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CEFIC Workshop on methods to determine dermal permeation for human risk assessment

Held in Utrecht 13-15th June 2004

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We report on a workshop, held with the support of CEFIC (European Chemical Industry Council), designed to bring together relevant expertise and to reach a consensus recommendation on a standardised protocol for in vitro determination of permeability coefficients. The proposed protocol is intended, in the first instance, for testing aqueous soluble chemicals only. The Workshop also proposed a strategy to extend the scope of the methodology to the full range of industrial chemicals, and made recommendations for the use of the permeability coefficients in risk assessment.

The rationale for organising the workshop is the European Commission proposal known as REACH, which will require extensive risk assessments of all existing chemicals, including exposure via dermal contact. It is impractical to measure dermal permeation for the many thousands of industrial chemicals in use today. An alternative approach is to base predictions of permeation on statistically derived relationships between physical-chemical properties and the permeability coefficient of representative chemicals, relationships known as QSARs (quantitative structure-activity relationships). However, existing QSARs have been derived from data obtained by a variety of experimental methods, which makes the prediction less reliable. The reliability is to be improved by using a standardised widely-adopted experimental protocol.

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SUMMARY

The meeting was held to address the need to reach a consensus on methods that should be used to determine dermal permeation in anticipation of information required by a proposed new European chemicals strategy involving **R**egistration, **E**valuation, **A**uthorisation (and restriction) of **Ch**emicals (REACH) to control risks. There are currently in excess of 30,000 industrial chemicals in use throughout the EU to produce many hundreds of thousands of products. Data on the potential for dermal uptake are available for only a small fraction of these chemicals and products. Moreover, these data are most often not obtained according to a standardised test protocol.

While there should ideally be human *in vivo* data on how all these chemicals and mixtures are likely to permeate the skin, this is not feasible. The approach of using human skin *in vitro* to determine absorption has been promoted by the EU and recent studies have shown similarity to *in vivo* data. *In vitro* tests are now accepted by many regulatory authorities as a replacement for *in vivo* experiments (e.g. OECD (2004a), EC [Sanco] (2002)). However it is not feasible to make *in vitro* measurements for such a large number of chemicals. The European Chemical Industry (through CEFIC [European Chemical Industry Council; Conseil Européen de l'Industrie Chimique; www.cefic.org]) recognises that there is a need to establish a systematic tiered approach to predicting dermal permeation of chemicals for risk assessment. Prior to the meeting there were concerns that some of the existing data on chemical dermal permeation rates used for QSAR (quantitative structure-activity relationship) predictions may be unreliable because they had been produced from *in vitro* tests conducted using a variety of different methods over more than a decade. The original aim of this workshop was to reach a consensus recommendation on a protocol for an *in vitro* method that would serve to produce reliable and consistent data. Subsequently, the data derived from testing chemicals using this protocol would be used to develop a QSAR linking physico-chemical properties to permeation data.

The meeting attempted to achieve these aims in the context of the process of risk assessment, with presentations and discussions spanning the assessment of dermal exposure, permeation measurements in the laboratory, and application of the permeation data to risk assessment. The discussions led to the conclusion that the existing databases (for the permeability coefficient, k_p), despite some inherent variability due to methodological differences, were acceptable for derivation of the existing QSARs. It was also agreed that it would be surprising if further data produced any significant revision of the QSARs. However, the existing database is primarily for chemicals from specialised sectors or selected on the basis of physicochemical properties and the meeting recommended that generation of *in vitro* data and k_p on a range of relevant industrial chemicals would be valuable in reassuring all stakeholders of the validity and relevance of QSARs within the broad application area of REACH.

The data currently used for QSARs are from infinite dose *in vitro* absorption studies. Such studies determine the maximum flux (for the applied concentration), and from that flux a permeability coefficient k_p is calculated. The permeability coefficients for a set of chemicals are related by QSARs to physical-chemical properties. However, realistic risk assessment scenarios usually correspond to finite dose conditions.

The main immediate need identified was to establish the link between finite and infinite dose experiments, thus linking the QSAR derived information with the inputs required for risk assessment. The meeting recommended this should be done using a standardised protocol, in order to obtain a database with best internal consistency. It was also recognised that it would

be possible, and most useful for the chemical industry, to conduct these tests with chemicals selected as being of greatest relevance to high volume production chemical manufacture. These chemicals may well provide a sufficiently good coverage of the range of physical chemical properties needed to produce data that will support the development of QSAR models.

The linkage between finite and infinite dose experiments relies on mathematical modelling and the associated relevant and reliable experimental data. These techniques enable a sound theoretical basis to be used in the interpretation of the data, and this should improve the reliability of parameters calculated from experimental data. The models also enable extrapolation to predict absorption under different dosing conditions.

The main outcomes of the meeting were:

- definition of a standardised protocol for an *in vitro* method for measuring dermal absorption of industrial chemicals after infinite and finite doses, to be used to produce data for the development of predictive relationships;
- recommendations on the existing status and reliability of QSAR data;
- recommendations on the role of model predictions in generating absorption data for risk assessment;
- recommendations for a strategy for using measurements and predictions of dermal permeation to meet the requirements of REACH;
- suggestions on the steps that will be needed to develop this strategy.

1. INTRODUCTION

1.1 THE WORKSHOP

The Workshop was organised on behalf of CEFIC (European Chemical Industry Council; Conseil Européen de l'Industrie Chimique; www.cefic.org) through a steering committee comprising the authors of this report. The Institute of Occupational Medicine (IOM) acted as principal organiser, with other members of the committee (in particular the Health and Safety Laboratory (HSL)) preparing material for the workshop, leading the work groups and scientific discussion.

The meeting arose from industry's need to develop an efficient methodology to meet the requirements of a proposed European regulatory regime for chemicals; **R**egistration, **E**valuation and **A**uthorisation (and restriction) of **Ch**emicals – REACH. This will involve Registration (of safety data), Evaluation (of risks), Authorisation and restrictions on the use of chemicals of high concern, both old and new. Under the proposals, all chemicals produced or imported into the European Union in quantities above 1 tonne per year would be registered in a central database. Dermal absorption data will be required for substances produced or imported in quantities greater than 10 tonnes per annum. This approach implies addressing the potential for dermal uptake for about 10,000 chemicals and many more mixtures in diverse use across the EU.

Data on percutaneous absorption are required to predict the systemic risk from dermal exposure to chemicals. For certain groups of compounds, such as agrochemicals and cosmetic ingredients, specific guidance on the performance of studies has been prepared by international bodies (OECD (2004a, 2004b), EPA (2004), SCCNF (2003), EC (2001)). This guidance starts from certain assumptions on the exposure scenarios (e.g. exposure time, dose applied) and the results are normally presented as relative absorption (percentage of applied dose). For many industrial chemicals, these exposure scenarios are often not known and the number of chemicals that need to be addressed within REACH far exceeds the number that can be tested economically.

Prior to the meeting, the expectation had been that the workshop would focus mainly on producing a consensus protocol that would be suitable for producing a data set with the best consistency and reliability, and thus would enable the development of relationships between chemical properties and the dermal permeation rates (known as quantitative structure-activity relationships (QSARs)). Therefore, the papers circulated prior to the meeting included a draft protocol (for an *in vitro* method) with notes on the contrasting specifications in the various published methods, protocols and guidelines.

1.2 THE PURPOSE

The broad aim of the Workshop was to achieve recommendations on the best way forward with regard to methods for determining dermal permeation rates and the use of this type of data within human risk assessment. More specifically the workshop aimed at producing a recommendation on a consensus protocol for *in vitro* determination of k_p values for further development of QSARs. This was to be done within the context of the possible future regulatory framework for chemical risk assessment (REACH).

1.3 THE STRUCTURE OF THE WORKSHOP

The Workshop was held over two days, and it involved initial presentations setting set out the issues and the background information. Then discussions were held in working groups, with

reporting back to general discussions of the whole Workshop, where recommendations were adopted in general discussion of the whole group.

The discussions were held in three working groups, with chair and rapporteur from the steering committee. The working groups were:

- *in vitro* methods, chaired by Dr Dick, rapporteur Dr Van de Sandt (which is described in Chapter 3);
- QSAR methods, chaired by Dr Cronin (described in Chapter 4);
- Selection of chemicals, chaired by Dr ten Berge (summarised in Chapter 8).

The process of discussion continued after the meeting through comments on draft reports.

1.4 THIS REPORT

This report summarises the conclusions that were reached in the discussions. As a draft, it was circulated and agreed by the Steering Committee. The Steering Committee's agreed draft was then circulated to all participants who were asked to raise any important omissions or to inform us if they dissented from any of the conclusions as reported. The report thus reflects a final consensus view of the Workshop participants.

The structure of the report follows the course of the meeting, modified slightly by the logical linkage of the topics. (A glossary is provided as Appendix 1).

- Chapter 2 describes the presentations, and the concepts of what dermal permeation involves and what the meeting should seek to achieve in recommending a protocol and a strategy for its application;
- Chapter 3 describes the consensus view of the current methods of measuring dermal permeation, which is obviously the necessary background to agreeing recommendations on a method;
- Chapter 4 outlines the recommendations from the QSAR workgroup as finalised from general discussion of the full Workshop;
- Chapter 5 describes the current developments in the application of mathematical modelling to relating permeability coefficients (from idealised infinite dose conditions) to the finite dose conditions that are more typical of occupational exposure;
- Chapter 6 summarises the current situation with respect to the linkage between infinite dose and finite dose results;
- Chapter 7 discusses the application of the permeability coefficients to occupational scenarios;
- Chapter 8 summarises the recommendations from the Chemical selection Working Group, as further discussed and accepted by the whole Workshop;
- Chapter 9 summarises the recommendations from the *in vitro* working group as further discussed and accepted by the whole Workshop;
- Chapter 10 summarises the limitations to the scope of the recommended protocol;
- Chapter 11 describes the Workshop's recommendations for a strategy as to how best to use the protocol, and how to address the limitations of the current methodology. The use of the recommended protocol should enable production of a data set that could enable predictions (of dermal permeation in occupationally relevant conditions) for a wide range of chemicals. The second part of the strategy proposes how to extend the methodology to deal with chemicals that are outside that range.

The protocols are included as Appendices 2 and 3. Appendix 4 contains a report from QSAR work Group. Points of important detail raised in comments that have not been fully covered

in the report are listed in Appendix 5. Appendix 6 contains a summary of information relating to one of the presentations.

The participants in the Workshop are listed in Chapter 13. Their positive and constructive contributions, at the workshop and in comments on the draft report, were essential to achieving this consensus report.

2. PRESENTATIONS AND CONCEPTS

2.1 PRESENTATIONS

The workshop involved eight presentations which:

- set out the objectives of the workshop (Dr Wil ten Berge);
- summarised current experience in *in vitro* methods (Dr Ian Dick);
- described the key findings from the recently completed EU funded project EDETOX (Evaluation and Prediction of Dermal Absorption of toxic chemicals) (Professor Faith Williams);
- outlined the state of the art in the application of quantitative structure-activity relationships (QSARs) in relating measured permeability coefficients (k_P) to properties of the molecule (Dr Mark Cronin);
- summarised the current regulatory practice (Dr Cees de Heer);
- outlined a recent research project on the measurement of dermal exposure (Dr John Cherrie);
- described the mechanisms of dermal permeation with a comparison of techniques using human and pig skin (Dr Winfreid Steiling);
- described the application of mathematical modelling to obtain permeability coefficients from finite dose experiments where the data had been sufficient for this purpose (Dr Jacob Krüse).

Additionally, data from modelling the permeation rate from finite dose experiments were presented (Professor Annette Bunge).

Video recordings of the presentations were made and are available on DVD.

2.2 CONCEPTS AGREED FOLLOWING THE PRESENTATION

2.2.1 Scope of the method

Following the presentation on the objectives, it was agreed that it would be impracticable to endeavour to undertake tests for every chemical at doses and vehicles corresponding to diverse exposure scenario potentially arising in industrial use. The meeting would seek to recommend a method that could be used to produce data to develop the QSAR approach.

However, it was suggested that the numbers of chemicals that might need to be addressed could be substantially reduced by excluding those for which testing (for dermal permeation) would be inappropriate, such as corrosive or irritant chemicals (which would damage the skin and would probably not be left in contact with the skin in normal use). One recommendation from the workshop was that it would probably be very informative to compile a list of the chemicals that would remain after such exclusions. Even after reductions, the number of chemicals likely to remain would be much more than could be reasonably dealt with as individual cases (with test conditions chosen to mimic a specific exposure scenario).

2.2.2 Concepts of skin permeation

The skin may be considered as comprising of three layers, the external layer is the stratum corneum which is approximately 10 to 20 µm thick, comprises dead cells and is lipophilic. Then there is the viable epidermis which is approximately 100 µm thick, comprises viable cells, and is hydrophilic although cell membranes are lipophilic. The third layer is the dermis

which is richly perfused with blood. Skin appendages such as hair follicles, sebaceous glands and sweat glands may also act as transport routes for absorption of chemicals. However, the skin can be viewed as two barrier layers, the lipophilic stratum corneum and the hydrophilic epidermis. The principal barrier function of the skin is provided by the stratum corneum, although the viable epidermis acts as an additional barrier for lipophilic chemicals.

The mechanism by which chemicals cross each barrier is diffusion. Fick's law describes the net rate of transport due to diffusion where there is a concentration gradient in a given medium, and it states that the rate of transport is proportional to the concentration gradient. Where a chemical is in two different media (e.g. in the aqueous vehicle on skin surface and in the lipophilic stratum corneum), then its stable concentrations in the two media may be different, and the ratio of these concentrations (at equilibrium across the boundary) is described by a partition coefficient.

The absorption rate or flux (J) measured in *in vitro* tests describes the net rate of transport once equilibrium (steady-state) conditions have been reached. The permeability coefficient (k_p) is the flux divided by the total concentration difference across the skin membrane, for measurements made with an infinite dose (i.e. a volume of dose large enough such that the loss of permeant to the skin has negligible effect on solution concentration). The permeability coefficient thus describes the net effect of both partition between media and diffusion through the skin. These two effects can be distinguished by using the partition coefficient to calculate the concentration at the skin surface (i.e. on the skin side of the boundary between two media), and then using this concentration to calculate the concentration gradient across the skin. (The concentration gradient is the concentration difference divided by the path length.) The diffusion coefficient (D) is the flux divided by this concentration gradient at steady state. Where the receptor concentration is negligible compared to the external concentration (as is usually the case in test conditions), this leads (as shown in Chapter 5) to a simple relationship between the permeability coefficient (k_p), the partition coefficient between the vehicle and skin (K_m), and the diffusional pathlength (l_m).

From Fick's Law, it follows that the rate of transport through the skin will be dependent on the concentration of the chemical applied. For a given vehicle, concentrated chemical solutions will cause much more rapid absorption than dilute chemical solutions. Consequently, the risks of significant internal dose are greater where concentrated chemicals are being used. The exposure situations which may give rise to significant internal dose from dermal exposure have been discussed in the review by Semple (2004).

The extent of dermal absorption of a chemical is dependent on its form (solid, liquid, vapour) and the vehicle in which the chemical is presented to the skin. Water is considered an appropriate vehicle for chemicals which are water soluble; if such a chemical is in solid form, then an aqueous solution may mimic the likely dissolution in the moisture on the skin. For chemicals which are not water soluble, the choice of a suitable organic solvent as a vehicle is more complex. Many organic solvents are themselves chemicals for which dermal exposure would be a concern, e.g. ethanol, isopropanol, acetone, toluene, xylene, or mixtures of these. For such solvents, dermal exposure can be a major contributor to total uptake (Semple, 2004). The vehicle can also substantially affect the skin's integrity, and therefore the choice of vehicle can be both important and complex.

In principle, provided the vehicle does not affect the integrity of the skin, the flux of a given chemical from different vehicles should be the same for thermodynamically equivalent (e.g. saturated) solutions.

