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Dermal absorption for pesticide health risk assessment: Harmonization of study design and data reporting for North American Regulatory submissions

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ABSTRACT

Although an internationally-adopted *in vitro* dermal absorption test guideline is available (OECD Test Guideline 428), the replacement of the *in vivo* approach in North America for pesticide formulations has not occurred due to concern over the reliability and consistency of the *in vitro* results. A 2012 workshop convened a panel of experts in the conduct of *in vitro* studies used for pesticide risk assessment, together with North American regulators, to examine techniques for *in vitro* dermal absorption testing. Discussions led to the recommended "best practices" for the conduct of *in vitro* dermal absorption studies provided herein. The workshop participants also developed recommendations for reporting study results in order to improve the quality and consistency of the data submitted to regulatory agencies in North America. Finally, a case study is presented that illustrates the use of the "triple-pack" approach; the studies, conducted for the registration of sulfoxaflor, follow the standardized recommendations provided at the workshop. In addressing the concerns of these regulators and of the regulated community, and providing harmonized recommendations to facilitate comparative data analyses, it is anticipated that wider acceptance of *in vitro* dermal absorption studies alone can be achieved for pesticide risk assessment.

1. Introduction

The assessment of exposures and the dermal absorption potential of regulated products, including, for example, pharmaceuticals, personal care products, and pesticides, is an important consideration for toxicologists and risk assessors. For scientific and animal welfare reasons, the assessment of dermal absorption *in vitro,* using human or animal skin sources, has become more common, and in some sectors, such as for cosmetics and personal care, *in vivo* dermal absorption studies have been completely replaced by *in vitro* methods. However, due to the potential toxic effects of some pesticides (e.g., cholinesterase inhibitors) and the fact that exposure can occur unintentionally (e.g., drift), there is compelling interest in ensuring that pesticide dermal absorption

Abbreviations: DAF, Dermal Absorption Factor; CDPR, California Department of Pesticide Regulation; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; NAFTA, North American Free Trade Agreement; OECD, Organization for Economic Cooperation and Development; PMRA, Health Canada Pest Management Regulatory Agency; SD, standard deviation; TEER, trans-epithelial electrical resistance; TEWL, trans-epidermal water loss.

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values are not underestimated, in order to be protective of human health.

The use of excised skin preparations for the purpose of estimating dermal absorption via *in vitro* methods capitalizes on the understanding that the process of passive diffusion of substances through the *stratum corneum* does not require a metabolically active *in vitro* test system. It has been established that human *stratum corneum* is a much more effective barrier to absorption compared to rat skin for most chemicals examined in these models (Aggarwal et al., 2014, 2015; Dumont et al., 2015; Fasano et al., 2005). Indeed, literature reviews and prospective studies found that the dermal absorption potential of pesticides, industrial chemicals, and cosmetic ingredients was higher in rat skin than human skin, usually by factors ranging from 5 to 100-fold and in some cases up to 500-fold (Bartek et al., 1972; Jung and Maibach, 2015; Ross et al., 2001; van Ravenzwaay and Leibold, 2004). OECD Test Guideline 428 (OECD, 2004c) provides a protocol for using excised human or rat skin for the purpose of assessing the dermal absorption of chemicals and formulated products and/or dilutions. However, as is the case with most OECD test guidelines, this test guideline and its accompanying guidance, Guidance Document No. 28 (OECD, 2004a), were written for general use, and generally do not include sector- or chemical property-specific protocol recommendations.

Since that time, a number of organizations, including the OECD, have published guidance documents on the conduct and interpretation of *in vitro* dermal absorption studies (OECD, 2011). The WHO International Programme on Chemical Safety recommendations state that human skin should be the "gold standard" in human health risk assessment for all chemical classes (WHO, 2006), and recommends the development of "consistent and well controlled studies with human skin in order to predict dermal absorption in humans." More recently, the EFSA has published guidance for the conduct and interpretation of dermal absorption studies and also state that *in vitro* studies performed with human skin are preferred (EFSA, 2012). This EFSA guidance document is used for the registration of pesticide-containing products in the European Union, and is currently under revision. A recent review by Dumont and coworker summarizes some of the similarities and differences in these guidance documents (Dumont et al., 2015).

While data from *in vitro* studies alone are fully accepted by European pesticide regulators, in North America an *in vivo* study, usually conducted using rats, continues to be required by the US EPA, CDPR, and PMRA to determine dermal absorption values for pesticides. In 2008, the NAFTA Dermal Absorption Working Group (EPA, PMRA and CDPR) (NAFTA, 2008) issued a policy statement outlining the triple pack approach, which was recommended as the preferred testing method(s) for new pesticidal active ingredients that are submitted for registration to regulatory authorities in North America. In this approach, three studies—an *in vitro* rat, an *in vivo* rat, and an *in vitro* human—may be submitted together in order to set a DAF for use in human health risk assessments for pesticides (NAFTA, 2008). The purposes of this recommended approach were: 1) to help improve/standardize the quality of *in vitro* studies both in terms of conduct and reporting to the NAFTA regulatory agencies and 2) to allow the assembly of a comparative database (with existing and new data), which is critical to determining whether the *in vitro* human skin method is predictive of *in vivo* dermal absorption, with the ultimate goal being the acceptance of *in vitro* studies alone.

