Methods for Assessing Percutaneous Absorption

The Report and Recommendations of ECVAM Workshop 13


Preface

This is the report of the thirteenth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become better informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of in vitro tests and their potential uses, and make recommendations about the best ways forward (1).

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The European Centre for the Validation of Alternative Methods. This document represents the agreed report of the participants as individual scientists.
The workshop on Methods for Assessing Percutaneous Absorption was held in Angera, Italy, on 30 May–3 June 1994, under the co-chairmanship of Doug Howes (Unilever Research, UK) and Jean-Paul Marty (Université Paris XI, France). The participants comprised scientists working in both academia and industry. The current status of in vivo and in vitro methods used for studying the absorption of compounds through the skin was reviewed, with particular emphasis given to recommending ways of reducing the number of animal studies needed and of maximising the use of scientifically valid in vitro methods.

Introduction

There are many complex interactions which may occur during the movement of molecules from the outer surface of the skin into the systemic circulation. There have been numerous attempts to produce simple models of these processes, which have resulted in the development of various types of in vitro systems (Figure 1). An appreciation of the limitations of these is now enabling the data generated by using in vitro methods to be applied with increasing confidence for predicting the likely effects of chemicals in vivo.

Figure 1: Physiological hierarchy of methods for measuring percutaneous absorption

<table>
<thead>
<tr>
<th>Confidence level</th>
<th>Human</th>
<th>Primate</th>
<th>Swine</th>
<th>Guinea-pig</th>
<th>Rat</th>
<th>Hairless strains</th>
<th>Mouse</th>
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<td>In vivo</td>
<td>Perfused skin</td>
<td>Whole skin (viable)</td>
<td>Whole skin (non-viable)</td>
<td>Keratome slices</td>
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The methods employed for studying percutaneous absorption range from in vivo systems, in which the full range of physiological processes are operative, to simple in vitro methods and modelling approaches (Figure 1).

The following aspects pertaining to percutaneous absorption were discussed at the workshop: model systems, mechanisms, modelling of structure–activity relationships (SAR), the quality of published data, limitations of existing databases, protocols, in vitro/in vivo correlations, and the validation of new test methods. A general strategy for the investigation of percutaneous absorption, which could be applied to all hazard assessments, has been proposed. However, it is recognised that specific protocols are needed depending upon the type of chemical or product (industrial chemical, agrochemical, pharmaceutical, cosmetic, etc.) being tested.

Regulatory Requirements for Percutaneous Absorption Measurements

There is an explicit regulatory requirement for the submission of relevant data showing the amount of a chemical which is absorbed by the skin. Data are generally required for risk assessments of chemicals which may come into contact with the skin either by design or by accident, and for therapeutic assessments of topical drug delivery systems. The regulatory requirements vary worldwide, from the very precise and demanding in vivo (rat) protocols of the US Environmental Protection Agency (EPA) for dermal absorption studies of pesticides, to the absence of any guidelines other than the statement that relevant toxicokinetic data should be provided. At present, there are no approved guidelines for the conduct of in vitro methods for measuring percutaneous absorption. However, draft guidelines for in vivo and in vitro percutaneous absorption studies have recently been circulated for comment by the Organisation for Economic Cooperation and Development (OECD; 2). Several discussion documents have also been produced by the US EPA and the US Food and Drug Administration (FDA), and these have prompted much discussion about the principles to be adopted when conducting
percutaneous absorption measurements (3–5).

In Europe, the requirement for percutaneous absorption data is usually fulfilled as part of the absorption, distribution, metabolism and excretion (ADME) studies (pharmacokinetics/toxicokinetics) undertaken for new chemicals, drugs and pesticides. In addition, experimental data on the delivery of novel topical formulations of drugs are required, and similar studies may be undertaken to assess the toxic potentials of substances used in cosmetics or in other consumer products. Due to the strong pharmacokinetics component of these studies, it is less common for in vivo experiments to be designed solely to obtain penetration data; in most cases, studies on tissue distribution, metabolism and excretion will be undertaken alongside percutaneous absorption measurements.

Since there is increasing pressure for industry to reduce the numbers of animals used in safety testing, and because of the practical limitations of conducting in vivo human studies, there is an urgent need for the development, evaluation and use of appropriate and reliable in vitro methods. At present, in vitro studies are not required by regulators; nevertheless, in vitro data are increasingly being submitted in combination with in vivo data to regulatory authorities, to illustrate formulation effects on skin absorption. Obviously, any in vitro protocols employed should clearly define the scientific rationale for the conditions used, and should indicate the limitations of the data with regard to their interpretation in terms of assessing human risk. If in vitro data are to be used with confidence for risk assessment purposes, it is critical that the methods have successfully undergone rigorous scientific validation. As with any other data which are submitted to regulators, compliance with Good Laboratory Practice (GLP) procedures needs to be demonstrated.

Mechanisms and Modelling of Skin Penetration

It is useful to be able to predict the way in which materials penetrate the skin for both toxicological and therapeutic reasons. The amount of a chemical which is able to penetrate the skin, and its concentration-dependent distribution within the skin, are both important factors for risk assessment purposes and in the development of formulations for topical application and transdermal delivery.

The skin is a good, but only partial, barrier to xenobiotics. When materials come into contact with the skin, they have to pass through both the stratum corneum and the viable tissue before they reach the systemic circulation (Figure 2). Their uptake into the blood and their subsequent distribution are usually rapid relative to the permeation process, unless the material itself constricts the blood vessels and thereby decreases its own clearance.

For all but the most lipophilic substances, the barrier properties of the skin reside in its outer layer, the stratum corneum, which is approximately 15–20 μm thick in normal human skin. The stratum corneum is composed of flattened dead cells, which are hexagonal in shape (about 20 μm across but only 1 μm thick) and which overlap to form the “bricks” in a “bricks and mortar”-type arrangement of cells (the intercellular lipids being the “mortar”). Several studies have shown that, as a consequence, the diffusional path length through the stratum corneum is greater than would be the case in the absence of this type of structure. These investigations include an assessment of the permeation of nicotinate esters (6) and of the diffusion of water (7). Visualisation studies have demonstrated that the permanent accumulates in the intercellular space (8). Thus, the evidence suggests that some molecules are able to permeate the skin via the intercellular lipid channels.

Modern analytical techniques have been used to show that the “mortar” is a complex mixture of lipids, including ceramides, free fatty acids, and cholesterol and its sulphate derivative (9). The lipids form structured arrays of bilayers, with characteristic phase transition temperatures which can be determined by using differential scanning calorimetry (10). The lowest phase transition temperature, which is not always present, has been attributed to the presence of a small lipid domain; the next phase transition temperature results from the melting of alkyl chains, and the highest one has been assigned to the presence of lipid-protein complexes.

Fourier transform infra-red (FTIR) techniques have been used to show that there is
Figure 2: General model for skin absorption

Evaporation ➔ Applied dose ➔ Stratum corneum ➔ Epidermis ➔ Dermis ➔ Blood ➔ Systemic disposition/metabolism ➔ Excretion ➔ Lung ➔ Kidney ➔ Liver ➔ Secretory glands ➔ Sebum

Desquamation ➔ Pilosebaceous system
a direct correlation between the permeability of the skin and the degree of disorder of the lipid alkyl chains (11). This disorder is measured by examining the symmetric and asymmetric stretching frequencies of the C-H bonds. The phase transition temperature associated with melting of the lipids can be lowered by penetration enhancers, such as oleic acid or laurocapram (Azone®). Recent studies have suggested that oleic acid is not homogeneously distributed within the lipids, but forms discrete pools (12). The permeant then passes through the more-liquid pools, which have lower diffusional resistances, or through the interfacial defects at the edges of the pools. Further information about the nature of the lipid bilayer structure has been obtained using X-ray diffraction (13) and electron microscopy (14) techniques.

The barrier properties of the skin result, therefore, from the combination of a tortuous route of diffusion and the requirement that the molecule possesses properties which will enable it to pass sequentially across the various bilayers. In so doing, it comes into contact with regions that are both hydrophilic and lipophilic. Thus, it is not surprising that the types of molecules which penetrate the skin most readily are those which show good solubility in both oils and water. There has been some debate as to whether both polar and non-polar routes exist within the intercellular channels.

