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Measurement and prediction of the rate and extent of drug delivery into and through the skin

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Skin diseases are prevalent and can significantly affect quality of life. Empirical mathematical models retrospectively analyse data to predict skin permeation from the physico-chemical properties of drugs. Quantitative structure permeability relationships are discussed, along with alternatives to linear modelling. Mechanistic mathematical models derived from first principles are also considered. Further, *in vitro* experiments allow predictions to be made using suitable membranes (cultured cell lines or excised skins). *In vivo* methods to assess (trans)dermal drug delivery aim to minimise clinical studies, especially to determine whether formulations are bioequivalent. Microdialysis is discussed, together with the FDA-approved skin blanching (pharmacodynamic) assay for corticosteroids. The progress made with the tape stripping methodology is reviewed. Two distinct strategies have emerged, the first where the total amount of drug in the stratum corneum (SC) at one uptake and one clearance time are compared; and the second which generates drug permeation profiles across the SC, and allows dermatopharmacokinetic parameters to be derived.

Keywords: Dermatopharmacokinetics, excised skin, keratinocyte cultures, mathematical models, pharmacodynamics, skin, stratum corneum, tape stripping

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1. Introduction

The skin is the largest organ of the human body. Skin forms an extremely effective barrier, preventing both the loss of heat and water, and the ingress of micro-organisms and chemicals. Drugs may be applied topically to the skin for the local treatment of inflammation, infections (bacterial, viral or fungal) or to anaesthetise an area. The target site may be the outermost layer of the skin, the stratum corneum (SC), or deeper layers. Potent drugs may also be administered transdermally, travelling through the skin to the dermal blood vessels and into the systemic circulation.

Assessing drug permeation into or penetration through the skin is important both to evaluate the usefulness of a drug for topical or transdermal delivery, and to compare different formulations to assess their bioequivalence.

Prediction of drug movement through skin layers is logistically, ethically and economically preferable to *in vivo* measurements. With respect to permeation prediction, mathematical models, based on empirical measurements or mechanistic deductions, have been explored. The most important predictors of permeation are lipophilicity and molecular weight. *In vitro* measurements of skin penetration using diffusion cells, separated by an appropriate membrane, either synthetic or *ex vivo*, provide important empirical measurements to use in the development of mathematical models. Bioengineered skin is being developed, but currently excised pig skin offers the most frequently chosen match to human skin.

Predictions help to reduce the number of *in vivo* measurements required, by focusing experimental strategy, but the latter are currently still required. The

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in vivo strategies developed are intended to provide objective measurements of drug permeation, and minimise the need for expensive, subjective clinical trials. Three prominent methods are discussed: microdialysis, phamacodynamic approaches and tape stripping. Within tape stripping, two improved metrics for evaluating the data are presented, alongside the approach originally proposed by the US Food and Drug Administration (FDA).

2. The prediction of topical and transdermal drug delivery

The main benefits of predictions over measurement of drug delivery are clear: it is usually cheaper and quicker. Furthermore, there are no problems with ethical issues, recruiting volunteers, or housing animals.

Ideally, predictions should provide a reliable indication of the extent to which drugs will permeate the skin, either to the target skin layers (for topical products) or to the systemic circulation (for transdermal products). Predictions are also useful to select and design the best drug molecules for these purposes.

Mathematical models, developed using existing experimental results, self-evidently provide the most convenient predictive tools. *In vitro* experiments can assess drug permeability through various membranes, which bear increasing relevance to human skin.

2.1 Mathematical models

Two main approaches have emerged for the prediction of drug permeation: empirical models and mechanistic models. The former are derived from a retrospective analysis of experimental results, and correlate skin permeation data with drug properties. Mechanistic models are derived from first principles. Permeation is predicted and modelled based on skin compartments and diffusion pathways. Within these broad categories, several other classes of models may be differentiated and are reviewed here.

2.1.1 Empirical models

The most intensively researched area involves quantitative structure permeability relationships (QSPRs) which correlate experimental permeation data with (i) physico-chemical properties, or (ii) molecular structural properties of the permeant.

2.1.1.1 Quantitative structure permeability relationships

Before any empirical modelling based on permeability can begin, the permeability of the chemical through the skin must be measured. The permeability coefficient, k_p (units: distance/time) is defined as:

$$k_p = \frac{K \cdot D}{H} \quad (1)$$

Where K is the drug's SC-vehicle partition coefficient; D is the diffusivity of the drug in the SC; and H is the SC thickness.

In vitro experiments are performed to generate data, as shown in **Figure 1**. Using the appropriate version of Fick's second law (**Figure 1**) for non-steady state diffusion, the data in **Figure 1** may be modelled and K and D/H^2 derived [2,3].

2.1.1.1.1 Databases of κ_p values

Databases of k_p values have been created retrospectively. The most extensively used of these is the Flynn database [4]. It comprises 97 k_p values, for 94 compounds, covering a broad range of molecular weights (MW) (18 – 765), and octanol–water partition coefficients (-3 to +6). The values are mostly from human skin *in vitro*, with the drug usually delivered from an aqueous solution. The database's principal shortcomings are: the proportionally small number of both hydrophilic compounds (9/94 with log oil–water partition coefficient ($K_{o/w}$) $\leq$ 0) and highly lipophilic compounds; and the lack of consideration of the chemical's ionisation state and of the temperature at which measurements were made [5].

Several other compilations of log k_p values have been assembled, often using some values from the original Flynn database. The value of these other databases have been described succinctly by Vecchia and Bunge [5].

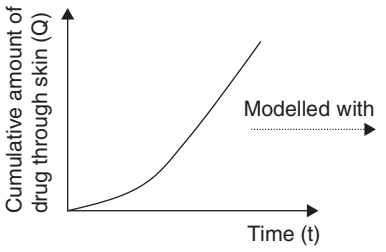
Recently, a European Union-funded study, 'Evaluations and Predictions of Dermal Absorption of Toxic Chemicals' (EDETOX), produced a database containing permeation data for 300 compounds [6]. The work also generated new data for the *in vitro* transport of 60 chemicals across human skin [7]. The experiments were well-designed and allowed comparison of: rat versus human skin; static versus flow-through diffusion cells; finite versus infinite dose; skin thickness; vehicle composition; and physico-chemical properties (MW, log $K_{o/w}$ solubility) [7]. The results have been modelled using QSPR and a novel mechanistic model, the Kruse model [8], developed.

