic ortho-phenylphenol, the amount systemically available was overestimated by a factor of 2 to 4. Human/rat full-thickness viable skins have been reported to underestimate the amount systemically available in the early time periods by factor of 7 to 33, although the prediction at later times was better.

8.3 Vehicle components

Small differences in the vehicle formulation can greatly influence *in vitro* penetration profile. Further, partitioning can be enhanced or evaporation of vehicle may impede penetration, with white-spirit based formulations having greater effects than acetone (Dick et al 1997). Griffin et al 1999 reporetd that the skin penetration of chlorpyrifos (amount recovered in receptor fluid) was about 1.5 times greater for commercial concentrate vehicle than ethanol vehicle. The vehicle components can alter skin permeability properties. Evaporation differences can change concentrations at skin surface.

A major and frequently mentioned obstacle is the difficulty of estimating dermal absorption of very lipophilic substances by *in vitro* methods, since they are poorly soluble in most receptor fluids and partitioning will be inhibited. It is expected that some accumulation in skin will occur. Even if the whole amount in skin is included (as generally recommended for *in vitro* studies), this may give an inaccurate estimate of dermal absorption, because it is not known if further uptake of the substance will be affected by the amount bound to skin already.

A key question that must always be asked is whether the concentration of solutes being measured in the receptor phase of any in vitro study is less than a tenth its

solubility in the phase. Ramsey et al 1994, in studies with human epidermal membranes, showed that *in vitro* skin penetration results using an aqueous ethanol receptor fluid predicted *in vivo* human results. However, *in vitro* receptor solutions consisting of tissue culture medium (TCM) and polyethylene glycol (PEG) underestimated human *in vivo* absorption.

8.4 Lipophilic chemicals

One point of contention that needs addressing is when does the chemical in the skin need to be included in the overall amount considered as having been absorbed. This is an issue relevant to both *in vivo* as well as *in vitro* testing, either for direct assessment of absorption or for the estimate of the dermal absorption factor. Overall, it is uncertain as to what extent an *in vitro* study with a lipophilic substance will accurately reflect *in vivo* conditions and, thus, results should be usually considered as not sufficiently reliable even if reference substances of similar lipophilicity had been included.

In general, this *Guidance Note* suggests that all of the chemical in the skin should be included in dermal absorption studies as potentially systemically available, as already argued in the OECD test guideline. However, some consideration also needs to be given to the lipophilicity of the applied chemical.

Uncertainties about the uptake of lipophilic compounds and their possible accumulation in certain skin layers will be probably outweighed by a general overestimation of dermal penetration for humans when the *in vivo* study is conducted on rats.

Overall, for chemicals that form a reservoir in the skin, such as diethanolamine and lawsone, the *in vitro* skin residue levels are likely to be unimportant for the estimation of *in vivo* systemic absorption (Bronaugh, 2007). For other compounds systemic levels from 24-h *in vivo* absorption studies would ideally be conducted. Then *in vitro* absorption experiments can be performed that determine the extent of retention in the skin and whether or not these residual levels should be included in estimates of systemic absorption (Bronaugh, 2007)

Some experimental data/evidence summarised below indicates both situations, those where all of the skin-bound residue should be counted in and in others where it probably should not:

- Hood et al. (1996) investigated the importance of permeant levels remaining in the skin i*n vitro* percutaneous penetration of a finite dose of fragrance musk xylol (lipophilic compound) into excised human skin. A 24-h penetration experiment was continued for an additional 6 days. Release of permeant from the skin into the receptor fluid continued during this period, such that on the 7^{th} day 6% of the applied dose remained in the skin. Most of the permeant absorbed into the skin within the first 24h could be available systemically *in vivo* within a week following the application. For this compound skin levels should be considered as potentially systemically absorbed levels.

- Yourick et al. (2004) studied the skin penetration of Disperse Blue 1 (DB1) incorporated into a hair dye formulation and applied to excised human skin. After 24h about 0.2% of the applied dose had absorbed into the receptor fluid, and over 10 times that amount remained in the skin. An extended experiment showed that DB1 levels in the receptor fluid did not increase significant beyond the % of applied dose at 24h.

- The *in vitro* and *in vivo* penetration of retinol into rats was compared by Yourick et al. (cited in (Bronaugh, 2007)). Data obtained after 24h showed that the *in vivo* levels agree closely with the *in vivo* systemic levels, supporting the idea that the amount of permeant remaining in the skin in the *in vitro* experiments should not be counted as potentially absorbed material. A study by Kraeling et al.(2004) on the absorption of consumer doses of the hydrophilic compound diethanolamine (DEA) also showed that after 24h, most of the absorbed dose from various vehicle remained in the skin, with very little penetrating into the receptor fluid. Thus receptor fluid levels of DEA are sufficient for estimates of the potential systemic absorption. The same conclusion was reached for the hydrophilic compound lawsone (Kraeling et al.2007).