Analysis of permeability data, obtained almost exclusively from in vitro studies, indicates that the results can be explained without there being a need for parallel pathways (15). The predominant physicochemical factors which are required to describe permeability are partitioning behaviour and molecular size (16).

The water solubilities of some very lipophilic materials are extremely low, and so the rate of partitioning from the stratum corneum to the viable tissue can become rate-limiting (17). The overall penetration rate of some chemicals can also be affected by their metabolism in the viable tissue (18); the significance of this will be greater, the lower the permeability of the chemical. There are, however, few data illustrating the significance of metabolism during skin permeation, since it is difficult to assess this in vivo, and few studies have been conducted in which skin has been maintained in a viable condition in vitro.

The skin also possesses appendages which may provide alternative, and sometimes important, pathways for penetration (19, 20). Recently, an electric current has been used to enhance permeation (iontophoresis); this forces molecules through hair follicles and sweat ducts (21).

Although the skin is a heterogeneous membrane, approximations can be made so that relatively simple solutions to Fick’s laws of diffusion can be obtained. These can indicate possible mechanisms of skin penetration. When a permeant is placed on the skin surface, it will partition into the lipids and diffuse through the intercellular channels. Following a short period of exposure, there will be a non-linear concentration gradient across the stratum corneum, the shape of which is described by Fick’s second law of diffusion. Evidence suggests that there is a non-uniform resistance to diffusion, with the outermost lipids being more permeable (22). This can be modelled, and the resulting profiles compared with data obtained from skin stripping experiments, in which the stratum corneum has been removed by using adhesive tape (23). The non-steady-state conditions give rise to the lag time found in in vitro skin diffusion experiments. From Fick’s laws, it is easily shown that the lag time (τ) is related to the diffusion coefficient (D) by the simple expression \( \tau = L^2/6D \), where \( L \) is the diffusional path length. It is often difficult to obtain accurate values for \( \tau \) and the precise value for the path length is a matter of debate.

When a linear concentration gradient has been established, steady-state conditions exist and the flux per unit area (J) of material across the skin is proportional to the concentration gradient:

\[
J = \frac{DK(c_0 - c_i)}{L}
\]

where \( c_i \) is the concentration applied to the outer surface of the skin, \( c_o \) is the concentration at the inner surface of the skin, and \( K \) is the partition coefficient for the chemical with respect to its distribution between the skin lipids and the formulation. In practice, \( c_i \) is very small compared to \( c_o \), and the above equation is often simplified to

\[
J = K_p c_o
\]

where \( K_p \) is the permeability coefficient and relates to the partitioning and diffusion behaviour of the permeant. These factors are...
formulation-dependent and, therefore, evaluations of permeability coefficients for developing SARs should be conducted using a common solvent. Water is the best solvent to use, since it will not damage the skin, but it will not be suitable for use with highly lipophilic compounds.

Mathematical models of skin penetration

A general model for skin absorption is outlined in Figure 2. However, it is very rare to have sufficient data to be able to quantify the contribution of the individual compartments to the overall process.

Kasting et al. (24) investigated the relationships between simple physicochemical parameters and skin permeability measured in vitro. Subsequently, Potts & Guy (16) analysed a data set of in vitro skin permeability coefficients published by Flynn (25), and demonstrated that there is a relationship between the permeability coefficient, $K_p$, and both the octanol/water partition coefficient, $K_{ocw}$, of the permeant and its molecular weight (MW). $K_{ocw}$ was chosen in view of the large database available and the ease with which the parameter can be computed; it reflects the way in which the permeant will partition between the skin lipids and water. The MW of a chemical is a good indicator of its molecular size, which, in turn, is related to the diffusion coefficient. The relationship found by Potts & Guy (16) is as follows:

$$\log K_p = -2.7 + 0.71 \log K_{ocw} - 0.0061 \text{ MW}$$

($r^2 = 0.67$)

where $r^2$ is the square of the correlation coefficient and the units of $K_p$ are cm/hour.

This equation can be employed to provide estimates of skin permeabilities of materials from two simple, easily obtained, physicochemical parameters. In combination with data on the aqueous solubility of the material, it can be used to provide a good estimate of the maximum attainable enhanced flux across the skin. This has considerable value for both the assessment of transdermal drug delivery systems and for risk assessment purposes. The results obtained should be confirmed by undertaking a skin permeability study, either in vitro or in vivo.

A recent re-evaluation of the same database has shown that permeability coefficients for the compounds can be determined using constants derived for separate fragments (26). Thus, $K_p$ values can be derived in a similar way to that in which organic molecules are broken into fragments, with each fragment then being assigned a value for its contribution to the $K_{ocw}$. This is useful in determining $K_p$ values ab initio, and also in assessing the ways in which the introduction of functional groups will affect skin permeability.

It is often desirable to calculate the expected plasma levels following topical application of a compound. Rough estimates can be obtained using values for $K_p$, the area of application (A) and the applied concentration ($c_{app}$); if the clearance (CL) is known, it is also possible to calculate the steady-state plasma levels ($c_p$) as follows:

$$\text{input} = \text{output}$$

$$A \times K_p \times c_{app} = \text{CL} \times c_p$$

A kinetic model has been developed for specific application to the transdermal delivery of compounds (15). In this model, rate constants are estimated which describe the diffusion and partitioning within the stratum corneum and viable tissue; MW and $K_{ocw}$ values are required (27). The input to the skin surface from the transdermal or topical delivery system, and an estimate of any solubility constraints within the skin lipids, are also needed. The former can be gauged from an assessment of the in vitro release characteristics of the preparation; the latter can be estimated from the octanol solubility or from the melting point of the penetrant (28). A number of comparisons have been made between estimated blood levels and those obtained following transdermal delivery. There is good agreement between the two, but the model requires further validation with a larger number of permeants.

Another useful way of predicting potential blood levels is to combine in vitro skin penetration data with the known clearance parameters of the xenobiotic. The in vitro permeability results provide a good estimate of the input into the systemic circulation, provided that there are no confounding factors, such as significant metabolism within the skin. If the compound is known to be metabolised extensively by enzymes in the epidermis, experiments can be undertaken using viable skin samples in vitro to provide appropriate data. However, such experiments are not easy to conduct and other possibilities, such
as the use of skin cultures, are currently being investigated.

**In Vivo Tests for Measuring Percutaneous Absorption**

**Animal studies**

Despite the importance of dermal exposure to chemicals, the majority of toxicological studies performed in laboratory animals use the oral route of administration. This can make assessing the likely hazard following dermal exposure rather difficult. The animal models used for studying percutaneous absorption are physiologically and metabolically intact systems. Animal skin can be treated in a manner which mimics very closely the conditions of human exposure (29), and such studies are particularly useful for hazard assessments of new chemicals which may come into contact with the skin, especially when oral or inhalation toxicity data are available but dermal toxicity data are not (30). Many animal species and strains have been used for studying skin absorption, with the rat being used most frequently. Nevertheless, it is important to recognise that animal skin is often more permeable than human skin and, therefore, animal studies will typically overestimate the human systemic levels of chemicals following dermal exposure.

Estimates of percutaneous absorption have been made by monitoring the extent of elimination of the chemical and/or its metabolites in urine and/or faeces, and then correcting these levels for incomplete excretion of the compound via these routes. To undertake this procedure, a knowledge of the pharmacokinetics of both the test material and its metabolites is essential. However, percutaneous absorption can be measured more accurately by undertaking a mass-balance study in which radioactivity is quantified in excreta, expired air, blood, at the dosing site and in the animal carcass (31). It is also necessary to measure the amount of the chemical: in the stratum corneum (removed by tape stripping); associated with the application site protective device; and in any solutions which were used to wash the application site.

The protocol outlined below relates to measurements of the percutaneous absorption of chemicals in the rat. However, the basic principles may be applied to other species. Regulatory test guidelines in some countries may differ from this general procedure.