2.1.1.1.2 QSPR models

The most cited QSPR proposed is that reported by Potts and Guy [9]. Their equation was derived from linear regression of the k_p, MW and log $K_{o/w}$ values in the Flynn database (93 compounds). The best fit equation was:

$$\log k_p(cm.h^{-1}) = -2.72 + 0.71(\log K_{o/w}) - 0.0061(MW) \quad (2)$$

which had an overall correlation coefficient (r^2) of 0.67. The model clearly shows that small, lipophilic chemicals are those with the greatest skin permeabilities. Given the typical level of variability in skin permeability measurements, the Potts and Guy equation performs remarkably well and certainly provides reasonable predictions. However, the approach focuses on the SC as the exclusive rate-limiting barrier and therefore significantly over-estimates k_p for highly lipophilic compounds. This issue was successfully addressed by Cleek and Bunge [5,10], whereby a corrected k_p value of lipophilic compounds ($k_{p\ (lip)}$) may be calculated using Equation 3:



$$Q = \{KH\}C_v\left[\frac{D}{H^2}t - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(\frac{-Dn^2\pi^2 t}{H^2}\right)\right]$$

Figure 1. The cumulative amount of drug permeating the skin, Q, under non-steady state conditions, as a function of time, t, may be modelled using the appropriate version of Fick's second law (equation as shown above); where K is the drug's SC–vehicle partition coefficient; H is the stratum corneum (SC) thickness; C_v is the drug concentration in the vehicle; and D is the diffusivity of the drug in the SC.

$$K_{p(lip)} = \frac{K_p}{1 + \left(\frac{K_p + \sqrt{MW}}{2.6}\right)} \quad (3)$$

where k_p is that derived from the Potts and Guy equation (Equation 2). The rationale behind this correction equation is discussed further below.

Many other models have been proposed and compared [5]. Inconsistencies in the Flynn database relating to ionisation state (which affects $K_{o/w}$), inter-dependence of MW and $K_{o/w}$ in some homologous series, and temperature (which affects diffusivity) have been identified. These factors have been explored, suitable adjustments to the permeability prediction equations have been proposed [5,11-13] and slight improvements recorded. For example, using the logarithm of the chemical's diffusion coefficient, log D, instead of log $K_{o/w}$, allows the effects of pH on ionisation to be incorporated [14], although sufficient data does not yet exist for ionisable compounds. A comprehensive review of these factors is presented elsewhere [11]. If reliable data regarding log $K_{o/w}$ are not readily available, estimates can be derived from the molecular structure, incorporating the contributions which various groups make to the overall lipophilicity [15]. Diffusion has also been shown to be related to partial charge interactions (such as H bonding), which may be predicted [16-19].

2.1.1.2 *Quantitative retention permeability relationships*

Quantitative *retention* permeability relationships (QRPRs) relate chromatographic parameters (rather than molecular parameters as in QSPRs) to permeability coefficients derived experimentally. Biopartitioning micellar chromatography uses modified high performance liquid chromatography (HPLC) columns as surrogates for biological barriers [20-24]. Solute capacity factors, directly calculated from retention times on the column, are used to derive QRPR models.

The first modified HPLC column used cell membrane-mimicking phospholipids on a silica surface, the so-called Immobilised Artificial Membrane (IAM) columns. The resultant retention times correlated well with (human derived) log k_p values for steroids, and slightly better in fact than the corresponding log $K_{o/w}$ values [22]. Phenolic and ionic compounds showed a poorer correlation [22].

A second column, with keratin immobilised on a silica support, was produced to mimic solute interaction with keratin in skin [23]. When a series of solutes were run on both this column and an IAM column, the resulting capacity factors were incorporated into one QRPR model which showed a good correlation ($r^2 = 0.87$) with experimental log k_p values for 17 compounds [23].

Theoretically, by manipulating the HPLC method, the effects of pH, temperature, ionisation and presence of excipients on permeability may also be measured [20,21]. This would be a potentially significant advantage when attempting to optimise formulations.

2.1.1.3 *Artificial neural networks*

In QSPRs, multiple linear regression is used to determine correlations between physico-chemical properties (such as log $K_{o/w}$ and MW) and permeability. Both linear and non-linear correlations may be investigated using artificial neural networks (ANNs). ANN modelling uses computer algorithms to learn from data in a manner similar to the brain's use of webs of interconnected neurons [25].

ANNs have been successfully used [25] to predict skin permeation of 40 compounds (MW range ~ 30 – 390 and log $K_{o/w}$ = -0.77 – 2.94). Further work, using different input parameters [26,27], has again shown an improvement over multiple linear regression models.

2.1.1.4 *Fuzzy modelling*

Fuzzy models cluster data for different compounds into appropriate subsets based on their properties. For example, a subset may contain compounds with a log $K_{o/w}$ around +2. Each compound will have a degree of membership of this set, between 0 and 1; that is, a compound with a log $K_{o/w}$ exactly equal to 2 would be assigned a membership degree of 1.0; if log $K_{o/w}$ = +1.8, then the membership degree may be ~ 0.9; for compounds with log $K_{o/w} \leq +1$ or $\geq +3$, the membership

degree is zero. Of course, compounds may be members of several subsets, as these may not be mutually exclusive.

The Fuzzy model algorithms consider all the compounds in the data set and, after a learning period, cluster the data points into appropriate subsets and determine the degrees of membership of each compound to each subset. Fuzzy modelling can simplify complex models by revealing patterns in the data. The algorithms are numerous and complex but they essentially find links between inputs and outputs via Fuzzy clustering techniques [28].

Fuzzy modelling has been used [28] to analyse the molecular parameters (including MW, log $K_{o/w}$, H-bond donor activity, H-bond acceptor activity, and polarisability) from three sources [4,29,30]. The correlation between the (Fuzzy) predicted and experimental log k_p was very good, with r^2 of 0.83, 0.97 and 0.96 for three distinct data sets (with 94, 37 and 54 compounds per dataset, respectively). In addition, fewer molecular parameters were required for the Fuzzy model compared with the original multiple linear regressions.

2.1.2 Mechanistic models

Mechanistic models predict permeation based on knowledge of skin compartments and diffusion pathways.

2.1.2.1 Stratum corneum and viable epidermal contributions to barrier function

To evaluate the normalised cumulative mass of drug absorbed into the SC, a series of three mechanistic models were derived [10], all related to exposure time, and such that unsteady state exposure may also be estimated.

The three models (**Figure 2**) consider: i) the SC alone as a single finite membrane; ii) the SC alone where it is assumed that the membrane is sufficiently thick and the exposure time short enough, such that it behaves as a semi-infinite membrane; and iii) both the SC and viable epidermis (VE) as a finite two-membrane composite, incorporating the partition, diffusivity and path length variables for both skin layers.

For unsteady state (short) exposure times, the drug will not have reached the VE to any significant extent and Models 1 and 2 are applicable.