8.5 Source of skin sample

The permeability of human skin is very different, depending on the site of body surface. Thus, the source of the samples used for *in vitro* investigations might alter the results leading to either over- or underestimation of dermal absorption *in vivo*. However, as exposure of very permeable skin sites such as scrotum or forehead to pesticide formulations will usually occur accidentally and for a short time only (and these skin areas are relatively small), whereas skin on arms, legs or the back can be certainly expected to be exposed to a larger amount and over a longer time period, but is less permeable. It is important to consider the variation in the barrier function between individuals and anatomic sites (van de Sandt, 2004) and differences in permeabilities between human dose sites may be a source for the differences between *in vivo* and *in vitro* human studies (Dick et al, 1995). As stated earlier, difficulty in applying a homogeneous (finite dose) film of formulation on skin (Mavon et al 2007) may also be a variable.

Cnubben et al 2002 have suggested that perfused ear pig model provides a good prediction of k_p and lag time relative to human skin. In contrast, they suggest that rat skin is more permeable than human skin and *in vivo* absorption in rats (amounts systemically available and k_p value) is 1.5-2.5 greater than in human skin. However, full-thickness rat skin is slightly less permeable than human skin.

8.6 Metabolism in the skin

The assessor should also consider the likelihood of the compound being metabolised on passage through the viable epidermis, recognising that *in vitro* studies will invariably underestimate such effects. In vitro studies with fresh skin using a flow through diffusion cells with a receptor fluid containing appropriate skin nutrients is more likely to adequately define metabolism than a static system. However, Dick et al, 1997 suggest that the degree of penetration is less with flow-through diffusion cells

than in static cells and also less comparable with *in vivo* data - especially for lipophilic compounds combined with a very polar receptor fluid.

8.7 Mass balance issues

The OECD Test Guidelines 427 and 428 require a mean mass balance of between 90-110 % and the OECD Guidance document 28 contain the same recommendation with a caveat that for volatile active substances a range of 80-120% is acceptable. Normalisation of this data is not required by these guidelines.

If the recoveries exceed the accepted maximum range, the data generated should not be normalised. This will result in potentially conservative absorption values**.** If these absorption values are not acceptable when used in a Risk Assessment, then the study should be repeated to address any bias resulting from excessive recoveries.

8.8 Values - mean, median or range

Comment [l8]: Note: should the Guidance Notes contain this proposal? The alternatne approaches include: discarding the text, or stating that if the study contains large variability, then it should not be relied on.

Results obtained from dermal absorption studies can show a high level of inter-animal and inter-sample variation. The underlying cause is often unknown. In performing human health risk assessments involving the dermal absorption of pesticides, mean values are used routinely. This is consistent with the reporting recommendations in the OECD test guidelines.

It has been argued that when there is a large variability in individual results, the use of mean values is not necessarily appropriate, particularly if the number of samples is small or from a limited number of sources (e.g. membranes used *in vitro*). Dermal penetration in humans shows considerable variation between individuals and between anatomical sites (Taylor and Francis date) and there is a need to take account of variability in study results from closely matched samples.

Alternatives to use of the mean values include the median; the mean plus one or more standard deviations (SD); a high percentile value; or the upper end of the overall range. To increase consistency the following approach may be used in evaluating dermal absorption studies in deriving absorption values for use directly in the risk assessment[2] the following is proposed:

- If the standard deviation is <50% of the mean value[3], the mean should be used;
- If the standard deviation is >50% of the mean but less than the mean, the mean plus one standard deviation should be used. [This should give *ca* 87% ile value for normally distributed results.]

[2] Values used uncorrected or the value from an animal in vivo study that is subsequently to be corrected for animal to human differences.
[3] It is assumed that clear outliers will have been excluded from the calculation of the mean values.

- If the standard deviation is greater than the mean, the highest individual value (excluding clear outliers) should be used. [NB -Variation of this extent indicates the overall reliability of the study results should be questioned / evaluated with particular care]

This approach is not based on a detailed statistical treatment but a pragmatic approach. It will tend to be more conservative for studies with low mean absorption values, where the potential for error/variation can have an impact of the greatest magnitude.

8.9 Defining completion of absorption

If dermal absorption studies are performed for only a relatively short time and absorption is not complete there may be a need to consider whether some or the entire residue at the application site is available for absorption.