The major problem in the past in attempting to link QSARs with risk assessment is that there have been, and still are, different priorities which have influenced the choice of experimental

procedure to collect permeability data. For development of QSARs, the priority is to avoid bias or variation from factors other than the properties of the chemical substance under test. Whereas, for risk assessment, the variability in work environment and exposure scenario needs to be taken into account (either in the calculations made using QSAR derived parameters or in the choice of experimental technique). Factors which can be important include solvents (vehicles), concentration, volume of dose, washing methods, mixtures, and temperatures; and they all need to be taken into consideration in the overall risk assessment. However, whilst duplication of all realistic exposure scenarios is impracticable for the range of industrial chemicals in diverse situations, risk assessments will be needed for these multiple scenarios. The challenge for this meeting was to develop a test protocol suitable to provide an efficient strategy that would meet this need.

2.2.3 Dermal exposure

The presentation on the measurement of dermal exposure (by Dr Cherrie) described a conceptual model of the processes involved with dermal exposure (Schneider *et al*, 1999). This recommends that data on the mass and concentration of contaminant chemicals in the skin contamination layer (the fluid layer covering the *stratum corneum*) should be measured along with the area of skin contaminated and the duration of exposure. However, currently available methods for measuring exposure do not provide this information and, in particular, there are no methods currently available to measure the concentration of contaminants in the skin contamination layer.

An extensive set of measurement data have recently been obtained as part of a multi-centre research project funded by the EU (RISKOFDERM). This project has been summarised in a number of papers and in overview by Rajan *et al* (2004). The measurements were made in many different workplaces for several different substances but only provide data for the mass of chemical - either landing on the skin or remaining on the skin after some period of exposure.

Some recent research has focused on an improved method of measuring exposure that is intended to mimic the skin and provide a “biologically relevant” measure of exposure (Lindsay *et al*, 2003). Although not yet commercially available, this methodology provides the possibility of making exposure measurements that are more relevant to risk assessments. In the meantime it is important to ensure that there is compatibility between the data that are collected during exposure assessments and the methodology used to estimate dermal uptake by skin permeation.

3. VIEWS ON CURRENT METHODS OF MEASURING DERMAL PERMEATION *IN VITRO*

The current internationally-recognised methods approved for measuring dermal permeation using skin *in vitro* are not very prescriptive and include several options (e.g. OECD, 2004b). There are options for the type of skin that may be used (human skin from surgery or from cadavers, or animal skin). The preparation of the skin may vary with some methods using full thickness skin and others using dermatomed skin (*stratum corneum* and epidermis with partial layer of dermis) or epidermal membranes. The storage process may also vary (refreezing being advised against but not excluded) as may the composition of the receptor fluid used to mimic the capillary blood circulation (in which the chemical must be soluble). This variability, allowed in the OECD guidelines, arose from the practical need to enable testing in a range of laboratories world wide in order to supply data needed to produce conservative risk assessments for a range of exposure scenarios. However, for the current exercise, the purpose is different; a data set with best internal consistency is required. Minimisation of all differences in experimental procedures is important if the best internal consistency of the data is to be obtained from a study (Van de Sandt *et al* 2003, Chilcott *et al* 2004)

The results from the EU funded EDETOX project defined the robustness of the *in vitro* method with human skin between laboratories and identified that the factors that contributed most to inter and intra laboratory variability were skin thickness (of skin samples used in the study) and human variability in skin permeability (Van de Sandt *et al*, 2003). The importance of directly parallel *in vivo* studies in volunteers in validating the *in vitro* methods was confirmed and it was shown that inter laboratory and inter individual variation in measurements of dermal penetration *in vivo* need to be taken in to account (Jakasa *et al*, 2004). For use of *in vitro* dermal absorption measurements directly for risk assessment, selection of dose and vehicle to reflect the exposure scenario was important mainly because of the lack of understanding of the relationship between finite and infinite dose and the effects of vehicle on absorption (Williams *et al*, 2004). A conclusion from the EDETOX study was that currently, data generated from a QSAR prediction is a good guide but a well designed *in vitro* absorption study may still be needed (in some circumstances) to examine actual and specific exposure conditions. The EDETOX project has produced a mechanistic model that is well suited to modelling dermal uptake of chemicals from actual exposure scenarios (Krüse *et al*, 2004). Further development of this model was encouraged as it could mathematically determine the profile of dermal penetration through skin *in vivo* and *in vitro* following low level short term doses.

In the EDETOX project, k_p values were determined for 21 new substances following application of infinite doses in saturated aqueous solution to human skin. This data set was not of sufficient size on its own to define a statistically significant QSAR. However, when the EDETOX data was combined with that of Patel *et al* (2002), it did not change the fit to the widely accepted Potts and Guy type equations which relate permeability coefficient and the two physicochemical parameters, the molecular weight and the octanol-water partition coefficient (P_{ow}) (Fitzpatrick *et al* 2004, Golden *et al* 2004)

The meeting recognised that the Potts and Guy relationship is scientifically sound in that a combination of molecular size (described by molecular weight) and the stratum corneum-aqueous vehicle partition coefficient (estimated from the octanol-water partition coefficient (P_{ow})) would be expected to be the main characteristics of the chemical which would affect its dermal permeation. The goodness of the statistical fit is supportive of the validity of the data. Where slight improvements in statistical fit have been achieved by including extra physicochemical descriptors such as hydrogen bonding and terms for the strength of crystal

forces (melting point), it is quite likely that they are not real improvements in the model. The existing data also span a wide range of molecular weight, diffusion coefficient, and log P_{ow}.

Much of the data (used for the QSAR models) have been generated for chemicals that are relevant to specialised sectors (such as pharmaceuticals and pesticides) or selected on the basis of their physicochemical properties and may therefore not be typical of the chemicals used in other sectors of industry. Therefore it was recommended that further dermal permeation measurement should be conducted for industrial chemicals that are produced in high volumes. It would be useful to demonstrate the validity of the existing QSAR models for more commonly used chemicals. The supposition is that inclusion of the new data (together with the EDETOX data) will strengthen the relationship and the fit of the existing QSAR, and would be important in providing evidence to support a general acceptance of its application to a wider range of chemical groups.

Furthermore, it was noted that the diversity of experimental procedures used in producing the data in the Cronin database may still be a cause for concern, and consequently the proposed additional high quality data would greatly improve confidence in the QSAR.

One important limitation to the application of the present QSARs for risk assessment is that they are based on aqueous solutions. The reasons for this and the implications are outlined in Chapter 10. The recommendations from this meeting proposed that aqueous solutions should continue to be used. Two phases (or themes) of work were proposed; they could be undertaken in parallel. Phase 1 would consist of developing the dataset for substances that could sensibly be tested in aqueous solution. Phase 2 would address the issues involved in dealing with the highly lipophilic chemicals and the effects of alternative vehicles.

4. QSAR (QSPR) ANALYSIS

4.1 ROLE OF QSARS

Quantitative structure-activity relationships (QSARs) are generally used to relate properties of chemicals to biological effects or transport properties; when applied to estimating dermal permeation, they are sometimes known as quantitative structure-permeability relationships (QSPRs or QSPeRs). In this report, QSAR is used to embrace general issues; QSPR is used for a QSAR that predicts a permeability coefficient. QSARs are involved at two levels in the relationship between chemical structure and permeation property. Firstly, the octanol-water partition coefficient (P_{ow}) has been measured for some chemicals but determined for others from a QSAR, although probably quite well predicted. The skin permeability coefficient (k_p) is predicted from molecular weight (MW) and P_{ow} by a second QSAR.

4.2 WORKGROUP DISCUSSIONS

The work group on QSARs produced a detailed report on their discussions (Appendix 4). Their main recommendations are summarised here.

4.2.1 Scope of QSARs and interaction with mathematical models

A QSAR attempts to relate statistically the biological fate or activity of a chemical (or series of chemicals) to its physico-chemical properties. It is an observation of the association between an outcome and the properties likely to affect that outcome. It is not an expression of a theoretical relationship, and is therefore complementary to mathematical models which express theoretical relationships (such as describe diffusion or other thermodynamic effects). A QSAR is complementary to mathematical modelling in providing predictions of coefficients needed to estimate absorption for untested chemicals. Conversely, mathematical models can be used (as described in Chapter 5) to produce estimates of k_p from finite dose experimental data, and thus can provide further input to QSARs.

Given that the complementary roles of mathematical models and QSPRs can serve to optimise the use of data, the QSAR Workgroup recommended that **more effort should be made to link mathematical modelling and QSPRs**.

4.2.2 Data requirements

The development of a QSAR involves establishing a statistical relationship between a biological effect (e.g. toxicity or permeability) and appropriate physico-chemical and /or structural properties available from either analytical measurements on the chemicals (e.g. octanol-water partition coefficients) or from fundamental properties of the molecule (e.g. molecular weight). The main requirements for constructing a reliable QSAR are that the data (observed permeation) should be consistent, produced from standardised experimental procedures, and be obtained for a set of chemicals that cover the domain of relevant chemical properties.

4.2.3 Outputs from QSPR

The permeability coefficient (k_p) has been the measure of skin permeability that has been most widely used for QSPR modelling. The work group recognised that k_p may not be directly suitable for application in risk assessment, but that (as described in Chapter 7) it can be used in conjunction with measured (or estimated) solubility to predict a maximum flux through the skin. This coefficient k_p has been preferred for QSPR modelling because it is a

measure that characterises the intrinsic steady state properties of the chemical and the membrane. The effects of variation in experimental conditions (solvent, temperature etc) on k_p are more difficult to characterise by a statistical relationship (from the existing database).

Other types of permeability data may, in principle, be predictable from QSARs. For example, maximum flux data could be modelled with QSARs. Data for "Percentage absorbed" are more specific to the particular conditions, and are less likely to be amenable to a QSAR model. Nevertheless there may be scope for using mathematical models to predict such percentages using permeability coefficients provided from QSPRs (as discussed more in Chapter 5).

The workgroup recommended that **the permeability coefficient k_p should continue to be used in QSPR development, as it is a steady-state parameter amenable to QSPR modelling and there is already a significant database available**.

4.2.4 Ionised compounds

The development of many QSARs, in general and for skin permeability in particular, has assumed and required that the compounds are not ionised. Whilst ions permeate the skin at a reduced rate (compared to the neutral molecule), ionised compounds have greater solubility and that tends to increase permeation. Such opposing effects lead to apparent inconsistencies in the data. Furthermore, the presence or absence of salts in an ionised solution affects transport. The workgroup recommended that **the solution (in which the chemical is presented to the skin) should be buffered to prevent ionisation**.

Furthermore, the workgroup noted that are other specific issues with certain groups of chemicals, such as the zwitterions which are effectively ionised but not charged.

The workgroup also recommended that **the permeability coefficients for use in QSPR analysis should be for the non-ionised compounds, and the QSPR predictions should be made only for the non-ionised compounds. As discussed at other times in the meeting, more work is required to elucidate the effect of ionisation on skin permeability.**

4.2.5 Consistency of protocol used in generating permeation data

The work group recommended that not only should a consistent protocol be used for generating future data (for QSPR development), but that also **existing data should be checked for consistency and any differences in protocol identified.** This recommendation was supported by other discussions at the meeting.

4.2.6 Potential accuracy of predictions from QSPRs

The accuracy of predictions from QSPR are limited by the inherent large variability in the experimental measurements. Knowledge of the experimental error should be incorporated into the development of QSPR, into the comparison of models with data, and into the expression of predictions. **Confidence intervals should be given in order to enable realistic application of QSPR predictions.**

4.2.7 Descriptors of physico-chemical properties

The workgroup concluded that the parameters used by Potts and Guy (1992), the molecular weight and log P_{ow} are a good basis for modelling permeability coefficients. The use of additional parameters in a QSPR (to improve statistical fit) should not be discounted but should be treated with caution. The workgroup noted that log P_{ow} has error associated with it,

both as a measured value and as an estimated value (Dearden and Bresnen, 1988). Furthermore, estimated (predicted) values of log P_{ow} can depend on calculation procedures. The workgroup recommended that **consistency should be ensured in the calculation of (predicted) values of log P_{ow}. Furthermore, attention should be given to the applicability of the value of log P_{ow} to the conditions of the test or exposure (e.g. pH of the vehicle) and that is a necessary part of ensuring that predictions fall within the applicability domain of the model** (see recommendation in Chapter 4.2.12 on defining and using an applicability domain).

The workgroup recommended that while molecular weight (*MW*) provides a simple and unambiguous estimate of molecular size, it is relatively unproven as the relevant size parameter for high density chemicals (because relatively few of them are included in existing databases). Their absence from existing databases may be because k_p is predicted to diminish rapidly with increasing chemical size and so very low permeation is expected for high molecular weight compounds (unless the log P_{ow} is high). Nevertheless, **the modelling of such high molecular weight chemicals requires further attention.**

4.2.8 Dataset quality

The quality of the biological data upon which a QSAR or QSPR is based largely determines the quality of the resultant model. Therefore, future reporting of such biological data (e.g. skin permeability coefficients) should include comprehensive notes on the procedures and experimental conditions. Other factors such as the purity of the compound being assessed, metabolism and degradation should also be considered.

There has been a general view that some of the historical skin permeability data were of questionable quality (cf. Moss and Cronin, 2002). However when the EDETOX infinite dose dataset (generated with standardised robust methodology) was combined with that of Patel *et al* (2002), it did not change the fit to the widely accepted Potts and Guy type equations which relate permeability coefficient and the two physicochemical parameters, the molecular weight and the octanol-water partition coefficient (P_{ow})

The approach applied in the EDETOX project to collating skin permeability information from the literature is important and may have widespread applicability. The EDETOX database www.ncl.ac.uk/edetox (Soyei and Williams, 2004) illustrates the wide variability in methods used to generate *in vitro* data and assesses the quality of the data against a set of criteria. Much of the available data is not generated using infinite dose conditions and is not appropriate for inclusion in the existing QSAR datasets

It is also recognised that regulatory agencies could potentially be a good source of data for modelling (Bronaugh 2004, personal communication). However QSAR modelling of regulatory data for other (acute toxicity) endpoints has illustrated the problems that may be faced (Lessigiarska *et al*, 2004). These include finding data for single organic substances in aqueous solution and the problems in variability of experimental protocols that may introduce uncertainty into the dataset.

The work group recommended that **a minimum set of criteria are required to describe the experimental protocol, these should be established and applied to the datasets modelled.**

4.2.9 Mechanism of action

A requirement for "high quality" QSARs is that they should be based on an established mechanism of action. The definition of a mechanism of action will also be a key point in the validation of QSPRs for regulatory use (Worth *et al*, 2004). For percutaneous absorption,

there is a general agreement on a single mechanism of action; and that will assist in the successful building of models for permeability coefficients. The mechanism is generally considered to involve partition of molecules on the basis of their lipophilicity and diffusion on the basis of their size. It is assumed that the mechanism is not class specific; and if that is valid then there is considerable potential for one global QSPR.

A single "global" QSPR would be the ideal situation. A "global" QSPR is one that could be applied to any chemical class (within the applicability domain of the model). As well as making the model more general to use, this would also eliminate the problematic derivation and utilisation of class specific models.

4.2.10 Statistical analysis (linear vs non linear) methods

Diverse statistical methods may be applied to the building of QSARs, some are linear in their nature and others non-linear (Livingstone, 1995). For many QSARs, including those for skin permeability coefficients, regression analysis is the statistical method of choice as it is simple, transparent and highly portable (Cronin and Schultz, 2001). However, there are a number of drawbacks in the use of regression analysis, including that it is by its very nature a linear technique, and that it is adversely affected by collinearity between independent variables (e.g. log P_{ow} and *MW*). For QSPR, there are two specific issues relating to the use of linear modelling techniques: whether regression analysis is a suitable technique for the development of QSPRs; and whether linearity is appropriate for modelling of highly hydrophilic and hydrophobic molecules. The other multivariate options include partial least squares and neural networks.

The work group recommended that **a high quality dataset (e.g. EDETOX dataset or data to be generated in this project) be modelled by a variety of methods to determine the relative merits of, for instance, regression analysis, partial least squares, and neural networks**.