**Protocol for measuring percutaneous absorption in the rat**

The test substance, preferably radiolabelled, is applied to animal skin at an appropriate dose level and in a suitable vehicle. The compound is allowed to remain in contact with the skin for a defined period of time. The dose site is protected by a device which prevents the animal from grooming that particular area. At the end of the exposure period, this protection device/cover is removed and the skin is washed with an appropriate cleansing agent; the cover and the cleansing materials are retained for analysis, and a fresh cover may be applied.

The animals are housed in individual metabolism cages and the excreta are collected for analysis. At the end of the experiment, the animals are killed; blood is collected for analysis, the dermal application site is removed for analysis, and the amount of substance remaining in the carcass is determined. The extent of percutaneous absorption is then estimated from knowledge of the total amount of compound excreted and of that present in the carcass.

1. The test substance should be radiolabelled in a metabolically stable position, preferably with $^{14}$C of high radiochemical purity (preferably >98%). The specific activity and radiochemical purity of the test substance must be known. A non-radiolabelled compound may be used, provided that there is a validated procedure for analysing the compound in each of the sample types taken, and that the metabolism of the compound has been well-characterised.

2. The test substance should be applied to the skin in a vehicle which is appropriate to the risk scenario. It should be stable in this preparation for the duration of the experiment. The experimental conditions must prevent the animal from ingesting the test material.

3. The study should be carried out using rats of the same strain as those used to generate other toxicological data. Young rats (8–14 weeks old), within the resting hair phase (dermal telogen), should be
used. The use of four animals per group is normally regarded as adequate. In general, the use of one sex is sufficient for a percutaneous absorption study (the more sensitive sex based on toxicological data should be used), although sex differences may be important in pharmacokinetic studies.

4. On the day before treatment, an area of hair on the shoulders and back of the rat should be removed with clippers, taking care to avoid abrasions. The rats should then be placed in their individual metabolism cages.

5. A known amount of test chemical should be applied to a defined area of skin. This area should be protected from grooming by a non-occlusive covering (for example, by a nylon gauze glued to a rubber O-ring at the dosing site), unless the objectives of the study or the pattern of use of the chemical indicate otherwise. The rats must be observed frequently to check for leakage or damage to the protective device. The dose administered and the duration of exposure should reflect the purpose of the experiment. For a radiochemical study, application of 10μCi/curies (0.37MBq) of 14C over a 10cm² area of skin is normally sufficient to detect penetration of less than 0.1% of the dose applied.

6. At the end of the exposure period, each animal should be anaesthetised and the gauze removed and retained for analysis. The treated area of skin should be washed at least three times; the cleansing agent used should have minimal effects on the uptake of the test substance, while effectively removing it from the skin. All swabs and washings from each animal should be retained for analysis. Care must be taken to ensure that the skin outside the O-ring is not contaminated during the washing procedure.

7. Groups of animals should be killed at various times, according to the objectives of the experiment. Excreta should be monitored throughout the experiment; when appropriate, expired air should be monitored continuously for 14CO₂ or volatile 14C-metabolites, urine should be collected at 8, 24, 48 and 96 hours after dosing, and faeces should be collected daily. In addition, cage washings, swabs and skin washings should be analysed using appropriate techniques (for example, liquid scintillation counting). Blood samples, major organs (if required), skin taken from the dosing site, and the entire carcass should be analysed after the animals have been killed. Carcasses can also be examined by whole-body autoradiography to assess the complete tissue distribution of the compound.

8. For each rat, the following determinations should be made. Results should be expressed in mg equivalents of active ingredient and/or degradation products or metabolites, as appropriate.

a) Quantity of the compound in/on the protective device.
b) Quantity of the compound washed from the skin.
c) Quantity of the compound at the site of application which could not be removed by washing (this may incorporate data obtained by using tape stripping and excised skin).
d) Quantity of the compound excreted in the urine and faeces (and as 14CO₂ and/or volatile 14C-compounds, if applicable).
e) Quantity of the compound remaining in the carcass.
f) Concentration of the compound which has been absorbed, or the amount of radioactivity in the blood.

From these values, the total absorbed dose at the time of sacrifice (and the percentage of the applied dose which was absorbed) should be calculated. Knowledge of the area of skin treated (that is, the area of skin enclosed by the O-ring) enables an estimate of the absorbed dose to be expressed as the unit weight of compound systemically absorbed per cm² skin, under the specific conditions used in the experiment.

Benefits and limitations of animal studies

There are several benefits and limitations of using animals for measuring percutaneous absorption. The benefits include the following:

a) Laboratory animals are readily available and represent a reproducible, physiologically and metabolically intact, test system.
b) Radiochemicals can be used to simplify the analyses.

c) Animals of the strain used in other toxicology/metabolism studies on the same substance can be used.

d) All chemical types (pharmaceuticals, agrochemicals, industrial chemicals, cosmetics, etc.) can be studied.

e) Animal procedures are recognised as being of value by regulatory authorities.

The limitations of animal studies include the following:

a) The permeability of human skin is often lower than that of animal skin.

b) Animal tests are viewed as being unethical by some people.

c) The dosing site must be protected to prevent removal of the chemical by normal animal grooming habits; this may cause the animal some distress. Data from comparisons of open and protected models are not available.

d) Rate terms are difficult to calculate without knowledge of basic pharmacokinetic data.

Recommendations

With respect to the use of laboratory animals for measuring percutaneous absorption, the following recommendations are made:

1. The number of laboratory animals used for this type of experiment should be kept to a minimum, and the pharmacokinetic and toxicokinetic data obtained from each experiment should be maximised.

2. Animal studies are best undertaken with chemicals of known oral or inhalation toxicity but of unknown dermal toxicity.

3. The value of these studies for direct extrapolation to percutaneous absorption in man is questionable, because of the higher permeability of rat skin. Thus, subsequent risk assessments will always be highly conservative.

Human volunteer studies

Human studies are the "gold standard" against which all methods for measuring percutaneous absorption should be judged. In this respect, it is important that the ethical issues of exposing individuals to radiochemicals and/or to toxic chemicals are adequately addressed. The purpose of any human percutaneous absorption study must be clearly stated (for example, occupational health investigations, the development of new pharmaceuticals or consumer products), and the protocol should be drawn up to meet these requirements. Practical and ethical considerations mean that in vivo human studies can address only specific questions. The necessity and value of the results which are likely to be obtained must be carefully considered; there must be no doubt that the objectives of the protocol can be met. Multiple dosing may be required in some cases.

The conduct of human volunteer experiments is closely regulated (32, 33); the study protocols and accompanying toxicological data must be submitted to an ethics committee for approval. Medical supervision of the studies is necessary. In the USA, 14C-labelled compounds may be applied to the skin for absorption experiments, in accordance with the Atomic Energy Commission's guidelines. The use of radiolabelled compounds is also possible in other countries, subject to compliance with various regulations (34–36). The use of stable isotopes may offer an alternative, but less convenient, method of analysis.

A known amount of the test substance (liquid or solid) is applied directly to the skin, or an area of skin (for example, an arm or the whole body) is exposed to the compound as a vapour in an inhalation chamber. At the end of the exposure period, the skin is washed to remove unabsorbed test compound. The rinsing procedure should relate to the risk scenario, since rinsing rarely removes all of the dose applied. The treated area of skin may be subjected to tape stripping, to remove stratum corneum which has absorbed the test material, but this will obviously affect skin permeability. Throughout the experiment, blood, urine, expired air and faeces may be collected at regular intervals, for subsequent analysis for the presence of the test compound and/or its metabolites.

1. The test substance should, ideally, be of a purity in excess of 98%. For radio-labeled materials, the radiochemical purity should be >98% and the radio-label should be in a metabolically stable position. The higher the purity, the greater the confidence in the data generated, especially in the case of poorly absorbed materials.

2. The material tested should be as similar as possible to the preparation which it is
intended to market, and it should be in the form in which it is likely to come into contact with the skin. It may be applied neat or, if analytical sensitivity permits, applied at the "in-use" concentration. Irritation studies on the same formulation should be performed in animals, and then in humans, before conducting a percutaneous absorption study.