A workshop held on May 1–2, 2012 in Gaithersburg, Maryland, USA, convened a small international panel of academic and industry experts in the field of dermal absorption, together with North American pesticide regulators and non-governmental representatives, to determine the barriers to acceptance of stand-alone *in vitro* dermal absorption studies. An expected outcome of the workshop was to build consensus around best practices for the conduct and reporting of *in vitro* dermal absorption studies for pesticide risk assessment and to increase comparability of *in vitro* studies across different laboratories. Steps outlined as part of this Workshop are captured herein with the aim of evaluating the predictive power of the *in vitro* method in typical in-use conditions, and to help North American regulatory agencies define the criteria by which *in vitro* study values can be used in future risk assessments. To help illustrate the type of data that is contained within a triple pack and its use in deriving values for risk assessments in North America, a brief review of a triple pack of studies with the pesticidal active ingredient sulfoxaflor is provided as a case study.

2. Regulatory agency considerations

For pesticides to be approved in North America, regulatory agencies are required to ensure there are no human health (or environmental) risks of concern when used according to the label directions. Dermal absorption values are used in estimating systemic exposure via the dermal route in order to facilitate comparison with critical effect levels derived from oral toxicological studies. Chemical-specific dermal absorption studies are used to determine the DAF, where possible.

Currently, *in vitro* dermal absorption studies on pesticides are not accepted in the absence of an *in vivo* dermal absorption study in North America for regulatory decisions, but can be accepted as part of a triple pack, as mentioned previously. North American regulators state that differences in test protocols have led to variable results such that a range of *in vitro* dermal absorption values would be obtained for the same test substance depending on the study methodology. This results in a lack of confidence in using *in vitro* data as standalone. Incomplete test reports or data dossiers also contribute to this uncertainty.

During the workshop all three North American regulatory agencies reported that a major barrier to acceptance of the *in vitro* dermal absorption method is the high degree of flexibility in the test protocol parameters that are described in established test guidelines and guidance (OECD, 2004a; OECD, 2004c; OECD, 2011). This prevents comparison between laboratories and studies, and creates uncertainty when reviewing individual study submissions, including the extent to which such variations may affect the study outcome. Some of the variable protocol elements identified include:

- Skin used, source, thickness, separation procedures
- Receptor fluid choice
- Barrier function testing method and criteria for exclusion of a skin sample
- Tape stripping methods and other post-exposure activities
- Numbers of individual donors, and samples per donor per test

US EPA described the *in vivo* dermal absorption rat study protocol, which uses four male rats per dose per time point (EPA, 1998). Studies usually contain at least three dose levels and four to six exposure times, using 80–120 rats, though reduced protocols are also accepted. The OECD *in vivo* dermal absorption test guideline (TG 427) is also accepted (OECD, 2004b). As the conditions for *in vivo* dermal absorption studies are somewhat standardized and reproducible, confidence in the use of *in vivo* dermal absorption studies for pesticide regulatory purposes is higher. However, *in vitro* dermal absorption studies using human skin offer several advantages, such as the use of skin from the relevant species of interest (i.e., human vs. rat) while avoiding human testing, the ability to better capture volatile chemicals, enhanced control over

the skin exposure site during the procedure, lower cost, and the avoidance of the use of any animals for testing purposes.

During the workshop Health Canada's PMRA reported receiving several triple pack submissions, but have only been able to apply the triple pack approach in setting a dermal absorption value in a few situations. This is typically due to limitations with the *in vitro* data, which include:

- Inadequate reporting of important details, such as raw data, skin preparation details (e.g. site, thickness, barrier integrity testing), and skin storage details
- Lack of evidence of adequate solubility of the test material in the receptor fluid
- Inadequate justification for deviations from test guidelines

The CDPR reported that they are not currently accepting use of *in vitro* dermal absorption data alone, and that *in vitro* data even when submitted with the triple pack approach is rarely used because of a lack of empirical validation of the *in vitro* dermal absorption approaches submitted. Problems cited include:

- Protocol differences between the *in vitro* rat and *in vitro* human studies
- Insufficient description of materials and methods
- Questions of body region from which skin samples are derived

All three regulatory agencies also reported that the acceptance of *in vitro* dermal absorption data in lieu of *in vivo* dermal absorption data is a desirable goal for the future. This is dependent upon the assurance that studies are routinely conducted according to standardized protocol procedures, study report submissions emulate best data reporting practices, and that the relationship between *in vitro* and *in vivo* dermal absorption methods and results is established in comparative studies submitted to the Agencies and in future publications.