4.2.11 Chemical selection

The selection of chemicals (for further tests on dermal permeation) will rely on a number of factors (see Chapter 8). However, the process of the selection of chemicals should also use a chemometric analysis to ensure that those selected compounds provide the maximum possible information. To enable successful application of chemo-metric analysis, the appropriate physico-chemical descriptors for the QSPR must be established. If this is restricted to log P_{ow} and *MW*, selection of chemicals is easier than for a more multivariate situation. Chemical selection should also be driven by an assessment of how representative the current database is, and what deficiencies it has.

It was further noted that there may not be a linear relationship between permeability coefficient and hydrophobicity for the complete range of log P_{ow}. In particular highly hydrophobic compounds may not be modelled well by a linear QSAR (*this is reflected in EC guidelines (EC, 2002; where a reduced default absorption is assumed for risk assessment if log P_{ow} is either <-1 or > 4)*. However, currently, there are insufficient data to assess this effect. The work group also recommended that **the modelling of highly hydrophobic compounds should be emphasised, especially if further testing is undertaken.**

The work group recommended that **the selection of chemicals for further testing should be underpinned by chemometric analysis of the current database to determine the areas in which knowledge is currently lacking as well as a need to include high volume use chemicals.**

4.2.12 Applicability domain for QSPR

The applicability domain of a QSAR is defined as "the physico-chemical, structural, or biological space, knowledge or information on which the training set of the model has been developed, and for which it is applicable to make predictions for new compounds" (Jaworska *et al* 2003). However, no formal methods currently exist to define the applicability domain of a structure-based prediction method (although work is in progress in this area).

It is accepted practice that a QSAR should not be used to make predictions outside of its applicability domain (Cronin and Schultz, 2003). If a global QSPR can be based on log P_{ow} and *MW*, then an applicability domain may be defined relatively easily and may be shown graphically on a 2-dimensional plot. Such an applicability domain will probably be elliptical in shape (because there are few, if any, low molecular weight molecules that are hydrophobic). The work group recommended that **the applicability domain should be defined for any QSPR developed; and all predictions should be only for chemicals within the applicability domain.**

5. MATHEMATICAL MODELLING

The principle underlying the calculations of dermal absorption is that if the transport properties of the chemical are known, and the resistance of the barrier is known, then the amount crossing the barrier can be calculated for any scenario of changing concentration. The skin is a complex organ, with hair follicles and sweat glands which can act as routes for chemicals to reach the dermis (and some of the more detailed models include simulation of these routes). Nevertheless, the treatment of the skin as a mechanical barrier is a very useful approximation. The structure of the skin is described in Chapter 2. The main barrier, the stratum corneum is about 20 µm thick. The next layer, the viable epidermis, is about 100 µm thick and is living tissue (where the lymph channels have an affinity for lipophilic compounds). Next, there is the dermis which is about 1000 µm thick and, *in vivo* would have been perfused with blood in capillaries.

There are several models which have been used for calculating skin permeation. The simplest treats the model as a single barrier with the permeation calculated from an equation of the same form as Fick's Law of Diffusion.

$$Flux = k_p \cdot (C_{outside} - C_{inside}) \qquad mg/cm^2/hour, \quad C_x = mg/cm^3$$

$$k_p = \frac{K_m D_m}{l_c}, \; if \; C_{inside} = 0$$

$$K_m = partition\ coefficient\ skin/vehicle$$

$$D_m = diffusion\ coefficient\ in\ stratum\ corneum\ \ (cm^2/hour)$$

$$l_c = thickness\ stratum\ corneum\ \ (cm)$$

$$k_p = permeability\ coefficient\ \ (cm/hour)$$

Once the steady state conditions are reached, the cumulative amount absorbed increases linearly with time.

There has been substantial work on the development of mathematical models for describing percutaneous penetration, (see, for example, Hadgraft 1987, McCarley and Bunge 1998, Redddy *et al* 1998). However, many existing models do not appear to have been applied to the task of predicting dermal uptake in a manner suitable for risk assessors to use. This section describes two applications of modelling which do attempt to produce such information.

A more detailed model as used by Krüse and Kezic (2004) addresses the effects of differences in the solubility of the chemical in the different media (the water, the stratum corneum, and the epidermis). These differences are expressed as partition coefficients (the ratio of the concentration in one medium compared to that in the next medium at equilibrium) for the aqueous solution /stratum corneum boundary and for the stratum corneum /epidermis boundary. The model is based on Fick's Law of diffusion within each layer (and based on a diffusion coefficient for the chemical in each layer). The model has more parameters (to be fitted), but it describes the time course of permeation more thoroughly, and is in principle more appropriate to predicting the consequences of non-steady state doses. It has been fitted to data describing the time course of permeation of chemical into the skin, *in vitro.*

The rate limiting step of permeation is usually diffusion through the stratum corneum. However, for some compounds (highly lipophilic compounds), that have a much higher

solubility in the skin than in water (e.g. by a factor of 1000), the diffusion within the aqueous vehicle towards the skin may become a rate limiting step. For compounds that have higher solubility in the skin than in water (but not necessarily by such a large factor) the diffusion through the viable (aqueous) epidermis may become a rate limiting step.

Mathematical modelling has a key role in linking the permeability coefficient obtained from tests under idealised, infinite dose conditions (i.e. steady-state conditions) to the permeation that will occur under the finite dose conditions more typical of occupational exposure (i.e. non-steady state conditions).

6. RELATING FINITE-DOSE AND INFINITE-DOSE RESULTS

An infinite dose is defined as the amount of test preparation applied to the skin being such that a maximum rate of absorption of the test substance (per unit area of skin) is achieved and maintained (OECD, 2004). That is, a maximum rate for whatever chosen concentration of test substance is applied. Thus, infinite dose is the situation where the volume of donor fluid (i.e. the aqueous solution of the chemical being tested) is large enough that, although chemical is taken up into the skin during the test, the donor fluid concentration of the chemical is not depleted.

Finite dose is the converse, where the volume of donor fluid is small enough that it will influence take-up of chemical into the skin. The maximum absorption rate may be reached for some of the time, but it is not maintained or may not be achieved. The concentration of the chemical in the donor fluid changes due to uptake of chemical into the skin, and may change due to evaporation of donor fluid. This situation occurs in the real occupational exposure scenario as well as in the *in vitro* test (unless the test cell is occluded).

The infinite dose assay allows the permeation rate to reach a steady state. This enables the slope from a plot (as shown in Figure 1) of the amount penetrating versus time to be read and used to calculate a steady state permeability coefficient (k_p). There is usually an interval between applying the dose and the steady state being reached. The "lag time" is derived from a graph of the cumulative absorbed dose versus time (Figure 1), and it is the intercept (on the time axis) of the tangent to the linear part of the absorption profile.

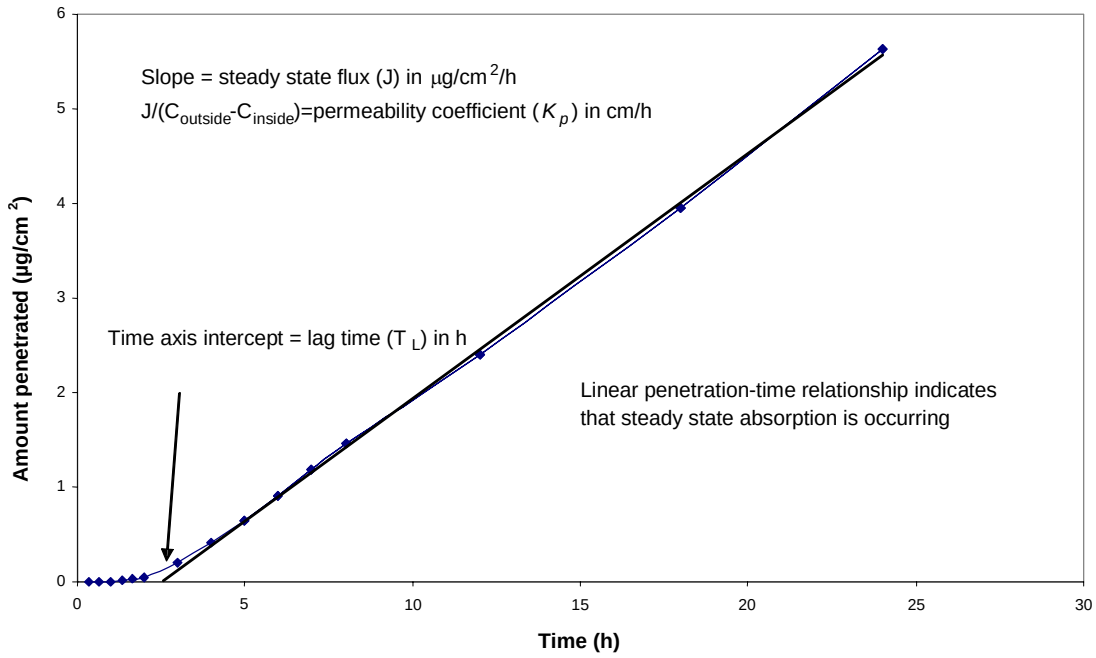

Figure 1 Illustration of steady state flux, permeability coefficient, and lag time.

The presentation from Dr Jacob Krüse explained how mathematical models can use the permeability coefficient (determined with an infinite dose in an *in vitro* test) to calculate the flux and the dose received in a finite dose situation. He also explained how the model could use finite dose test data to obtain values for k_p. Using this approach, experimental data obtained by the finite dose test (Wilkinson *et al*, in the EDETOX project) were analysed by his new numerical model implemented using the Berkley Madonna Package (Krüse and Kezic 2004, Golden *et al* 2004) using two alternative weightings. This gave results which showed

that the values of the two fitted parameters (partition coefficient K_m stratum corneum/water and diffusion coefficient in the stratum corneum) were slightly different according to whether greater weighting was given to the early time points (by expressing the data as flux rates) or to the later time points (by expressing the data as cumulative dose) (Krüse and Kezic, 2004). The differences in the estimates were of the order of a factor of 1.5. Assuming the fitted parameters are constant over the exposure scenario, the fitted model is capable of predicting the permeation under any scenario including the infinite dose and thus links finite and infinite dose experiments. However, only a limited number of substances (*in vitro* data for five substances, and *in vivo* data for two) have been modelled so far, so the reliability and accuracy of such predictions needs to be established. Also, the domain where extrapolation is valid has yet to be established.

These data were also fitted with an implementation of an existing pair of mathematical models developed by Anissimov and Roberts (Golden *et al*, 2004). The two approaches gave acceptably consistent results; and showed that the transport parameters (such as k_p) derived were better (i.e. give a more realistic physical description) than those calculated when simplifying assumptions (e.g., that a steady state has been reached) are made in the interpretation of apparently infinite-dose data. These observations give confidence that these methods can determine reliable transport parameters from infinite and finite dose experiments.

There are two further consequences flowing from this: firstly the data upon which any resultant QSAR may be based will be more reliable and this reliability may be the key to good predictions for untested chemical entities; and secondly when reliable parameters have been derived for a substance, Krüse's model allows for the modelling of a range of pertinent absorption regimes including those relevant to real exposure scenarios such as finite doses at multiple exposures.

The application of finite dose data to the calculation of k_p depends on the adequacy of the experimental data. The key feature was the collection of data at short exposure times that characterised the changing permeation rate at the start of the experiment (i.e. non-steady state kinetics). This required measurement of low concentrations of chemicals in the small samples of receptor fluid which was achieved by using a radio-labelled chemical. The early time points at which data should be collected will vary between chemicals. It was recommended that the selection of time points for collecting data should be designed for each chemical either by modelling (prior to the test) or by pilot tests.

To use the more detailed model to predict the dermal permeation for an exposure scenario with non-steady state conditions, additional information would be needed: the partition coefficients, and the lag time from the *in vitro* measurements. The lag time (t_{lag}) is related to the diffusion coefficient (D_m) and the diffusion path length (l_c):

$$t_{lag} = \frac{l_c^2}{6 \cdot D_m} \qquad K_p = \frac{K_m D_m}{l_c}$$

Usually, the values of D_m and l_c are based on the approximation that the stratum corneum can be treated as an homogenous layer. The effective diffusion path length is then estimated from the thickness of the stratum corneum; and the effective diffusion coefficient is then derived from the measured permeability coefficient. As the stratum corneum is not a uniform thickness, the values assigned (to both $D_{effective}$ *and* $l_{c\text{-}effective}$) may be refined using the above relationship with the measured permeability coefficient.

The meeting noted that there had been a recent analysis (by Frasch and Barbero; 2003) who modelled the diffusion through a more complex and realistic representation of the stratum corneum structure. Their more detailed simulations gave results which they found to be well approximated by appropriate solution of the diffusion equation for a homogenous membrane. More explicitly, they considered that the homogenous medium representation may be thought of as the homogenous barrier which has the same permeability per unit area and lag time as the heterogeneous stratum corneum lipid pathway.

Frasch and Barbero (and others) have pointed out that the effective diffusivity is not the same as the intrinsic diffusivity of the permeant in the lipid medium, and the effective path length is not the same as the actual pathlength along the tortuous lipid channels through the stratum corneum. Frasch and Barbero found that the effective pathlength may be about 10 times larger than the physical thickness of the stratum corneum. So the term l_c^2 in the lag time might produce an error up to a factor of 10 or more, especially for lipophilic compounds which would diffuse mainly through the lipid channels, if calculated based upon the thickness of the stratum corneum.

This error can be diminished by the following approach:

- measuring or estimating the K_m , the partitioning between the vehicle and the skin;
- estimating the true lag time by frequent analysis of the receptor fluid after first skin contact.

Dr Bunge presented data for a range of pesticides showing how predictions of absorption (based on QSAR predicted k_p) compare with systemic absorption *in vivo* measured in rats. She commented on how such data could be used to develop a safety factor such that a safety factor times the predicted systemic absorption would encompass the majority of the set of measured values for *in vivo* systemic absorption. Dr Bunge commented that as rat skin is between 2 to 3 times more permeable than human skin, a factor of 10 would really be a safety factor of between 2 to 5. The data presented by Dr Bunge are in Appendix 6. In this data (Table A6.1), the final four columns show the systemic absorption measured in rats exposed dermally for 10 hours at three (or four) dose levels. For comparisons, predictions are made from the maximum flux (measured in human skin *in vitro*) in column 5. The maximum flux (in $\mu g/cm^2/h$) multiplied by 10 hours gives the values in column 6 (in $\mu g/cm^2$) and that predicted value is then multiplied by factors (of 3, 10 and 100) to give columns 8, 9 and 10. The tenfold times predicted-value (in column 9) is higher than the measured systemic absorption (columns 11 to 14) in 49 out of 58 results (12 out of 18 at the highest dose).

It was recommended that the confidence intervals estimated for the calculated results should be reported, and indeed would be essential if the results are to be used properly. (For example, if confidence intervals are not reported, then regulators and others may apply their own arbitrary safety factor.)

7. APPLICATION OF PERMEABILITY COEFFICIENTS TO OCCUPATIONAL SCENARIOS

The Technical Guidance Documents (TGD) for the European Risk Assessment of Substances (EUR 20418 EN, 2003) recommends, that, in the case of a compound with a molecular weight smaller than 500, a dermal absorption of 100% should be selected as the default starting point for human risk assessment for exposure via the dermal route. A 10% default absorption is recommended only if the molecular weight is larger than 500 and the log P_{ow} is either less than -1 or greater than 4. The justification, in the TGD, for the 10% default for substances in this extreme range of molecular weight and partition coefficient was that there is published evidence that such substances can cross the skin, albeit to a limited extent. The TGD also states that: "*If data are available (e.g. data on water solubility, ionogenic state, 'molecular volume', oral absorption and dermal area dose in exposure situations in practice) which indicate the use of an alternative dermal absorption percentage is appropriate, then this alternative value can be used. Scientific justification for the use of alternative values should be provided*". In the experience of European risk assessments, the authorities are using the default guidance as a binding dermal risk assessment procedure which can be overruled only by good accepted experimental data.