3. Normally, five volunteers should be adequate to produce a standard deviation of 30%, but metabolic differences between individuals need to be considered. In general, volunteers should be healthy males, aged between 18 and 60 years old. The medical status of all volunteers must be known and fully recorded. A medically qualified person must be included in the project team and should decide on any specific parameters to be included in the medical examination. The volunteers should be informed about all the procedures to be undertaken, and about any known risks and areas of uncertainty; they should sign a statement indicating that they have understood this information. Volunteers should be closely supervised throughout the study.

4. The test site should be relevant to the anticipated exposure, taking into account any practical limitations. The dose applied should be appropriate to the requirements of the experiment and should be justified in the protocol. Every effort should be made to avoid inadvertent physical removal of the test material. The duration of exposure will depend upon the objectives of the study.

5. The exact times at which blood and excreta samples are taken will depend upon the results from oral/intravenous administration of the compound in humans, if these are available. Blood samples should, ideally, be taken when exposure commences, at one or more times on the ascending portion of the concentration curve, at one or more times close to the peak concentration (at $T_{\text{max}}$), and at three or more times after this. Substances which are slowly absorbed and rapidly excreted may never reach sufficiently high blood or plasma concentrations to achieve this ideal situation; they may only be detectable around the peak concentration. In the absence of pharmacokinetic data, it is suggested that blood samples are taken at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 60 and 72 hours following application. If samples can be analysed immediately, sampling can cease when the concentration falls below the limit of detection. Blood should be taken from a site which is unlikely to be contaminated with test substance, for example, the contralateral arm. Depending on the number of samples to be taken, a needle or cannula should be used. It is reasonable to withdraw 5-20ml of blood per collection, which should be transferred immediately to tubes; these should contain anticoagulant and, in some cases, stabilisers, if the analysis is to be carried out on whole blood or plasma.

6. Urine collection should cover 12-hour periods, except on the first day, when collections should be over shorter periods. Volunteers should be encouraged to take fluids in reasonable quantities during the first 12 hours in order to give a good flow of urine. Volunteers should be asked to empty their bladders into inert containers at the end of each sampling period. The total volume collected at each time-point should be recorded. Ideally, sampling should be continued until the detection limit has been reached. Urinary creatinine levels may also be measured, to provide an indication of the completeness of urinary collections. Faeces can usually be collected in polyethylene bags.

7. Skin rinsings should be collected as quantitatively as possible. Tape strips from treated skin should be extracted with suitable solvents, to enable the analysis of any parent compound residing in the stratum corneum. For some compounds, skin decontamination may be required at the end of the exposure period; the method to be used for this should be defined in the protocol.

8. Whatever the nature of the biological sample, it should be ascertained in preliminary studies that the compound and/or its metabolites can be extracted and analysed, and that they are stable under the storage conditions to be used.

9. For each subject, the following determinations should be made. Results should be expressed as mg of active ingredient
and/or degradation products, as appropriate.

a) Concentrations of the compound and/or its metabolites in blood.
b) Quantity of the compound excreted in the urine, faeces and expired air.
c) Quantity of the compound rinsed from the treated area of skin.
d) Quantity of the compound removed by tape stripping.
e) Quantity of the compound in/on the protective device which may have been used to protect the application site.
f) Any results from the measurement of relevant biochemical markers in urine and blood.

From these values, the total amount of compound absorbed at each time-point can be calculated, provided that pharmacokinetic information relating blood and urinary concentrations to the dose administered are available.

Non-invasive methods

Residual analysis — "difference" or "disappearance" methods

In these procedures, the amount of drug absorbed into the skin is evaluated by determining the difference between the amount applied and that recoverable at a subsequent time-point. Two methods have been used: a) single-point measurements of drug disappearance; and b) continuous or periodic monitoring of drug uptake.

In the first method, following drug application for a fixed period of time, any residual formulation is washed from the skin surface and the amount removed is analysed (37). The amount absorbed is determined by subtracting the amount remaining from that which was applied. The advantages of this procedure are that it requires very small amounts of active formulation, and that it is inexpensive, relatively rapid, and applicable to clinical studies. The use of a radio-isotope is feasible, since very low levels are required. However, as only one assay per site per application is conducted, full characterisation of the drug uptake profile requires multiple measurements to be carried out. Uniform recovery from different sites must be demonstrated; for example, drug removed inadvertently from the surface (on clothing, etc.) may be considered erroneously to have been absorbed. The application technique is critical with respect to the sensitivity of the method: for poorly penetrating compounds, attempts are made to quantify a very small difference between two much larger values.

In the second method, following application of the formulation, the amount of the drug in the outer skin layers is monitored as a function of time by using spectroscopic (for example, infra-red) or radio-isotope techniques (38–40). Penetration kinetics are assessed from the decrease in the radio-isotope or spectral signal. Attenuated total reflectance infra-red (ATR-IR) spectroscopy has recently been shown to be useful for following the penetration of model compounds across human stratum corneum in vivo (39).

The amount of a radiolabelled penetrant at different depths in the stratum corneum was measured by sequential spectral recording. The stratum corneum was then tape stripped and the radioactivity profile through the stratum corneum compared with the spectral results. The correlation was good, and the procedure was able to distinguish the dermal delivery from two formulations, one of which contained a known penetration enhancer.

The relatively non-invasive nature of the approach, together with its ability to discriminate between vehicles of differing release behaviour, suggest that ATR-IR could become a powerful tool for assessing the bioavailability of topicaly applied chemicals. The fact that the technique can be conducted in vivo in humans increases its potential utility. Very low doses of drugs (at pharmacologically ineffective concentrations) can be used, and complete characterisation of the drug uptake profile from a single experiment is possible. The methods used are relatively sensitive, non-invasive, precise and objective. Nevertheless, at present there are limited data available for the technique. Only compounds which have a unique spectrophotometric signature, distinct from that of the stratum corneum, can be used, and the equipment required is specialised and expensive.

Tape stripping method

In this procedure (Table 1), drug penetration is estimated from the amount recovered from the stratum corneum by adhesive tape stripping, at a fixed time-point following drug application (41–44). The procedure is often referred to as the "reservoir" technique (45). The underlying basis of the method is the
Table I: Tape stripping method

1. Choose a bald area.
2. Clean gently with tissue paper and water.
3. Stick the cell (1cm²) with silicone glue.
4. Apply the formulation for 30 minutes.
5. Wash with 300μl of the appropriate medium (for example, solvent, shampoo).
6. Dry gently with cotton swab.
7. Wash as in 5.
8. Dry as in 6.
9. Rinse with 300μl of water.
10. Dry as in 6.
12. Dry as in 6 (twice).
13. Remove the cell.
14. Perform one stripping (micropore 3M) and discard it.
15. Strip 15 times (human) or six times (hairless rat) using invisible adhesive tape, 3M.

Radiolabelled compound

16. Dissolve the stripings in 15ml of Soluene 350 (United Technology Packard, Chicago, IL, USA).
17. Leave at room temperature for 2 days.
18. Sample 2ml of Soluene and add 15ml of Hionic Fluor (United Technology Packard).
19. Count in scintillation counter after 24 hours (to prevent chemiluminescence).

Non-radiolabelled compound

16'. Extract the stripping using 15ml of the appropriate solvent.
17'. Choose the appropriate analytical technique.

Results

— Express the amounts of product found in the stratum corneum stripings (B) in moles.
— Total penetration within 4 days (A) for a 30-minute application will be \( A = 1.8B \).
— For longer times of application (t minutes), total penetration within four days (\( A' \)) will be \( A' = A \times t/30 \).

From Rougier & Lotte (46).

correlation between short-term uptake and steady-state permeability. It is hypothesised that, if a compound is applied to the skin for a limited time (for example, for 30 minutes) and is then removed, the amount of chemical in the upper layers of the immediately stripped stratum corneum will be predictive of the bioavailability of the compound when the skin is left undisturbed. In other words, given a limited exposure period, the fraction of the dose which enters the stratum corneum to the extent that it cannot be removed by simple washing, will equal the fraction that ultimately reaches the systemic circulation and is subsequently excreted. Thus, it follows that a short-term application followed by skin stripping can adequately predict in vivo topical bioavailability.