Model 3 is important for longer exposure times, when steady state may be reached, and the overall drug flux may be affected by the hydrophilic VE. This is indeed the case for highly lipophilic chemicals which will partition slowly from the SC and eventually become rate-limited by the VE. The extent to which the VE contributes to the total resistance to transport may be captured in a modified expression to calculate the normalised cumulative mass of drug absorbed into the SC, incorporating a SC–VE permeability ratio, B [10]. For lipophilic compounds, for which $B \geq 100$, it is apparent that the VE affects the skin permeation process [31].

Experimentally, however, B is difficult to measure and various approximations have been introduced to enable its use in Model 3 [31]. For example, the $K_{o/w}$ is used to estimate the chemical's SC/VE partition coefficient, and molecular weight is used to correct diffusivities. Both parameters are extracted from the Potts and Guy equation [29].

The correction introduced in this way places a limit on the calculated values of the cumulative mass of drug absorbed into the SC. The impact is greatest for lipophilic compounds to which the skin is exposed for long application times (and longer than the conventional lag time for diffusion across the skin) [32].

2.1.2.2 Scaled particle theory

Scaled particle theory calculates the work required for solutes to be incorporated into lipid bilayers [33]. This theory has been applied to transdermal permeation, and used to calculate diffusion and partition coefficients of solutes [33]. These parameters will depend on lipid density and lipid disorder (assumed constant) [33].

The model first assumed a tortuous pathway for diffusion across the SC [34]. Empirical data was used to derive the relationship $K = (K_{o/w})^{0.7}$ which was then used to estimate K in the model [33]. The solute's diffusivity through the SC was estimated from the molecular radius (r, (Å)) which, in turn, was calculated empirically from the MW [33,35], using data from Mitragotri *et al* (Table 1 of [34]) to derive:

$$r = \sqrt[3]{0.22 \times MW} \tag{4}$$

The final expression for the permeability coefficient was:

$$k_p(cm.hr^{-1}) = (K_{o/w})^{0.7} * 0.02\exp(-0.46r^2) \tag{5}$$

Or, in logarithmic form:

$$\log k_p(cm.hr^{-1}) = 0.7K_{o/w} - 1.70 + 0.20r^2 \tag{6}$$

This model and the Potts and Guy equation give very similar results [12]. Although they are derived in very different ways, by re-substituting MW for in place of the radius (using Equation 4), Equation 6 can be rewritten as:

$$\log K_p(cm.hr^{-1}) = 0.7\log K_{o/w} - 1.70 + 0.072MW^{2/3} \tag{7}$$

which bears remarkable similarity to the Potts and Guy equation (Equation 2) [12].

The model was tested using a compilation of experimental values [34], and a slightly lower mean absolute error was found for the mechanistic model (5%) as compared with Potts and Guy (6.3%) [33]. Notable deviations from Equation 6 are molecules with MW > 400 Da.

Subsequently, the model in its original form (which focussed on solute permeation through the intercellular lipid domains of the SC) was expanded to include alternative,

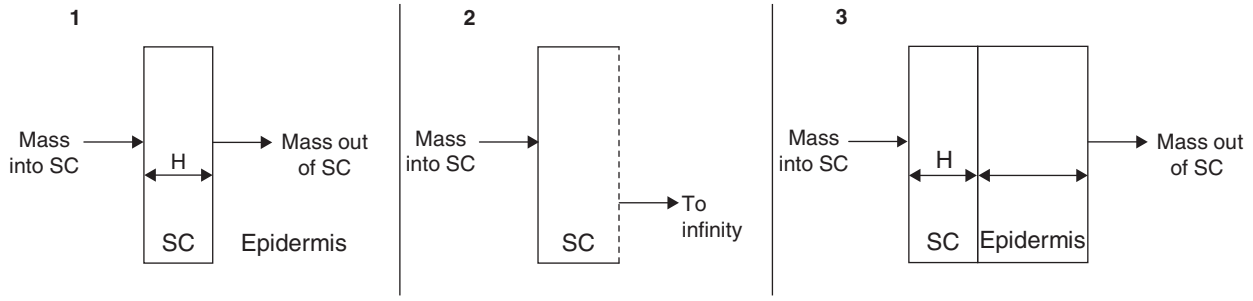


Figure 2. Three mechanistic models proposed by Cleek and Bunge. Models based on: **1**. Single finite membrane; **2**. semi-infinite membrane; **3**. finite two-membrane composite. H, stratum corneum (SC) thickness. Redrawn from [28].

parallel pathways, including transport via putative aqueous 'pores' and in various shunts, such as hair follicles and sweat glands [36]. This more complex approach was not only able to match the predictions of the Potts and Guy algorithm, but could also fit the results for larger hydrophilic compounds, the permeability of which is usually underestimated by earlier models. In contrast, it should be pointed out that the 'price' for this improvement is a more complex model containing an increased number of adjustable parameters.

In summary, the most straightforward and widely used mathematical model is the empirical Potts and Guy equation (Equation 2). It provides an adequate prediction based on two easily obtainable physicochemical properties, MW and $\log K_{o/w}$. Modifications and refinements have been proposed, and the adaptation for lipophilic compounds [10,31,32] is a sensible and necessary development. Introduction of additional parameters or incorporation of other parallel pathways can improve the overall correlation between predicted and experimental results, but there is a cost in terms of simplicity and practicality.

2.2 *In vitro* experiments

In vitro experiments use vertical or side-by-side diffusion cells, with the receptor fluid separated from the solution/formulation by an appropriate membrane. Measurements of drug flux through the membrane are obtained from the cumulative amount transported as a function of time (**Figure 1**). Versions of Fick's first and second laws can then be used to derive the permeability coefficient, or other representative transport metrics, in steady or unsteady state situations, respectively [2]. *In vitro* experiments provide a quantitative measure of drug penetration, and are cheaper and less complicated to perform than *in vivo* studies.

2.2.1 Choice of membranes

The best membrane for *in vitro* studies is human skin, which may be obtained from surgical procedures. However, a regular supply is not always available; the use of cadaver skin is not generally possible and frequently leads to highly variable data. As a result, alternatives have been investigated.

2.2.1.1 Cultured cell lines

Cultured cell lines represent a development from the field of tissue engineering i) to provide biomimetic, non-immunogenic skin layers to protect, and aid the healing of, acute and chronic wounds, burns and ulcers; and ii) as a model for permeation and toxicological assessment of cosmetic and medicinal products. Research has accelerated because, from 2009, European Union directives will prohibit the use of animals in toxicity studies for cosmetic products [37]. In addition, the use of donated human tissue for purposes of financial gain is prohibited [38].