The OECD test guideline recommends that the residue present at the application site is included in the total amount absorbed. There is also practical experience that by including the application site residue, the variation between individual wells is reduced. However, the OECD test guideline indicates that it would be possible to base absorption on the receptor fluid alone if it could be demonstrated that this was appropriate.

For studies that have included monitoring for several days, the inclusion of the entire application site residue is likely to produce a conservative value for dermal absorption. At present there is no definitive guidance on defining when absorption can be considered complete and, if not how the residue at the application site should be addressed.

The cells of the epidermis migrate upwards, until as the stratum corneum they are sloughed off. This process means that chemical residues remaining in the stratum corneum after washing at the end of a dermal absorption study might not be bioavailable, as the residue will be lost with the cells. The inclusion of such residues in the calculation of the amount absorbed could lead to an overly conservative estimate.

Techniques such as tape stripping can be used to determine if the residue at the application site is in the dermis, epidermis or the stratum corneum. If the sampling period in the dermal absorption study was such that absorption was essentially complete or had declined significantly, it is reasonable to assume that the residue in the stratum corneum, especially the outer layers typically removed by the tape strips, would not be bioavailable and can be excluded from calculations of the amount absorbed. However, at present there is no definitive guidance on the application of tape stripping data.

9 COMPARATIVE VALUE OF DATA FOR THE ESTIMATION OF THE DERMAL ABSORPTION FACTOR

This section will be developed to give the assessor at-a-glance idea of the value of each type of approach in estimating dermal absorption. Against each type of approach will be a comment (qualitative) on:

- precision of quantitation
- possibility of outliers
- relevance to humans
- possible underestimate
- possible overestimate
- etc

To be developed with the Expert Group

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APPENDIX I : Case studies

Comment [l11]: Draft section for Expert Drafting Group discussion

Experience with some recently assessed pesticides may be useful to highlight practical issues that assessors face in determining values for dermal absorption.

Case 1

Chemical A is an active used as a spot-on for dogs to control fleas and ticks. It was applied at the concentration in the product (12.5%) to human skin *in vitro* for 24 hours. Only a very small portion (0.57% of the dose) was detected in the receptor fluid. The experimental method separated the skin into two layers, the stratum corneum (31.01% of the dose, and the remaining part of the skin (8.22% of the dose). The residues retained in stratum corneum could not be washed off with soap and water. Hence, the dermal absorption for humans was considered to be the sum (approximate 40% of the dose) of the amounts in the receptor fluid (0.57%), the stratum corneum (31.01%) and the rest of the exposed skin layers (8.22%).

According to OECD Guideline 428, since the chemical remaining in the skin (including the stratum corneum) at the end of study may continue to be absorbed, this portion should be included in the total amount of skin absorption unless it can be demonstrated that absorption can be determined from fluid values alone.

The dermal estimate figure of 40% was used for risk assessment purposes.

Case2

Chemical B is a herbicide. Biological monitoring of body doses involved calculations from analysis of lawn care specialists' urine collected over a 72 hour interval after the application of Chemical B and ranged from 9.22×10^{-6} to 8.13×10^{-5} mg/kg/lb applied (2.03×10^{-5} to 1.79×10^{-4} mg/kg/kg applied) with a mean body dose of 4.6×10^{-5} mg/kg/lb applied (1.01×10^{-4} mg/kg/kg applied).

The passive dosimetry body dose estimates using dermal deposition measurements corrected for skin penetration and simulated inhalation measurements were calculated for the two clothing scenarios observed in the worker tests. Passive dosimetry measurements for the 18 replicates corrected for dermal penetration and normalized for body weight and amount of chemical handled averaged 8.09×10^{-5} mg/kg/lb applied (1.78×10^{-4} mg/kg/kg applied) for the long-sleeved scenario and 3.62×10^{-4} mg/kg/lb applied (8.14×10^{-4} mg/kg/kg applied) for the short-sleeved scenario. Despite monitoring workers in performance of their full day's work, all urine specimens contained less than 5.4 ppb. As indicated above, skin penetration was corrected. This was done with the use of pharmacokinetic studies in Rhesus monkeys.

The results of an intravenous study indicated that dithiopyr is rapidly metabolized and cleared (>94%) from the body within a 72 hour period. Chemical B is excreted primarily in the urine (64.8%) and the remainder (29.4%) in the faeces. From the dermal application study, it was observed that the majority of the dose, up to 83%, was washed off after a 12 hour exposure period. The skin was excised from the application site and analysed. No significant residue was found in the skin. A portion of the dose was accounted for by related in-vitro monkey skin experiments as losses

due to volatilization. The study data indicated that the amount of percutaneous absorption was less than 1% of the topical dose. This value was used to correct the passive dosimetry data for dermal penetration.