Initial validation studies were conducted using the hairless rat (44). Subsequently, these findings were reproduced in man for a set of four chemicals of varying physical properties (46). The approach would appear to have broad applicability, enabling ques-
tions about the effects on skin penetration of, for example, vehicle, anatomic site, age, etc. to be addressed. Measurements can be undertaken using drug doses which do not elicit pharmacological effects. The procedure is non-invasive, straightforward and inexpensive. It is not essential to use radio-labelled drugs, although the quantification of the amount present in stratum corneum tape strips has, to date, been limited almost exclusively to radio-isotope counting. Comparisons between formulations are easily performed, and can be well-controlled. However, correlations between the amount of drug in the stratum corneum and total drug absorption have been established for a limited number of drugs and formulations. Preparation of the skin for stripping is a critical determinant of drug recovery. Kinetic information could be obtained by using more complex protocols. The procedure needs to be validated by testing a wide range of chemicals of different properties, including poorly penetrating compounds, to determine its value in predicting percutaneous absorption in man.

**Pharmacodynamic responses for assessing topical bioavailability**

Pharmacodynamic changes (for example, vascular responses) in skin function can serve as indicators of local absorption. Several such assays (for example, the skin blanching or vasoconstriction assay for glucocorticoids) are worthy of further consideration; the development of standard, validated, procedures is required (47, 48).

With glucocorticosteroids, good correlations with clinical effects have been demonstrated. The methods are internally consistent, qualitatively reliable, inexpensive and easy to carry out; they are particularly useful for testing vehicle effects on drug absorption. However, only a limited number of compounds (for example, topical corticoids) can be evaluated using a pharmacodynamic method. For most topically active compounds, the dose-response curves have yet to be defined. Pharmacodynamic responses are difficult to validate; objective methods for quantifying pharmacological responses are needed. In this respect, recent results obtained by using a chromameter have been encouraging (49). Methods for quantifying the "area-under-the-curve" (that is, response versus time) need to be developed.

**In Vitro Methods for Measuring Percutaneous Absorption**

Percutaneous absorption can be studied by using in vitro methods, since it is possible to maintain the barrier properties of the stratum corneum in excised skin. There is good evidence that in vitro data are predictive for in vivo percutaneous absorption in both animals and man (50-57). Such studies are becoming increasingly acceptable to regulatory agencies with respect to the provision of percutaneous absorption data for drugs, cosmetics, pesticides and industrial chemicals. However, the current database is limited.

There are many advantages of using in vitro methods, not least that human skin can be used and that procedures are not conducted on living animals. There is a great opportunity to reduce, refine and replace animal tests for measuring dermal absorption by using meaningful and validated in vitro alternatives. The distribution of test chemicals in the skin can also be measured in vitro, by using techniques such as tape stripping of the epidermis (58) and tangential skin slicing (59, 60). Disadvantages of using in vitro methods for studying percutaneous absorption include the lack of provision of pharmacokinetic data, and the difficulty of obtaining human skin in sufficient quantities. Artificial skin systems (human skin models) are of limited use for predicting absorption in man. It was agreed that only human skin should be used to provide data for risk assessment purposes, or for the prediction of therapeutic effects, because there are major differences in the skin permeability properties of animal and human skin (50, 51, 61, 62). Thus, the use of animal skin often results in an overprediction of likely human percutaneous absorption.

With respect to the problem of human skin availability, it was recognised that in vitro models using skin from other species could be of value if they were fully validated. Animal skin can also be a useful surrogate when relative skin permeabilities are being determined, such as for comparative assessments with different formulations. However, it is recommended that there should be a concerted move toward using human skin as the primary in vitro model for skin permeability studies, to complement the increasing number of in vivo studies with human volunteers which are being undertaken.
From a physiological perspective, the use of perfused skin is attractive. However, only a limited number of techniques have been found to be useful, including use of the perfused pig flap (63), which is surgically demanding, the perfused rabbit ear (64), and the perfused pig ear (65). These have rarely been used for routine measurements of percutaneous absorption, because they are technically demanding and the preparations are not usually viable for more than a few hours. Nevertheless, they may be very useful models for studying short-term dermal exposure to chemicals which are metabolised extensively by the skin. To date, there is a very limited data set available.

In vitro procedure for assessing percutaneous absorption

The method outlined below is based upon protocols described previously (30, 66), and upon other published methods for measuring percutaneous absorption in vitro (3, 67).

Skin membrane

Human skin samples should be used for the prediction of dermal absorption in man. Animal skin can be used for assessing relative permeabilities. Human skin may be obtained fresh, but is more likely to be obtained from cadavers. Safe working practices for handling human tissues should be strictly adhered to. To minimise variability in the skin permeability properties between different anatomical sites (which have varying thicknesses of stratum corneum and follicle densities), standardisation by removal of the skin from a single site, such as female abdomen, is most appropriate. Whole skin, with the subcutaneous fat removed, or epidermal membranes prepared by a heat separation technique, can be used. Membrane separation can be achieved by the immersion of whole skin in water at 60°C for 45 seconds, after which the epidermis can be peeled off from the dermis. Alternatively, skin slices (200–400μm) can be cut from whole skin by using a dermatome. Once prepared, skin samples can be stored frozen (−20°C) on aluminium foil.

Diffusion cell

There are several types of glass, teflon and stainless steel diffusion cells, which consist of donor and receptor chambers between which the skin is positioned. The chamber should be designed so that it provides a good seal around the skin, and enables easy sampling, a good mixing of the receptor solution in contact with the underside of the skin, and good temperature control of the receptor chamber and its contents. Static and flow-through diffusion cells are both acceptable; flow-through cells may offer advantages by enhancing “sink” conditions when the absorption rate is high. Furthermore, if metabolically active skin is required, flow-through cells with a physiological receptor phase, for example, tissue culture medium, are necessary.

Receptor fluid

The following should be taken into account when selecting an appropriate receptor fluid: a) it must not adversely alter the barrier properties of the skin; b) the physicochemical properties of the test chemical; and c) analytical considerations. Various receptor phases can be used, including tissue culture medium (this is often used with freshly resected skin), saline/aqueous saline, polyethylene glycol/water, and ethanol/water. Typically, aqueous phases are used with polar chemicals and ethanolic phases with lipophilic chemicals.

It is important that the concentration of the penetrant in the receptor solution remains low at all times, to prevent significant back diffusion. In general, the thermodynamic activity should never exceed 10% of that in the donor formulation to maintain an adequate diffusion gradient. It is important that any potential damaging effects of the receptor solution on the skin are assessed, by checking the barrier properties of the skin membrane used.

Temperature and humidity

The diffusion of chemicals, and therefore their absorption, is affected by temperature. The diffusion chamber and skin should be maintained at a constant temperature (thermostatically controlled), close to the normal skin temperature of 32°C. Different cell designs will require different water bath or heated block temperatures to ensure that the skin is at its physiological norm. It is necessary to ensure adequate hydration of the skin, particularly if the preparation is to be maintained for more than just a few hours. Donor chambers may or may not be occluded during exposure to the test chemical, depending on the objective of the experiment.
Skin integrity
Although most human skin samples can be assessed visually for evidence of gross damage or disease prior to their initial preparation, in order to avoid abnormally permeable samples it is recommended that the integrity of all skin membranes is checked prior to the application of test chemical. This can be done in various ways, including measurement of the transfer of low MW markers (for example, tritiated water), the appearance of visual markers (for example, fluorescein), electrical conductivity, or transepidermal water loss. All of these methods give a relatively quick assessment of abnormally high permeability, thereby enabling skin samples of poor quality to be rejected.