There are two principal categories of cultured skin tissues: living skin equivalents comprising a dermis, epidermis and partially differentiated SC, but without skin appendages; and human reconstructed epidermis which consists simply of keratinocytes grown on a substrate. Examples of the latter are more common (EpiSkin® [L'Oréal, France], SkinEthic® [SkinEthic Laboratories, France], EpiDerm® [MatTek Corporation, USA]) than the former (Apligraf, Graftskin [Organogenesis, USA]).

The morphology and biochemistry of human reconstructed epidermis has been compared with *ex vivo* human tissues [39-41]. Both have a differentiated epithelium, with stratum corneum, stratum granulosum, stratum spinosum and stratum basale, but several histological and biochemical differences are apparent [39]. The SC is typically thicker and more variable that human skin, and is not always fully keratinised [39]. The morphology, lipid composition and biochemistry of Epiderm, SkinEthic and EpiSkin have been reviewed, together with their applications in phototoxicity, irritancy and absorption testing [41].

Generally speaking, chemical penetration across human reconstructed epidermis is quite reproducible, but significantly higher than through human, pig or rat skin [40-44]. They have thus been proposed as useful tools with which to compare and optimise formulations [45], but care must be exercised if excipients are present which may alter barrier function (and result in artificially amplified penetration results).

Living skin equivalents, despite their greater similarity with human skin [41], have shown to similarly overestimate chemical penetration [42,43,46].

Overall, therefore, while valuable, for example, for skin irritation assessment, skin cell culture models are not yet able to provide quantitative predictions of percutaneous penetration.

2.2.1.2 *Excised skins*

Many animal models have been suggested for evaluating percutaneous penetration, including primates, ungulates (pigs, including miniature pigs), rodents (mice, rats and guinea pigs) and reptiles (snakes).

Rodent skin is widely available and is often used. Hairless species are preferred since potentially damaging shaving or depilation is avoided, and the follicular density resembles human skin more closely. Results show that penetration rates are higher for rodent skin than human skin, with rat skin being up to ten times more permeable [47,48]

Pig skin has been repeatedly shown to be a relevant and convenient animal model for *in vitro* penetration studies [42,43]. When compared to human skin, pig skin is similar in terms of histology and dimensions, but the diameter of porcine hair follicles is larger [49].

Flux profiles of diverse compounds across pig skin are similar to those through the human barrier [42,50] and it appears unlikely that the use of the porcine model will overestimate the results in man. Equally, when using the tape stripping methodology discussed below, the concentration profiles of drugs (such as ibuprofen) across pig SC closely resemble those observed *in vivo* in humans [51].

3. Measurement of (trans) dermal drug delivery *in vivo*

Clearly, for transdermal products, whose target site is the systemic circulation, well-established *in vivo* pharmacokinetic studies, taking blood and/or urine samples at particular times, after application and removal of the product, provides the most reliable measurement of transdermal drug penetration. These pharmacokinetic studies, similar to those of controlled release formulations, are accepted from a regulatory perspective for transdermal products [52,53].

Despite the advances made in predictive models and *in vitro* testing, and their inherent advantages, the ultimate evaluation of topical drug delivery into and though the skin has to be performed *in vivo*, in man. For new chemical entities, the necessity of clinical trials is clear. In the case of generic products, while testing of efficacy remains a requirement, there is considerable effort currently directed at the assessment and validation of alternative approaches with which to assess bioequivalence.

3.1 Microdialysis

Microdialysis is an *in vivo* technique for sampling free drug concentrations in the extracellular fluid within tissues or organs. A thin dialysis membrane tube is inserted into the site of interest and perfused with a site-compatible physiological solution (**Figure 3**). Molecules of interest (usually small and water soluble) diffuse across the membrane, are collected and measured.

With respect to (trans)dermal drug delivery, the microdialysis probe is inserted subcutaneously or intradermally. The technique boasts many advantages: i) continuous measurements of drug concentration with time are possible, allowing pharmacokinetics to be followed; ii) large molecules are size-excluded by the dialysis membrane, limiting analytical interference and enzymatic degradation [54,55]; iii) no tissue or bodily fluids are removed, permitting an unlimited number of samples to be taken; and iv) endogenous compounds, such as lactate, glucose, neurotransmitters, etc, may also be measured.

Limited experiments have assessed drug concentrations in subcutaneous tissue and underlying muscle after topical and oral administration of ibuprofen [56]. Dermal concentration profiles of lidocaine [57], salicylic acid [58] and 8-methoxypsoralen [59] have been measured, including comparisons with plasma levels for 8-methoxypsoralen.

The drawbacks of the technique include tissue reactions as the probe is inserted [60]; the choice of flow rate and sampling times requires fine tuning [61]; analytical techniques require high sensitivity due to dilution with perfusate [62]; and selection of perfusate and dialysis membrane depends on the drug of interest, with lipophilic drugs posing particular problems [63]. The mathematical extrapolation of extracellular fluid concentrations from the dialysate concentration is also challenging, often requiring the co-administration of a reference molecule (reviewed, [64]). Inter- and intra- individual variability is relatively large, with coefficients of variation between 50 – 100% [60].

3.2 Pharmacodynamic assessment of topical corticosteroids

Corticosteroids typically exert a quantifiable blanching effect on the skin caused by vasoconstriction of dermal microvasculature after the drug has permeated through the epidermis. The FDA has adopted this pharmacodynamic effect as an acceptable method for assessing the bioavailability and bioequivalence of topical corticosteroids [65]. Originally the colour change of the skin was assessed by a trained operator, but the development of the chromometer has allowed objective, sensitive and quantifiable measurements to be made. This apparatus assesses (skin) colour based on the L-scale (light–dark), the a-scale (red–green) and the b-scale (yellow–blue). The FDA guidance document specifies only the use of the a-scale [65]; however, the L-score [66,67] and all three [68] have also been used successfully.

The main advantage of the skin blanching assay is its non-invasive nature and that, as a result, many measurements may be made over an extended time period on the same site. Corrections for changes in skin colour unrelated to the formulation are made at an adjacent site. Further advantages, compared with clinical trials, are that formulations may be quickly and objectively assessed in a relatively small number of healthy participants, avoiding the problems associated with patient selection.

The FDA protocol involves two stages [65]. First, a pilot study determines the effect of dose duration (0.25 – 6.0 h) on the blanching response for all the formulations of interest.