3. Test method user/industry considerations

During the last decade, EU Member States have been accepting the *in vitro* dermal absorption approach using human skin as a stand-alone model for assessing dermal absorption for the registration of new pesticide formulations. This has allowed testing laboratories to develop significant expertise in *in vitro* methodologies as underlined by published protocols and data (Aggarwal et al., 2014, 2015; Fabian et al., 2017).

The basic structure and set-up of the *in vitro* dermal absorption system has some variability in the permitted methods, but certain aspects are standard. In one commonly used approach, a static diffusion cell apparatus (Fig. 1) is used to hold the skin tissue for each sample, apply the test material, and obtain aliquots from receptor fluid to measure the absorbed concentration of the test material. Another equally valid set-up uses a flow-through apparatus (Fig. 2) (Moore et al., 2014a); both systems provide equivalent data (Clowes et al., 1994).

It is important when using either cell type that solubility in the receptor fluid is not rate limiting to absorption. The advantage of the flow-through diffusion cell is that the receptor fluid is continuously being replaced so that it does not become the barrier to absorption. Excised tissue from any species can be used, but *in vitro* studies in the triple pack approach typically use clipped rat skin and human dermatomed skin of thickness of 200–400 μm. Human tissue is obtained from donors according to ethically approved procedures (e.g., cosmetic surgery), and is normally frozen before use. One common approach has been to use heat-separated or chemically-separated epidermal membranes, which provide a

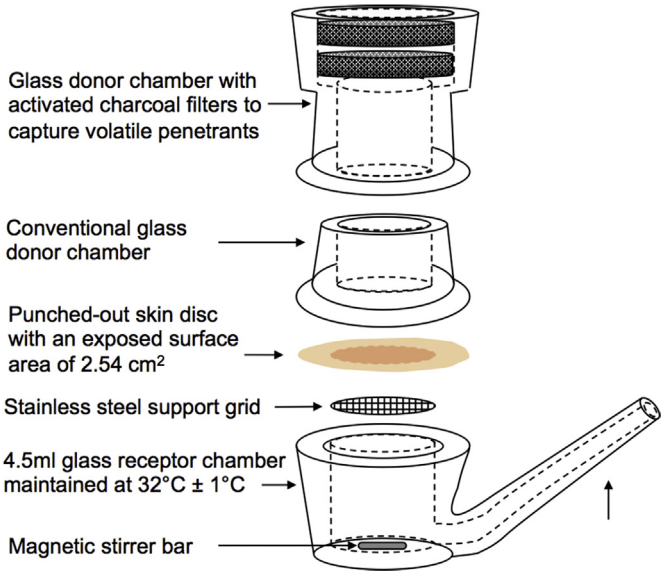

Fig. 1. A Franz static diffusion cell apparatus. Drawing by Dermal Technology Laboratory, Ltd.

conservative model because they are relatively thin (100–200 μm) and consist of the *stratum corneum* and epidermis. In the last several years, there has been a dedicated move towards split-thickness dermatomed skin, which is slightly thicker (200–400 μm) and contains *stratum corneum*, epidermis, and a small portion of the remaining dermis. The integrity of each skin sample is assessed and quantitatively determined (Diembeck et al., 1999; Guth et al., 2014; Meidan and Roper, 2008; OECD, 2004a; OECD, 2004c). The test material is applied as a finite dose for a pre-determined amount of time (usually six, eight, or ten hours (representing a normal work duration), though 24-hr exposure can be used) and the skin surface is sponge-washed at the end of the exposure period. The test continues to run for 24 h with multiple sampling times, and the receptor fluid is analyzed to determine the absorbed concentration of the test material. At the end of the test period, the upper layers of *stratum corneum* are removed by tape stripping (Trebilcock et al., 1994). Historically, pieces of adhesive tape are applied to the skin, pressed, and pulled away. However, currently many laboratories perform tape stripping using a sophisticated device which applies the same pressure on each tape-disc (Fig. 3). A major point of discussion during the workshop was whether to include the portion of the test material remaining in the tape strips, remaining epidermis, dermis, or whole skin in the final calculation of absorbed test material; workshop participants recommended providing all values in the study report to facilitate regulatory review and verification of the absorbed dose.

Presentations and discussions during the workshop focused on experience and data generated in expert dermal absorption laboratories to identify preferred options for pesticide studies where existing guidance offered less specificity. Variables discussed included skin integrity measurements, species, sample location, age of tissue donor, donor and receptor fluid solubility, storage conditions, and skin thickness and removal of *stratum corneum* by tape-stripping.