Application of test chemical
The study design will differ depending on whether the chemical is to be applied in its normal physical state, as a solution in a simple vehicle, or as a formulation containing excipients which may have their own intrinsic effects on the skin (for example, surfactants). The conditions of application may also vary. The exposure may involve different occlusion conditions, and may be short or last for many hours. Obviously, the procedure used for the application of the test chemical must be appropriate to the objectives of the study. If the chemical is not being applied neat, the choice of vehicle is an important consideration; ideally, the vehicle should not affect the barrier properties of the skin. Also, the solubility of the chemical in the vehicle, particularly at the extremes of polarity, will predetermine its partitioning between the vehicle and the stratum corneum lipids, and thus influence dermal kinetics.

Dose and volume of test chemical
The amount of chemical applied may be very small (for example, with a potent drug) or very high (for example, with pesticide concentrates and inert cosmetics). The dose applied should mimic the “in-use” conditions. It should be given as an appropriate volume spread on the skin surface. This often depends on the viscosity of the application, but is typically 5–100μl/cm². With large volumes, some of the material applied may adhere to the donor cell, and will therefore be unavailable for absorption. Small volumes are difficult to spread, may be less accurate, and are sometimes limited to the more-soluble test chemicals. An important consideration here is whether a finite or an infinite dose is applied, since the absorption parameters are not the same under these different conditions.

Duration of experiments
The integrity of the skin can be maintained for many hours under appropriate conditions, and can be checked as described previously. Experiments should normally be conducted over a 24-hour period, and should be completed within 48 hours. When the lag phase is found to be particularly long, the duration of an experiment may be extended to 72 hours or even longer, especially if steady-state fluxes are to be obtained from infinite dose experiments.

Sampling times
Samples of the receptor fluid should be taken from each diffusion cell at regular intervals. Generally, sampling will be required less frequently as the exposure period increases during the “assumed” steady-state period. A typical experiment may incorporate ten or more time-points. Following sampling, the change in volume of the receptor solution should be corrected for. Flow-through cells should deliver reliable and accurate small volumes of receptor solution, as programmed. Care should be taken to ensure that the test chemical does not bind to tubing, etc., and that none of the analytical procedures are compromised.

Analysis of test chemical
The sensitivity and specificity of the analytical procedure, the stability of the test chemical, the nature of the receptor phase, and the volume of sample will all affect the analysis. The stability and purity of the test chemical should be defined; for radiolabelled chemicals, the specific activity and the position/nature of the label should also be defined. In some cases, a mass balance of the test chemical following its application may be undertaken. This is most easily accomplished when radiolabelled test chemical is available. The quantities which can be recovered by mild detergent wash from the skin surface, the donor chamber, tape strips or epidermis, the underlying dermis, and the receptor phase are indicative of the chemical distribution in vitro.

Results
The manner in which absorption data are expressed is an area of considerable debate.
Several methods of calculation are acceptable, and often the type of data analysis required is specified in regulatory guidelines. The profile of absorption should be characterised for a minimum of 24 hours following application. A common measure is the steady-state flux or absorption rate, which is usually expressed as μg test chemical per unit area of skin per hour. This should relate to an infinite dose of test chemical where steady-state has been achieved, and it allows the Kₚ value (cm/hour) to be calculated by dividing the steady-state absorption rate by the concentration of chemical applied. The amount absorbed over a given time-period, either as the absolute amount or as a percentage of the dose applied, is a commonly accepted measure of percutaneous absorption. In some cases, where a mass balance of the test chemical has been undertaken in the different layers of skin, together with determination of the unabsorbed and absorbed fractions, the total recovered dose can be given.

**Statistical analysis and experimental design**

Careful consideration of the scientific integrity of percutaneous absorption data is important. Sufficient replicates (a minimum of five) for the determination of any one variable should be used. Since some skin preparations may be damaged inadvertently during the exposure period, it is good practice to ensure that an adequate group size is specified prior to commencing the study. Definition of any change in skin permeability which is unrelated to the test chemical should be clearly described. For example, flux data which are three standard deviations higher than the mean of the group would be regarded as abnormal, and therefore should be omitted from the data set.

**Skin Metabolism**

The skin is able to metabolise a wide range of substrates. For example, various dermal cytochrome P450 isozymes have been identified, many of which have been shown to be inducible (68, 69). Evidence indicates that the cytochrome P450 and Phase II metabolising enzymes in skin may be localised within specific cell types, particularly in the epidermis and pilosebaceous system (70). Enzymes in the stratum corneum, and probably in the sebum, are able to hydrolyse certain chemicals (for example, esters). Lipase, protease, phosphatase, sulphatase and glycosidase activities have been identified in stratum corneum; these activities have been linked with the maturation and desquamation processes (71–73).

Thus, the skin can metabolise compounds before they enter the bloodstream. Knowledge of this "first-pass" effect is important for risk assessment purposes and in terms of drug delivery. However, the importance of skin metabolism with respect to the measurement of percutaneous absorption is open to debate. If the chemical of interest undergoes biotransformation during its passage through the stratum corneum to metabolites which penetrate the skin to a greater extent than the parent compound, metabolism is obviously a critical determinant of its percutaneous absorption. However, if metabolism occurs after the normal rate-limiting passage of the material across the stratum corneum into the pilosebaceous system or epidermis, the material is usually regarded as having penetrated the skin and as being systemically available. Thus, for materials which are stable in the stratum corneum, metabolism will not be rate-limiting.

**In vivo** percutaneous absorption studies are unable to distinguish between the dermal and systemic metabolism of a compound, whereas **in vitro** studies enable skin metabolism to be assessed directly. If isolated skin is not kept under tissue maintenance conditions, it will have a reduced metabolic capability; some regulatory authorities claim that this may affect the penetration flux. However, the data comparisons which have been undertaken, albeit limited, suggest that this point is equivocal; for example, Boehnlein *et al.* (74) showed that the percutaneous absorption of methyl salicylate was very similar in both viable and non-viable skin from the hairless guinea-pig, although, in this case, metabolites were recovered in the receptor solution, not the parent compound. Interestingly, even the "non-viable" skin appeared to have retained some metabolic activity. In contrast, Collier *et al.* (75) showed that, for phenylazophenol, the penetration barrier presented by the epidermis was affected by its viability.

It is thought that there could be active transport of some compounds between the stratum corneum and the dermis. This has not been demonstrated, but could be particu-
larly important for compounds with specific binding sites in the epidermis or which are very lipophilic.

It was concluded that, while skin metabolism is an important aspect of risk assessment, it is of less importance in experiments which are designed principally to quantify the amounts of compounds able to penetrate the skin. Dermal metabolism should be investigated separately from skin absorption, by using dermatome slices or skin homogenates. If the skin is shown to have a significant ability to metabolise the chemical of interest in such studies, the metabolites produced should be identified, and this knowledge should be incorporated into the risk assessment for that particular chemical.

**Production of a Protocol for Assessing Percutaneous Absorption In Vitro**

*In vitro* percutaneous absorption studies with animal or human skin membranes are an elegant tool for obtaining data on the flux of test substances through the skin as a function of time (that is, the amount of chemical crossing the skin membrane per cm² per hour). The rate and extent to which a chemical penetrates the skin depend on a variety of factors: the physicochemical properties of the penetrant, the vehicle of application, the application and exposure conditions, and the type and status of the skin (Table II).

A single study design is not feasible for assessing the percutaneous absorption of diverse types of chemicals and formulations (pharmaceuticals, pesticides, cosmetics, etc.). Nevertheless, it is important that the application and exposure conditions mimic the human *in vivo* exposure situation as closely as possible.