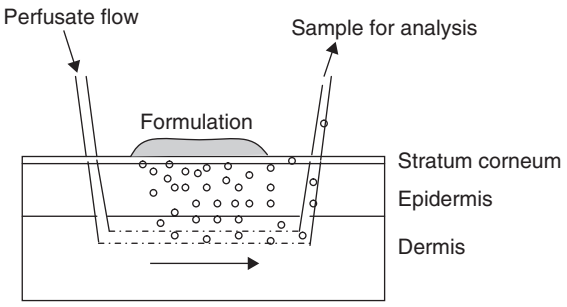


Figure 3. Schematic of microdialysis membrane used for dermatopharmacokinetics.

Second, the 'pivotal *in vivo* bioequivalence study' evaluates the pharmacodynamic changes that occur (over 24 – 28 h) after the formulation has been removed and the skin cleaned.

The degree of skin blanching has been correlated with drug potency [67] and with the permeation of the corticosteroid through the skin [66,67-71]. Bioavailability has also been assessed using the technique [69] and formulations have been compared [66,70,72,73]. Attempts to correlate skin blanching results with clinical results [69,74] have also been reported.

However, the method has its limitations. The precision of chromometer readings has been unfavourably compared with the visual assessment of blanching [68,75]. Skin colour may be affected by the pressure with which the chromometer is applied, by inhomogeneity in the initial skin colour and by circadian variation [75]. There is evidence that the blanching response can be saturated, preventing differences between formulations from being observed [76,77].

Clearly, the vasoconstrictor assay is only applicable to drugs eliciting the appropriate pharmacological response, and it is approved by the FDA uniquely for topical corticosteroids. No other drug has a 'surrogate' measure of this type, although there have been efforts to determine whether other types of compound may be amenable to a pharmacological assay.

For example, while non-steroidal anti-inflammatory drugs (NSAIDs) do not produce a comparable visual response *per se*, their ability to diminish the action of a subsequently applied vasodilator (such as an ester of nicotinic acid) may be assessed objectively using a chromometer [67,78] or a laser Doppler velocimeter [79].

Equally, topical retinoids typically induce an increase in transepidermal water loss (TEWL) [80-83], a response which can again be quantified with existing biophysical tools. The same drugs also cause visible irritation [80], which can be assessed instrumentally, as described above, and this action has been reported to be better quantifiable than the change in TEWL [80]. These two measurements should also be correlated, although this has not been shown unequivocally.

3.3 Tape stripping

The removal of sequential layers of the SC using adhesive tapes can be performed with minimum discomfort and relative ease *in vivo*, usually on the volar forearm of healthy volunteers.

When a formulation has been applied to the skin prior to tape stripping, drug may be extracted from the tapes to quantify the amount of drug which has permeated into the SC. Since the SC is the principal barrier to drug absorption, it may be assumed that the (dermatopharmaco)-kinetics (DPK) of drug passage through this layer are related to topical bioavailability [84]. Formulations may be applied for various application times, or stripping delayed after formulation removal to measure SC clearance, and DPK parameters derived. The latter can be used to compare topical formulations and to assess bioequivalence. In the original application of tape-stripping to assess chemical uptake across the skin, good correlations were observed between those amounts recovered on the strips and that which penetrated through to the systemic circulation [85].

Three approaches, summarised schematically in **Figure 4**, have been proposed for the analysis of tape stripping data.

3.3.1 *Original FDA dermatopharmacokinetic metric*

In 1998, the FDA issued draft guidance for the DPK method to assess the bioequivalence of topical products for application to the skin, except those causing severe SC disruption [86]. The draft guidance required quantification of the total amount of drug in the SC as a function of time, akin to the typical concentration–time profiles of traditional pharmacokinetic studies using blood sampling. Four application times, and four elimination times were required per formulation. From these profiles, the area under the curve (AUC), the maximum drug concentration achieved in SC (C_{max}) and the application time after which maximum concentration measured (T_{max}) were evaluated per unit area of skin (**Figure 4A**). For the test products to be bioequivalent, the 90% confidence interval for the ratio of population geometric log means of test to reference should be 80 – 125% for AUC and 70 – 143% for C_{max}.

The draft guidance was withdrawn in 2002 [87] following the results of two studies which compared tretinoin gel formulations, specifically Retin-A, the innovator, and Avita, an approved generic product. Avita is qualitatively and quantitatively different, and has been found to be therapeutically inequivalent to Retin-A [88-90]. Both investigations concluded that the formulations were bio*in*equivalent but, worryingly, one measured higher retinoid levels from Retin-A than Avita [89,90], while the other found the opposite [88]. Naturally, these opposing results prompted concern about reproducibility; furthermore, the adequacy of the method to assess drugs whose target site is beyond the SC was questioned.

Parenthetically, it should be noted that the divergent tretinoin findings were probably due to differences in the areas of SC stripped by the two laboratories. One group stripped an area exactly equal to the application area, while the other stripped an area larger than the treated site. Because one of the formulations was subsequently shown to spread radially on the skin surface