The TGD (EUR 20418 EN, 2003) indicates the percentage of dose absorbed, and dose absorbed per day, as the only default guidance for risk assessment based on absorbed dose per day. However, the time scale of dermal absorption may be more important than simply the daily exposure. In particular, the lagtime (time to constant permeation rate) and the time for 90% absorption may vary by a factor of 1000. For example, although the percentage absorption might be 100 percent, this 100% absorption may occur in 3 or 3000 minutes. So the rate of absorption and the dose rate may vary greatly at a fixed absorption percentage. The effect of evaporation (on the period when absorption can take place) can be taken into account as described in Appendix 1F of the TGD on Risk Assessment (ECB, 2003). The best estimate of dermal absorption should take account of the lag time and the effect of evaporation on the residence time of the compound on the skin surface.

The skin permeability coefficient may play a role in the risk assessment for exposure to substances via the dermal route. The water solubility is the maximum possible concentration of substance (in an aqueous layer on the skin surface). Therefore, the product of the water solubility, the permeability coefficient, the skin contact area and the exposure time predicts a maximum absorbed quantity of the substance. Another constraint is that a feasible estimate of maximum absorbed quantity cannot exceed the total deposited quantity on the skin contact area. For this calculation, the duration of dermal exposure should be taken as being not less than 6 times the lag time (even for cases of short duration dermal exposure) in order to allow for the residence time of permeant in the stratum corneum (Roberts *et al*, 1999). This procedure has the advantage of taking account of the rate of absorption, which is equivalent to the dose rate. The internal dose rate, together with the effects of distribution and excretion, determines the dose rate at any specific target organ, and thus controls the final effect and response.

Where there is a lack of information (or lack of good information) about the toxicity of a chemical, then there may be an advantage in being able to show that the dermal dose is low. Dermal permeation may be more readily determined than new toxicity data generated.

The internal dose is determined by both dermal exposure and dermal absorption, and both need to be considered in a risk assessment. The importance of proportionate attention to each component is discussed in Chapter 11.

8. CHEMICALS TO BE SELECTED FOR TESTING

The chemical selection Working Group discussed both the application of the permeability coefficient in risk assessment leading to the conclusions described above and the chemicals that should be selected for further testing using the agreed *in vitro* method. For selection of chemicals for developing the acceptance of QSARs as a valid and reliable method of predicting the permeability coefficient for industrial chemicals, the recommendation was that the chemicals should:

- span the physical chemical space of the parameters log P_{ow} from -3 to 4 and molecular weight from 30 to 1000 (an elliptical space on a two dimensional plot of chemicals by these two parameters);
- be chosen from chemicals which are produced in high volume in industry;
- be chosen in conjunction with industry;
- if possible (for time-effectiveness), be radiolabelled versions of the production chemicals (by selecting chemicals for which radioactive versions are available);
- if some non-radiolabelled chemicals are used (to obtain the range of physical/chemical characteristics), then the chosen chemical needs to be suitable for sensitive chemical analysis.

9. *IN VITRO* PROTOCOL

9.1 INTRODUCTION AND PURPOSE

It was agreed that the protocol should be designed to minimise unnecessary variation within a data set which is to be used as a basis for establishing the applicability of the QSAR (QSPR) approach to industrial chemicals. Although the protocol may not be directly applicable to risk assessment, it was considered expedient to agree a standard that would be suitable for generating data for developing a sound QSAR model. The draft protocol circulated prior to the meeting listed the potential options from various current guidelines and methods. For each aspect, the meeting selected a particular option wherever there was a basis for saying that it was more directly relevant to human exposure (e.g. human skin), and the choice of skin was narrowed to material likely to be in best condition (elective surgery skin, not cadaver skin). Where the current options within a method were known to be equivalent in terms of the results or relevance (for example static-cell or flow-through cell), the specification was that the same choice should be adhered to (as far as technically feasible) throughout.

The recommended protocol for the *infinite* dose method is attached in Appendix 2.

9.2 EXISTING GUIDELINES

The draft protocol (circulated prior to the meeting) listed the recommendations from existing published guidelines next to each part of the test procedure. However, these guidelines were not specifically designed for producing data to support a QSPR model, and were therefore not always relevant.

9.3 TEST SUBSTANCE

Accurate analysis of the quantity of test substance will be essential, and therefore the preference was for reliably radiolabelled substances (if available). If unlabelled compounds are needed, then suitable methods for analysis of test substance dissolved in the relevant receptor fluid need to be available for measuring concentrations at early time points.

9.4 LOCATION OF THE STUDY

Inter-laboratory variation within the study data set would be avoided by using just one laboratory. Therefore it was recommended that ideally the study would be performed at one laboratory. However, the use of more than one laboratory would expedite the production of data. There are also other practical issues that may need to be considered; for example, the use of more than one (Principal) laboratory might also help safeguard the future availability of the same technical service.

It would also be important to understand the potential impact of inter-laboratory differences (for example to enable extension of the data set in future). Therefore in addition to the Principal laboratory, a minimum of two (Secondary) laboratories will be used to test compounds selected from amongst those tested by the Principal Laboratory. The selected compounds should span the range of key properties (e.g. lipophilicity, molecular weight). For the inter-laboratory comparison, the effect of laboratory difference would be distinguished from variability in skin samples by using artificial (e.g. silastic) membranes, from the same production batch, in those inter-lab comparison tests.

9.5 SKIN SAMPLES

Pig skin is a generally accepted and valuable alternative to human skin, especially where human skin is not available. However, human skin was the preferred option especially as the existing QSPR databases are based on data obtained exclusively with human skin.

Human variability in skin, between individuals and location on the body, was recognised as important. Only skin from the breast or abdomen should be used; however, specifying only one or the other would restrict the availability of skin. Therefore, tests should include both. The specification was that 9 replicate tests should be conducted, with skin from a minimum of 3 subjects, and with at least two (but not more than 3) replicate tests per subject. Data describing the donor characteristics (but not individual identity) should be collected and reported. All 9 replicate samples would be run at the same time. (For the proposed new data set, it would be important to ensure that differences between chemicals are not completely confounded with differences between individuals and that could be achieved by using a balanced statistical design specifying which donor skin would be used for which chemical,. Therefore, a robust statistical design should be set out for the programme of tests to be conducted under the protocol.)

The preparation of the skin should be standardised: dermatomed skin set to a fixed target with the OECD guideline range (0.2 to 0.4 mm) but closer to the 0.4 mm (e.g. dermatome set at 0.35 mm). This is an internationally accepted skin preparation used in this type of study, and it will provide membranes containing the epidermis and a limited amount of dermis.

9.6 SKIN CONDITION (VISUAL AND BARRIER INTEGRITY)

Procedures that might damage the skin were excluded (i.e. no refreezing). The skin would be checked visually for damage and rejected if any damage found.

The tests of integrity (using electrical resistance) would be made before and after the test, but used only to justify exclusion of outliers rather than reject skin samples. An exclusion principle has yet to be decided.

9.7 DIFFUSION CELLS AND SETTINGS

Flow-through cells enable continuous collection of samples of the receptor fluid. Therefore flow-through would be the preferred cell type. However, if the continuous dilution of the flow through receptor fluid would dilute the concentration of the test substance too much for adequate analytical sensitivity, then the use of a static cell would be appropriate and necessary. Variations in technique should be minimised, and therefore the same cell type should be used throughout if technically possible.

As solubility is dependent on temperature and diffusion is driven by the thermal energy of the molecules, permeation rates would be expected to depend on temperature. Furthermore, the state of the skin may change with temperature, and therefore the temperature in the cell at the skin surface should be maintained at the temperature representative of normal external skin surface temperature (i.e. 32 ±1 °C).

9.8 RECEPTOR FLUID

It was agreed that a single receptor fluid would be specified as Physiologically Buffered Saline (PBS, pH 7.4), containing bovine serum albumin (BSA, ca 5%, w/v). Adequate solubility of each chemical in the receptor fluid must be demonstrated. It is recognised that very lipophilic chemicals will not be suitable for testing in this phase of the study and further

work will be needed to bridge to more lipophilic receptor fluids and data for lipophilic chemicals.

9.9 PREPARATION OF THE DOSE SOLUTION

Where possible, all dose solutions would be produced as saturated solutions in order to obtain solutions with similar thermodynamic activity. Saturated solutions would be prepared and the concentration measured at the temperature of the skin surface (i.e. 32°C). When applied to the skin, additional solid matter would be included to ensure saturation.

The chemical to be applied dermally should be prepared as an aqueous solution, buffered to maintain pH 5.5 (i.e. the same as the pH of normal skin), and as a saturated solution, or up to a maximum of a 25% w/v solution for very water-soluble substances. An ionisable compound might overwhelm the buffer, but that would be accepted as part of the characteristics of the chemical. The pH of donor solutions will be measured, as the value of log P_{ow} changes with pH for ionisable chemicals.

Where possible, all dose solutions would be produced as saturated solutions in order to obtain solutions with the same thermodynamic activity. Saturated solutions would be prepared and the concentration measured at the temperature of the skin surface (i.e. 32°C). When applied to the skin, additional solid matter would be included to ensure saturation.

9.10 APPLICATION OF THE DOSE SOLUTION

The test cell would be equilibrated by pumping the receptor fluid through the chambers for long enough to settle any transient effects; a period of about 30 minutes was thought to be more than adequate.

The doses would be applied in a plentiful but practicable quantity (e.g. 1 ml/cm^2). The cell would be occluded to eliminate the complications of evaporation. The dose solution may need to be topped up or replaced, especially for the more lipophilic compounds, to maintain a stable concentration for faster penetrants.

9.11 SAMPLE COLLECTION

Sample collection would include controls to determine the level of background readings in blank samples from the receptor fluid, and for receptor fluid run through the test cell with skin in place.

The collection (of samples) during the test should include samples taken during the initial non-steady state period to help relate infinite-dose studies to the non-steady state data from finite dose tests. The appropriate time intervals would depend on the kinetics of the particular substance, and should be chosen for each substance based either on preliminary modelling or on pilot test data.

9.12 CHEMICAL ANALYSES

Quality control procedures are important in all stages, including the chemical analysis; substantial variation has been found attributable solely to the non-biological parts of the test (e.g. the order of fivefold variation between labs in tests on permeation of silastic membranes; Chilcott *et al*, 2004). Therefore it will be important to have the samples analysed in a laboratory with appropriate quality standards (such as UKAS accreditation). If more than one laboratory is involved in the analyses, then quality control samples should be distributed to

the laboratories for analysis. Inter-laboratory differences should be included in the data reported.

The obtaining of full mass recovery data was considered to be a valuable confirmation of the validity of an experiment. The steady state permeation rate is measurable (in the infinite dose experiment) without obtaining the full mass recovery data, but the recovery data is an important corroboration of the consistency of the data.

9.13 REPORTING DATA

The reported data must include full characterisation of the test substance. The test results should include the time dependence of the absorption rates (i.e. absorption rate as a function of time), the lag time, and the steady state permeability coefficient. The procedures used for calculating the absorption rates and permeability coefficient should be described fully.

All data for individual diffusion cells will be reported, including the skin sample.

The sources of each skin sample will be reported (in terms of sample code, code for the individual, site of origin and type of skin).

9.14 FINITE DOSE PROTOCOL

The main general recommendation for the finite dose method was that it should be as consistent as possible with the infinite dose method, to maximise the comparability of the two datasets. One of the main issues of the finite dose method is the choice of sample volume. A volume of 10 μl per cm^2, as mentioned in the OECD guidelines for skin absorption, will be used. The applied volume affects the results if they are expressed as % absorbed; at one extreme the infinite dose gives the maximum absorption rate (mass per unit time) from a saturated donor solution but by definition the change in concentration of the donor solution is negligibly small and the percentage absorbed is indeterminate. At the other extreme, a finite dose will (by definition) give a much lower mass absorption rate (due to a depleting concentration in the donor fluid) but a higher percentage absorbed due to the small divisor.

It was recommended that the reported results should always include confidence intervals.

In order for data on the transient phase to be useful for modelling, it is important that the inherent delays in the experimental system be quantified and reported, for example the time delay while the receptor fluid travels from test cell to collecting device.

Diffusion is a process that is driven by the thermal energy of the system, and the condition of the skin barrier is likely to be temperature dependent. Therefore, the temperature of the system (especially in each test cell at the skin barrier) must be monitored and controlled.

The recommended protocol for the finite dose method is attached in Appendix 3.

10. LIMITATIONS TO THE PROTOCOL (VEHICLE AND CHEMICAL RANGE)

A consistent set of data exists for chemicals in aqueous solution, albeit mainly chemicals from particular sectors. It is believed that new data for industrial chemicals is likely to demonstrate that the same relationship applies to chemicals from other sectors. The confirmation of this relationship was recommended as being readily feasible. However, extension of the relationship to chemicals that are not soluble in water was considered more complex. Various possibilities were discussed.

One possibility would be to test a range of highly lipophilic chemicals (P_{ow} >5) in a suitable organic liquid as the vehicle. However, the choice of a suitable oil is not simple. EPA has promulgated a final rule (Federal Register 26 April 2004) for testing the skin permeation of 34 industrial chemicals. For the testing of poorly water soluble compounds, isopropyl myristate (IPM) is recommended as a vehicle by the EPA. However, the EPA does not explain the logic behind the choice of IPM. The liquid vehicle may be liable to have effects on the skin which:

- can change the physical structure of the stratum corneum;
- can enhance the solubility of lipophilic compounds by simultaneous permeation with water (e.g. as seen in tests with ethanol-water);
- may be more extensive under the prolonged contact in the test than would be representative of industrial use conditions.

Water itself can hydrate the skin, but is arguably less foreign to the skin than organic liquids. One criterion for choosing the liquid might be that it should enable some overlap with the range of chemicals testable in aqueous solution. For example, if IPM were the other vehicle being tested, then some of the tested chemicals should be soluble in both IPM and in water. However, it was generally considered that the overlap would probably be relatively unimportant, and that a completely independent data set for the highly lipophilic chemicals (P_{ow}>5) would suffice. However, there is not a good basis for making that choice of vehicle at present, nor for endorsing or rejecting the isopropyl myristate (IPM) specified in the EPA method.

The case for using IPM may be argued as being that:

- water is not an appropriate solvent for very lipophilic chemicals, because of the disproportionate preference of the chemical for the lipid rich stratum corneum;
- the structure of isopropyl myristate is more or less similar to certain natural lipids but less sticky than natural oils;
- using IPM safeguards a constant level (of lipid) at the surface of the stratum corneum.

An alternative, and well published approach, is to dissolve the test chemical in acetone and then apply at 4 $\mu g/cm^2$ in 10 $\mu l/cm^2$.

The effects and interactions of vehicles and receptor fluid on chemical absorption were recognised as being complex. There will be a need to clarify these effects. That will involve acquiring data for a matrix of chemical and vehicle combinations.

A study of the effects of the vehicle may also clarify the importance of water solubility. The main resistance to the absorption of lipophilic compounds may be their low solubility in the aqueous layer on the surface of the skin and a low solubility (or low partition coefficient) in the water rich part of the viable epidermis. Theoretically, the effects of the vehicle can be explained and predicted from the thermodynamics, as long as the vehicle does not affect the integrity of the stratum corneum.

It was noted that the EPA protocol specifies application of "neat chemical" to the skin, without specifying just what that means for solid compounds. The OECD specifies that the applied product should represent the real workplace situation. It is often assumed that water soluble chemicals form an aqueous solution on the skin surface, and that testing in aqueous solution was generally relevant for substances soluble in water. However, this may not always be valid. Also, relatively large splashes of chemicals on the skin may overwhelm the skin surface layer (sweat and oils). The alternative assumptions regarding whether chemicals on the skin form aqueous solutions or are in direct contact with the *stratum corneum* need to be considered in producing assessments of dermal exposure and risk assessments.

11. STRATEGY RECOMMENDATIONS

11.1 SUMMARY OF THE RECOMMENDATIONS

The workshop recommended that two main programmes of work are needed to enable efficient and reliable risk assessments to be made for industrial chemicals. These programmes, called Stage 1 and Stage 2, could be undertaken in parallel or sequentially.