While it is technically feasible to maintain skin under conditions in which it is viable, the procedure could not be applied with human skin for the routine screening of chemicals or delivery systems. Demonstrat-

<table>
<thead>
<tr>
<th>Parameter</th>
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<tbody>
<tr>
<td>Test compound</td>
<td>— molecular weight — water/lipid partition coefficient — ionisation</td>
</tr>
<tr>
<td>Vehicle</td>
<td>— solubility/polarity — volatility — concentration — distribution in stratum corneum — excipients — penetration enhancer — pH</td>
</tr>
<tr>
<td>Skin</td>
<td>— species — age, sex, race — anatomical site — temperature — hydration of stratum corneum — damage to stratum corneum — metabolism</td>
</tr>
<tr>
<td>Application</td>
<td>— skin area dose (film thickness, concentration) — total skin area in contact with vehicle — duration of exposure</td>
</tr>
</tbody>
</table>

Table II: Factors influencing percutaneous absorption
ing skin viability to the satisfaction of GLP requirements would impose prohibitively expensive overheads on such experiments. Most of the in vitro percutaneous absorption studies which have been published have employed non-viable skin. The data have proved to be reproducible in most cases, and have provided a better understanding of the diffusion processes involved in skin penetration. The use of non-viable skin is a valid model for assessing the percutaneous absorption of many compounds, and should therefore be used as the basic screening method.

From percutaneous absorption data obtained from either an in vitro or an in vivo experiment, the systemic exposure (body burden or systemic load) can be estimated, for a given dermal exposure situation, as the amount of chemical per kg body weight per day. By comparing the estimated or extrapolated systemic exposure following dermal exposure with the no-observed adverse effect level (NOAEL) derived from animal systemic toxicity studies, a margin of safety can be calculated.

The following sections outline those aspects of a protocol for measuring in vitro percutaneous absorption which are particularly important.

Protocol title

The title should be as informative as possible, incorporating details about the hypothesis to be investigated, the test compound, and the methodology to be used (the type of diffusion cell, skin membrane, and analytical method).

Aim of the study

The objectives of the study should be described clearly, and the choice of methodology should be justified with respect to its suitability for providing answers to the questions raised.

Description of the in vitro method

Skin membrane

The type of skin membrane (species, source, regional site, possible pretreatment, etc.), the method of preparation (use of a dermis, procedure used to isolate the epidermis), and the conditions and time of storage should be described. The integrity of the membrane should usually be assessed prior to the start of the experiment.

Diffusion cell

The type of cell and the experimental set-up (finite or infinite dose experiment, static or flow-through cell, dimensions of cell, manufacturer, etc.) should be described, or reference made to a Standard Operating Procedure (SOP). The number of cells to be used in the experiment should also be justified.

Receptor fluid

The choice of receptor fluid (saline, buffer, ethanol/water, polyethylene glycol/water, etc.; addition of antibiotics, sodium azide, bovine serum albumin, etc.) should be explained. The solubility of the penetrant, the volume exchange and the flow rate should not be rate-limiting with respect to the diffusion of penetrant into the receptor phase. In order to prevent a back-diffusion from the receptor fluid into the skin, the penetrant concentration should not exceed 10% of its saturation level. The receptor fluid must be stirred continuously and thermostated, so that the membrane is maintained at 32°C.

Test substance and dosing

Test substance

The composition, purity and stability of the test compound must be defined. The provision of physicochemical data, such as pKa and Kow values, may aid in the interpretation of the results obtained.

Formulation

The composition of the vehicle, the concentration of test compound (total concentration and, if possible, that of the dissolved portion), and its pH should be defined. The formulation should be prepared in a reproducible manner according to established procedures. For radiolabelled substances, non-labelled and labelled compounds should be mixed in solution, to guarantee uniform labelling. For solid and semi-solid formulations and for suspensions, compound uniformity within the formulation should be checked.

Dose

The amount or volume of the test formulation to be applied to a defined area of skin, and the procedure for its application, should be defined. The area dose (that is, the amount of test chemical/formulation per cm² of skin) determines the thickness of formula-
tion film on the skin surface. With general-purpose cosmetic creams, the area dose has been estimated to be $1\text{mg}$ of formulation per cm$^2$ of skin, which corresponds to a film layer of 10$\mu$m. For dermatopharmaceuticals, the area dose during therapeutic use is 2–4$\text{mg}$ formulation per cm$^2$ of skin.

**Exposure and sampling time**

The exposure time (that is, the time elapsing until the final change of the receptor fluid) should reflect in-use conditions; it will be very short for rinse-off products, whereas an eight-hour exposure period with agrochemicals and industrial chemicals, and a 24-hour exposure, or longer, with leave-on products, may be appropriate. The procedure to be used to remove formulations from the skin surface at the end of the exposure period (washing, use of cotton swabs, etc.) should be described. Sampling should be continued for a minimum of 24 hours. Determination of the actual frequency of receptor fluid sampling should be based upon information about the absorption rate, lag time, etc., and should be such that the absorption profile can be described adequately (that is, 6–8 points for a 24-hour exposure period).

**Analysis**

The amount of test compound in the receptor fluid should be analysed by an appropriate, sensitive and validated method. With radiolabelled compounds, radiochromatograms of pooled fractions of the receptor fluid can provide an indication of skin bio-transformation. Where dermal metabolism is suspected, the experiment can be repeated employing receptor phases which maintain the viability of freshly prepared animal skin for 24 hours.

**Data presentation and evaluation**

The protocol should also contain information on how to present the results. Cumulative amounts of the test substance in the receptor fluid should be plotted as a function of the exposure time. From the slope of the curve, the substance flux through the skin can be calculated for any time-point. Expressing the cumulative amount of the test substance which has reached the receptor compartment at the end of the exposure time as a percentage of the dose applied is only meaningful for finite-dose experiments. The results obtained relate to the specific dose, formulation and exposure conditions employed; extreme care should be taken if these are extrapolated to other data sets or exposure conditions.

Many factors which can affect the penetration of materials through the skin have been identified. Thus, provision of the details listed in Table III is critical with respect to evaluating the results from *in vitro* percutaneous absorption studies, and for subsequent use of the data obtained for risk assessment purposes.

**In vitro/in vivo comparisons**

*In vivo* measurements of skin absorption in humans are rare and information about *in vitro/in vivo* correlations is, therefore, limited. However, *in vitro/in vivo* comparisons have been undertaken with data from laboratory animals; many of these have shown good correlations (30, 57). Nevertheless, for some chemicals, there is poor agreement between *in vitro* and *in vivo* percutaneous absorption data, with *in vitro* methods usually underestimating absorption (51, 76, 77).

In general, it was felt that if the amounts and rates of penetration determined following identical applications were similar, this represented a good correlation. Differences in flux of a factor of two or three were considered to be reasonable agreements. The type of data which should be used to quantify penetration *in vitro* was discussed. The material recovered in the receptor solution can be regarded as having penetrated. In some cases, the material remaining in the skin (sometimes after removing the stratum corneum by tape stripping) after terminal rinsing has been added to the amount measured in the receptor solution. This has usually improved the *in vitro/in vivo* correlation, especially when full thickness skin samples were used or with highly lipophilic compounds.

In general, most poor *in vitro/in vivo* correlations have been found with highly lipophilic compounds ($\log K_{oc} > 4$). This is particularly noticeable when full thickness skin samples are used with compounds which are able to interact with skin. The binding of chemicals to skin components is a possible reason for the underestimation of percutaneous absorption with *in vitro* methods. Another reason may be the inability of physiological receptor solutions to remove
Table III: Information required for evaluating *in vitro* percutaneous absorption data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test compound</td>
<td>purity, physicochemical properties, nature of any impurities</td>
</tr>
<tr>
<td>Vehicle</td>
<td>solvent or formulation used to deliver test substance to skin surface</td>
</tr>
<tr>
<td>Cell type</td>
<td>design, material of construction, receptor solution, dynamics of flow cell</td>
</tr>
<tr>
<td>Exposure conditions</td>
<td>volume, duration of contact, occlusive nature of donor compartment, rinsing conditions</td>
</tr>
<tr>
<td>Skin membrane</td>
<td>source, preparative procedures (cleaning, keratome slicing, heat preparation, etc.), storage, metabolic capabilities, surface temperature</td>
</tr>
<tr>
<td>Receptor solution</td>
<td>identity, properties, solubility of test material</td>
</tr>
<tr>
<td>Terminal procedures</td>
<td>rinsing of skin, removal of skin by stripping, preparation for analysis</td>
</tr>
<tr>
<td>Analytical procedures</td>
<td>methods used to determine concentration of test material in donor solution, receptor solution, skin samples and rinsings</td>
</tr>
<tr>
<td>Results</td>
<td>tables showing individual (as appendices) and mean data from each cell; data should show overall percentage recoveries and the mass penetrating per unit area with time. Graphs showing changes in flux with time (illustrative of absorption profile). Permeability constants are only valid for steady-state situations.</td>
</tr>
</tbody>
</table>

...penetrated material from the epidermis/dermis.