3.1. Triple pack case study: sulfoxaflor

The NAFTA Dermal Absorption Working Group (NAFTA, 2008) has recommended that registrants submit three dermal absorption studies for new pesticidal active ingredients: *in vitro* rat and human

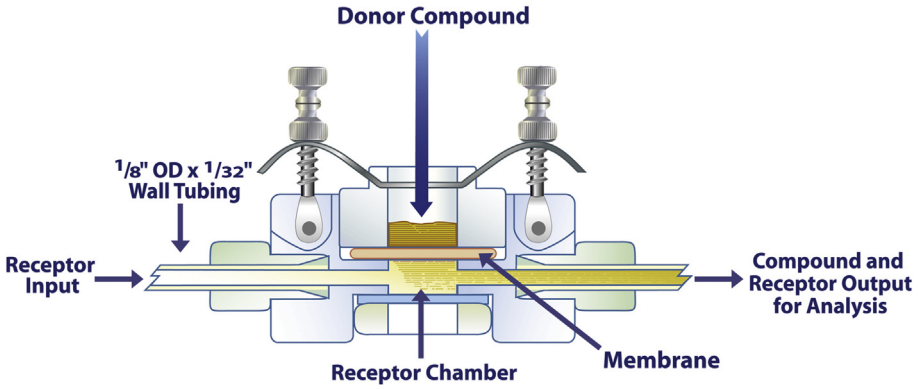

Fig. 2. Cross-sectional view of a flow-through diffusion cell apparatus. Image courtesy of PermeGear.

Fig. 3. D-Squame is a spring-loaded device that delivers the same amount of pressure every time on the D-Squame tape disc. The use of this device is not required, though it increases the consistency of the tape-stripping procedure. Image courtesy CuDerm Corporation.

studies and an *in vivo* rat study, to support the derivation of a DAF from an *in vitro* human dermal absorption study. It is expected that if the ratio of absorbed dose in *in vitro* and *in vivo* rat studies is close to one, then the *in vitro* human study will be reflective of the human *in vivo* absorption and that data from that study can be used to set the DAF (Fig. 4).

Dow AgroSciences submitted three studies according to the triple pack approach as part of a registration package for sulfoxaflor. The studies were done according to applicable OECD test guidelines and their design elements were similar to those recommended in the current publication. Results are presented in Table 1 as taken

$$\text{IF} \quad \frac{\text{Animal } \textit{in vitro}}{\text{Animal } \textit{in vivo}} \approx 1 \quad \text{THEN} \quad \text{Human } \textit{in vitro} \approx \text{Human DAF}$$

Fig. 4. Equation representing the procedure originally proposed by the NAFTA Dermal Absorption Working Group for assessing the utility of the *in vitro* human study based on the ratio between the rat *in vitro* and *in vivo* studies (NAFTA, 2008).

from US EPA human health risk assessment review for sulfoxaflor (EPA, 2012). Each study used 3 equal doses of the same formulation consisting of the undiluted product and 2 water-based dilutions representing the field spray concentrations. The rat *in vivo* absorbed dose (excreted dose plus dose retained in the body, excluding the application site skin) was about 11%, and the rat *in vitro* absorbed dose (dose in the receptor fluid plus skin including whole *stratum corneum* but not tape strips 1 and 2) was about 9%. Percent absorbed was much lower in both studies for the highest dose, which represented the undiluted formulation.

The US EPA review (EPA, 2012) concluded that when accounting for standard deviations, the *in vivo* and *in vitro* rat studies gave an equal absorbed dose, leading to the use of the *in vivo* human absorbed dose of 2.4%. However, criteria chosen for percent absorbed dose calculation for *in vivo* and *in vitro* studies (exclusion or inclusion of the radioactivity seen in the skin) as well as comparison time point (192-h absorption from *in vivo vs* 24-h absorption from *in vitro* studies) were different.

This sulfoxaflor example provides a demonstration of the central thesis of the 2012 workshop discussions and this paper: standardization of the parameters of the *in vivo* and *in vitro* studies provides data which can be used to demonstrate the utility of the *in vitro* dermal absorption test for deriving values for use in a pesticide risk assessment. In addition, presenting results in a transparent manner according to the principles in this manuscript allowed the NAFTA regulatory agencies (e.g. US EPA in the case study above with sulfoxaflor) to form their own conclusions on the appropriate values for a DAF to use in human risk assessments.

4. Recommendations

4.1. Protocol elements

Based on the presentations and discussions during the workshop, the regulators and experts present made data-driven protocol recommendations aimed at harmonizing the conduct of *in vitro* dermal absorption studies. These recommendations can be found in Table 2. While variations allowed within the guidelines remain acceptable for regulatory purposes, minimization of variation by following the protocols in this paper is recommended. This will allow easier interpretation of studies within any one triple pack approach and also facilitate data analysis between studies with multiple chemicals, both of which are needed to expedite future acceptance of *in vitro* dermal absorption data alone in setting dermal absorption values for exposure assessments. For comparisons within a triple pack approach, it is desirable to harmonize protocol elements such as skin location, storage and preparation,

Table 1
Sulfoxaflor dermal absorption study data.