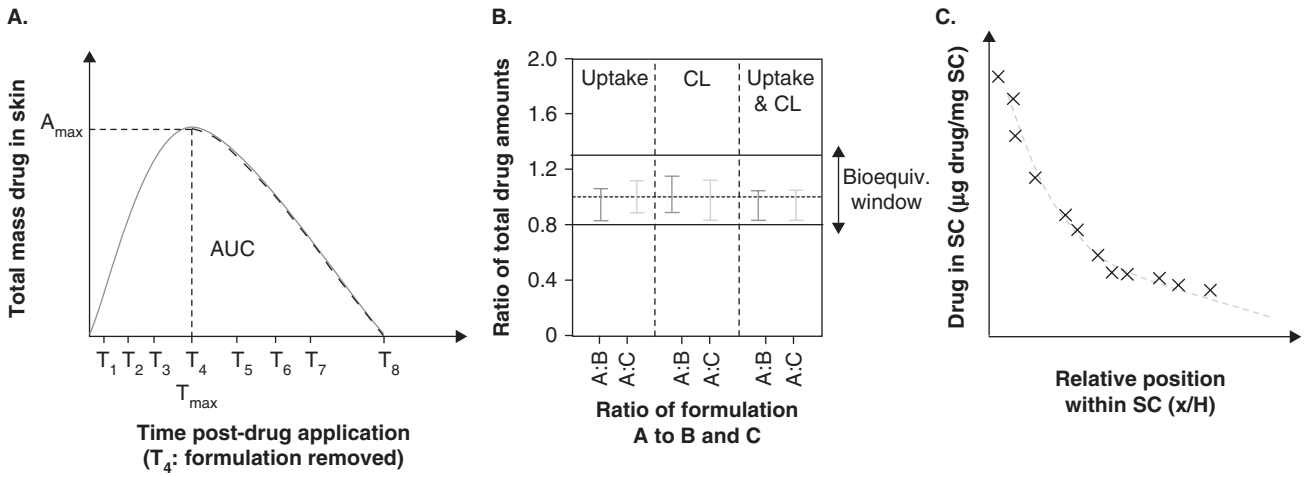


Figure 4. Three approaches for the analysis of dermatopharmacokinetic (DPK) data. A. Original FDA metric. The total amount of drug in the stratum corneum (SC) after four application times (—) and four clearance times (-----) are determined and plotted as a function of time. The maximum amount of drug in the SC (A_{max}), the time at which A_{max} was achieved (t_{max}), and the area under the curve (AUC) are found and used to evaluate and compare formulations. **B.** 'Two-time' method. Ratio of drug amounts (± 95% confidence interval) for an innovator drug A, compared with two generic formulations, B and C. Two application times (one uptake and one clearance (CL)) and uptake + CL data combined are considered. To be bioequivalent, the ratio should lie in the 0.8 – 1.25 bioequivalence window. **C.** 'Drug in SC profile' method. For each tape, the concentration of drug in SC is plotted against the corresponding relative position within the SC (x/L). The curve is fitted to Equation 9 to generate a best fit profile (---) and to extract partitioning and diffusivity parameters.

during the application period, the drug amounts removed were different, did not match and the two laboratories revealed opposite conclusions.

With this observation in mind, a subsequent large-scale study was performed comparing the innovator tretinoin gel with therapeutically inequivalent and equivalent formulations [89]. Following the FDA guidance, eight time points were examined per formulation in 49 volunteers, resulting in a total of 1176 sites. The results confirmed the clinical findings for the different gels. In contrast, another DPK study did not confirm the clinical findings for two miconazole nitrate vaginal creams [91], when compared using the FDA's DPK protocol.

In addition to ensuring that the area of SC stripped is less than, or equal to, the area of application, a further modification of the FDA guidance encourages analysing drug content from all tapes. The FDA proposal to discard the first two tape-strips was never validated and typically results in a significant amount of compound being ignored. This is important because the first tape-strips invariably take off the largest amounts of SC [92]. Including all tapes, on the other hand, requires that the cleaning of the skin surface at the end of the application period is efficient to ensure that unabsorbed drug (i.e., that not taken up into the SC) is completely removed. This is particularly important for real formulations which may lodge in the 'furrows' of the skin [93]. It appears that an alcohol wipe, similar to that used to sterilise the skin before an injection, may offer a useful solution to this challenge [94].

A further stipulation of the 1998 FDA draft guidance was that, having discarded the first two strips, drug should be quantified in the subsequent ten tape-strips. Two obvious weaknesses of this approach are clear: i) ten strips do not remove the same amount, nor the same fraction of the SC, in each volunteer; and ii) ten strips will not remove (in most instances) the entire barrier layer and will not necessarily 'capture', therefore, all the drug taken up into the SC at the sampling time. A simple approach to overcome this problem is to continue stripping until most, if not all, of the SC is removed. TEWL measurements provide a convenient means with which to ensure that this is the case [93,95], as shown in **Figure 5**.

Other criticisms of the original FDA guidelines are apparent. For example, the requirement for eight time points (four for uptake, four for clearance) is very burdensome in terms of study time and analytical work. Unlike the situation with oral drug administration, for which the pharmacokinetic parameters are determined by a complex function of absorption, distribution, metabolism and excretion, the uptake phase in a DPK experiment depends only upon drug partitioning and diffusion in the SC, while clearance is only dependent upon diffusion. Likewise, and specific to DPK, the clearance phase only begins after formulation removal at the longest application time [93]. It is a reasonable question, therefore, to ask whether the comparison between formulations requires such a large number of time points. The concomitant difficulty with a requirement for such a large number of test sites is that, for drugs which do not penetrate the SC well, it may be necessary to increase the surface area of application (and hence the area stripped) to have enough drug in the tape strips for reliable quantification. At some point, as a result, there may be insufficient skin surface to perform the experiment according

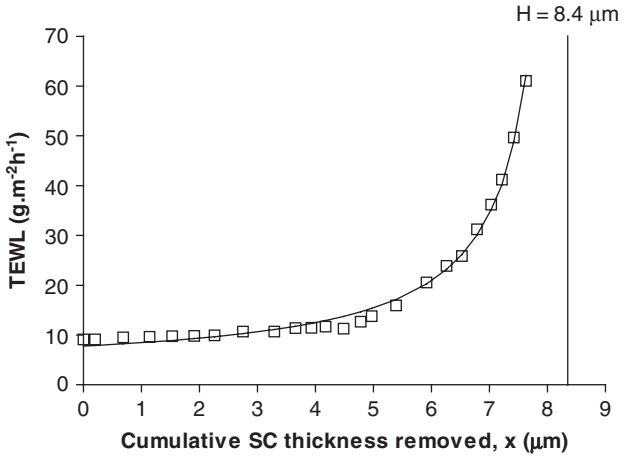


Figure 5. Example of experimental data (□) of TEWL against cumulative stratum corneum (SC) thickness removed, x. A non-linear fit to Equation 7 produces the best fit (—) and an estimate of the total SC thickness, H. TEWL, transepidermal water loss.

to the FDA guidance. Furthermore, as no replicates are performed, the number of volunteers required to achieve statistical power is large.

Research into alternatives has focused on two areas: i) comparing the total amounts of drug in the SC at one uptake and one clearance time; and ii) modelling drug in SC permeation profiles showing drug concentration at progressive depths within the SC.

3.3.2 *The 'two-time' metric*

This method examines only one uptake and one elimination time per formulation. This allows replicate sites to be studied, and higher statistical power to be achieved with fewer volunteers [96]. No tapes are discarded, but tapes are combined and extracted in groups to achieve high concentrations for analysis. The total amount of drug in the SC is measured for each site. The ratio of total drug amounts in the SC from formulation A to B or A to C is determined (**Figure 4B**). The two-time method correctly predicts bioequivalence or bioinequivalence of retinoids [93,96,97] and econazole [93,96].

The retinoid data produced to satisfy the full DPK metric [89,98] has been reanalysed using four alternative metrics: i) the sum of total drug amount from all four uptake time sites; ii) the sum of total drug amount from all four clearance time sites; iii) the sum of total drug amount from all uptake and clearance sites; and iv) the total drug amount from each uptake and clearance time separately [93,97]. Ratios of each of these total amounts of drug in the SC were calculated and used to assess bioequivalence of formulations B (therapeutically inequivalent) and C (therapeutically equivalent) to the innovator (Retin-A). All four metrics confirmed the clinical results. Most interestingly, the fourth metric showed that the ratios were similar for each of the time periods examined individually, suggesting that information regarding bioequivalence may be gleaned from just one uptake and one clearance time. Considering the bioinequivalent gel, the re-analysis highlighted a greater difference from the innovator in the clearance phase than during the uptake phase.