*In vitro* methods have been widely used for studying the relative percutaneous absorption of compounds from different vehicles. Typically, the results of these *in vitro* experiments accurately reflect the vehicle effects seen *in vivo* (3).

Consideration of a recent publication by Ramsey *et al.* (78), in which some excellent data are presented, illustrates many of the problems encountered in designing *in vitro* experiments. The authors reported data from *in vivo* and *in vitro* penetration studies in humans and rats with fluazifop-butyl (FB). They concluded that the *in vitro* data adequately predicted the *in vivo* situation. In all experiments, the compound (5μl/cm²) was applied to the skin surface at three concentrations in the same dosing formulation. The *in vivo* experiment in rats was conducted using a pharmacokinetic protocol, with animals being sacrificed at 4, 8, 12, 24, 48 and 72 hours. Six human volunteers were treated topically with the compound and the skin was washed after eight hours. Blood and urine samples were taken throughout the experiment up to 9 days and 11 days, respectively, after dosing. The absorbed dose was calculated from the urinary and blood levels of FB and its metabolites by using known pharmacokinetic parameters to calculate the total dose absorbed with time. *In vitro* studies were undertaken with static diffusion cells, using human and rat skin membranes. Three receptor solution formulations were used: 50% aqueous ethanol (AE), 6% polyethylene glycol 20-oleyl ether in saline...
(PEG), and tissue culture medium (TCM). The receptor solution AE consistently gave higher absorption values than the PEG or TCM receptor solutions, both of which gave remarkably similar results. With rats, the best in vitro predictions of the in vivo data were obtained by using PEG or TCM as the receptor solution; for humans, the use of AE as the receptor solution gave the best correlation. The in vitro data did not completely reflect the reduced penetration seen in the in vivo studies as the applied concentration increased.

The data presented by Ramsey et al. (78) indicate that, by using inappropriate in vitro experimental conditions, in vivo skin absorption could be underestimated by up to 30-fold with human skin, or could be overestimated 50-fold by directly extrapolating rat skin penetration data.

Conclusions and Recommendations

1. Percutaneous absorption can be measured by a variety of techniques. Specific protocols need to be drawn up which best meet the requirements of pharmaceutical, veterinary, environmental and cosmetic scientists for data which need minimal extrapolation to the in-use assessments of hazard/risk/benefit. These protocols have many features in common, the principles of which have been described in this report. A simple strategy for assessing percutaneous absorption is outlined in Figure 3.

2. There are numerous techniques available which give estimates, of varying accuracies, of skin absorption in man. Directly measured in vivo human data are unequivocal, but their limited availability, due, in part, to pharmacokinetic and analytical limitations, requires that less direct methods are employed. Indirect in vivo human data obtained by using the skin stripping technique, by measuring pharmacological responses, by residual analysis, and by using other non-invasive techniques require careful interpretation and the methodologies require further validation. The tape stripping technique, in particular, has potential, but its applicability to a wider range of compounds should be investigated.

3. The amount of quantitative data available on the in vivo percutaneous absorption of chemicals in humans is limited. There are more in vivo data available for laboratory animals, and the amount of in vitro data is considerable. This situation reflects the technical and regulatory requirements of the different methods. Examples of good and poor in vitro/in vivo correlations have been published in the literature. Efforts should be made to make unpublished “in-house” data more widely accessible, to improve the ability to undertake in vitro/in vivo evaluations.

4. In vivo animal procedures have regulatory approval with respect to the production and marketing of new chemicals and require no further development. Results from these studies should be extrapolated to man with care. There is considerable pressure to replace these procedures with in vitro methods.

5. In vitro techniques for measuring the ability of a chemical to penetrate the skin are quite diverse and they are useful screens. It was concluded that the simple diffusion cell is an appropriate model for this type of assessment, and that human skin is the membrane of choice. At the workshop, there were advocates for both static and flow-through in vitro systems. While attractive, the use of metabolically active skin for routine screening purposes would not appear to be feasible. Typically, good in vitro/in vivo correlations have been found, but poor correlations have been reported for highly lipophilic compounds, in particular, where the absorption was lower in vitro than in vivo, and the lag time was shorter in vitro than in vivo.

6. The limited availability of human skin (especially of viable tissue) is a severe restriction. Post-operative human skin, and that from cadavers, should be made more readily available. Pig skin and hairless guinea-pig skin may be acceptable alternatives to human skin. The barrier properties of synthetic membranes (human skin models) are not yet adequate for them to be used routinely.
Figure 3: Strategy for assessing percutaneous absorption

1. Chemical under consideration

2. Purity/stability — suitable for measurement?
   - Yes
   - No

3. Physical properties

4. Can sufficient be delivered?
   - K_p < 10^{-5} (Low penetration)
   - K_p > 10^{-2} (High penetration likely)

5. Calculate likely permeability coefficient (K_p)

6. Risk/benefit scenario acceptable?
   - Yes
   - No

7. In vitro experiment using appropriate vehicle

8. Is penetration sufficient for toxicity/efficacy?
   - Yes
   - No

9. Confirm with appropriate in vivo studies
   - Confirm?

10. Can absorption be controlled?
    - Yes
    - No

11. Reject drug/chemical
    - Confirm?
7. Skin metabolism was recognised as being of great importance in understanding the first-pass effect in skin, but, in general, it was not felt that metabolism was rate-limiting in the penetration process. The ability of the skin to metabolise chemicals should be investigated in separate experiments. The potential for changes to chemicals on or in the stratum corneum due to hydrolysis, oxidation, etc. must be carefully assessed before measuring absorption. Evidence indicates that simple diffusion is the primary process limiting percutaneous absorption. The existence of active transport mechanisms has not been demonstrated, but these may exist for certain highly lipophilic compounds and for compounds which have strong receptor sites within the epidermis/dermis.

8. Perfused skin systems are attractive in that the blood supply is maintained and acts as the physiologically relevant "sink". However, such systems are technically demanding and are not suitable for routine assessments. Nevertheless, they have an important role with respect to certain, specific, investigations and as part of an overall strategy for measuring percutaneous absorption.

9. Standardisation of in vitro systems is required. Standards should be used which assess the integrity of the membrane, enable the test substance to be ranked with a penetrant of known characteristics, and give added quality and confidence in the data in GLP terms. A list of standard chemicals should be drawn up; these chemicals should cover a range of physicochemical properties, should not affect the skin barrier properties, should be readily available and should be supported by reproducible penetration data. Possible standards include hydrocortisone, benzoic acid, salicylic acid, caffeine, water, mannitol, sucrose, and non-ionic, water-soluble, polymers which do not penetrate the skin (for example, PEG1000), since some in vivo data are available for these compounds. If possible, the penetration of these standards should have been, or should be capable of being, studied in man, to enable complete assessment of their penetration characteristics.

10. Methods for calculating the potential of a chemical to penetrate the skin from its physicochemical properties are improving, and will continue to do so provided that data of sufficient quality are produced. Since the vehicle can greatly affect penetration, in ways which are not completely understood, the complete modelling of skin absorption will require a lot more development if it is to be a reliable predictor. Ideally, for selected chemicals of importance, sufficient data should be generated to enable the construction of models and testing schemes, such as that outlined in Figure 3.

11. The establishment of centres which could provide high-quality scientific and technical training for those involved in conducting percutaneous absorption studies was seen as desirable by some of the workshop participants. Approved training courses could be held, which would fulfil the GLP requirement for demonstrating technical competence.

12. Quality control aspects of percutaneous absorption studies are a very important consideration, especially for data submitted to regulatory authorities. The quality of the data generated in some of the early studies is poor. Guidelines should be drawn up in this area.

References

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