The mean total urine output, normalised for body weight and the amount of active ingredient applied, was 1.01×10^{-4} mg/kg/kg of product applied. Therefore, the conclusion of the assessment was that very little Chemical B is likely to be absorbed by workers during mixing and application exposure.

Case 3

Chemical C is an active in products to be applied topically on dogs and cats as a single spot-on application for the control of fleas and ticks. As the products are for domestic use, the potential for public exposure due to transfer of product from the pet's hair coat to humans through animal handling and stroking activities post-application (dermally and/or orally via hand-to-mouth transfer) is one of the major considerations for the risk assessment.

A "triple pack"of dermal absorption studies was available for evaluation and consisted of rat *in vivo* and *in vitro* and human *in vitro* studies. In all studies, Chemical C was applied to skin as a suspension in tap water (solubility of 0.27 μg/mL) and not in the product formulation.

Results of these studies were utilised for the purposes of interspecies comparison and for establishing a dermal absorption factor for risk assessment. Of the two dose levels tested *in vivo* (0.0024 and 2.4 mg/cm^2 equivalent to approximately 0.1 mg/kg bw and 100 mg/kg bw respectively), the highest level of Chemical C absorption (2.6%) was displayed for the more dilute solution. A similar observation was noted *in vitro*. Absorption of Chemical C across human skin was found to be 6 times lower than that absorbed across rat skin *in vitro*.

Using this information, the predicted *in vivo* absorption in humans after dermal application of Chemical C in aqueous suspension was concluded to be 0.43%.

The level of Chemical C absorption following exposure to the formulation could not be determined from the submitted data, but is suspected to be significantly higher than this value, since both product formulations (for cats and dogs) contain appreciable quantities of known skin penetrant enhancers. The applicant has provided evidence to indicate that the solvents present in the products will evaporate but, as the conditions used were not simulating those in use, the assessment could not determine as to how rapid this may be after dermal application on the animal skin. Exposure of humans to Chemical C though post-application petting activities was considered to be likely in the dry residue form only, when no contact is made with the animal during the evaporation period.

APPENDIX II: Examples for the use of human skin in vitro studies as "stand alone" information to predict dermal absorption of pesticides – inclusion of the stratum corneum levels in the estimate.

The general approach taken in these *Guidance Notes* is that *in vitro* human skin absorption data alone can be sufficient to estimate the dermal absorption percentage to be used for risk assessment. When only rat skin data are available, the most conservative approach would be to assume that human skin absorption would be equal to rat skin absorption. There is no biological reason why absorption through skin *in vivo* should be significantly different from absorption through the same appropriately prepared, viable skin *in vitro*.

A chemical found in the skin *in vitro* should be considered as absorbed and should be added to the amount recovered from the receptor fluid, with the exception of the portion recovered from the stratum corneum.

- In 2005, the Belgian agency (designated Rapporteur Member State in the EU) concluded that the dermal absorption of the herbicide **Bifenox** should be assumed to be 1% (concentrate) or 4% (dilution 1:225) and proposed these values for the EU re-evaluation of this compound. In the lack of any *in vivo* data, these values had been derived from an *in vitro* study on human skin including 8 hours exposure and a total study duration of 24 hours. Recently (January 2006), the same study had been assessed in Germany revealing similar but more precise (*i.e.*, not rounded for uncertainties) values of 3.8% and 0.5%. The EU evaluation has not been finished yet.

- In 2005, an EU expert meeting (EPCO) decided on dermal absorption rates for the insecticide **Fenitrothion** of 3.9% (concentrate) or 20.9% (dilution) on the basis of an *in vitro* study with a microencapsulated formulation on human skin that had thoroughly reviewed by the UK PSD before. In deviation from the UK evaluation, the whole amount in skin was included as a worst-case assumption. There were no other experimental data on dermal absorption available. Is the EU evaluation finalised?

- In 2001 the insecticidal compound **Esfenvalerate** was included in Annex 1 of Directive 91/414/EEC. As a result of the evaluation process on community level, a dermal absorption rate of 10% was estimated solely on basis of *in vitro* studies. A study on rat skin had revealed 44% dermal absorption whereas penetration through human epidermis was much lower (0.6%). Because of this large difference, 10% was agreed on.

- When the active ingredient **Propineb** was included in Annex I in 2004, the following statement was made in the "Final Review Report" with regard to a plant protection product: "The relative skin absorption of technical Antracol 70 WP 24 hours after application – if any - was low and ranged *in vitro* from 0.04 to 1.37% in human skin and from 0.05 to 1.18% in rat skin." Although this is not very precise and, maybe, not very helpful for risk assessment, it can be seen that again *in vitro* studies were taken as the basis for estimation of dermal absorption.