Parameters	*In Vivo* Rat			*In Vitro* Rat			*In Vitro* Human		
Test Material	SC Formulation (24%)			SC Formulation (24%)			SC Formulation (24%)		
Exposure duration	10 h			10 h			10 h		
Study duration	192 h			24 h			24 h		
Dose tested (μg/cm^2)	0.25[a]	4.8 [a]	2360 [b]	0.25 [a]	4.8 [a]	2360 [b]	0.25 [a]	4.8 [a]	2360 [b]
% Absorbed dose*	10.77[c]	11.35[c]	1.22[c]	8.02[d]	8.72[d]	1.67[d]	2.38[d]	2.44[d]	0.35[d]
DAF (*In Vivo* rat) ≈ DAF (*In Vitro* rat) ≈ 11%									
In Vitro Human dermal absorption = 2.4%									

*Values shown represent the final values (% absorbed dose) based on the USEPA review of these studies (EPA, 2012): % absorbed dose for *in vivo* study is excreted dose plus dose retained in the body, excluding the entire application site skin while for *in vitro* studies is dose in the receptor fluid plus entire skin but excluding Tape-strips 1 and 2. In the final reports for these studies, % of dose values in each compartment from the three studies were separately tabulated.

[a] Dilution of the formulation with water in ratio representing the product label.
[b] Undiluted formulation.
[c] Absorption in 192 h.
[d] Absorption in 24 h.

dose, nature of the receptor fluid, and exposure times among studies as much as possible.

For some protocol elements, variations (between studies with different chemicals) were considered acceptable provided adequate documentation and justification was given in the study report. For example, there are a few different methods to check integrity of the barrier function of the skin sample before the study. The methods presented included determining impedance (TEER), tritiated water flux, and TEWL. Each method has advantages and disadvantages (Davies et al., 2004; Guth et al., 2014, 2015; OECD, 2004a); therefore the workshop participants declined to specify a recommendation, though participants agreed that reporting a particular lab's capability to effectively determine barrier function integrity was extremely important.

For other elements, workshop participants expressed clear preferences, based on the data presented and specific pesticide risk assessment considerations. For example, dermatomed skin preparations with a thickness of 200–400 μm were preferred. This is in line with European guidance (EFSA, 2012), and has become the state of the art in recent years for several reasons. Other methods of skin preparation, such as chemical separation, may alter the barrier function of the sample; also, it is not possible to perform tape stripping of heat-separated rat epidermal membranes which are biologically very thin. Therefore, the use of dermatomed skin in both rats and humans allows direct compartment:compartment comparisons. Finally, epidermis-only models may overestimate or underestimate (in the case of highly lipophilic compounds) actual *in vivo* absorption values. Special interest should be given to the quality of the skin preparation and the washing process after exposure and after the experiment to allow easier and better tape-stripping. Presenters also emphasized that the composition of the receptor fluid can have a major impact on study results. The solubility of the test material in the receptor fluid must be adequate and not limit the absorption process; however, some fluids especially containing ethanol may increase absorption and may represent a "worst case scenario" (Challapalli and Stinchcomb, 2002; Guth, 2013).

4.2. Data reporting guidelines

During the workshop, regulatory representatives expressed that technical details in the reports submitted to the regulatory agencies were lacking in many cases, preventing informed decisions about the validity of the study results. Workshop participants derived the reporting template provided in Table 3 from previous OECD guidance (OECD, 2004a) as a tool for registrants that would provide sufficient detail to allow an unambiguous description of the study procedures and findings as well as facilitate data interpretation and acceptance. One key piece of any study is the reporting of the amounts of compound found in each "compartment", including donor chamber and wash, dermis/epidermis and *stratum corneum* (i.e., tape strips), skin swabs, and in the receptor chamber. The group agreed that since regulatory agencies may wish to categorize amounts in each of these compartments differently, it is more appropriate for the study report to include all individual results rather than just the final recoveries. The full mass balance recovery of the test material should also be provided.

5. Literature reviews comparing *in vitro* and *in vivo* dermal absorption values

The aim of this workshop was to harmonize variable elements of existing *in vitro* dermal absorption test guidelines, which are used in setting dermal absorption values for pesticide exposure and risk assessment, in order to facilitate regulatory acceptance. A harmonized dataset of *in vivo* and *in vitro* dermal absorption studies would aid in the analysis of the relationship of these studies for a range of pesticides. Recently, Aggarwal and colleagues compiled 295 OECD TG 428- and Good Laboratory Practice-compliant *in vitro* human dermal studies performed with pesticide formulations which resulted in more than 750 dermal absorption values (Aggarwal et al., 2015). Based on this database, the authors proposed guidance on (1) potential default values in absence of a compound-specific study, (2) a read-across approach in absence of a formulation-specific study, and (3) a novel approach for extrapolation for an untested dilution of a formulation (Aggarwal et al., 2015). More recently, the same authors developed a method to create a dry residue of the formulation spray in laboratory conditions and to transfer it to the skin surface for dermal absorption testing (Aggarwal et al., 2016). The dermal absorption data generated with dry residue of the spray would be relevant for risk assessment for workers entering treated fields once the spray is dried. Depending on the situation, workers could potentially come in contact with dry residue of the spray present on the foliage surface.