Both programmes are based on the principle that QSARs should be used to relate the physical and chemical properties of groups of chemicals to their dermal permeation characteristics. Stage 1 builds on the already extensive data set for aqueous soluble chemicals, and the QSARs that have been constructed for such chemicals, and uses the protocols recommended by this workshop. Stage 2 extends the same approach to other (non-aqueous soluble) chemicals, and to other vehicles.

The application to risk assessment would benefit from improved information on exposure.

11.2 STAGE 1: WATER SOLUBLE CHEMICALS

For the first stage, the recommendations are:

- to promote the recognition of the likely reliability of QSAR predictions of dermal permeation for industrial chemicals by having a selection of chemicals tested by the proposed *infinite* dose protocol. On the basis of experience from the EDETOX project and elsewhere, it was estimated that about 50 chemicals would be needed;
- to establish the validity of using the mathematical models to extrapolate from *infinite* dose to *finite* dose. The model should be used to extrapolate from the infinite dose to predict the results of a finite dose experiment before the finite dose experimental results are available. Then the reported prediction should be compared with the subsequently measured value.

The protocol specifies that variability should be minimised, wherever possible, in collecting data. Optimally, that would involve using one laboratory to do the measurements; however, to generate sufficient data for statistical analysis and QSAR development, more than one laboratory might be necessary to expedite the rate of data production. In either case, it would be an essential safeguard to gather information on inter-laboratory comparability with selected comparator substances. That would provide important support for the robustness of the data and could be related to the recent studies of robustness and variability (van de Sandt *et al*, 2004 and Chilcott *et al*, 2004). This would also provide for continuity of standards in the generation of further data as and when needed in the future. All laboratories must adhere to the essence of Good Laboratory Practice.

The first stage would use a wide range of chemicals with the standard aqueous solution protocol. The infinite dose data would be directly comparable to the existing data set that has been used for the development of QSARs, and could be extrapolated using mathematical models for comparisons with the finite dose data (generated by the recommended protocol).

11.3 STAGE 2: OTHER CHEMICALS, AND OTHER SOLVENTS

The second stage would involve more detailed testing and development using a subset of the chemicals tested in stage 1, along with more lipophilic chemicals that cannot be tested in the aqueous solution protocol. It would include investigations of the effects of various donor fluids, receptor fluids, and mixtures. The objective would be to enable development of models that bridge from the first stage results (standard aqueous solution test conditions) to the second stage (more complex and more realistic exposure conditions for an extended range of chemicals).

This stage would also address the effects of the vehicle in which the chemical is presented to the skin. An objective would be to enable a standardised protocol to be defined for chemicals in solution in other liquids. The possibilities would be expected to depend on whether or how the vehicles affect the skin.

11.4 USE OF THIS DATA IN RISK ASSESSMENTS

A tiered approach, which enables efficient risk assessments for dermal exposures using dermal permeability coefficients, was recommended by the workshop. This approach would be to:

- as under current guidelines, assume either 100% absorption, or 10% default assumption for high molecular weight and extreme log P_{ow} (log P_{ow} <-1 or log P_{ow} >4); and then, if necessary,
- use saturated water concentration and k_p to calculate an estimate of maximum flux, allowing for any effects from the vehicle; then, if necessary,
- use the more complete mathematical model with diffusion coefficients and partition coefficients to obtain a best estimate of the flux and dose for the likely occupational exposure concentration (i.e. finite dose).

Partition coefficients need to be obtained to support the latter stage of the above calculations for some industrial chemicals.

11.5 EXPOSURE, DERMAL PERMEATION AND RISK ASSESSMENT

Measurement of dermal exposure is complex and there are a number of scientific issues that need to be resolved. Future developments in determining dermal permeation should be conducted in close liaison with developments in dermal exposure assessment. The internal dose, received via the dermal route, is a function of dermal exposure (in terms of amount and concentration deposited, and area of skin over which this deposition occurs) as well as the ability of the material to pass through the skin. Current methods only focus on the mass of contaminant on the skin. Recent studies (Kromhout *et al*, 2004) have shown that variability in dermal exposure (for nominally similar exposure scenario) can be very large, with differences in geometric mean potential dermal exposures possibly being 3 to 5 orders of magnitude. These researchers concluded that such differences in dermal exposure arose from differences in local conditions, such as the actual handling of the agent, the control measures, and the training and attitude of workers. Actual data on the dermal exposures can be used in the risk assessment procedures but the data must be compatible with the permeation models. The dermal permeation rates need to be reliable, but risk assessment should address both exposure and permeation with proportionate effort if the assessments are to use resources efficiently.

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APPENDIX 1: CLARIFICATIONS AND GLOSSARY

Various issues relating to dermal permeation were clarified during the meeting.

Scope of this protocol and validity of other protocols

The protocol specified here is designed for the specific aim of developing a consistent database, minimising avoidable variations, in order to minimise uncertainty in the development of mathematical models. The protocol is not suitable for generating data directly applicable to risk assessment, but it is hoped and expected that this standardised approach integrated with mathematical modelling will allow efficient assessments of risk to be achievable eventually.

Terminology

The OECD give definitions (OECD, 2004), and the EPA and EU skin absorption documents state that these terms should be used. The OECD glossary (as below) is followed here.

GLOSSARY OF TERMS (OECD)

Absorbed dose (in vivo): *comprises that present in urine, cage wash, faeces, expired air (if measured), blood, tissues (if collected) and the remaining carcass, following removal of application site skin.*

Absorbed dose (in vitro): *mass of test substance reaching the receptor fluid or systemic circulation within a specified period of time.*

Absorbable dose (in vitro and in vivo) *represents that present on or in the skin following washing.*

Absorption (Dermal, Percutaneous and Skin absorption*): diffusion of chemicals from the outer surface of the skin to the receptor fluid or systemic circulation.*

Absorption profile*: a graphical representation of cumulative absorption as a function of time.*

Absorption rate*: mass of test substance passing through a unit area of skin into the receptor fluid or systemic circulation, per unit time (in* $\mu g/cm^2/h$*).*

Adsorption*: reversible binding or adherence the test substance to any component of the test system.*

Applied dose*: mass of test preparation containing a specified mass of test substance applied per* cm^2 *of skin.*

Dermal delivery: *sum of the applied dose found in the treated skin and the absorbed dose at the end of the experiment.*

Dislodgeable dose*: mass of test substance that is removable from the application site.*

Exposure period*: time from application of test preparation to removal at skin washing.*

Finite dose*: amount of test preparation applied to the skin where a maximum absorption rate of the test substance may be achieved for a certain time interval but is not maintained.*

Flux: *mass of test substance passing through a unit area of skin per unit of time under steady-state conditions (in* $\mu g/cm^2/h$*).*

'in-use' preparation: *the preparation of test substance which relates directly to potential human exposure (e.g. cosmetic or agrochemical formulations and dilutions thereof, a mixture of industrial chemicals in a solvent, etc.).*

Infinite dose*: amount of test preparation applied to the skin where a maximum absorption rate of the test substance is achieved and maintained.*

Lag time: *derived from a graph of cumulative absorbed dose and time. Intercept of the tangent of the linear part of the absorption profile with the x-axis.*

Penetration enhancer: *adjuvant, which facilitates penetration of the test substance through skin.*

Percentage absorption*: the mass of test substance absorbed (over a given time period) divided by the mass of test substance applied multiplied by 100.*

Permeability coefficient (k_p***)****: a value, in units of cm/h, that represents the rate at which a chemical penetrates the skin. This is calculated from the flux divided by the applied concentration. [Division by the applied concentration is correct only in the case of an infinite dose and where the concentration in the receptor fluid is extremely small compared to that in the vehicle. And the calculation of* k_p *should be from the steady state flux.].*

Steady-state*: the part of an absorption profile where the absorption rate remains constant.*

Test substance*: a single chemical entity whose penetration characteristics are under investigation.*

Test preparation*: actual material that is applied to the skin. Usually the test preparation will be the 'in-use' preparation that reflects actual use conditions; alternatively it may be a mixture of the test substance in a carrier or solvent to facilitate application to the skin.*

Unabsorbed dose*: represents that washed from the skin surface after exposure and any present on the non-occlusive cover, including any dose shown to volatilise from the skin during exposure.*

APPENDIX 2: INFINITE DOSE PROTOCOL

The *In Vitro* Percutaneous Absorption of Substances through Human Skin: Data provision for QSPR modelling

PROTOCOL A : INFINITE DOSE

CONTENTS

A2.1. INTRODUCTION AND STUDY OBJECTIVES

A2.1.1 The rate and extent of absorption following topical application to human skin will be assessed. It is noted that human skin is not always available and that pig skin is a generally acceptable alternative. However, for comparative purposes with published QSPR databases where human skin has been exclusively used, human skin is the preferred model.

A2.1.2 The *in vitro* technique outlined below is compliant with as many elements as possible contained within the OECD Guideline 428 and Guidance Document No. 28 (see below) which have been accepted by the Regulatory Authorities for this type of study. Where there are differences, this is mainly due to procedural differences between the finite and infinite dose experiments.

A2.1.3 The test substance will be applied as a saturated aqueous solution (or a maximum of 25% w/v for very water-soluble substances)) in excess (infinite dose). The experiment will be run for sufficient time for steady-state absorption to be maintained and assessed. Sufficient receptor fluid samples will be taken and analysed to permit determination of steady-state flux, k_p and lag time.

A2.1.5 The performance of the study will be overseen by a designated study co-ordinator.

A2.2. STUDY OUTLINE

The absorption of the test substance will be assessed after dermal application to excised human dermatomed skin mounted in flow-through diffusion cells. The test substance will be applied in water as a saturated solution (or up to a maximum of 25% w/v for very water-soluble substances).

Following a single application, dermal absorption will be monitored by sampling the receptor fluid bathing the under-surface of the skin at regular intervals (e.g. hourly). The duration of the exposure will be sufficient to ensure steady-state absorption is maintained (e.g. 24 hours, but may be longer for slower penetrants).

At the end of the study, unabsorbed dose will be removed, the diffusion cell will be dismantled. If a full recovery measurement is required, then the amount of material (radioactivity) not absorbed into the receptor fluid will be measured.

The amount of test substance absorbed per unit area of skin (e.g. $\mu g/cm^2$) into the receptor fluid with time (hr) will be graphically represented and the absorption rates calculated from the steady-state region of the absorption profile (e.g. $\mu g/cm^2/hr$).

A2.3. TEST GUIDELINES

A2.3.1 OECD Guideline for Testing of Chemicals, Guideline 428: Skin Absorption: *In Vitro* Method (2004).

A2.3.2 OECD Environmental Health and Safety Publications Series on Testing and Assessment No. 28. Guidance Document for the Conduct of Skin Absorption Studies (2004).

A2.3.3 EPA Final rule (OPPT-2003-0006) *In Vitro* Absorption Rate Testing of Certain Chemicals of Interest to the Occupational Safety and Health Administration (2004).

A2.3.4 SCCNFP Opinion concerning Basic Criteria for the *In Vitro* Assessment of Cosmetic Ingredients (2003)

A2.3.5 EC Guidance Document on Dermal Absorption, Directorate E1 – Plant Health (Sanco/222/2000 rev. 6, November 2002)

A2.3.6 It should be noted that these guidelines are not intended for producing data to support QSPR models and are not necessarily complementary to this aim.

A2.4. MATERIALS

A2.**4.1** Test Substance

A2.4.1.1 Preference will be to use radiolabelled test substance, although unlabelled substance may have to be employed if radiolabelled compound is unavailable.

A2.4.1.2 If radiolabelled, the position of labelling on the test substance is one that is considered to be chemically and metabolically stable. The purity of the radiolabelled compound must be > 98%.

A2.4.1.3 If unlabelled, methods should be available for analysis of test substance dissolved in the designated receptor fluid and should have adequate specificity and limits of detection sufficient to observe absorbed chemical at early time-points.

A2.4.1.4 The supplied test substance will be accompanied by a Certificate of Analysis that will include data on the chemical and radiochemical purity, and specific activity (where appropriate). Radiochemical purity will be confirmed in the dose preparation. The specific activity of radiolabelled test substance will not be confirmed.

A2.4.1.5 Method of preparation of aqueous solution will be agreed in advance.

A2.4.1.6 The test substance and dose solutions will be stored under appropriate conditions.

A2.**4.2** Other materials

A2.4.2.1 Chemicals will be of analytical grade where available.

A2.5. LOCATION STUDY

A2.5.1 The study will (ideally) be performed by one (Principal) laboratory to reduce inter-laboratory variability. However, it should be noted that use of more than one laboratory will expedite production of data.

A2.5.2 Other (Secondary) laboratories (minimum of two) will be employed to assess any inter-laboratory variability/bias using silastic (artificial) membranes (same batch) and selected reference compounds from those tested at the Principal laboratory, and spanning the range of lipophilicities available.

A2.6. SKIN SAMPLES

A2.6.1 Human Skin Samples

A2.6.1.1 Human skin samples will be obtained from elective plastic surgery. Only abdominal and / or breast skin should be used. Ideally, to enable comparison between the skin permeability of the two anatomical sites, there should be 2 abdominal and 2 breast donors per chemical. (See section A2.6.2.4 for description of replicates.).

A2.6.1.2 The donor will give prior written consent for the skin to be used for scientific research. The donor's age and sex will be recorded.

A2.6.1.3 Full thickness skin will be obtained, cleaned of subcutaneous fat and muscle, and stored flat at ca -20°C before use. The duration of storage of selected skin samples will not exceed one year. Larger samples may need dividing up to negate the need to thaw and re-freeze, which is not acceptable for this study.

A2.6.1.4 The date of receipt together with the anatomical site from which the skin was obtained and date of use of each skin sample will be recorded.

A2.6.2 Preparation of skin

A2.6.2.1 Split thickness skin (0.2 - 0.4 mm) will be used. The ability of the dermatome setting to cut this thickness will need to be confirmed by histological determination.

A2.6.2.2 Any membranes visibly damaged during the preparation procedure will be discarded.

A2.6.2.3 The split thickness membranes will be used on the day of preparation.

A2.6.2.4 A minimum of 9 replicates with no more than 3 samples from each donor will be used for determination of percutaneous absorption for each substance.

A2.7. DIFFUSION CELL AND SETTINGS

A2.7.1 Flow-though cells will be employed in order to standardise the equipment used. Static cells may be used only when the use of flow-through cells do not provide the required analytical sensitivity.

A2.7.2 Skin surface must be maintained at 32± 1ºC due to diffusion being a temperature-dependent process. This can be performed by assessing the temperature of the temperature-maintaining system (e.g. water bath) required to deliver the correct skin surface temperature for the typical thickness of membrane used. The temperature of the maintaining system must be routinely checked accordingly.

A2.7.3 For flow-through cells, a standard flow rate will be used, preferably with the capability of simultaneously stirring the receptor fluid contents. For static cells, the receptor fluid must be stirred and regularly sampled.

A2.7.4 The concentration of chemical in the receptor fluid must never exceed 10% of the initial concentration of chemical in the dose solution.

A2.8. RECEPTOR FLUID

A2.8.1 The recommended receptor fluid of choice for both hydrophilic and lipophilic substances will be phosphate-buffered physiological saline (PBS, pH 7.4) containing bovine serum albumin (BSA, *ca* 5%, w/v).

A2.8.2 Adequate solubility of the test substance in the receptor fluid will be demonstrated to confirm that it did not result in limiting the amount absorbed into it at any time during the absorption process. This should be at least 10 times greater than the anticipated maximum concentration in the receptor chamber during the experiment. If this is found not to be the case subsequent to the experiment being performed, the experiment will need to be repeated with a different receptor fluid in which the penetrant has adequate solubility.

A2.8.3 The receptor fluid will be degassed or sonicated to reduce the possibility of air bubble formation in the receptor chamber during the experiment.

A2.9. BARRIER INTEGRITY ASSESSMENT

A2.9.1 This will be performed prior to application of the dose solution. This will involve assessment of electrical conductivity across the skin membrane using an AC supply at up to 2V.

A2.9.2 Details of rejection criteria will be provided to the study co-ordinator and considered in light of the need to include the wide range of human skin permeabilities from different donors, sites, etc.