- The compound **Flufenacet** (Fluthiamide) was included in Annex I in 2004. On the basis of *in vitro* studies on human skin, 10% dermal absorption was estimated for the concentrate (to be considered for mixing/loading in the exposure calculation and risk assessment) and 60% for a ready-to-use dilution.

Note:
This list is not comprehensive. At least in some of these cases (*e.g.*, Fenitrothion, Esfenvalerate or Flufenacet), no experimental data were available when the EU evaluation process started. The *in vitro* data were submitted, evaluated and, in time, accepted during this process. When the so-called "triple pack" (*in vivo* rat + *in vitro* comparison human vs. rat skin) is available, these results are usually taken into consideration instead of the *in vitro* study on human skin alone.

APPENDIX III: Formulation effects and skin penetration

Enhancers in formulations can affect skin penetration by: (a) disruption of the stratum corneum bilayer lipids to reduce resistance; (b) altered thermodynamic activity of permeant in stratum corneum – solvent effect; (c) swelling and increased hydration of intracellular keratin.

Below is a table of general effects on skin penetration for a range of chemicals commonly used as non-active ingredients and formulated vehicles for pesticides.

Vehicle component	Mechanism	Notes	Effect	Ref
Mineral oils co-solvents	Increase permeant solubility in vehicle		Increase solubility of lipophilic permeant vehicle – can reduce thermodynamic activ and skin permeation of lipophilic permear	1
water	Increased hydration	Occlusion, hig humidity environment	In general increase penetration of hydroph and lipophilic compounds (up to 100-fold under occlusion compared to no occlusion steroid [3]). Note effect of hydration on animal skin varies to human membranes v rodent skin permeability increasing substantially more when hydrated than hu skin.	2-4
DMSO, DM DCMS	Aprotic solver alter keratin a bilayer lipids	Effect is concentration dependent. >6 needed for substantial penetration increase.	High % causes increase penetration of hydrophilic and lipophilic permeants and skin irritation and damage (erythema and wheals). 15-fold increase caffeine penetra reported with DMF Note effect of DMSO on animal skin varie to human membranes with rodent skin permeability increasing substantially more than human.	5-8
Pyrrolidones e.g. N-methy pyrrolidone (NMP)	Aprotic solver enhance solubility in stratum corne		Enhancement greater with hydrophilic tha lipophilic permeants examples: 200 to 450-fold increase flux mannitol 30-fold increase flux 5-FU 275-fold increase flux sulfaguanidine Cause irritancy and erythema	9-12
Fatty acids e lauric acid, c acid	Alter bilayer lipids	Effective at lo concentrations <10% particul with propylen glycol	Enhancement greater with hydrophilic tha lipophilic permeants examples: Oleic acid: 28-fold increase flux salicylic acid, 56-fold increase flux 5-FU, 10-fold increase estradiol	13-15
Alcohols	Enhance solubility in vehicle and stratum corne lipid extractio	Ethanol is enhancer at up approx 60%; l conc cause dehydration a	Ethanol permeates skin rapidly; common solvent 5-10-fold increase nitroglycerin flux 10-fold increase estradiol flux	16-18

	on prolonged exposure	reduce permeation		
Propylene glycol	As above		Poor enhancement as stand alone vehicle, synergistic effect with other enhancers suc as oleic acid and terpenes: 6-fold increase	19
Surfactants	Solubilise lipi in stratum corneum, inte with keratin		Increase TEWL *in vivo*. Nonionic surfactants (e.g. Tween) have minimal effect compared with ionic surfactants e.g. SLS Note effect of surfactants on animal skin varies to human membranes with rodent s permeability increasing substantially more than human.	20,21
Urea	Increased hydration, keratolytic on prolonged cor or high concentration		Mild enhancement effect at low concentrations e.g. 10%	19
Terpenes – components essential oils	Increase solubility with stratum corne disrupt bilaye lipids		Substantial increase of hydrophilic but no increase of lipophilic permeants: 34-fold increase 5FU by eucalyptus oil (human skin *in vitro*) 95-fold increase 5FU by 1,8-cineole No increase estradiol with 1,8-cineole QSAR important – polar group containing terpenes such as monoterpenes (e.g. mentl cineole) most active penetration enhancers hydrophilic compounds; non-polar group containing terpenes (e.g.limonene) better enhancers for lipophilic permeants. Synergistic effect between terpenes and propylene glycol	19,22

Further general reference for the use of rodent skin *in vitro* are included at reference 25

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