Support for the approach of harmonizing protocol elements to enable comparison between *in vivo* and *in vitro* studies can be found in a retrospective evaluation of 92 data sets that reported *in vitro* and *in vivo* dermal absorption results in humans (Lehman et al., 2011). When considering all 92 data sets, the authors found correlation between *in vitro* and *in vivo* values, but with %-absorbed ratios (*in vitro*/*in vivo* ratios) that ranged from 0.18 to 19.7 (mean 1.6) (Fig. 5). However, two major factors that influenced the concordance between the *in vitro* and *in vivo* studies were

Table 2
Preferred harmonized design elements of *in vitro* dermal absorption studies for North American submission.

Protocol Elements		Consensus Recommendations
Skin Variables	Type	• Preparation: Dermatomed split thickness skin preferred • Surgical vs cadaver: either are appropriate if integrity verified • The panel is aware of issues with regard to the limited availability of surgical skin
	Species	• Human and rat preferred • In vivo and in vitro rat should be same strain
	Derivation [anatomical location]	• Rat: Dorsal only • Human: abdomen, breast, back, upper leg
	Number of individuals used as donors	• At least 6 evaluable samples per concentration should be available at end of study • Human: 4 donors with 2 replicates each would provide information on inter-individual and assay variability (per concentration) • In vitro rat: 8 skin replicates from minimum number of animals, as rats are inbred • In vivo rat: 4 animals per dose per time-point
Skin Preparation	Thickness	• 200–400 μm
	Skin type/storage procedures	• Human: dermatomed split thickness skin preferred • Fresh vs frozen: either are appropriate if integrity verified
	Pre-test skin sample treatment	• Skin should be properly hydrated before integrity testing and use
	Method for testing skin integrity	• Several methods were discussed; the method chosen should be supported by argument/literature and should follow current existing guidance (OECD, 2004c) • Concurrent integrity testing with radiolabeled standard is appealing but not yet proven
Model Preparations	Static vs flow-through method	• Both methods equally acceptable • Solubility of test compound in the receptor fluid is critical in choosing method
	Choice of receptor fluid	• Physiological saline supplemented with surfactant is a typical receptor fluid • Per existing guidance, solubility should drive choice of fluid (OECD, 2004a) • The solubility of the test material in the receptor fluid is a critical component of the study. Inadequate solubility can underestimate dermal absorption.
	Volatile and Semi-Volatile materials testing	• Any device used to trap volatile or semi-volatile compounds should not create occlusive conditions • Evidence should be provided to support that the compound is volatilizing to avoid adding loss into % absorbed
Substance application	Composition of test substance(s)	• Refer to given exposure scenario (e.g., according to product label of use) and provide a rationale for the doses used that takes into consideration the amount that may get on a person's skin for a given scenario (e.g. while mixing/loading) rather than just the concentration of the product used in that scenario. • For microencapsulated compounds, etc., careful thought should be given to the location of the radiolabel and that the test substance is representative of the actual product
	Appropriate application doses/ procedures	• For viscous compounds, capture details of how uniform application is achieved • Care should be taken to spread pastes evenly over the skin surface • Important to identify nominal vs. actual dose
	Exposure duration	• 6–10 h • Important to ensure that in vitro rat and human and in vivo rat are the same exposure duration, ideally from the same dose preparation
	Washing procedures	• Follow existing guidance
Measure-ment	Tape stripping	• Specific methods should be reported and justified. For example, as mentioned in Fig. 3, the D-squame pressure device can be used along with D-squame tape-strips. • Useful additional information would include typical amounts of stratum corneum removed with successive tape strips for the particular tape stripping method used • First and second strips analyzed together or individually then third onward pooled or analyzed separately
	Receptor fluid	• Serial collection at 1–2 h intervals until 24 h • Calculate lag phase and maximum flux
	Measurement of bound material	• As long as values are reported, any differences in assessment preferences can be taken into account
	Sampling duration	• Normally 24 h
	What is included in % absorbed?	• As long as values are reported, any differences in assessment preferences can be taken into account
	Mass balance of applied material	• Follow OECD guidance (100 ± 10%)
	Volatile Test materials	• Test for volatility and loss of test material in preliminary experiments; if loss due to volatility will be significant (recoveries <90%), include non-occlusive traps for volatiles

identified:

- Use of skin from an anatomical site other than that used *in vivo*, and
- Use of a vehicle to dilute and apply the test article that differed from the *in vivo* study vehicle.