A2.10. PREPARATION OF DOSE SOLUTION

A2.10.1 Dose solutions will be in pH 5.5 buffer (at 32ºC) for all solutions.

A2.10.2 All solutions will be prepared as saturated or up to a maximum of 25% w/v for very water-soluble substances. The use of saturated solutions ensures that all dose solutions have the same thermodynamic activity.

A2.11. APPLICATION OF DOSE SOLUTION

A2.11.1 An equilibration period of *ca* 30 min will be allowed while receptor fluid is pumped through the receptor chambers prior to dosing.

A2.11.2 Dose solution will applied in excess (e.g. 1 ml/cm^2) and will be occluded for duration of the study. Any air bubbles present at the vehicle/skin interface will be removed by gentle pressure using the dose solution.

A2.11.3 Dose may need to be topped up or replaced at least once a day to ensure infinite dose scenario, particularly for low solubility, lipophilic substances. This will need to be considered in advance of the study on a case-by-case basis.

A2.12 SAMPLE COLLECTION

A2.12.1 A blank sample of receptor fluid will be collected prior to application of the test preparation.

A2.12.2 For cold analysis, control, undosed diffusion cells will be set up in parallel to demonstrate that there is no interference from endogenous chemicals in the skin.

A2.12.3 The main objective of the sampling regime will be to assess the rate of absorption during steady-state. However, it would also be beneficial to obtain data for the non-steady-state kinetics to enable comparison with the finite dose studies (where comparison is intended for that compound). For this reason, absorption will be assessed by collecting more frequent samples at the start: typically, 15-minute fractions for the first hour, 30-minute fractions for the next 3 hours and hourly fractions 4 to 24 h post dose. For compounds with very short lag times or very long lag times, the sampling regime will need to be modified accordingly. Optimum times should be determined by initial modelling or pilot experiments.

A2.12.4 Any time delays involved in the transit of sample from receptor chamber to fraction collector must be taken into account. For example, the time display for the fraction collector could be offset to account for the delay.

A2.12.5 The donor solution should be sampled at the end of experiment to check that excess loading has been maintained, particularly for lipophilic substances (cf. Section 10.3).

A2.12.6 At the end of the selected exposure time (e.g. at 24 hours), the dose solution will be removed and the diffusion cells will be dismantled. The dose recovery exercise will consist of measuring the total radioactivity in the following :

- receptor fluid;
- remaining dose solution;
- total skin content;
- cell washes (donor chamber and receptor chamber analysed separately);
- carbon gauze (where appropriate).

A2.12.7 The skin sample will then be extracted or solubilised.

A2.12.8 The donor chamber and the receptor chamber will be extracted and analysed separately.

A2.13. STORAGE OF SAMPLES

A2.13.1 Samples not analysed immediately will be stored frozen (ca -20°C) until taken for analysis. After analysis, samples will be returned to storage at ca -20°C.

A2.14. CHEMICAL ANALYSIS

A2.14.1 Analyses will be performed by laboratories of an appropriate standard, e.g. UKAS accredited.

A2.14.2 Analysis of a given substance may be performed by a single laboratory and samples sent there for analysis. If more than one laboratory is performing the analysis of a substance, QCs may be distributed to those involved to assess consistency in analytical results. Details of transport arrangements and conditions will be provided.

A2.14.3 Exact details of chemical analysis will be provided with each protocol dependent on the chemical selected.

A2.15. PROTOCOL CHANGES

A2.15.1 Changes to this protocol will be documented and the reason for the change stated. Where possible, any changes will be agreed with the study co-ordinator.

A2.16. REPORTING DATA

A2.16.1 The following will be reported for the test substance:

- CAS number;
- molecular formula;
- lipophilicity (log K_{ow});
- molecular mass (and molecular mass of radiochemical, where appropriate);
- molecular volume (if available);
- structure of chemical (and site of label, where appropriate);
- batch number of test substance and source;
- pK_a of test substance.

Experimental conditions should be fully described, as these are relevant to modelling of the data. These must include:

- concentration of the dose solution (at the pH used);
- pH used;
- temperatures of the test system.

The reported measurements and results must include, the following for the receptor fluid measurements:

- absorption profiles for chemical (e.g. $\mu g/cm^2$ vs. time);
- steady-state maximum absorption rate (e.g. $\mu g/cm^2/hr$);
- lag time (hrs);
- k_p (cm/hr);

and the following from the dose recovery measurements:

- % total recovery.

A2.16.2 All data for individual diffusion cells will be included. Skin sample, cell number and date of experiment will be reported for each set of individual data. Means, SDs and CVs will be calculated for the data set as a whole for each substance (for each laboratory, if appropriate) as well as for each individual skin donor.

A2.16.3 The study co-ordinator will be responsible for collation and statistical analysis of all data.

A2.16.4 Standard pro forma for results should be prepared in advance for participating laboratories to use in compiling their data (cf. EDETOX experience).

A2.16.5 On completion of the study, the draft report will incorporate:

- Description of materials and methods followed;
- Results – both hard copies and electronic copies (Excel files).

A2.16.6 DEFINITIONS: Will comply with those given in the OECD Guidance Document.

APPENDIX 3: FINITE DOSE PROTOCOL

The *In Vitro* Percutaneous Absorption of Substances through Human Skin: Data provision for QSPR modelling

PROTOCOL B : FINITE DOSE

CONTENTS

A3.1. INTRODUCTION AND STUDY OBJECTIVES

A3.1.1 The rate and extent of absorption following topical application to human skin will be assessed. It is noted that human skin is not always available and that pig skin is a generally acceptable alternative. However, for comparative purposes with published QSPR databases where human skin has been exclusively used, human skin is the preferred model.

A3.1.2 The *in vitro* technique outlined below is compliant with as many elements as possible contained within OECD Guideline 428 and Guidance Document No. 28 (see below) which have been accepted by the Regulatory Authorities for this type of study.

A3.1.3 The test substance will be applied using the same concentration as the infinite dose study but as a finite dose (usually a volume sufficient to just cover the skin surface). Sufficient receptor fluid samples will be taken, particularly at early time-points, to permit determination of percentage absorbed with time and non-steady state kinetics.

A3.1.4 The intention of these experiments is to compare the data from finite dose exposure with infinite dose kinetics and provide definitive data for mathematical modelling. This will provide an important relationship between k_p (determined from infinite doses) and the more occupationally-relevant finite dose exposure scenario.

A3.1.5 In addition, the amount of test substance on the skin surface (swabs), in the stratum corneum (tape-strips) and in the skin (tape-stripped skin) will be assessed at the end of the experiment.

A3.1.6 The performance of the study will be overseen by a designated study co-ordinator.

A3.2. STUDY OUTLINE

The absorption of the test substance will be assessed after dermal application to excised human dermatomed skin mounted in flow-through diffusion cells. The test substance will be applied in water as a saturated solution (or up to a maximum of 25% w/v for very water-soluble substances).

Following a single application, dermal absorption will be monitored by sampling the receptor fluid bathing the under-surface of the skin at regular intervals (e.g. hourly).

At the end of the study, unabsorbed dose will be removed using dampened swabs prior to dismantling the diffusion cell. The skin will be tape-stripped and then digested/extracted. This will be considered absorbed dose for risk assessment purposes. The initial tape-strips (1-2) will be considered as unabsorbed dose whereas subsequent tape-strips represent removal of the stratum corneum and associated chemical and will be considered absorbable dose. The diffusion cell will also be soaked in an appropriate solvent as part of accounting for the applied dose.

The cumulative amount of test substance absorbed per unit area of skin (e.g. mg/cm^2) into the receptor fluid will be plotted against time (hr). The cumulative percentage of dose absorbed into the receptor fluid will also be plotted similarly.

A3.3. TEST GUIDELINES

A3.3.1 OECD Guideline for Testing of Chemicals, Draft New Guideline 428: Skin Absorption: *In Vitro* Method (2002).

A3.3.2 OECD Environmental Health and Safety Publications Series on Testing and Assessment No. 28. Guidance Document for the Conduct of Skin Absorption Studies (2004).

A3.3.3 EPA Final rule (OPPT-2003-0006) *In Vitro* Absorption Rate Testing of Certain Chemicals of Interest to the Occupational Safety and Health Administration (2004).

A2.3.4 SCCNFP Opinion concerning Basic Criteria for the *In Vitro* Assessment of Cosmetic Ingredients (2003)

A3.3.5 EC Guidance Document on Dermal Absorption, Directorate E1 – Plant Health (Sanco/222/2000 rev. 6, November 2002)

A3.3.6 It should be noted that these guidelines are more complementary to performing finite dose studies than infinite dose studies.

A3.4. MATERIALS

A3.4.1 Test Substance

A3.4.1.1 Preference will be to use radiolabelled test substance. Although use of unlabelled compound may be unavoidable, the procedures required for determining dose recovery may be quite demanding.

A3.4.1.2 If radiolabelled, the position of labelling on the test substance is one that is considered to be chemically and metabolically stable. The purity of the radiolabelled compound must be > 98%.

A3.4.1.3 If unlabelled, methods should be available for analysis of test substance present in the designated receptor fluid, the skin and any surface washes. The analytical method should have adequate specificity and limits of detection sufficient to observe the distribution of chemical at early time-points.

A3.4.1.4 The supplied test substance will be accompanied by a Certificate of Analysis that will include data on the chemical or radiochemical purity, and specific activity (where appropriate). Radiochemical purity will be confirmed in the dose preparation. The specific activity of radiolabelled test substance will not be confirmed.

A3.4.1.5 Method of preparation of aqueous solution will be agreed in advance. Ideally, the same dose preparation used for the infinite dose studies will be used in these studies.

A3.4.1.6 The test substance and dose solutions will be stored under appropriate conditions.

A3.4.2 Other Materials

A3.4.2.1 Chemicals will be of analytical grade where available.

A3.5. LOCATION OF STUDY

A3.5.1 The study will (ideally) be performed by one (Principal) laboratory to reduce inter-laboratory variability. However, it should be noted that use of more than one laboratory will expedite production of data.

A3.6. SKIN SAMPLES

A3,6.1 Human Skin Samples

A3.6.1.1 Human skin samples will be obtained from elective plastic surgery. Only abdominal and/ or breast skin should be used. Ideally, to enable comparison between the skin permeability of the two anatomical sites, there should be 2 abdominal and 2 breast donors per chemical. (See section 6.2.4 for description of replicates.)

A3.6.1.2 The donor will give written consent, prior to elective surgery, for skin to be used for scientific research. The donor's age and sex will be recorded.

A3.6.1.3 Full thickness skin will be obtained, cleaned of subcutaneous fat and muscle, and stored flat at ca -20°C before use. The duration of storage of selected skin samples will not exceed one year. Larger samples may need dividing up to negate the need to thaw and re-freeze, which is not acceptable for this study.

A3.6.1.4 The date of receipt together with the anatomical site from which the skin was obtained and date of use of each skin sample will be recorded.

A3.6.2 Preparation of skin

A3.6.2.1 Split thickness skin (0.2 - 0.4 mm) will be used. The ability of the dermatome setting to cut this thickness will need to be confirmed by histological determination.

A3.6.2.2 Any membranes visibly damaged during the preparation procedure will be discarded.

A3.6.2.3 The split thickness membranes will be used on the day of preparation.

A3.6.2.4 A minimum of 9 replicates with no more than 3 samples from each donor will be used for determination of percutaneous absorption for each substance.

A3.7. DIFFUSION CELL AND SETTINGS

A3.7.1 Flow-though cells will be employed in order to standardise the equipment. Static cells may be used only when the use of flow-through cells do not provide the required analytical sensitivity.

A3.7.2 Skin surface must be maintained at 32 ± 1ºC due to diffusion being a temperature-dependent process. This can be performed by assessing the temperature of the temperature-maintaining system (e.g. water bath) required to deliver the correct skin surface temperature for the typical thickness of membrane used. The temperature of the maintaining system must be routinely checked accordingly.

A3.7.3 For flow-through cells, a standard flow rate will be used, preferably with the capability of simultaneously stirring the receptor fluid contents. For static cells, the receptor fluid must be stirred and regularly sampled.

A3.7.4 The concentration of chemical in the receptor fluid must never exceed 10% of the initial concentration of chemical in the dose solution.

A3.8. RECEPTOR FLUID

A3.8.1 The recommended receptor fluid of choice for both hydrophilic and lipophilic substances will be phosphate-buffered physiological saline (PBS, pH 7.4) containing bovine serum albumin (BSA, *ca* 5%, w/v).

A3.8.2 Adequate solubility of the test substance in the receptor fluid will be demonstrated to confirm that it did not result in limiting the amount absorbed into it at any time during the absorption process. This should be at least 10 times greater than the anticipated maximum concentration in the receptor chamber during the experiment. If this is found not to be the case subsequent to the experiment being performed, the experiment will need to be repeated with a different receptor fluid in which the penetrant has adequate solubility.

A3.8.3 The receptor fluid will be degassed or sonicated to reduce the possibility of air bubble formation in the receptor chamber during the experiment.

A3.9. BARRIER INTEGRITY ASSESSMENT

A3.9.1 This will be performed prior to application of the dose solution. This will involve assessment of electrical conductivity across the skin membrane using an AC supply at up to 2V.

A3.9.2 Details of rejection criteria will be provided to the study co-ordinator and considered in light of the need to include the wide range of human skin permeabilities from different donors, sites, etc.

A3.10. PREPARATION OF DOSE SOLUTION

A3.10.1 Dose solutions will be in pH 5.5 buffer (at 32°C) for all solutions.

A3.10.2 All solutions will be prepared to the same concentration as used in the infinite dose study). This will ensure that all dose solutions have a similar thermodynamic activity.

A3.10.3 Ideally, the same dose preparation used for the infinite dose study will be used in this study.

A3.11. APPLICATION OF DOSE STUDY

A3.11.1 An equilibration period of *ca* 30 min will be allowed while receptor fluid pumped through the receptor chambers prior to dosing.

A3.11.2 Dose solution will applied to enable a thin film covering of the skin surface (e.g. 10 ml/cm^2) and will be unoccluded for the duration of the study. The dispensed dose will be determined from mock doses (QCs) taken into suitable containers (e.g. scintillation vials) before, during and after the dosing procedure.

A3.11.3 In the case of a suspected volatile substance, an activated carbon gauze may be fitted above the skin to trap any evaporated organic material. For vehicles that do not readily spread across the surface of the skin, a spreading device may be used to

ensure the dose covers the entire surface of the exposed skin sample. The spreading device will need to be analysed separately and deducted from the dispensed dose to give the actual dose applied.

A3.12. SAMPLE COLLECTION

A3.12.1 A blank sample of receptor fluid will be collected prior to application of the test preparation.

A3.12.2 For cold analysis, control, undosed diffusion cells will be set up in parallel to demonstrate that there is no interference from endogenous chemicals in the skin.

A3.12.3 The main objective of the sampling regime will be to assess the amount absorbed during non-steady state. For this reason, absorption will be typically assessed by collecting 10-minute fractions for the first 3 hours, 30-minute fractions from 4 to 8 h post dose, and hourly fractions between 8 and 24 hours. For compounds with very short lag times or very long lag times, the sampling regime will need to be modified accordingly.

A3.12.4 At the end of selected exposure time (e.g. at 24 hours), the diffusion cells will be dismantled for a dose recovery exercise, which will consist of measuring the dose contained in the following :

- receptor fluid;
- skin surface swabs;
- tape-stripping (removal of the stratum corneum);
- skin content;
- cell washes (donor chamber and receptor chamber analysed separately);
- carbon gauze (where appropriate).

A3.12.5 The skin will be swabbed using natural sponge as this material is readily soluble in organic solvents. The sponge will be soaked in a suitable aqueous soap solution (e.g. 1% Tween 80) and used to gently remove unabsorbed substance, and dried using a final swab. To ensure that the surface dose is removed, the washing regime will need to be tested using a mock dose on a skin sample immediately followed by repetitive swabs until a negligible amount of test substance is removed in the final swab. (Note: this test of the effectiveness of the swabbing may have been performed as part of the parallel infinite dose experiment).