The simplified approach was further tested using econazole nitrate creams, for which the drug's site of action is the SC. The innovator product (Spectazole®, Johnson & Johnson , USA)) was studied alongside two therapeutically bioequivalent generics (made by Taro Pharmaceuticals Inc., Canada and Clay-Park Laboratories Inc., USA). The general (controlled strip area; no tapes discarded; site cleaned with alcohol swab; number of tapes determined by TEWL) and specific (one uptake and one clearance time measured; duplicate measurements; tapes combined for analysis) modifications discussed above were all incorporated.

In a preliminary study [93], all creams were studied on three occasions (separated by at least four weeks) in a single volunteer to check the proposed methodology for the subsequent 'pivotal' study, and to assess the intra-subject variability when measurements were made at different times. It was found that using TEWL, to determine when to terminate stripping, reduced variability in the amounts of SC and drug collected. The variability between sites studied on different occasions was also low compared with the retinoid data discussed above. In the preliminary study only, the tapes were weighed before and after stripping, and the SC thickness was determined at a separate skin site. However, as the SC was almost completely removed (as determined by TEWL), it was not considered necessary to quantify the SC on the tapes in the pivotal study.

The subsequent, larger, 'pivotal' study involved just 14 volunteers and confirmed the promising results obtained in the tretinoin re-analysis [93,99]. When uptake and clearance were considered separately, the 90% confidence interval of the ratio of total drug amount in the SC (generic to innovator), conclusively lay within the 80 – 125% bioequivalence window, consistent with the clinical studies. The subject-to-subject variability was higher than the intra-subject variability, which may support the comparison of products within each subject [93].

3.3.3 *Drug permeation profiles across SC*

In this approach, the drug concentration profile across the SC is determined by quantification, on each tape, of i) the amount of drug; and ii) the amount of SC removed, and hence the depth within the barrier at which each drug amount is determined. Analysis of the concentration profile permits parameters describing the partitioning and diffusivity of the drug (reflecting the rate and extent of permeation, respectively) to be estimated and used to compare different formulations.

Quantification of the SC removed on each tape is assessed by weighing the tape pre- and post-stripping. From the mass of SC, and its published density ($1 g/cm^3$) [100], the volume of tissue removed is found. Then, given that the area stripped is known and controlled, the average SC thickness (x)

removed on each tape (and hence the cumulative depth sampled) can be calculated. If measurements of TEWL are made during the sequential tape-stripping process, the total thickness of the SC (H) can be found by fitting the results to a recently published non-linear model which describes how transepidermal water loss increases as the SC barrier is removed [101]:

$$TEWL = B + \frac{D \cdot K \cdot \Delta C}{H - x} \quad (8)$$

where B is a constant, D is the diffusivity of water in the SC, K is the SC-viable tissue partition coefficient of water, and ΔC is the water concentration gradient across the SC. A typical fitted profile is shown in **Figure 5**.

This analysis represents a refinement of the originally reported approach [102]. The advantage of the new method is that the non-contribution of the stratum disjunctum to the SC barrier can be taken into account (by the parameter B) and a more precise value of H determined as a result. Furthermore, by analysing the original values rather than reciprocal values, the errors associated with each point are not correspondingly distorted. Both the new approach and the method used earlier have the important benefit of allowing H to be found without having to completely remove the SC. Once TEWL has increased four- or fivefold (which corresponds to ~ 75% removal of the SC), sufficient information has been obtained to reliably find H.

This procedure to evaluate H is typically performed at a site adjacent to the treatment site, to avoid any potential artefacts affecting TEWL, for example evaporation of residual excipients from the formulation. Knowledge of H for each volunteer permits all SC concentration profiles to then be expressed as a function of *x*/H, or relative depth reached within the SC. This value varies, therefore, between 0 at the skin surface, to 1 at the SC–stratum granulosum interface.

However, the drawbacks of the gravimetric (weighing) approach for the determination of the SC thickness are significant: the procedure is laborious and tedious; reproducibility and precision can be low as the mass of SC is small relative to the mass of the tape; and measurements can be influenced by environmental conditions (e.g., humidity) and static electricity.

Other methods have been investigated. The UV pseudo-absorption of corneocytes has been extensively researched [103], but the correlation between this measurement and the mass of SC is unsatisfactory unless those tapes with an inhomogeneous layer of SC are ignored. Hence, this strategy is inappropriate to accurately measure the position reached within the SC, where the contribution from every tape is required.

The extraction and quantification of proteins from tapes can also measure the SC removed by tape-stripping. However, this is a destructive test, often incompatible with drug extraction and quantification. While such an approach may be not useful for dermatopharmacokinetic studies, a sensitive protein assay would be valuable to validate other techniques. Unfortunately such a sensitive technique does not currently exist.

Attempts have also been made to correlate spectroscopic absorbance (using infra-red light at 850nm) with masses of protein determined from SC extraction and assay [104] Correlation coefficients were high ($r^2 = 0.85$) with all 238 tapes included, but issues regarding sensitivity remain, as expressed above.

Most recently, a novel imaging method to quantify SC on tapes has been investigated. High-resolution images are taken of each tape under carefully controlled optical conditions. An image is composed of ~ 64000 pixels, each of which has a greyscale value associated with it when examined using image analysis software. Statistical analysis on the distribution of these pixels provides a mean greyscale value that offers a relative measure of SC content of the tapes. The approach has been shown to be rapid, simple, sensitive and precise. Further, the greyscale values have been shown to be a useful relative measure of SC amount per tape for the determination of SC total thickness in drug permeation experiments, and for full dermatopharmacokinetic studies of acyclovir creams [105].