Other factors were also noted, such as differences in the total dose between studies and differences in length of exposure or wash time. When data sets that carefully harmonized the two major factors cited above were evaluated, a much closer correlation between *in vitro* and *in vivo* human dermal absorption was observed. This set of 11 harmonized data sets from 2 studies reflected compounds with a similarly wide range of dermal absorption values, but the *in vitro* to *in vivo* ratios only ranged from 0.58 to 1.28 (mean 0.96), indicating a very close approximation of the *in vivo* human absorption when *in vitro* conditions were designed to match (Fig. 6).

Although these two harmonized studies were conducted with non-pesticidal substances and include one of the OECD recommended reference compounds (testosterone), the importance of harmonized protocols when comparing *in vivo* and *in vitro* studies is considered to extend to all substances.

Table 3
Recommended study report contents.

Product Variables	• Material/product tested (name/code number) • Type of formulation • Concentration of active substance in the formulation with Certificate of Analysis • Vehicle used (if any)

Tissue Variables	• Species/strain
	• Number of donors/animals, sex and age
	• Sample site
	• If cadaver sourced, date of death and date of collection of skin
	• If non-human, housing and treatment conditions
	• Skin collection protocol (report on pre-operative cleaning procedures, if available)
	• Skin preparation used (e.g. split/full thickness skin, heat or chemical separation/dermatomed)
	• Storage conditions, including time stored
	• Integrity testing method used
	  • Data for individual cells
	  • Acceptance criteria/cut-off value, supported by literature reference
	  • Time between integrity testing and start of test
	• Optional: Historical data ranges for reference compounds (May be useful for context)

Experimental set up & conduct	• Group size/number of wells
	• Type of Franz cells e.g. flow-through or static
	• Cell construction details including stirring process
	• Formulation concentrate and spray dilution rates
	• Finite dose used adequately covering the whole skin surface
	• Application rates both in micrograms active substance per milliliter of test preparation and micrograms active substance per cm^2 of skin surface
	• Nominal and actual dose applied (e.g. actual dose would not include amount left in pipette if any)
	• Exposure time
	• Sampling frequency and duration (time of last sample)
	• Demonstrated consistent temperature (32° C) and humidity
	• Receptor fluid composition, as well as a justification for use, and a statement of whether the chosen receptor fluid provides adequate solubility of the test material
	• Adequate solubility: provide supporting evidence or test results. It is not sufficient to state that the material was found to be soluble in the receptor fluid.
	• Receptor fluid volume or rate of flow of receptor fluid
	• Discussion of receptor fluid sink conditions relative to the amount of active compound dosed, and its solubility limits, to assure that either static or flow-through diffusion cells are fit for purpose
	• If static diffusion cell, frequency of sampling and sample volume should be reported.
	• Detail skin swabbing or washing process
	• Tape stripping:
	  • Evidence that tape striping method is appropriate with laboratory evidence or literature sources.
	  • ID model number and brand of tape used
	  • Fully describe tape stripping method: force of press, pressing instrument if used, etc.

Data Reporting	• If dermatomed skin is used, skin can be split into dermis and epidermis and separate analysis can also be performed • Total recovery (%, mean ± SD) • Mean of maximum flux (μg/cm^2/h, mean ± SD) • Lag time (h) • Amount absorbed (%, mean ± SD) • Absorption rate and time course profile of absorption • Which samples contribute to the amount absorbed • Amount in donor chamber wash • Amount in skin surface washes (%, mean ± SD) • Amount in tape strips 1 + 2 (%, mean ± SD) • Amount in tape strips 3 to ∞ (%, mean ± SD) • Amount in stripped skin sample (%, mean ± SD); present dermis and epidermis data separately, if available • Amount in receptor fluid and receptor chamber wash (%, mean ± SD)

6. Future outlook

The field of *in vitro* dermal absorption is now scientifically well established, and *in vitro* human skin is used by some regulators to assess the dermal absorption potential of chemicals, as well as to assess the dermal absorption potential of chemicals and provide values suitable for human risk assessments or other compound-specific calculations in a variety of industries including cosmetic (Hoppel et al., 2015), pharmaceutical and agrochemical (Aggarwal et al., 2014, 2015; Moore et al., 2014b).

In 2008, the NAFTA Dermal Absorption Working Group (NAFTA, 2008) had proposed an ongoing comparative analysis of the reliability and relevance of the *in vitro* method by encouraging registrants of new pesticidal active ingredients to submit a triple pack of rat *in vivo*, rat *in vitro*, and human *in vitro* data. To further increase the reliability of the *in vitro* methods and the data interpretation by dossier reviewers, the 2012 workshop participants agreed on a series of protocol and reporting recommendations, which were then communicated to the pesticide registrant community.