A3.12.6 The (swabbed and dried) skin sample will then be tape-stripped using a low adhesive tape (e.g. 3M “Magic” tape) until the stratum corneum is removed, indicated by the glistening appearance or loss of adhesion associated with the viable epidermis. The initial strip will be removed and analysed separately as it may contain residual surface dose. Subsequent tape-strips will be extracted or the skin debris solubilised collectively.

A3.12.7 The tape-stripped skin sample will then be extracted or solubilised.

A3.12.8 The donor chamber and the receptor chamber will be extracted and analysed separately.

A3.13. STORAGE OF SAMPLES

A3.13.1 Samples not analysed immediately will be stored frozen (ca -20°C) until taken for analysis. After analysis, samples will be returned to storage at ca -20°C.

A3.14. CHEMICAL ANALYSIS

A3.14.1 Analyses will be performed by laboratories of an appropriate standard e.g. UKAS accredited.

A3.14.2 Analysis of a given substance may be performed by a single laboratory and samples sent there for analysis. If more than one laboratory is performing the analysis of a substance, QCs may be distributed to those involved to assess consistency in analytical results. Details of transport arrangements and conditions will be provided.

A3.14.3 Exact details of chemical analysis will be provided with each protocol dependent on the chemical selected.

A3.15. PROTOCOL CHANGES

A3.15.1 Changes to this protocol will be documented and the reason for the change stated. Where possible, any changes will be agreed with the study co-ordinator.

A3.16. REPORTING DATA

A3.16.1 The following will be reported for the test substance:

- CAS number;
- molecular formula;
- lipophilicity (log K_{ow});
- molecular mass (and molecular mass of radiochemical, where appropriate);
- molecular volume (if available);
- structure of chemical (and site of label, where appropriate);
- batch number of test substance and source;
- pK_a of test substance.

Experimental conditions should be fully described, as these are relevant to modelling of the data. These must include:

- concentration of the dose solution (at the pH used);
- pH used;
- temperatures of the test system.

The reported measurements and results must include the following for the receptor fluid measurements:

- cumulative-absorption profiles for chemical (i.e. $\mu g/cm^2$ vs. time and % absorbed vs. time);
- non-steady state maximum absorption rate (in $\mu g/cm^2/hr$) *;
- pseudo lag time (hrs);

and the following from the dose recovery measurements:

- % chemical remaining in the surface compartment (swabs plus donor chamber washings);
- % chemical in the stratum corneum (tape-strippings);
- % chemical absorbed into the tape-stripped skin;
- % dermal delivery (cumulative receptor fluid plus receptor chamber washings plus viable skin content);
- % total recovery.

* A standard procedure will be defined

A3.16.2 All data for individual diffusion cells will be included. Skin sample, cell number and date of experiment will be reported for each set of individual data. Means, SDs and CVs will be calculated for the data set as a whole for each substance (for each laboratory, if appropriate) as well as for each individual skin donor.

A3.16.3 The study co-ordinator will be responsible for collation and statistical analysis of all data.

A3.16.4 Standard proforma for results should be prepared in advance for participating laboratories to use in compiling their data (cf. EDETOX experience).

A3.16.5 On completion of the study, the draft report will incorporate:

- Description of materials and methods followed
- Results – both hard copies and electronic copies (Excel files)

A3.16.6 DEFINITIONS : Will comply with those given in the OECD Guidance Document

APPENDIX 4: REPORT FROM QSAR WORKING GROUP

Report on the use of Quantitative Structure Penetration Relationships (QSPRs) to Predict Skin Permeability Coefficients.

Prepared by Mark TD Cronin

CONTENTS

A4.1. INTRODUCTION

Quantitative structure-activity relationships (QSARs) attempt to relate statistically biological effects from physico-chemical and structural properties. There has been considerable interest in the development of QSARs for the ability of chemicals to penetrate the skin. Efforts to predict skin permeability are well reviewed by Moss *et al* (2002) and Geinoz *et al* (2004); more general guidelines for the development of QSARs for chemical toxicity and fate are described in Cronin and Livingstone (2004). The development of QSARs, in general and for skin permeability in particular, ideally requires a number of fundamental criteria to be met Cronin (2004). These include the necessity of high quality data that form a consistent and reliable data set. Modelling processes must be appropriate to the endpoint being modelled and, ideally, based on a mechanism of action. Further, transparent models are preferable for regulatory use (Worth *et al* 2004). Special issues for the development of skin permeability QSARs relating to these issues are dealt with below.

A4.2. TYPE OF DATA FOR QUANTITATIVE STRUCTURE PERMEABILITY RELATIONSHIPS (QSPRS)

A number of measurements of skin permeability are possible and it is relevant to QSPR to identify and model the most appropriate endpoint. It is assumed that the permeability coefficient is most suited to the development of QSPRs. A relatively large amount of data (for permeability coefficients) is available in compilations such as Flynn (1990), Patel (2002) and from the EDETOX project. Whilst such data are available, it is recognised that they may not be directly suitable for use in risk assessment; however, their combination with measures (or estimates) of solubility may allow for calculations of maximal flux. The permeability coefficient is appropriate as it provides a measure that characterises the intrinsic steady state properties of the chemical and membrane. It is considered that it is more difficult to characterise the experimental conditions (e.g. solvent, temperature etc.) noted below.

Whilst permeability coefficients may be preferred for modelling purposes, other values of permeability may be utilised. Maximum flux data may be modelled and so provide more applicable measures for risk assessment. However, fewer maximum flux data are available for modelling. It is less likely to be possible to predict percentage absorbed data; whilst many data may be available, they may not form a consistent data set due to the many time endpoints at which they have been measured. Despite this, the percentage absorbed may be related to the solubility properties of a molecule and thus be capable of modelling (for absorption from a fixed vehicle). The likelihood of modelling such data may be possible when significant data are available. It is noted however that there are practical difficulties in QSPR modelling of percentage absorbed data due to the limited range of values as compared to permeability coefficient.

A number of other types of data may be modelled. These include skin-water partition coefficients. Whilst potentially useful, the disadvantage of these data is that they may be difficult to measure and therefore less practical. It is noted that some literature are available for modelling purposes. A further parameter available for modelling is the diffusion coefficient, which again is useful for risk assessment. However, this may be difficult to measure experimentally as it requires many data points at the beginning of an analysis.

Recommendation: currently the permeability coefficient is recommended for use in QSPR development as a significant database is available and it is a steady-state parameter applicable to QSPR modelling.

Consistent data are required for QSPR development. Any differences in protocols used for measurement will result in a collection of values that are not wholly consistent and thus will introduce variability into the resultant QSPR. To ensure a consistent protocol, experimental details must be standardised and there must be appropriate data analysis again to a consistent method. The protocol should be designed to provide a relevant endpoint and the following points should be addressed.

Skin permeability assessments must be performed at a consistent temperature. Temperature is related directly to solubility, and thus affects skin permeability coefficients. A protocol therefore requires a standard temperature to be stated including the temperature of liquids coming into the cell and that of the receptor fluid. For existing data, many of the measurements have been made at different temperatures, which often may not even be known (e.g. for the receptor fluid). There are a number of different approaches to managing a data set that contains values at different temperatures. Abraham and Martins (2004) corrected skin permeability, on the basis of pre-defined criteria, for temperature. Another option is to include a term for temperature in a model, this would not require a pre-defined knowledge of how temperature may effect permeability and may provide an understanding of how temperature relates to permeability.

The development of many QSARs in general, and those for skin permeability in particular assumes (and requires) that the compounds considered are not ionised. With regard to skin permeability coefficients, an ionised compound will permeate the skin at a reduced rate, however aqueous solubility is higher providing an opposing effect. This will inevitably result in inconsistencies in data. Further, the presence or absence of salts in an ionised solution will influence transport, although the effect is largely unknown. It is recommended therefore that where possible a buffer should be chosen to ensure a compound is not ionised. There are also other specific problems that it has not yet been possible to address, for instance the effect of zwitterions (which are effectively ionised but not charged) is unknown.

Aqueous solubility plays a very important role in skin permeability. Compounds which are hydrophobic may be highly toxic, and thus still require skin permeability measurement for risk assessment purposes. The issue of whether it will be possible to obtain a skin permeability coefficient for poorly soluble compounds requires further effort. A possible practical approach in the short term may to set limits for hydrophobicity and / or water solubility. This could allow the creation of applicability domains for the QSPRs developed and even for the test methods.

Any solvent utilised in skin permeability assessment has an effect on the permeability coefficient. As yet there is a lack of fundamental knowledge of the specific nature of that effect i.e. it is unpredictable. This includes the effects of different solvents (when measuring the permeability coefficient of a single compound), or for the measurement of the permeability coefficient for a selection of compounds using the same solvent. Solvents are known to affect the barrier properties of skin in a number of ways, they may extract skin lipids, they may enter skin lipids or they may alter the structure of skin lipids. With these considerations in mind, it is recommended **that permeability coefficients measured (and used for QSPR development) should either not utilise a solvent, or utilise a solvent that does not interact with the skin.**

Many substances are presented as formulations. It is not possible to predict the permeability of a compound from a formulation. In order for this to be possible, further fundamental research is required into the effects of formulations on skin permeability.

In order to gain reliable data from skin permeability assessments, a consistent and appropriate method for the treatment of the raw data is required. Sufficient data and its proper analysis will ensure that steady state conditions have been achieved. The protocol should ensure that experiments be run long enough such that steady-state is achieved. In addition, to ensure data quality, full raw data should be provided for experiments e.g. for steady state, report calculation of lag time, and how permeability coefficients are determined. It is recommended that steady state values should not be extrapolated, and they should be reported only when steady state conditions have been achieved. It is also clear that permeability data should not be extrapolated from the results obtained in one vehicle to another.

Recommendation: In order to ensure that a database is created for the development of a high quality QSPR, new measurements should be made using the same highly prescriptive protocol, and existing data should be checked to ensure consistency (and highlight any differences in protocol).

A4.3. THE POTENTIAL ACCURACY OF PREDICTIONS FROM QSPRS

One of the key aspects of the use of QSARs is that expectations should be realistic. Therefore predictions from QSPRs should not be expected to be any more accurate than the experimental measurements on which they are based. It is acknowledged that the measurement of the skin permeability coefficient is variable and this must be appreciated in the modelling. The accuracy of the predictions is dependent on the required use of the prediction; therefore, all that may be required for risk assessment purposes is a prediction of “high” or “low” penetration.

It would be useful to apply confidence limits and intervals to predictions from QSPRs. Regression analysis is a statistical technical that can provide such limits (most standard statistical programs allow for this). It was noted that the US EPA had applied confidence limits for the assessment of Superfund sites. In order to apply confidence limits to predictions successfully, it would be useful to have some assessment of experimental error. There may be varied error associated with experiments, in particular measurements made at the extremes of the experimental values are likely to have greater error than values taken in the middle of the experimental range of values. In terms of comprehending the data and QSPR modelling, it would be useful to have a knowledge of the factors contributing to experimental error. As noted above, a knowledge of the experimental error should be incorporated into development of QSPRs.

Recommendation: QSPRs should be developed that do not exceed the experimental error of the system. Confidence intervals should be applied that are variable and allow for realistic application of the QSPR.

A4.4. MATHEMATICAL MODELS

Mathematical models are not necessarily QSPR approaches *per se*, but are attempts to model permeability data on the basis of experimental variables and conditions. These so-called mathematical models could provide a tool for regulators (once correctly parameterised) to predict effects e.g. the amount of penetration after specific time

periods. In addition they may be used to relate, link and allow for the calculation of infinite dose data from finite doses.

QSPRs and mathematical models can provide mutually relevant information. In particular QSAR values could input into mathematical models (e.g. K_p) to predict lag time and flux. The mathematical models are often complex and typically can have many parameters as inputs (e.g. concentrations, volumes etc) many parameters are "pre-definable" or may be assumed. However, mathematical models are likely to be specific to the chemical tested and therefore may still require biological data as inputs. One such input is clearance into the receptor (blood), although there are some attempts to model this parameter. It is acknowledged that mathematical models may provide predictions, each made up of a number of predictions. Caution should be taken in assessing the combined errors from all the predictions.

Recommendations: Mathematical models may provide a method to calculate infinite dose data from finite doses and therefore provide input into QSPRs. Conversely QSPRs and QSARs may provide estimations as inputs into mathematical models. More effort is required to link QSPRs and mathematical models successfully and effectively.

A4.5. DEVELOPMENT OF QSPRS

A4.5.1 Descriptors

A variety of descriptors have been applied in the development of QSPRs. Examples of such descriptors can be found in the extensive reviews of Moss *et al* (2002) and Geinoz *et al* (2004). It is acknowledged that in terms of QSPRs for skin permeability coefficients, the most commonly used descriptors are for hydrophobicity and molecular size. Since the work of Potts and Guy (1992) the parameters most frequently used are the logarithm of the octanol-water partition coefficient (log P_{ow}) and molecular weight to describe hydrophobicity and molecular size respectively. As noted below, these two parameters have a strong mechanistic basis and are unambiguous as well as being easily calculated. A large number of additional parameters have been applied in the development of QSPRs for percutaneous absorption (cf Patel *et al* 2002) or completely different approaches, such as the solvatochromic parameters (cf Abraham and Martins, 2004).

Recommendation: QSPRs developed in the same manner as the Potts and Guy (1992) i.e. utilising log P_{ow} and molecular weight are a good starting point for the modelling of permeability coefficients. The use of additional parameters in a QSPR (to improve statistical fit) should not be discounted but should be treated with caution.

It has been acknowledged that the descriptors in QSAR will have error associated with them as well (Cronin and Schultz, 2003; Seward *et a,l* 2001). It is well established that log P_{ow} will have error associated with it, both as a measured and then as an estimated value (Dearden and Bresnen, 1988). In terms of modelling QSPRs, there would be a benefit in reviewing the accuracy of measurements and predictions of log P_{ow}. Further, in terms of developing models, the calculation of log P_{ow} must be made with the same the software. Further, in using the QSPR to calculate skin permeability, the same calculation method should be used for the predictions. The inclusion of measured and estimated log P_{ow} values in the same model has been little addressed in QSAR as a whole. Ideally, measured values for log P_{ow} would be preferred to calculated values; however, predictions will inevitably be based on

calculated log P_{ow} values, which may increase the uncertainty associated with measurement.

Recommendation: Some formal assessment of the utility of log P_{ow} in QSPR development is required. Consistency should be ensured in the calculation of log P_{ow}.

After hydrophobicity, the second parameter in the Potts and Guy approach will help encode information relating to molecular size. As described by Patel *et al* (2002) there are a rich variety of descriptors for molecular size, including molecular weight, volume and surface area; topological indices such as molecular connectivities; molecular dimensions etc. There is a high collinearity between all of these parameters. Because of its simplicity and lack of ambiguity, molecular weight has been the parameter of choice for the development of QSPRs for percutaneous absorption. Whilst it is simple to calculate, molecular weight is recognised to have some drawbacks. It does not account well for high density chemicals (which may have a low molecular volume compared to their molecular weight). Unfortunately this problem is little recognised as current databases are deficient in high density chemicals. It is possible to calculate molecular volume, but such calculations tend to be complex and are conformationally dependent. In order to investigate the effects of high density chemicals, a possible solution could be to calculate the ratio of molecular weight (*MW*) and molecular volume (MV) and concentrate on compounds that deviate from the general ratio (e.g. chlorinated alkanes). Another approach could be to develop QSPRs with log K_{ow} and *MW* and log K_{ow} and MV and see if the model based on MV provides better predictions for "high density" compounds.

Recommendation: Molecular weight provides a simple and unambiguous estimate of molecular size in the development of QSPR. However, it may parameterise the properties of high density chemicals only poorly, the modelling of such chemicals requires further attention.