The concentration profiles of a drug as a function of the relative position in the SC (x/H) (**Figure 4C**) have typically been fitted to a solution of Fick's second law of diffusion assuming the following boundary conditions: i) that an infinite dose is applied; ii) that the SC is initially drug-free; iii) that the SC is homogeneous in its barrier properties; and iv) that the viable epidermis provides a perfect sink for permeating drug. Under these circumstances, the concentration (C_x) of drug, as a function of time (t) and of position (x) in the SC of total thickness (H) is given by:

$$C_x = KC_v\left[\left(1-\frac{x}{H}\right)-\frac{2}{\pi}\sum_{n=1}^{10}\frac{1}{n}\sin\left(n\pi\cdot\frac{x}{H}\right)\exp\left(-\frac{D}{H^2}n^2\pi^2 t\right)\right] \quad (9)$$

where C_v is the drug concentration in the vehicle. By fitting the concentration profile to this equation, estimates of the drug's SC–vehicle partition coefficient (K) and its characteristic diffusion parameter (D/H^2, which has units of $[time]^{-1}$ like a first order rate constant) can be obtained. If H has been separately evaluated, as described above, then the drug's permeability coefficient (k_p) across the SC, when delivered from the vehicle under consideration, can be calculated from the three measured parameters

$$k_p = \frac{KD}{H} = K\left(\frac{D}{H^2}\right)H \quad (10)$$

Integration of Equation 9 permits the entire amount of drug in the SC (Q) at time, t, to be found:

$$Q = \int_0^1 C_x d\left(\frac{h}{H}\right) = K_m C_V \left[\frac{1}{2} - \frac{4}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left(-\frac{(2n+1)^2\pi^2 Dt}{H^2}\right)\right] \quad (11)$$

This estimation can be compared with the total drug recovered experimentally to check that the SC was indeed mostly removed during the tape stripping process. It can also be appreciated that K, D/H^2 and Q provide useful pharmacokinetic parameters with which to characterise and compare formulations. The value of K will impact significantly on the extent of uptake (Q), and D/H^2 offers a metric with which to characterise the rate of drug absorption across the SC. It will also be appreciated, once K and D/H^2 have been found from the concentration profile at a specific time, t, that these values can be substituted into Equation 11 to predict the evolution of Q as a function of time (offering thereby a means with which to simplify a DPK study considerably).

Experiments from which drug concentration profiles across the SC have been obtained, are robust and reproducible even with relatively small numbers (n = 4 – 6 per experiment) of subjects and sites [51,106-109]. By judiciously using the data derived from individual tapes, information on the rate and extent of drug permeation has been derived both *in vivo* in man and *ex vivo* using porcine skin.

These experiments have allowed both the practical comparison of formulations (such as ibuprofen gels [109]) and the deduction of mechanistic information. For example, the addition of oleic acid to a formulation containing terbinafine enhanced drug uptake into the SC by increasing the value of D/H^2, but had no effect on K [92]. In contrast, the role of a co-solvent (propylene glycol) on ibuprofen formulations clearly had a major impact on K, while D/H^2 was unaltered [110,111]. In this instance, it was possible to deduce that the excipients had increased the solubility of the drug in the SC, thereby significantly modifying their uptake into the skin [110,112]. The effect of formulating betamethasone-17-valerate in novel microemulsions (with significant concentrations of different surfactants) showed that Q was dependent on both concentration in the vehicle, and the degree of saturation of the drug in the formulation [106].

Furthermore, as mentioned above, it has also been possible to use values of K and D/H^2, derived from SC concentration profiles observed following relatively short periods of exposure, to predict the uptake kinetics over time up to the attainment of steady state conditions using Equation 11 [84,94,108]. The value of this approach, of course, lies in the potential to significantly simplify a DPK protocol for the comparison of formulations. As fewer sites are required than for the original FDA guidance, replicates can also increase statistical power.

An important caveat to this strategy, however, is that it is sensitive to the application time chosen for the determination of K and D/H^2. This period must be long enough that a measurable profile of drug across the SC is achieved, but should not be too close to steady state because the information about D/H^2 will be lost. The use of too short an application period not only brings with it a potential analytical chemistry problem, it also risks the introduction of an artefact if the exposure time is similar to the time required to effectively strip the skin [113]. Selection of the optimal time at which to conduct the experiment needs to be confirmed by the drug's diffusion lag time across the SC, a parameter largely determined by its molecular volume (or molecular weight) [93].

4. Conclusion

The empirical mathematical model proposed by Potts and Guy provides an adequate prediction of drug permeation based on two easily obtainable physicochemical properties, MW and $\log K_{o/w}$. Drug flux is the product of the permeability coefficient and drug solubility. There are numerous software programs that provide estimates of $\log K_{o/w}$ and solubility, although experimental values are most certainly preferred. Mathematical models consider solely the drug molecule of interest, and cannot predict the significant effects of formulation on permeation.

For *in vitro* penetration studies, the use of porcine skin is favoured and provides a useful indication of *in vivo* penetration. The influence of formulation is measurable, and can thus be used for development work.

From a regulatory perspective, *in vivo* measurements are required, and there is certainly enthusiasm to replace subjective clinical trials with a sensitive objective method such as tape stripping. *In vivo* measurements offer scope for objectively comparing formulations. The pharmacodynamic assessment of corticosteroids is approved and improvements made to the tape stripping methodology have shown promise for comparing many drugs, and may offer explanations as to why formulations perform differently.

5. Expert opinion

The use of predictive models and *in vitro* diffusion cells to estimate the percutaneous absorption of drugs are now relatively mature fields. Recent advances in theoretical approaches have been mostly incremental and have not added significant new insight into the transport of chemicals across the skin. *In vitro* experiments mainly use either human or porcine skin, and the 'dos and don'ts' of the methodology are well-known and mostly followed. While cell cultures have had noticeable impact on skin irritation/sensitisation testing, and other aspects of dermal toxicity, these systems have yet to produce a permeability barrier which is quantitatively similar to human skin. This clearly remains a challenge for the future.

In terms of *in vivo* methods, the focus will remain on human studies. Despite an unconvincing start, the tape stripping approach to DPK has shown its usefulness for the assessment of topical bioavailability of a drug class

whose site of action is on the skin's surface and/or within the SC. This was clearly the necessary first step in terms of validating the method's potential. It remains to be seen whether the principle can be extended to compounds which act in deeper skin layers (eg, corticosteroids) – recent work in this regard certainly encourages further efforts to test the hypothesis. Microdialysis, as a concept, is extremely attractive and offers, perhaps, the most intellectually satisfying strategy with which to quantify a topically applied drug's concentration profile within the skin. In addition, the potential for its use in diseased skin adds to the chemical relevance of data obtained in this way. However, for the moment, the method is beset by technical challenges, and by sensitivity and reproducibility issues. The future of the method depends, therefore, on these obstacles being resolved sooner rather than later.

Overall, there is no question that the measurement and prediction of drug delivery will remain an important and intensively studied area of research. The potential for 'deep delivery' to subcutaneous tissues has yet to be fully exploited; rational, optimised formulation of topical drugs is an unmet medical need, the importance of which is steadily increasing (the dramatic increase in the incidence of atopic eczema in children is an example); and the tightening of regulations with respect to safety evaluations of both an 'active' and the associated excipients, means that well-validated methodology, of proven predictive ability, will be absolutely essential for many years to come.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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