This paper provides an overview of those discussions and the resulting recommendations, demonstrates a successful triple pack case study that was acceptable to the US EPA reviewers (allowing use of the human *in vitro* values), and encourages further collaborative action on the part of regulatory agencies, companies, and other stakeholders to facilitate the use of *in vitro* dermal absorption methods for risk assessment. A natural first step is the analysis of any existing data. Because *in vitro* dermal absorption methods are accepted as stand-alone by regulatory agencies in the European Union, there are more recent publications on the use of the *in vitro* approach for pesticides across different laboratories (e.g. Aggarwal

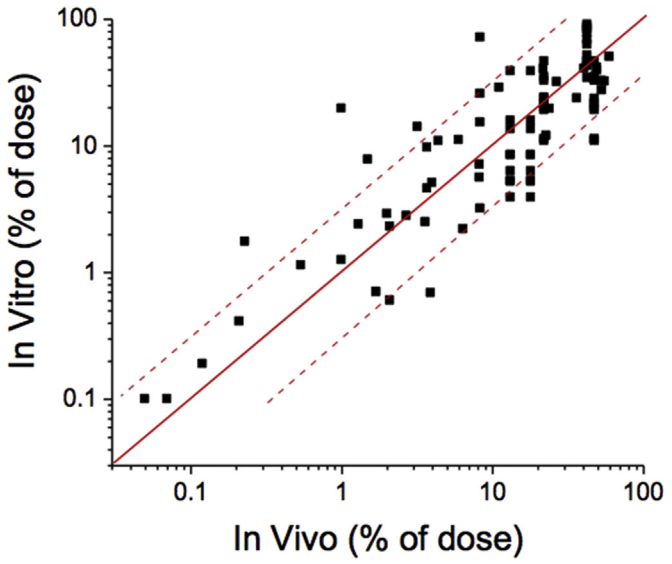

Fig. 5. From (Lehman et al., 2011): *In vitro*/*in vivo* correlation for total absorption for all 92 data sets plotted on log-log scale. Solid line represents the ideal one-to-one correlation and dashed lines represent ± three-fold difference from ideal. The *in vitro*/*in vivo* ratios ranged from 0.18 to 19.7 with an overall mean of 1.6. [reprinted with permission].

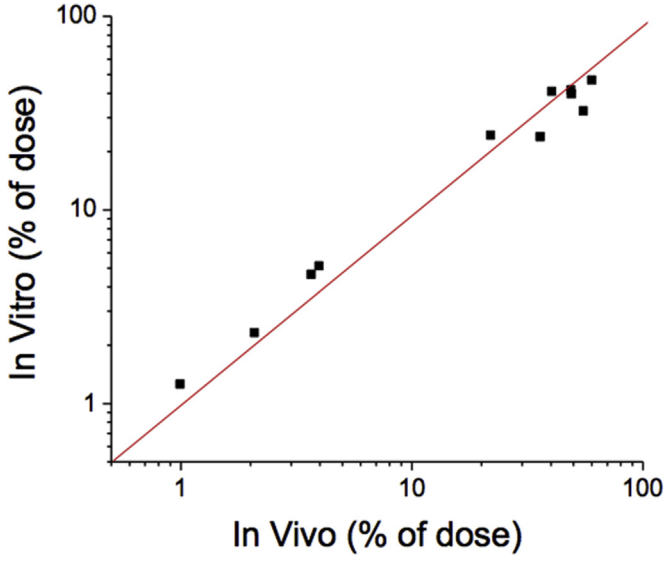

Fig. 6. From (Lehman et al., 2011): *In vitro*/*in vivo* correlation for total absorption for 11 fully harmonized data sets plotted on log-log scale. Line represents the ideal one-to-one correlation. The *in vitro*/*in vivo* ratios ranged from 0.58 to 1.28 with an overall mean of 0.96. [reprinted with permission].

et al., 2014, 2015, 2016). Other groups continue to develop approaches for reducing inter-study variability and exploring the utility of *in vitro* or *in silico* models to help replace the use of *in vivo* animal models (i.e. in rats) (Aggarwal et al., 2016; Challapalli and Stinchcomb, 2002; Desmedt et al., 2015; Dumont et al., 2015; Guth, 2013; Lehman et al., 2011), particularly when the goal is to derive human-relevant measurements for dermal absorption potential. It is hoped that the harmonized approach described in this current publication, in combination with the database of existing triple packs that NAFTA regulators have received for pesticidal formulations since 2008, will provide a good basis for determining the best way to estimate human dermal penetration. The extent of available and appropriate comparable data is currently unknown, and collecting and analyzing this data is a clear next step. Continued active participation from all stakeholders, especially from North American regulatory agencies, is essential to ensure sustained progress in this area, where ideally the animal approach will ultimately be replaced by scientifically valid *in vitro* human dermal absorption studies.

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