Ionisation is an important effect to take into account when attempting to model biological activity. It is recognised that ionised compounds enter into, and thus penetrate biological (phosolipid) membranes at a much slower rate that non-ionised molecules. Conversely in skin permeability measurements, ionised compounds will be drawn into an aqueous receptor fluid more rapidly than non-ionised compounds. The relative "strength" of each of these two effects has not been quantified and so is difficult to assess. As a rule, most QSARs assume that no compounds are ionised, and this is the case with skin permeability. There are a number of possible solutions to the problem of ionisation. The most pragmatic is simply to ensure that all permeability coefficients are measured for non-ionised compounds. Alternatively Abraham and Martins (2004) illustrated how ionisation could be accounted for in the measured value. Additionally log P may be corrected for ionisation (the so-called distribution coefficient, D) (Cronin and Livingstone 2004). The problem of ionisation is generally made worse in QSAR modelling as *pKa*, a fundamental physico-chemical property, is only poorly predicted, especially for molecules with multiple ionisable functional groups. A further group of compounds that are not well characterised, and which have been little addressed in QSPR, are zwitterions – such compounds are very difficult to parameterise accurately in terms of their physico-chemical properties.

Recommendations: Permeability coefficients for use in QSPR analysis should be for the non-ionised compound, and it should be recognised that predictions may be made only for non-ionised compounds. More work is required on the effect of ionisation on skin permeability.

A4.6. QUALITY OF DATABASES

As noted the quality of the biological data upon which a QSAR or QSPR is based underpins the quality of the resultant model. Ideally when reporting, for instance, skin permeability coefficients, a minimal set of criteria and experimental conditions should be noted. Other factors such as the purity of the compound being assessed, metabolism and degradation should also be considered.

There has been a general feeling that some of the historical skin permeability data were of questionable quality (cf. Moss and Cronin, 2003). Therefore the approach applied in the EDETOX project to collating skin permeability information is important and may have widespread applicability. The analysis of the EDETOX database illustrates that wide variability in methods; however, there is now a general agreement that EDETOX database is of "acceptable quality", and it will be difficult to improve upon this data set. The success of the QSAR modelling of the database illustrates the high quality of the data being modelled.

It is also recognised that there could potentially be a good data source from regulatory agencies (Bronaugh, 2004, personal communication). However QSAR modelling of regulatory data for other (acute toxicity) endpoints has illustrated the problems that may be faced (Lessigiarska *et al*, 2004). These include finding data for single organic substances, and the problems in variability of experimental protocols that may introduce uncertainty into the dataset.

Recommendation: A minimum set of criteria are required to describe the experimental protocol, these should be established and applied to the datasets modelled.

A4.7. MECHANISM

A requirement for "high quality" QSARs is that they should be based on an established mechanism of action. The definition of mechanism of action will also be a key point in the validation of QSPRs for regulatory use (Worth *et al* 2004). For percutaneous absorption, there is a general agreement in a single mechanism of action, and that will assist in the successful building of models for permeability coefficients. The mechanism is generally considered to involve partition of molecules on the basis of their lipophilicity and diffusion on the basis of their size. It is assumed that the mechanism is not class specific, therefore there is considerable potential for one global QSPR.

With regard to the possibility of a single "global" QSPR, this is an ideal situation. A "global" QSPR is one that could be applied to any chemical class (within the applicability domain of the model). This makes the model more general to use, and also eliminates the problematic derivation and utilisation of class specific models.

A4.8. NON-LINEAR VS LINEAR METHODS

There are a wide variety of statistical methods that may be applied to building QSARs, some of which are linear in their nature and others non-linear (Livingstone, 1995). In terms of QSPR there are two issues relating to linearity. The first is whether regression analysis is a suitable technique for the development of QSPRs; the second relates to the modelling of highly hydrophilic and hydrophobic molecules.

For many QSARs, including those for skin permeability coefficients, regression analysis is the statistical method of choice as it is simple, transparent and highly portable (Cronin and Schultz, 2001). For these reasons regression analysis is probably the statistical technique of choice for skin permeability coefficients. However, there are a number of drawbacks in the use of regression analysis, including that it is (by its very nature) a linear technique, and that it is adversely affected by collinearity between independent variables (e.g. log P_{ow} and *MW*). Many more multivariate options are available including partial least squares and neural networks.

Recommendation: A high quality dataset (e.g. EDETOX dataset) could be modelled by a variety of methods to determine the relative merits of, for instance, regression analysis, partial least squares, and neural networks.

It is further noted that there may not be a linear relationship between permeability coefficient and hydrophobicity for the complete range of log P_{ow}. In particular highly hydrophobic compounds may not be well modelled by a linear QSAR. At the present time, there is insufficient information to determine the nature of this effect.

Recommendation: The modelling of highly hydrophobic compounds should be emphasised, especially if further testing is to be considered.

A4.9. CHEMICAL SELECTION

Should further tests for skin penetration be undertaken, the selection of chemicals will rely on a number of factors. However, the selection of chemicals should also include a chemometric analysis to ensure that those compounds selected provide the maximum possible information. To allow for the successful application of chemometric analysis, the appropriate physico-chemical descriptors for the QSPR must be established. If, this is restricted to log P_{ow} and *MW*, selection of chemicals is easier than for a more multivariate situation. Chemical selection should also be driven by an assessment of how representative the current database is, and what deficiencies it has.

Recommendation: Selection of chemicals for further testing should be underpinned by chemometric analysis of the current database, and the areas in which knowledge is currently lacking.

A4.10. APPLICABILITY DOMAIN

The applicability domain of a QSAR is defined as “the physico-chemical, structural, or biological space, knowledge or information on which the training set of the model has been developed, and for which it is applicable to make predictions for new compounds” (Jaworska *et al*, 2003). As yet, no formal methods exist to define the applicability domain of a structure-based prediction method.

However, work is currently being performed in this area. It is accepted practice in QSAR that predictions should not be made outside of its applicability domain (Cronin and Schultz, 2003). If it may be assumed that a global QSPR will be based on log P_{ow} and *MW*, then an applicability domain may be defined relatively easily and may be shown graphically on a 2-dimensional plot. It is likely that an applicability domain will be elliptical in shape (i.e. there are few, if any, low molecular weight molecules that are hydrophobic). The applicability domain should be defined and used for any QSPR.

Recommendation: The applicability domain should be defined for any QSPR developed and all predictions should be made for chemicals within the applicability domain.

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APPENDIX 5: ADDITIONAL POINTS

This Appendix contains additional notes. Mostly, they arise from comments on draft versions of the report.

A5.1. RELEVANT TO ACCEPTANCE OF INFINITE DOSE DATA

1. Sanco document stipulates that k_p is not sufficient for regulatory approval required by the agricultural products community.

A5.2. RELEVANT TO EXPANSION OF DETAILS IN THE PROTOCOL

1. Most vehicles do affect the skin to some degree. However, some will affect skin permeability much more than others.
2. Acceptable buffering pH range will need to be provided.
3. Issue of mixtures of isomers and/or oligomers was raised (limited data on their dermal absorption) and this may be relevant to the selection of chemicals.
4. It has been demonstrated that an application of 10 $\mu l/cm^2$ can be difficult to spread evenly over the surface of skin in the test cell (unreported findings from EDETOX project and was the justification for using a volume of 25 µl (per cm^2) in the EDETOX project.
5. The term "essence of Good Laboratory practice" does not require the use of GLP compliant laboratories, but that GLP principles are followed. Good Laboratory Practice also needs further consideration, e.g. as to whether formal Quality Assurance is considered appropriate, or whether routine data checking within and between laboratories would be sufficient.

A5.3. FURTHER INFORMATION REQUIRED

1. Stipulation of exclusion criteria for outliers in data will be needed.

APPENDIX 6: PRESENTATION BY PROFESSOR BUNGE - SUMMARY OF INFORMATION

Pesticide Absorption Data Compared to Absorption Estimates Made Using Maximum Flux

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In its regulatory role, the United States Environmental Protection Agency (US EPA) is the repository for a large collection of dermal absorption data supplied by pesticide registrants in compliance with US federal regulations. This database contains almost 300 dermal absorption studies of more than 160 different pesticides (Zendzian, 2000a). Many of these studies followed the Zendzian protocol (Zendzian, 1994), which prescribes procedural details of *in vivo* rat experiments measuring dermal absorption as a function of both the amount of pesticide applied and the exposure time. Recently, Zendzian (Zendzian, 2000b) reported data collected using the Zendzian protocol for representative pesticides from each of three classifications described as volatile, skin damaging, and neither volatile nor skin damaging. In this investigation we examine dermal absorption data obtained using the Zendzian protocol for 17 pesticides including data published by Zendzian (Zendzian, 2000b) and data from study summaries provided to us by Zendzian (personal communication). Most of this study was conducted by Micaela Reddy (Reddy, 2000; Reddy and Bunge, 2002). I have recalculated the maximum flux results, so the numbers differ slightly from those given in Reddy (Reddy, 2000) and in Reddy and Bunge (Reddy and Bunge, 2002). The absorption data are the mean values for 4 rats. Unfortunately, we do not have information on standard deviation. Zendzian did not supply this information and we do not have the raw numbers.

For the 17 pesticides examined in this study, the relationship between systemic absorption and applied dose was different for pesticides that are liquids and those that are solids at skin temperature. For both groups, the amount of pesticide in skin increased proportionally with applied dose. We think this is because washing is never complete. Systemic absorption of liquid pesticides also increased with applied dose. However, for solid pesticides systemic absorption was a weaker function of applied dose and in some cases was independent of applied dose. A simple method for estimating the maximum systemic absorption using a pesticide's permeability coefficient and water solubility under-estimated the amount of dermal absorption for most doses of many of the pesticides investigated in this study. However, in most cases a safety factor of 10 was sufficient to estimate a larger internal dose than experimentally observed.

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Table A6.1. Comparison of estimates for the "maximum" penetration to systemic absorption determined experimentally in male rates *in vivo* for 3 or sometimes 4 applied doses. Information about the applied dose is given in Table 2. Input parameters for the calculation are listed. [a]

Pesticide	*MW*	*log*$K_{o/w}$	[b] $K_{p,w}$ *cm/hr*	[c] S_w *µg/cm³*	[d] Max Flux *µg/cm²/h*	[e] $M_{max,10}$ *µg/cm²*	3 x $M_{max,10}$ *µg/cm²*	10 x $M_{max,10}$ *µg/cm²*	100 x $M_{max,10}$ *µg/cm²*	[f] Experimental Systemic Absorption in 10 h *µg/cm²*			
										LL	L	M	H
Acetochlor	270	3.03	0.00609	223.0	1.36	13.6	40.7	136	1360	0.59	9.8	25	**130**
azinphos-methyl	317	2.96	0.00280	28.0	0.0785	0.785	2.36	7.85	78.5	--	0.22	1.4	2.7
Diclofop-methyl	341	4.58	0.02829	0.8	0.0226	0.226	0.679	2.26	22.6	--	0.89	*15	**78
Diniconazole	326	4.3	0.02210	4.0	0.0884	0.884	2.65	8.84	88.4	--	0.41	2.2	2.0
"	"									--	0.17	0.40	2.0
Disulfoton	274	3.95	0.02589	25.0	0.647	6.47	19.4	64.7	647	--	0.22	2.8	21
EPTC	189	3.2	0.02507	375.0	9.40	94.0	282	940	9400	4.3	6.6	40	430
Imazalil	297	3.82	0.0152	180.0	2.73	27.23	81.8	273	2730	1.7	9.8	68	*1000
Iprodione	330	3.0	0.00249	13.0	0.0324	0.324	0.973	3.24	32.4	--	1.0	0.80	1.5
Isoxaflutole	359	2.32	0.000534	6.	0.00331	0.033	0.0993	0.331	3.31	--	0.030	0.040	0.024
Lindane	291	3.72	0.0136	7.3	0.0989	0.989	2.97	9.89	98.9	--	3.6	*17	*56
(S_w @ 35C)				12	0.164	1.64	4.92	16.4	164.2				
Metolachlor	284	2.9	0.00404	488.0	1.97	19.7	59.2	197	1970	--	3.3	20	70
Mevinphos	224	0.127	0.000101	[g] 1.24×10^6	125	1250	3760	12500	125000	--	0.059	0.33	2.0
Molinate	187	2.88	0.0153	88.0	1.34	13.4	40.3	134	1340	--	2.1	34	*310
Phosmet	317	2.95	0.00276	25.0	0.0690	0.690	2.07	6.90	69.0	--	3.4	3.5	2.4
Thiobencarb	258	3.42	0.0136	30.0	0.409	4.09	12.3	40.9	409	--	3.1	26	*85
Tribufos	315	3.23	0.00449	2.3	0.0103	0.103	0.309	1.03	10.3	--	0.38	*1.4	**13
Vinclozlin	286	3	0.00463	2.6	0.0120	0.120	0.361	1.20	12.0	0.27	0.86	1.0	< 4.4

a Experimental systemic absorption was measured in male rats *in vivo*. There is some evidence that on average the permeability through rat skin is approximately 3-fold larger than through human skin. Dose was applied in 10 $\mu l\ cm^{-2}$ of formulation. Test protocol specified that the exposed area is not smaller than 10 cm^2. Most water solubility and K_{ow} data were taken from *The Pesticide Manual*, 11th Ed., Ed. CDS Tomlin, British Crop Protection Council, 1997.

b Permeability coefficient through the stratum corneum from a water vehicle: $\log k_{p,w}\ [cm\ h^{-1}] = -2.73 + 0.71\ \log K_{o/w} - 0.0061\ MW$

c Water solubility

d Maximum flux = flux through skin at solubility limit = $k_{p,w} S_w$

e Maximum penetration in 10 h = Max flux × 10 h

f Black = Experiment < 10 × $M_{max,10}$; *10 × $M_{max,10}$ < Experiment < 100 × $M_{max,10}$; **100 × $M_{max,10}$ < Experiment

g Completely miscible in water. Density of pure mevinphos was used as the water solubility limit.

Table A6.2. Applied doses and a brief qualitative description of the absorption experimental results for the absorption data in Table 1.

	Applied Dose of Active, *µg cm*$^{-2}$					Did the amount in skin reach a maximum	At 10 h, % on skin for all doses[f]	
Pesticide	**LL**	**L**	**M**	**H**	**S / L**	**and then decrease?[e]**	**<50%[d]**	**>50%**
Acetochlor	3.0	42	270	2940	L	No		
azinphos-methyl	--	0.951	9.19	93	S	No		
diclofop-methyl	--	9.9	100	1000	?[b]	No	X[d]	
diniconazole	--	4.9	50	500	S	No		X (?)[c]
disulfoton	--	0.85	8.5	85	S	Yes, all doses > 1h	X[d]	
EPTC	94	196	902	8760	L	Yes, all doses > 1h	X[d]	
Imazalil	4.0	40	400	4010	S	No		
iprodione	--	31	310	3100	S	No		X
isoxaflutole	--	0.87	7.3	79	S	No		X
Lindane	--	20	200	2000	S	Yes, dose L & M > 4h		
metolachlor	--	10	100	1000	L	Yes, dose L > 4 h		
mevinphos	--	0.45	2.5	12.5	L	No	X[d]	
Molinate	--	9.0	89	890	L	Yes, all doses > 4h	X[d]	
Phosmet	--	58	520	2670	S	No		X (?)[c]
thiobencarb	--	5.2	50	500	L	Yes, doses L & M > 2h		
Tribufos	--	2.0	10	100	L	No		
vinclozlin	2.0	20	200	2000	S	No		X

[a] -- = not measured. S / L denotes solid or liquid at skin temperature (~32ºC).

[b] The melting point of pure diclofop-methyl is slightly higher than skin temperature (i.e., 39 - 41ºC), but the formulation is not pure pesticide and could have a lower melting point.

[c] The ? indicates that the percent of applied dose on the skin was not reported, but it probably remained > 50% because the percent in skin and absorbed systemically were low and it is not likely these pesticides evaporated.

[d] The percent of applied dose on the skin at 10 hours was less than 50% for all applied doses (i.e., the exposed dose changed rapidly).

[e] This column notes the doses and exposure times at which the amount in the skin started to decreased due to a decrease in the exposed dose.

[f] This column notes whether the % absorbed was greater than 50% for all doses, less than 50% for all doses. If neither column has an X, then the % absorbed was less than 50% for H and perhaps M (or L if there was an applied dose of LL).

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